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DEVELOPMENT AND VALIDATION OF A HPLC WITH SOLID-PHASE CLEAN-UP AND PRE-COLUMN DERIVATIZATION METHOD FOR THE SIMULTANEOUS DETERMINATION OF THREE β- LACTAM ANTIBIOTICS RESIDUES IN EGGS

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ABSTRACT

An accurate and sensitive HPLC method with UV detector was developed for the simultaneous determination of three β- lactam antibiotics residues in eggs. The residues in the eggs samples were extracted with acetonitrile, cleaned up with C_{18} solid phase cartridge and derivated with 1, 2, 4 – triazole. The analytes were separated on an ODS-BP C_{18} column. The mobile phase was a mixture of acetonitrile – 0.1mol/L phosphate buffer pH 6.5 (25:75; V/V) containing 0.015 mol/L tetrabutyl ammonium bromide and sodium thiosulfate. The flow rate was 1.0 mL/min and the wavelength was set at 325nm. The method was validated according to the Directive 2002/657/EC and subsequently applied to twenty commercial eggs samples. The method showed good linear relationship over the concentration range of 2.5 to 100.0μg/kg for amoxicillin, ampicillin and 30.0 to 300.0 μg/kg for oxacillin with the coefficient of variation was over 0.99. The average recoveries of amoxicillin and ampicillin residues in eggs spiked from 2.5 μg/kg to 25.0 μg/kg ranged from 69.3% to 81.8 % with the relative standard deviations of less than 9.2 %, while the average recoveries of oxacillin residues in eggs spiked from 30.0 μg/kg to 300.0 μg/kg ranged from 72.4% to 82.7 % with the relative standard deviations of less than 9.6%. The limit of detection and quantification were 2.5 μg/kg, 2.5μg/kg and 30.0μg/kg for amoxicillin, ampicillin and oxacillin, respectively. Only one egg sample was found to contain amoxicillin

at concentration of 7.2±3.6 µg/kg. The proposed HPLC method could be applied to the determination of amoxicillin, ampicillin and oxacillin residues in eggs.

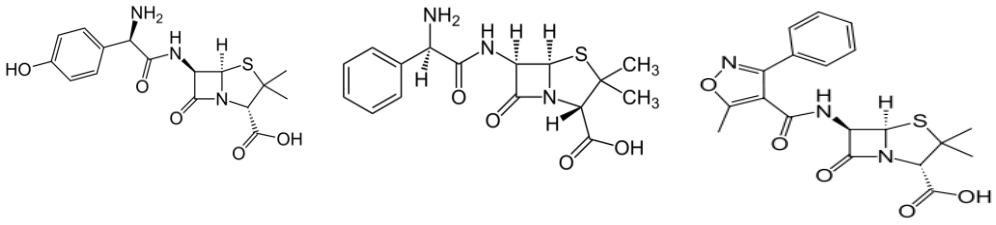
KEYWORDS: HPLC, UV, Amoxicillin, Ampicillin, Oxacillin, Residues, Eggs.

INTRODUCTION

β-Lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contains a β-lactam ring in their molecular structures. This includes amoxicillin, ampicillin, oxacillin and so on (Fig. 1.). Nearly all of these antibiotics work by inhibiting bacterial cell wall biosynthesis [1]. β- Lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by Gram-positive and Gram-negative bacteria in animal husbandry. Such wide application may lead to problems with residues in the animal derived food products. The overmuch residue could bring about adverse drug reactions to people which include diarrhea, nausea, rashes, fever, vomiting, erythema, sensitivity to lights and sounds. Serious adverse reactions include immunologically mediated adverse reactions and anaphylaxis. The onset of allergic reaction to β-Lactam antibiotics can be very sudden and intense emergency [2]. Many countries have taken strict measures to control the residues of β-Lactam antibiotics in foodstuffs of animal origin in order to protect the health of consumer. The maximum residue limits (MRLs) for β- Lactam in bovine milk and other animal derived food products have been regulated in several countries and organizations. For example the MRLs were laid down in EEC regulation 2377/90, and subsequent modifications. The European Union has laid down MRLs of 4.0 µg/kg for ampicillin and amoxicillin and 30.0 µg/kg for oxacillin in raw milk. The MRLs of β- Lactam antibiotics in animal derived food products have also been set by Ministry of Agriculture of the People's Republic of China [3-5], but no β- Lactam antibiotics MRLs have been established yet for eggs by now. It is necessary to develop a method to determine the residues of β- Lactam antibiotics in eggs because the residues could bring about unpredictable adverse drug reactions.

Many methods which include microbiological methods, HPLC-MS, HPLC and HPCE have been reported to determine β- Lactam antibiotics in samples at present [6-17]. To the best of our knowledge, only a few methods has been developed in the literature for the simultaneous determination of β- lactam antibiotics residues in eggs [18-20], and the research was only focused on the amoxicillin and ampicillin residues. Taking the above mentioned considerations into account, the aim of this study was to develop and validate an accurate and sensitive HPLC method that used for the determination of the residues of amoxicillin,

ampicillin and oxacillin in eggs. The analytical method was validated considering specificity, linearity, limits of detection (LOD) and quantification (LOQ), accuracy and precision.



(a) **(b)** **(c)**

Figure 1: Typical chemical structures of amoxicillin (a), ampicillin (b) and oxacillin (c).

MATERIALS AND METHODS

Reagents and Chemicals: Amoxicillin, ampicillin and oxacillin were all of sodium salt and received as gifts from Zhengzhou Zhongzhou Pharmaceutical Co., Ltd (Zhengzhou, China). HPLC grade acetonitrile and all other chemicals were purchased from Xinshiji Chemicals Co., Ltd (Xinxiang, China). AccuBond ODS C_{18} solid phase extraction (SPE) cartridges had a 3 ml capacity packed with a 500 mg solid phase were purchased from Agilent Technologies China Ltd (Shanghai, China). The distilled water in the study was purified with Smart2 Pure 12 UV/UF purification system (Thermo Fisher Scientific, USA).

Preparation of Solutions

Standard Solutions: The individual standard solutions of the pure chemicals were prepared by dissolving accurately 10 mg amoxicillin, ampicillin and oxacillin in a 10 mL volumetric flask using water. Intermediate and working solutions were prepared by appropriate dilution of stock solutions with water prior to use. The solutions were stored at or below 4℃ in refrigerator for further use.

Solutions for Derivatization Reaction

1.378g 1, 2, 4 – triazole was accurately weighted and dissolved with 6 mL water in a 10 mL volumetric flask. 1 mL 0.01 mol/L mercuric chloride solution was added into the volumetric flask. The pH was adjusted to 9.0 with 2.0 mol/L sodium hydroxide solution. The solution was made up to the mark with water and stored at or below 4℃ for derivatization reaction.

Sample Preparation

Sample Extraction: The eggs samples were purchased from supermarket. The whole egg was homogenized thoroughly with a high-speed food blender. 5g eggs sample was transferred

into a 50 mL glass centrifuge tube, spiked with the analytes followed by vortex- mixing at least 1min. 15 mL acetonitrile was added into the tube. The mixture was homogenized thoroughly for 3 min and then centrifuged at 4000 rpm for 5 min at 4℃. The upper layer was carefully transferred into another centrifugation tube. The same extraction procedure was repeated with 10mL acetonitrile, and the supernatants were combined into the same centrifuge tube. 5 mL N-hexane was piped into the combined extracted solution. After vortex-mixing for 5 min and centrifuging at 4000 rpm for 5 min at 4℃, the upper N-hexane was discarded, and the extracted solutions were evaporated to dryness under vacuum in a water bath at 40℃. The residues were dissolved with 5 mL 0.1 mol/L phosphate buffer solution for SPE clean-up procedure.

Sample SPE clean-up

The residues solution was loaded onto the C_{18} SPE columns which were preconditioned with 3 mL acetonitrile and 3 mL 0.1 mol/L phosphate buffer solutions. The column was washed with 3mL water and then dried under vacuum for 1 min. The analytes were eluted with 5mL acetonitrile and evaporated to dryness under a stream of nitrogen at 40℃. The residues were dissolved with 0.5 mL 0.1 mol/L phosphate buffer solution for further derivatization reaction.

Sample Derivatization

30 µL acetic anhydride solution prepared with acetonitrile was transferred to the residues solution. The mixture was homogenized thoroughly for at least 2 min. The reaction was maintained for 10 min at 40℃. 0.5 mL 1, 2, 4 – triazole- mercuric chloride solution was added into the tube. After keeping in a water bath at 70 ℃ for 10 min, the tube was rapid cooled to room temperature with ice water. The mixture was filtered with 0.45µm microporous membrane for HPLC analysis.

HPLC Conditions

Chromatographic analyses were performed using a Shimadzu system that comprised of a LC-20AT pump, SPD 20A UV-Visible absorbance detector connected to Shimadzu Spin Chrome software. The UV detector was set at a wavelength of 325 nm. ODS-BP C_{18} column (5µm, 4.6mm×250mm) was used for separation coupled with a 2mm C_{18} guard column at 30℃. The mobile phase consisted of acetonitrile – 0.1mol/L phosphate buffer pH 6.5 (25:75; V/V) containing 0.015 mol/L tetrabutyl ammonium bromide and sodium thiosulfate. The mixture was degassed by an ultrasonic bath and filtered through a 0.45µm membrane filter under

vacuum before use. The flow rate was 1.0 mL/min. A 20µL aliquot was injected onto the HPLC column.

Method Validation

The method was validated according to the Decision 2002/657/EC under Council Directive 96/23/EC and the analytical parameters were specificity, linearity, LOD, LOQ, accuracy and precision.

Specificity

The specificity of the proposed HPLC method was performed by analyzing ten eggs samples from different sources to evaluate possible endogenous interferences in samples. The sample preparation, clean-up, derivatization reaction and chromatographic conditions were optimized to guarantee that no interferences incurred around the retention time of the tested residues.

Linearity

Linearity of the method was established as described above through a calibration curve obtained by triplicate analysis of the three chemicals at five concentration levels in the matrix. The spiked concentrations were 2.5, 5.0, 10.0, 50.0 and 100.0 µg/kg for amoxicillin and ampicillin and 30.0, 50.0, 100.0, 150.0 and 300.0 µg/kg for oxacillin. The responses were measured as peak areas.

LOD and LOQ

The LOD and LOQ were established with spiked eggs samples ranging from 2.0 µg/kg to 50.0 µg/kg at five concentration levels. The LOD was calculated by the comparison of the threefold variation of signal to noise ratio (S/N=3:1) obtained from analysis extract of blank eggs samples, while the lowest level that gave reasonable accuracy and precision was designated to be LOQ.

Accuracy and Precision

The accuracy of the method was expressed as the average recoveries of spiked analytes in eggs samples. The average recoveries were measured in eggs samples known to be noncompliant spiked at three different levels (2.5, 5.0, 25.0 µg/kg for amoxicillin and ampicillin and 30.0, 50.0, 300.0 µg/kg for oxacillin) with five replicates over a period of five days. The samples were prepared and analyzed as described above. The recoveries were calculated by comparing peak areas of measured and spiked concentrations. The precision

was defined by the relative standard deviation (RSD) and evaluated in terms of intra-day precision and inter-day precision. Intra-day precision was conducted at three fortification levels by the analysis of spiked samples in five replicates on the same day. Inter-day precision was determined for the same fortification levels of spiked samples in five replicates on five consecutive days.

RESULTS AND DISCUSSION

Chromatographic Conditions: Chromatographic conditions were studied in order to achieve the best separation and retention for the analytes, minimizing analysis time. Amoxicillin, ampicillin and oxacillin are very hydrophilic compounds. Ion-pair reversed phase HPLC is first preferred to separate the three analytes on a C_{18} column. The use of ion-pairing reagent in the mobile phase is indeed useful for HPLC analysis. Acetonitrile, methanol and water are often used as mobile phases for reversed-phase HPLC separation of various compounds. Therefore the separation effect of the three composition added with ion-pairing reagent were tested in this study. Acetonitrile- water system was applied to be as mobile phase which the percentage of acetonitrile ranged from 10.0 % to 40.0 % to guarantee its separation. Tetrabutylammonium hydrogen sulfate was added to the acetonitrile- water system as ion-pairing reagent. The separation effect was recorded with resolution, retention time and tailing factor. The satisfactory separation of the three analytes were achieved when using the mixture of acetonitrile and phosphate buffer (pH was adjusted to 6.5) (25:75; V/V) consisting of 0.015 mol/L tetrabutyl ammonium bromide and sodium thiosulfate. The pH of the selected mobile phase was another important factor affecting the chromatographic separation of the three β- Lactam antibiotics in eggs samples. It could influence the dissociation degree, the retention time and signal of the studied compounds. The pH of the mobile phase was adjusted to 6.5 in order to prevent the degradation of the residues at a lower pH. The monitoring wave length was set at 325 nm which was the maximum absorption wave length of the derivatives. No interference peak around the three analytes was observed in the chromatogram. Under these conditions, elution of analytes was completed in less than 15.0 min.

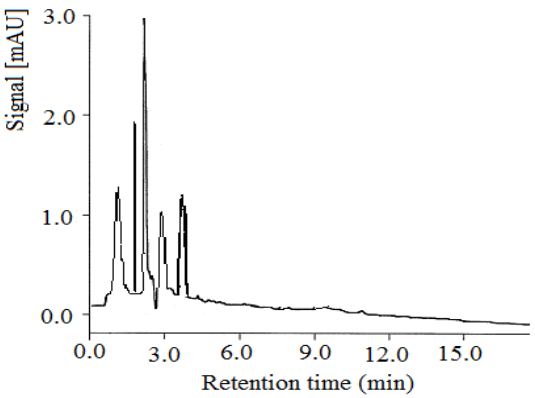
Sample Extraction and Clean-Up: Different kinds of solvents were used to extract the drugs from eggs samples spiked with individual β- Lactam antibiotics. Acetonitrile was selected as extraction solvent because less protein was found in the extracted solution and higher recoveries were obtained. Furthermore, N –hexane was found more effective to

remove adipose in extracted solution. The further purification procedure was necessary because some interference was in the extracted solution. C_{18} SPE cartridges were selected for purification according to the literature [7]. The results showed that there was not any interference at the same retention time of the three analytes. It was superior with good recovery (>90%) and little matrix interference. Acetonitrile was used as elution solution showed a higher elution power to acquire good recoveries and little interference.

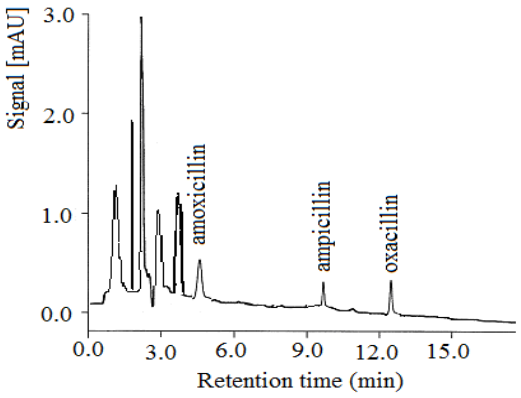
Derivatization Reaction: The maximum absorption wavelength of β- Lactam antibiotics were always in the range of 200～235 nm because they generally do not had specific strong UV absorption groups. There are always endogenous substances in the samples that was not helpful for the determination of resides. Pre-column derivatization can improve the sensitivity of the detection. Benzaldehyde, formaldehyde, o-phthalaldehyde and salicylic aldehyde were always used as derivatization reagents in HPLC connected with a fluorescence detector. 1, 2, 4- triazole solution made with 0.01mol/L mercuric chloride (pH was adjusted to 9.0±0.5 with sodium hydroxide) was used in HPLC connected with a UV detector. First the free amino group of the chemicals was quickly acylated with acetic anhydride at room temperature. After the reaction, the derivatization reaction between β- Lactam antibiotics and 1, 2, 4- triazole in mercuric chloride solution was performed at 70℃ in water bath. The derivative was proved to be stable in one day after being analyzed eight times every one hour by HPLC.

Method Validation

Specificity validation was performed by analyzing the different sources blank eggs samples. No interfering peaks could be observed at the expected retention time of the three β- Lactam antibiotics residues as shown in the chromatograms (Fig.2.). It indicated that the method was highly selective.



(a)



(b)

Figure (2): Chromatograms of blank eggs (a) and spiked eggs (b) at the concentrations 2.5 µg/kg for amoxicillin, ampicillin and 30.0 µg/kg for oxacillin.

Five different concentrations ranging from 2.5 to 100.0 µg/kg for amoxicillin, ampicillin and 30.0 to 300.0 µg/kg for oxacillin were derivatized and analyzed. Typical regression equations were calculated as follows: y = 0.4878x + 0.3869 for amoxicillin, y = 0.5302 x +0.1876 for ampicillin and y = 0.2254x + 0.4986 for oxacillin, where y is peak area based on three parallel measurements and x is the concentration (µg/kg) for the three residues spiked in the eggs samples. The correlation coefficient values were over 0.99 which indicate a good linear relationship between peak area and concentration over a wide range.

The LOD and LOQ of the method were determined according above procedure. The LOD was calculated by the comparison of the threefold variation of signal to noise ratio (S/N=3:1) obtained from analysis extract from blank eggs samples, while the lowest level that gave reasonable accuracy and precision was designated to be LOQ. The LOD and LOQ of the three analytes extracted from eggs were 2.5µg/kg for amoxicillin and ampicillin and 30.0 µg/kg for oxacillin. The results showed that the method could be helpful to determine amoxicillin, ampicillin and oxacillin in eggs.

Accuracy and precision were two essential criteria to evaluate an analytical method. Generally, the accuracy of the proposed HPLC method was performed by analyzing the spiked known concentration and comparing the determination results with the true value. Intra-day precision was conducted on the same day. Inter -day precision was determined by repeating the study for five consecutive days. The results of accuracy and precision are summarized in Table 1. The overall recoveries ranged from 69.3% to 82.7%; and the RSD values were all below 9.6%.

Table 1. Accuracy and precision of the method for amoxicillin, ampicillin and oxacillin in the eggs.

Analyte	Spiked concentration (μg/kg)	Average recovery (%)	Inter-day RSD (%)
Amoxicillin	2.5	69.3±8.3	7.8
	5.0	73.1±6.8	8.1
	25.0	80.2±7.4	7.5
Ampicillin	2.5	71.2±5.6	8.4
	5.0	78.4±6.3	9.2
	25.0	81.8±5.3	7.7
Oxacillin	30.0	72.4±8.1	9.6
	50.0	78.1±5.6	7.9
	300.0	82.7±7.3	8.5

The results of the method validation indicated that the method was selective and accurate enough for the determination of three β- Lactam antibiotics residues in eggs.

Application for the Analysis of the Commercial Eggs Samples

The optimized method was applied to the analysis of twenty commercial eggs samples from local market to validate the method. The samples were analyzed using the above described HPLC method. The residue amounts of the three β- Lactam antibiotics residues in eggs were calculated by the matrix-matched calibration curves, and only one sample was found to contain amoxicillin at concentration of 7.2±3.6 μg/kg.

CONCLUSION

An ion-pair HPLC method with UV detector for the separation and simultaneous determination of amoxicillin, ampicillin and oxacillin in eggs in a single assay was developed. The samples were extracted with acetonitrile followed by cleaned up through a C_{18} cartridge. After derivatization with 1, 2, 4- triazole, the residues were analyzed by ion-pair HPLC method with UV detector. The method has been successfully validated in terms of specificity, linearity, LOD, LOQ, accuracy, precision and subsequently applied to twenty commercial eggs samples. The obtained results indicate that the combination of acetonitrile extraction, C_{18} cartridge clean-up, precolumn derivatization and an ion-pair HPLC can provide sufficient sensitivity and quantitativity for simultaneous determination of amoxicillin, ampicillin and oxacillin in eggs.

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