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Qualitative and Quantitative Drug residue analyses: Florfenicol in white-tailed deer (*Odocoileus virginianus*) and supermarket meat by liquid chromatography tandem-mass spectrometry

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ABSTRACT

A method for confirmation and detection of Florfenicol amine residues in white-tailed deer tissues was developed and validated in our laboratory. Tissue samples were extracted with ethyl acetate and cleaned up on sorbent (Chem-elut) cartridges. Liguid chromatography (LC) separation was achieved on a Zorbax Eclipse plus C_{18} column with gradient elution using a mobile phase composed of ammonium acetate in water and methanol at a flow rate of 300 μL/min. Qualitative and quantitative analyses were carried out using liquid chromatography – heated electrospray ionization(HESI) and atmospheric pressure chemical ionization (APCI)-tandem mass spectrometry in the multiple reaction monitoring (MRM) interface. The limits of detection (LODs) for HESI and APCI probe were 1.8 ng/g and 1.4 ng/g respectively. Limits of quantitation (LOQs) for HESI and APCI probe were 5.8 ng/g and 3.4 ng/g respectively. Mean recovery values ranged from 79% to 111% for APCI and 30% to 60% for HESI. The validated method was used to determine white-tailed deer florfenicol tissue residue concentration 10-days after exposure. Florfenicol tissue residues concentration ranged from 0.4 to 0.6 μg/g for liver and 0.02-0.05 μg/g for muscle and a trace in blood samples. The concentration found in the tested edible tissues were lower than the maximum residual limit (MRL) values established by the federal drug administration (FDA) for bovine tissues. In summary, the resulting optimization procedures using the sensitivity of HESI and APCI probes in the determination of florfenicol in white-tailed deer tissue are the most compelling conclusions in this study, to the extent that we have applied this method in the evaluation of supermarket samples drug residue levels as a proof of principle.

1. Introduction

The combination of Liquid chromatography/Mass spectrometry provides two powerful analytical tools for the determination of tissue residues of environmental contaminants and pharmaceuticals. The pharmaceutical florfenicol and other broad-spectrum antibiotic that exhibits activity against both gram negative and gram-positive bacteria. These antibiotics are frequently utilized as feed additives for food-producing animals due to low toxicity profile, and more significantly, low cost [1]. Structurally florfenicol resembles chloramphenicol and is a fluorinated analogue of thiamphenicol. While florfenicol has similar activity to chloramphenicol and thiamphenicol, it is less susceptible to bacterial resistance due to the fluorine atom present on the C-3 position instead of the –OH group that is present in chloramphenicol and thiamphenicol. Florfenicol does not induce the irreversible aplastic anemia or reversible bone marrow suppression seen with the use of chloramphenicol and thiamphenicol [2]. Furthermore, florfenicol is resistant to the actions of acetyltransferase, the enzyme that bacteria use to develop resistance to thiamphenicol and chloramphenicol [3,4]. Florfenicol was developed to be administered intramuscularly on every-other-day. In the US, it is approved for treatment of bovine respiratory disease caused by *Pasturella hemolytica* and *Haemophilus somnus* [5]. Florfenicol also is approved for use in agricultural industry including farm animals and fish [6]. Respiratory infections are a common cause for death within the agricultural

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industry [7,8]. Florfenicol amine (FFA) the primary metabolite of florfenicol is used as a biomarker residue for animal species and is commonly distributed primarily to the liver as well as feces, urine, muscle, and kidney [9]. Monitoring of drug residue concentration is vital for the food production industry. The maximum residue limits (MRLs) for florfenicol has been set by the Food and Drug Administration (FDA) to regulate residue levels for food safety. The MRLs of florfenciol depends on the animal species and type of tissue and varies according to country specific standards. The majority of drugs used in the white-tailed deer industry are used extra-label (off-label) because none is approved for white-tailed deer including florfenicol. Extra-label users as well as health authorities have to deal with the difficult question of the risk for public health resulting from the use of these drugs or antibiotics. Therefore, it is important that we optimized and validated these precise analytical methods for the determination of drug residues.

Various analytical techniques have been reported for the determination of florfenicol and its metabolites in biological samples such as: capillary electrophoresis [10,11] and fluorescence spectroscopy [12], HPLC-UV [13,14], HPLC-PDA[15], UV–vis spectroscopy [16]. However, FFA analyses are adoptable to liquid chromatography methods [17–23]. These reports have also included the determination of florfenicol in various biological matrices such as plasma [16], fish [9,14,16,24], cattle [4,19–21,23,25,26], chicken [17,18,27], swine [15,18,27]. Currently there are no published analytical method for drug residue concentration in white-tailed deer; this manuscript will focus on developing a method using heated electrospray ionization (HESI) and atmospheric pressure chemical ionization (APCI) for the determination of drug residue such as florfenicol in white-tailed deer.

2. Materials and method

All reagents were HPLC grade unless stated otherwise. Methanol (LC–MS and HPLC grade), ethyl acetate, water (LC–MS), and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Phosphoric acids, hydrochloric acid, sodium hydroxide, ammonium acetate, and florfenicol amine (FFA) were purchase from Sigma (St. Louis, MO, USA). Buffers and solutions were prepared using Milli-Q deionized water. SPE filter syringe vials, glass round-bottomed flask (100 mL), polypropylene centrifuge tubes (50 mL) were from VWR International. Chem Elut CE1020 sorbent columns (20 mL) were from Varian/Analytichem International.

2.1. Standard preparation

A stock solution of 100 μg/mL (ppm) was prepared by dissolving 10.0 mg of FFA in 100 mL of methanol. Working standard solutions of 0.001, 0.005, 0.010, 0.025, 0.050, 0.1, 0.2, 0.5, and 1 μg/mL (ppm) were prepared by diluting the stock solution with methanol. The stock solutions were stable for 6 months at −20 °C. The standard solutions were stable for 1 month at 4 °C.

2.2. Animal care and treatment

This experiment was performed in compliance with the appropriate laws and institutional guidelines approved by the Texas Tech Institutional Animal Care and Use Committee, protocol number 14039-08. The experiment was conducted in 15 healthy white-tailed deer with an age ranging from 2 to 6 years old, which weighed 42–47 kg. They were kept in high fenced pens. The animals were fed drug-free deer feed ad libitum with free access to water. Lab personnel as well as the university's veterinarians monitored the health of the animals. We randomly assigned the animals to their respective treatment groups. Five animals were administered intramuscularly a single dose of florfenicol (FF) at 20 mg/kg of body weight. Five animals were in the untreated control group and five were assigned to the saline solvent control group. Blood samples were taken at the beginning of the study and at the end of the study (after the 10 days exposure). Representative tissue samples were collected for drug residue analyses from all animals 10 days after exposure to florfenicol. In an effort to support our data local meat market samples of liver and muscle from bovine, porcine, shrimp, lamb and chicken were purchased and analyzed in our laboratory.

2.3. Sample preparation

A United States Department of Agriculture (USDA) protocol (2010) [28] was modified and used for optimization in our procedure. Briefly, frozen tissue samples (muscle and liver) were thawed at 4 °C and weighed (2 g) in a 50 mL centrifuge tube. Milli-Q deionized water (2 mL) was added to the tissue samples and homogenized. Hydrochloric acid (8 mL 6N) was then added and the mixture vortex and placed in a water bath at a temperature between 95 and 100 °C for 2 h. The samples were vortexed at 30 min interval until digestion was completed. Ethyl acetate (20 mL) was added to the mixture and vortexed for 2 min and then centrifuged for 5 min at 2000 RPM. The organic layer (upper layer) was discarded. Care was taken not to disturb the black tarry interface. After centrifugation, the hydrolyte was adjusted to a pH of 12.5 or higher by adding 30% sodium hydroxide (~8 mL). The sorbent column (Chem-elut) was pre-conditioned with ethyl acetate (20 mL) and the mixture was then poured into the column. The column was washed with 20 mL ethyl acetate twice. The eluate was transferred to a 125 mL round bottom flask and evaporated to dryness at 50 °C under vacuum using a rotary evaporator. The residue was reconstituted in 2 mL of methanol, vortex and sonicated for 30 s each and filtered into an autosampler vial using Acrodisc LC25 PVDF 0.2 μm filters.

2.4. Chromatographic conditions

We optimized the conditions for white-tailed deer tissue using a Heated Electrospray Ionization (HESI) and Atmospheric Pressure Chemical Ionization (APCI) probes. Similar liquid chromatographic parameters, mobile phase conditions, and columns (analytical and guard) were used for both probes. Chromatographic separation was carried out using a refrigerated autosampler (CTC PAL) system with a Zorbax Eclipse plus-C18; 3.5 μm; 4.6 × 150 mm with a Zorbax Eclipse plus guard column-C18; 4.6 × 12.5 mm, 5 μm (Agilent Technologies, USA) on column heater coupled to a Quantum Access MAX triple stage quadrupole (TSQ) mass spectrometer (Thermo Scientific) for optimization and validation. Column temperature was set to 25 °C. The following conditions were used: elution solvents were 10 mM ammonium acetate in water (A), methanol (B) mobile phase composition (A:B; v/v) was 95:5 at 0 min, 95:5 at 3 min, 20:80 at 5 min, 20:80 at 10 min, 95:5 from 12 to 14 min with a flow rate of 300 μL/min and an injection volume of 20 μL.

2.5. LC–MS/MS analysis

Florfenicol amine was optimized for HESI and APCI probes with positive polarity. A 5 μg/mL FFA standard was used to determine the optimum counts per seconds (cps) in MS and MS/MS scans. The parent ion and product ion transitions and other MS parameters for HESI and APCI probes are outlined in Table 1. The MS parameters for HESI and APCI probes differed accordingly as per the probe selection and florfenicol optimization. FFA analyte specific mass spectrometer parent and product ions settings are presented in Table 2. The calibration curves, carry-over determination, and intra-day standard precision were performed for both HESI and