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Application News

SSI-LCMS-089

Liquid Chromatography Mass Spectrometry

LCMS-8060 Determination of Three Polypeptide Antibiotics in Pork

Liquid Chromatograph Mass Spectrometer

LCMS-8060

Summary: Residues of three types of polypeptide antibiotics in pork were monitored using Shimadzu's Nexera ultra high performance liquid chromatograph coupled with the LCMS-8060 triple quadrupole mass spectrometer. Using optimized UHPLC-MS/MS analysis and matrix matched calibration, linearity was obtained from 0.5 to 100 µg/L. Correlation coefficients were greater than 0.999. Limits of quantitation ranged from 0.40-0.70 µg/kg, and limits of detection were 0.14-0.24 µg/kg. Percent recoveries were between 72.9-82.0%. Intraday/interday precision did not exceed 12.7% and 13.9% respectively. This method provided a highly sensitive and accurate analytical solution for polypeptide antibiotics.

Background: Polypeptide antibiotics are often used for bacterial infections. They are widely used in animal feed for domestic fowl, pigs, cattle, etc. As the use of polypeptide antibiotics as additives in feed and for veterinary drugs increases, monitoring proper intake and prescription length has become a challenge. Many non-compliant incidents have occurred. Human health can be at risk with the consistent intake of antibiotics through meat. Due to this concern, vancomycin has been banned by certain regulatory bodies for use as a veterinary drug. In addition to the use of vancomycin, its demethylation form is widely used. Therefore, It is crucial to consider the residual effect of both vancomycin and norvancomycin. Bacitracin, composed of similar small peptides, is effective on treating gram-positive bacteria. Bacitracin A is the most active form. Long-term intake of low-dosage drug residues of the product may lead to the slow accumulation in the body and risk human health. In China, bacitracin residue in animal feed is limited to 500 µg/kg (MRLs). Attention to establishing limits of polypeptide antibiotic residues in animal feed has increased.

Method: This method provides simultaneous analysis of vancomycin, norvancomycin, and bacitracin. A positive pressure SPE pretreatment technique without nitrogen drying step was employed. The elimination of nitrogen drying reduced sample loss and sample preparation time. In addition, it increased bacitracin recovery. Quantitation limit was 0.40-0.70 µg/kg. A highly selective

and sensitive method was created using the LCMS-8060. Thanks to the high recovery from the sample preparation step, a highly sensitive and accurate method resulted.

Molecular formula and structures of the compounds of interest can be found in Table 1. Shimadzu Nexera UHPLC and LCMS-8060 triple quadrupole mass spectrometry were used for this analysis. (Parts including: LC-30ADx2, DGU-20A5R, SIL-30ACMP, CTO-20AC, and CBM-20A.) LabSolutions (V. 5.82) was used for data analysis.

An ACQUITY UPLC HSS T3 C18 (2.1 mm x 100 mm, 1.8 µm) column was used. Mobile phase A was water with 0.01% formic acid. Mobile phase B was acetonitrile with 0.01% formic acid. Flow rate was set at 0.4 mL/min and the column oven was set at 40 ºC. Injection volume was 5 µL. A gradient chromatography method was used where the initial condition was 5%B. (Table 2)

Table 1. Compounds of interest information

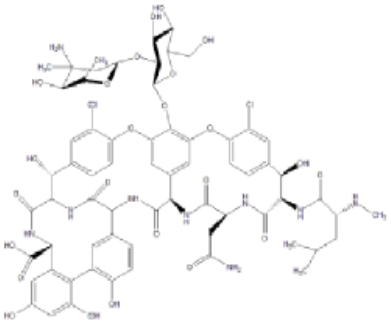
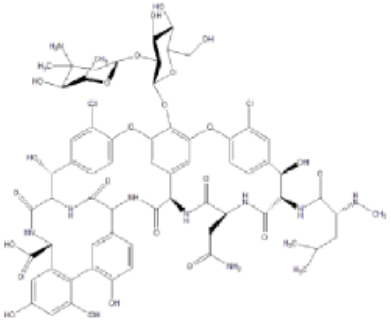
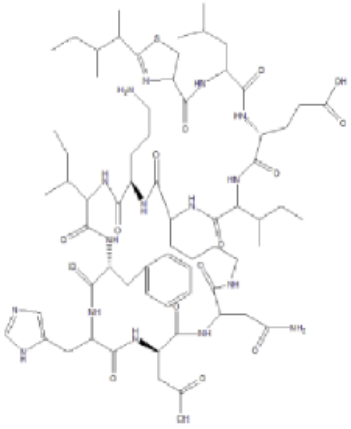
Compound Name	CAS Number	Formula	Structure
Norvancomycin	91700-98-0	$C_{65}H_{72}Cl_2N_9O_{24}$	
Vancomycin	1404-90-6	$C_{66}H_{75}Cl_2N_9O_{24}$	
Bacitracin A	1405-87-4	$C_{66}H_{102}N_{17}O_{16}S$	

Table 2. Gradient Conditions

Time (min)	Module	Command	Value
3.00	Pumps	Pump B Conc.	15
6.00	Pumps	Pump B Conc.	90
7.00	Pumps	Pump B Conc.	90
7.10	Pumps	Pump B Conc.	5
8.50	Controller	Stop	

ESI positive mode was used for this analysis. Nebulizing gas was 3 L/min, heating gas was 15 L/min, and drying gas was 5 L/min. Interface temperature was set at 250 ºC, DL temperature at 250 ºC, and heating block at 300 ºC. ESI capillary was at position +1.0 mm. Multiple reaction monitoring (MRM) mode was used for this analysis. MRM transition information can be found in Table 3.

Table 3. MRM Transitions

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Q1 Bias (V)	Collision Energy CE(V)	Q3 Bias (V)
Norvancomycin	718.80	144.05*	-26.0	-15.0	-25.0
		100.00	-26.0	-33.0	-18.0
Vancomycin	725.75	100.00*	-26.0	-41.0	-18.0
		144.05	-26.0	-16.0	-26.0
Bacitracin A	712.05	199.00*	-26.0	-45.0	-21.0
		85.90	-26.0	-43.0	-15.0

* Quantifying Ion

Norvancomycin, vancomycin, and bacitracin A were dissolved in water to make 1 mg/mL stock solutions. Stock solutions were stored at 4 ºC. Blank sample matrix was used to make 0.5, 1.0, 5.0, 10, 50, and 100 ng/mL working solutions. 5.0 g of mixed sample were weighted into a centrifuge tube (50 mL). 5 mL of 7:3 (v/v) 0.1% Formic acid in water and ACN were added. Sample was vortexed for 3 minutes, sonicated for 10 min, and centrifuged at 5000 r/min for 5 minutes. Extraction process was repeated again and the supernatants were combined. 5 mL of extract was taken and mixed with 5 mL of hexane. The sample was vortexed for 2 minutes, and centrifuged at 12000 r/min for 3 minutes. Hexane layer was discarded and the process was repeated once again to eliminate lipids. 2mL of the extract was then add to 4 mL of 0.1% formic acid in water before going on the Pharma FF cartridge. 2 mL of 9:1 (v/v) 0.1% formic acid in water and ACN were used to for washing, then 4 mL of 4:6 (v/v) 0.1 formic acid in water and ACN used for elution. Sample was filtered (0.22 µm) prior to analysis.

Results and Discussion: Mass spectrum of norvancomycin, vancomycin, and bacitracin A along with product ion scans are shown in figure 1-6. Blank matrix and spiked standard MRM chromatograms are shown in figure 7-8.

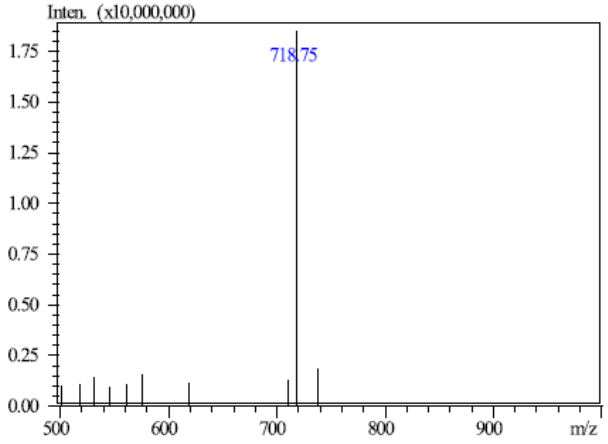


Figure 1. Mass spectrum of norvancomycin

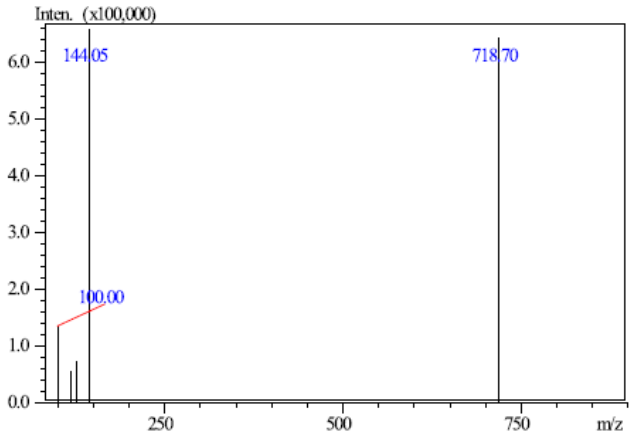


Figure 2. Product ion scan of norvancomycin (CE= -15V)

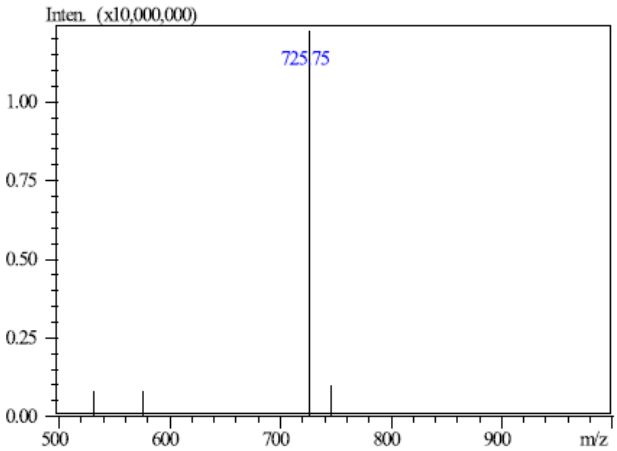


Figure 3. Mass spectrum of vancomycin

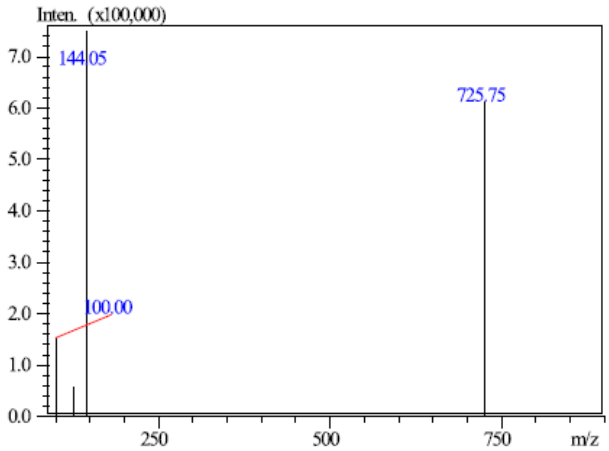


Figure 4. Product ion scan of vancomycin (CE= -15V)

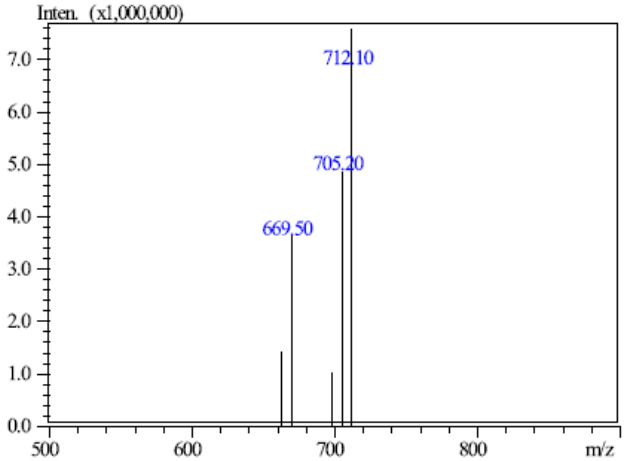


Figure 5. Mass spectrum of bacitracin A

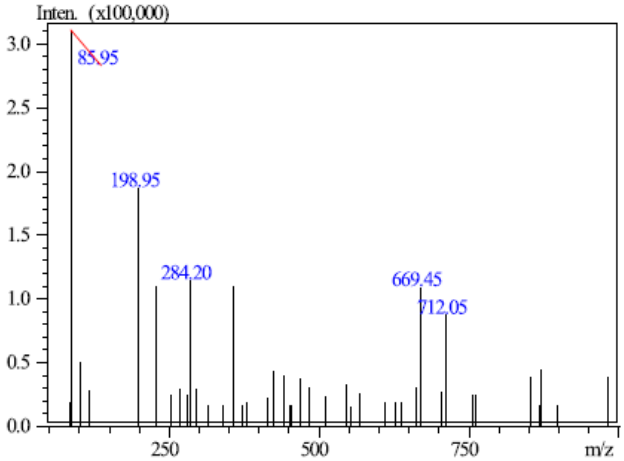


Figure 6. Product ion scan of bacitracin A (CE= -30V)

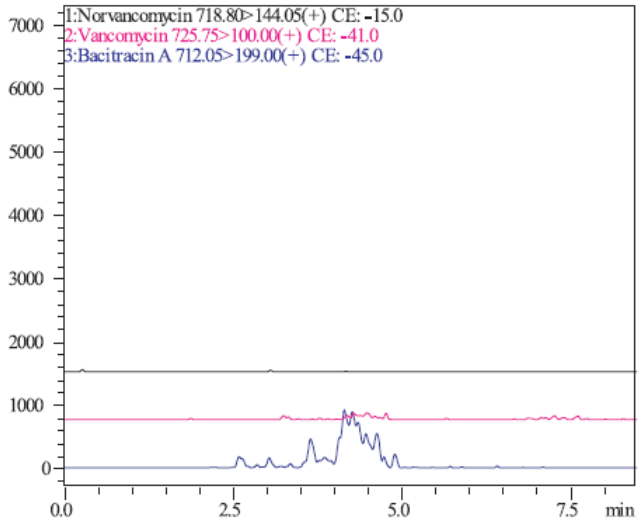


Figure 7. Blank pork matrix MRM chromatogram

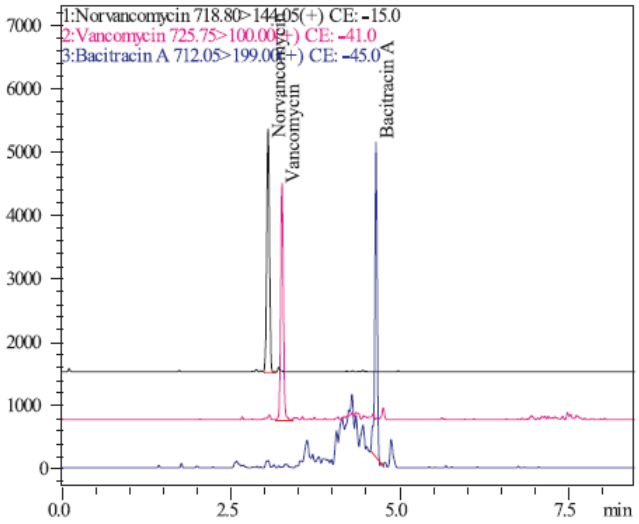


Figure 8. Spiked matrix MRM chromatogram (5ng/mL)

Since the extraction solvent for the SPE was 4:6 (v/v) 0.1 formic acid in water and ACN, chromatography of vancomycin and norvancomycin were compromised. (Figure 9b) In order to improve chromatography to achieve a higher quantitation accuracy, a co-injection technique was included. (Figure 9a) A 1:1 dilution with 0.1% formic acid in water was automatically performed to obtain sharper chromatography peaks for vancomycin and norvancomycin. (Figure 9c)

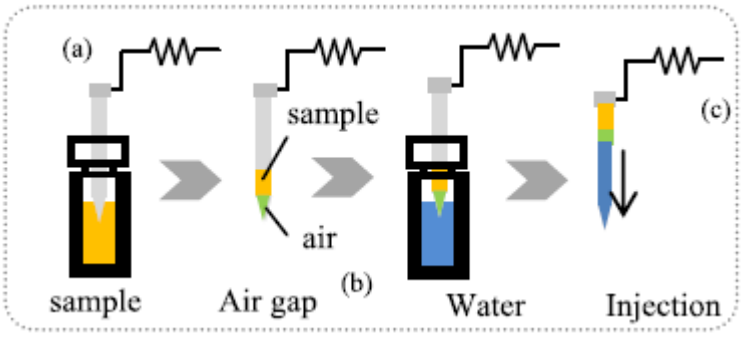


Figure 9a. Co-injection method

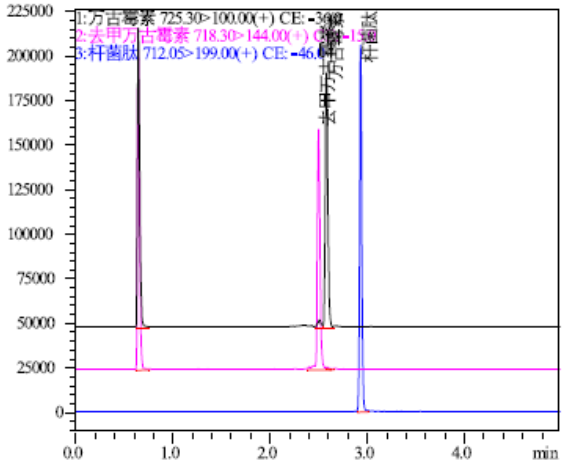


Figure 9b. Direct sample injection

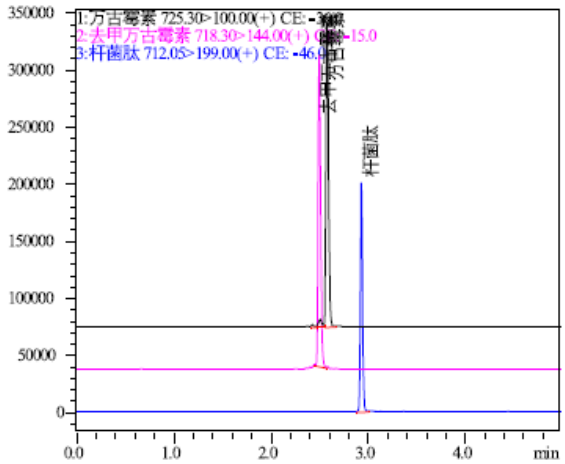


Figure 9c. Sample introduction with co-injection

Matrix effect was evaluated by checking the ratio between matrix matched and neat standard calibration curves. Calibration levels were in the range from 0.5 to 100 µg/L. Norvancomycin and vancomycin showed matrix evaluation ratio close to 1. This indicated that low matrix effect was observed. Bacitracin A had the ratio of 0.343, which means a strong matrix effect was observed.

To compensate for the matrix effect, matrix matched calibration was utilized in this study. Calibration curve can be found in Figure 10. LOQ (S/N=10) for norvancomycin, vancomycin, and bacitracin A were 0.40, 0.66, and 0.70 µg/kg. LOD (S/N=3) were 0.14, 0.22, and 0.24 µg/kg, respectively. Calibration information can be found in Table 4.

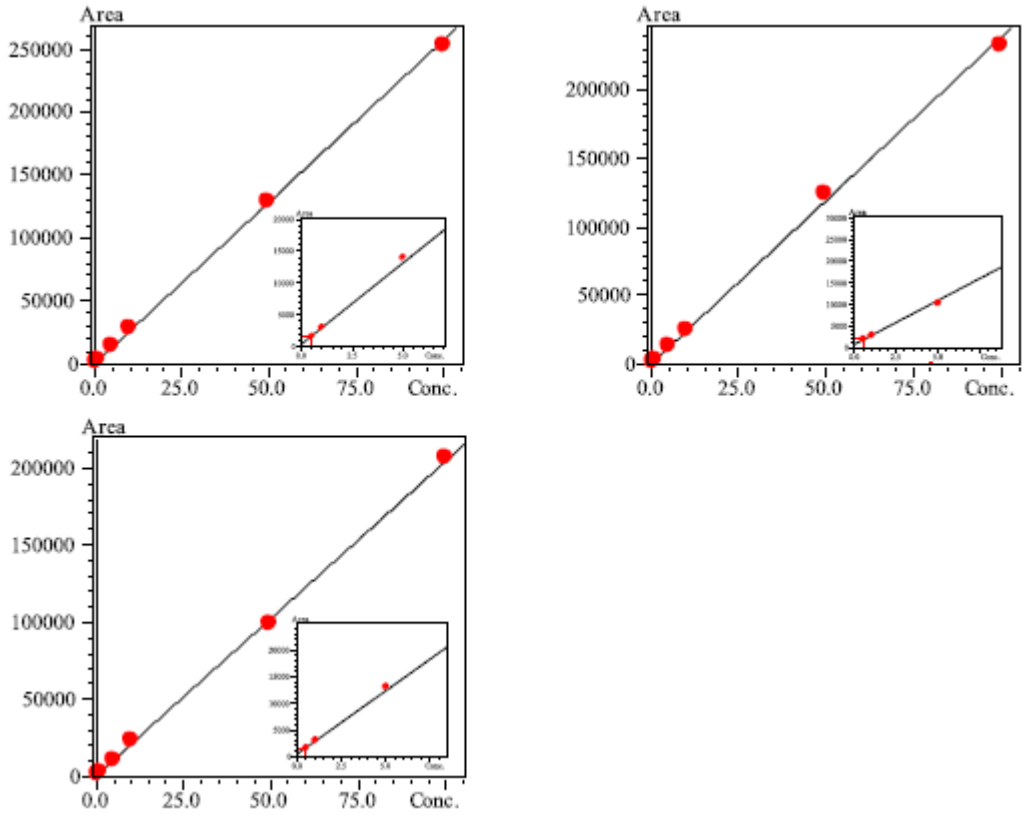


Figure 10. Calibration curves for norvancomycin, vancomycin, and bacitracin A

Table 4. Calibration curves information for norvancomycin, vancomycin, and bacitracin A

Compound Name	Linear range (µg/L)	Calibration equation	R^2	Accuracy range (%)
Norvancomycin	0.5~100	Y = (2564.90)X + (383.031)	0.9995	86.1~109.5
Vancomycin	0.5~100	Y = (2376.55)X + (534.851)	0.9995	85.4~106.2
Bacitracin A	0.5~100	Y = (2033.81)X + (804.551)	0.9995	92.6~106.8

Using pork meat as the blank matrix, sample preparation was done using the procedure described above. Three different concentrations: 1 µg/kg, 5 µg/kg, and 25 µg/kg (2X, 10X, and 50X LOQ) were used for recovery and accuracy analysis. (N=6) Results of the recovery and accuracy study can be found in Table 5. Market sourced pork subjected to this analysis did not exhibit residues of the three polypeptide antibiotics.

Table 5. Calibration curve information for norvancomycin, vancomycin, and bacitracin A

Compounds	Conc. (µg/kg)	Recovery (%)	Intraday Acc. (%)	Interday Acc. (%)
Norvancomycin	2	76.3	7.11	12.4
	10	80.3	7.59	9.33
	50	82.0	3.76	10.1
Vancomycin	2	77.0	12.7	12.5
	10	75.6	8.53	12.0
	50	80.5	2.57	10.0
Bacitracin A	2	72.9	10.9	13.9
	10	77.0	7.74	8.50
	50	79.6	7.30	7.85

Conclusion: A method using positive pressure SPE combined with UHPLC-MS/MS was created to analyze vancomycin, norvancomycin, and bacitracin A in pork. Linearity was obtained at 0.5-100 µg/L where R^2>0.999. Limit of quantitation were 0.40-0.70 µg/kg and limit of detection were 0.14-0.24 µg/kg. Percent recovery were between 72.9-82.0%. Intraday/interday precision did not exceed 12.7% and 13.9%, respectively. An accurate and highly sensitive method using the Shimadzu LCMS-8060 for the analysis of polypeptide antibiotics in pork was demonstrated.

Please note that this document was translated and summarized into English from Chinese (LCMSMS-238; SSL-CA14-285).

LCMS-8030 LCMS-8040 LCMS-8050 LCMS-8060 LCMS-2020 LCMS-IT-TOF

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