Simplified High-Performance Liquid Chromatographic Determination of Residual Amprolium in Edible Chicken Tissues

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Abstract

A simplified determining method for the routine monitoring of residual amprolium in edible chicken tissues (muscle and liver) is developed using a high-performance liquid chromatographic (HPLC) method with a photodiode-array detector after sample cleanup by an Ultrafree-MC/PL centrifugal ultrafiltration unit. For the HPLC determination and identification, a Mightysil RP-4 GP column and a mobile phase of an ethanol–5mM 1-heptanesulfonic acid sodium salt solution (35:65, v/v) using an ion-pairing system with a photodiode-array detector are used. Average recoveries (spiked at 0.3–3.0 µg/g) are > 90%. The inter- and intravariabilities are 1.9–2.4%. The limits of quantitation are 0.22 µg/g for muscle and 0.25 µg/g for liver. The total time and solvent required for the analysis of one sample are < 20 min and < 2 mL of ethanol, respectively. No toxic solvents and regents are used.

Introduction

Amprolium, 1-[(4-amino-2-propyl-5-pyrimidinyl)methyl]-2-methylpyridinium chloride hydrochloride, (APL) (Figure 1) is most frequently used for therapeutic or prophylactic purposes for chicken coccidiosis. Chicken feed containing APL is routinely fed to chickens as a starter feed to prevent diseases. The use of APL in poultry industries may result in APL residues being present in poultry products if adequate withdrawal times for the animals have not been observed or if these drugs have been improperly administered.

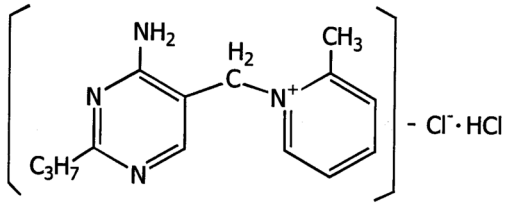

Figure 1. Chemical structure of APL.

Because consumers have no way of knowing what veterinary drugs may be in the food, they rely on the passed inspection of chickens in order to assure safety and wholesomeness.

In order to prevent these residues in chicken products, the U.S. Food and Drug Administration (FDA) has established tolerances for APL in the muscle (0.5 ppm) and liver (0.3 ppm) in the Code of Federal Regulations (21 CFR Part 556). In order to confirm rigidly whether APL residues are retained in poultry products, an analytical method for the routine monitoring of APL residues in the products must be precise, simple, economical in cost and time, and capable of detecting the residues below the tolerances to permit the monitoring of large numbers of samples, with negligible harm to the environment. At present, discharging the waste of toxic organic solvents is a severe problem. Analytical methods should avoid the use of toxic solvents and reagents (1–3).

Several techniques involving high-performance liquid chromatography (HPLC) using a reversed-phase ion-pair system with UV or fluorescence detection have been reported for the determination of APL from different materials such as chicken feeds, plasma, and muscles (4–8). However, these methods have the following problems: (*a*) the extraction and cleanup involves numerous and varying analytical steps that are time consuming, do not permit the monitoring of a large number of samples, and have recoveries that are sometimes low and variable and (*b*) some toxic solvents (such as methylene chloride, acetonitrile, and methanol) are required as extraction solvents, an HPLC mobile phase, or both, which may be harmful to the environment.

In this study, we developed an accurate and sensitive method for determining and identifying APL in chicken tissues. This study presents a rapid and simple procedure for sample preparation, avoiding successive sample manipulations and the use of toxic solvents. The determination is performed by an HPLC equipped with a photodiode-array detector because it measures retention time and absorption spectrum simultaneously, thus allowing for instant identification.

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Experimental

Reagents

Standard APL was obtained from Sigma (St. Louis, MO).

A stock standard solution of APL was prepared by accurately weighing 10 mg and dissolving it in 100 mL distilled water. The working standard solutions were prepared by diluting the stock solutions with distilled water. These solutions can be kept in a refrigerator for up to one month. A 0.5-mol/L volume of a 1-heptanesulfonic acid sodium salt solution (low UV type, ion-pairing chromatograph grade) (HSA), which is an ion-pairing agent for the HPLC detection of APL and ethanol (HPLC grade), were purchased from Wako Pure Chem. Ltd. (Osaka, Japan).

Instrument

An HPLC analysis of the target compound was conducted using a Jasco (Tokyo, Japan) Model PU-980 pump and a DG-980-50 degasser (Jasco) equipped with an SPD-M10A$_{VP}$ photodiode-array detector (Shimadzu, Kyoto, Japan) interfaced with a Fujitsu (Tokyo, Japan) FMV-5133D7 personal computer. The separation was performed on a Mightysil RP-4 GP (end-capped) (5 µm) column (4.6 × 150 mm) (Kanto Chem. Co., Inc., Tokyo, Japan) with a guard column (4.6 × 5 mm) (Kanto Chem.) using a mixture of ethanol–5mM HSA (35:65, v/v) as the mobile phase at a flow rate of 0.9 mL/min at ambient temperature.

The following apparatuses were used in the sample preparation: an ultrasonic homogenizer (Model HOM-100, 2-mm-i.d. chip) (Iwaki Glass Co., Ltd., Funabashi, Japan); a microcentrifuge (Biofuge fresco, Kendo Lab. Products, Hanau, Germany); and centrifugal ultrafilter units—three membrane types of the Ultrafree MC series (nominal molecular weight limit = 5000 amu, capacity ≤ 0.5 mL), which were Ultrafree-MC/Biomax (Biomax high-flux polysulfone ultrafiltration membrane), Ultrafree-MC/PL (regenerated cellulose ultrafiltration membrane), and Ultrafree-MC/PT (polysulfone ultrafiltration membrane) (Millipore, Bedford, MA).

Samples

Chicken tissues (muscle and liver) from meat breeder chickens that were kept in individual cages and given an APL-free basal diet continuously were used as blank samples. In order to validate this study's method for routine monitoring, the tissues with residual APL from meat breeder chickens that were fed a diet containing 200 ppm APL for 7 days were also used.

Procedure

An accurately weighed 0.2-g sample was placed into a microcentrifuge tube and homogenized in 0.4 mL of a 20% (v/v) ethanol solution (in water) with an ultrasonic homogenizer for 30 s. After 30 s, the tube was centrifuged at 10,000 × g for 5 min. A 0.4-mL portion of the supernatant liquid was put into an Ultrafree-MC/PL and centrifuged at 3000 × g for 5 min. A 20-µL portion of the ultrafiltrate was injected into the HPLC system.

Recovery test

The recoveries of APL from blank samples spiked at 0.3, 0.5, 0.8, 1.5, and 3.0 µg/g were determined. The spiked samples were allowed to stand at 4°C for 12 h after the addition of APL followed by mixing. In the test, the coefficients of variation (CVs) determined for each spiked concentration were then averaged, which resulted in a mean ± standard deviation (SD). This was defined as interassay variability. Intraassay variability was defined as the CV for the mean of five replicates of an identical sample and represented the variability associated with the analytical procedure used.

Results and Discussion

Acetonitrile, methanol, and methylene chloride are usually used in the mobile phase for reversed-phase HPLC analyses or the extraction solution of various veterinary drugs, including APL. These organic solvents are handled as toxic solvents in Swiss Toxicity Classification (9). In the HPLC fluorometric detection of APL, APL was detected by postcolumn reaction with ferricyanide containing NaOH (4,8). These reagents are also handled as toxins or harmful substances. In contrast, environmental and human toxicity for ethanol and HSA (used as the extraction solution and HPLC mobile phase) are negligible (Table I).

Sample preparation

One advantage of the proposed extraction is that the operation used an ultrasonic homogenizer (handy type), which is especially easy. The device was able to homogenize smaller chicken tissue samples (0.2 g) easily with a little of the extraction solution (0.4 mL) in a microcentrifuge tube (capacity ≤ 1.5 mL). The extract (processed with a 20% (v/v) ethanol solution (in water)) did not form an emulsion that would hinder the recovery of APL. After centrifugation, it was completely recovered in the supernatant liquid. The extract obtained was further purified to remove interfering materials. The MolCut ultrafiltration unit has previously been used as a simple cleanup technique for the determination of veterinary drugs in animal tissues (10–12). This process enabled easy deproteinization of the extractant with syringe pressure. In order to simplify the procedure further, in this study the Ultrafree centrifugal ultrafiltration unit was used. The recoveries of APL from three types of the Ultrafree-MCs were examined and compared. A 0.4-mL portion of a standard solution containing 0.2 µg of APL was applied to three types of the Ultrafree-MC filters,

Table I. Swiss Toxicity Classification* of Solvents and Reagents Using the APL Analysis

Substance	Poison class*
Acetonitrile	Very strong toxin
Ethanol	Not subject to toxicity
Methanol	Strong toxin
Methylene chloride	Harmful substance
NaOH	Very strong toxin
Potassium hexacyanoferrate (III)†	Harmful substance
1-Heptanesulfonic acid sodium salt‡	Not subject to toxicity

* Reference 9.
† Fluorometric reaction agent.
‡ Ion-pairing agent.

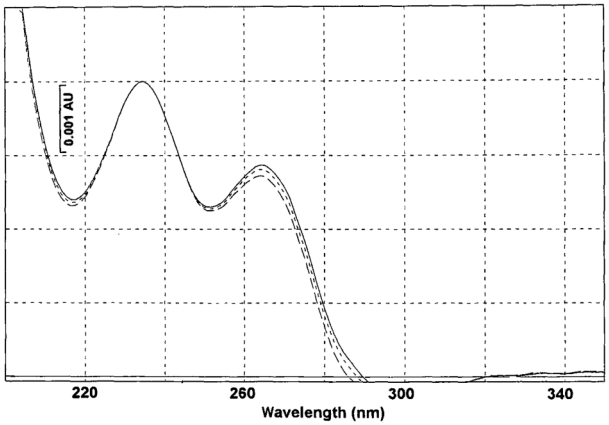

Figure 2. UV spectra of standard APL (solid line), APL (spiked 0.3 µg/g) in a chicken muscle sample after isolation (dotted line), and APL (spiked 0.3 µg/g) in a chicken liver sample after isolation (dashed line), which were all obtained by photodiode-array detection.

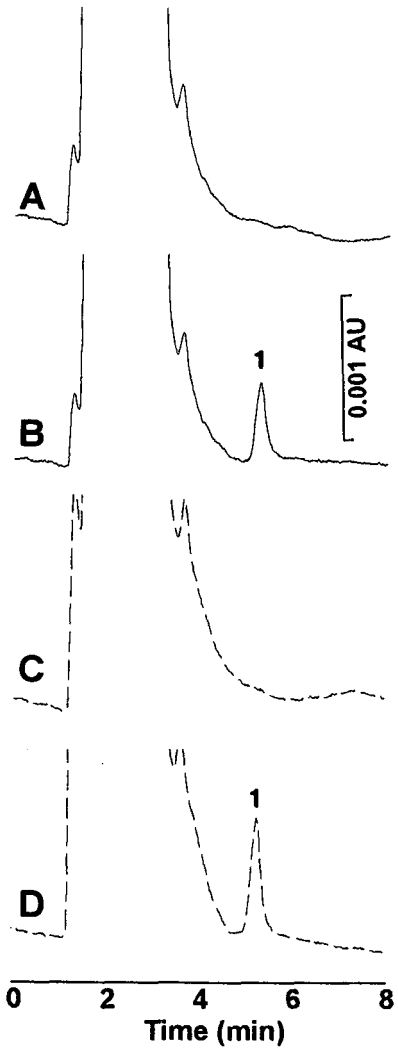

Figure 3. HPLC chromatograms obtained from chicken tissue samples (photodiode-array detector set at 234 nm): (A) a blank chicken muscle sample; (B) spiked (0.8 µg/g of APL) chicken muscle; (C) a blank chicken liver sample; and (D) chicken liver sample from a chicken fed a diet containing 200 ppm of APL for 7 days. Peak 1 is APL (R_t = 5.2 min).

respectively. The ultrafiltrate was examined by the HPLC. An Ultrafree-MC/PL gave the best recovery (96.1%) and precision (CV = 0.8%) for APL. The procedure enabled rapid and excellent reproducibility with a considerable saving of time.

In order to determine the effect of ethanol concentrations in the extraction solution on the recoveries of APL and the forming of emulsions, concentrations over the range of 0% to 30% were tested. Optimum results were obtained when using concentrations ≥ 10% ethanol.

HPLC

As a result of separating the APL using a Mightysil RP-4GP column and a combination of ethanol and 5mM HSA as the mobile phase while using a reversed-phase ion-pairing system, the best separation was obtained with the C_4 column and ethanol–5mM HSA (35:65, v/v) as the mobile phase when the APL monitoring was adjusted to 234 nm (a maximum absorption spectrum for APL, Figure 2). A flow rate of 0.9 mL/min gave a favorable retention time under the conditions examined over the range of 0.7 to 1.2 mL/min. The solvent (ethanol) consumption per sample was < 2 mL.

Figure 3 indicates that the procedure allows for the rapid and efficient purification of APL, thus resulting in freedom from interfering compounds as observed in the HPLC chromatograms. Figure 3D demonstrates that this method is valid for practical usage.

The HPLC method in this study made it unnecessary to use a gradient system to improve the separation and did not require "precolumn washing" after an analysis. In the proposed conditions, the target compound was detected at 5.2 min (Figure 3B). Because HPLC was performed in a serial manner, the length of time per run became more critical in routine analysis. The short run time not only increases sample throughput for analysis but also affects the method development time.

Method validation

Table II summarizes the recoveries from chicken muscle and liver samples at five different spiking levels (0.3–3.0 µg/g for APL), correlation coefficients (r) of calibration graphs, and inter- and intraassay variabilities of APL isolated from spiked chicken muscle and liver samples, respectively. Average recoveries were greater than 90.1%, with SDs between 1.5 and 2.9%. Inter- and intraassay variabilities ranged from 1.9% to 2.4%. As the calibration lines, the spiked recovery graphs were generated by plotting peak areas of fortified sample extracts ranging from 0.3 to 3.0 µg/g. The graph was constructed from five points, and each point represented the mean of the five injections. The resulting r values for APL in the muscle and liver samples were highly significant statistically ($P < 0.01$) (Table II). A good linearity and reproducibility of the determination were obtained in the concentration range examined.

The limits of quantitation (LOQs) of APL in chicken muscle and liver samples were calculated by measuring the analytical background response. Based on the peak areas in HPLC chromatograms, LOQ was defined as the average background plus 10 times the SD. Five different blank muscle or liver samples known to be near the LOQ were analyzed in duplicate. In a practical analysis for the residue monitoring, the LOQs for the muscle and liver

samples were 0.22 and 0.25 µg/g, respectively (Table II). The LOQs were well-below the tolerances (0.5 µg/g for the muscle and 0.3 µg/g for the liver).

Selectivity

In HPLC analysis for residual drug monitoring, a photodiode array gives spectral information and is an easy way to confirm peak identity. HPLC combined with the diode-array system proved to be able to detect a wide range of molecules and ensure identification of the target compounds. The retention time and spectrum allow for peak identification. The APL examined could be identified in the tissue sample with its retention time and absorption spectrum. The spectra of APL obtained from chicken muscle and liver samples were practically identical with that of the standard, as can be seen in Figure 2. The present sample preparation allowed a reliable confirmation.

A shorter analysis time and the non-use of toxic solvents and reagents were achieved by the proposed procedure. The proposed procedure is negligible in toxicity to the analysts and environment and also significantly reduces cost per sample. The total analytical time and solvent consumption were < 20 min per sample and < 2 mL of ethanol per sample, respectively. The analytical time was shortened to < $^1/_6$ of that required for the referenced procedures (4–7).

Monitoring residue in marketing chicken tissues

Thirty different samples of edible chicken tissues that were available in Osaka City were analyzed by using the method in this study. No APL was detected. There were no interfering peaks in the resulting chromatograms.

Table II. Recoveries of APL from APL-Fortified Chicken Tissue Samples

	%Recovery (mean ± SD, *n* = 5)	
Spiked (µg/g)	**Muscle**	**Liver**
0.3	91.2 ± 2.1	90.4 ± 2.5
0.5	92.5 ± 2.0	91.6 ± 2.9
0.8	92.2 ± 1.5	91.4 ± 2.2
1.5	90.1 ± 2.3	93.5 ± 1.8
3.0	93.3 ± 1.9	92.2 ± 1.6
Calibration graphs		
r* (*n* = 5)	0.999	0.998
%Assay variability		
Interassay (*n* = 25)	2.0 ± 0.3	2.4 ± 0.5
Intraassay (*n* = 5)	1.9	2.2
LOQ		
(µg/g)	0.22	0.25

* Mean of five determinations using spiked samples for standard curves (range of concentration between 0.3 and 3.0 µg/g).

Conclusion

The following characteristics prove that the proposed procedure is useful for the routine residue monitoring of APL in chicken tissues: (*a*) a shorter analytical time (total < 20 min per sample); (*b*) it is highly precise (inter- and intraassay variabilities were 1.9–2.4%); and (*c*) no toxic solvents were used and there was low solvent consumption (total solvent consumption < 2 mL of ethanol per sample).

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Manuscript accepted March 14, 2002.