Talanta 131 (2015) 163–169

Contents lists available at ScienceDirect

Talanta

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

Determination of α- and β-boldenone sulfate, glucuronide and free forms, and androstadienedione in bovine urine using immunoaffinity columns clean-up and liquid chromatography tandem mass spectrometry analysis

Luca Chiesa [a], Radmila Pavlovic [a], Guglielmo Dusi [b], Elisa Pasquale [a], Alessio Casati [c], Sara Panseri [a], Francesco Arioli [c,*]

[a] Department of Veterinary Science and Public Health, University of Milan, Milan, Italy
[b] Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna "B. Ubertini", Brescia, Italy
[c] Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy

ARTICLE INFO

Article history:
Received 8 May 2014
Received in revised form
9 July 2014
Accepted 11 July 2014
Available online 12 August 2014

Keywords:
Boldenone sulphate
Boldenone glucuronide
Boldenone
Androstadienedione
Bovine urine
Food safety

ABSTRACT

The debate about the origins of boldenone in bovine urine is ongoing for two decades in Europe. Despite the fact that its use as a growth promoter has been banned in the European Union (EU) since 1981, its detection in bovine urine, in the form of α-boldenone conjugate, is considered fully compliant up to 2 ng mL^{-1}. The conjugated form of β-boldenone must be absent. In recent years, the literature about boldenone has focused on the identification of biomarkers that can indicate an illicit treatment. β-boldenone sulfate is a candidate molecule, even if the only studies currently available have taken place in small populations. In this study, a method for the determination of sulfate and glucuronate conjugates of β-boldenone was developed and validated according to the European Commission Decision 2002/657/EC and applied to α-boldenone sulfate and glucuronide, α- and β-boldenone free forms and androstadienedione (ADD), too. The clean-up with immunoaffinity columns enabled the direct determination of the conjugates and free forms and allowed specific and sensitive analyses of urine samples randomly selected to verify this method. The decision limits (CCα) ranged between 0.07 and 0.08 ng mL^{-1}, the detection capabilities (CCβ) between 0.08 and 0.1 ng mL^{-1}. Recovery was higher than 92% for all the analytes. Intra-day repeatability was between 5.8% and 17.2%, and inter-day repeatability was between 6.0% and 21.8% for the studied free and conjugated forms. This method has been developed as a powerful tool with the aim to study the origin of boldenone in a trial on a significant number of animals.

1. Introduction

The use of substances that have hormonal activity for growth promotion in farm animals has been prohibited in the European Union (EU) since 1981 [1]. The bans on the use of such substances, on the trade of treated animals and their meat within the EU, and also on the import from third countries was confirmed in 1988 [2,3]. A typical substance with hormonal action is 17β-boldenone (1-dehydrotestosterone or androsta-1,4-dien-17β-ol-3-one) (β-bold), an anabolic steroid that differs from testosterone only by the double bond between carbons 1 and 2 of the steroid A ring as shown in Fig. 1. Arts et al. [4] reported the natural occurrence in calf urine of 17α-boldenone (α-bold) at concentrations ranging from <0.1 to 2.7 ng mL^{-1}. Since then, a number of studies and regulations followed, aiming to explain the presence of boldenone (bold) in bovine urine, to indicate a biomarker metabolite for illicit treatment, and to establish levels of the hormonal substance that could exclude administration to the animal [5,6]. In particular, in September 2003, the thesis of the natural production of this steroid was proposed by the experts within the EU, who stated that scientific knowledge was sufficient to conclude that the presence of α-bold in urine and faeces of bovine animals has a natural origin. They set the 'natural threshold' of 2 ng mL^{-1} in the urine of veal calves below which α-bold conjugate (boldenone conjugates are water soluble forms of boldenone bound to e.g. glucuronic acid formed by metabolism in the animals) come from sources other than illegal treatment. The presence of β-bold

* Correspondence to: Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria, 10 - 20133 Milan, Italy. Tel.: +390250317877; fax: +390250317890.
E-mail address: francesco.arioli@unimi.it (F. Arioli).

http://dx.doi.org/10.1016/j.talanta.2014.07.035
0039-9140/

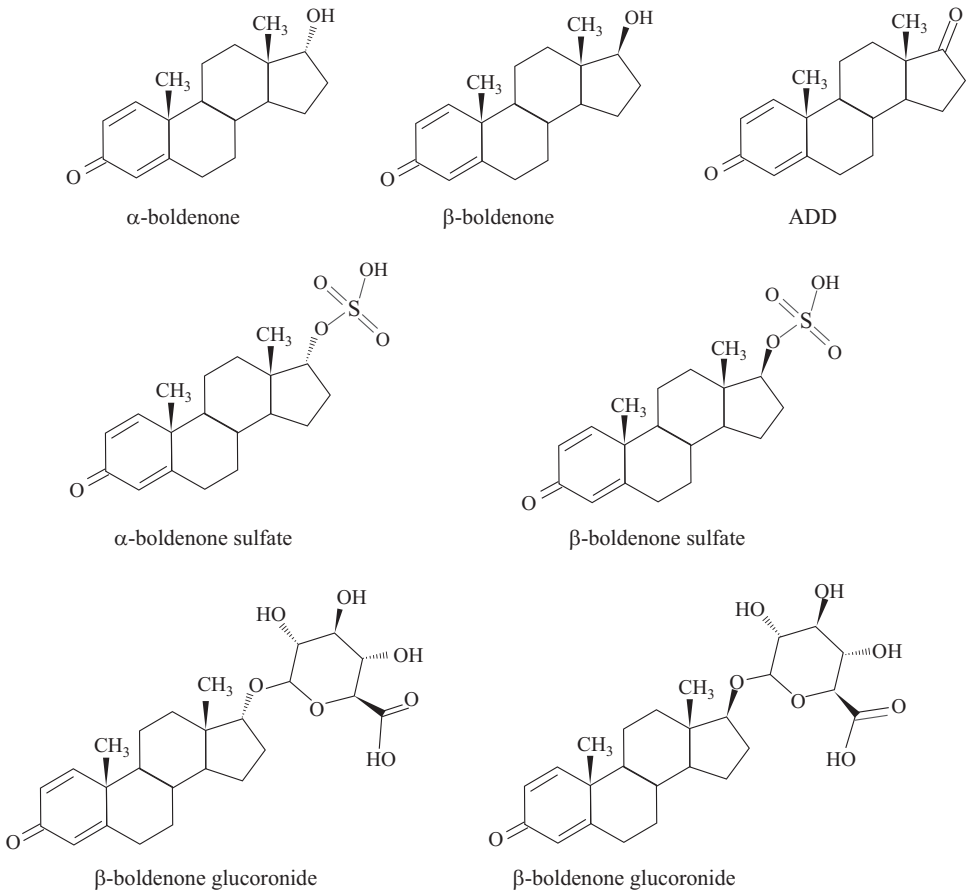

Fig. 1. Chemical structures of the seven analytes.

conjugates at any concentration in the urine of veal calves was indicated as the result of an illegal treatment [7]. The presence of conjugates of α- and β-bold, without specifying the nature of the ionized group (sulphate, glucuronide) is not the only option considered by the scientific community. Biddle [8] performed a study on beef cattle treated with three preparations of bold: intra-muscular bolus administration of β-bold, followed by oral administration of the supplement androstadienedione (1,4-androstadiene-3,17-dione) (ADD), and finally intra-muscular administration of β-bold undecylenate. He concluded that highly sensitive methods would be required to detect the abuse of bold using β-bold glucuronic acid conjugate as a marker; they could not confirm the EU recommended level of 2 ng mL^{-1} for α-bold glucuronic acid conjugate due to the lack of the reference standard. Finally, two markers, present in the glucuronate fraction regardless of route of administration, were specially indicated in this study: 6β-hydroxy-17α-bold and 5z-androst-1-ene-3z-ol-17-one (the letter 'z' indicates position α or β, due to the lack of the reference standard). Another study [9] investigated the metabolites of bold in treated cattle after intramuscular and oral treatment with bold, bold esters and ADD. The authors showed that the majority of metabolites, analysed by GC–MS, were glucuronide conjugates and that β-bold sulfate was present only in urine from treated animals (this last result obtained by LC–MS/MS). They therefore suggested to use β-bold sulfate as an indicator of bold administration, after larger scale studies. However, the study was conducted in a predominantly qualitative way, the analytical limits in the LC–MS/MS were not reported; therefore, the question: "Who is to say that as analytical limits decrease, (particular steroids) will not be discovered as endogenous at a lower concentration?" [6] has a fundamental importance. Destrez et al. [10] performed a study on treated male calves with oral administration of ADD or with intra-muscular injection of bold undecylenate. The analytical limits for β-bold sulfate were set by both LC–MS/MS (negative ESI, SRM acquisition, triple quadrupole) and LC-HRMS (negative ESI, *R* 30,000, Orbitrap™): the decision limits (CCα) were 0.2 and 0.1 ng mL^{-1} and detection capability (CCβ) 0.4 and 0.2 ng mL^{-1}, respectively. The authors concluded that once again β-bold sulfate demonstrated to be the candidate marker of a treatment. In an effort to develop a study on an extended population, deemed necessary also by the authors cited above, we developed an LC–MS/MS method with triple quadrupole technology that had the lowest analytical limits possible for the detection of β-bold sulfate in bovine urine. The method was also developed for α-bold sulfate, α- and β-bold glucuronide, ADD, α-bold and β-bold (Fig. 1). The validation was made according the Decision of Commission 2002/657/EC [11].

2. Materials and methods

2.1. Materials

All solvents were of HPLC or HPLC–MS grade quality and supplied by Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) was from Riedel-de Haën (Sigma-Aldrich, St. Louis, MO, USA). The chemicals for the preparation of artificial urine were from Sigma-Aldrich (St. Louis, MO, USA). β-bold sulfate (triethylamine salt), β-bold glucuronide, and α-bold were from LGC Standards (Teddington, UK), and ADD and β-bold were from Fluka (Sigma-Aldrich, St. Louis, MO, USA). The sulfate and glucuronide forms of α-bold, provided by research partners, were prepared by a two-step synthesis: the epimerization of β-bold (Steroid SpA, Cologno Monzese, Milan, Italy) using a modified Mitsunobu