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Determination of acetylsalicylic acid and its major metabolites in bovine urine using ultra performance liquid chromatography

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ABSTRACT

A new method based on ultra high performance liquid chromatography (UPLC) with photometric and fluorometric detection for the determination of acetylsalicylic acid and its main metabolites, namely gentisic, salicylic and salicyluric acids, in bovine urine samples is reported. Photometric detection was used for acetylsalicylic acid determination, whereas the native fluorescence of the metabolites was monitored using fluorometric detection. The separation was performed under isocratic conditions, using acetonitrile–phosphate solution (3.5 mM, pH 3.5) (26:74, v/v) as the mobile phase. The retention times of the four compounds were lower than 2 min, which are shorter than those achieved using conventional HPLC. Under the optimum separation conditions, the dynamic ranges and detection limits (ng mL^{-1}) were: 0.2–2500, 0.09 for gentisic acid; 0.2–2500, 0.08 for salicylic acid and 2.5–15,000, 1.1 for salicyluric acid, using fluorescence detection, and 10–25,000, 2.2 for acetylsalicylic acid, using UV detection. Intra-day and inter-day precision data were assessed at two levels of concentration of each analyte using both detection systems. The selectivity of the method was checked by assaying different drugs of veterinary use showing that most of them did not interfere with the determination of the analytes. The method has been applied to the analysis of bovine urine samples, which only required a simple clean up step of the samples prior to injection in the UPLC system. A recovery study was performed, which provided values in the range of 80–100%. This fact proves the practical usefulness of this method as an ultrafast analytical tool for the therapeutic control of acetylsalicylic acid administration in bovine animals intended for food production.

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1. Introduction

Acetylsalicylic acid (ASA) is a non-steroidal anti-inflammatory drug, which therapeutic action happens both at locally and peripheral inflammatory sites by inhibiting cyclooxygenase enzymes [1]. ASA has a really widespread use because it also has other beneficial therapeutic properties, such as analgesic and anti-pyretic action. ASA is rapidly hydrolyzed to salicylic acid (SAL), which is the active metabolite and has a half-life of 2.5–4 h in the body. It can be further conjugated to glycine to give rise to salicyluric acid (SAU) or to glucuronic acid to form glucuronide conjugates or being excreted as unchanged SAL [2]. Another SAL metabolite is gentisic acid (GEN), which is obtained after SAL oxidation. These metabolites are eliminated from the body by urinary excretion, their ratio and concentration being dependent on urinary pH. The renal elimination of SAL in ASA overdose cases is favored by increasing the pH to alkaline values. Owing to this behavior, although excretion of ASA metabolites follows zero-order kinetics, there is not always a clear correlation between their serum and urine concentrations. However, urine is a specimen easy to obtain that can provide an initial estimation of ASA intake, being ferric chloride used as reagent when overdose is suspected [3]. If positive, these results can be later confirmed by analyzing serum samples.

ASA and SAL are used for veterinary purposes, the extent of their use depending on the region of the world considered. For instance, the European Union allows their use for veterinary purposes in all food producing species except fin fish and animals intended for egg or milk production [4]. The US Food and Drug Administration totally disapproves their use in cattles and calves because the lack of evidence about their therapeutic action in these animals [5]. The occurrence and fate of SAL in calf urine samples have been previously studied [6], finding that the prevalence of

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the different metabolites depends on the administration route. The intravenous injection leads to high SAL concentration in urine samples, whereas oral administration enhances SAU concentration in this biological fluid. It has been reported that the concentration of glucuronide conjugates in bovine urine samples is almost negligible, which simplifies the assay and eliminates the need of using glucuronidase enzyme for the determination of total ASA metabolites in this species.

Although some sensors have been developed for the determination of ASA [7] and SAL in urine samples [8], the determination of ASA and its metabolites is usually carried out using a separation technique, such as capillary electrophoresis (CE) [9–11], HPLC [2,12–19] and ultra high performance liquid chromatography (UPLC) [20–22]. CE methods mainly involve laser-induced fluorescence detection to overcome the sensitivity problems associated to injecting only some nL of each sample. Numerous HPLC methods have been described using photometric [2,12–14], MS [2,16,17] and, in less extension, fluorometric [18,19] and amperometric [15] detection. These chromatographic methods have been applied to the analysis of human biological fluids, such as serum [12,15,19], plasma [13,14,17,18] and urine [13,14] and most of them only determine ASA and SAL [12,17–19]. An HPLC method with fluorometric detection has involved a post-column hydrolysis reaction to achieve the determination of ASA, which needs the use of a second pump and all lines and connectors associated that complicate the experimental set-up [19].

UPLC with UV detection has been used to determine ASA and SAL in human plasma samples in less than 3 min under isocratic conditions [20,21], which was applied to perform pharmacokinetic studies. Another UPLC method has been reported for the determination of ASA and other anti-inflammatory drugs (ketoprofen, diclofenac, naproxen and ibuprofen) in human urine samples, using microextraction by packed sorbent, to perform the sample preparation [22]. Also, UPLC with tandem MS has been applied to the determination of drug residues, including SA, in milk and dairy products [23] and infant formula samples [24]. However, the use of UPLC for the fast determination of ASA and its metabolites SAL, SAU and GEN in bovine urine samples has not been reported up to date.

The method presented here describes the separation of these compounds in less than 2 min under isocratic conditions. Photometric and fluorometric (FL) detectors have been used to perform the determination, which provide complementary information. A solid phase extraction (SPE) with polymeric cartridges has been included to clean up the samples prior their introduction into the chromatographic system. Afterwards, a simple dilution step was performed in order to match the dynamic range of the calibration curves, which were obtained by using external calibration with aqueous standards. The low detection limits obtained enable a reliable detection of ASA and its metabolites in bovine urine samples, which can be used as part of the process of monitoring and surveillance of animal origin products.

2. Experimental

2.1. Instrumentation and chromatography conditions

The UPLC system used consisted of the high pressure pump unit (LC-30AD), a degasser (DGU-20A5), an autosampler (SIL-30AC) equipped with a sample cooler set to 15 °C, a column oven (CTO-10AS vp), an UV/vis detector (SPD-20A) and a fluorescence detector (RF-20AXS) (Shimadzu, Kyoto, Japan). A Syncronis C18 column (100 mm × 2.1 mm I.D., particle size 1.7 μm; Thermoscientific, Inc., Walthan, MA) was used as stationary phase for the separation. A 24-port VisiPrep vacuum device and HLB cartridges (60 mg/3 mL) from Supelco (Bellefonte, PA) were used to perform SPE experiments.

2.2. Reagents and solutions

All reagents were of analytical grade. Gentisic acid was purchased from Sigma (St. Louis, MO, USA), salicylic acid from Aldrich (Milwaukee, USA), salicyluric acid from Zerenex molecular (Greater Manchester, UK) and acetylsalicylic acid from Fluka (Buchs, Switzerland). Stock solutions of these compounds (1000 mg L^{-1} each) were prepared in acetonitrile (ACN) and stored at 4 °C. Working solutions were prepared by dilution of the stock solutions in doubly deionized water. ACN, methanol (both of HPLC-gradient grade) and 85% ortho-phosphoric acid were supplied by Panreac (Castellar del Vallès, Spain). The pH of the phosphate solution (3.5 mM) was adjusted to 3.5 by adding sodium hydroxide and the solution was filtered using a 0.22 μm nylon membrane filter (Millipore, Bedford, USA). Bovine urine samples were kindly donated by Cooperativa Ganadera del Valle de los Pedroches (Córdoba, Spain).

2.3. Procedures

2.3.1. Determination of acetylsalicylic acid and its metabolites by UPLC

A volume (8 μL) of a standard mixture of analytes or diluted sample extract, in the ranges of 0.2–2500 ng mL^{-1} for GEN and SAL, 2.5–15,000 ng mL^{-1} for SAU, and 10–25,000 ng mL^{-1} for ASA, was injected onto the chromatographic system. The analytes were isocratically separated using a mobile phase composition of (26:74, v/v) ACN – 3.5 mM phosphate solution (pH 3.5) at a flow-rate of 0.63 mL min^{-1}. The temperature was kept at 40 °C in the column oven. Chromatograms were obtained using photometric and fluorometric detectors. UV chromatograms were recorded at 240 nm using a response time of 1 s and the temperature of the detector was set at 40 °C. Fluorescence detection was performed using a wavelength program, as follows: λ_{ex} 320 nm λ_{em} 444 nm from 0 to 1.14 min and λ_{ex} 295 nm λ_{em} 410 nm from 1.15 to 3 min, with a spectral bandwidth of 20 nm and a time response of 1 s in each case, and the measurements were obtained at 40 °C. Each standard or sample extract was assayed in triplicate. Chromatograms were processed by using LabSolutions LC/GC software (release 5.42 SP5).

2.3.2. Assessment of precision

The precision of the method using photometric and fluorometric detection was evaluated at two different concentration levels, 100 and 500 ng mL^{-1} and 15 and 200 ng mL^{-1}, respectively. Precision values were obtained by injecting six solutions on the same day, which were analyzed in triplicate (intra-day data), and one solution, which was analyzed in triplicate for 15 different days (inter-day data). The percentage of relative standard deviation (%RSD) was calculated for retention times and areas in each case.

2.3.3. Estimation of limits of detection

The estimation of the limits of detection (LODs) was done following IUPAC recommendations [25], which involve the use of a signal-to-noise ratio of 3. The signal considered as the blank signal was the *y*-intercept of the calibration curve made using 15 mixtures of aqueous standards of the compounds determined.

2.3.4. Analysis of bovine urine samples

A volume (1.0 mL) of urine sample was acidified to pH 1.3 with 1 M HCl and immediately applied to HLB cartridges previously conditioned using 5 mL methanol and 5 mL of 0.045 M HCl (pH 1.3). Cartridges were washed with 2 mL of 0.045 M HCl (pH 1.3) to remove interferences and the analytes were eluted with 1 mL of 90% ACN. Afterwards, extracts were diluted in ratios (1:500–1:2000) to match the dynamic range of the calibration curves. A volume of the diluted extract was treated as indicated in Section 2.3.1. The recovery study was carried out using urine samples spiked with different

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