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Analytical Methods

High-resolution mass spectrometry applied to the study of metabolome modifications in various chicken tissues after amoxicillin administration

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ABSTRACT

The performance of high resolution accurate mass spectrometry (HRMS) operating in full scan MS mode was investigated for the quantitative determination of amoxicillin (AMX) as well as qualitative analysis of metabolomic profiles in tissues of medicated chickens. The metabolomic approach was exploited to compile analytical information on changes in the metabolome of muscle, kidney and liver from chickens subjected to a pharmacological program with AMX. Data consisting of *m/z* features taken throughout the entire chromatogram were extracted and filtered to be treated by Principal Component Analysis. As a result, it was found that medicated and non-treated animals were clearly clustered in distinct groups. Besides, the multivariate analysis revealed some relevant mass features contributing to this separation. In this context, recognizing those potential markers of each chicken class was a priority research for both metabolite identification and, obviously, evaluation of food quality and health effects associated to food consumption.

1. Introduction

Poultry are one of the most consumed foods by humans and their intakes have been positively associated with some healthy effects because of their dietary relevance. Prior to commercialization, comprehensive quality controls of poultry products are necessary to ensure a high level of protection of human health. For this purpose, regulation concerning the control of food from animal origin is enshrined in the Annex I of Commission Regulation 37/2010 (Commission Regulation (EU) No 37/2010, 2010). In the cited text, legislated MRLs of antibiotics allowed for veterinary use are given, including those of β-lactamic drugs.

Nowadays, there is an increasing interest in monitoring regulated compounds and their metabolites. However, beyond the quantification of exogenous components, researchers have been pointed out the need of studying the influence of such xenobiotics on the endogenous metabolism from the evaluation of changes in metabolite levels (e.g., up- and down-regulations). As a result, metabolic modifications generated "in vivo" might be of great interest in the research of new potentially toxic or healthy compounds and conclusions extracted can be applied to further studies on food regulations (García-Reyes, Hernando, Molina-Díaz, & Fernández-Alba, 2007). At this point, considering the scarce information about metabolomic alteration caused to the use of antibiotics in veterinary and human medicine, thorough studies to assess possible biomarkers of the pharmaceutical treatments are increasingly demanded.

In this work, the metabolic profile of amoxicillin (AMX) has been studied. AMX is a penicillin drug sometimes administered to farm animals due to its high antimicrobial activity. As a result, AMX might be detected in biological fluids and tissues of animals subjected to therapeutic treatment (De Baere, Cherlet, Baert, & De Backer, 2002; Reyns et al., 2009).

High-resolution mass spectrometry (HR–MS) has become the current approach of choice to face some challenges raised in metabolomic studies. In particular, mass spectrometry time-of-flight (MS-TOF), and more recently, linear ion trap quadrupole-Orbitrap MS (LQT-Orbitrap MS), both coupled to liquid chromatography, have proved their excellent performances for metabolomic research (Berendsen et al., 2013; Bousova, Senyuva, & Mittendorf, 2013; Hurtaud-Pessel, Jagadeshwar-Reddy, & Verdon, 2011; Szultka, Krzeminski, Szeliga, Jackowski, & Buszewski, 2013; Zubarev & Makarov, 2013). Qualitative analysis corresponding to exact mass measurements and elemental composition assignment are fundamental for a more feasible characterization of small pharmacologically active substances (Hermo, Gómez-Rodríguez, Barbosa, & Barrón, 2013; Pérez-Parada et al., 2011). In parallel, MS^n experiments have been exploited successfully to confirm fragmentation routes and to elucidate of structures of target and unknown compounds (Nägele & Moritz, 2005). However, the assessment of metabolic changes in biological matrices is a complex task, and full scan chromatograms may result in an excellent source of high quality data to evaluate variations in the chemical

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composition in a comprehensive manner without losing statistically significant information. Nowadays, the most used strategy for data treatment relies on Principal Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLS-DA) and related methods (Marquez, Albertí, Salvà, Saurina, & Sentellas, 2012). Such chemometric methods allow noise filtering and the concentration of information into a reduced number of latent variables. Raw data from HPLC-MS measurement of samples is taken to construct a data matrix focused on the differentiation of positive and blank samples. As a result, PCA-based methods have demonstrated to be highly efficient to extract and visualize the useful information using, for instance, scatter plots of samples (scores plot) and variables (loadings plot) on the principal components (PCs) (Serrano-Lourido, Saurina, Hernández-Cassou, & Checa, 2012). The most discriminat MS features, characterized by their retention time and m/z values, may result in chemical markers to define the different categories or classes of samples.

One of the aims of this study was to improve the AMX detection in different biological tissues using LTQ-Orbitrap mass spectrometry, to quantify the active compound in several positive chicken muscle, liver and kidney samples from animals medicated with AMX. Besides, multivariate analysis with PCA was exploited to study the distribution of samples and variables, to associate veterinary treatments with m/z, and to find reliable indicators, drug related compounds and up- and down-regulated endogenous metabolites in the biological tissues.

2. Experimental

2.1. Reagents and materials

Unless specified, all reagents were of analytical grade. Amoxicillin (AMX) was supplied by Sigma–Aldrich (St. Louis, MO, USA) and piperacillin (PIP), used as internal standard (IS) was supplied by Fluka (Buchs, Switzerland). Acetonitrile (MeCN, MS grade), ammonium acetate, ammonia, formic acid, potassium dihydrogenphosphate, methanol (MeOH) and sodium hydroxide were from Merck (Darmstadt, Germany). Ultrapure water was generated by the MilliQ system of Millipore (Billerica, MA, USA).

The SPE cartridges used in this study were ENV + Isolute (3 cm^3/200 mg) purchased from Biotage AB (Uppsala, Sweden).

Nylon microcon centrifugal filter membranes of 45 μm pore size (Millipore) were used to filter the extracts before the injection into the chromatographic system.

2.2. Preparation of standard solutions

Individual stock solutions of AMX and PIP (IS) were prepared at a concentration of 100 μg ml^{-1} by dissolving the exactly weighed quantity of each compound in MilliQ water. The working solutions used to spike the chicken tissue samples were prepared from the individual stock solutions by appropriate dilution to obtain concentrations of 10, 5, 1 and 0.5 μg ml^{-1} AMX. For the extraction procedures, 50 mM dihidrogenphosphate solution (adjusted to pH 5 with sodium hydroxide 0.1 M) and hydroorganic solutions consisting of MeCN:H_2O (91:9, v:v) and MeCN:MeOH (50:50, v:v) were also prepared.

2.3. Instrumentation

The LC-ESI-LTQ-Orbitrap MS method was carried out using an Accela HPLC system from Thermo Fisher Scientific (Hemel Hempstead, UK) equipped with an autosampler injector, a thermostatically controlled column compartment and a linear ion trap quadrupole-Orbitrap-mass spectrometer LTQ-Orbitrap-MS from Thermo Fisher Scientific (Hemel Hempstead, UK). The analytical column was a reversed-phase Pursuit UPS 2.4 μm (50 × 2.0 mm) C18 column from Agilent Technologies (Waldbronn, Germany).

Auxiliary equipment was as follows: A CRISON 2002 potentiometer (±0.1 mV) from Crison S.A. (Barcelona, Spain) using a CRISON 5203 combination pH electrode was used to measure the pH of the buffers. A centrifuge 460R of Hettich Zentrifugen (Tuttlingen, Germany) was used to perform the extractions and obtain the final extracts. SPE was carried out on a SUPELCO vacuum manifold for 24 cartridges connected to a SUPELCO vacuum tank (Bellefone, PA, USA). TurboVap LV system with nitrogen stream was used for the evaporation of the extracts from Caliper LifeSciences (Hopkinton, MA, USA).

2.4. Procedures

2.4.1. Sample preparation procedures

2.4.1.1. Blank and positive samples. An amount of 4 g (±0.1 mg) of minced chicken muscle (blank or positive) or 2 g (±0.1 mg) of minced chicken kidney and liver was introduced into a 50 ml centrifuge tube (Macarov et al., 2012). The I.S., PIP, was added at a concentration of 300 μg kg^{-1}. Analytes were extracted from the muscle tissue with 2 ml water (1 ml for kidney and liver tissues) by shaking for 1 min. Then, 20 ml MeCN were added to muscle (10 ml to kidney and liver) in order to precipitate the proteins. Extracts were shaken for 1 min and the resulting mixtures were centrifuged at 3500 rpm for 5 min at 20 °C. Subsequently the organic solvent (MeCN) was eliminated by evaporation under nitrogen current at 35 °C. To improve the retention of penicillins on the SPE cartridge, 25 ml of 50 mM dihydrogenphosphate at pH 5.0 solution were added to the final muscle extracts (12.5 ml to liver and kidney).

2.4.1.2. Spiked samples. An amount of 4 g (±0.1 mg) of minced blank chicken muscle or 2 g (±0.1 mg) of chicken kidney and liver was introduced into a 50 ml centrifuge tube (Macarov et al., 2012). Samples were directly spiked with suitable volumes of AMX working solutions to provide the desired concentrations. PIP was also added at a concentration of 300 μg kg^{-1}. The samples were allowed to stand in the dark for 20 min at room temperature to promote the interaction between the antibiotics and chicken matrix. The recovery of AMX and its metabolites was as detailed above for blank and positive samples.

2.4.2. Solid phase extraction (SPE)

ENV + Isolute cartridges were activated with 2 ml of MeOH, 2 ml of MilliQ water and 2 ml of 50 mM dihydrogenphosphate (pH 5) solution. Sample extracts, as prepared in 2.4.1, were passed through the SPE system. Cartridges were then cleaned with 3 ml of dihydrogenphosphate and 1 ml of MilliQ water. The analytes were eluted with 4 ml of MeCN:MeOH (50:50; v:v). The samples were evaporated to dryness at 35 °C under current of nitrogen. 200 μl of MilliQ water were added to muscle (100 μl to kidney and liver) in order to redissolve the residue. The samples were stored in a freezer at −80 °C until analysis. Prior to injection into the chromatographic system, samples were thawed and filtered with microcon filter.

2.4.3. Liquid chromatography-mass spectrometry (LC-ESI-LTQ-Orbitrap MS)

LC-MS conditions were established by multiple injection of individual standard of AMX using a Pursuit UPS C18 column. The separation was carried out under the elution gradient given in Table 1 using 5 mM ammonium acetate adjusted at pH 2.5 with formic acid and MeCN as the eluents. The flow rate the mobile phase was maintained at 0.3 ml min^{-1} and the injection volume was 10 μl.

The ESI-LTQ-Orbitrap MS conditions were optimized by automatic gain control (AGC) by direct infusion of 100 μg ml^{-1}