Development and Validation of an LC-MS/MS Assay for the Quantification of a Therapeutic Peptide, MPCAP 120-146WH5RMP, in Rat Serum

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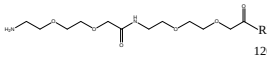
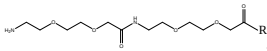
INTRODUCTION

- MPCAP 120-146WH5RMP, a multifunctional peptide based on the cationic antimicrobial protein of 37 kDa (CAP37, also known as Azurocidin or heparin-binding protein), demonstrates strong bactericidal activity, chemotactic activity, and significant corneal wound healing properties.
- Extremely low extraction recovery of MPCAP antimicrobial peptide analogs from plasma/serum was observed in previous studies. The purpose of the current study is to develop a sample preparation method with improved extraction efficiency, and to validate an LC-MS/MS assay for the quantification of this therapeutic peptide MPCAP 120-146WH5RMP in rat serum.



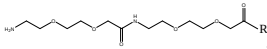
RRRRG TRCQV AGWGS WHSGG RLSRF PRFVN VR

120-146WH5RMP



RRRRN QGRHF CGGAL IHARF VMTAA SCFQR

BCC01



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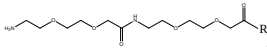
BCC02



RRRRN QGRHF CGGAL IHARF VMTAA SSFQR

BCC03

METHODS

- *Instrument:* AB Sciex (Ontario, Canada) API-2000 triple quadruple mass spectrometer coupled with Agilent (Santa Clara, CA) Series 1100 HPLC System and Phenomenex (Torrance, CA) Kinetex C18, 2.6 micrometer, 4.6 x 100 mm column were used for quantification of therapeutic peptide. Barnstead (Lake Balboa, CA) Nanopure Water Systems was used through the study.
- *Materials*: Acetonitrile, Formic Acid (98%) and from EMD Millipore; Methanol from BDH HiPerSolv. MPCAP 120-146WH5RMP and its stable isotope labeled internal standard were synthesized by CS Bio.
- *Chromatographic and MS Conditions:* Mobile Phase A: 0.2% Formic Acid in 10% Acetonitrile/Water; Mobile phase B: 0.2% Formic Acid in Acetonitrile. Column Temp: 40ºC; Flow Rate: 0.5 mL/min. Electro Spray Ionization and positive mode MS detection Gradient:

Method Time	% Mobile Phase - B
0 – 0.5 min	5%
0.5 – 1.5 min	5 – 11%
1.5 – 2.5 min	11%
2.5 – 2.7 min	11 – 95%
2.7 – 5.5 min	95%
5.5 – 5.6 min	95 – 5%

Electro Spray Ionization and positive mode MS detection:

Operating Parameter	Setting
Polarity	Positive
Drying Gas Temperature	450 ˚C
Gas 1 Pressure	35 PSI
Gas 2 Pressure	65 PSI
Ion Spray Voltage	4.5 kV
CAD Gas Pressure	5 PSI
CUR Gas Pressure	50 PSI
IHE	On

Operating Parameter	Setting
Resolution Q1	Unit
Resolution Q3	Open
Scan Type	MRM
Declustering Potential	40 V
Focusing Potential	200 V
Entrance Potential	9 V
Collision Energy	30 V
Collision Exit Potential	5 V

RESULTS

Table 1. The representative sample cleanup and extraction procedures were evaluated

Peptide Analogs	Matrix	Additive Prior to Extraction	Sample Clean and Extraction	Extraction Recovery
MPCAP37 120-146 WH5RMP	Rat Serum	200 µg/mL polylysine	Protein Precipitation with Methanol	<2%
MPCAP37 120-146 WH5RMP	Rat Serum	None	Protein Precipitation with Methanol	Not Detected
MPCAP37 120-146 WH5RMP	Rat Serum	2% Formic Acid	Protein Precipitation with Methanol	~70%
MPCAP 37 BCC01, BCC02, BCC03	Rat Plasma	None	Solid Phase Extraction with Phenomenex Strata-X	Not Detected
MPCAP 37 BCC01, BCC02, BCC03	Rat Plasma	Diothiothreitol	Protein Precipitation with 1% Formic Acid in Acetonitrile	Not Detected
MPCAP 37 BCC01, BCC02, BCC03	Mouse, Monkey and Human plasma	4M urea	Protein Precipitation	Not Detected
MPCAP 37 BCC01, BCC02, BCC03	Mouse, Monkey and Human plasma	0.25M Arginine	Protein Precipitation	Not Detected
MPCAP 37 BCC01, BCC02, BCC03	Human and Baboon Plasma	Dithiothreitol	Thermo Scientific Rapid Equilibrium Dialysis (8,000 Da)	Not Detected

Plasma/Serum spiked with MPCAP antimicrobial peptide analogs were extracted with different protein precipitation solvents. The impact of addition of arginine, urea, poly-lysine, surfactant, protease inhibitor or formic acid into plasma/serum prior to protein precipitation was evaluated as well. By acidifying serum by formic acid then extracting by methanol, ~70% extraction recovery was achieved in comparison to less than 10% by other tested extraction procedure.

Fig 1. Representative chromatography of MPCAP37 120-146 WH5RMP (A) in rat plasma at 4 °C and (B) in water at room temperature on T0 (dark) and T4 hour (red). Significant degradation of peptide was observed in rat serum.

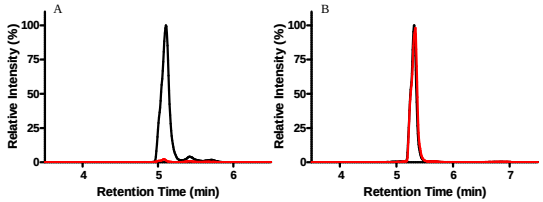


Fig 2. Representative (A) total ion chromatography and (B) mass spectrum of degradant after spiking MPCAP37 120-146 WH5RMP in rat serum for 1 hour.

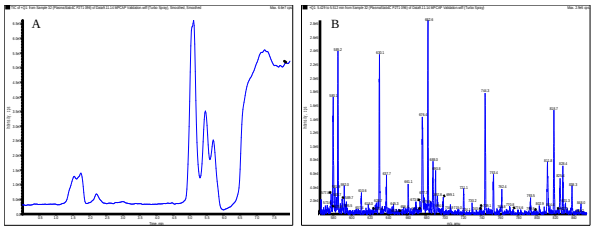


Fig 3. Representative chromatography of MPCAP37 120-146 WH5RMP spiked into rat serum at 70 µg/ml before extraction (red) and after extraction (dark).

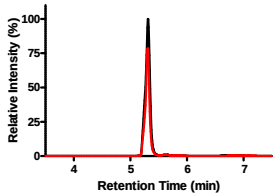


Fig 4. The calibration curve of LC-MS/MS assay was established for the concentrations range of 2–1000 µg/ml for MPCAP37 120-146 WH5RMP with a coefficient of determination (r2) of 0.9986.

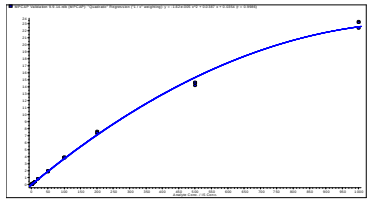


Table 2. The accuracy and precision of LC-MS/MS assay for MPCAP37 120-146 WH5RMP

Expected Amount (µg/mL)	Calculated Conc. In Nine Replicated Sample Preparation			Average Conc. (µg/mL)	% of Expected	% RSD
7	6.2	6.0	6.2	6.2	88.7%	4.2%
	5.9	6.6	5.9			
	6.2	6.6	6.3			
70	67.3	70.3	69.2	67.3	96.1%	4.1%
	70.9	67.2	63.9			
	62.6	68.2	65.9			
700	655.3	542.8	624.7	595.6	85.1%	7.1%
	635.5	614.8	531.2			
	561.8	597.5	597.2			

CONCLUSIONS

- Non Specific binding and degradation was determined as the major cause of low spike recovery for MPCAP37 120-146 WH5RMP in rat serum.
- The extraction recovery of MPCAP 120-146WH5RMP is significantly improved. The results indicated that this validated LC-MS/MS method is robust, rugged, sensitive and suitable for monitoring the therapeutic peptide in rat serum for pharmacokinetic and/or toxicokinetic study with the desired precision and accuracy.