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# Quantitation of 17β-nandrolone metabolites in boar and horse urine by gas chromatography–mass spectrometry

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**Abstract**

A method to quantify metabolites of 17β-nandrolone (17βN) in boar and horse urine has been optimized and validated. Metabolites excreted in free form were extracted at pH 9.5 with *tert*-butylmethylether. The aqueous phases were applied to Sep Pak $C_{18}$ cartridges and conjugated steroids were eluted with methanol. After evaporation to dryness, either enzymatic hydrolysis with β-glucuronidase from *Escherichia coli* or solvolysis with a mixture of ethylacetate:methanol:concentrated sulphuric acid were applied to the extract. Deconjugated steroids were then extracted at alkaline pH with *tert*-butylmethylether. The dried organic extracts were derivatized with MSTFA:NH4I:2-mercaptoethanol to obtain the TMS derivatives, and were subjected to analysis by gas chromatography mass spectrometry (GC/MS). The procedure was validated in boar and horse urine for the following metabolites: norandrosterone, noretiocholanolone, norepiandrosterone, 5β-estran-3α, 17β-diol, 5α-estran-3β, 17β-diol, 5α-estran-3β, 17α-diol, 17α-nandrolone, 17βN, 5(10)-estrene-3α, 17α-diol, 17α-estradiol and 17β-estradiol in the different metabolic fractions. Extraction recoveries were higher than 90% for all analytes in the free fraction, and better than 80% in the glucuronide and sulphate fractions, except for 17α-estradiol in the glucuronide fraction (74%), and 5α-estran-3β, 17α-diol and 17βN in the sulphate fraction (close to 70%). Limits of quantitation ranged from 0.05 to 2.1 ng $mL^{-1}$ in the free fraction, from 0.3 to 1.7 ng $mL^{-1}$ in the glucuronide fraction, and from 0.2 to 2.6 ng $mL^{-1}$ in the sulphate fraction. Intra- and inter-assay values for precision, measured as relative standard deviation, and accuracy, measured as relative standard error, were below 15% for most of the analytes and below 25%, for the rest of analytes. The method was applied to the analysis of urine samples collected after administration of 17βN laureate to boars and horses, and its suitability for the quantitation of the metabolites in the three fractions has been demonstrated.


*Keywords:* Boar; Horse; Urine; 17β-Nandrolone metabolites; Gas chromatography/mass spectrometry

## 1. Introduction

The use of anabolic agents is prohibited in sports and in horseracing, as well as in food producing animals in the European Union (96/22/EC Directive) [1]. 17β-Nandrolone (17βN) is one of the most common anabolic steroids used. Detection of the illegal use of 17βN is difficult as a result of the extensive metabolism and the possibility of interference with endogenous compounds, which are species dependent.

The metabolism of 17βN has been studied in humans [2] and in different animal species [3–11]. Norandrosterone (NorA) and noretiocholanolone (NorE) excreted in the glucuronide fraction are the main metabolites in humans; norepiandrosterone (NorEpiA) is also excreted in the sulphate fraction [2]. In horses, isomers of estranediol (mainly 5α-estrane-3β,17α-diol, *aba*, and 5α-estrane-3β,17β-diol, *abb*) and the metabolite resulting from epimerization in $C_{17}$, 17α-nandrolone (17αN), have been detected as main metabolites, 17α isomers mainly in glucuronide fraction and 17β isomers, mainly in sulphate fraction [3–6]. In bovines, 17αN, *aba* and NorE have also been detected as main metabolites in cows and calves [7–9]. 17βN, isomers of 3-hydroxyestran-17-one (NorA, NorE, NorEpiA) and isomers of estrane-3, 17-diol (5β-estrane-3α,17β-diol, *bab*, and 5α-estrane-3β,17β-diol, *abb*), have been identified in miniature pigs after administration of 17βN laureate [10,11]. For most of the animal species, quantitative data on 17βN and its metabolites has never been reported.

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In recent years, different authors reported data to demonstrate the endogenous presence of 17βN or some of its metabolites in different species [10–20]. However, comprehensive data on the endogenous presence of all 17βN metabolites was not reported.

Detection of the consumption of anabolic agents is performed by identification of specific metabolites using gas chromatography or liquid chromatography coupled to mass spectrometry (GC/MS, LC/MS) or to tandem mass spectrometry [21–23]. These techniques are effective to detect the administration of exogenous compounds; however they are not able to distinguish the exogenous origin of compounds normally present in urines. In order to detect consumption on the basis on concentrations detected in urine and to define the time window to obtain the maximum retrospectivity of consumption and to discard endogenous production, a suitable target metabolite needs to be selected. A deep knowledge on the metabolism and the elimination kinetics of the different metabolites as well as their concentrations endogenously present in urine is needed in order to select the suitable target metabolite to be analyzed by GC/MS or LC/MS. For this reason, quantitative methods able to quantify 17βN and the metabolites in samples obtained after administration of 17βN to different animal species or in samples obtained from non-treated animals need to be developed.

The aim of the present study was to optimize and to validate a procedure for the quantification of 17βN and its metabolites in different animal species to help in the selection of the best target analyte to detect the administration of 17βN esters for fraudulent purposes.

## 2. Experimental

### 2.1. Reagents and solvents

Methanol and ethyl acetate (both of HPLC grade), *tert*-buthylmethyleter, 25% ammonia, ammonium chloride, sodium hydroxide pellets, di-sodium hydrogen phosphate, sodium hydrogen phosphate, potassium carbonate, ammonium iodide and 2-mercaptoethanol (all analytical grade), and sulphuric acid (extra pure grade) were purchased from Merck (Darmstadt, Germany). *n*-Hexane (HPLC grade) was supplied by Scharlau (Barcelona, Spain). β-Glucuronidase from *Escherichia coli* K12 was supplied by Roche Diagnostics GmbH (Mannheim, Germany). *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased by Macherey-Nagel (Düren, Germany). Cartridges Sep Pak $C_{18}$ Vac RC (500 mg) were supplied by Waters (Milford, MA, USA). Milli Q water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

The solid-phase extraction (SPE) was performed on a Vacuum manifold (Biochem Diagnostics, Edgewoodm, NY, USA). Organic layers were evaporated to dryness under nitrogen stream with a Turbo-Vap LV evaporator from Zymark Corporation (Hopkinto, MA, USA). Bulk human blank urine was supplied by BioRad (USA).

### 2.2. Standards and reference material

Norandrosterone (NorA), noretiocholanolone (NorE), 19-noretiocholanolone glucuronide, methyltestosterone, 17α-nandrolone (17αN), norandrosterone-$d_4$ (NorA-$d_4$), testosterone (T) and epitestosterone (epiT) were supplied by NARL, Reference Materials (Cottesloe, Australia). Norepiandrosterone (NorEpiA), 17α-estradiol (a-E2), 5α-estran-3β, 17β-diol (*abb*), 5β-estran-3α, 17β-diol (*bab*), 5α-estran-3β, 17α-diol (*aba*) were purchased by Steraloids Inc. (Newport Rhode Island, USA). Dehydroepiandrosterone-3-sulphate, 17β-estradiol (b-E2), 17β-nandrolone (17βN) and estrone were supplied by Sigma (St. Louis, Mo, USA). 5(10)-Estren-3β, 17α-diol was supplied by Hong Kong Jockey Club and norandrostenedione was purchased by Research Plus Inc. (Manasquan, NJ, USA).

Stock standard solutions (1 mg $mL^{-1}$, in free base form) of each analyte were prepared by dissolving 10 mg of the free base form in 10 mL of methanol. Working solutions of 100, 10 and 1 μg $mL^{-1}$ were prepared by 1:10, 1:100 and 1:1000 dilutions of the 1 mg $mL^{-1}$ stock solutions with methanol. All solutions were stored at −20 °C.

### 2.3. Extraction of steroids from urine matrix

Three metabolic fractions were extracted: free, glucuronide and sulphate fraction.

#### 2.3.1. Free fraction extraction

Aliquots of urine samples (10 mL for boars and 5 mL for horses) were added with a concentration of 20 ng $mL^{-1}$ of methyltestosterone and norandrosterone-$d_4$, used as internal standards, and adjusted to pH 9.5 with $NH_4Cl/NH_3$ buffer (100 μL). Free steroids were extracted with 5 mL of *tert*-butylmethylether by shaking at 40 mpm for 20 min. After centrifugation (3500 rpm, 5 min), organic layers were evaporated to dryness under nitrogen stream in a water bath at 50 °C. The extracts were kept in desiccators containing $P_2O_5$ and maintained under vacuum for at least 30 min before derivatization.

#### 2.3.2. Extraction of the glucuronide fraction

The small volume of organic solvent still present on top of the aqueous phase was evaporated under nitrogen stream. The aqueous phase was applied to Sep Pak $C_{18}$ cartridges previously conditioned with methanol (2 mL) and water (2 mL). Two protocols of cartridge washing and elution were compared: A, washing with water (2 mL), drying for 2 min, and eluting with methanol (2 mL); and B, washing with water (2 mL) and *n*-hexane (5 mL), drying for 2 min, and eluting with a mixture of methanol/ethyl acetate (30:70, v/v) (5 mL).

The organic extracts were evaporated to dryness under nitrogen stream in a bath at 50 °C. Residues were reconstituted in 1 mL of sodium phosphate buffer (0.2 M, pH 7), and subjected to enzymatic hydrolysis with 50 μL of β-glucoronidase from *E. coli* and incubation at 55 °C for 1 h. Then, samples were made alkaline with 250 μL of 5% solution of $K_2CO_3$ and extracted with *tert*-butylmethyleter (5 mL) by shaking at 40 mpm for