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Development and validation of a multi-residue method for the detection of a wide range of hormonal anabolic compounds in hair using gas chromatography–tandem mass spectrometry

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Abstract

The monitoring of anabolic steroid residues in hair is undoubtedly one of the most efficient strategies to demonstrate the long-term administration of these molecules in meat production animals. A multi-residue sample preparation procedure was developed and validated for 28 steroids. A 100 mg hair sample was grinded into powder and extracted at 50 °C with methanol. After acidic hydrolysis and extraction with ethyl acetate, phenolsteroids, such as estrogens, resorcyclic acid lactones and stilbens in one hand, are separated from androgens and progestagens in the other hand. Solid phase extractions were performed before applying a specific derivatisation for each compound sub-group. Detection and identification were achieved using gas chromatography–tandem mass spectrometry with acquisition in the selected reaction monitoring mode after electron ionisation. The method was validated according to the 2002/657/EC guideline. Decision limits (CCα) for main steroids were in the 0.1–10 μg kg^{-1} range.

Keywords: Anabolic; Steroid; Validation; Hair; Gas chromatography–tandem mass spectrometry (GC–MS/MS)

1. Introduction

The use of anabolic agents for improving the growth and feed conversion rates of food producing animals has been banned in the European Union since 1988 and no residues of these anabolic substances should be present in animal products. Many biological matrices such as tissue or urine samples can be used for the control of anabolic steroids misuse but hair samples remain as amongst the most judicious matrix due to its easiness of collection and sometimes the long-term detectability of administered substances.

Originally used to evaluate human exposure to heavy metals [1], hair analysis is used for criminal court proceedings, clinical purposes and doping control [2]. While urine analysis allows the detection of residues during a period ranging from several hours to 2–3 weeks, hair testing permits long-term detection sometimes up to several months [3]. In 2000, Gaillard et al. [4] published a study on the compared interest between hair analysis and urine analysis in doping controls. Thirty cyclists were sampled and tested for the two matrices. For corticosteroids, urine sample was preferred whereas for to anabolic steroids and amphetamines, hair appeared more as a powerful diagnostic matrix.

Most of the papers reporting the analysis of steroids in hair were published in the human field and often focused on a single anabolic compound such as testosterone [5], nandrolone [6], methenolone [7], stanozolol [8] or on several steroids simultaneously [9,10]. Several publications described a multi-residue method allowing the analysis of a wide range of anabolic steroids: in 1999, Gaillard et al. [11] and more recently Marcos et al. [12] published a method permitting the analysis of more than 12 analytes in hair samples.

To establish the presence of anabolic agents and their metabolites in hair, it is essential to base the measurement on analytical techniques [13] able to detect ultra-trace amounts of these compounds. Indeed, even if the observed concentrations are in the

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0.1–10 μg kg^{-1} range, the limited sample size implies detection of picogram level of steroids. Gas chromatography–tandem mass spectrometry (GC–MS/MS) was chosen as detection technique, especially in the SRM mode, because of its specificity and sensitivity, compatible with the target concentration range.

Thereafter will be presented our sample preparation strategy with the justifications of the choices, the monitored signals for the multi-analyte survey, the performances of the method taking into account the CCα and CCβ concept, and finally selected examples of application on real hair samples have been introduced and discussed.

2. Experimental

2.1. Reagents and chemicals

Most of the reagents and solvents were of analytical grade quality and provided by VWR International (Pessac, France) and Solvants Documentation Syntheses (SDS, Peypin, France). The solid-phase extraction (SPE) columns were from SDS (Peypin, France) (silica, 1 g; aminopropyl, 0.5 g). The derivatisation reagents *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), dithiothreitol (DTE) and trimethyliodosilane (TMIS) were purchased from Sigma–Aldrich (St. Quentin Fallavier, France). Iodine (I_2) was from Panreac (Barcelona, Spain). The reference steroids were from Interchim (Montluçon, France), Sigma–Aldrich (St. Quentin Fallavier, France), AGAL/NARL (Australia) and RIVM (Bilthoven, The Netherlands). The internal standards used were DES-d_6, 17β-estradiol-d_3, 17β-nandrolone-d_3 (NT-d_3), zeranol-d_4, taleranol-d_4, 17α-methyltestosterone-d_3 (MT-d_3), medroxyprogesterone-d_3 (MP-d_3), melengestrol-d_3 (MLG-d_3), 17β-testosterone-d_2 (T-d_2), 17β-trenbolone-d_2 (Tb-d_2) and megestrol-d_3 (MG-d_3).

2.2. Extraction and purification procedure

The developed procedure for the extraction–purification of steroids in the hair sample is shown in Fig. 1.

2.2.1. Hair sample extraction and hydrolysis

- Hair grinding and methanolic extraction procedure [14]:
  The hair was grinded, sonicated 1 h with 5 mL of methanol and incubated one night at 50 °C. After centrifugation the methanolic extract was removed. Same volume of methanol was added to the grinded hair then removed for completing steroids extraction. The methanolic extracts were spiked with a mixture of internal standards and evaporated before further hydrolysis.
- Three main hydrolysis procedures with a repetition of four were tested and compared, standards being added just before the studied hydrolysis step:
  (A) Hair grinding and methanolic extraction then sodium methylate (MeONa) hydrolysis (500 μL MeONa 1% in methanol, 30 min at 50 °C, then addition of 1 mL 1 M NaOH).

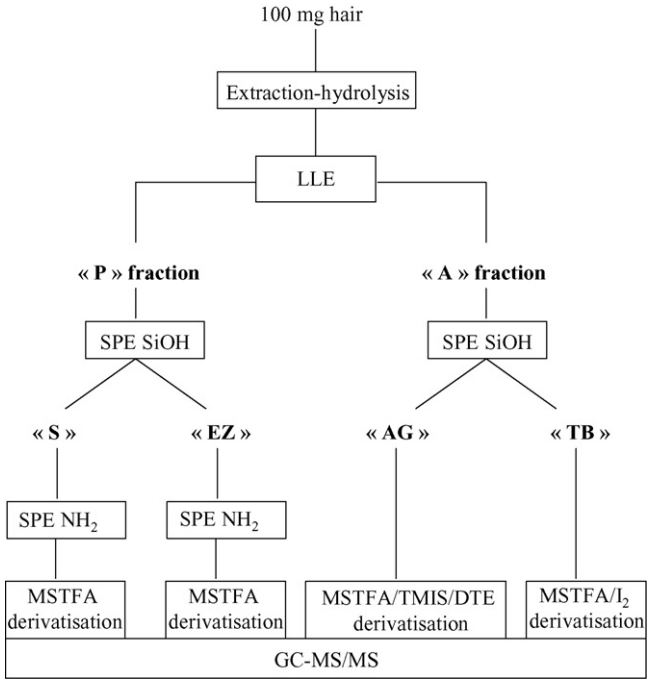

Fig. 1. General analytical procedure for steroid analysis in hair samples (SPE, solid phase extraction; LLE, liquid–liquid extraction).

  (B) Hair grinding and methanolic extraction then HCl hydrolysis (2 mL MeOH and 3 mL 1 M HCl, 4 h at 50 °C before extraction with ethyl acetate, then evaporation and addition of 4 mL 1 M NaOH).
  (C) NaOH hydrolysis directly on non-grinded hair (4 mL 1 M NaOH, 25 min at 85 °C), before further clean up and analysis by GC–MS/MS. The most efficient method was then studied in terms of ester hydrolysis efficiency then optimized.

2.2.2. Hair sample clean up

2.2.2.1. Separation of phenolic and Δ4-3-one compounds. After hydrolysis, two liquid–liquid extractions in alkaline medium phase performed with 5 mL hexane/diethylether (70:30, v/v) permitted to extract Δ4-3-one compounds "A" (androgens and progestagens). Glacial acetic acid and 1 mL of 0.2 M acetate buffer were added to the aqueous phase to obtain a pH of 5.2. The extraction of phenolic compounds "P" (stilbens, resorcylic acids and phenolsteroids) was realized twice with 5 mL diethyl ether.

2.2.2.2. Purification of Δ4-3-one compounds. After evaporation, the dry residue was dissolved in 500 μL hexane/dichloromethane (60:40, v/v) and applied onto a SPE silica column conditioned with hexane. The column was washed with 3 mL hexane/ethylacetate (75:25, v/v) then 8 mL hexane/ethylacetate (85:15, v/v). Analytes were eluted with 20 mL hexane/ethylacetate (60:40, v/v) and then splited in two groups: trenbolone (alpha and beta) ("TB") and other androgens–progestagens ("AG").