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Development of a robust, sensitive and selective liquid chromatography-tandem mass spectrometry assay for the quantification of the novel macrocyclic peptide kappa opioid receptor antagonist [D-Trp]CJ-15,208 in plasma and application to an initial pharmacokinetic study

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Abstract

Selective kappa opioid receptor (KOR) antagonists may have therapeutic potential as treatments for substance abuse and mood disorders. Since [D-Trp]CJ-15,208 (*cyclo*[Phe-D-Pro-Phe-D-Trp]) is a novel potent KOR antagonist *in vivo*, it is imperative to evaluate its pharmacokinetic properties to assist the development of analogs as potential therapeutic agents, necessitating the development and validation of a quantitative method for determining its plasma levels. A method for quantifying [D-Trp]CJ-15,208 was developed employing high performance liquid chromatography-tandem mass spectrometry in mouse plasma. Sample preparation was accomplished through a simple one-step protein precipitation method with acetonitrile, and [D-Trp]CJ-15,208 analyzed following HPLC separation on a Hypersil BDS C8 column. Multiple reaction monitoring (MRM), based on the transitions m/z 578.1 $\rightarrow$ 217.1 and 245.0, was specific for [D-Trp]CJ-15,208, and MRM based on the transition m/z 566.2 $\rightarrow$ 232.9 was specific for the internal standard without interference from endogenous substances in blank mouse plasma. The assay was linear over the concentration range 0.5–500 ng/mL with a mean $r^2 = 0.9987$. The mean inter-day accuracy and precision for all calibration standards was 93–118% and 8.9%, respectively. The absolute recoveries were 85 $\pm$ 6% and 81 $\pm$ 9% for [D-Trp]CJ-15,208 and the internal standard, respectively. The analytical method had excellent sensitivity with a lower limit of quantification of 0.5 ng/mL using a sample volume of 20 μ L. The method was successfully applied to an initial pharmacokinetic study of [D-Trp]CJ-15,208 following intravenous administration to mice.

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Keywords

Macrocyclic peptide; kappa opioid receptor antagonist; [D-Trp]CJ-15,208; LC-MS/MS; multiple reaction monitoring; peptide quantitation

1. Introduction

The macrocyclic tetrapeptide CJ-15,208 (*cyclo*[Phe-D-Pro-Phe-Trp]) was first isolated from the fermentation broth of a fungus, *Ctenomyces serratus*, and reported to exhibit kappa opioid receptor (KOR) antagonism *in vitro* [1]. We synthesized both *cyclo*[Phe-D-Pro-Phe-Trp], which appears to be the natural product and its D-Trp isomer [2], and found that both exhibited promising *in vivo* opioid activity following central (intracerebroventricular) administration to mice [3]. These peptides are also active after oral administration and appear to penetrate into the central nervous system [4, 5], and hence can serve as lead compounds for further development. A growing body of preclinical evidence suggests that selective KOR antagonists may have therapeutic potential as treatments for drug abuse and mood disorders [6]. KOR antagonists have shown promising results in preclinical models, preventing stress-induced reinstatement of drug seeking behavior for cocaine and nicotine and also for increased ethanol consumption [7, 8, 9]. Since [D-Trp]CJ-15,208 is a potent KOR antagonist *in vivo* it is imperative to evaluate the pharmacokinetic properties of this lead compound to assist in the design and development of analogs as potential therapeutic agents. This necessitated the development and validation of a quantitative method for determining its plasma levels.

In recent years, the combination of high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection has become an important technique in bioanalytical research. The LC-MS/MS quantitation of macrocyclic peptides has not been extensively reported, with the only exceptions being the determination of cyclosporine A and apicidin plasma levels by LC-MS/MS quantitation [10, 11]. The quantitation of the macrocyclic tetrapeptide [D-Trp]CJ-15,208 in biological samples has not been reported. Therefore, the objective of this study was to develop a fully-validated method for the quantitation of [D-Trp]CJ-15,208 in plasma that could be applied to pharmacokinetic studies of the lead peptide. Herein, we describe for the first time an LC-MS/MS method for quantification of [D-Trp]CJ-15,208 in plasma utilizing a simple one-step protein precipitation method that is sensitive, reproducible, and selective. This method was successfully applied to an initial pharmacokinetic study of the peptide following intravenous (i.v.) administration.

2. EXPERIMENTAL

2.1. Materials

[D-Trp]CJ-15,208 and the structurally related analog [D-NMeAla²]CJ-15,208 (*cyclo*[Phe-DNMeAla-Phe-Trp], Figure 1) used as the internal standard were synthesized as reported previously [2, 12]. Drug-free (blank) mouse plasma was obtained from Bio-Reclamation Inc (Westbury NY). HPLC-grade acetonitrile was obtained from Fischer Scientific (Pittsburgh,

PA, USA), and deionized water was obtained from a Millipore water purification system. Solutol HS 15 was a kind gift from BASF.

2.2. Instrumentation and LC-MS/MS conditions

Liquid chromatography was performed on a Hypersil BDS C8 column (3 μ , 2.1 mm $\times$ 50 mm) with a flow rate of 0.2 mL/min and an injection volume of 20 μ L using a Waters Acquity Classic UPLC (Waters Corp., Milford MA) coupled to a triple quadrupole mass spectrometer (Quattro Ultima Micromass Ltd. Manchester UK) operating in the positive-ion mode. The peptides were separated using the following gradient of water (solvent A) and acetonitrile (solvent B), both containing 0.08% formic acid: 0–2 min (20% B), 2–3 min (20–50% B), 3–6 min (50–80% B), 6–7 min (80% B), 7–8 min (80–20% B) and 8–10 min (20% B).

The precursor ions of [D-Trp]CJ-15,208 and its internal standard were first isolated and then subjected to collision-induced dissociation to give their fragment ions. Data acquisition was carried out with Mass Lynx 4.1 software with the following settings: capillary voltage, 3800 V; cone voltage, 80 V; source temperature, 100 $^{\circ}$ C; desolvation temperature, 150 $^{\circ}$ C; cone gas flow, 279 L/h; desolvation gas flow, 1157 L/h. Q1 and Q3 resolution were 0.8 u FWHH. The argon filled collision cell pressure was 1.63×10^{-3} mbar on a guage in-line with the cell. Multiple-reaction-monitoring was used for [D-Trp]CJ-15,208 $[M + H]^+$ m/z 578.1 $\rightarrow$ 217.1 and 245.0, collision energy 30 eV and for the internal standard $([M + H]^+ m/z 566.2 \rightarrow 232.9)$, collision energy 22 eV with a dwell time of 0.3 s (Figure 1).

2.3. Sample preparation

Stock and working solutions of [D-Trp]CJ-15,208 and internal standard were prepared in acetonitrile. The calibration standards and quality control (QC) samples were prepared in blank (drug-free) mouse plasma using the [D-Trp]CJ-15,208 working solution. The plasma calibration curve consisted of eight standards in plasma: 0.5, 5, 10, 25, 50, 100, 250, and 500 ng/mL. QC samples consisted of four different concentrations: 0.5, 10, 50, and 100 ng/mL. The calibration standards and QC samples were prepared fresh daily. Calibration curves were constructed using spiked plasma samples. The linear regression of the peak area ratios of [D-Trp]CJ-15,208 to IS versus analyte concentrations was fitted over the concentration range of 0.5–500 ng/mL. A typical equation for the calibration curve was: $y = 376.73x - 4.55$, $r^2 = 0.9987$, where y represents plasma concentration (in ng/mL) and x represents the ratio of the peak areas of [D-Trp]CJ-15,208 to IS. Ice-cold plasma aliquots (50 μ L) were processed by a simple one-step protein precipitation using ice-cold acetonitrile (100 μ L) containing the internal standard (3 μ g/mL). The sample was centrifuged at 10,000 rpm for 10 minutes, and supernatant (50 μ L) diluted with water (115 μ L), stored overnight (-20° C), and analyzed by LC-MS/MS as described above.

2.4. Pharmacokinetic study

Adult male C57BL/6J mice (5 per time point), weighing 20–25 g obtained from the Jackson Laboratory (Bar Harbor, ME, USA), were selected for this study because of their use in our pharmacological studies [3, 5]. All mice were housed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. [D-Trp]CJ-15,208 (0.1

mL) was administered to mice by intravenous injection (10 mg/kg) in 10% Solutol in saline. The animals (5 mice per time point) were sacrificed 2, 5, 15, 30 and 60 min following administration, and blood drawn by heart puncture; the blood was immediately centrifuged at 15,000 rpm for 15 min to harvest the plasma. The plasma samples were processed as described above, and the plasma [D-Trp]CJ-15,208 concentration versus time was analyzed by Non-compartmental WinNonLin software.

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS method development

A method was developed for the rapid and robust quantitation of [D-Trp]CJ-15,208 in mouse plasma. A one-step protein precipitation method was employed to facilitate rapid sample processing. Ice-cold acetonitrile containing the internal standard was added to the ice-cold plasma samples which were analyzed by LC-MS/MS following centrifugation and dilution with water. This simple rapid sample processing method has advantages over solid-phase and tedious liquid-phase extraction methods. The ionization and fragmentation of [D-Trp]CJ-15,208 was first studied to identify appropriate electrospray ionization-tandem mass spectrometry conditions for the method. The intensity of the sodium adduct $[M+Na]^+$ (m/z 600.0) in the Q1 mass spectrum was substantially higher than the molecular ion $[M+H]^+$. However, the sodium adduct gave rise to low intensity fragment ions, and therefore was not used for quantitation. The collision-induced dissociation of the $[M+H]^+$ precursor ion (m/z 578.1) produced intense and stable fragments at m/z 217.1 and 245.0 at the optimum collision energy of 30 eV. The product ion mass spectra for the analyte and internal standard are shown in Figure 2A and 2B, respectively.

Initially, the analyte and a chemically similar internal standard were eluted from a C18 column. Owing to their hydrophobic nature, however, they were not well resolved which increased carry-over and the occurrence of cross-talk in the MS/MS spectrum. To address this problem, we used a C8 column and a less hydrophobic but chemically similar internal standard which gave rise to a unique fragment ion (m/z 232.9) in MRM mode. The gradient was started at a much lower concentration of MeCN than needed to elute the analyte and internal standard to minimize the possibility of polar components (e.g. salts) in the sample precipitating on the column. Both peptides were eluted from the C8 column using a relatively high concentration (60–80%) of MeCN with negligible carry-over and cross talk.

Figure 3A shows the lower limits of quantitation (LLQ) chromatogram for the MRM transitions m/z 578.1 $\rightarrow$ 217.1 and 245.0 of blank plasma spiked with 0.5 ng/mL [D-Trp]CJ-15,208; Figure 3B and 3C shows the chromatograms for [D-Trp]CJ-15,208 plus the internal standard with retention times of 5.72 and 5.06 min in the post-protein precipitation spiked samples and standard protein precipitation method, respectively thus confirming the absence of any matrix effect. A typical chromatogram from blank mouse plasma (Figure 3D) shows that multiple reaction monitoring based on the m/z 578.1 $\rightarrow$ 217.1 and 245.0 transitions was specific for [D-Trp]CJ-15,208 and that the transition m/z 566.2 $\rightarrow$ 232.9 was specific for the internal standard. Evaluation of six plasma without either [D-Trp]CJ-15,208 or internal standard (blank plasma) verified the lack of interfering endogenous components with either peptide, and thus the specificity of the method.

3.2. Method validation

The method was validated in terms of linearity, specificity, matrix effects, LLQ, recovery, intra- and inter-day accuracy and precision. Each analytical run included a blank sample without internal standard, a blank sample with internal standard, eight standard concentrations of [D-Trp]CJ-15,208 for calibration and four QC samples. The mean extraction recoveries of [D-Trp]CJ-15,208 and the internal standard were $85 \pm 6\%$ and $81 \pm 9\%$, respectively, at 100 ng/mL plasma. The matrix effect of [D-Trp]CJ-15,208 was measured by comparing the peak response of the sample spiked after protein precipitation (Figure 3B) with that of the pure standard containing the same amount of [D-Trp]CJ-15,208 added to plasma in the protein precipitation solvent (Figure 3C). The results indicate that the LC-MS/MS data is not affected by any component in plasma.

The method precision was assessed by the coefficient of variation (%CV). The assay was linear over the above concentration range. The correlation coefficients for the calibration curves ranged from 0.9974 to 0.9996 with a mean of 0.9987 ± 0.0002 (0.0158% CV). The mean inter-day accuracy for all calibration standards ranged from 93–118%, and the mean inter-day precision for all standards was 8.9% (Table 1). The %CV values for the inter-day evaluation of QC samples ranged from 2.2 to 17.0% (Table 2). The mean intra- and inter-day assay accuracies, determined at each QC level throughout the validated runs, remained below 11% and 4.0%, respectively. Thus, the described method has satisfactory accuracy, precision and reproducibility for the quantification of [D-Trp]CJ-15,208 in mouse plasma.

3.3. Application of the method in a pharmacokinetic study

The method was successfully applied to a pharmacokinetic study of [D-Trp]CJ-15,208 in mice. The mean plasma concentration-time curve after intravenous administration of 10.0 mg/kg [D-Trp]CJ-15,208 is shown in Figure 4 and the pharmacokinetic parameters from non-compartmental model analysis are summarized in Table 3. The mean maximum plasma concentration ($C_{\max}$) was found to be 1020 ng/mL. The plasma concentration of [D-Trp]CJ-15,208 declined by over 50% within 15 minutes ($t_{1/2} = 14.2$ min), and the compound was almost completely eliminated from plasma within 1 hour (Figure 4).

4. CONCLUSIONS

A robust and sensitive LC-MS/MS assay was developed to quantitate [D-Trp]CJ-15,208 in mouse plasma for the first time. The method has a wide linear dynamic range from 0.5 to 500 ng/mL. The method utilizes a simple one-step protein precipitation by acetonitrile and does not require solid-phase or time-consuming liquid-liquid sample extraction procedures. The method has high sensitivity, specificity and reasonable sample throughput. This simple, robust method was successfully applied to an initial pharmacokinetic analysis of [D-Trp]CJ-15,208 in mice.

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blood collection in mice and the calculation of the pharmacokinetic parameters, respectively. This research was supported by the National Institute on Drug Abuse grant R01 DA032928.

Abbreviations

CV	coefficient of variation
[D-Trp]CJ-15,208	<i>cyclo</i> [Phe-D-Pro-Phe-D-Trp]
KOR	kappa opioid receptor
LLQ	lower limit of quantitation
MRM	multiple reaction monitoring
MS/MS	tandem mass spectrometry
QC	quality control

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Highlights

- The macrocyclic tetrapeptide [D-Trp]CJ-15,208 was quantified in plasma by LC-MS/MS.
- Quantitation was specific without endogenous interference in mouse plasma.
- The validated method was linear over 0.5–500 ng/mL (mean $r^2 = 0.9987$).
- The limit of quantitation was 0.5 ng/mL.
- Mean inter-day accuracy and precision standards were 93–118% and 8.9% respectively.

Figure 1.
Precursor ions and resulting fragment ions of [D-Trp]CJ-15,208 and the internal standard

Figure 2.

(A) Product ion scan of [D-Trp]CJ-15,208, $[M+H]^+$ at m/z 578.1 (collision energy 30 eV) and (B) Product ion scan of the internal standard, $[M+H]^+$ at m/z 566.2 (collision energy 22 eV)

Figure 3.

Chromatograms monitoring [D-Trp]CJ-15,208 and the internal standard in mouse plasma. (A) Chromatogram for the MRM transitions m/z 578.1 $\rightarrow$ 217.1 and 245.0 of blank (drug free) mouse plasma spiked with 0.5 ng/mL [D-Trp]CJ-15,208; (B) Chromatogram for the MRM transitions m/z 578.1 $\rightarrow$ 217.1 and 245.0 of blank mouse plasma spiked with 100 ng/mL [D-Trp]CJ-15,208 (t_r 5.68 min) and internal standard (t_r 5.03 min) after protein precipitation; (C) Chromatogram for the MRM transitions m/z 578.1 $\rightarrow$ 217.1 and 245.0 of blank mouse plasma spiked with 100 ng/mL [D-Trp]CJ-15,208 followed by standard sample preparation; (D) Chromatogram for the MRM transitions at m/z 578.1 $\rightarrow$ 217.1 and 245.0, and 566.2 $\rightarrow$ 232.9 in blank mouse plasma

Figure 4. [D-Trp]CJ-15,208 levels in plasma ($\pm$ SEM) over 1 hour following intravenous administration of 10 mg/kg to mice (n=5).

Table 1

Summary of the back-calculated calibration standards for [D-Trp]CJ-15,208 in mouse plasma (n=4)

Nominal conc. (ng/mL)	Interday validation summary			
	Mean	SD	% CV	Accuracy (%)
0.5	0.47	0.10	20.4	93.5
5	5.92	0.46	7.8	118
10	11.1	0.9	8.3	111
25	27.2	1.8	6.7	109
50	55.8	5.2	9.4	111
100	111	10	9.0	111
250	258	14	5.3	103
500	495	24	4.9	99.1

Table 2

Summary of the quality control validation for [D-Trp]CJ-15,208 in mouse plasma (n=4)

Interday validation summary				
Nominal conc. (ng/mL)	Mean	SD	% CV	Accuracy (%)
0.5	0.49	0.08	17.0	97.2
10	11.3	0.25	2.2	113
50	55.0	1.9	3.4	110
100	98.2	11.2	11.4	98.2

Table 3

Pharmacokinetic parameters after intravenous administration of 10 mg/kg i.v. [D-Trp]CJ-15,208 to mice

Parameters	Estimate
Half-Life (min)	14.2
C _{max} (ng/mL)	1017.3
C ₀ (ng/mL)	1129.5
AUC _{0-t} (min*ng/mL)	19124.7
AUC _{0-∞} (min*ng/mL)	20240.3
Volume of Distribution (mL/kg)	10142.6
Clearance (mL/min/kg)	494.1