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Determination of Penicillins Residues in Livestock and Marine Products by LC/MS/MS

Ji Young Song, Soo Jung Hu, Hyunjin Joo, Joung Boon Hwang, Mi Ok Kim, Shin Jung Kang, Dae Hyun Cho.

***Abstract*—**Multi-residue analysis method for penicillins was developed and validated in bovine muscle, chicken, milk, and flatfish. Detection was based on liquid chromatography tandem mass spectrometry (LC/MS/MS). The developed method was validated for specificity, precision, recovery, and linearity. The analytes were extracted with 80% acetonitrile and clean-up by a single reversed-phase solid-phase extraction step. Six penicillins presented recoveries higher than 76% with the exception of Amoxicillin (59.7%). Relative standard deviations (RSDs) were not more than 10%. LOQs values ranged from 0.1 and to 4.5 ug/kg. The method was applied to 128 real samples. Benzylpenicillin was detected in 15 samples and Cloxacillin was detected in 7 samples. Oxacillin was detected in 2 samples. But the detected levels were under the MRL levels for penicillins in samples.

***Keywords*—**Penicillins, livestock product, Multi-residue analysis, LC/MS/MS

I. Introduction

PENICILLINS are widely used in veterinary medicine for preventing and treating bacterial infections and feed additives because of their economic advantages [1-3]. Improper use of penicillins may lead to residue problem in livestock and marine products and cause a serious health hazard [4-6]. Therefore, maximum residue limits (MRLs) have been established to provide safe products for consumers through quantification of these residues in livestock and marine products. Generally, the most of current methods for the measurement of penicillins residue are complicated, time consuming and cannot be used to analyze multi-residue [7-12]. This study has been undertaken to determinate multi-residues of penicillins, including amoxicillin, ampicillin, oxacillin, bezylpenicillin (Penicillin G), cloxacillin, dicloxacillin, and nafcillin (Figure 1), using liquid chromatographic tandem mass spectrometry (LC/MS/MS).

II. Materials and Methods

A. Chemicals and Materials

Amoxicillin (trihydrate), Ampicillin (trihydrate), Cloxacillin (sodium salt), Dicloxacillin (sodium monohydrate salt) and Oxacillin (sodium monohydrate salt) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Benzylpenicilln (Penicillin G, potassium salt) was purchased from Wako Pure Chemical Industries Inc. (Osaka, Japan) Nafcillin (sodium monohydrate salt) was purchased from Dr. Ehrenstofer GmbH (Augsburg, Germany). Disodium hydrogen phosphate (Na2HPO4), potassium dihydrogen phosphate (KH2PO4) and formic acid were obtained from Wako Pure Chemical Industries Inc. (Osaka, Japan). Tricloroacietic acid was obtained from Merck(Darmstasdt, Germany). And, all reagents were LC grade. The solid phase extraction (SPE) catridge used in this study was Oasis HLB (500mg, 6ml) obtained from Waters.

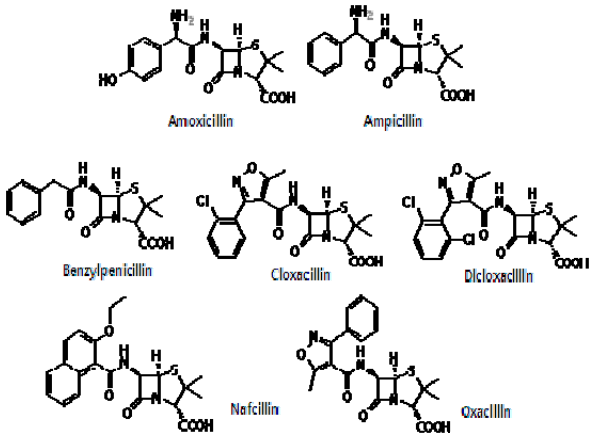

Fig. 1 Penicillin antibiotics

B. Instrument

Chromatographic analysis was performed using a Agilent 1200 LC system (Santa Clara, CA, USA) equipped with an auto sampler. Mass spectrectrometry was performed on API 4000 (Appied Biosystem, USA) triple quardrupole mass spectrometer in positive electrospray ionisation (ESI+) mode.

C. Sample preparation

The validation was performed with some foods such as bovine muscle, chicken muscles, milk and flatfish as major matrices. The samples were homogenized and stored at -20℃ until analysis. The steps of sample preparation are presented in Figure 2.

D. Optimization of the chromatographic Condition and the MS conditions

Mobile phase A was distilled water containing 0.1% formic acid and10mM ammonium formate. Mobile phase B was methanol. The flow rate was set at 0.2ml/min and the injection

Authors are with Hazardous Substances Analysis Division, Gyeoin Regional Food and Drug Administration, 217 Juanyeok-Gil, Nam-gu, Incheon, 402-835, South Korea (phone: 82-32-450-3254; fax: 82-32-442-4622, e-mail: songpeony@korea.kr, sjhu01@korea.kr, concerto@korea.kr, toda77@korea.kr, miok95@korea.kr, sapium@korea.kr, dhcho5@korea.kr).

volumn was 10ul. The total run time for each injection was 15min. The gradient conditions were as follows, base on time(t) set at the pump : t=0.0-2.0 min, hold 20% B ; t=2.0-2.1 min, ramp linearly to 100% B ; t=2.1-12.0 min, hold 100% B ; t=12.0-12.5 min, drop to 20% B ; t=12.5-15.0 min, hold 20% B to re-equilibrate of the column. The mass spectrometer was operated in the positive ion mode. The capillary temperature was set to 350℃ and the ion spray voltage to 5.5kV. The mass spectrometric parameters are summarized in Table I.

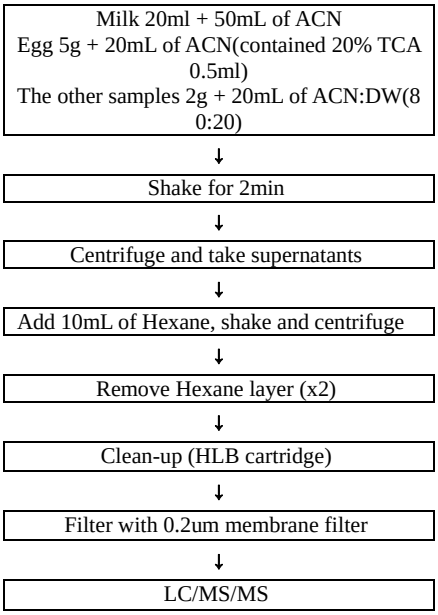

Fig. 2 Flow chart for extraction of analytes

TABLE I
THE MRM TRANSITIONS AND PARAMETERS

Analyte	Parent ion	Cone Voltage	T 1	C.E. 1	T 2	C.E. 2
Ampicillin	350	56	106	25	160	17
Amoxicillin	366	46	114	25	208	19
Penicillin G	335	66	160	15	176	17
Cloxacillin	436	66	277	19	160	19
Dicloxacillin	470	76	160	19	311	21
Nafcillin	415	46	199	19	256	19
Oxacillin	402	66	160	17	243	17

Abbreviations: T, transition; C.E., collision energy.

III. Results and Discussion

The developed method was validated for specificity, precision, recovery and linearity in bovine muscle, chicken muscle, milk, and flatfish.

E. Linearity and Calibration Curve

The linearity of the method was evaluated at five different concentration levels. The range of the curve was from 0.075 to 0.125 ug/ml. The calibration curves for all analytes are linear in given range with a correlation of 0.999. (Table II).

TABLE II
CALIBRATION CURVE OF EACH ANALYTE TOGETHER WITH THEIR CORRELATION COEFFICIENT

Analyte	Calibration curve	*r*
Ampicillin	Y = 5220x + 3010	0.9999
Amoxicillin	Y = 26000x - 47000	0.9995
Penicillin G	Y = 79900x - 90000	0.9999
Cloxacillin	Y = 51100x -11700	0.9999
Dicloxacillin	Y = 43200x + 33100	1.0000
Nafcillin	Y = 198000x + 261000	0.9995
Oxacillin	Y = 69900x + 25900	1.0000

F. Limit of Quantification (LOQ)

To estimate LOQ, samples were spiked with 0.01ug/ml. Limit of quantification (LOQ) was calculated at a signal to noise ratio of 10. LOQs values ranged from 0.1 and to 4.5 ug/kg. The results for LOQs are shown in Table III.

TABLE III
LOQ OF PENICILLINS IN BOVINE MUSCLE, CHICKEN, FLATFISH, AND MILK

Analyte	Bovine muscle	Chicken	Flatfish	Milk
Amoxicillin	3.13	4.55	2.60	1.65
Ampicillin	0.21	0.41	1.57	0.18
Benzylpenicillin	0.09	0.09	0.32	0.63
Cloxacillin	0.14	0.27	0.46	0.27
Dicloxacillin	0.34	0.17	0.18	0.16
Nafcillin	0.15	0.21	0.24	0.23
Oxacillin	0.29	0.45	0.44	0.54

G. Recovery Study

Samples were spiked with the MRL concentration of amoxicillin, ampicillin, oxacillin, bezylpenicillin, cloxacillin, dicloxacillin, and nafcillin. Six penicillins presented recoveries higher than 76% with the exception of Amoxicillin and Ampicillin (Table IV). Relative standard deviations (RSDs) were not more than 10%.

TABLE IV
RECOVERY OF PENICILLINS IN BOVINE MUSCLE, CHICKEN, MILK, AND FLATFISH

Analyte	Sample	Fortified Conc. (mg/kg)	Recovery (%)	CV (%)
Amoxicillin	Bovine muscle	0.05	59.66	9.2
	Chicken	0.05	59.93	1.5
	Flatfish	0.05	61.25	2.6
	Milk	0.004	77.96	10.4
Ampicillin	Bovine muscle	0.05	112.48	3.1
	Chicken	0.05	61.82	1.3
	Flatfish	0.05	90.87	5.4
	Milk	0.004	88.00	7.3
Benzylpenicillin (Penicillin G)	Bovine muscle	0.05	92.03	6.4
	Chicken	0.05	102.61	2.7
	Flatfish	0.05	78.39	6.1
	Milk	0.004	106.33	5.2
Cloxacillin	Bovine muscle	0.3	86.91	6.4
	Chicken	0.3	84.82	2.6
	Flatfish	0.3	85.50	4.0
	Milk	0.03	90.56	6.0
Dicloxacillin	Bovine muscle	0.3	79.62	5.5
	Chicken	0.3	76.26	3.1
	Flatfish	0.3	81.28	1.7
	Milk	0.03	81.67	6.8
Nafcillin	Bovine muscle	0.3	81.19	5.9
	Chicken	0.3	76.02	3.4
	Flatfish	0.3	103.20	2.4
	Milk	0.03	89.44	4.2
Oxacillin	Bovine muscle	0.3	89.37	7.1
	Chicken	0.3	87.08	3.5
	Flatfish	0.3	89.59	3.9
	Milk	0.03	89.72	6.0

IV. CONCLUSION

A method for multi-residue analysis of penicillins has been developed and validated in bovine muscle, chicken, flatfish, and milk by liquid chromatography tandem mass spectrometry (LC/MS/MS). The method was validated for penicillins in the samples, including linearity, precision, specificity, LOQ, and recovery.

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