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A validated analytical method to study the long-term stability of natural and synthetic glucocorticoids in livestock urine using ultra-high performance liquid chromatography coupled to Orbitrap-high resolution mass spectrometry

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ABSTRACT

Due to their growth-promoting effects, the use of synthetic glucocorticoids is strictly regulated in the European Union (Council Directive 2003/74/EC). In the frame of the national control plans, which should ensure the absence of residues in food products of animal origin, in recent years, a higher frequency of prednisolone positive bovine urines has been observed. This has raised questions with respect to the stability of natural corticoids in the respective urine samples and their potential to be transformed into synthetic analogs. In this study, a ultra high performance liquid chromatography–high resolution mass spectrometry (UHPLC–HRMS) methodology was developed to examine the stability of glucocorticoids in bovine urine under various storage conditions (up to 20 weeks) and to define suitable conditions for sample handling and storage, using an Orbitrap Exactive™. To this end, an extraction procedure was optimized using a Plackett–Burman experimental design to determine the key conditions for optimal extraction of glucocorticoids from urine. Next, the analytical method was successfully validated according to the guidelines of CD 2002/657/EC. Decision limits and detection capabilities for prednisolone, prednisone and methylprednisolone ranged, respectively, from 0.1 to 0.5 $\mu g\,L^{-1}$ and from 0.3 to 0.8 $\mu g\,L^{-1}$. For the natural glucocorticoids limits of detection and limits of quantification for dihydrocortisone, cortisol and cortisone ranged, respectively, from 0.1 to 0.2 $\mu g\,L^{-1}$ and from 0.3 to 0.8 $\mu g\,L^{-1}$. The stability study demonstrated that filter-sterilization of urine, storage at −80 °C, and acidic conditions (pH 3) were optimal for preservation of glucocorticoids in urine and able to significantly limit degradation up to 20 weeks.

1. Introduction

The well-known anti-inflammatory properties of the natural glucocorticoid cortisol, has led to the development of synthetic glucocorticoid analogs, which exert even higher anti-inflammatory activities [1]. Nowadays, the commonly used therapeutic glucocorticoid drugs in veterinary medicine comprise betamethasone, dexamethasone, methylprednisolone and prednisolone, with prednisone as prodrug. Beside the anti-inflammatory properties, these drugs also induce body weight gain in production animals by improving feed intake and lowering feed conversion. However, due to their growth-promoting effects and potential consumer's health risks [2,3], the use of glucocorticoids in livestock has been strongly restricted within the European Union [4].

The analysis of glucocorticoids, which is of critical importance in light of the national control plans within the EU, remains, however, a challenging task. After all, glucocorticoid residues are typically present in urine at very low concentrations in a background of a wide range of more abundant primary and secondary metabolites [5]. Besides, chromatographic separation of these compounds is not straightforward due to their similar chemical configurations and

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chromatographic behavior [6,7]. Because of the identical molecular masses of cortisone and prednisolone, co-elution may result in a loss of selectivity. Indeed, the natural prevalence of the glucocorticoids cortisol and cortisone in urine at low concentrations may hamper the analysis of prednisolone and prednisone [5]. Nevertheless liquid chromatography coupled to mass spectrometry has been proven suitable to enable sensitive detection of glucocorticoids in urine [6,8–10]. Because conventional liquid chromatography presents some limitations in terms of a longer run time and lower chromatographic resolution, ultra-high performance liquid chromatography (U-HPLC) using columns with sub 2 μm particles, is more commonly used these days [11–13]. Due to the very narrow peaks produced by U-HPLC, a compatible fast scanning MS device is required. The most applied mass spectrometric technique in hormone and veterinary drug residue analysis is quadrupole tandem mass spectrometry (QqQ-MS/MS) [14], relying on the high sensitivity and selectivity of the selected reaction-monitoring (SRM) mode of QqQ-MS/MS. This technology has certain limitations since there is no possibility of post-acquisition re-interrogation of data and only a limited number of compounds can be measured within one run (max. 100–120 using timed SRM). Also the screening for unidentified and unknown compounds is not possible because the fragmentation behavior of the compound is unknown [15,16]. Because of these limitations, there is currently a trend toward full scan high resolution MS analysis using amongst others ToF (time of flight) instruments, with mass deviations below 5 parts per million (ppm) and resolutions of about 15,000 full width at half maximum (FWHM) [11,15]. However, in complex matrices this resolution is inadequate for accurate mass measurements. One of the most attractive and relatively new techniques is the Fourier Transform Orbitrap mass spectrometric technology with a resolving power up to 100,000 FWHM and a precise mass deviation below 2 ppm [15], allowing fast, reproducible and reliable analytical results for multiple residue analysis [12].

Prior to HRMS analysis, it is common to apply a generic extraction to allow as much as possible relevant analytes to be retained in the extract, but at the same time remove potential matrix interferences. The Plackett–Burman experimental design is a highly efficient and useful tool to screen for the main variables within a large number of variables that may affect the extraction yield [17,18]. This time saving approach, providing the opportunity to identify the optimal conditions for extraction of a certain number of analytes from a matrix by evaluating a large number of variables with a minimum of experiments. It also permits estimation of random error variability and testing the statistical significance of the variables [17,19].

The European Commission reported in 2012 in the Commission Staff Working Document on the implementation of national residue monitoring plans in the member states in 2010 that 0.14% of the bovine urine samples were non-compliant for prednisolone (3.12–179.72 $\mu g\,L^{-1}$) [20]. This high frequency of bovine urine samples found positive for prednisolone without any direct evidence for illegal use, has raised many questions [8–10,21]. A possible hypothesis is the conversion of the natural glucocorticoid cortisol into prednisolone, during inappropriate storage [9,10]. The prevalence of fecal microbiota in urine may indeed alter the endogenous concentration of steroid hormones [22,23]. A well-known example of such a process is the microbial transformation of testosteron to boldenone by a Δ^1-dehydrogenation [24,25]. Due to structural similarities, similar reactions may be expected from cortisol to prednisolone and cortisone to prednisone (Fig. 1) [23]. Research with respect to the long-term stability of these compounds and possible changes occurring during storage is, however, scarce and mainly targeted oriented.

Therefore, the present study examined the changes in glucocorticoid concentrations of bovine urine samples during a long-term storage experiment, in which the effect of different storage conditions such as pH and temperature were considered. Additionally, the preservation under aerobe and anaerobe environments, as well as the contamination with fecal bacteria, was evaluated. Furthermore, this stability study included the determination of losses during multiple freeze–thaw cycles. The compounds of interest were the natural glucocorticoids cortisol, cortisone and dihydrocortisone and the synthetic glucocorticoids prednisolone, prednisone and methylprednisolone. To this extent, a generic extraction and targeted U-HPLC–Orbitrap-HRMS approach, with the possibility of untargeted screening, was developed and validated according to the guidelines of CD 2002/657/EC [26].

2. Materials and methods

2.1. Reagents and chemicals

Standards of prednisolone, prednisone, cortisone, cortisol, dihydrocortisone and methylprednisolone were purchased from Sigma–Aldrich (St. Louis, MO, USA). Internal standards were cortisol-d4 (Sigma–Aldrich) and prednisolone-d8 (TRC, Canada). Reagents were of analytical grade when used for extraction purposes and obtained from VWR International (Merck, Darmstadt, Germany). The reagents were of LC–MS Optima grade for U-HPLC–HRMS application. These were obtained from Fisher Scientific UK (Loughborough, UK). Ultrapure water was obtained by usage of a purified-water system. For filter-sterilization of urine, membrane filters of polyvinylidene fluoride (0.22 μm pore size) were purchased from Millipore (Billerica, USA).

Primary stock solutions were prepared in ethanol at a concentration of 200 $\mu g\,mL^{-1}$ and stored in dark glass bottles at −20 °C. Working solutions were made in ethanol at a range of 0.1–10 $\mu g\,mL^{-1}$.

2.2. Instrumentation

Analyses were carried out on an U-HPLC system, which consisted of an Accela U-HPLC pump, an Accela Autosampler and Degasser (Thermo Fisher Scientific, San José, CA, USA). Separation of the glucocorticoids was carried out on a reverse phase Nucleodur C_{18} Isis U-HPLC column (1.8 μm, 100 mm × 2 mm, Macherey-Nagel, Düren, Germany) at a column oven temperature of 30 °C. The elution gradient was carried out with a binary solvent system consisting of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) at a constant flow rate of 0.3 $mL\,min^{-1}$. Optimized separation of all analytes was obtained using a linear gradient starting with a solvent mixture (v/v) of 75% A and 25% B, which was held for 4.0 min. The percentage of acetonitrile was increased to 95% in 0.1 min, and further to 100% in 1.4 min and held there for 2.0 min. Between samples, the column was allowed to re-equilibrate at initial conditions for 1.5 min. A 10 μL aliquot of each sample was injected for analysis. High-resolution mass spectrometric analysis was performed on an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization probe (HESI II), operating in both the positive and negative ionization mode. Ionization source working parameters were optimized and are reported in Table 1. The resolution was set at 100,000 FWHM at 1 Hz and a scan range of *m/z* 150–800 was chosen. The automatic gain control (AGC) target was set at balanced ($1 \times e^6$ ions) and the High Energy Collision Dissociation (HCD) cell was turned off. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific).

2.3. Samples

Urine was collected from five healthy, adult Holstein-Friesian cows, housed at the Institute for Agricultural and Fisheries Research