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Quantification of doping compounds in faecal samples from racing pigeons, by liquid chromatography-tandem mass spectrometry☆

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ABSTRACT

The use of performance enhancing drugs is not only common in humans, but also in animal sports, including racing of horses, greyhounds and pigeons. The development of accurate analytical procedures to detect doping agents in sports is crucial in order to protect the fair-play of the game, avoid financial fraud in the attribution of eventual awards and, even more important, to protect the animals from harmful drugs and/or dangerous dosage regimens. The present study aimed to develop and validate, a method that enabled the screening and confirmation of the presence of a beta-agonist (clenbuterol) and three corticosteroids (betamethasone, prednisolone and budesonide) in faeces from pigeons. The extraction procedure entailed the combination of liquid-liquid extraction with solid-phase extraction and the analysis was performed by liquid- chromatography coupled to tandem mass spectrometry, with a single 15 minute chromatographic run-time. The method was validated concerning selectivity, linearity (with coefficients of determination always > 0.99), accuracy (87.5–114.9%), inter-day and intra-day precisions, limits of detection (0.14–1.81 ng/g) and limits of quantification (0.49–6.08 ng/g), stability and extraction recovery (71.0%–99.3%). The method was successfully applied for the analysis of samples from two pigeons that had been orally administered betamethasone, demonstrating its suitability for doping control purposes.

1. Introduction

Over the last decades, the interest on the detection of forbidden substances in biological matrices of animals has grown exponentially. The two major reasons to perform these analytical studies are the control of misused drugs as growth promoters in food-producing animals [1–4], and the detection and punishment of the artificial increase of mental and physical abilities of animals involved in sports, commonly known as doping [5–7]. The potential practice of negative doping, in order to sabotage other competitors, has also been addressed in some studies, in which compounds such as sedatives were included in the anti-doping analysis [8,9]. The motivation for doping sport animals goes well beyond recreational issues, since many times the breeders seek to ensure financial benefits from prizes and gambling [10]. As well as with human athletes, it is acknowledged that animals might be doped with a diverse number of substances including, β2 agonists [11], corticosteroids (anti-inflammatory agents) [12], anabolic steroids (muscle building agents and promoters of reduced recovery times after energy expenditure) [13] and opioids (due to their analgesic effects with the purpose of recovering the animal after the competition and also for masking pain during competition) [14]. Apart from the ethical judgement owing to the lack of equality of opportunity, and the unfair financial gains of cheaters, the reasons for banning the misuse of drugs, both as growth promoters and in sports, also include the health risks of performance-enhancing substances both for animals and for consumers [15]. Horse, greyhound, and pigeon racings are thought to be the animal sports that raise more suspicions about the possible introduction of substances to improve their performance.

Pigeon racing is an ancient sport, very popular in many European countries, the United States of America (USA), Taiwan and China, where over 3 million pigeons are registered for gambling purposes, only in Beijing [16]. However, scientific literature about doping analysis in

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racing pigeons (*Columba livia*) is still scarce. It is therefore important to develop and improve analytical procedures for the screening of doping agents in these animals, ultimately promoting the compliance with the regulations of the different competent authorities/federations of several countries, namely Portugal [17], USA [18], Belgium [19] and Australia [20]. These federations foresee severe penalties for the use of doping drugs such as corticosteroids, β2 agonists, beta-blockers, anabolic steroids, non-steroidal anti-inflammatory drugs and masking agents. Indications for use of corticosteroids in birds are similar to mammals, and dosages and modes of application are derived from those of mammals. Glucocorticoids, such as dexamethasone, betamethasone, prednisolone, and hydrocortisone, are often used as a doping agent in pigeons in Europe. They are thought to enhance racing performance by inhibiting molt, which results in a full wing during competition. These doping glucocorticoids are administered orally, in the drinking water, or as ophthalmic drops [21].

To our knowledge, the only two articles published about doping analysis in racing pigeons date from 1996 [22] and 1997 [23]. In both cases, faeces were the chosen biological matrix and analysis was performed by high performance liquid chromatography coupled to enzyme-linked immunosorbent assay (HPLC-ELISA). As with other animal species, the identification of drugs in pigeon faeces is very difficult, due to the complexity of this matrix that hinders the extraction and recovery of the analytes [24]. Furthermore, the metabolic pathways of these drugs in pigeons are unknown and considering the size and weight of pigeons, the administered doses of most drugs are minimal, making their detection difficult. Considering these constraints, conventional matrices such as blood and urine would probably be preferred. Nevertheless, these matrices are not available in pigeons: urine is combined with faeces in the cloaca and the volume of blood that would be available to be collected is insufficient for an anti-doping analysis [22]. Therefore, faeces are an important alternative sample that has to be considered, owing to its ready and non-invasive availability in relatively large amounts.

The present work represents a successful attempt to simultaneously quantify the β2 agonist drug, clenbuterol and three corticosteroids (budesonide, prednisolone and betamethasone), in pigeon faeces using liquid chromatography tandem-mass spectrometry (LC-MS/MS). Even though our test substances are exogenous to the animal and nowadays strictly forbidden in racing pigeons, due to their potential therapeutic use, threshold values may be assigned as is the case of some beta-agonist drugs [17]. On the other hand, given the nature of the analytical matrix, and the low dosages applied to these small animals, highly sensitive quantitative methods are preferred over broader screening methods that may fail to detect the expected very low concentrations. To this end, a sensitive, fast and reliable method, able to detect trace amounts of doping agents in pigeon faeces, was developed and validated.

2. Materials and methods

All reagents used in the present work were of analytical grade or of the highest grade available. Sodium acetate, sodium chloride (NaCl), sodium hydroxide (NaOH), ethyl acetate, methanol, acetonitrile, ammonium acetate, and formic acid were obtained from Merck (Darmstadt, Germany). Prednisolone, betamethasone, clenbuterol hydrochloride, budesonide, methyltestosterone (internal standard, IS), and β-glucuronidase (≥100,000 units/mL) from *Helix pomatia* were obtained from Sigma (St. Louis, MO, USA). HPLC-grade water was obtained through a MilliQ system (Millipore, Lisbon, Portugal). OASIS HLB 3 cm^3 columns were purchased from Waters Technologies (Lisbon, Portugal).

2.1. Biological specimens

Drug free faeces were collected from four racing pigeons (weight ranging between 300 and 350 g) kept in appropriate cages under a controlled drug-free diet. Tap water was available ad libitum. The faeces were collected daily into an aluminum foil and kept at −20 °C until analysis. These blank samples were previously checked to ensure the absence of the analytes and were used to prepare the calibrators and quality control samples.

For proof of applicability, two racing pigeons were administered orally with a daily dose of 0.075 mg of betamethasone. This drug was chosen since it is one of the most frequently used for doping racing pigeons [21] and the dose corresponds to the dose range that was admitted by the pigeon breeders that were interviewed for this purpose under anonymity. The dosage also corresponds to the adaptation of the usual dosage in mammals, to the weight of the birds, as already stated in previous published works [21]. One of the pigeons received four daily doses while the other received three daily doses. Faeces were collected daily. During the three days that followed the final administration, faeces were also collected daily to investigate whether betamethasone could still be detected after ceasing drug administration. The samples collected from the second pigeon were also submitted to an enzymatic hydrolysis prior to the extraction procedure to check whether the drug was excreted conjugated with glucuronide and/or sulfate. For this purpose, aliquots of 2 g were transferred into a 25 mL glass tube and 1 mL of 0.2 M sodium acetate pH 5.2 and 50 μL of β-glucuronidase from *Helix pomatia* were added. The samples were then incubated at 37 °C for 24 h and afterwards submitted to the extraction procedure described below.

All procedures involving animals were reviewed, approved, and performed in accordance with the Ethics Committee guidance of the Faculty of Pharmacy of the University of Porto.

2.2. Calibrators and quality control samples

Standards and internal standard stock solutions of 1 mg/mL were prepared in methanol and stored at −20 °C. All intermediate solutions were also prepared in methanol and stored at −20 °C. Working calibrators were prepared by spiking 5 mL of blank faeces homogenates (prepared as described below) with the methanolic stock solutions of the different drugs to obtain the final concentrations of 6.12, 12.24, 24.53, 49.05, 98.11, 196.21, 392.43 ng per gram of faeces of betamethasone; 6.72, 13.43, 26.91, 53.82, 107.63, 215.27, 430.53 ng per gram of faeces of budesonide, 4.32, 8.65, 17.32, 34.65, 69.30, 138.60, 277.19 ng per gram of faeces of clenbuterol and 5.62, 11.25, 22.53, 45.06, 90.11, 180.22, 360.44 ng per gram of faeces of prednisolone. For all studies performed for the method validation, the internal standard concentration was set to 37.81 ng/g.

2.3. Extraction procedure

Samples of 2.0 g of faeces were transferred into 50 mL falcon tubes and vortex mixed with 5 mL of 0.2 M sodium acetate solution pH 5.2. When preparing working calibrators and quality control samples, these blank samples were then spiked with appropriate amounts of the methanolic stock solutions to obtain the desired concentrations. After spiking, the samples were continuously shaken for 10 min (min) using a VV3 mixer (VWR, Lisbon, Portugal). After homogenization, 0.5 g of NaCl was added to the samples, followed by pH adjustment to pH 9.5 with 10 M NaOH. The analytes were extracted by adding 7 mL of ethyl acetate. The tube was vortex mixed for 1 min, continuously shaken for 20 min, and centrifuged for 20 min at 3220*g* in a refrigerated centrifuge at 4 °C. The organic phase was transferred into a clean tube, and the aqueous phase was kept. The extraction process was repeated and the organic phases were combined. The samples were evaporated to dryness under a gentle nitrogen stream. The dry residue was dissolved with 4 mL of water/methanol (5:1). For solid phase extraction (SPE) the Supelco™ Visiprep SPE vacuum manifold device was used (Sigma-Aldrich). The totality of the suspended sample was transferred onto the