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Review

Analytical possibilities for the detection of stanozolol and its metabolites

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Abstract

In sports doping, as well in man as in horseracing, stanozolol (Stan) was abused and became the subject of metabolism research. Also in veterinary practice, stanozolol became an important misused anabolic steroid.

Like most other anabolic steroids, stanozolol has poor gas chromatographic behavior. It is difficult to detect in urine, because of low urinary excretion and renal clearance. This is due to the rapid metabolization, leading to low concentration levels of the parent compound found in urine. Therefore, most research studies have focused on the detection of its urinary metabolites.

For the identification of the metabolites, different methods of extraction and detection are described in the literature. These are reviewed in this article. Most authors use a hydrolysis to free the phase II metabolites. Extraction procedures vary from solid-phase extraction (SPE), liquid–liquid (L–L) extraction to immunoaffinity chromatography (IAC). For the final detection, the use of gas chromatography (GC)–mass spectrometry (MS) can be compared with liquid chromatography (LC)–MS^n. Different metabolites are identified depending on the administration of stanozolol in the animal experiment (oral or intramuscular). Analyses for these analytes in other matrices are also briefly discussed.

Keywords: Anabolic steroids; Stanozolol; Metabolites; GC–MS; LC–MS

1. Introduction

Androgens are drugs, derived from the natural male sex hormone testosterone, with high anabolic

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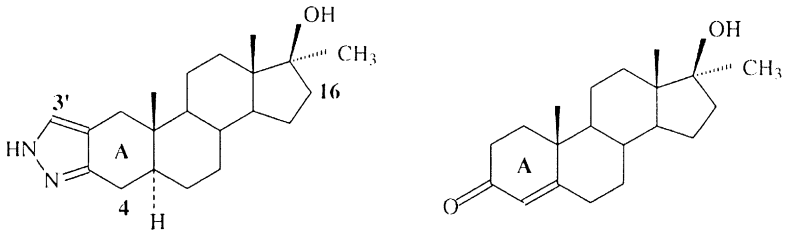

Fig. 1. Structure of stanozolol (left) and methyltestosterone (right).

potential and minimized androgenic activity [1]. Anabolic steroids stimulate protein synthesis, resulting in an acceleration of the food conversion rate and increasing muscle growth, body mass and enhanced performance [2]. Androgens can be used as therapeutics, because they accelerate the recovery of protein deficiency and protein-wasting disorders (e.g. osteoporosis) [3,4], but they are also widely abused in doping, as well in animals as in men. This led to the prohibition of the drugs by the International Olympic Committee (IOC) in 1974.

Stanozolol (Stan) (5α-androstane-17α-methyl-17β-ol [3,2-c] pyrazole) was first synthesized by Clinton et al. [5] in 1959, as a heterocyclic anabolic androgenic steroid. The structure of Stan differs from endogenous steroid hormones and most commercially available anabolic steroids [6]. It most closely resembles methyl testosterone (Fig. 1). Instead of the 3-ketogroup in methyltestosterone, there is a pyrazole ring fused to the androstane ring system. This slightly different structure has the disadvantage of making extraction and isolation of the molecule from matrices more difficult [7]. Like most other anabolic steroids, Stan has poor gas chromatographic behavior and is difficult to detect in urine, because of renal clearance and low urinary excretion [8]. This is due to the

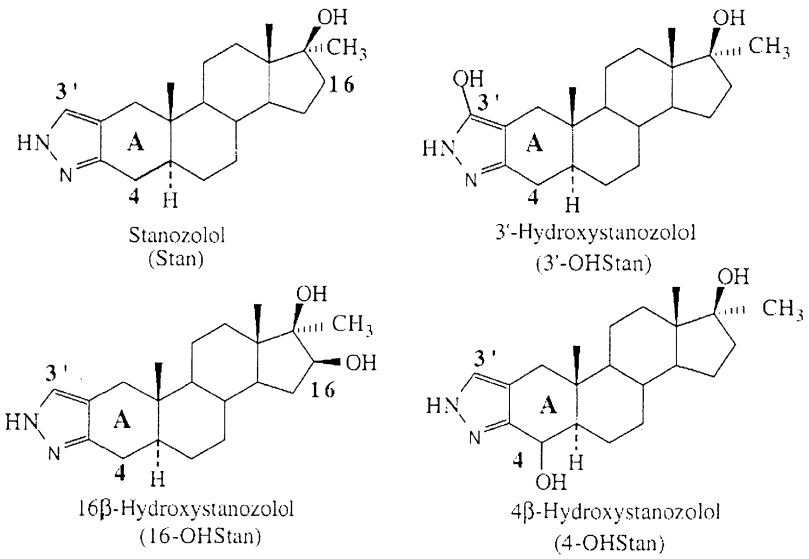

Fig. 2. Structures of stanozolol and its main metabolites.

rapid metabolization, leading to low concentration levels of the parent compound found in urine [9]. Therefore, most research studies had focused on the detection of urinary metabolites [8,9] (Fig. 2). This review discusses the different methods of detection of stanozolol and the formation of different metabolites in urine. Also analysis for these analytes in other matrices is briefly discussed.

2. Metabolization

In 1989, Massé et al. [6] published the first extensive report describing the major urinary metabolites of stanozolol in humans. Stanozolol and 11 urinary metabolites were detected after administration of Stan to humans. Gas chromatography–mass spectrometry (GC–MS) data illustrate that the biotransformation of stanozolol was characterized by the formation of mono- and dihydroxylated metabolites. Most of them are excreted in urine in the form of conjugates [6]. Less than 5% of the metabolites are found in the unconjugated fraction [10]. The free metabolites were extracted from urine by solid-phase extraction (SPE) followed by a liquid–liquid (L–L) extraction. The conjugated metabolites were hydrolyzed with a β-glucuronidase/sulfatase enzymatic preparation prior to extraction. The most abundant metabolites identified in the conjugated fraction were 16α- and 16β-hydroxystanozolol (16-OHStan), stanozolol and 3′-hydroxystanozolol (3′-OHStan) [6].

Already in 1990, Mück and Henion [1] developed a LC–MS procedure for stanozolol in human and equine urine. This approach was also based on an enzymatic hydrolysis to release the conjugated metabolites, L–L extraction and single- or coupled-column reversed-phase LC combined on-line with tandem MS (atmospheric pressure ionization (API) coupled with a triple-quadrupole mass spectrometer). The author concluded, as already described by Massé et al. in 1989, that human urine contained unmetabolized Stan, mono- and dihydroxylated metabolites. Data showed that the human urine levels of 3′-OHStan were lower than the levels of Stan itself and 16-OHStan, which had the highest level.

In equine urine, unmetabolized Stan as well as dihydroxylated Stan could not be detected, only monohydroxymetabolites were present.

In 1990, Chung et al. [12] succeeded in identifying the main conjugated form. He described that Stan metabolites were present in a glucuronide form exclusively in human urine. The concentration of the parent ion of Stan measured in the free fraction was negligible and non-detectable in gas chromatograms. The author also reported that the main metabolites of Stan analyzed by GC–MS with selected ion monitoring of their characteristic ions were found to be 3′-OHStan and its epimer. This does not completely correspond to the conclusion of Mück and Henion [1] who found Stan and 16-OHStan were present in a higher concentration than 3′-OHStan.

In 1996, Schänzer et al. [11] studied the elimination of the stanozolol metabolites in urine of athletes. He concluded that the metabolites could be detected much longer than the parent compound. The metabolites, all conjugates, were identified as 3′-OHStan, 4β-OHStan and 16β-OHStan.

Ferchaud et al. [7] in 1997 studied the metabolization of Stan in cow-urine. The author was the first to demonstrate the difference in presence of Stan and its metabolites depending on the way of administration. When Stan was administered orally, there was only an identification of Stan and the metabolite 16-OHStan, while two hydroxymetabolites, 16-OHStan and 4,16-diOHStan, were found after subcutaneous injection.

16-OHStan was found to be the major metabolite in veal calf urine. In a multi-laboratory study [9], all five laboratories found that the concentration of 16-OHStan in function of the time was similar. The first 4 days a stable concentration of 16-OHStan (between 1 and 4 $\mu g\,kg^{-1}$) was found, from day 4, there was an increase of the detected concentration until a maximum (between 5 and 11 $\mu g\,kg^{-1}$) was found between days 8 and 10. The results of the concentration were depending on the laboratory. Later there was a decrease in concentration, but after day 14, 16-OHStan was still observed. In addition to 16-OHStan, also small amounts of Stan were observed in the first few hours after injection. The metabolite 3′-OHStan was only found in low concentration by some laboratories. This is the first paper indicating that, depending on the detection method used (GC–MS or LC–MS), there can be different interpretations concerning the identification of metabolites.

3. Overview of the different detection methods

3.1. GC–MS

Horning and Donike introduced the use of modern high resolution mass spectrometry (HRMS) in 1993 [13]. Schänzer et al. [11] also implemented HRMS screening and identification of stanozolol metabolites. The assembled metabolites were derivatized to trimethylsilyl derivates for GC–MS analysis.

GC–MS was also used by Ferchaud et al. [7] in 1997 in electronic impact (EI) mode. The author used a derivatization procedure of the residue with MSTFA–TMIS–DTE (*N*-methyl-*N*-(trimethylsilyl)-triluoroacetamide–trimethyliodosilane–dithiothreitol). Changing the derivatization method by using heptafluorobutyryl anhydride (HFBA) instead of MSTFA–TMIS–DTE allowed the detection of lower concentrations (around $1\,ng\,l^{-1}$) of Stan in cow-urine after oral administration.

With GC–MS in selected ion monitoring (SIM) mode, Delahaut et al. [14] detected two of the urinary metabolites, 3′-OHStan and 16-OHStan. There was no doubt of the identity of the metabolites because the spectra for both metabolites contained more than four diagnostic ions at the correct retention time and in the correct ratios. However, it was obvious that the detection power of 16-OHStan was lower than that of 3′-OHStan. There was also a response of 4-OHStan at the correct retention time, but because of a lack of diagnostic ions the data were considered as non-relevant.

The detection power difference between the two metabolites, 3′-OHStan and 16-OHStan, could be due to the derivatization. A possible reason for incomplete derivatization could be steric hindrance of the hydroxylic groups present at the positions 16 and 17 in 16-OHStan [9]. Choi and Chung [15] developed in 2000, a method for improving the detection of 3′-OHStan and its 17-epimer as the main metabolites of Stan in human urine by GC–MS. The extraction was based on extractive isobutyloxycarbonylation (isoBOC reaction) combined with a subsequent pentane extraction. This extraction with an isoBOC reaction led to excellent recoveries of both metabolites. The injection of *N*-isoBOC-O-TMS derivates of the metabolites resulted in two chromatographic peaks with identical mass spectra in the electron ionization (EI) mode. In 2001, Haber et al. [16] reported a method for automatization of the sample preparation and GC–MS analysis for human urinary androgenic anabolic steroids.

3.2. LC–MS

In 1998, a multi-laboratory study [9] was performed to study the analytical procedure and the kinetics of Stan and its metabolites in calves treated with Stan. The animal experiment comprised three male calves that were injected intramuscularly with a single dose of Stan. Different laboratories examined urine samples with different extraction and clean-up procedures and evaluated different analytical techniques like GC–MS in the negative ionization mode and LC–MS–MS. As mentioned above, the detection power for 16-OHStan with GC–MS is inferior to that of 3′-OHStan. In LC–MS–MS there was no difference in detection power between 3′-OHStan and 16-OHStan. The LC–MS–MS signals of injection of equal amounts of standards were of the same magnitude for both metabolites. However, in the liquid chromatogram, 16-OHStan and 3′-OHStan co-eluted. From the spectra, it could be concluded that the presence of a smaller amount of 3′-OHStan was masked by a larger amount of 16-OHStan [9]. The main conclusion from this multi-laboratory study was that LC–MS–MS seemed to be the detection method of choice for determination of the 16-OH metabolite of Stan.

In 2000, Van de Wiele et al. [17] reported the optimization of the detection of Stan and its major metabolite 16β-OHStan in faeces and urine from cattle by LC–MS. The clean-up and extraction procedure consisted of a direct liquid–liquid extraction for faeces. Urine was enzymatically hydrolyzed prior to a SPE and an acidic back extraction. The intention of this acidic back extraction was to produce a much clearer extract. Without this extraction the dirty matrix caused early deterioration of the chromatographic column and blocking of the heated capillary of the spectrometer. The authors discussed two different methods of detection with LC–MS–MS. In a first approach, the final extract was detected without derivatization, while in a second approach, a derivatization step for 16β-OHStan was included. The detection method was optimized by the approach without

derivatization. Electrospray ionization (ESI) seemed to give better results than atmospheric pressure chemical ionization (APCI). By testing different mobile phases they illustrated that the mobile phase containing formic acid was twice as good as the mobile phase containing acetic acid.

Without the derivatization step, the MS–MS results showed a lot of diagnostic ions that were present in a specific pattern of clusters (Fig. 3). The derivatization step in the second approach was first based on a reaction of 16-OHStan with phenylboronic acid (PBA) (which was specific for binding diol-containing compounds in solution). A derivative was formed, which in APCI mode produced one intense ion peak and in MS–MS, a limited number of diagnostic ions (Fig. 4). This procedure resulted in chromatograms with no interference of the matrix in contrast with the first method. The advantage of the method without derivatization was that no extra derivatization step was needed and also that Stan could be determined. The spectrum of 16-OHStan was difficult to interpret due to matrix interferences. The derivatization reaction had the advantage of producing spectra with abundant ions in more stable ion ratios. Only the metabolite 16-OHStan could be derivatized with phenylboronic acid.

A reliable method for the confirmation of Stan and its major metabolite 16β-OHStan in bovine urine by LC–MS–MS was developed in 2001 by Draisci et al. [18]. The sample procedure consisted of an enzymatic hydrolysis, a L–L extraction in duplicate and purification using an amino solid-phase extraction column. APCI in positive ion mode was used for ionizing the analytes. The protonated molecules $[M+H]^+$ of Stan (*m*/*z* 329) and 16β-OHStan (*m*/*z* 345) generated, served as precursor ions for collision induced dissociation (CID) in the MS–MS experiments. Three

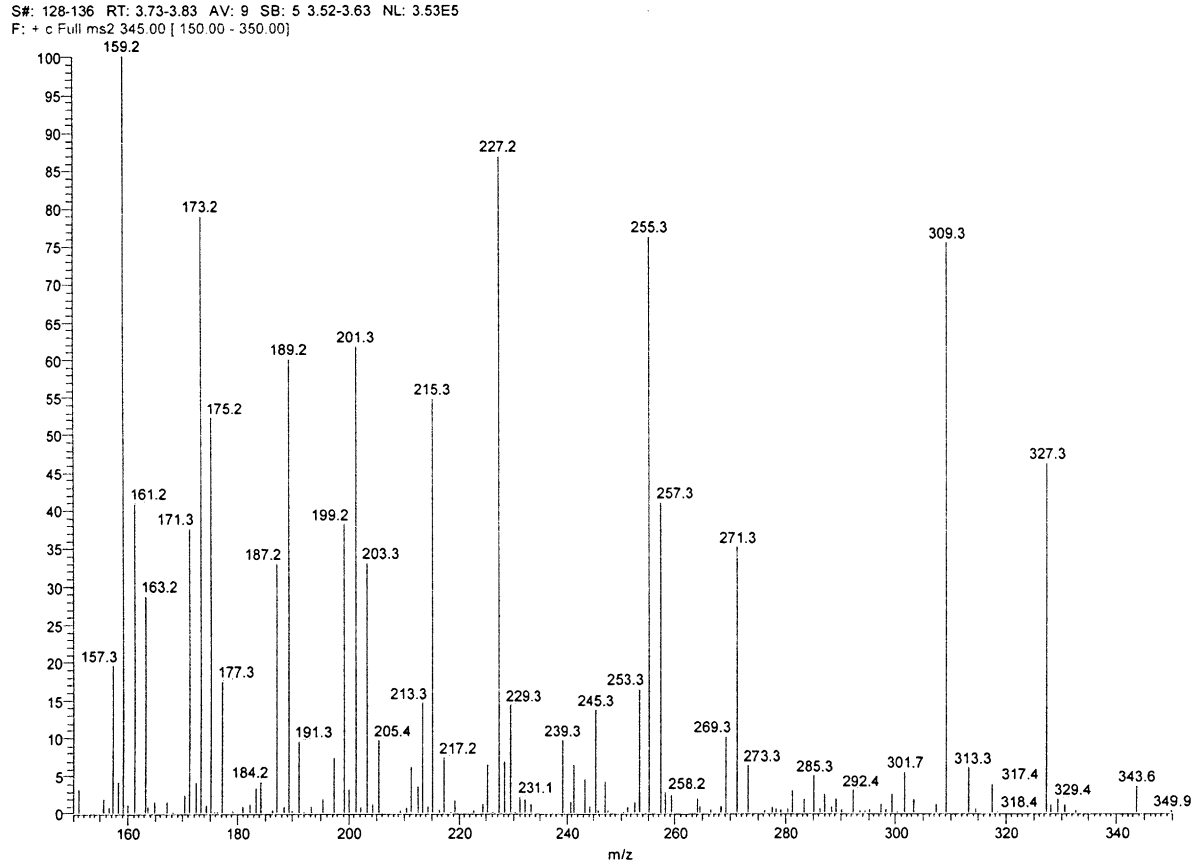

Fig. 3. LC–MS^2 spectrum of 16-OHStan.

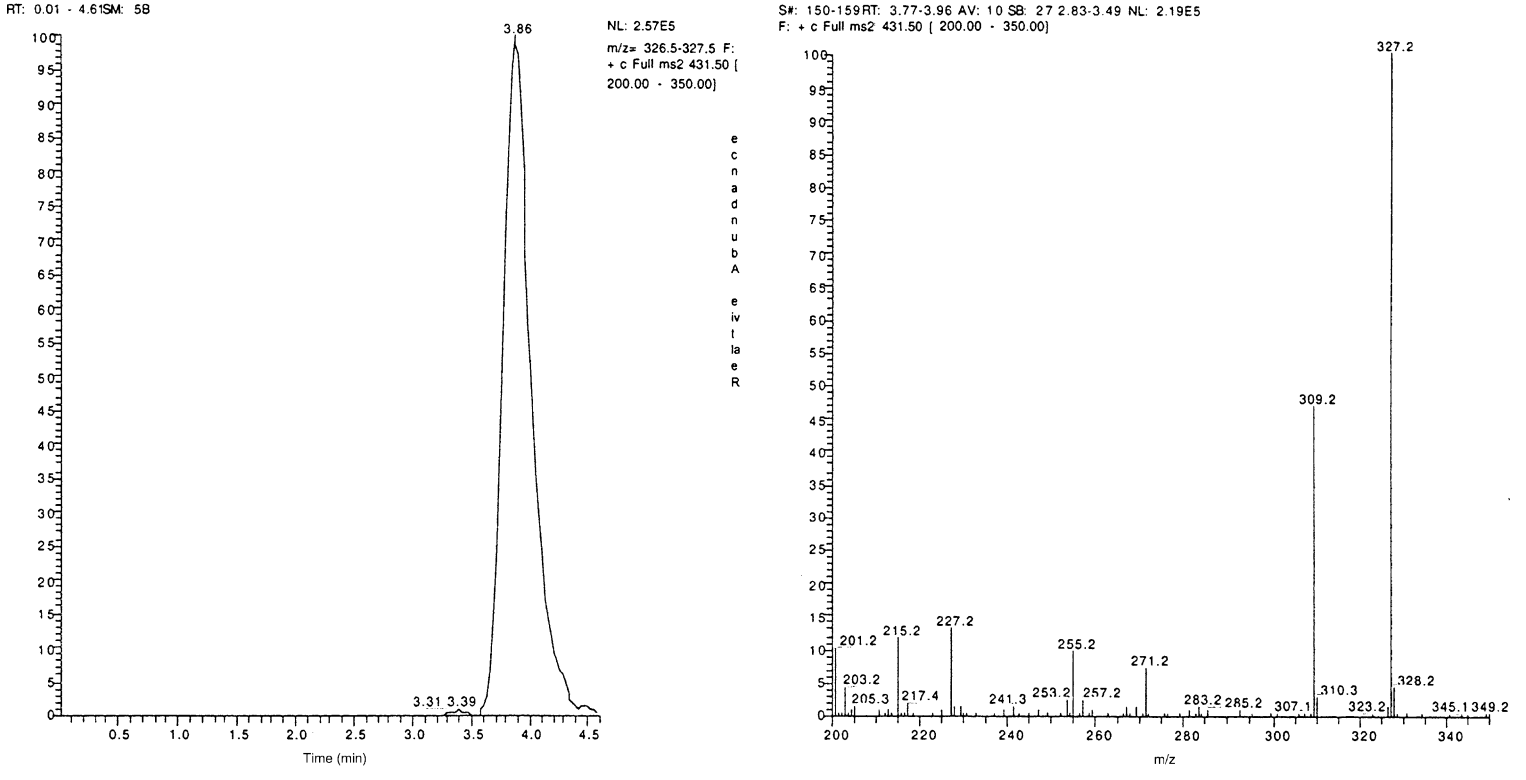

Fig. 4. LC–MS^2. Chromatogram and spectrum of 16-OHStan with PBA derivatization.

diagnostic ions for each analyte were identified for the confirmation by selected reaction monitoring (SRM) LC–MS–MS.

3.3. Limit of detection

The clean-up procedure can be a limiting factor when determining the limit of detection (LOD). Schänzer et al. [11] and Delahaut et al. [14] reported the use of IAC to eliminate the biological background that co-elutes with Stan metabolites. An acidic back extraction as reported by Van de Wiele et al. [17], also efficiently eliminated matrix interferences compared to the SPE extraction without back extraction.

Instrumental factors can also play an important role. Working in MS–MS, the LOD depends on a number of factors as collision induced dissociation (CID) efficiency, intensity of the precursor ion, fragmentation efficiency and collection efficiency. When all of these parameters were considered, Johnson et al. [19] in 1990, reported a 15-fold detection advantage of an ion trap over a quadrupole mass filter, due to the collection efficiency of the ion trap. While Thomson and co-workers [20] in 1995, were able to achieve a similar efficiency with a quadrupole mass filter when a "high pressure" collision cell design was used. MS^3 was used for TMS-enol-TMS ether derivates because for 3′-OHStan only a single MS^2 fragment ion was obtained as reported by Bowers and Borts [21].

The need for derivatization when using GC–MS was a negative factor for the detection of 16-OHStan [9]. However, in LC–MS, the PBA derivatization improved the detection efficiency [17] and facilitated the identification conditions because of the stable ion ratios of two intense MS^2 fragment ions.

4. Detection of Stan and its metabolites in other matrices

4.1. Edible bovine matrices

A large variety of bovine matrices was investigated for Stan and its major metabolite 16β-OHStan by De Wasch et al. [22]. The matrices of an animal trial muscle, liver, kidney, kidney fat and heart, were collected at the slaughterhouse. Detection was performed with LC–ESI–MS^2. The authors concluded that liver was the target matrix. Stanozolol and 16-OHStan were both detected. The concentration of the parent compound compared to the metabolite depended on the amount of stanozolol that was administered and the period between administration and slaughter.

4.2. Hair

Hair has been proposed as an alternative matrix to urine for detecting drug use and might be useful for the detection of anabolic steroids [8]. Urine analysis provides short-term information of the use of drugs, whereas long-term histories are accessible through hair analysis [23]. This possibility of detecting drugs in hair for a long period of time after ingestion is an important feature [8,24].

4.2.1. Animal hair

In 1996, Höld et al. [8] reported the detection of Stan in rat hair by negative ion chemical ionization–mass spectrometry. Stan was easily detected in both non-pigmented and pigmented hair. The concentrations found in the pigmented hair ($362.4\,\mu g\,kg^{-1} \pm 332.4\,\mu g\,kg^{-1}$) were 3.4 times greater than found in the non-pigmented hair ($90.0\,\mu g\,kg^{-1} \pm 46.9\,\mu g\,kg^{-1}$). The importance of pigmentation for the incorporation of certain drugs in hair is well established [25–27]. It is probable that, for certain drugs, pigmented hair has specific binding sites of the polyanionic polymer melanin. Nakahara et al. [26] showed that a drug increase of basicity would increase its incorporation into pigmented hair. Because of the presence of the basic nature of its pyrazole ring, Stan would be preferentially distributed in pigmented hair, as was experimentally proved [8]. The binding has an ionic character, which suggests that methanolic sonication of a powdered hair sample should be an effective pretreatment of the matrix when testing for anabolic steroids [24].

In 1999, Gaillard et al. [23] reported the analysis of hair as a possible application in meat quality control instead of analyzing meat.

4.2.2. Human hair

Gaillard et al. [23] also tested human hair for the presence of anabolic steroids and their esters with GC–tandem MS. Application in human doping control was demonstrated. Some individuals were isolated as steroid users by the analysis of their hair, while urine analysis gave negative results.

In 2000, Cirimele et al. [24], tested the presence of Stan in human hair by GC–negative ion chemical ionization MS. The hair was obtained from a bodybuilder who declared to be a regular user of Stan. Stan was identified and quantified at a concentration of 15 $\mu g\,kg^{-1}$, its urinary metabolite 3′-OHStan was not detected in hair. This concentration of Stan was largely lower than those determined by Höld et al. [8] in the hair of rats as described earlier. This was due to the high dose of Stan administered to the rats. Thieme et al. [28] determined concentrations of 180 $\mu g\,kg^{-1}$ and 6 $pg\,mg^{-1}$ for Stan and 3′-OHStan, respectively, and Kintz [29] reported concentrations of two bodybuilders of 135 and 156 $\mu g\,kg^{-1}$. This could explain why 3′-OHStan was not detected by Cirimele et al. They reported that it was generally the case with hair analysis that the parent drug was found in higher concentrations than the metabolites.

5. Other detection methods

Besides GC–MS and LC–MS other detection techniques can be applied for the determination of Stan and its metabolites.

An accurate HPLC–UV method was developed for anabolic steroids in human serum by Lampert and Stewart [2]. An enzyme-linked immunosorbent assay (ELISA) test for the screening of Stan and 16β-OHStan in urine samples was reported by Douglas et al. [30]. High-performance thin-layer chromatography (HPTLC) was also proposed, but it showed poor sensitivity and low specificity for Stan in comparison with other anabolic steroids [31]. Preliminary steps in the development of a radioimmuno assay (RIA) for the detection of stanozolol were presented by Barret et al. in 1995 [32]. The development of a biochip for the detection of drug residues was described by McConnell et al. [33].

6. Conclusions

Extensive knowledge of the metabolic transformation of stanozolol and the development of efficient analytical methods to identify and determine parent drug and/or metabolic products in different matrices are necessary for achieving evidence of the illegal use of drugs in athletics and in animals.

The metabolization of Stan indicates a quick production of mono- and dihydroxylated metabolites in humans and animals that are mainly present in a glucuronide form. The most abundant metabolites identified in human and animal urine are 16β-OHStan, 3′-OHStan and 4β-OHStan. 3′-OHStan was the main metabolites used in routine detection methods analysing human urine. 16-OHStan was the main metabolite after administration to bovine. It should also be noted that depending on the way of administration, oral or subcutanous, a difference can be observed in the identity of the metabolites.

Because of the conjugated presence of the metabolites, the extraction procedure for Stan and its metabolites from urine always consisted of an enzymatic hydrolysis and a liquid–liquid or solid-phase extraction. IAC isolation or an acidic back extraction can be used to eliminate background interferences.

Also the derivatization, prior to GC–MS analysis, plays an important role in the detection. The derivatization works better for 3′-OHStan than for 16β-OHStan, so the detection is in favor of 3′-OHStan. Meanwhile in LC–MS is no need to derivatize. The detection power of the metabolites gave better results using LC–MS. Intensive fragmentation in MS^2 has led to the development of a simple PBA derivatization resulting in more stable spectra.

Next to urine also other matrices as edible animal matrices and hair were briefly discussed. Liver was the target matrix for analysis of slaughterhouse samples and depending on the period between administration and slaughter the balance was shifted toward larger concentrations of 16-OHStan instead of Stan. Hair analysis allowed the detection of long-term histories of abuse. It was generally the case for hair that the parent drug was found in higher concentrations than the metabolites. Stan was preferentially distributed in pigmented hair.

Based on the papers as cited in the reference list, a complete overview has been discussed of the analytical possibilities for analyzing stanozolol and metabolites in a variety of matrices.

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