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

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## OVERVIEW

- Cystine-Dense Peptides (CDP) have vast applications for diagnostic and therapeutic applications.
- CDP structural stability is an important factor for biological function and delivery to target (e.g., oral delivery, etc.).
- Developed a general workflow on CDP screening for translational medicine development using High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS)
- CDP compounds that are expressed using adapted Daedalus lentivirus transduction system in HEK293 cells were evaluated.
- Robust multiplex CDP screening method is developed based on chromatographic peak elution, plasma stability and sensitivity.
- The optimized and accurate HPLC-MS/MS method developed for screening can be easily utilized in preclinical therapeutic discovery studies when the selected compounds move further down into the translational medicine development pipeline.
- HPLC-MS/MS CDP quantitation performed on intact peptides (not denatured or digested) with an LLOQ of 10.0 ng/mL for each CDP and accuracies within 20%.

## INTRODUCTION

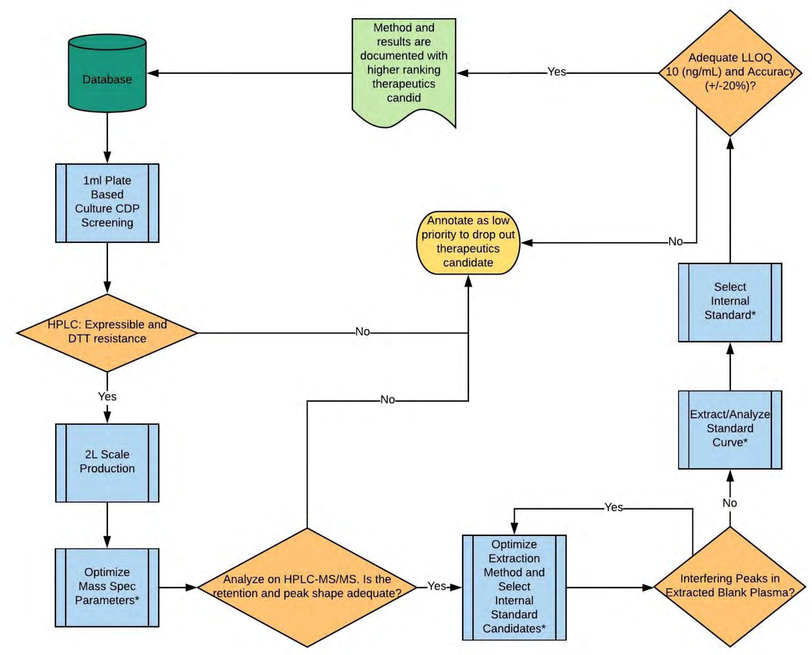
Cystine-Dense Peptides (CDP) can be found as naturally expressed, small proteins from all Kingdoms. CDPs have a highly disulfide cross-linked structure and some may exhibit drug-like properties.[1,2] As an example, the protein CTXL_LEIQU from the Egyptian deathstalker scorpion has a known biological function such as 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 us to further classify CDPs based on connectivity.[3] A high-throughput platform expression screening method (up to 20 µg in 1mL scale plate culture) and large-scale production (up to 10 mg/L in 2 L cell culture) has been developed for further translational medicine 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, we hypothesized that intact CDP stability (e.g., keeping disulfide cross-linked structural integrity) in plasma or therapeutic target tissues will increase its natural bioavailability and we can further prioritize them 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 CDP based therapeutics development schema in prioritizing the therapeutic development candidates including 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 Lower Limit of Quantification (LLOQ) which can then be used in future preclinical discovery PK studies. The criteria for candidate selection can be found in **Figure 1**.

**Table 1. CDP Compound Sequence, Source Organism and Fully Disulfide Bonded Monoisotopic Mass**

| ID | Target #30 | Target #9 | Target #12 | Target #91 | Target #62 | Target #19 |
|---|---|---|---|---|---|---|
| **Sequence** | GSEGDCPISEAIKCVEKCK EKVEVCEPGVCKCSG | GSVRIPVSCKHSGQCLKPC DAGMRFGKCMNGKCDCTPK | GSQKILSNRCNNSSECPHC IRIFGTRAAKCINRKCYCYP | GSQFCGTNGKPCVNGQCCG ALRCVVTYHYADGVCLKMNP | GSQIDTNVKCSGSSKCVKIC IDRYNTRGAKCINGRCTCYP | GSGHACYRNCWREG NDEETCKERC |
| **Source Organism** | Lychas mucronatus (Chinese swimming scorpion) | Androctonus australis (Sahara scorpion) | Buthus occitanus israelis (Common yellow scorpion) | Macrothele gigas (Japanese funnel web spider) | Tityus discrepans (Venezuelan scorpion) | Heterometrus fulvipes (Indian black scorpion) |
| **Mass** | 3539.04 | 4165.93 | 4543.24 | 4089.68 | 4337.94 | 2799.96 |
| **Q1 Ion** | 885.5 | 834.1 | 909.3 | 818.7 | 868.4 | 560.7 |
| **Product Ion** | 1131.2 | 1152.9 | 1067.5 | 954.2 | 1017.1 | 664.2 |
| **RT (min)** | 4.22 | 2.73 | 3.49 | 3.83 | 2.57 | 2.83 |
| **Recovery (%)** | 90 | 34 | 63 | 43 | 50 | 80 |
| **r** | 0.997 | 0.995 | 0.998 | 0.997 | 0.997 | 0.995 |

## FIGURE 1

**CDP Therapeutic Candidate Priority Schema by Assays.**
**The process annotated with "*" are addressed by HPLC-MS/MS assay.**




## METHODS

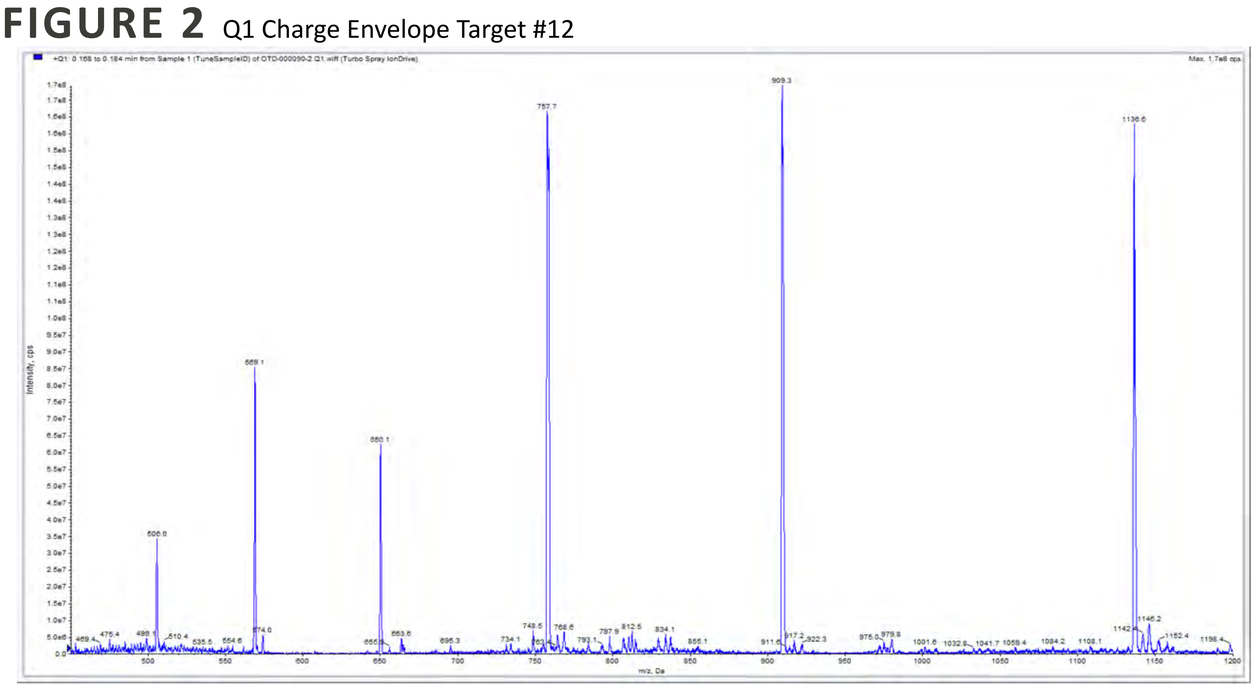
### SPE Extraction

- Add 100 µL plasma to 96 DWP
- Add 25 µL of internal standard (IVTD-F*-SVIK)
- Add 100 µL water 0.1% formic acid
- Condition MAX SPE plate with Methanol then water
- Add entire sample volume to SPE plate
- Wash with 500 µL water then 100 mL water 10% methanol
- Elute with 250 µL 2% TCA in methanol (2 times)
- Blown down eluent and reconstitute with 100 µL water 0.1% formic acid

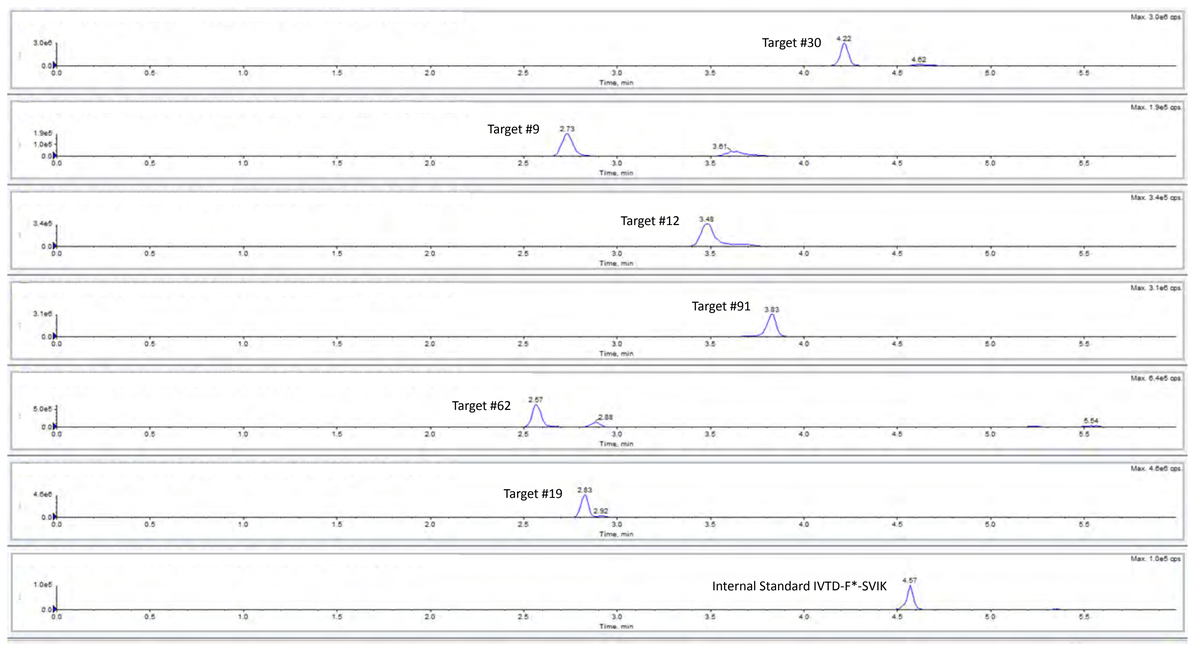
### LC-MS/MS

- Shimadzu Binary LC Systems
- Gradient using acetonitrile and water with formic acid
- Flow rate: 700 µL/minute
- Column: Pursuit 5 Diphenyl (100 X 2.1 mm, 5 µm)
- Column temperature: 50°C
- Sciex 6500 QTRAP® and 6500 QTRAP + operating in MRM mode
- ESI
- Positive ion mode

## FIGURE 2 Q1 Charge Envelope Target #12




## FIGURE 3 Representative Chromatogram 1000 ng/mL CDPs extracted from Human Plasma




## 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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