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Development of a high-performance liquid chromatography method for the determination of florfenicol in animal feedstuffs

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ABSTRACT

An effective thin layer chromatography (TLC) purification procedure coupled to high-performance liquid chromatography (HPLC) method was developed for the determination of florfenicol (FF) in pig, chicken and fish feedstuffs. The feedstuff samples were extracted with ethyl acetate, defatted with n-hexane saturated with acetonitrile, and further purified by TLC. The chromatographic separation was performed on a Waters Symmetry C_{18} column using an isocratic procedure with acetonitrile-water (35:65, v/v) at 0.6 mL/min. The ultraviolet (UV) detector was set at a wavelength of 225 nm. The FF concentrations in feedstuff samples were quantified using a standard curve. Good linear correlations ($y = 159075\,x - 15054$, $r > 0.9999$) were achieved within the concentration range of 0.05–200 μg/mL. The recoveries of FF spiked at levels of 1, 100 and 1000 μg/g ranged from 80.6% to 105.3% with the intra-day and inter-day relative standard deviation (RSD) less than 9.3%. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.02 and 0.06 mg/kg for pig feedstuffs, 0.02 and 0.07 mg/kg for chicken feedstuffs, and 0.02 and 0.05 mg/kg for fish feedstuffs, respectively. This reliable, simple and cost-effective method could be applied to the routine monitoring of FF in animal feedstuffs.

1. Introduction

FF is a broad-spectrum amphenicol antibacterial agent developed specifically for veterinary use. In China, FF is mostly administered to pig, poultry and fish by incorporation into feedstuffs for the control of bacterial diseases. The misuse of FF can cause the presence of its residues in animal-derived foods. For consumer protection, the application of FF is strictly controlled in China [1]. The routine monitoring of FF in animal feedstuffs is also performed to ensure compliance with corresponding regulation. Reliable analytical method is required for this purpose.

Several analytical methods have been published for determining FF in animal feedstuffs based on HPLC [2–4], liquid chromatography-mass spectrometry (LC–MS) [5,6], planar chromatography [7] and enzyme-linked immunosorbent assay [8,9]. The HPLC method has its own advantages over the others, such as low cost and acceptable specificity, accuracy and precision. Nevertheless, there is a need to optimize the existing HPLC methods because (i) these methods are developed for pig and fish feedstuffs, their applicability to poultry feedstuffs needs to be demonstrated, (ii) an additional solid-phase extraction (SPE) purification procedure is required to achieve acceptable specificity, which increases the cost of analysis.

TLC is a classic chromatographic separation technique. It has been widely used for the separation of bioactive components from complex natural product extracts [10]. In previously published study, TLC has been successfully used to separate tiazofurin [11], FF [12] and methyl-3-quinoxaline-2-carboxylic acid [13] from body fluids. Compared with SPE, TLC has the advantages of high separation efficiency, low cost and less consumption of organic solvents.

The aim of this study was to develop a reliable, simple and cost-effective HPLC method for the routine monitoring of FF in pig, chicken and fish feedstuffs. The possibility of using TLC as a purification

Abbreviations: TLC, thin layer chromatography; HPLC, high performance liquid chromatography; FF, florfenicol; UV, ultraviolet; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantification; LC–MS, liquid chromatography–mass spectrometry; SPE, soild-phase extraction

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technique for complex biological samples was also investigated.

2. Materials and methods

2.1. Reagents

The analytical standard of FF (purity > 99.7%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from Tedia Company Inc. (Fairfield, OH, USA). Ethyl acetate, hexane, dichlormethane, acetone and ammonium hydroxide (analytical grade) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Deionized water used through the study was purified using a Milli-Q Millipore Water System (Millipore Corp., Bedford, MA, USA).

2.2. Materials

Pre-coated silica gel 60 GF254 TLC plate was purchased from Merck KGaA (Darmstadt, German). Oasis HLB cartridges (60 mg, 3 cm^3) and vacuum manifold were from Waters (Milford, MA, USA). PTFE syringe filter (0.22 μm, 13 mm) was from Lizhu Biological Technology Co., Ltd. (Guangzhou, China).

The pig feedstuffs were obtained from Wens Animal Husbandry Co., Ltd. (Yunfu, China) and Wannianqing Animal Husbandry Co., Ltd. (Wuhan, China). The chicken feedstuffs were from Feeds Inspection Point (Huazhong Agriculture University, Wuhan, China) and Aohua Feedstuff Co., Ltd. (Huizhou, China). The fish feedstuffs were from Aohua Feedstuff Co., Ltd. (Huizhou, China). All feedstuff samples were ground, thoroughly mixed and passed through a 1 mm sieve, and then stored in cool, dry containers.

2.3. Standard stock and working solutions

Stock standard solution (10000 μg/mL) was prepared by dissolving FF in acetonitrile. It was stable for six month at −20 °C. Working standard solution (5000 μg/mL) was prepared by dilution of the stock standard solution in acetonitrile, and was stable for at least two weeks at 4 °C.

2.4. Sample preparation

Feedstuff samples (5.0 g) known to be free of FF were accurately weighed into 100 mL polypropylene centrifuge tubes. These samples were spiked with working standard solution to yield final concentrations of 1, 100 and 1000 mg/kg, respectively. Before extraction, the spiked samples were thoroughly mixed and air-dried in a fume hood for at least 20 min at room temperature.

Fifty milliliters of ethyl acetate was added to the spiked sample. The mixture was vortexed for 2 min, followed by centrifugation at 6000 *g* for 5 min. One milliliter of the supernatant was carefully transferred into an Eppendorf tube and evaporated to dryness under a stream of nitrogen at 45 °C. The dried residue was reconstituted in 50 μL of acetonitrile and then defatted once with 1 mL of n-hexane saturated with acetonitrile. Further sample purification was performed by TLC using a GF-254 silica gel plate. Twenty microlitre of stock standard solution and all the feedstuff sample extracts were applied separately on the TLC plate. The plate was developed in a 20 cm × 10 cm twin-trough chamber to a distance of 8 cm with a mixture of dichloromethane, acetone, ammonium hydroxide (5:5:0.25, v/v/v). After the developing solvent was dried at room temperature, the TLC plate was scanned at 254 nm, and the FF standard spot on the plate was outlined. The silica gel band, which corresponded to the feedstuff extracts and simultaneously located at the same horizontal position as that of FF standard spot, was carefully scrapped into an Eppendorf tube and extracted once with 1 mL of the acetonitrile-water mixture (35:65, v/v). The supernatant was filtered through a 0.22 μm PTFE syringe filter. An aliquot of 50 μL was analyzed by HPLC.

2.5. HPLC anlaysis

All analyses were performed on a Waters 1525 HPLC system (Waters Co., Milford, MA, USA) that consisted of a 1525 binary pump, 2489 UV detection and 2707 auto sampler. The chromatographic separation was performed on a Waters Symmetry C_{18} column (250 mm × 4.6 mm I.D, 5 μm) (Waters Co., Milford, MA, USA). The mobile phase consisted of acetonitrile-water (35:65, v/v), which was pumped at 0.6 mL/min. The column temperature was maintained at 32 °C. The UV detector was set at a wavelength of 225 nm.

2.6. Method validation

The analytical method was validated according to the performance criteria established by U.S. Food and Drug Administration [14]. The validation parameters included specificity, sensitivity, linearity, range, accuracy, precision, and proof of performance.

The specificity of the method was assessed by comparing the chromatograms of blank samples with those of spiked samples. All the matrices (the pig, chicken and fish feedstuffs) were investigated. For each matrix, five blank samples were analyzed as described above.

The LOD and LOQ were determined using blank feedstuff extracts spiked with working standard solution at nine concentration levels from 0.02 to 0.1 mg/kg. For each concentration level, five samples were analyzed as described above. The LOD was calculated by the comparison of the threefold variation of signal to noise ratio (S/N = 3:1), while the LOQ was calculated by using a signal-to-noise of 10.

The FF concentrations in feedstuff samples were quantified using a standard curve, which was obtained using serial dilutions (0.05, 1, 10, 50, 100 and 200 μg/mL) of working standard solution. These concentration levels were established considering the working range of FF and the LOD of the equipment. Each concentration level was injected into HPLC in triplicate. The standard curve was generated by plotting the peak area of FF (y) versus the corresponding concentration (x).

The accuracy and precision of the method were evaluated by analyzing the spiked feedstuff samples at the concentrations of 1, 100 and 1000 mg/kg (five replicates for each concentration level) over a period of three days. The accuracy was represented by the mean recoveries of spiked FF in various feedstuff samples. The mean recovery was calculated by dividing the concentration measured with the concentration spiked in blank feedstuff samples. The precision was expressed as the intra-day and inter-day RSD.

The performance of the method was further evaluated by analyzing five batches of commercial medicated feedstuff samples. For each batch, five replicate samples were analyzed as described above.

3. Results and discussion

3.1. Chromatographic conditions

Several previously published HPLC methods employed acetonitrile-sodium acetate buffer [2–4], acetonitrile-aqueous ammonium acetate [15,16] and acetonitrile-water mixtures [17,18] to elute FF from C_{18} reverse-phase HPLC columns. In this study, the influence of various mobile phase compositions on the chromatographic behavior of FF was comprehensively investigated. There was no significant difference in peak shape, retention time and resolution when comparing acetonitrile-water mixtures with acetonitrile-sodium acetate buffer and acetonitrile-aqueous ammonium acetate mixtures. To simplify the method, the acetonitrile-water mixtures were used as the mobile phase. The acetonitrile content in the mobile phase was also optimized from 15% to 40% (v/v). The maximum sensitivities and satisfactory chromatographic separation were achieved on Waters Symmetry C_{18} column with the mobile phase consisted of acetonitrile-water (35:65, v/v) (Fig. 1).