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Detection of mono-hydroxylated metabolites of stanozolol by HPLC-ESI (+) MS/MS in Indian sports persons

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The abuse of stanozolol is quite widespread in Indian sport. Its analysis is challenging and this has led to the development of new methods to improve its detection. A method was developed and validated for the detection of the three main monohydroxylated metabolites of stanozolol. The excretion profile of these metabolites was studied in four healthy male volunteers. The excretion study samples, after a single oral dose of drug, showed that 3′-OH-stanozolol was excreted at the highest concentration, followed by 16β-OH stanozolol, with 4β-OH stanozolol as the least excreted. Ninety-eight old doping samples with adverse analytical findings for 3′-OH-stanozolol were reanalysed using this method. This showed 3′-OH-stanozolol and 16β-OH stanozolol in all the 98 samples whereas 4β-OH-stanozolol was identified in 90 samples. The percentage of positive identifications of stanozolol in Indian sportspeople has increased markedly in the last five years, from 31.9% in 2004 to 81.8% in 2009; however, this may be due to the more effective detection of stanozolol metabolites. It can thus be concluded that the marked increase in percent positive of stanozolol in Indian sportspersons in 2009 may be due to the improved detection by a more effective LCMS/MS method.

Keywords: LC-MS/MS; minimum required performance limit; stanozolol; World Anti Doping Agency

Introduction

The use of androgenic anabolic substances (AAS) for performance enhancement in sports is fairly common.[1–2] Stanozolol (17β-hydroxy-17α-methyl-5α-androst-2-eno (3, 2-C)-pyrazole) has been one of the most abused anabolic steroids and the analysis of this compound is very challenging. It must be detected and confirmed at the minimum required performance limit (MRPL) of 2 ng/ml.[3–4] Stanozolol is metabolized to a large extent and the main metabolic products in urine are the mono hydroxylated metabolites 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol (Figure 1).[5–6] Traditionally, doping control analysis for stanozolol and its metabolites in urine involves basic extraction followed by derivatization and identification using gas chromatography-mass spectrometry (GC-MS).[7–8] However due to its pyrazole structure, stanozolol and its metabolites form bonds with any active site in the gas chromatographic system, leading to difficulties in its detection – especially at low concentration. This led to the use of the high-resolution mass spectrometer (HRMS) for the detection of stanozolol in sports.[9–11] Later, various extraction procedures were employed for the detection of stanozolol and its metabolites, including the use of Oasis cartridges and immunoaffinity chromatography to reduce matrix interference and improve detection.[12–14] The lengthy extraction protocols and expensive high-resolution magnetic sector instruments demonstrated the need to explore an alternative approach. It was then observed that liquid chromatography-mass spectrometry (LC-MS/MS) offers an attractive solution for the detection of stanozolol and its metabolites.[15–20]

The objective of the present paper was to explore the possibility of including 3′-OH-stanozolol, 4 β-OH-stanozolol and 16β-OH-stanozolol in an existing routine method employed for the detection of glucocorticosteroids, a few anabolic steroids and anti-oestrogenic agents on ESI-LC-MS/MS. The method was validated and applied for the detection of stanozolol metabolites in human urine after oral administration of stanozolol to four healthy volunteers. Ninety-eight old doping-control samples with adverse analytical finding for 3′-OH-stanozolol, which had been reported earlier to the National Dope Testing Laboratory India lab from 2006 to 2009, were reanalysed using this method.

Stanozolol is the most abused AAS in Indian sports. A record has been kept of the percentage of positive findings for the past few years. A statistical evaluation for total stanozolol positive cases in India from 2000 to 2009 was performed.

Experimental

Materials and chemicals

Certified reference standards of 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol were purchased from the National Measurement Institute (Australia). The organic solvents and reagents were of HPLC grade. Acetonitrile, ethanol and ethyl acetate were obtained from Qualigens (Mumbai, India), tertiary butyl methyl ether (TBME) from Acros Organics (New Jersey, US), and formic acid from Merck (Mumbai, India). β-Glucuronidase *(E. coli)* enzyme was purchased from Roche Diagnostics Corporation (Indianapolis, US). Deionized water was prepared in a Milli-Q laboratory plant (Millipore, Bedford, US).

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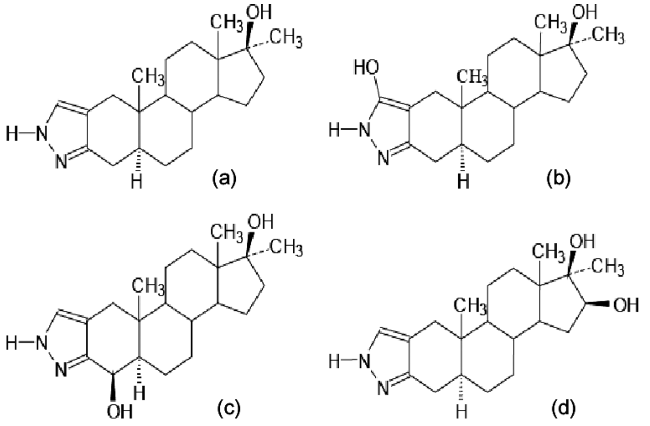


Figure 1. Structures of (a) stanozolol; (b) 3′-OH-stanozolol; (c) 4β-OH-stanozolol and (d) 16β-OH-stanozolol.

All standard solutions were prepared in ethanol at a concentration of 1 mg/mL. These solutions were stored at 4 °C and standard working solutions were prepared freshly each day by dilution with ethanol.

Sample pretreatment

The sample extraction procedure involves enzymatic hydrolysis and liquid-liquid extraction. Two/four ml of urine sample aliquots based on specific gravity were taken and 500 ng/mL of internal standard (17-α-methyl testosterone) was added. Hydrolysis was carried out by the addition of the β–glucuronidase *(E. coli)* enzyme at pH 7.0 using phosphate buffer at 60 °C for an hour. After hydrolysis, the pH was adjusted to 9–10 with 7% K_2CO_3. First extraction was performed using TBME solvent and organic layer was separated. The pH of the aqueous layer was then adjusted between 2 and 3 and the second extraction was done with ethyl acetate. Both the organic layers were mixed and evaporated under nitrogen gas at 60 °C. The residue was redissolved in 100 µL mobile phase (1% formic acid and Acetonitrile-50 : 50 (v/v)) and was transferred into conical autosampler vials for analysis.[2]

High performance liquid chromatography

The HPLC system used consisted of Agilent 1100 series (Agilent Technologies, Waldron, Germany) equipped with G1311A high-pressure gradient pumping system, G1329A Autosampler, G1379A degasser and G1316A column compartment. An Inertsil® C-18 ODS-3 column (3.0 µm, 50 mm × 4.6 mm) from GL Sciences Inc., Tokyo, Japan was used. The mobile phase was 1% formic acid in water (solvent A) and Acetonitrile (solvent B). The flow rate was set to 0.7 mL/min. The gradient was 15% B to 100% B in 7.00 min and then reducing back to 15% B by 11 min. The injection volume was 10 µL

Mass spectrometry

The Sciex API 3200 (Applied Biosystems, Canada) triple quadrupole mass spectrometer with electro spray ionization source was used. The ionization source was operated in positive mode at 550 °C using spray voltage of 5000 V. Nitrogen was used for curtain gas (setting 10 psi), nebulizer gas (setting 35 psi) and heater gas (setting 30 psi). The MRM transition, collision energy and the declustering potential used are summarized in Table 1. The CAD gas setting was 3 psi. The HPLC and MS/MS were controlled using the PE Sciex Analyst 1.4.1 Software.

Table 1. Compound ionization details

Compound	MRM transitions (quantifier ion)	Declustering potential	Collision energy
3′-OH-stanozolol	345-97	35	55
4β-OH-stanozolol	345-145	35	35
16β-OH-stanozolol	345-81	35	55

Method validation

The analytical method was validated in accordance with the requirements of WADA ISL (version 5.0), keeping in view linearity, accuracy, precision, recovery, specificity, limit of detection (LOD) and limit of quantitation (LOQ).

The four-level calibration curve was made by adding defined volumes of ethanolic solution of the reference standards of 3-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol. Spiked quality-control samples were prepared in five replicates at four concentration levels. The concentrations of calibration standard and quality-control samples were 1 ng/mL, 2 ng/mL, 4 ng/mL and 10 ng/mL.

Recovery was calculated by comparing the areas of spiked quality control samples with areas of direct standard in ethanol.

Acceptable specificity was defined as the area of possible interferences in blank urine samples. Blank urine samples had to be below 1/3 of the area of calibration standard of 1 ng/mL or not detectable.

The LOD was defined as the lowest concentration of analyte that can be identified, measured and reported. It was calculated using two diagnostic ions with a signal-to-noise ratio greater than 3. The LOQ of the method was defined as the lowest concentration of an analyte for which results can be obtained with reasonable statistical certainty.

Excretion study

The excretion study was performed with four healthy male volunteers after the administration of a single dose of 4 mg of drug. The study protocol was reviewed and approved by the ethical committee of NDTL, India. Each volunteer signed a statement of informed consent prior to drug administration. Samples were collected before and after drug administration for 80 hours and stored at −20 °C prior to analysis.

Positive doping control samples

The efficacy of the present method was confirmed by testing 98 old doping-control samples reported to the NDTL, India, from 2006 to 2009, for adverse analytical findings for 3′-OH-stanozolol.

Results

All the three metabolites of stanozolol, namely 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol, are efficiently ionized using positive ESI and give rise to characteristic product ions. When the parent ion was chosen as m/z 345 the ESI, the product ions m/z 97, m/z 145 and m/z 81 were obtained as quantifier ions for 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol respectively (Table 1). The spectra obtained for all the three metabolites from their certified reference materials were matched with the spectra

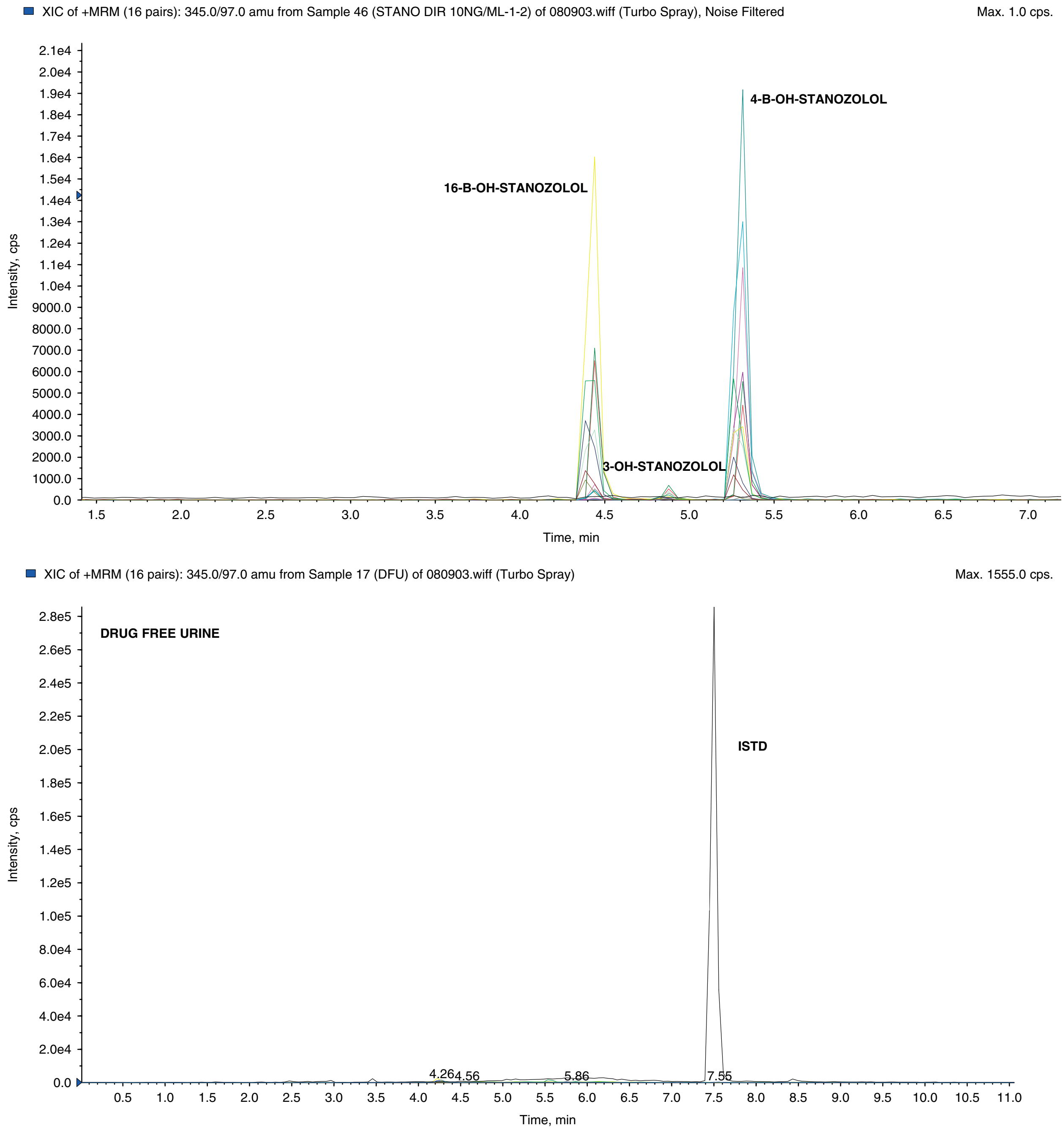


Figure 2. (a) Total ion chromatogram showing 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol and (b) total ion chromatogram showing specificity of the method with no interference in urine blank.

of metabolites in stanozolol positive urine samples. The MRM transitions used for the ion matching of the three metabolites passed the criteria set by WADA.[21]

Method validation

Calibration curve

The calibration curve was found to be linear in the range from 1 to 10 ng/ml for all the three metabolites. The calibration equation obtained is $y = 1.1700+0.0005$ $r^2 = 0.9998$, $y = 0.0003+0.0043$ $r^2 = 0.9997$, $y = 0.017+0.288$ $r^2 = 0.9969$ for 3-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol respectively.

Recovery precision and accuracy

The percentage recovery of three metabolites at various concentrations ranged from 80.5% to 93.3%. The method showed good precision and accuracy (Table 2).

Specificity

Ten different blank urine samples prepared and analysed by the same procedure show the absence of any interfering signals (Figure 2b).

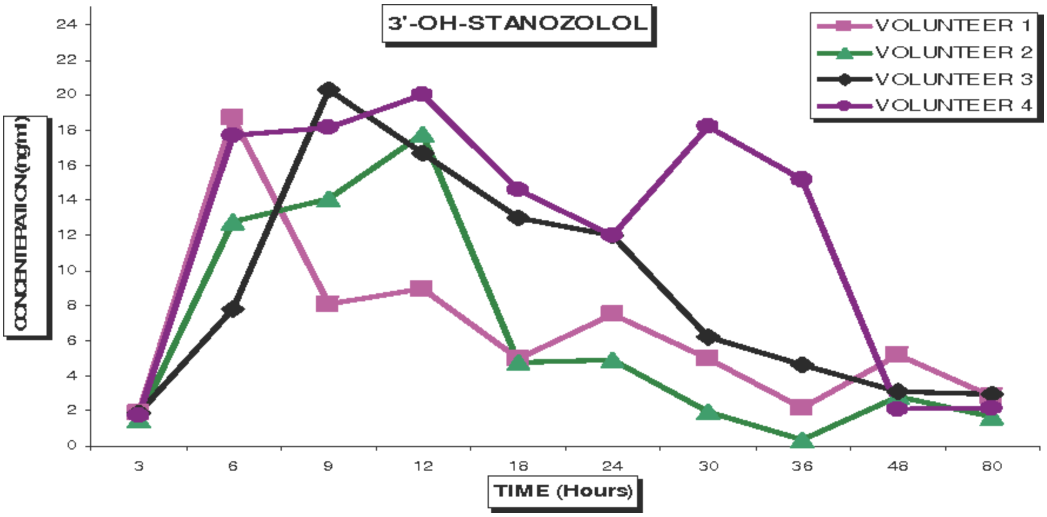


Figure 3a. Excretion profile of 3′-OH-stanozolol, n = 4, in human urine after administration of stanozolol (4 mg).

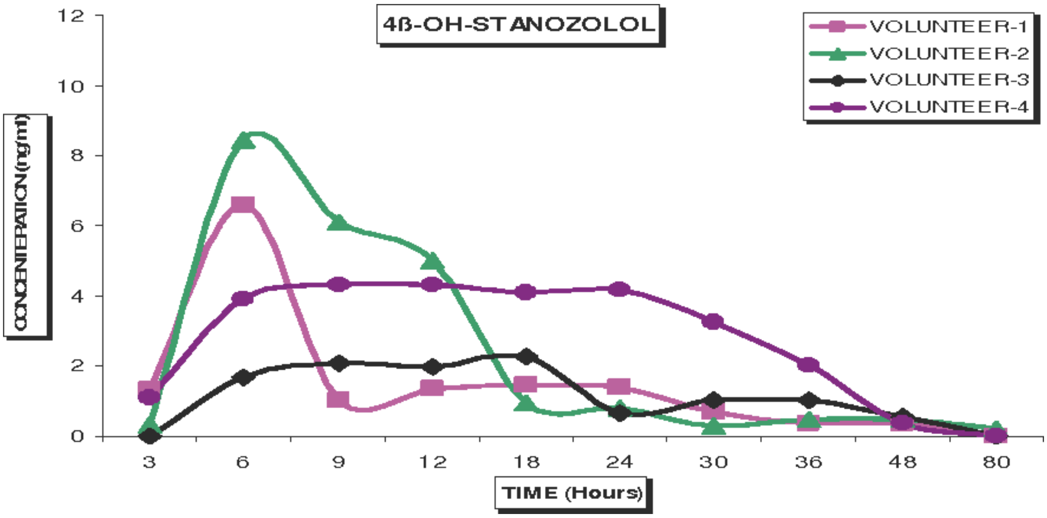


Figure 3b. Excretion profile of 4β-OH-stanozolol, n = 4, in human urine after administration of stanozolol (4 mg).

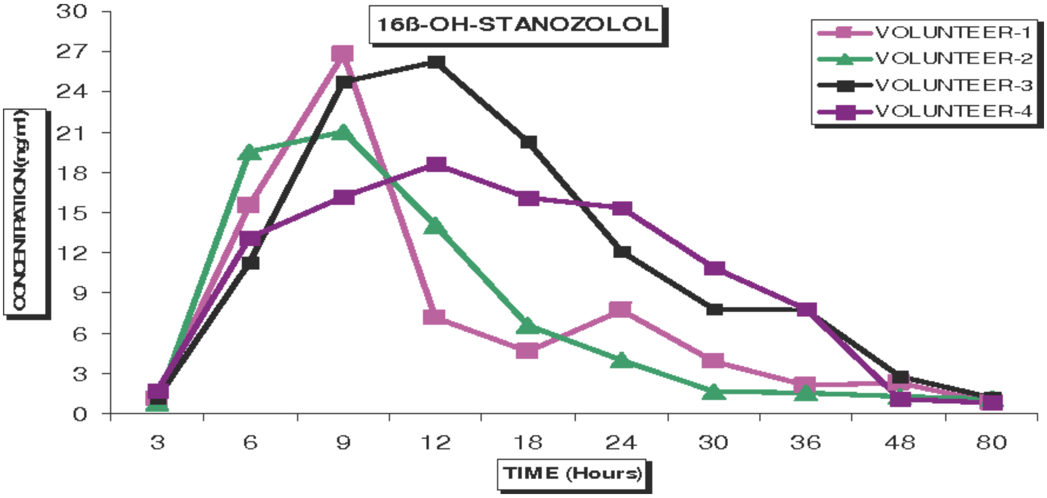
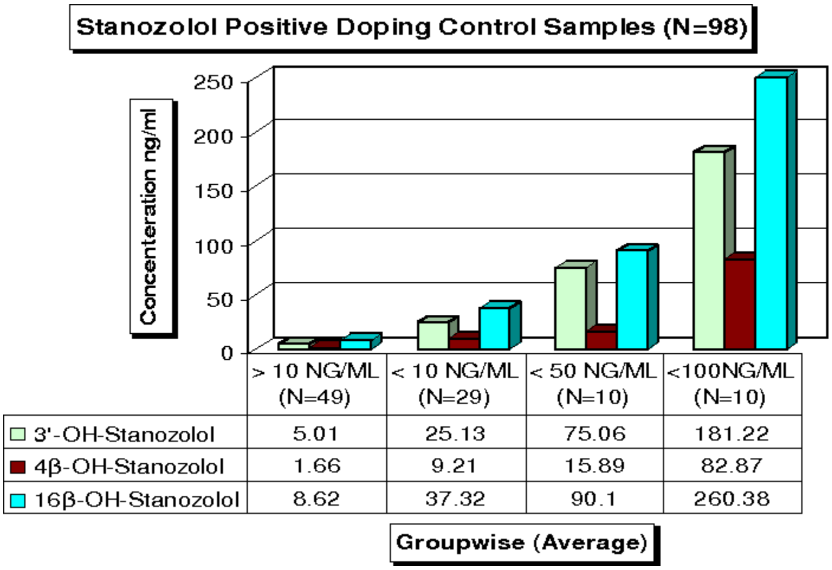


Figure 3c. Excretion profile of 16β-OH-stanozolol, n = 4, in human urine after administration of stanozolol (4 mg).



	> 10 NG/ML (N=49)	< 10 NG/ML (N=29)	< 50 NG/ML (N=10)	<100NG/ML (N=10)
☐ 3'-OH-Stanozolol	5.01	25.13	75.06	181.22
■ 4β-OH-Stanozolol	1.66	9.21	15.89	82.87
■ 16β-OH-Stanozolol	8.62	37.32	90.1	260.38

Figure 4. Graphical representation for 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol in positive doping-control samples.

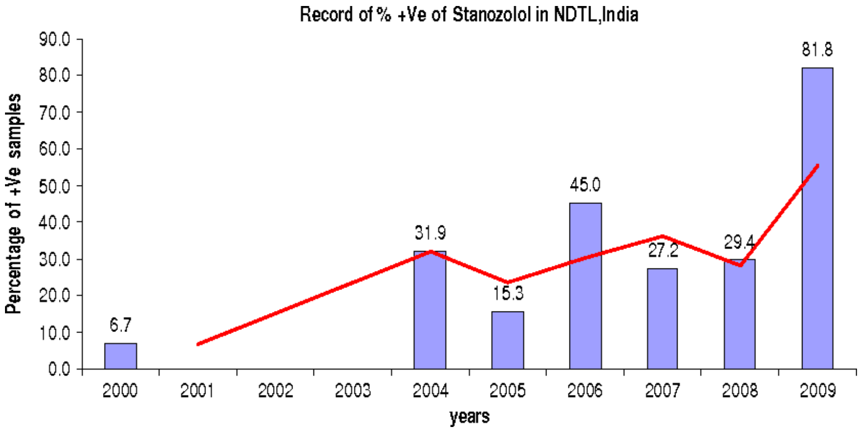


Figure 5. Yearwise record of adverse analytical finding for stanozolol at NDTL, India.

Matrix effect

The ion suppression and ion enhancement effects were determined using post-column infusion analysis for the target analytes and then introducing the matrix. No significant increase or decrease in signal sensitivity at expected retention times was observed for all the three analytes.

Limit of detection and limit of quantitation

The LOD was found to be 0.5 ng/mL and the LOQ was calculated to be 1 ng/mL.

Excretion study

The excretion study of hydroxylated products of stanozolol in human urine after a single oral administration of drug (4 mg) showed peak levels of 16β-OH-stanozolol as highest (22.22 ± 4.65 ng/mL) followed by 3′−OH-stanozolol (15.86 ± 4.80 ng/mL) and 4β-OH-stanozolol (5.15 ± 2.96 ng/mL). The excretion profile of all the metabolites in 0–80 hours is shown in Figures 3a to 3c.

Positive doping control samples

The results of 98 old doping samples with adverse analytical findings for 3′-OH-stanozolol showed the presence of 3′−OH-stanozolol and 16β-OH-stanozolol in all the 98 samples and 4β-OH-stanozolol could be detected in 90 samples. The quantitation values of stanozolol metabolites in 98 old doping-control samples showed that 50% of positive samples had less than a 10 ng/ml concentration (Figure 4) and 15% of these samples were tested in the year 2009 whereas rest of the samples were tested during the period from 2006 to 2008. The record of adverse analytical finding for stanozolol at NDTL, India, shows 81.8% of positive identifications of stanozolol in Indian sportspeople in the year 2009 (Figure 5). However, statistical analysis of stanozolol positive cases by event (Figure 6) shows that the highest percentage of positive identifications (34.76%) occurred in weightlifting, followed by athletics (25%), body building (18.7%) and power lifting (16.4%).

Discussion

Stanozolol, a potent anabolic androgenic steroid, is the most misused anabolic steroid in Indian sports. It is metabolized to a

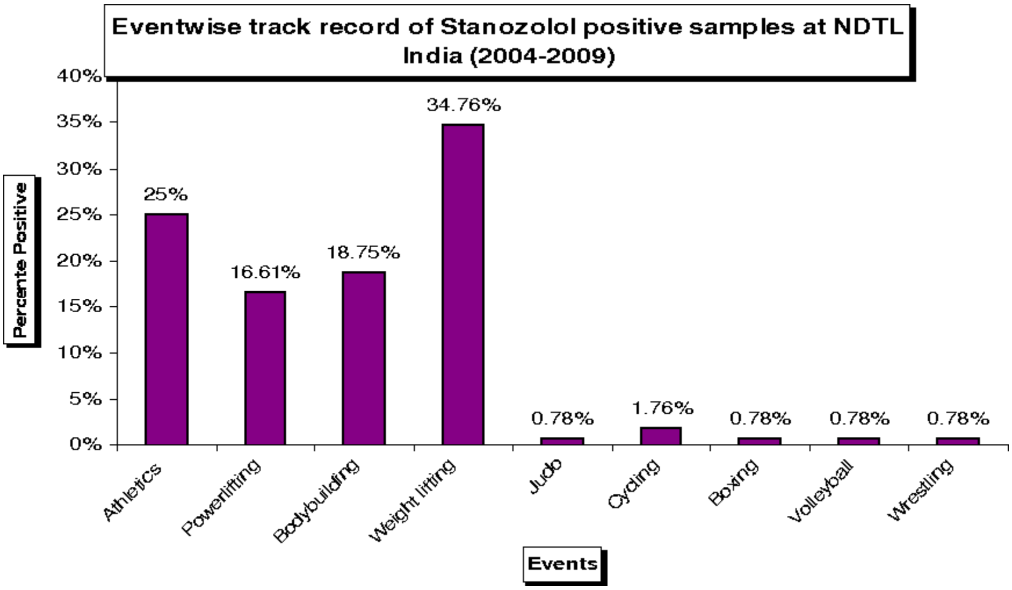


Figure 6. Eventwise record of adverse analytical finding for stanozolol at NDTL, India.

Table 2. Recovery percentage, precision and accuracy for all the metabolites

Metabolite	Expected concentration (ng/ml)	Measured concentration (mean ± SD)	Recovery percentage (mean ± SD)	% CV	Accuracy
3′-OH-stanozolol	1	0.8 ± 0.05	86.3 ± 8.07	6.1	81.0
	2	1.8 ± 0.14	87.3 ± 5.52	8.2	88.8
	4	3.8 ± 0.04	89.3 ± 7.14	1.1	95.1
	10	10.1 ± 0.46	92.4 ± 2.37	4.6	101.4
4β-OH-stanozolol	1	1.1 ± 0.05	87.9 ± 6.45	5.3	107.8
	2	1.90.05	87.6 ± 4.17	2.8	92.6
	4	3.9 ± 0.08	90.8 ± 4.93	2.1	96.9
	10	10.10.47	92.5 ± 3.82	4.7	100.6
16 β-OH-stanozolol	1	1.0 ± 0.06	80.5 ± 2.70	5.8	102.2
	2	1.8 ± 0.09	88.8 ± 3.13	5.3	88.9
	4	3.1 ± 0.43	91.0 ± 2.59	9.6	99.9
	10	10.0 ± 0.15	93.3 ± 1.57	1.5	100.4

large extent and the main metabolic products in human urine are 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol. The detection of stanozolol metabolites presents many difficulties for gas chromatography.[5–6] In order to improve detection of stanozolol and its metabolites at low concentration, various analytical strategies have been applied both in sample preparation and instrumentation. The present method involves a strategy to further improve detection of stanozolol metabolites.

The method developed was sensitive enough to detect 3′-OH-stanozolol, 4β-OH-stanozolol and 16β-OH-stanozolol at a low concentration of 0.5 ng/mL. However, the sample extraction method is an extension of the existing method being used for glucocoticosteroids, a few anabolic steroids and anti-oestrogenic agents in our lab and hence it was economical in terms of time and money.[2] Earlier, screening of stanozolol metabolites was carried out using XAD-2 extraction and HRMS analysis. The suspicious samples were further confirmed using Oasis cartridges for extraction and HRMS analysis. It was very time consuming, causing delays in reporting adverse analytical findings.[11] The detection of stanozolol metabolites using LC-MS/MS screening improved the detection limit of stanozolol and its metabolites. This may account for the increase in the percentage of positive findings of stanozolol in India in recent years.

The abuse of stanozolol is prevalent in Indian sports. Ninety-eight old positive samples reported for 3′-OH-stanozolol by testing with HRMS analysis were retested using the present method. All the samples show the presence of 3′–OH stanozolol and 16β-OH-stanozolol whereas 4β-OH-stanozolol could be detected in 90 samples. The evaluation reveals that, in all the cases, 3′-OH-stanozolol and 16β-OH-stanozolol were present in the highest amount followed by 4β-OH-stanozolol. The excretion profile of stanozolol metabolites after a single oral dose of 4 mg of drug in four healthy volunteers also showed a similar pattern – i.e. 3′–OH stanozolol is excreted at a slightly higher concentration than 16β-OH-stanozolol followed by 4β-OH-stanozolol. This study shows that, in the Indian population, 3′–OH stanozolol and 16β-OH-stanozolol can be treated as the marker metabolites for stanozolol abuse. The results of the excretion study are consistent with the results of analyses of old positive doping-control samples and contradict the results of Thevis *et al.*[15] where 4β-OH-stanozolol was found in higher concentration.

Conclusion

The marked increase in the percentage of positive findings of stanozolol in Indian sportspeople in 2009 may be due to improved detection by the more effective LC-MS/MS method. Further work is in progress to explore detection of other reported stanozolol metabolites in old doping samples at NDTL, India.

Acknowledgement

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