Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 588 (2007) 20–25
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Rapid multi-residue method for the quantitative determination and confirmation of glucocorticosteroids in bovine milk using liquid chromatography–electrospray ionization–tandem mass spectrometry ☆

Mark McDonald [a,*], Kristina Granelli [b], Pernilla Sjöberg [b]

[a] *Central Meat Control Laboratory, Department of Agriculture, Backweston Campus, Young's Cross, Celbridge, Co. Kildare, Republic of Ireland*
[b] *Chemistry Division 1, National Food Administration, Box 622, SE-75126 Uppsala, Sweden*

Received 16 June 2006; received in revised form 19 December 2006; accepted 29 January 2007
Available online 7 February 2007

Abstract

Dexamethasone, betamethasone and prednisolone are synthetic glucocorticosteroids authorised for therapeutic use in bovine animals within the European Union. Dexamethasone and betamethasone are used mainly for the treatment of metabolic and inflammatory diseases. Prednisolone is used to treat bovine mastitis. Maximum residue limits (MRLs) of 0.3 $\mu g\,kg^{-1}$ for both dexamethasone and betamethasone and 6.0 $\mu g\,kg^{-1}$ for prednisolone in bovine milk have been established. 6α-Methylprednisolone and flumethasone are not authorised for use in bovine animals and are completely banned in bovine milk. The proposed method is based on deprotenisation of milk using 20% (w/v) trichloroacetic acid. Samples are filtered using glass microfibre filters and subject to clean-up using OASIS HLB solid phase extraction. Separation was achieved on a Hypercarb 100 mm × 2.1 mm × 5 μm column. Mobile phase was: 90/10 acetonitrile/0.1% formic acid in water; flow rate was 600 $\mu L\,min^{-1}$. The method allowed the rapid identification and confirmation of the five glucocorticosteroids according to the criteria laid down in Commission Decision 2002/657/EC. Matrix calibration curves for all compounds were linear in the interval 0.0 MRL to 2.0 MRL with a correlation coefficient (r^2) higher than 0.96. Relative recoveries ranged from 97% for betamethasone to 111% for prednisolone. Precision at the MRL ranged from 3.8% for prednisolone to 13.8% for betamethasone. Decision limits, CCα, and detection capability, CCβ have been calculated for all compounds.

Keywords: Synthetic glucocorticosteroids; Dexamethasone; Betamethasone; Prednisolone; 6α-Methylprednisolone; Flumethasone; Milk; Liquid chromatography tandem mass spectrometry; Validation

1. Introduction

Synthetic glucocorticoids are widely used in veterinary medicine, particularly for the treatment of anti-inflammatory diseases, shock and circulatory collapse and acetonemia. Dexamethasone, betamethasone and prednisolone are specifically authorised for therapeutic use in bovine animals within the European Union. Under Community Regulation 2377/90 [1], the European Medicines Agency (EMEA) set maximum residue limits of 0.3, 0.3 and 6.0 $\mu g\,kg^{-1}$, respectively for these residues in bovine milk [2]. Residues of these catabolic components along with 6α-methyl prednisolone and flumethasone are also known for their growth promoting properties [3]. They increase live weight gain even at low concentrations. This practise is banned by European legislation. From both an animal welfare and consumer protection perspective, there is an urgent need to develop comprehensive control measures to monitor glucocorticosteroids at low levels. A major analytical challenge for such control is the development of rapid analytical methodology that cannot only detect such compounds at 0.5 MRL as now required by legislation, but also the chromatographic separation of dexamethasone and betamethasone which has proven to be difficult in the past.

Initial methods for the detection of such residues were based on liquid chromatography (LC) with ultra violet (UV) detection. Such methods lacked specificity and were soon replaced by gas chromatography (GC) linked to mass spectrometry (MS) [4,5]. The need for derivatisation prior to GC–MS analysis made this methodology very time consuming. Improvements in liquid

☆ This paper was intended for inclusion in the Drug Residue Analysis SI but is now too late

* Corresponding author. Tel.: +353 1 6157358; fax: +353 1 615 7359.
E-mail address: mark.mcdonald@agriculture.gov.ie (M. McDonald).

0003-2670/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2007.01.075

chromatography–tandem mass spectrometry (LC–MS-MS) now means that the detection of such residues using triple quadrupole tandem mass spectrometers, particularly in multiple reaction monitoring mode (MRM), is possible.

Antignac et al. [6] demonstrated the ability of LC–MS-MS to detect glucocorticosteroids in hair/urine/tissue samples using positive electrospray. Van den Hauwe et al. [7] have descried the detection of these compounds in similar matrices using negative electrospray. This was possible due to the ionisation of these compounds with formic acid, producing $[M + \text{formate}]^-$ adducts which were then used as the precursor ion in multiple reaction monitoring. Methods using atmospheric pressure chemical ionisation (APCI) and ion trap LC–MS-MS have also been published in the literature [8–19]. Cherlet et al. [20] have been reported a method for the detection of dexamethasone in bovine milk by LC–APCI-MS-MS.

No multiresidue method for the detection and confirmation of glucocorticosteroids in bovine milk has been published. This paper presents a rapid method for the detection, quantification and confirmation of dexamethasone, betamethasone, prednisolone, flumethasone and 6α-methylprednisolone in bovine milk. Sample preparation is based on protein precipitation using trichloroacetic acid. A required performance level (RPL) of 0.3 μg kg^{-1} was set for 6α-methylprednisolone and flumethasone. Clean-up was carried out using Oasis HLB solid phase extraction. Rapid separation is achieved using a Hypercarb column. Mass spectrometric detection is performed using LC–ESI-MS-MS in negative mode with multiple reaction monitoring. Analytical limits are determined and validation data is presented in accordance with Commission Decision 2002/657/EC [21]. The method may be used for the detection of all 5 residues at 0.5 MRL/RPL.

2. Experimental

2.1. Chemicals

Chemicals were all of analytical-reagent grade unless otherwise stated. HPLC-grade acetonitrile was from Lab Scan (Dublin, Ireland). Gradient grade methanol, *n*-hexane and trichloroacetic acid were from Merck (Darmstadt, Germany). A 20% trichloroacetic acid (TCA) solution (w/v) was prepared by dissolving 200 g TCA in 1000 mL of water and a solution of 20 mM NaOH (Eka Nobel, Suite, Sweden) was prepared by dissolving 0.8 g in 1000 mL of water. Dexamethasone (DEX), betamethasone (BETA), flumethasone (FLU), 6α-methylprednisolone (MPRE) and prednisolone (PRE) were obtained from Sigma–Aldrich (Stockholm, Sweden). Deltafludrocortisone (DFUD) was from Steraloids (Newport, USA). OASIS HLB 3cc, 60 mg columns were supplied by Waters (Milford, USA). Whatman GF/B filters were supplied by Whatman (GmbH, Dassel, Germany).

2.2. Standard solutions

Individual stock standard solutions at 1 mg mL^{-1} of each standard were made. From these stocks, 100 μg mL^{-1} solutions were prepared for PRE and DFUD (internal standard) and 10 μg mL^{-1} solutions for FLU, MPRE, DEX and BETA. A mixed working standard was then prepared by diluting FLU, MPRE, DEX and BETA to 50 ng mL^{-1} and PRE to 1 μg mL^{-1}. The individual working standard solution of DFUD had a concentration of 1 μg mL^{-1}. All solutions were prepared in methanol. The standards are stable for a minimum of 1 year when stored in the dark at −20 °C.

2.3. Equipment

The water used was purified by a Milli-Q plus 185 water system, Millpore. Multifuge 35R and Biofuge 13 centrifuges were from Hereaus. Whirlimixer was from Fisons and shaker from Edmund Büchler GmbH. A Micromass Quattro Ultima mass spectrometer (Waters) connected to a Waters Alliance 2695 Liquid Chromatograph system was used. Instrument control was carried out using Masslynx software (Version 4.0). Separation was achieved using a hypercarb column (100 μm × 2.1 μm × 5 μm), mobile phase was isocratic: 90/10 acetonitrile/0.1% formic acid in water: flow rate was 600 μL min^{-1}. Column temperature was ambient room temperature. The mass spectrometer was operated in negative electrospray ionisation (ESI^-) mode. During method development standards were infused directly into mass spectrometer at a concentration of 1 μg mL^{-1} and a flow rate of 10 μL min^{-1} in electrospray negative mode. The $[M + \text{formate ion}]^-$ was selected as the precursor ion for all transitions. The two strongest MRM transition signals were selected for each compound. Cone voltage was 30 V and collision energies were optimised and are given in Table 1. Hexapole 1 was optimised to 30 V and hexapole 2 was optimised to 0.1 V, with the aperture set to 0.2 V and the multiplier to 650 V. Nitrogen was used as desolvation gas at a flow of 500 L h^{-1} and cone gas at a flow of 100 L h^{-1}.

Table 1
MRM transitions, optimised cone voltages and collision energies

Analyte	Cone (V)	Transition 1	Collision energy (eV)	Transition 2	Collision energy (eV)
Dexamethasone	30	437.2 > 361.3	16	437.2 > 345.3	25
Betamethasone	30	437.2 > 361.3	18	437.2 > 345.3	25
Prednisolone	30	405.2 > 295.1	30	405.2 > 280.1	36
Deltafludrocortisone	30	423.0 > 347.1	15	423.0 > 293.2	30
6α-Methylprednisolone	30	419.2 > 343.3	15	419.2 > 309.2	30
Flumethasone	30	455.1 > 379.3	30	455.1 > 325.3	15