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Article

Simultaneous Determination of Amoxicillin and Ampicillin in Eggs by Reversed-Phase High-Performance Liquid Chromatography with Fluorescence Detection using Pre-Column Derivatization

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A sensitive and robust method is presented for the simultaneous determination of amoxicillin (AMO) and ampicillin (AMP) in eggs by reversed-phase high-performance liquid chromatography with fluorescence detection (RP-HPLC–FLD). This method used a simple liquid–liquid extraction of the samples with acetonitrile and dichloromethane as precipitation of proteins and extraction solvent. AMO and AMP reacted with salicylaldehyde to form fluorescent derivatives, which were then analyzed with RP-HPLC–FLD. Separation was carried out on an Athena C18 column with a mobile phase consisting of 0.01M potassium dihydrogen phosphate, adjusted to pH 5.5 by 2M potassium hydroxide and acetonitrile. The detector response was linear over the tested concentration range from 5.0 to 800 ng/mL for AMO and AMP. The recovery values ranged from 78.4 to 88.7% for AMO and from 77.6 to 82.0% for AMP. The limits of detection were 1.2 for AMO and 0.4 μg/kg for AMP. The limits of quantification were 3.9 for AMO and 1.5 μg/kg for AMP. The corresponding intra-day and inter-day variation (relative standard deviation) were found to be less than 9.6 and 14.8%, respectively.

Introduction

Amoxicillin and ampicillin are two broad-spectrum β-lactam antibiotics that are widely used in veterinary medicine because of their broad spectrum and low cost. However, this use can result in the presence of residues in food that can cause allergic reactions in people with hypersensitivity to penicillins, and may lead to the emergence of penicillin-resistance bacterial strains. To ensure the safety of food for consumers, regulation 508/99 of the European Union (EU) Commission (1) has laid down maximum residue limits of 50 μg/kg of ampicillin and amoxicillin in animal tissues and 4 μg/kg in milk.

Traditionally, pencillins are determined by microbiological assay. Although sensitive, these assays are not specific or quantitative because it is very difficult to distinguish amoxicillin (AMO) from ampicillin (AMP) (2). Capillary electrophoresis is one of the techniques used for the determination of penicillin in feeds (3–6). Many chromatographic methods are also used with diode array and fluorescence detection (7–12) or liquid chromatography–mass spectrometry (LC–MS) (13–15) for the determination of penicillin in food of animal origin. However, no current public reports describe a method for the simultaneous determination of AMO and AMP in eggs. Therefore, a specific and sensitive method for the simultaneous determination and confirmation of AMO and AMP residues in eggs is urgently needed.

The purpose of this study was to develop a simple, specific and sensitive method using reversed-phase high-performance liquid chromatography with fluorescence detection (RP-HPLC–FLD) for the simultaneous determination of AMO and AMP in eggs.

Experimental

Chemicals and reagents

AMO (98% standard) and AMP (99% standard) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile of HPLC grade was purchased from Tedia (Fairfield, OH). HPLC-grade salicylaldehyde was the product of Aladdin Chemistry (Shanghai, China). Other reagents of analytical grade were supplied by Sinopharm Chemical Reagent Co. (Shanghai, China). Water was purified using a PURELAB Option-Q synthesis system from ELGA Lab Waters (High Wycombe, Bucks, UK).

Standard stock and working solutions

Stock solutions for AMO and AMP at different concentrations were prepared by dissolving each analyte in water. Working standard solutions at different concentrations for AMO and AMP were prepared by diluting the stock solutions with water. The stock solutions were stable for 6 months at −20°C. Fresh working solutions were prepared by an appropriate dilution of the stock solution before use.

Equipment

The HPLC system consisted of a Waters Alliance 515 LC System and a Waters multi λ 2475 fluorescence detector (Waters, Milford, MA). An ART MICCRA D-9 homogenizer (ART-moderne Labortechnik, Müllheim, Germany), a model G560E vortex mixer (Scientific Industries, Bohemia, NY) and a model 5810R centrifuge (Eppendorf, Hamburg, Germany) were also used.

Chromatographic conditions

HPLC separation was achieved on an Athena C18 column (250 × 4.6 mm i.d., 5 μm; Hanbon Sci. & Tech., Huan'an,

Table I
Chromatographic Gradient Conditions for the Separation of AMO and AMP

Time (min)	A (%)	B (%)	Flow rate (mL/min)
0	65	35	1.0
10	65	35	1.0
11	40	60	1.0
18	40	60	1.0
19	65	35	1.0
22	65	35	1.0

China). The column temperature was thermostatted at 40°C. The mobile phase was 0.01M potassium dihydrogen phosphate, adjusted to pH 5.5 by 2M potassium hydroxide (mobile phase A) and acetonitrile (mobile phase B). The flow rate was 1.0 mL/min. The fluorescence detector was set at 354 nm for excitation wavelength and 445 nm for emission wavelength. The injection volume was 200 μL manually with a 200 μL quantitative ring. The gradient conditions are shown in Table I.

Optimization of derivative reaction conditions

To optimize the derivative reaction conditions used in the analytical procedure, we investigated the effects of the reaction time, the water bath temperature, the concentration of the 200 μL of trichloroacetic acid and the amount of salicylaldehyde added to the formation of the fluorescent derivatives of both AMO and AMP. To optimize the reaction time, nine centrifuge tubes, each containing 1 mL of spiked samples of the authentic egg extract solution (100 ng of AMO and 100 ng of AMP), were each mixed with 200 μL of 30% (w/v) trichloroacetic acid aqueous solution and 20 μL of salicylaldehyde. The nine tubes were heated in a 100°C water bath and then individually removed from the water bath after 0, 15, 25, 35, 40, 45, 50, 55 and 60 min reaction time. The fluorescent derivatives formed in each tube were quantitatively determined by HPLC with FLD. The effects of heating temperature (with heating time, 45 min; salicylaldehyde, 20 μL; 30% trichloroacetic acid, 200 μL), different amounts of salicylaldehyde (with heating time, 45 min; temperature, 100°C; 30% trichloroacetic acid, 200 μL) and concentration of the 200 μL trichloroacetic acid (with heating time, 45 min; temperature, 100°C; salicylaldehyde, 20 μL) were determined in a similar manner.

Sample preparation and extraction

A 5.0-g sample of homogenized eggs was weighted into a 50-mL polypropylene centrifuge tube. Five mL acetonitrile was added to the sample and the mixture was vortexed for 1 min. After that, 15 mL of acetonitrile was added and the mixture was vortexed for 2 min. After centrifugation at 8,000 × g for 10 min, the supernatant was transferred to another 50-mL polypropylene centrifuge tube. Twenty mL of dichloromethane was added to the mixture and it was vortexed for 2 min. After centrifugation at 6,500 × g for 10 min, 1 mL of the supernatant was transferred to a 10-mL glass test tube.

Derivative reaction

A 20-μL aliquot of salicylaldehyde and 200 μL of 25% trichloroacetic acid were added to the concentrate in centrifuge tube, which was then vortexed for 10 s. The centrifuge tube was capped loosely and heated in a boiling water bath (100°C) for 60 min. After cooling to room temperature, the contents in the graduated centrifuge tube were brought to 500 μL with 50% acetonitrile in water and repeated twice, then diluted to 2 mL. Before analysis, the resulting solution was centrifuged at 12,100 × g for 15 min and poured through a 0.22-μm filter, and then 200-μL of supernatant was injected manually into the HPLC system.

Linearity

The calibration curves were prepared on the basis of the peak areas and the working solution concentrations. To establish calibration curves, a series of working standard solutions at concentrations of 5.0, 25, 50, 100, 200, 400 and 800 ng/mL for AMO and AMP were prepared by diluting the stock solutions. One milliliter of each standard solution was reacted with salicylaldehyde and trichloroacetic acid as described previously. After dilution to 2 mL, 200-μL of supernatants was injected into the HPLC system. Triplicate injections were also performed.

Accuracy and precision

A standard solution containing both of AMO and AMP was used to spiked egg samples at the levels of 5, 25 and 125 μg/kg. After the spiked egg samples were analyzed by the procedure described previously, percentage recoveries were calculated using the daily calibration standards as external standards for quantitation. Six replicates of spiked samples at concentrations of 5, 25 and 125 μg/kg were analyzed to determine the recoveries and intra-day variations of the analytical procedure within the work day. Spiked egg samples were also analyzed on different days to determine the inter-day variation of the analytical procedure.

Results and Discussion

Optimization of sample derivatization

To obtain a high fluorescent response, the derivatization reaction conditions were optimized and the results of these experiments are shown in Figure 1.

The optimum combination of the reaction conditions consisted of 1 mL of standard solution mixed with 200 μL of 25% trichloroacetic acid and 25 μL of salicylaldehyde and then heated at 100°C for 60 min.

Specificity and sensitivity

The specificity of the method was evaluated by preparation and analysis of blank and spiked samples. No interfering peaks were detected at the retention times of AMO and AMP. Representative chromatograms of blank samples and spiked samples are shown in Figure 2.

The sensitivity of the method was estimated by limits of detection (LODs) and limits of quantitation (LOQs), as shown in

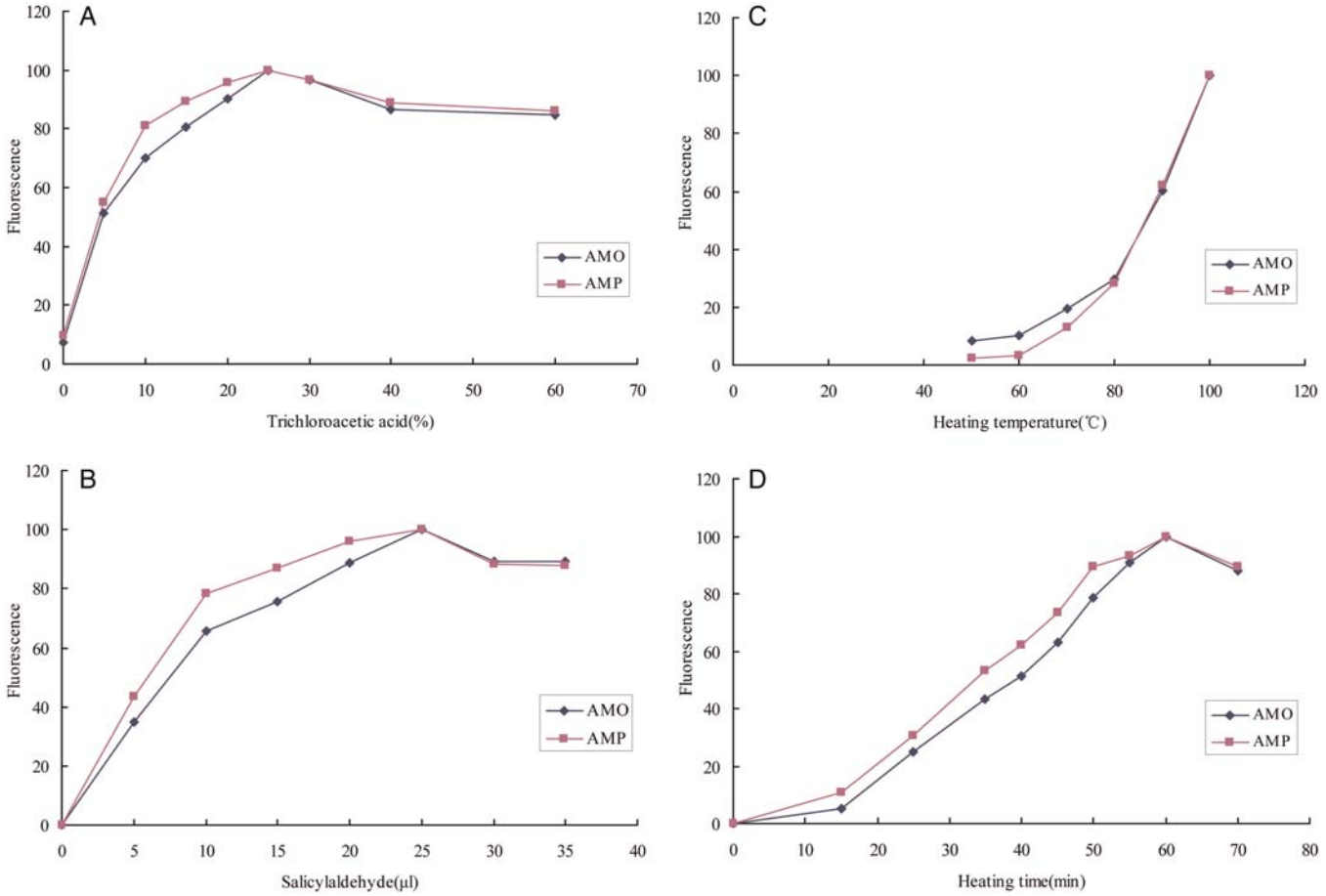

Figure 1. Effects of concentration of trichloroacetic acid (A); amount of salicylaldehyde (B); heating temperature (C); and heating time (D) on the formation of fluorescent derivatives of AMO and AMP.

Table II. The LODs, defined as the spiking lever in eggs producing a signal-to-noise ratio of 3:1 of the weaker transition for each of the two analytes were 1.2 μg/kg for AMO and 0.4 μg/kg for AMP. The LOQs, the lowest point on the calibration curve for each of the two analytes, were 3.9 μg/kg for AMO and 1.5 μg/kg for AMP, based on a signal-to-noise ratio of 10:1.

Linearity

The linearity of the standard curves was assessed with the slope (a), intercept (b) and correlation coefficient (r) and their variations in the range of 5.0–800 ng/mL. Typical values for the regression parameters a, b and r and the linear ranges of AMO and AMP are shown in Table III.

Accuracy and precision

As shown in Table IV, mean extraction recoveries from fortified egg samples at levels of 5, 25 and 125 μg/kgranged from 78.4 to 88.7% for AMO and from 77.6 to 82.0% for AMP. The corresponding intra-day and inter-day variations (relative standard deviation; RSD) were found to be less than 9.6 and 14.8%, respectively, as shown in Table V,.

Optimization of sample extraction and HPLC analysis

Sample extraction, precipitation of proteins and chromatographic separation were the key steps in this method. Therefore, selection of a solvent system was critical. Acetonitrile, water–methanol and potassium dihydrogen phosphate were used to optimize the extraction efficiency. Then, acetonitrile, trichloroacetic acid, perchloric acid, sodium tungstate and sulphuric acid were used to optimize the deproteinization efficiency. Excellent results were reached with extraction and deproteinization of the analytes by acetonitrile. The use of dichloromethane after acetonitrile deproteination not only removed some organic substances from the sample, but also absorbed the acetonitrile, thereby leaving only the aqueous mixture for assay. The choice of acetonitrile and dichloromethane as the solvent for precipitation of proteins and extraction was based on the method described by Yoon (16).

The mobile phase of the method for the determination of pencillins is usually potassium dihydrogen phosphate–acetonitrile. The chromatographic separation was optimized by testing different concentrations of potassium dihydrogen phosphate (0.01M, 0.02M and 0.05M) and pH (4.5, 5.0 and 5.5) of the mobile phase. By choosing a mobile phase composed of 0.01M potassium dihydrogen phosphate, adjusted to pH 5.5 by 2M potassium hydroxide (A) and acetonitrile (B), reasonable

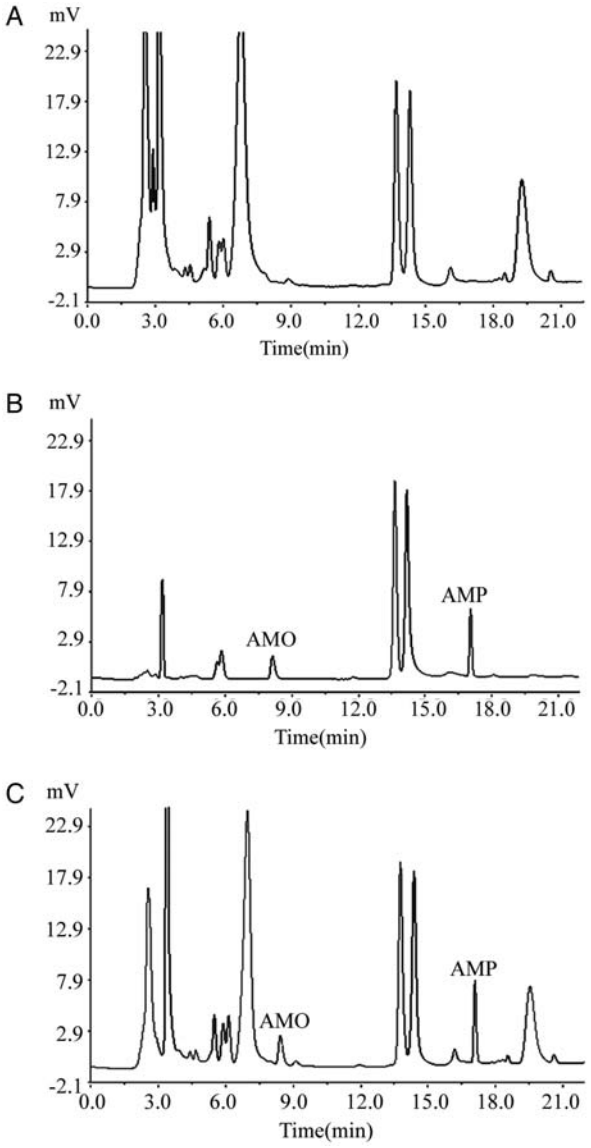

Figure 2. HPLC chromatograms of: blank egg (A); standard solution containing AMO (20 ng/mL) and AMP (20 ng/mL) (B); blank egg spiked with AMO and AMP (C).

Table II
LODs and LOQs

Analyte	LODs (μg/kg)	LOQs (μg/kg)
AMO	1.2	3.9
AMP	0.4	1.5

Table III
Regression Lines, Relative Coefficients (r) and Calibration Range of AMO and AMP

Analyte	Regression lines	Relative coefficients (r)	Calibration range (ng/mL)
AMO	$Y = 3.1159X - 1.8771$	0.9997	5.0 ~ 800
AMP	$Y = 2.1432X - 6.2902$	0.9994	5.0 ~ 800

Table IV
Accuracy and Precision Data of the Method ($n = 6$)

Analyte	Spiked level (μg/kg)	Mean result (μg/kg)	Recovery (%)	RSD (%)
AMO	5	3.7 ± 0.2	79.5 ± 4.6	5.8
	25	19.7 ± 0.6	78.4 ± 2.5	3.2
	125	109.0 ± 7.3	88.7 ± 6.0	6.7
AMP	5	4.0 ± 0.2	77.6 ± 4.9	6.3
	25	20.00 ± 1.3	79.6 ± 5.1	6.4
	125	102.6 ± 8.9	82.0 ± 7.1	8.7

Table V
Performance of the Method in Terms of Precision ($n = 6$)

Analyte	Spiked level (μg/kg)	Intra-day RSD (%)	Inter-day RSD (%)
AMO	5	9.6	14.8
	25	9.6	11.0
	125	8.6	11.9
AMP	5	9.3	8.7
	25	9.6	12.5
	125	8.0	11.4

retention times for AMO and AMP were obtained with good peak shape.

Application to real samples

To evaluate the applicability and reliability of the proposed method, AMO and AMP were determined in real samples. A total of 60 egg samples from local supermarkets were analyzed by the described method. One incurred egg sample was analyzed by the described method and 6.8 μg/kg of AMO was detected. None of the other analyzed egg samples showed residues of targeted compounds at detectable levels.

Conclusion

In this study, we developed a specific, simple, reliable, widely applicable and low-cost RP-HPLC–FLD method for the simultaneous determination and confirmation of AMO and AMP residues in eggs. This method can be performed under the most common experimental conditions without adding expensive special equipments, and fulfils the requirement of the confirmatory criteria according to the European Commission.

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