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Detection of selected corticosteroids and anabolic steroids in calf milk replacers by liquid chromatography–electrospray ionisation – Tandem mass spectrometry

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Cortisol (PubChem CID: 5754)
Cortisone (PubChem CID: 222786)
Prednisolone (PubChem CID: 5755)
Prednisone (PubChem CID: 5865)
Dexamethasone (PubChem CID: 5743)
Testosterone (PubChem CID: 6013)
Epitestosterone (PubChem CID: 10204)
Nandrolone (PubChem CID: 9904)
Trenbolone (PubChem CID: 25015)
Boldenone (PubChem CID: 13308)

ABSTRACT

The use of corticosteroids and anabolic steroids in food producing animals is regulated or banned in the European Union (EU). However, their use as growth promoters cannot be excluded. Milk replacers, considered by EU legislation as feeds, may be a good way of administration of these compounds. In order to improve the control of growth promoter utilization in animal husbandry and preventing possible consequences to animal welfare, we developed a method for multiresidue analysis of prednisolone, prednisone, dexamethasone, cortisone, cortisol, 17α- and 17β-boldenone and their precursor androstadienedione (ADD), testosterone, epitestosterone, 17α- and 17β-nandrolone, and trenbolone in powdered milk for calves. All analytes were extracted, after a common deproteinization and defatting sample pretreatment, by a unique immunoaffinity column and analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS) in both positive and negative electrospray ionization (ESI) modes. The method was validated according to the criteria of the Commission Decision 2002/657/CE. The analytical limits were from 0.39 to 0.73 ng mL^{-1} for the decision limit (CCα) and 0.46–0.99 ng mL^{-1} for detection capability (CCβ). The analysis of 50 samples of milk replacers for calves, always revealed the presence of cortisol and cortisone (average concentrations 2.56 and 1.06 ng mL^{-1}, respectively), frequently testosterone and epitestosterone (1.24 and 0.63 ng mL^{-1}, respectively), occasionally β-nandrolone (0.82 ng mL^{-1}) and prednisolone (0.41 ng mL^{-1}). The other anabolic steroids were never found.

1. Introduction

Successful calf growth depends on a combination of many factors related to health, management and nutrition (Heinrichs, Wells, & Losinger, 1995). From the alimentary aspect, natural milk, as a wholesome food, is the most important source of nutrition for young mammals before they are able to digest other types of food. Powdered milk is commonly used for the daily feeding of calves, as it is an adequate alternative to the mother's dairy milk and an economically feasible source of all essential nutriments. Feeding with high quality milk replacers allows healthy growth in calves equal to that attainable with whole milk (Jorgensen, Hoffman, & Nytes, 2006). Since manufacturing powdered milk directly from whole milk is an expensive process, the bulk of the constituents of commercial calf milk replacers are either by-products of dairy processing or non-dairy products. Powdered milk replacers are generally made up of ingredients such as skim milk powder, vegetable or animal fat, whey protein, soy lecithin and vitamin-

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mineral premix (Geiger et al., 2014). Protein levels in dry milk replacers range from 18% to 30% with an average value of approximately 20–22%, preferably of diary origin, but can also include soy protein, soy flour, wheat proteins, potato and animal plasma protein. Fat levels range from 10% to 28–30%, with 18%–22% being the most common fat levels, mainly added as tallow, lard or coconut oil (Bamn, 2014 and Ontario veal association, 2015).

The inclusion of veterinary drugs in calf milk replacers is a matter of concern, particularly if their administration is not fully regulated and especially when legislation varies across the Countries. For example, in the USA medications (decoquinate, lasalocid, oxytetracycline, chlortetracycline, and neomycin) are approved for inclusion in milk replacers, but the U.S. Food and Drug Administration (FDA, 2013) recommended a three-year judicious period (starting from December 2013) during which utilisation of antibiotics in animal husbandry has to be reduced. European legislation does not treat milk replacers individually, but sets out the conditions under which medicated animal feeds may be prepared, placed on the market and used within the Community (European Union, 1990 and European Union, 2010 a).

The use of steroids in food-producing animals for therapeutic purposes is regulated (corticosteroids) or banned (anabolic steroids) in the European Union; however, their use as growth promoters cannot be excluded (Pavlovic et al., 2012). Cortisol, cortisone, testosterone and epitestosterone are endogenous, prednisolone (Bertocchi et al., 2013), boldenone (Chiesa et al., 2014) and nandrolone (Glenn Kennedy et al., 2009) are considered pseudoendogenous steroids while dexamethasone and trenbolone are well-known synthetic steroids. A faster feed conversion rate, improvement of the carcass with improved meat quality, fat reduction, and increase in milk production are some of the notable features that could be achieved by treatment with these substances. Thus, regulations on steroid residues with hormonal activity in food of animal origin are essential in order to safeguard animal welfare and ascertain any fraud. In the case of therapeutic use of regulated substances, a prescription by a veterinarian is needed and a suspension period has to be respected between the end of treatment and slaughter or milk marketing. The European Commission has established the maximum residue limits (MRLs) for four corticosteroids in several matrices such as muscle, liver, kidney and milk from different animal species (European Union, 2010b).

On the basis of the regular protocol applied, there are a few principal techniques by which medication can be introduced into an animal: oral administration, intramuscular, subcutaneous and intravenous injection or implantation under the skin (Courtheyn et al., 2002). Unfortunately, some illegal growth-promoting agents are suspected of being administered with feed, despite the fact that they are not licenced as additives (Courtheyn et al., 1993 and European Union, 2004). Therefore, in order to achieve comprehensive surveillance and have insight into how a medication was delivered to an animal, analysis of the feed for the presence of steroids should be included as well. It should be emphasized that the presence of steroid hormones in feedstuffs can be also unintentional, due to cross-contamination or owing to the appearance of pseudo-endogenous substances such as prednisolone (Chiesa et al., 2014). Among feedstuffs used in animal husbandry, powdered milk replacers are perhaps most suitable for illegal tampering as drug distribution via this route is very simple: during the reconstitution of milk replacers, immediately before feeding. As hormones and steroids migrate to milk from the cow bloodstream, we need additional information about their physiological levels in milk related to milk replacers (Jouan, Sylvie, Gauthier, & Laforest, 2006). To the best of our knowledge, there has been neither a preliminary assessment of the status of endogenous or exogenous steroids, nor a fully validated method for their screening in powdered milk used in calf breeding.

Taking into account all the above mentioned factors, with the intention of improving residue control and preventing possible consequences for animal welfare and the consumer, our aim was to develop a liquid chromatography–tandem mass spectrometry (LC-MS/MS) analytical method for evaluating selected glucocorticosteroids and anabolic steroids in milk replacers used as dairy feed replacement in calf rearing.

Nowadays, LC-MS/MS is the most suitable technique for detecting veterinary drugs in feedstuffs because it provides unambiguous identification and a reliable confirmation. On the other hand, milk replacers are complex matrices, containing many solutes with different physico-chemical properties: fatty acids, proteins, neutral lipids (glycerides, phospholipids and sterols), glycides, vitamins and minerals, which may interfere with analyses. The removal of these compounds is necessary in LC-MS/MS methods, especially if low ng mL^{-1} of steroid levels are to be screened for. Applying adequate and efficient purification, ion suppression can be successfully avoided together with improvements in overall method performances such as the detection limit and reproducibility.

There have been just a few studies in the literature on powdered milk – infant formulae for human use (Romero-González, Aguilera-Luiz, Plaza-Bolaños, Frenich, & Vidal, 2011 and Zhan et al., 2013) and only one that described a multi-residue method for detecting 17 selected veterinary hormones in six different powdered ingredients derived from bovine milk used modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation (Ehling & Reddy, 2013). Other researchers devised a method for the detection of eight corticosteroids in milk replacers, through C18 SPE, but with relatively high detection limits (Fiori, Pierdominici, Longo, & Brambilla, 1998). Immunology-based pre-treatment techniques have been introduced recently, but have not yet been used in powdered milk analysis. For other matrices (urine, bile) this kind of purification in general has exhibited better selectivity than those obtained with common procedures (Chiesa et al., 2014, 2015). This is the reason we decided to take advantage of an immunoaffinity sample cleaning approach in the multi-drug method presented in this paper.

To this end, the main objective of this study was the establishment of a LC-MS/MS method able to identify steroids such as corticosteroids (prednisolone, prednisone, dexamethasone, cortisone and cortisol) and anabolic steroids (17α- and 17β-boldenone, their precursor androstadienedione (ADD), testosterone, epitestosterone, 17α- and 17β-nandrolone and trenbolone) in calf milk powder. All analytes were investigated after a common pretreatment step of deproteinization and defatting followed by immunoaffinity column (IAC) clean-up and LC–MS/MS analysis, validated according to Commission Decision 2002/657/EC (European Union, 2002). Finally, we applied the validated method to the analysis of 50 samples of commercially available powdered bovine milk.

2. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën.

(Sigma–Aldrich, St. Louis, MO, USA). Water was purified by a Milli-Q System. The IACs were provided by Randox (DM 2185, Randox Laboratories, Antrim, UK). Concentrated wash and storage buffers, diluted following the manufacturer's instructions before use, were supplied with the columns. ADD and β-boldenone were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA); α-boldenone was obtained from LGC Standards (Teddington, UK). Their