Development and Validation of Analytical Assays for Synthetic Antimicrobial Peptides Based on Cationic Antimicrobial Protein of 37 kDa (CAP37)

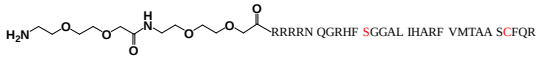
Qiang Liu[1], Douglas Bailey[1], Eliza Yeung[1], Chanelle Toerien-Hayes[1], Tiffany Hyde[1], Nicole Vu[1], Thomas Kupiec[1], and H. Anne Pereira[2]

[1]Analytical Research Laboratories, OK, USA [2]Department of Pharmaceutical Sciences, College of Pharmacy, University of Oklahoma Health Sciences Center, OK, USA

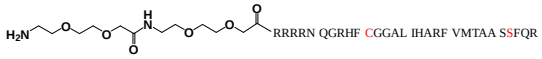
www.arlok.com

INTRODUCTION

- Cationic antimicrobial protein of 37 kDa (CAP37), also known as Azurocidin or heparin-binding protein, contributes to the oxygen-independent killing mechanism of the human neutrophil (PMN). CAP37 and its peptide derivatives demonstrate strong bactericidal activity for Gram-negative organisms, monocyte chemotactic activity, and also plays a significant role in corneal wound healing. [1-4]
- Comprehensive characterizations (including identification, assay, purity, and specific tests) are required for the regulatory approval of the therapeutic peptides. However, the development and validation of bio-analytical assays for therapeutic peptides remains a challenge due to their varying size, sequence and physical/chemical property. [5]
- The objective of this study is to develop and validate analytical methods for the comprehensive characterization and stability evaluation of CAP37-derived antimicrobial peptide BCC02 and BCC03 (MW 3775.9601 Da)



BCC02



BCC03

METHODS

- *Instrument:* Agilent (Santa Clara, CA) Series 1100 HPLC System equipped with Phenomenex (Torrance, CA) Aeris Peptide 3.6 µm XB-C18, 4.6 x 150 mm column was used for the stability indicating HPLC assay. Bruker (Billerica, MA) MicroTOF high resolution mass spectrometer coupled with an Agilent 1100 HPLC System and Agilent Zorbax SB-C18 5µ 4.6 x 150mm column were used for intact mass measurement and peptide mapping. Norlake (Hudson, WI) incubator (Model NSR|522WSW8) was utilized in sterility determination. Tecan (San Jose, CA) Sunrise plate reader and Endoscan V software (Charles River Laboratories, Wilmington, MA) were used in endotoxin assay. Barnstead (Lake Balboa, CA) Nanopure Water Systems was used throughout the study.
- *Materials*: 1N Sodium Hydroxide (NaOH) and 1N Hydrochloric Acid from Ricca; Dextrose and Isopropyl Alcohol (IPA) from Spectrum; Heptafluorobutyric Acid (HFBA) and Ammonium Bicarbonate from Fluka; Acetonitrile from Fischer Scientific; Trifluoroacetic Acid (TFA) from Acros; Chymotrypsin from Roche ; Steritest Compact Filter Device from Millipore; LAL reagent water, LAL plate and Control Standard Endotoxin from Charles River Laboratories; Proteinase K from Thermo Scientific; Sterile Fluid D from Beckton Dickinson; Testing Organisms (*Clostridium sporogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Candida albicans, and Aspergillus brasiliensis*) from Thermo Quanti-Cult Plus. CAP37-derived antimicrobial peptide BCC02 and BCC03 were synthesized by PeptiSyntha.
- *Chromatographic Conditions of stability indicating HPLC method:* Mobile Phase A: 0.1% HFBA in Nanopure Water; Mobile phase B: 0.1% HFBA in Acetonitrile. Gradient: 0-3 min (5% B), 3.01-13 min (32%-44% B), 13.01-15 min (95% B), 15.01-23 min (5% B). Column Temp: 35 ºC; Auto-sampler Temp: 4 ºC; Flow Rate: 1.5 mL/min. UV detection at 220 nm.
- *Chromatographic and MS Conditions of intact mass and peptide mapping LC-TOF MS assay:* Mobile Phase A: 0.1% TFA in 2% acetonitrile; Mobile phase B: 0.1% TFA in Acetonitrile. Gradient: 0-5 min (0% B), 10 min (20% B), 25 min (50% B), 30-35 min (95% B), 36 min (0% B). Auto-sampler Temp: 5 ºC; Flow Rate: 0.3 mL/min. Electro Spray Ionization and positive mode MS detection.

RESULTS

Table 1. Examining the specificity of stability indicating HPLC assay through forced degradation

Sample type	BCC02		BCC03	
	% Of Control	Relative Retention Time of Additional Peaks	% Of Control	Relative Retention Time of Additional Peaks
Peptide Control	N/A	N/A	N/A	N/A
Peptide + HCl	73%	0.85, 1.17, 1.21, 1.24	37%	1.04, 1.22, 1.23, 1.26
Peptide + NaOH	0%	0.83, 0.85, 0.86, 0.88, 0.92, 0.93, 0.95, 1.02, 1.17	0%	0.95, 0.96, 0.98, 1.22
Peptide + H2O2	62%	0.84, 0.90, 0.91, 1.16, 1.21, 1.22	49%	0.76, 0.80, 0.82, 0.85, 0.86, 0.87, 0.88, 0.89, 0.91
Peptide + Ambient Light	97%	N/A	98%	0.89
Peptide + Heat/Humidity	95%	N/A	94%	N/A

Almost complete degradation occurred in BCC02 and BCC03 samples exposed to NaOH, most likely due to hydrolysis of peptide bonds. In all stress conditions, the resolution between the analyte and any closely eluting degradant peaks were no less than 1.5. Peak Purity was assessed to verify that no additional impurities co-elute with the analyte of interest, and the API peak was spectrally pure.

Fig 1. The linearity of HPLC assay for BCC02 and BCC03. The plots of concentration vs. peak area were linear (R2 = 0.999) for both peptides between 25-125% of assay level (500 µg/mL), where the y-intercept were **(A)** 1.7% of BCC02 response and **(B)** 0.4% of BCC03 response at assay level.

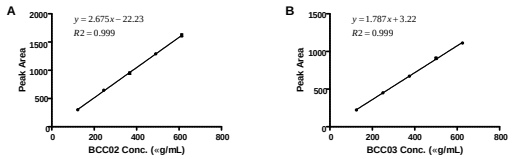


Table 2. The accuracy and precision of HPLC assay for BCC02 and BCC03

Accuracy and Precision of BCC02						
Expected Amount (µg/mL)	Calculated Conc. In Triplicate Sample Preparation			Average Conc. (µg/mL)	% of Expected	% RSD
	1	2	3			
367.50	368.86	371.67	379.91	373.48	101.63%	1.54%
490.00	502.20	506.92	500.15	503.09	102.67%	0.69%
612.50	629.67	624.06	625.73	626.49	102.28%	0.46%
Accuracy and Precision of BCC03						
Expected Amount (µg/mL)	Calculated Conc. In Triplicate Sample Preparation			Average Conc. (µg/mL)	% of Expected	% RSD
	1	2	3			
375.00	377.95	377.93	376.66	377.51	100.67%	0.20%
500.00	511.55	497.32	489.96	499.61	99.92%	2.20%
625.00	626.26	623.79	619.46	623.17	99.71%	0.55%

Table 3. Solution stability of the HPLC assay samples for BCC02 and BCC03

	T0 at 5° C	12 Hours at 5° C	72 Hours at 5° C
BCC02 Conc. (µg/mL)	507.0	508.5	511.0
% of Initial Concentration	-	100.3%	100.8%
	T0 at 5° C	24 Hours at 5° C	85 Hours at 5° C
BCC03 Conc. (µg/mL)	500.0	494.8	489.4
% of Initial Concentration	-	99.0%	97.9%

Fig 2. Intact mass measurement of BCC02. The molecular ions were observed at multiple charge states. The calculated intact molecular mass after deconvolution was 3775.9502 Da (-2.6 ppm).

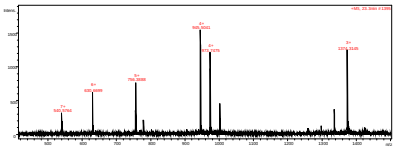


Table 4. Chymotryptic peptide mapping for BCC02

RT (min)	Measured Mass (Da)	Theoretical Mass (Da)	Accuracy (ppm)	Location	Sequence	Modifications
6.3	302.1703	302.1703	0	A(29-30)	QR	None
15.5	1046.608	1046.6057	2.2	A(1-5)	mPEG-mPEG-RRRRN	None
17.1	643.3211	643.319	3.26	A(6-10)	QGRHF	None
17.4	1672.906	1672.8982	4.66	A(1-10)	mPEG-mPEG-RRRRNQGRHF	Q-6 Deamidation
17.7	653.3503	653.3497	0.92	A(11-17)	SGGALIH	None
18.6	642.3613	642.3602	1.71	A(16-20)	IHARF	None
18.9	598.2421	598.2421	0	A(23-28)	TAASCF	None
19.4	844.3415	844.3459	-5.21	A(21-28)	VMTAASCF	Met-22 Oxidation
21.1	828.351	828.351	0	A(21-28)	VMTAASCF	None
22.2	1670.6804	1670.6813	-0.54	A(21-28) + A(21-28)	VMTAASCF + VMTAASCF	Cysteine disulfide bond (A27-A27) + Oxidation
22.4	1424.576	1424.5774	-0.98	A(23-28) + A(21-28)	TAASCF + VMTAASCF	Cysteine disulfide bond (A27-A27)
23.3	1654.6839	1654.6863	-1.45	A(21-28) + A(21-28)	VMTAASCF + VMTAASCF	Cysteine disulfide bond (A27-A27)

Table 5. Chymotryptic peptide mapping for BCC03

RT (min)	Measured Mass (Da)	Theoretical Mass (Da)	Accuracy (ppm)	Location	Sequence	Modifications
16.6	1046.611	1046.6057	5.06	A(1-5)	mPEG-mPEG-RRRRN	None
18.2	643.3222	643.319	4.97	A(6-10)	QGRHF	None
18.5	1672.883	1672.8982	-9.09	A(1-10)	mPEG-mPEG-RRRRNQGRHF	Q-6 Deamidation
18.8	642.3613	642.3602	1.71	A(16-20)	IHARF	None
19	582.2676	582.2649	4.64	A(23-28)	TAASSF	None
19.2	392.2175	392.2172	0.76	A(18-20)	ARF	None
19.5	828.3716	828.3688	3.38	A(21-28)	VMTAASSF	Met-22 Oxidation
20.9	812.3782	812.3738	5.42	A(21-28)	VMTAASSF	None
22.2	669.328	669.3268	1.79	A(11-17)	CGGALIH	None

The identification of peptides is further confirmed by the peptide mapping data collected from chymotryptic digestion. Specifically, the sequence of BCC02 has been completely covered by the digested peptides as shown in Table 4. And 93% of BCC03 sequence has been covered by the digested peptides as shown in Table 5. In addition, the oxidation products were detected in both intact mass measurement and peptide mapping.

Table 6. Endotoxin levels and spike recoveries in BCC02 samples pre-treated with and without a proteinase k digestion step

Sample	Proteinase K Digestion	Endotoxin, EU/mL						Spike Recovery		
		- Spike			+ Spike					
		EU/mL	AVG	SD	EU/mL	AVG	SD	%	AVG	SD
BCC02	-	<0.100	<0.100		0.07	0.227	0.136	6%	22%	14%
		<0.100			0.309			30%		
		<0.100			0.3			29%		
	(+)	<0.100	0.307	0.213	0.829	1.01	0.156	82%	98%	14%
		0.295			1.104			107%		
		0.526			1.095			104%		
Sample Buffer	(+)	0.288	0.338	NA	1.08	1.273	NA	105%	124%	NA
		0.388			1.467			143%		

The average spike recovery of 21.7% was below the acceptable range of 50% - 200% for samples without digestion. It was most likely due to the peptide binding with endotoxin and rendering them unavailable for the assay.

Table 7. Organisms, media and conditions used during sterility validation of the closed membrane filter system for BCC03 samples

Organism	Classification	Culture Media	Temperature (˚C)	Oxygen
C. sporogenes	Bacterium	Fluid Thioglycollate medium (FTG)	30 - 35˚C	Anaerobic/ Aerobic
S. aureus	Bacterium			
P. aeruginosa	Bacterium			
B. subtilis	Bacterium	Tryptic Soy Broth (TSB)	20 - 25˚C	Aerobic
C. albicans	Yeast			
A. brasiliensis	Mold			
Negative Control	A filter unit through which D5W and un-inoculated Fluid D had been passed, and medium added, was used for a negative control sample and was incubated along with the test units			
Positive Control	Filter units through which blank D5W and inoculated Fluid D had been passed, and medium added, were used for positive control samples			

No turbidity was observed in negative control after 14 days. In contrast, turbidity and the characteristic growth of the inoculated organisms was observed in all of the containers in which samples were filtered prior to inoculation.

CONCLUSIONS

- Complementary analytical methods have been developed and validated for therapeutic peptides BCC02 and BCC03, including stability indicating HPLC assay and identification with high resolution mass spectrometry. The microbiology quality of antimicrobial peptide solution were also evaluated. A proteinase K digestion step was critical to endotoxin measurement.

REFERENCES

1. Shafer et al., *Infect Immun* 1984; **2.** Spitznagel et al., *J Clin Invest* 1990; **3.** Gordon et al., *Curr Eye Res*. 2009; **4.** Griffith et al., *Invest Ophthalmol Vis Sci.* 2013; **5.** Aleksander et al., *BioPharm Int.* 2014