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New Sample Preparation Procedure for the Confirmation of Stanozolol and its Metabolites by Means of GC/MSMS and LC/ESI-MSMS

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The anabolic androgenic steroid stanozolol (17α-methyl-17β-hydroxy-5α-androstane-[3,2,c]pyrazole) (Fig. 1a) was first synthesized in 1959 by Clinton et al. [1]. In spite of its prohibition by the International Olympic Committee (IOC) since the mid-1970s, the drug is still one of the most misused substances in sports [2]. Numerous investigations of the stanozolol metabolism in humans have been performed focusing on the detection of its urinary metabolites [3-4]. Stanozolol is metabolized in a large extent and the main metabolic products in urine are the mono-hydroxylated 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol (Fig. 1, b-d) [4].

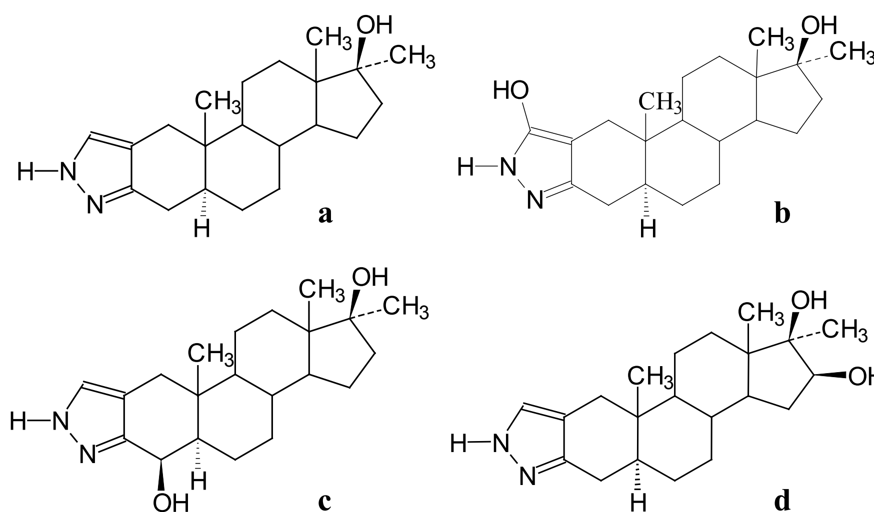

Fig. 1: Chemical structures of stanozolol (a), 3'-OH- stanozolol (b), 4ß-OH- stanozolol (c) and 16ß-OH- stanozolol (d).

For the identification of these analytes different methods of extraction and detection are described in the literature. The sample preparation procedures vary from solid phase

extraction, liquid liquid extraction (LLE) [3, 4] to immunoaffinity chromatography [5]. In doping control current detection methods for stanozolol misuse predominantly rely on gas chromatography (GC) coupled to low or high resolution mass spectrometry (MS) [5, 6]. However, due to its pyrazole structures stanozolol and its metabolites form bonds with any active sites in the gas chromatographic system leading to difficulties in its detection especially at low concentrations [7]. In combination with soft ionization techniques as electrospray ionization (ESI) and atmospheric pressure ionization (APCI) the liquid chromatography/mass spectrometry (LC/MS) nowadays is a powerful alternative in various fields of analytical chemistry as doping control and food safety [8]. Nevertheless, irrespective of the method of sample preparation and the kind of final detection, the identification of stanozolol and its metabolites has proved to be problematic, particularly in cases of low concentrations in combination with interfering matrix peaks. This paper describes a simple and fast sample preparation procedure allowing the simultaneous confirmation of stanozolol and its major urinary metabolites. The procedure consists of very efficient purification steps such as solid-phase extraction, several liquid-liquid extractions and acidic re-extraction followed by GC/MSMS and LC/ESI-MSMS detection, respectively.

Experimental

Chemicals, steroids and reagents: Hydrochloric acid (32%), potassium hydroxide, potassium carbonate and sodium hydrogen carbonate were obtained from Merck (Germany). t-Butyl methyl ether (distilled before use) and n-pentane (HPLC grade) were purchased from KMF (St. Augustin, Germany). Sodium acetate and stanozolol were from Sigma (Deisendorf, Germany). 3'-OH-stanozolol-d3 was obtained from Promochem (Wesel, Germany). N-Methyl-N-trimethylsilyltrifluoracetamide (MSTFA) was purchased from Macherey & Nagel (Düren, Germany). Serdolite PAD-I solid phase extraction (SPE) bulk material was obtained from Serva (Heidelberg, Germany). The enzyme ß-glucuronidase (E. coli) was supplied by Roche Diagnostics (Mannheim, Germany). The steroids 4,5-dehydrostanozolol, 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol were synthesized in our laboratory. All solutions and buffers were prepared using deionised water (Millipore, Eschborn, Germany).

Sample preparation. In Figure 2, the sample preparation procedure is shown.

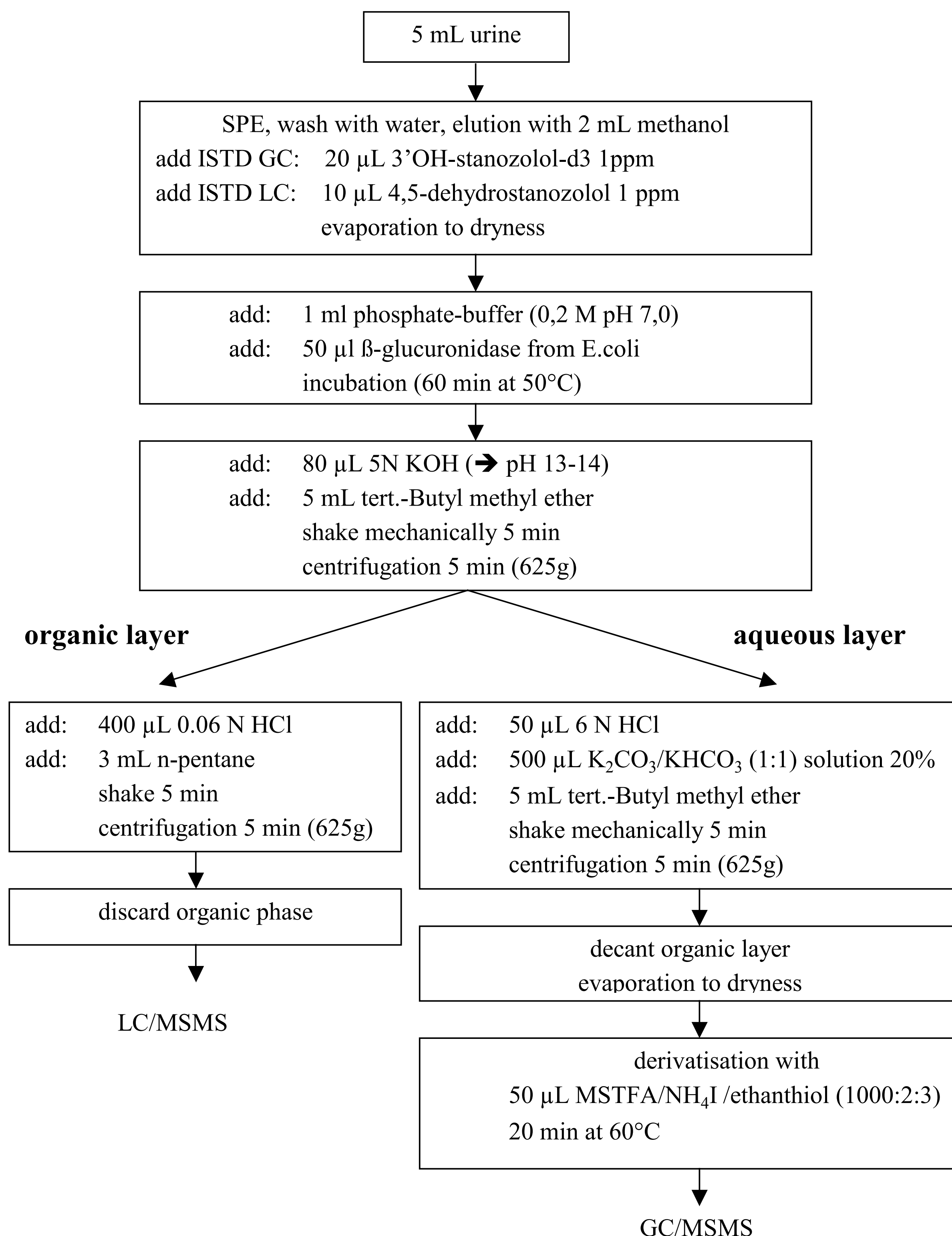

Fig.2: Sample preparation flow chart for the detection of stanozolol, 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol.

Liquid Chromatography-Tandem-Mass Spectrometry: Analyses were performed on an Agilent 1100 liquid chromatograph (Waldbronn, Germany) interfaced to an Applied Biosystems API Qtrap 4000 mass spectrometer with electrospray ionization. LC was done on an Agilent Zorbax XDB-C8 column (4.6 x 125 mm, 5 µm particle size). The mobile phase was A: 5 mM ammonium acetate buffer containing 1% of acetic acid and B: acetonitrile. The flow rate was set as 0.8 mL/min. The gradient was 10% B to 100% B within 12 min. The column was flushed for one minute at 100% B and re-equilibrated for 2.5 min at 10% B. The injected volume was 50 µL. The ion source was operated in the positive mode at 550°C using a spray voltage of 5500 V. All target analytes were detected by means of characteristic product ions using the multiple reaction monitoring (MRM) mode (Table 1).

Compound	**Declustering potential (V)**	**Ion transition (m/z)**	**Collision offset voltage (V)**	**Dwell time (ms)**
stanozolol	140	329-81	65	40
	140	329-95	59	40
	140	329-105	57	40
4ß-OH-stanozolol	71	345-327	21	40
	71	345-309	21	40
	71	345-145	41	40
16ß-OH-stanozolol	120	345-105	69	40
	120	345-91	85	40
	120	345-81	67	40
4,5-dehydrostanozolol	136	327-311	53	40
	136	327-145	59	40
	136	327-131	77	40

Table 1: Mass spectrometric parameters for the LC/MSMS identification of stanozolol, 4ß-OH-stanozolol, 16ß-OH-stanozolol and 4,5-dehydrostanozolol.

Gas Chromatography-Tandem-Mass Spectrometry: Analyses were performed using a Thermo TraceGC coupled to a PolarisQ Ion Trap mass spectrometer (Dreieich, Germany). The GC system was equipped with a Varian VF-1ms (length 25 m; i.d. 0.2mm; film thickness 0.1 µm), and the temperature program started at 200°C increasing to 310°C at 40°C/min. The injector and interface temperatures were set to 300°C, the ion source was operated at 225°C. Ionization was accomplished using EI (70 eV) and MS/MS analysis was realized using an isolation width of 1.5. A collision energy of 1.8 V was employed to dissociate selected

precursor ions using helium as the damping gas. A volume of 3 mL of each sample was injected into the system operating in the split mode (1:10). The mass spectrometric parameters are given in Table 2.

	Precursor ion m/z	**Product ion range m/z**	**Monitored ions m/z**
3'-OH-stanozolol	545	200-550	455, 387, 439, 277, 347
3'-OH-stanozolol-d3	548	200-550	458

Table 2: Mass spectrometric parameters for the detection of 3'-OH-stanozolol and 3'-OH-stanozolol-d3.

Validation: The validation was performed regarding specificity, linearity, recovery, lower limit of detection (LLOD), intraday and interday precision according to ICH guideline [9].

Specificity: For the examination of the specificity 10 different blank urine samples were prepared as described in the flow chart.

Calibration curves: A calibration curve was generated in the concentration range 0.1 to 10 ng per mL of urine. Each calibration point was prepared and analyzed once. The peak area ratios of analyte and ISTD were used to calculate the correlation coefficient, intercept and slope.

Recovery: The recoveries of all analytes were determined at 0.5, 2.0 and 8.0 ng/mL. Six urine samples were spiked with the target analytes in the beginning of the sample preparation. In other six urine samples the target analytes were added into the final extract. To all urine samples the internal standards were spiked into the final extract before injection into the GC/MSMS or LC/MSMS system, respectively.

Lower limit of detection: The lower limit of detection was calculated from the signal to noise ratio of six blank urine samples and six specimens spiked with 0.1 ng/mL and 0.3 ng/mL of target analytes, respectively.

Precision: Ten urine samples of low (0.5 ng/mL), medium (2.0 ng/mL) and high (8.0 ng/mL) concentrations of target analytes were prepared and analyzed. For the calculation of the intraday precision the coefficient of variation of each concentration level was calculated. The

corresponding inter-day precision was calculated from samples prepared and analyzed at three consecutive days.

Results and Discussion

The described sample preparation procedure for the detection of stanozolol and its major metabolites 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol in human urine is a fast and simple method for the elimination of biological matrix interferences by efficient purification steps. This results in highly purified extracts for sensitive GC/MSMS and LC/MSMS analysis.

In Fig. 3, typical LC/MSMS chromatograms generated from a) a blank urine sample and b) a urine sample spiked at 0.3 ng/mL of stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol are shown, demonstrating the efficiency of the concerted sample preparation and mass spectrometric assay. In Fig. 4 the corresponding GC/MSMS chromatograms resulting from a blank urine sample (a) and a urine sample spiked at 0.3 ng/mL of 3'-OH-stanozolol (b) are presented.

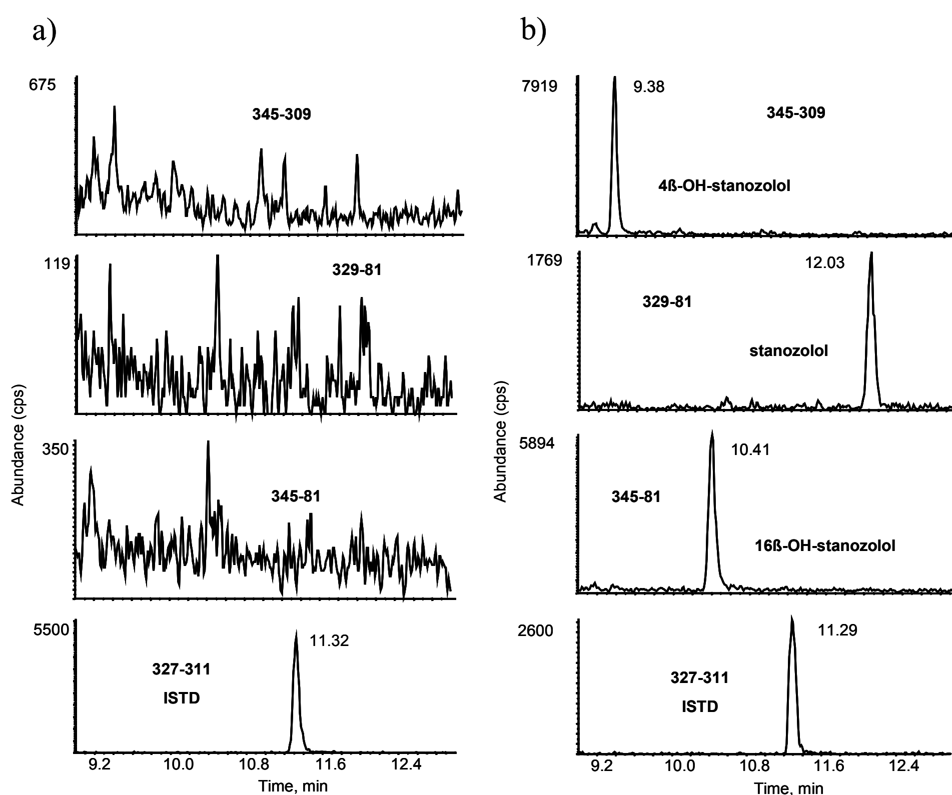

Fig. 3: LC/MSMS chromatograms of stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol in a blank urine sample (a) and a urine sample fortified at 0.3 ng/mL each (b).

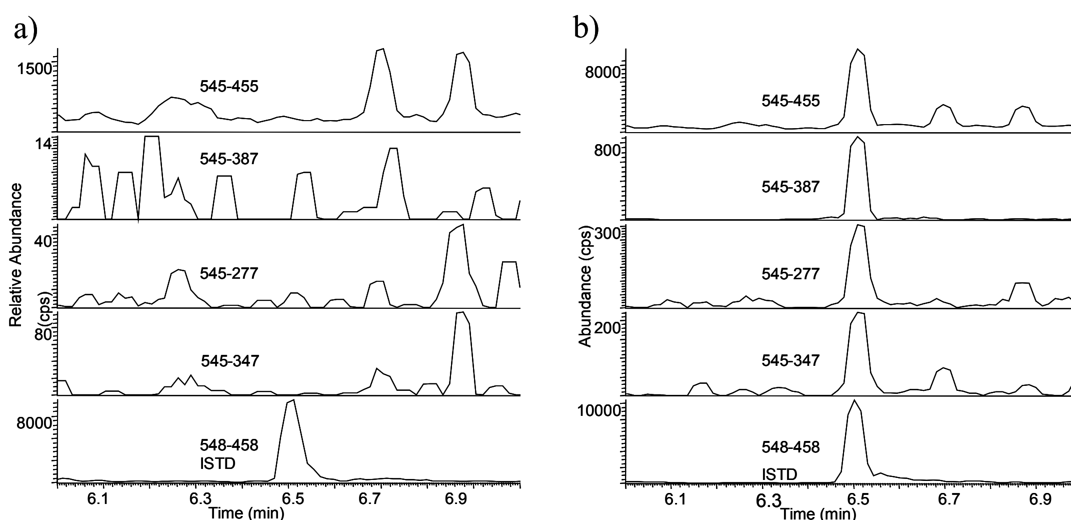

Fig. 4: GC/MSMS chromatograms obtained from a blank urine sample (a) and a urine sample spiked at 0.3 ng/mL of 3'-OH-stanozolol (b).

Validation results

Specificity: Specificity is shown by the absence of interfering signals.

Lower limit of detection: For all target analytes the LLODs were determined using three diagnostic ion transitions shown in Table 1 and 2. The LLODs are presented in Table 3.

Calibration curves: Linear calibration curves were obtained over a range of 0.1–10 ng/mL. The obtained calibration equations are shown in Table 3.

Compound	LLOD (S/N>3) (ng/mL)	calibration equation
3'-OH-stanozolol	0.3	y = 0.7780 + 0.043 r^2=09793
stanozolol	0.1	y = 2.1337 + 0.040 r^2=0.9935
4ß-OH-stanozolol	0.2	y = 3.8203 + 0.058 r^2=0.9940
16ß-OH-stanozolol	0.1	y = 3.6409 + 0.011 r^2=0.9885

Table 3: Detection limits and calibration equations for stanozolol, 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol.

Recovery, Precision: The results for recovery, intraday and interday precision are summarized in Table 4.

Compound	Concentration (ng/mL)	Recovery %	Intraday precision CV (%) n=10	Interday precision CV (%) n=30
3'-OH-stanozolol	0.5	77.9	10.6	10.0
	2.0	73.2	6.5	8.1
	8.0	81.1	7.1	7.8
stanozolol	0.5	6.3	14.1	14.9
	2.0	5.0	8.3	12.0
	8.0	5.0	10.5	15.7
4ß-OH-stanozolol	0.5	25.9	12.5	16.2
	2.0	22.6	11.3	13.3
	8.0	20.8	12.6	20.1
16ß- OH-stanozolol	0.5	28.0	13.1	13.0
	2.0	29.1	9.5	12.5
	8.0	27.2	9.7	15.5

Table 4: Validation results

Doping control samples: The described sample preparation procedure was applied to 13 doping control samples tested positive for stanozolol misuse. The results are illustrated in Figure 5. For standardization the concentration of 3'-OH-stanozolol is set as one and the abundances of the other target analytes are shown as ratios calculated relative to the concentration of 3'-OH-stanozolol.

Discussion

Due to its comparably good gas chromatic behavior after TMS-derivatization 3'-OH-stanozolol has been used in the past as target analyte for the GC/MS long-term detection of stanozolol misuse. A sensitive GC/MS detection of stanozolol itself, 4ß-OH-stanozolol and 16ß-OH-stanozolol is very difficult according to unstable TMS-derivatives or elevated background noise. In contrast, LC/MS is the favored method for the identification of stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol, whereas 3'-OH-stanozolol shows only a small sensitivity resulting from elevated background noise. Thus, the application of the presented sample preparation allows the determination of the target analytes each with the highest sensitivity by different detection methods.

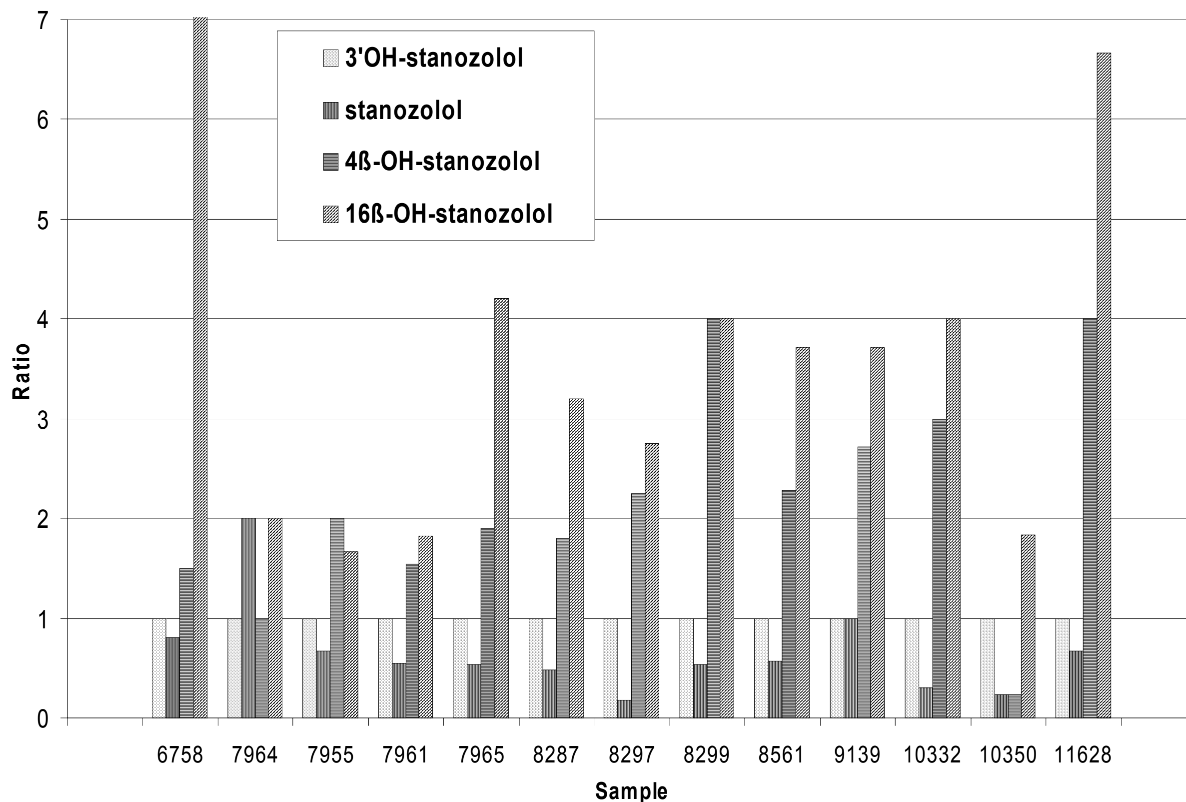

Figure 5: Concentration pattern of stanozolol, 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol in doping control samples with adverse findings regarding stanozolol misuse.

According to the fact that the concentration of stanozolol and its metabolites is very low in doping control samples complex and time-consuming purification methods as immunoaffinity chromatography had been used for their identification. The new sample preparation procedure based on consecutive solid-phase and liquid-liquid extraction with subsequent re-extraction is characterized by an efficient elimination of interfering biological matrix. Although the calculated recoveries are comparably low for stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol, the detection limits are great as a consequence of excellent signal to noise ratios. The re-extraction with a mixture of 0.06 M hydrochloric acid and n-pentane has proven to be the important purification step for the LC/MSMS analysis. In contrast, two consecutive liquid-liquid extractions at different pH values are responsible for highly purified extracts for GC/MS analysis. Thus, the presented sample preparation procedure is a rapid and simple alternative to established procedures using immunoaffinity chromatography.

The evaluation of the doping control samples tested positive for stanozolol misuse reveals that in most cases 4ß-OH-stanozolol and 16ß-OH-stanozolol are present in higher abundances

than the commonly employed long-term target analyte 3'-OH-stanozolol. In combination with its excellent detection limit of 0.1 ng/ml 16ß-OH-stanozolol seems to be the most appropriate analyte for the identification and long-term detection for a stanozolol misuse.

Conclusion

The sample preparation procedure allows the confirmation of stanozolol, 3'-OH-stanozolol, 4ß-OH-stanozolol and 16ß-OH-stanozolol in human urine. Based on SPE and LLE with subsequent re-extraction, this procedure makes it possible to generate highly purified extracts for GC/MSMS and LC/MSMS analysis, respectively. Excellent detection limits enable the long-term detection of stanozolol misuse in doping control. Particularly suitable for a prolonged traceability are the hydroxylated metabolites 4ß-OH-stanozolol and 16ß-OH-stanozolol.

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