Journal of Chromatography A, 1336 (2014) 76–86

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development and validation of a high-resolution mass-spectrometry–based method to study the long-term stability of natural and synthetic glucocorticoids in faeces

Nathalie De Clercq[a], Julie Vanden Bussche[a], Siska Croubels[b], Philippe Delahaut[c], Lynn Vanhaecke[a,*]

[a] Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health & Food Safety, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium
[b] Ghent University, Faculty of Veterinary Medicine, Department of Pharmacology, Toxicology and Biochemistry, Laboratory of Pharmacology and Toxicology, Salisburylaan 133, B-9820 Merelbeke, Belgium
[c] CER Groupe, Département Santé, Rue du Point du Jour 8, B-6900 Marloie, Belgium

ARTICLE INFO

Article history:
Received 11 October 2013
Received in revised form 11 February 2014
Accepted 11 February 2014
Available online 18 February 2014

Keywords:
Faeces
glucocorticoids
Plackett–Burman
stability study
ultra-high performance liquid chromatography-orbitrap-high resolution mass spectrometry

ABSTRACT

Faecal glucocorticoid analysis is a powerful non-invasive tool for the study of the animal endocrine status and stress physiology, which is mainly carried out by immunoassays, characterised by some limitations. In this study, an ultra high-performance liquid chromatography coupled to high resolution Orbitrap mass spectrometry (U-HPLC-HRMS) method was developed to confirm the presence of glucocorticoids in bovine faeces during a long-term stability study. Because of the complex nature of faeces, an appropriate extraction and purification procedure was developed. To this extent, a Plackett–Burman experimental design was successfully applied to determine the key conditions for optimal extraction of glucocorticoids from faeces. The targeted analysis, including natural and synthetic glucocorticoids, was successfully validated according to CD 2002/657/EC. Decision limits and detection capabilities for prednisolone, prednisone, methylprednisolone and the metabolites 20α-dihydroprednisolone and 20β-dihydroprednisolone ranged, respectively, from 0.15 to 2.95 μg kg^{-1} and from 0.40 to 5.20 μg kg^{-1}. Limits of detection and limits of quantification for the natural glucocorticoids dihydrocortisone, cortisol and cortisone ranged, respectively, from 0.55 to 2.10 μg kg^{-1} and from 0.70 to 5.00 μg kg^{-1}.

The stability study of glucocorticoids in faecal matrix demonstrated that lyophilising the faeces, storage at -80°C, and aerobic conditions were optimal for preservation and able to significantly ($p < 0.05$) limit degradation up to 10 weeks.

1. Introduction

The glucocorticoids cortisol and cortisone are steroid hormones naturally synthesized in the adrenal cortex. Their well-known anti-inflammatory properties have led to the development of synthetic glucocorticoid analogues, which exert even higher anti-inflammatory activities i.e. betamethasone, dexamethasone, methylprednisolone and prednisolone, with prednisone as prodrug [1]. In the European Union, these compounds are permitted for therapeutic use in livestock. Beside the anti-inflammatory properties, these drugs also induce body weight gain in production animals by improving feed intake and lowering feed conversion. Due to their growth-promoting effects and the potential consumer's health risks of residues thereof [2,3], the use of synthetic glucocorticoids in livestock has been strictly regulated in the European Union [4], by setting maximum residue limits for betamethasone, dexamethasone, methylprednisolone and prednisolone in selected tissues of animal origin [5].

In the frame of the National Residue Monitoring Plans, liver, urine and faeces are frequently analysed to ensure the absence of residues in food products of animal origin and to detect possible illegal use as growth-promoter [6,7]. The European Union Reference Laboratories made a consensus to set the minimum required performance limit (MRPL) for prednisolone in bovine urine at 5 μg L^{-1} [8]. While liver samples can be merely obtained upon slaughtering [9,10], urine and faeces are easily accessible through non-invasive sampling. The use of faecal samples has some advantages over that of urine when focussing on the hormonal status

* Corresponding author: Tel.: +32 9 264 74 57; fax: +32 9 264 74 92.
E-mail address: Lynn.Vanhaecke@ugent.be (L. Vanhaecke).

0021-9673/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2014.02.033

of the animals and their long-term endocrine profile [11]. The sampling is easy, does not interfere with the stress response and permits on-farm monitoring. Faecal analyses are increasingly being used to examine glucocorticoids as a potential indicator of adrenal activity and animal stress [12]. They reflect an average level of circulating glucocorticoids over a time period, rather than a point sample, since the levels in faeces are less affected by episodic fluctuations or the pulsatility of hormone secretion. Therefore the measured faecal glucocorticoid concentrations might represent the hormonal status of an animal more accurately than in a single plasma or urine sample. [12–15].

In recent years, a higher frequency of prednisolone-positive bovine urines has been observed [16,17]. Several hypotheses have been put forward for this finding, including the influence of stress evoked by handling before slaughter and the resulting conversion from cortisol and cortisone to prednisolone and prednisone, respectively. [18,19]. This has rendered the analysis of glucocorticoids into a complicated business, since besides the mere presence of residues, their origin (either endogenous and/or exogenous) has become a matter of debate. As the glucocorticoid metabolism gives rise to large number of derivates with similar chemical structures and molecular weights, the search for biomarker candidates proved quite challenging [20,21]. At this point, one of the principal metabolites of prednisolone, i.e. 20β-dihydroprednisolone, has been put forward as potential biomarker for specifying endogenous traces of prednisolone [21,22].

In the past, steroid analysis in faecal samples was mainly carried out by immunoassays [13,23]. Although these techniques have proven their usefulness in wildlife studies [24,25], some limitations exist with respect to specificity. Cross-reactivity of the specific antibody with other similar steroids can lead to controversial results [26,27]. In this context, liquid chromatography–mass spectrometry (LC-MS) techniques are more fit to distinguish similar glucocorticoid compounds [28]. Ultra-high performance liquid chromatography (U-HPLC) using columns with sub 2 μm particles, which results in a higher chromatographic resolution, are commonly used these days [29]. From literature, it may be concluded that tandem MS using selected reaction monitoring is currently the preferred detection method for glucocorticoid analysis [11,28]. An inherent limitation of this targeted approach is the inability to screen for unidentified and unknown compounds such as metabolites. Therefore in this study, the U-HPLC system was coupled to a high-resolution Orbitrap mass spectrometer, which allows the production of full-scan MS spectra with a resolving power up to 100,000 full-width half-maximum (FWHM) and a high mass accuracy (mass deviations below 2 ppm) [30]. This detection technique offers the possibility to simultaneously analyse a virtually unlimited number of compounds, provides sufficient selectivity for complex matrix extracts such as faeces and allows post-acquisition re-interrogation of data and screening for unidentified and/or unknown compounds.

Because of the complex nature of faeces, appropriate sample preparation procedures are required, but in terms of the metabolomic approach to be kept as generic as possible. To this extent, Plackett–Burman experimental design is a useful tool to screen for the main variables within a large number of variables that may affect the extraction yield [31]. This highly efficient design provides the opportunity to identify the significant extraction conditions with a minimum number of experiments.

Although the use of non-invasive sampling techniques has increased, several confounding factors inhibit its wide spread use [32]. A long-term stability study of glucocorticoids in urine has for example shown that the environmental conditions during preservation have a big influence on the recovery [33]. This is particularly true for urine contaminated with faecal material which can contain a microbial flora up to 10^{11} CFU/g faeces [34]. This microbial activity may seriously interfere with the concentration of the extracted compounds as it has been shown that bacteria and bacterial enzymes in faeces decompose steroid metabolites within hours in untreated faeces [23,35]. Therefore, in the present study, an extensive stability study of glucocorticoids in bovine faeces was performed in which the effect of different storage conditions such as lyophilisation and temperature were considered. Additionally, the preservations under aerobic and anaerobic environments, as well as the addition of ethanol 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 (4-pregnene-17α,20β,21-triol-3,11-dione) and the synthetic glucocorticoids prednisolone, prednisone and methylprednisolone, and several potential biomarker candidate prednisolone metabolites including 20α-dihydroprednisolone and 20β-dihydroprednisolone (Figure 1). To this extent, a generic extraction and analytical method to measure glucocorticoids and a number of their metabolites in faecal samples of cattle was developed and validated according to the guidelines of CD 2002/657/EC.

2. Experimental

2.1. Reagents and chemicals

Standards of prednisolone, prednisone, cortisone, cortisol, dihydrocortisone and methylprednisolone were purchased from Sigma-Aldrich (St. Louis MO, USA). The metabolites 20α-dihydroprednisolone and 20β-dihydroprednisolone were purchased from Steraloids (Rhode Island, USA). Internal standards were cortisol-d4 (Sigma-Aldrich, USA) 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 produced with an Arium 611 UV system (Sartorium Stedim Biotech, Aubagne, France). Isolute C18 (EC) (500 mg, 10 mL) cartridges were purchased from Biotage (Uppsala, Sweden). Strata-X (200 mg, 6 mL) cartridges were purchased from Phenomenex, Inc. (Torrance, USA).

Primary stock solutions were prepared in ethanol at a concentration of 200 μg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at a range of 0.1–10 μg 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 C18 Isis U-HPLC column (1.8 μm, 100 × 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}. Optimised separation of all analytes was obtained using a linear gradient starting with a solvent mixture (v/v) of 80% A and 20% B. The percentage of acetonitrile was increased to 25% in 1 min, and held there for 5.0 min. Next, a linear increase to 95% B in 1 min was performed, and further up to 100% in 1 min and held there for 2.0 min. In between samples, the column was allowed to re-equilibrate at initial conditions for 2 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