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LC–MS/MS based method development for the analysis of florfenicol and its application to estimate relative distribution in various tissues of broiler chicken

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ABSTRACT

Florfenicol, a broad spectrum bacteriostatic antibiotic belonging to amphenicol class, is widely used in poultry and livestock for the treatment of various infections. The major metabolite of florfenicol in different animal species is florfenicol amine which is exploited as the marker residue for the determination of florfenicol. Analysis of florfenicol merely by solvent extraction cannot determine the accurate amount of the drug present in incurred tissues (muscle, liver and kidney) of treated birds, as indicated by this study. Thus the methods solely based on solvent extraction may lead to false negative results. A reliable LC–MS/MS based confirmatory method for the analysis of florfenicol and its metabolites in chicken muscle was developed and validated according to the European Union Commission Decision 2002/657/EC. The method was based on acid hydrolysis to liberate non-extractable residues having presumably been covalently bound to tissues, and to convert all the florfenicol residues as well as its metabolites into florfenicol amine. The amine was subsequently recovered with ethyl acetate at pH 10.5, defatted and further cleaned up with dispersive solid phase extraction (dSPE). The LC separation was achieved on reverse phase C-18 column with isocratic elution using acetonitrile/water mobile phase and the analysis was performed on linear ion trap mass spectrometer. Calibration curve was obtained over a concentration range of 25–600 μg/kg for chicken muscles. The accuracy values ranged from 84 to 101.4% and the precision values for within day and between days ranged from 1.2-11.7%, respectively. Limit of detection (LOD), limit of quantification (LOD), CCα and CCβ values were 0.98, 3.2, 113 and 126 μg/kg, respectively. The developed method was highly robust and was further applied to estimate the relative distribution of solvent-extractable against solvent-non-extractable florfenicol drug residues in muscle, liver and kidney samples of broiler chicken after 5 days of oral dosing.

1. Introduction

Amphenicol family comprises of chloramphenicol (CAP), thiamphenicol (TAP) and florfenicol (FF); all of them are synthetic broad-spectrum antibiotics used against several bacterial species [1]. Chloramphenicol, the first broad spectrum antibiotic, has been used as the most effective drug against various bacterial strains, including many anaerobic organisms [2]. However, due to adverse side effects (such as blood dyscrasias) in humans, associated with nitro benzene group of chloramphenicol, many countries *e.g.* China, United States and European Union have prohibited its use in food producing animals [3]. Thiamphenicol and florfenicol were synthesized with a major structural modification in chloramphenicol replacing nitro with methylsulfone. However, the development of antibiotic resistance strains against thiamphenicol due to the presence of acetyltransferase, made this drug less effective. On the contrary, florfenicol owing to the substitution of OH with F is resistant to acetyltransferase transformation. This drug exhibit better bioavailability in several species as compared to other competing antibiotics, such as those from tetracycline and quinolone groups [4]. Florfenicol is now being widely used in aquaculture, livestock and poultry production for the treatment of many bacterial infections caused by *Pasteurella*, *Haemophilus, Actinobacillus*, *Bordatella, Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Salmonella typhimurium* and *Staphylococcus aureus* [4–6].

In treated animals, FF metabolizes into a number of intermediates such as florfenicol alcohol, monochloroflorfenicol and florfenicol

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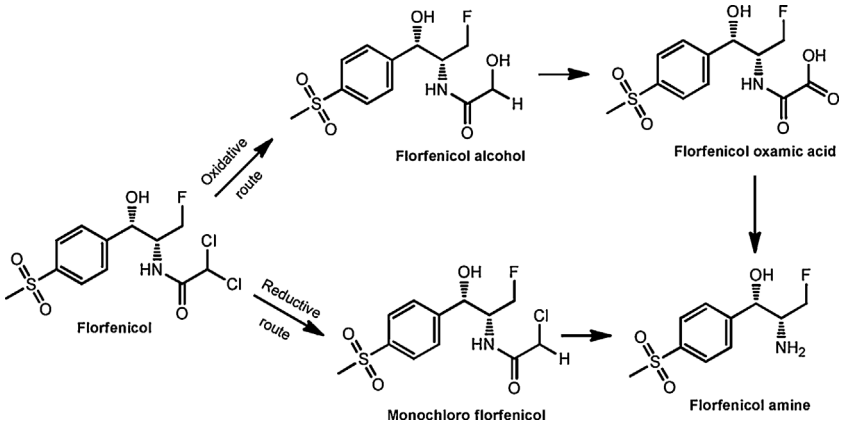

Fig. 1. The proposed metabolic pathways of florfenicol in chicken [8].

oxamic acid (Fig. 1), which are then rapidly converted to florfenicol amine (FFA) [7,8]. The major metabolite of FF in edible tissues of various species *i.e.* chicken, pigs and fishes, is FFA [9–11]. Hence, FFA has been considered as the marker residue of FF in various international legislations [12]. In most of the animal species, FF and its metabolites are excreted in urine, highlighting the fact that the kidneys are involved in the major clearance route of this drug [7,13].

Maximum Residual Limits (MRLs) of FF has been set for all food-producing animals (listed in Table I of the European Commission Regulation No 37/2010) [12]. The MRLs in poultry tissue, liver and kidney are 100 μg/kg, 2500 μg/kg and 750 μg/kg, respectively, with the marker residue defined as 'the sum of florfenicol and its metabolites measured as florfenicol amine' [9]. However, in Europe, FF is not permitted to be used in milk and egg producing animals, farmed for human consumption [12].

Various analytical methods have been published for the analysis of florfenicol and its major metabolite florfenicol amine (FFA), either alone or with other amphenicols, in a variety of food items. These analytical methods include ELIZA for chicken tissues [14], HPLC method for fish, chicken muscles and eggs [15–17], GC–MS based analysis for fish, poultry and porcine muscles [18,19], and LC–MS analysis for chicken, milk, bovine, swine and porcine muscles [1,20–22]. Most of the published methods for the analysis of florfenicol involve solvent extraction, either with acetone [16] or ethyl acetate [1,19,20], followed by solid phase extraction with Oasis MCX, C-18 or Oasis HLB. Metabolic studies of florfenicol in poultry, cattle, swine and porcine, however, have demonstrated that solvent non-extractable residues of florfenicol are present in higher concentrations as compared to solvent extractable residues [9,22]. These non-extractable metabolites could only be extracted with organic solvents such as ethyl acetate after strong acid hydrolysis. The acid hydrolysis completely digest the animal tissues liberating bound residues and converts all the metabolites and the parent compound into florfenicol amine. Consequently, the determination of FFA in hydrolyzed extracts can give more accurate measurement of total florfenicol residues as compared to that in the direct solvent extracts according to European Union legislations [6,22].

A few methods for the analysis of florfenicol involving acid hydrolysis have been reported in literature such as HPLC-UV method for channel catfish [6] and an LC–MS based analysis of FF in swine and porcine muscles [21,22]. The official method of USDA for the determination of total florfenicol in bovine, poultry and catfish muscle by HPLC also requires the acid hydrolysis [23]. However, in both of the above published methods [6,21] as well as in USDA official method, FFA stock solution was used as spiking standard solution for the method development and validation studies. The studies have not encompassed the utility of acid in the hydrolysis of FF to FFA in matrix matched standard preparation steps to mimic the chemical transformations occurring during incurred sample processing. The residues of florfenicol consists of parent drug, FFA and other metabolites present at different concentrations *in vivo* [9,22], which after acid hydrolysis, are converted to FFA. Hence, the spiking of FF standard to the negative tissue, followed by its acid hydrolysis to FFA (including the other method development steps), seems to be a comparatively better option. Drug standards would also get hydrolyzed in matrix calibrants and any losses that occur during chemical conversion can be accounted for. A recent method by Faulkner et al., [22] has also used the native FF standard for method development and estimation of non-extractable residues in porcine kidney.

The bioavailability, pharmacokinetic and tissue depletion studies of florfenicol and florfenicol amine in different species have been reported by many authors [5,8,24]. But the methods based on acid hydrolysis to recover the solvent non-extractable residues are scarce. The method reported by Kong et al., [21] and Faulkner et al., [22] are perhaps the only reference methods, which have used the acid hydrolysis for the analysis of FF as FFA during tissue depletion studies for the estimation of non-extractable residues in swine and porcine. However, to the best of the authors' knowledge, no LC–MS/MS based method is found in literature, which describes the relative distribution along with comparison of solvent extractable against solvent non-extractable FF residues in various tissues of broiler chicken.

Poultry industry together with other livestock industry has encountered numerous infectious diseases. The uncontrolled and non-regularized use of veterinary drugs results in extensive misuse of antibiotics, especially in tropical/sub-tropical under-developed countries like Pakistan. Exposure to these higher levels of antibiotics consequently results in extra health burden due to the emergence of drug resistant bacteria in developing countries [25]. The lack of governing rules and analytical facilities to screen and quantify drug residues in food products is one of the major hurdles to accurately assess the drug contamination level. The development of validated analytical methods can, therefore, contribute to address the health issues as well as can support the increase in export of veterinary and poultry products to the international market [26].

The aim of present work was to develop an LC–MS/MS based method for the analysis of FF residues as FFA in whole muscle tissues of broiler chicken. The method applications were further enhanced to estimate solvent extractable versus solvent non-extractable FF residues in liver, kidney and muscle tissues of broiler chicken during the depletion period. The results of the experiments showed that, proportion of solvent non-extractable FF residues were much higher in comparison to the solvent extractable residues in broiler tissue.

2. Material and methods

2.1. Chemical reagents and materials

All the chemical reagents used were at least analytical or HPLC/LC–MS grade. Ultrapure water with resistivity of 18 MΩ cm obtained from in house Smart 2 Pure water purification system (Thermo