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Quantitative determination of salicylic acid and metabolites in animal tissues by liquid chromatography–tandem mass spectrometry

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Abstract

In order to study the excretion pattern of salicylic acid and its metabolites in animals, a liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed. The method was used to study the biotransformation of salicylic acid in poultry (chicken and pigeon) after therapeutic use, i.e. in matrices such as plasma, excreta (=combined urine and faeces), kidney and liver. Thereafter, the method was also adopted for the analysis of residues of salicylic acid in porcine tissues, such as muscle, kidney, liver and skin + fat.

For the biotransformation study, salicylic acid, the oxidation and amino acid conjugation metabolites such as gentisic acid, salicyluric acid, and a double conjugated ornithine metabolite, together with the internal standard phenoxyacetic acid were extracted from 1.0 g of tissue or 100 μl of plasma into ethyl acetate, after acidifying using 1 M HCl. After centrifugation and for kidney and liver tissue, the analytes were back-extracted and subjected to a solid-phase clean-up on SAX columns. For the analysis of excreta, the sample preparation included only a dilution step, performed before LC–MS/MS analysis.

For the residue study, salicylic acid and the internal standard phenoxyacetic acid were extracted from 1.0 g of porcine muscle, liver, kidney and skin + fat with 6 ml of diethyl ether, after acidifying using 3 ml of 1 M HCl. After extraction and centrifugation, the ether phase was evaporated under N_2 at 40 °C. The residue was redissolved with 500 μl of 0.1% acetic acid in water. For skin + fat and when needed for the other tissues, a supplemental extraction of the redissolved residue with 500 μl of hexane was performed.

The analysis of the extracts was done on a Nucleosil 100-5 C18 column, using a gradient elution with 0.1% acetic acid in water and methanol. A Quattro Ultima® triple quadrupole instrument was used, equipped with an ESI z-spray source, which was operated in the negative ion MS/MS mode.

The methods were validated for the linearity (100–1000 ng ml^{-1} and 10–50 μg ml^{-1} for plasma; 5–250 μg g^{-1} for excreta; 100–1000 ng g^{-1} and 5–25 μg g^{-1} for the biotransformation study in poultry kidney and liver; 25–1000 ng g^{-1} for the residue study in porcine tissues); trueness and precision; specificity; limit of detection and limit of quantification. The limit of quantification for the residue analysis of salicylic acid in porcine tissues was set at 50 ng g^{-1}.

Keywords: Salicylic acid; Metabolites; Amino acid conjugates; Poultry; Porcine; Residues; LC–MS/MS

1. Introduction

Salicylic acid is the basic substance of the salicylates, which are non-steroidal anti-inflammatory drugs (NSAIDs). Salicylic acid, its sodium and aluminium salt and methyl salicylate are used topically in cattle, horses, sheep, goats and poultry. Acetylsalicylic acid, mainly as its sodium salt; acetylsalicylic acid DL-lysine, carbasalate calcium and sodium salicylate are substances that can be used orally in drinking water and feed for pigs, calves and chickens. Besides the therapeutic use of salicylates as anti-inflammatory, analgesic and antipyretic agents; some of these substances are also used as anti-clotting agents to obtain a more efficient

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bleeding. In the latter context, a possible misuse in poultry results in a more white colour of meat, relevant in some cases as a quality parameter.

The Committee for Veterinary Medicinal Products (CVMP) of the European Medicines Evaluation Agency (EMEA) concluded that there is no need to establish a maximum residue limit (MRL) for salicylic acid; sodium salicylate; aluminium salicylate, basic; and methyl salicylate, and therefore included these substances in Annex II of Council Regulation No. 2377/90, i.e. for topical use only in all food producing species, except fish. Acetylsalicylic acid, sodium acetylsalicylate, acetylsalicylate DL-lysine and carbasalate calcium are also included in Annex II, i.e. for all food producing species, except fish and animals from which milk or eggs are produced for human consumption. Recently, the CVMP also included sodium salicylate in Annex II, for oral use only in bovine and porcine species, but not in animals from which milk is produced for human consumption.

In humans, salicylic acid is metabolized to various compounds. The main important metabolites are salicyluric acid, also called the glycine conjugate; salicylic acid phenolic glucuronide; salicylic acid acyl glucuronide and the oxidation product gentisic acid. Salicyluric acid is also further conjugated with glucuronic acid to form salicyluric acid phenolic glucuronide. In animals however, the knowledge of the metabolism of salicylic acid is limited. The fate of the simplest aromatic carboxylic acid, benzoic acid, has been studied extensively as a model compound for biotransformation reactions in animals, and it has been demonstrated to undergo conjugation with sugars (glucuronic acid in vertebrates, glucose in insects) or amino acids [1]. For the amino acid conjugation, it is known that in birds belonging to the order of Galliformes (chicken, turkey, quail), Anseriformes (duck, goose) and in some reptilian species, the major metabolite of benzoic acid is ornithurate (α,δ-dibenzoylornithine) [2–4]. Whereas in most mammals and in some bird species belonging to the order of Columbiformes (pigeon, woodpigeon, dove), conjugation of benzoic acid with glycine resulting in the formation of hippurate (benzoylglycine) is present.

In order to study the biotransformation of salicylic acid, chromatographic methods able to detect the metabolites should be available. Several LC–UV methods were described for the determination of salicylic acid, together with its glycine and glucuronide conjugates in human plasma and urine [5–7]. However, no methods are available for the analysis of the ornithine metabolite. Capillary electrophoresis was also used for the determination of salicylic acid, gentisic acid and the glycine conjugate in human urine [8]. Mass spectrometry was only used in combination with gas chromatography for the determination of salicylic acid in plants [9,10], and in combination with liquid chromatography for the determination of salicylic acid in surface water and wastewater [11].

The aim of this research was two-fold. First, in order to study the differences in amino acid conjugation, especially between chicken and pigeon, a liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method able to detect the metabolites, was developed. The method was used to study the biotransformation of salicylic acid in poultry (chicken and pigeon), i.e. in matrices such as plasma, excreta (=combined urine and faeces), kidney and liver, where therapeutic concentrations (and no residue concentrations) were expected. No other tissues such as skin + fat or muscle were sampled.

Secondly, the method for the biotransformation study was adopted in order to perform the analysis of residues of salicylic acid in porcine tissues, such as muscle, kidney, liver and skin + fat, which can be useful for residue monitoring purposes.

2. Biological samples for the biotransformation study in birds

Sodium salicylate was administered by intravenous bolus injection in the *vena basilica* (wing vein) at a dose of 25 mg kg^{-1} BW to eight broiler chickens (1.8 ± 0.2 kg) and eight pigeons (0.45 ± 0.02 kg). Blood was taken from a leg vein at following time points after the administration: 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 30, 36 and 48 h from six pigeons and at 0, 0.33, 0.66, 1, 2, 3, 4, 6, 8, 12, 24 and 36 h from six chickens. All the combined urine and faeces samples were collected and pooled from six different birds at several time points after the administration, i.e. at 0, 2, 4, 6, 8, 12, 24 and 36 h for chickens and at 0, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h for pigeons. The kidneys and liver of the two other treated pigeons and chickens were collected at 28 and 3 h after administration, respectively.

3. Experimental

3.1. Chemicals

Salicylic acid (SA); the glycine conjugate of SA (salicyluric acid, SU); gentisic acid (GA, 2,5-dihydroxybenzoic acid, which is an oxidation product); and the IS phenoxyacetic acid (PAA) were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). The double conjugated ornithine metabolite of SA (OR) was not commercially available and was synthesized by the Laboratory of Medicinal Chemistry of the Faculty of Pharmaceutical Sciences (Ghent University, Belgium) [12]. The chemical structure of all analytes is shown in Fig. 1. Stock solutions of 10000 μg ml^{-1} SA, SU and GA; 3000 μg ml^{-1} OR; and 1000 μg ml^{-1} PAA in methanol were prepared and were stored in the dark at ≤−15 °C for at least 1 month. Appropriate working solutions were prepared in HPLC water and stored between 2 and 8 °C. All products used for the extraction procedure were of analytical grade (glacial acetic acid; Na_2CO_3 and $NaHCO_3$; NaH_2PO_4 and Na_2HPO_4 for the preparation of a carbonate and phosphate buffer, respectively; ethyl acetate; HCl 37%; hexane; trifluoroacetic acid or TFA). Methanol