Food Chemistry 172 (2015) 30–39

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Metabolic study of enrofloxacin and metabolic profile modifications in broiler chicken tissues after drug administration

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ARTICLE INFO

Article history:
Received 12 March 2014
Received in revised form 2 September 2014
Accepted 6 September 2014
Available online 16 September 2014

Keywords:
Chicken tissues
Enrofloxacin
Metabolites
Multivariate data analyses
Metabolic profile

ABSTRACT

In this work, the identification and distribution of the metabolites from enrofloxacin (ENR) in liver, kidney and muscle tissues from broiler chickens subjected to a pharmacological treatment was studied. In addition, qualitative analyses of changes in the metabolic profile in those tissues after drug administration were also investigated.

As a result, a total of 31 different metabolites from ENR were identified, which ciprofloxacin (CIP) and desethylene-ENR were the major metabolites. After four days of withdrawal period, most of the metabolites were excreted, but residues of ENR and CIP still persisted in tissues at a concentration under the permitted maximum residue limit (MRL). Non-medicated, medicated and post-treatment samples of chicken tissues were clearly clustered according to their metabolite profile by principal component analysis and partial least squares discriminant analysis, which indicates that endogenous metabolites have not returned to their original levels after the withdrawal period. A total of 22 relevant mass features contributing to this separation as potential markers of chicken samples were tentatively identified.

1. Introduction

In modern agricultural practice, veterinary drugs are extensively used and administered as feed additives or via the drinking water as therapeutic, prophylactic and growth promoting agents, but the inappropriate and abusive use of these substances can leave residues in food products from animals. Specifically in the case of antibiotics, the concern about their residues in foodstuff and the misuse in humans has increased as a result of the transfer of antibiotic-resistant bacteria to man, toxicity and allergy problems and their illegal use as growth promoters (Blasco, Torres, & Picó, 2007; Fàbrega, Sánchez-Céspedes, Soto, & Vila, 2008; Stolker & Brunkman, 2005).

In the last few years, the concern about the use of veterinary drugs in food-producing animals and their possible negative effects in the health of consumers has made the control of these residues in edible animal tissues mandatory at the EU. Maximum residue limits (MRLs) of antibiotics in foodstuffs of animal origin such as multiple animal tissues were established by the Commission Regulation (EU) No. 37/2010 (Commission Regulation (EU) No. 37/2010, 2010) for safe consumption.

In the last recent years, the interest in monitoring those regulated compounds has increased. Up to this time, a large number of published articles have been focused on the development and validation of analytical methods to determine target substances and their main metabolites in several matrices (Blasco et al., 2007; Bogialli & Di Corcia, 2009; Clemente, Hermo, Barron, & Barbosa, 2006; Hermo, Nemutlu, Barbosa, & Barron, 2011; Macarov et al., 2012; Marazuela & Bogialli, 2009; Martínez-Huélamo, Jiménez-Gámez, Hermo, Barrón, & Barbosa, 2009; Moreno-Bondi, Marazuela, Herranz, & Rodríguez, 2009; Romero-González, Aguilera-Luiz, Plaza-Bolaños, Garrido Frenich, & Martínez Vidal, 2011), but there are few studies focused on the identification and determination of unknown metabolites and degradation products (Hermo, Gómez-Rodríguez, Barbosa, & Barrón, 2013), which could lead to unknown harmful effects for human health. Consequently, the analysis of metabolites and degradation products of food contaminants, especially those that are considered genotoxic or carcinogenic, is of great interest at present.

Moreover, researchers have recently indicated the need of studying the influence of antibiotics on the endogenous metabolism to evaluate changes in metabolite levels (Drexler, Reily, & Shipkova, 2011; García-Reyes, Hernando, Molina-Díaz, & Fernández-Alba, 2007). Accordingly, metabolic alterations caused by the use of antibiotics in veterinary and human medicine might be of great interest in the research of new potentially toxic or

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http://dx.doi.org/10.1016/j.foodchem.2014.09.025
0308-8146/

healthy compounds and to determine possible markers of the pharmaceutical treatments. To the best of our knowledge, only two studies have been previously reported related to the evaluation on metabolome modifications after pharmacological treatment in animals. Sun et al. (2013) evaluated the effects of Penicillin V on the metabolome of rats and more recently, Hermo, Saurina, Barbosa, and Barrón (2014) estimated the metabolome modifications in chicken after amoxicillin administration. Accordingly, due to the scarce information obtained, more efforts need to be taken in evaluating the effect on metabolic changes in animal tissues intended for human consumption after a pharmacological treatment with antibiotics, which could be of great interest in food control and safety applications.

Enrofloxacin (ENR) is a widely used quinolone in poultry farms due its high antimicrobial activity against a wide variety of infections in animals. The present study was focused on the identification of residues of metabolites from ENR and the study of their distribution in liver, kidney and muscle tissues from broiler chickens slaughtered in different days after applying a pharmacological treatment with the antibiotic.

Thus, besides of the identification of metabolites from ENR, differences in the metabolome caused by the administration of the drug followed by the tentative identification of markers from non-medicated, medicated and post-treatment samples of chicken liver, kidney and muscle tissues after applying the pharmacological treatment with ENR was also carried out.

2. Experimental

2.1. Reagents and materials

Unless specified otherwise, all reagents were of analytical grade. Quinolones were purchased from different pharmaceutical firms: enrofloxacin (ENR) from Cenavisa (Reus, Spain), ciprofloxacin (CIP) from Ipsen Pharma (Paris, France) and norfloxacin (NOR), used as internal standard (IS), was supplied by Liade-Boral Quimica (Barcelona, Spain).

Formic acid (FA), trifluoroacetic acid (TFA), acetic acid (HAcO), ammonia (NH_3), potassium dihydrogenphosphate (KH_2PO_4), sodium hydroxide (NaOH), methanol (MeOH, HPLC grade) and acetonitrile (MeCN, HPLC and MS grade) were provided from Merck (Darmstadt, Germany). Ammonium acetate (NH_4AcO, MS grade) was supplied by Sigma–Aldrich (St. Louis, MO, USA).

Ultrapure water was obtained from a MilliQ system from Millipore (Billerica, MA, USA). Solid-phase extraction (SPE) cartridges Isolute ENV+ (3 mL/200 mg) were supplied by Biotage AB (Uppsala, Sweden).

2.2. Preparation of standard and working solutions

Individual ENR, CIP and NOR stock solutions were prepared at a concentration of 100 mg L^{-1} in HAcO 0.050 mol L^{-1}. The working solutions used to spike the chicken tissue samples were prepared from the individual stock solutions by appropriate dilution to obtain concentrations of 1 and 0.5 mg L^{-1}. For the extraction procedures, 0.050 mol L^{-1} dihydrogenphosphate solution (adjusted to pH 5.0 with NaOH 0.1 mol L^{-1}) and the hydroorganic solution TFA:H_2O:MeCN (2:23:75, v/v/v) were also prepared.

2.3. Pharmacological treatment with ENR

Chickens were medicated according to the pharmacological administration protocol fit for human consumption. The therapeutic treatment involved a daily dose of 10 mg/kg of ENR dissolved in the chicken drinking water during 4 days. Fresh pre-solutions of the antibiotic and the medicated water were prepared every day just before it is offered to the animals.

Four types of samples were analysed. Two male broiler chickens (A1 and A2, non-medicated chickens) randomly selected from the poultry farm were sacrificed and used as blanks, three male broiler chickens (A3, A4 and A5) slaughtered on the second day of the pharmacological treatment (2-day treated), three male broiler chickens (A6, A7 and A8) slaughtered on the fourth day of the pharmacological treatment (4-day treated) and two male broiler chickens (A9 and A10) slaughtered four days after pharmacological treatment ends (post-treatment). Recovery time was chosen according to the administered commercial product specifications, which indicate a withdrawal period of 4 days. All animals were handled and sacrificed according to the ethical protocols of the chicken producer farm. Chicken liver, kidney and muscle tissue samples from the 10 animals were analysed.

Meat was minced, homogenised and stored at −20 °C until sample treatment (Section 2.4.1). For each type of tissue (muscle, liver and kidney), three independent replicates of the 10 specimens were analysed. Each extract was injected twice.

2.4. Sample preparation

2.4.1. Medicated animal samples

An amount of 4 g (±0.1 mg) of minced chicken muscle or 2 g (±0.1 mg) of minced chicken kidney and liver was introduced into a 50 mL capped polypropylene centrifuge tube, adding then the IS (NOR) at a concentration of 300 μg kg^{-1} (Macarov et al., 2012; Morales-Gutiérrez, Hermo, Barbosa, & Barrón, 2014). Analytes were extracted with a mixture of 2 mL (1 mL for kidney and liver tissues) of MilliQ water and 20 mL of MeCN (10 mL for kidney and liver tissues). After shaking for 2 min, the mixture was centrifuged at 3500 rpm (5 min). The supernatant was then isolated and the organic solvent (MeCN) was eliminated by evaporation under N_2 stream in a TurboVap system at 35 °C until 2 mL (1 mL for kidney and liver tissues) as final volume. After adding 25 mL of 0.050 mol L^{-1} dihydrogenphosphate solution (12.5 mL for kidney and liver tissues) to the remaining aqueous extract, the resulting mixture was processed by solid phase extraction (SPE). The Isolute ENV+ cartridges were activated with 2 mL of MeOH, 2 mL of MilliQ water and 2 mL of 0.050 mol L^{-1} dihydrogenphosphate solution at pH 5.0. The mixture was then loaded to the cartridge and washed with 3 mL of dihydrogenphosphate solution at pH 5.0 and 1 mL of MilliQ water, followed by the elution of the analytes with 5 mL of the hydroorganic solution TFA:H_2O:MeCN (2:23:75, v/v/v) and 1 mL of MeCN. The produced SPE eluates were evaporated to dryness at 35 °C under N_2 stream and reconstituted with 200 μL of MilliQ water (100 μL for kidney and liver tissues). Prior to injection, samples were filtered and stored at −20 °C.

2.4.2. Quantification of ENR and CIP

The concentration of ENR and its major metabolite (CIP) were determined in the three studied tissues from the medicated chickens. Chicken liver, kidney and muscle blank samples obtained from a local supermarket in Barcelona (Spain) were firstly screened to ensure that they were free of antibiotics of interest. Blank samples were directly spiked at seven ENR and CIP concentrations levels in the range of 5–250 μg kg^{-1} and NOR (IS) at a concentration of 300 μg kg^{-1}. Extraction of analytes was accomplished following the same procedure described above (Section 2.4.1). Calibration curves were constructed using analyte/IS peak area ratios versus analyte/IS concentration ratios.

2.5. LC–MS and LC–MS/MS conditions

Identification of metabolites and metabolome modifications analyses were performed using an Accela HPLC system from