Multiplex Screening for Cystine-Dense Peptides (CDP) Therapeutics Proteins by HPLC-MS/MS

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Introduction

Cystine-Dense Peptides (CDP) can be found as naturally expressed, small proteins from all Kingdoms. CDPs have a highly disulfide cross-linked structure and some may exhibit drug-like properties. [1,2] As an example, the protein CTXL_LEIQU from the Egyptian deathstalker scorpion has a known biological function such as chloride channel inhibition and selective interaction with MMP2 to inhibit its enzyme activity. More than 680,000 putative CDPs have been bioinformatically identified and ~700 structures were available for further classification of CDPs based on connectivity.[3] A high-throughput platform expression screening method (up to 20 µg in 1 mL scale plate culture) and large-scale production (up to 10 mg/L in 2 L cell culture) has been developed for further translational medicinal development such as in-vivo biodistribution. While CDP classes that are found in nature provide great potential, determining each CDP mechanism of action in human biology and mapping out exact pharmacophores to the therapeutic binding partner is time consuming. Thus, a hypothesis that intact CDP stability (e.g., keeping disulfide cross-linked structural integrity) in plasma or therapeutic target tissues will increase its natural bioavailability can allow for further prioritization of CDPs in the therapeutic development pipeline. HPLC-MS/MS analysis is well suited for targeted protein quantification[4] and is the industry standard due to the selectivity and sensitivity of the instrumentation.[5,6] Here, we present a schema for prioritizing the development of CDP based therapeutics based on high throughput HPLC-MS/MS bioanalysis of several CDPs simultaneously using solid phase extraction (SPE) and HPLC-MS/MS. This accurate method was used to screen CDPs in order to determine the stability and Limit of Quantification (LOQ) which can then be used in future preclinical discovery PK studies. Candidates must exhibit adequate peak shape and sensitivity (10 ng/mL) when analyzed on the HPLC-MS/MS with accuracies within 20% of the nominal concentrations.

Methods

SPE extraction followed by HPLC-MS/MS analysis was developed for the simultaneous quantitative analysis of six CDPs. Several extraction methods were investigated. Strong anion exchange provided the highest recovery for the six peptides. Internal standard selection was conducted by the HPLC-MS/MS analysis of a 32 isotopically labeled peptide mixture (SpikeMix CEF Pool – heavy, jpt). Twelve of the peptides were selected as possible internal standards based on the peak shape and retention of the labeled peptides when analyzed on the optimized HPLC-MS/MS method developed for the CDPs. The internal standard for each CPD was selected by extracting and analyzing the labeled peptides from plasma containing the CPDs (eight concentrations in duplicate) and determining which labeled peptide provided the highest accuracy when a linear calibration curve was generated for each CPD and labeled peptide area

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ratio. The optimized extraction consisted of the following: 100 µL of plasma containing the six peptides was placed into a 96 deep well plate.25 µL of the internal standard SpikeMix was added to the plate along with 100 µL water. This entire volume was added to a pre-conditioned strong anion exchange plate (Waters, Oasis MAX) and vacuum was applied. The wells were washed with 500 µL water and then 100 µL water:methanol 9:1. The peptides were eluted by adding 250 µL of 2% Trifluoroacetic acid in methanol (twice) to each well. The solvent was evaporated and 100 µL of water containing 0.1% formic acid was added to each dry well. The sample was then analyzed by HPLC-MS/MS on API-6500QTRAP® and API-6500 QTRAP+ (Sciex) mass spectrometers operating in positive ESI mode. The HPLC systems were Shimadzu LC-30AD pumps operating with a binary gradient method and a flowrate of 0.700 mL/min. Separation was achieved using an Agilent Pursuit Diphenyl column (10 cm x 2.1 mm, 5 µm).

Results

The data indicated that with optimized extraction and HPLC-MS/MS methods a low LLOQ can be accomplished. The LLOQ for the method is 10 ng/mL with a dynamic range of 10.0-2,000 ng/mL, accuracy within 20% and an r value >0.995 for each CDP. Each CPD has been found to be stable in plasma for at least one hour with adequate recovery. No significant interfering peaks are present in the chromatograms of an extracted blank plasma sample. One stable label internal standard peptide was found to provide adequate accuracy for the quantitation of all six peptides (IVTDF*SVIK).

Conclusions and Discussion

A schema has been developed to screen CDP compounds for therapeutics translational medicine development based on chromatographic peak elution, plasma stability and sensitivity. We presented the best overall recovery method from plasma to establish the lowest Limit of Quantification (LLOQ) by SPE and HPLC-MS/MS (10 ng/mL). The developed method can be used in further therapeutics development pipelines (e.g, PK). Though we presented a “universal” method for CDP screening, the developed method was only successful for 6 out of the 7. CPDs tested. The most polar compound among the candidates was rejected during the selection stage due to poor retention on the LC column. Future investigation is needed to cluster CDPs into groups based on informatics biophysical predicted values (e.g. polarity) and to develop multiple extraction and HPLC-MS/MS methods to apply to varying therapeutically desirable properties.

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