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Urinary excretion profile of main mono hydroxylated metabolites of stanozolol by HPLC-ESI (+)-MS/MS

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Introduction

The use of androgenic anabolic substances (AAS) for performance enhancement in sports seems to be a ubiquitous topic of discussion.[1] Stanozolol has been one of the most abused anabolic steroids.[2] It is metabolized to a large extent and the main metabolic products in urine are the mono hydroxylated 3´-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol.[3] Doping control analysis for Stanozolol and its metabolites in urine includes basic extraction following derivatization and identification using Gas Chromatography-Mass Spectrometry (GC-MS) and High Resolution Mass Spectrometer (HRMS) for detection at low concentration. [3.6]. The traditional method used to detect these compounds faces some pitfalls as these compounds show a very poor gas chromatographic behavior and are difficult to detect the drug at low concentration. The alternative techniques like Liquid chromatography-Mass Spectrometry (LC-MS) offer an attractive solution for detection of stanozolol and its metabolites.[5]

The objective of the present paper was to develop and validate the method for the identification of 3´-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol on LC-MS/MS.

Experimental

The sample extraction procedure involves hydrolysis and Liquid-liquid extraction and analysis by LC-MS/MS.[6] The Analytical method was validated as per the requirement of WADA ISL (version 5.0) keeping in view linearity, accuracy, precision, specificity, recovery, limit of detection (LOD) and limit of quantitation (LOQ).[7] Excretion study was performed with four healthy male volunteers (Age-24-27, Weight-55-60) after administration of single dose of 4 mg of drug (Tanzol-Scortis). The study protocol was reviewed and approved by the

ethical committee of NDTL, India. The efficacy of the method was confirmed by testing seventy five old doping control samples reported with adverse analytical finding for 3´-OH-stanozolol from 2006-2009.

Result and Discussion

Method Validation: The calibration curve was linear in the range from 1ng/ml to 10ng/ml of 3´-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol in human urine. The calibration equation obtained was 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, 4ß-OH-stanozolol and 16ß-OH-stanozolol respectively. The recovery, accuracy and precision of all analytes is given in Table 1. Ten different blank urine samples prepared and analyzed by the same procedure, showed the absence of any interfering signals. LOD was found to be 0.25 ng/ml and LOQ was 0.5 ng/ml for all the metabolites.

Excretion Study: Excretion profile of 3´-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol in human urine is illustrated in Table 2. The results showed that 3´-OH-stanozolol was excreted at highest concentration followed by 16ß-OH-stanozolol and 4ß-OH-stanozolol as the least excreted.

Positive Doping Control Samples: The method applied to 75 old doping samples with adverse analytical finding for 3´-OH-stanozolol showed presence of 3´-OH-stanozolol and 16ß-OH-stanozolol in all the seventy five samples and 4ß-OH-stanozolol could be detected in sixty seven samples. The graphical representation is shown in figure-1.

The present method is sensitive enough to detect 3´-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol at low concentrations. The developed method is incorporated in routine screening procedure to detect stanozolol abuse. The marked increase in percent positive of stanozolol in Indian Sports persons in 2009 may be due to the improved detection by ESI-LC/MS/MS method (figure-2).

Conclusion: It can thus be concluded that liquid-liquid extraction followed by ESI-LC/MS/MS is an effective tool for the detection of stanozolol metabolites. However, 3-OH-Stanozolol and 16 β-OH-Stanozolol can be treated as a long term metabolite and effective marker for stanozolol abuse.

References

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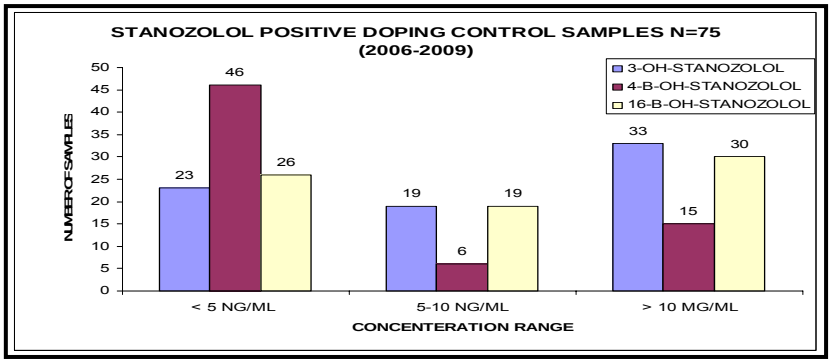

Figure-1: Graphical representation for 3´-OH-Stanozolol, 4ß-OH-Stanozolol and 16ß -OH-Stanozolol in positive doping control samples (n=75)

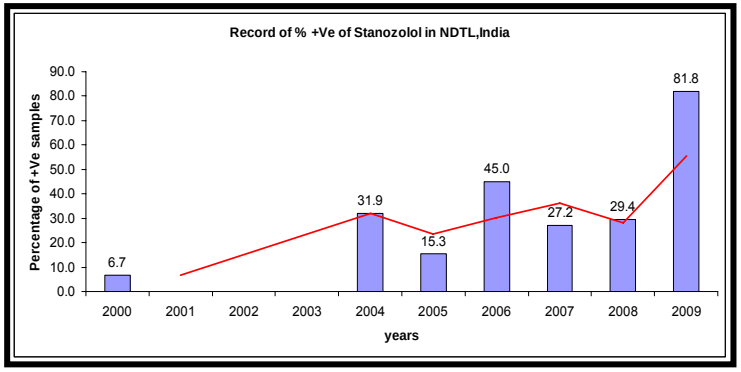

Figure-2: Yearwise record of adverse analytical finding for stanozolol at NDTL, India

Metabolite	Expected Conc. [ng/ml]	Measured Conc [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
4ß-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
16ß-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

Table-1: Recovery percentage, accuracy, and precision for stanozolol metabolites.

	Calculated Concentration [Mean±SD]		
Hours	**3-OH-Stanozolol**	**4-B-OH-Stanozolol**	**16-B-OH-Stanozolol**
3	6.93±6.72	1.84±1.96	8.50±9.31
6	14.25±5.01	5.15±2.96	19.90±11.05
9	15.15±5.33	3.38±2.27	17.26±7.72
12	15.86±4.80	3.17±1.76	16.54±8.01
18	9.33±5.19	2.20±1.37	15.92±9.02
24	9.12±3.48	1.75±1.64	9.79±5.00
30	7.84±7.13	1.06±0.84	6.07±4.11
36	6.59±7.54	1.21±1.22	4.83±3.43
48	3.80±1.57	0.35±0.24	1.89±0.78
80	2.39±0.60	0.05±0.11	1.00±0.15

Table-2: Quantitation values for stanozolol metabolites in excretion study samples