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Confirmatory analysis of stanozolol metabolites in bovine, pig and sheep urines using an optimized clean-up and liquid chromatography–tandem mass spectrometry

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ABSTRACT

This paper describes a new liquid chromatography–tandem mass spectrometric (LC–MS/MS) method for the analysis of three stanozolol metabolites (16β-hydroxystanozolol, 3′-hydroxystanozolol, and 4β-hydroxystanozolol) in urines of animal origins. The solid-phase extraction (SPE) clean-up procedure was optimized to reduce the matrix effects in the LC–MS/MS analysis and to enhance recovery. Four different approaches were tested to prepare the sample, which include anion, and cation mixed-mode ion exchange, reversed-phase and normal-phase SPE cartridges. Mixed-mode anion exchange Strata-XL-A SPE column with diethyl ether elution yielded the best values. The separation of metabolites was optimized on Kinetex XB column using isocratic elution. The best mobile phase composition was achieved at the acidic pH with 0.1% (v/v) formic acid in water and methanol composition. The main advantages of the approach applied in the present study over other known methods include the single step SPE clean-up, relatively fast separation on HPLC column packed with core–shell particles, and lowering the limit of detection of target metabolites to the range between 0.05 and 0.15 μg/l. Additionally, the developed method was successfully validated for the first time for three species in accordance with the European Union (EU) 2002/657/EC decision. Finally, the efficiency of method was demonstrated by analyzing incurred samples.

1. Introduction

Anabolic steroids are commonly used in human and animal therapy because they improve the protein synthesis and mass growth. However, the misuse of anabolic steroids in the past decade led to frequent detection of these compounds in doping analysis [1]. Stanozolol (5α-androstane-17α-methyl-17β-ol [3,2-c] pyrazole; STN) belongs to the exogenous androgenic/anabolic steroids category and is allowed for zootechnical use. However, STN is banned for fattening purpose for animals, intended for human consumption [2]. Due to rapid metabolization of STN, the abuse can mainly be detected via its hydroxy metabolites [3,4]. Numerous metabolites have been identified in urine samples of human and test mouse as well as in calves samples [3,5–12]. Among different detected hydroxy metabolites in samples, 16β-hydroxystanozolol (16-OH-STN), 3′-hydroxystanozolol (3-OH-STN), and 4β-hydroxystanozolol (4-OH-STN) (Fig. 1) are most commonly found in different urine samples of animal origin [8,9,12]. These metabolites are thus the focus of the present study.

The European Union Reference Laboratory (EU-RL, Rikilt, The Netherland) has set a recommended concentration level of 2 μg/l for 16-OH-STN in urine of animals, farmed for food producing. This level could be applied for validation purpose and the decision limit calculated in the validation should be lower than 2 μg/l [13]. Gas chromatography coupled to mass spectrometry (GC–MS) is preferred for the detection of stanozolol metabolites, but it has limitations due to the time-consuming derivatization and thermolability [4,14]. Another alternative method is the immunochemical determination, which can perform relatively fast analysis [10,15,16], but these methods are not able to confirm target compounds [17]. Consequently, the liquid chromatography–mass spectrometry (LC–MS) methods are preferable for analysis of hydroxystanozolol. Confirmatory or screening determinations are currently being performed using

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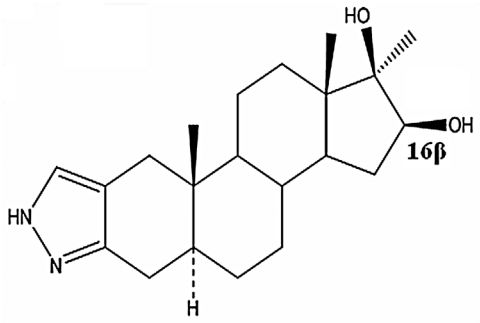
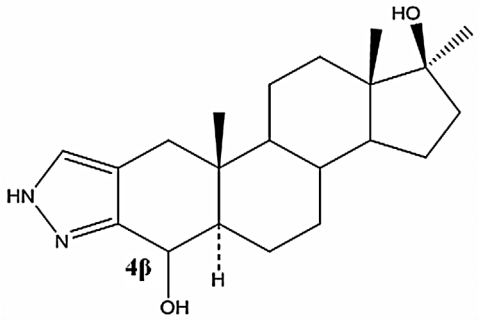
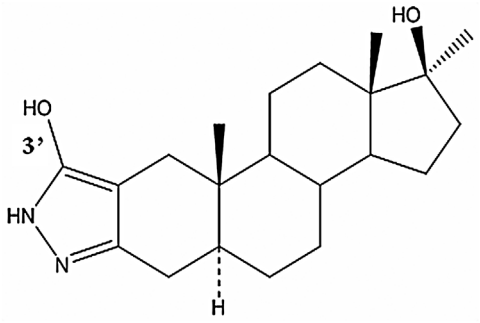


16β-hydroxystanozolol (16-OH-STN, pKa 3.05)



4β-hydroxystanozolol (4-OH-STN, pKa 4.0)



3'-hydroxystanozolol (3-OH-STN, pKa 5.35)

Fig. 1. Structures and pK_a values of investigated metabolites.

LC–MS/MS or LC–MS^n ion trap detectors [3,6,8,9,18–25]. However, quantification using LC–MS/MS methods is strongly influenced by the co-extracted and co-eluted matrices. Hence, improved clean-up approaches prior to analysis are critically required to reduce the effect of complex matrices of biological samples.

Metabolites of STN are excreted in conjugated form in urine samples and enzymatic hydrolysis is needed to liberate the metabolites, which can be carried out before and after the sample extraction [3,8]. Earlier methods applied either liquid–liquid extraction (LLE) or solid-phase extraction (SPE) for sample cleaning. Due to the basic characteristics of STN metabolites (see Fig. 1), the extraction efficiency of LLE can be enhanced using the approach of pH adjustment. Generally, acceptable extraction can be achieved at basic pH with immiscible organic solvents [8]. In an inter-laboratory comparison, two laboratories applied only LLE method using diethyl ether as an extraction solvent and no further clean-up was performed. The performance characteristics of methods described were not fully reported in the study, the limit of detections was in the range from 0.5 to 1 μg/l [8]. SPE clean-up could be carried out using reversed-phase, amino or cation exchange cartridges [9,12,20]. Conjugated stanozolol metabolites were extracted on weak anion exchange SPE column [3]. Most of the studies used LLE and subsequent SPE to get relatively clean sample before LC–MS or GC–MS analysis, and consequently, to improve the analytical limits [3,8,20–22]. Consecutive SPE steps for sample cleaning have been attempted [12].

The objectives of the present study are: (i) develop a simple and rapid clean-up approach for the simultaneous analysis of three main metabolites of stanozolol (16-OH-STN, 3-OH-STN, and 4-OH-STN) in urines of food producing animals, (ii) optimize method for a wide range of sample species (e.g. bovine, pig and sheep), (iii) demonstrate successful clean-up approach to reduce the matrix effect in the LC–MS/MS analysis in order to obtain enhanced analytical performance characteristics, (iv) conduct validation of newly developed method according to the EU 2002/657/EC decision for all investigated samples [26], and (v) evaluate the developed method using incurred bovine samples.

2. Experimental

2.1. Reagents and samples

Analytical standards of 4β-hydroxystanozolol (4β,17β-dihydroxy-17α-methyl-5α-androstando [3,2-c] pyrazole, 0.5 mg), 3′-hydroxystanozolol (3′,17β-dihydroxy-17α-methyl-5α-androstando [3,2-c] pyrazole, 1 mg), 16β-hydroxystanozolol (16β,17β-dihydroxy-17α-methyl-5α-androstando [3,2-c] pyrazole, 100 μg/ml 8.17 in 1,2 dimethoxyethane), and 3′-hydroxystanozolol-d3, 100 μg/ml in acetonitrile–water (70/30, v/v) were obtained from LGC Standards GmbH (Wessel, Germany). 3′-Hydroxystanozolol-d3 (3-OH-STN-d3) was used as internal standard (ISTD).

Stock solutions (100 μg/ml) of 3-OH-STN and 4-OH-STN were prepared separately in methanol and were stored and kept at −20 °C for one year. A 100 ng/ml working standard solution that contained all steroids, except the internal standard, was prepared by diluting the stock solutions with methanol. An ISTD (100 ng/ml) working standard solution was also prepared in methanol. These standard solutions were prepared weekly and were used for calibration and spiking, and were stored at −20 °C. Other steroids (17β-oestradiol, 17α-testosterone, methyl-testosterone, 17α-boldenone, nortestosterone, 17β-trenbolone and progesterone) were purchased from Sigma–Aldrich (Budapest, Hungary). HPLC grade methanol, acetonitrile, dichloromethane, diethyl ether, ethyl acetate, *n*-hexane, and acetone were obtained from Promochem (Budapest, Hungary). Suprapur formic acid (98%), acetic acid (100%), and ammonia solution (25%) were purchased from Merck (Budapest, Hungary). Ammonium acetate (99.999%) and sodium acetate were obtained from Sigma–Aldrich (Budapest,