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Monitoring of Low Concentration Anabolic Steroids in Urine Samples from the 13th Asian Games by Ion-trap GC/MS/MS

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Introduction

Detection of low level anabolic steroid compounds has been commonly performed by high resolution mass spectrometry (HRMS) especially for doping control during international competitions with large numbers of samples. Recently, it has been demonstrated in some laboratories that the anabolic agents in urine samples could also be detected by MS/MS at the low concentrations required by the IOC (1-3). However, the analysis has never been performed with large numbers of samples under restricted conditions of a major competition event. Application of the ion-trap MS/MS for the purpose is very intriguing with its advantages of simplicity in operation and maintenance, although the MS/MS is limited by sensitivity and reliability of mass fragmentation pattern compared to the HRMS.

During the doping control at the Bangkok 13th Asian Games (9-20 December 1998), it was decided to experiment a strategy combining ion-trap GC/MS/MS in screening of low concentration anabolic steroids with the confirmation of suspected samples by HRMS. The decision has provided us two-fold benefit, not only solving the problem of HRMS instrument inaccessibility but also reducing operational cost of relatively less sophisticated ion-trap instrument. This paper describes our protocols and screening results performed by ion-trap MS/MS for doping control during a large international competition.

Methods

Two positive controls were included in every batch of injection (UPC-2 and UPC-1). UPC-2 was a control urine spiked with clenbuterol, norandrosterone (nandrolone M1), epimetenediol (17β-methyl-5β-androst-1-ene-3α,17α-diol) 3ng/ml each, 5β-THMT (methyltestosterone M2, 17α-methyl-5β-androstane-3α,17β-diol) 5ng/ml, 3'-hydroxy-stanozolol 5ng/ml. UPC-1 was spiked with the same compounds including noretiocholanolone (nandrolone M2) 25ng/ml.. Both control solutions were prepared and kindly provided by Prof. P. Hemmersbach (Hormone Laboratory, Oslo, Norway) and by Prof. R. de la Torre (IMIM, Barcelona, Spain) respectively. A negative control urine (UNC) was spiked with only the internal standard. Methyltestosterone (50 ng/ml) was used as the internal standard (ISTD) and spiked into all the control and sample urines prior to the extraction.

Urine samples were processed according to the routine screening procedure previously described for extraction of the anabolic steroids(4,5). Five ml urine sample was spiked with the internal standard, followed by enzyme hydrolysis, t-butyl-methyl ether extraction and derivatization with 100 µl MSTFA / NH_4I / mercaptoethanol, finally divided into two vials (50µl each), one for GC/MS and the other for GC/MS/MS as well as GC-HRMS analyses.

Instrument conditions of the GC/MS/MS were adjusted with main objectives to optimize the sensitivity, reproducibility, accuracy and the throughput of samples to be analyzed during the Games. Fragmentation parameters for the analysis of each anabolic substance were explored and established using the software for ion preparation method (IPM). Some of the values were distinct from that previously described (2), in particular the collision energy and the excitation mode (Table 1). Clenbuterol, nandrolone metabolites and epimetenediol were detected with better sensitivity in resonant mode than non-resonant one. Increased collision energy usually reduced sensitivity due to increased fragmentation of the selected daughter ions. Maximum sensitivity was not achieved in this screening analysis in order to minimize trap contamination, therefore 2 µl sample were injected using a split ratio of 10:1. Two identical GC/MS/MS instruments were operated throughout the Games, one

instrument analyzed 27 sample batches without trap cleaning, whereas the other one analyzed 24 batches and was cleaned once due to leaking.

Instrumentation

The separations for all the instruments were carried out using the same type of methyl silicone fused silica capillary column (HP Ultra-1, length 17 m, ID 0.2 mm, film thickness 0.11μm).

GC/MSD

GC/MS Hewlett-Packard (GC6890 / 5973 MSD / Autosampler7673); Temperature program: 180°C(0min)--3°C/min--230°C(0min)--40°C/min--310°C(3min); Carrier gas:helium 0.8ml/min; Injector/interface temperatures: both at 280°C; Injection 2μl (split 10:1); Electron impact ionization with 70 eV; Acquisition mode: selected ion monitoring (SIM) with one or more ions selected for each substance.

GC/MS/MS

Varian Saturn2000 (GCStar3400CX / MS Ion-trap / Autosampler8200CX); Temperature program: 185°C(1min)--5.0°C/min--240°C(0min)--20°C/min--310°C(2min); Carrier gas: helium, 0.9ml/min; Injector temperature 280°C; Injection 2μl (split 10:1); Transfer-line temperature: 250°C; Ion-trap temperature: 200° C; Electron impact ionization with filament 50 μA; Axial modulation: 4.0 volts; AGC Target: 5000; Acquisition mode: MS/MS with the ions selected as shown in Figure 1.

GC/HRMS

FinniganMAT95S (GC Hewlett-Packard HP6890 / MS with magnetic sector / Autosampler Finnigan MAT A200S; Temperature program: 180°C(0min)--3.3°C/min--231°C(0min)--30°C /min--310°C(2min); Carrier gas: helium, 0.5ml/min; Injector/interface temperatures: both at 300°C; Injection 2μl (split 20:1); Electron impact ionization with 70 eV; Acquisition mode:MID; Resolution: >5,000.

Results and discussion

After the analysis of each batch, the screening results were analyzed using Saturn

View software (Window version 5.2). Due to lacking of proper macros for the Saturn software to visualize the print out results, the data were subsequently processed and printed out by Hewlett-Packard Chem Station software with suitable macros. MS/MS analysis of low concentration positive control (UPC2) generally produced results as shown in Figure 1. The retention times varied between the two instruments as the carrier gas flow rate of the Saturn2000 must be regulated by manual control of the column head pressure which was relatively inaccurate. However, the anabolic standard relative retention times obtained from each particular instrument were fairly reproducible for all batches throughout the whole Games (Table 2.) This was one important criteria for consideration if a suspicious signal appeared in the screening time window.

The sensitivity and reproducibility in detecting the anabolic standards in UPC2 by MS/MS were compared between the two instruments. The detectable amounts of the compounds in each screening batch were estimated from their corresponding peak areas using the internal standard peak area (50ng/ml) in the same batch as the reference. The anabolic standards were detected in comparable sensitivity between the two instruments (Figure 2). The average amounts of clenbuterol, norandrosterone, epimetenediol (3ng/ml each) and 5β-THMT, 3'-HO-stanozolol (5ng/ml each) from 24 batches screened by the Varian-I were 1.6 ± 0.5, 4.2 ± 1.1, 1.1 ± 0.2, 4.8 ± 2.1, 5.7 ± 2.0 ng/ml respectively, whereas the values from 27 batches screened by the Varian-II were 2.0 ± 0.5, 4.3 ± 1.1, 1.7 ± 0.4, 4.6 ± 1.3, 4.6 ± 1.5 ng/ml respectively. The data, although not quantified accurately, suggested having false negative results in the MS/MS screening for low concentration anabolic steroids were unlikely.

The sensitivity of the MS/MS screening was compared with the HP5973/MSD. Norandrosterone at 0.8 ng/ml and 3'-HO-stanozolol at 2.1 ng/ml were apparently undetectable in the MSD screening whereas suspicious result was obtained from the sample containing 10ng/ml norandrosterone (Table 3). In comparison, both cases were observed clearly in the MS/MS screening. All the samples demonstrated were subsequently confirmed and quantified by GC/HRMS, except the sample #0812 whose norandrosterone concentration (>600 ng/ml) was estimated from the MSD screening. Figure 3 demonstrates identification of a positive sample (#0759 in Table 3) by mass spectra comparison from MS/MS screening. A sample with low concentration 3'-HO-stanozolol (2.1 ng/ml, #0960 in Table 3) was also

detectable in the MS/MS screening (Figure 4). The concentration estimated initially from the MS/MS screening using UPC2 as reference was 1.7 ng/ml and subsequently quantified to be 2.1 ng/ml by MAT95S-HRMS confirmation.

Comparison between GC/MS/MS with the GC/HRMS was also examined. Randomly selected 12 batches (178 samples. 25% of total) from the MS/MS analysis were injected into the Finnigan MAT95S-HRMS using the same sample vial. 3'-HO-stanozolol (30 ng/ml, #0796 in Table 3) was detected in one sample similarly to the result obtained from the ion-trap MS/MS analysis and no anabolic steroid was detected by either HRMS or MS/MS in the remaining 177 negative samples. The data also support that having false negative results in the MS/MS screening for low concentration anabolic steroids should not have occurred.

Conclusion

Ion-trap GC/MS/MS was applied for the screening of the IOC prohibited low concentration anabolic steroids during the 13th Asian Games in Bangkok. Satisfactory results were obtained from the analyses of 718 urine samples of the athletes during the 12 day period of competition. Operational conditions of both sample preparation and instrument parameters were established to optimize rapidity, sensitivity, and accuracy of the analysis using two instruments. Due to relatively reproducible retention times and specific characteristic masses, urine samples containing anabolic steroids as low as 0.8 ng/ml norandrosterone and 2.1 ng/ml 3'-HO-Stanozolol were detectable even under the restricted conditions of the Games. The possibility of having false-negative result for all the competition samples was unlikely, although possible. Parallel analyses of randomly selected 12 batches (178 samples, ~25 % of total) using the same injection vials showed results without discrepancy between the ion-trap GC/MS/MS and the MAT95S-GC/HRMS. Therefore, our experience from the 13th Asian Games offers an alternative strategy for doping control analysis of low concentration anabolic steroids by applying the ion-trap GC/MS/MS for screening. We have found the approach was reasonably reliable, relatively inexpensive and low maintenance requiring. Extensive investigation should be further explored to improve instrumental conditions as well as analytical strategy for better application in doping control.

References

1. Garle,M. Ion-trap: Different problems and discussion. W. Schänzer (Editor), *Recent Advances in Doping Analysis(4)-Proceedings of the 14th Cologne Workshop on Dope Analysis 1996,* Sport und Buch Strauβ, Koln (1997). p 271-274.
2. Munoz-Guerra, J., D. Carreras, C. Soriano, C. Rodriguez, A.F. Rodriguez. Use of ion-trap GC/MS/MS mass spectrometry for detection and confirmation of anabolic substances at low concentration levels in doping analysis. W. Schänzer (Editor), *Recent Advances in Doping Analysis(5)-Proceedings of the 15th Cologne Workshop on Dope Analysis 1997,* Sport und Buch Strauβ, Koln (1998). p 169 –183.
3. Pascual J.A., X. de la Torre and J. Segura. High sensitivity analysis of anabolic agents: some alternative instrumental possibilities (1999). (submitted).
4. Bowers, L.D. and D.J.Borts. Separation and confirmation of anabolic steroids with quadrupole ion-trap tandem mass spectrometry. *.J Chromatog. B Biomed Appl.* (1996) **687(1)**: 69-78
5. Segura J., J.A.Pascual, R.Ventura, J.I.Ustaran, A.Cuevas, R.Gonzalez International cooperation in analytical chemistry: experience of antidoping control at the XI Pan American Games. *Clin. Chem.* (1993) **39 (5)**: 836-845.

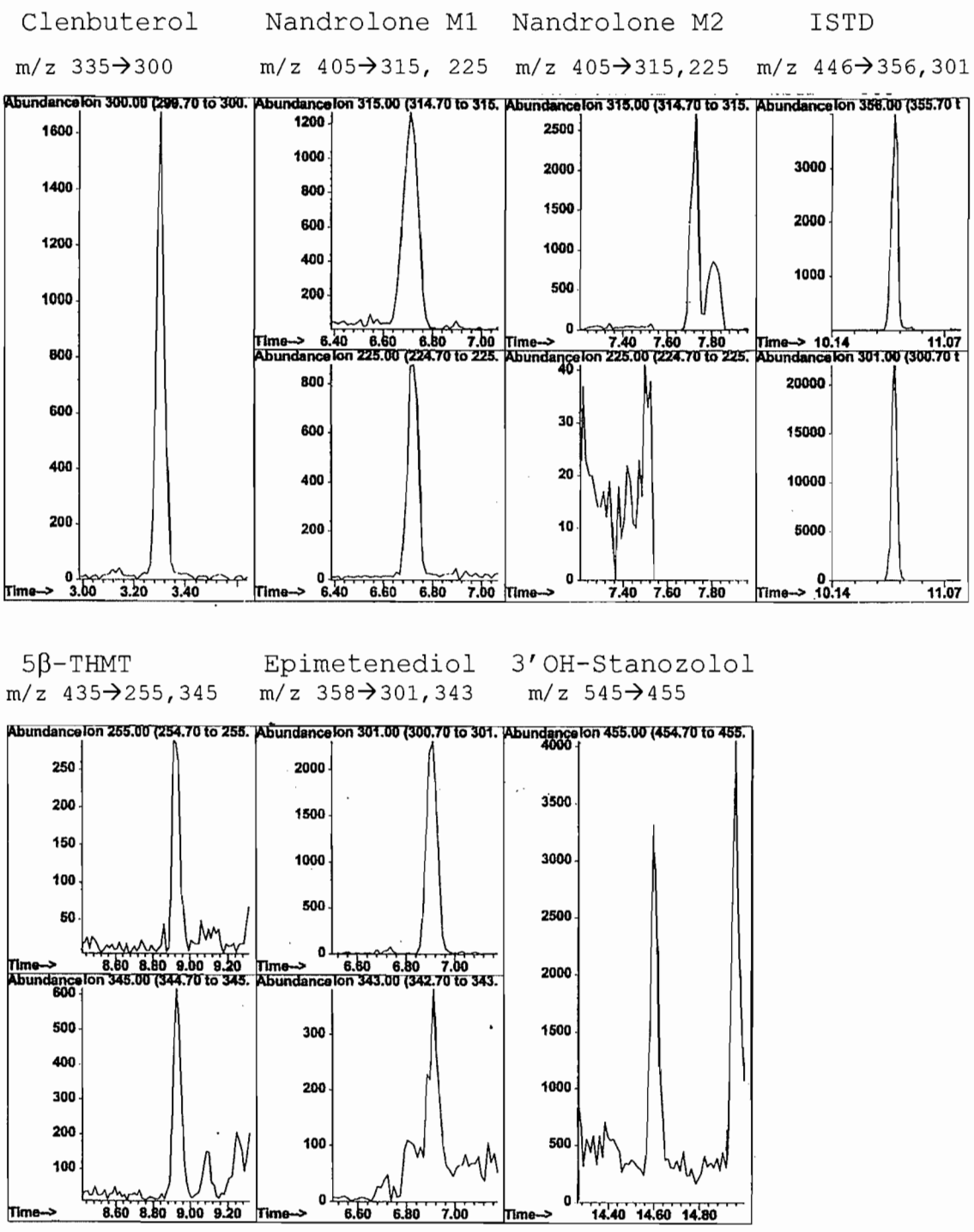

Figure 1: Typical result obtained from the MS/MS screening of the anabolic standards in UPC2. Noretiocholanolone (nandrolone M2) was not present in the solution.

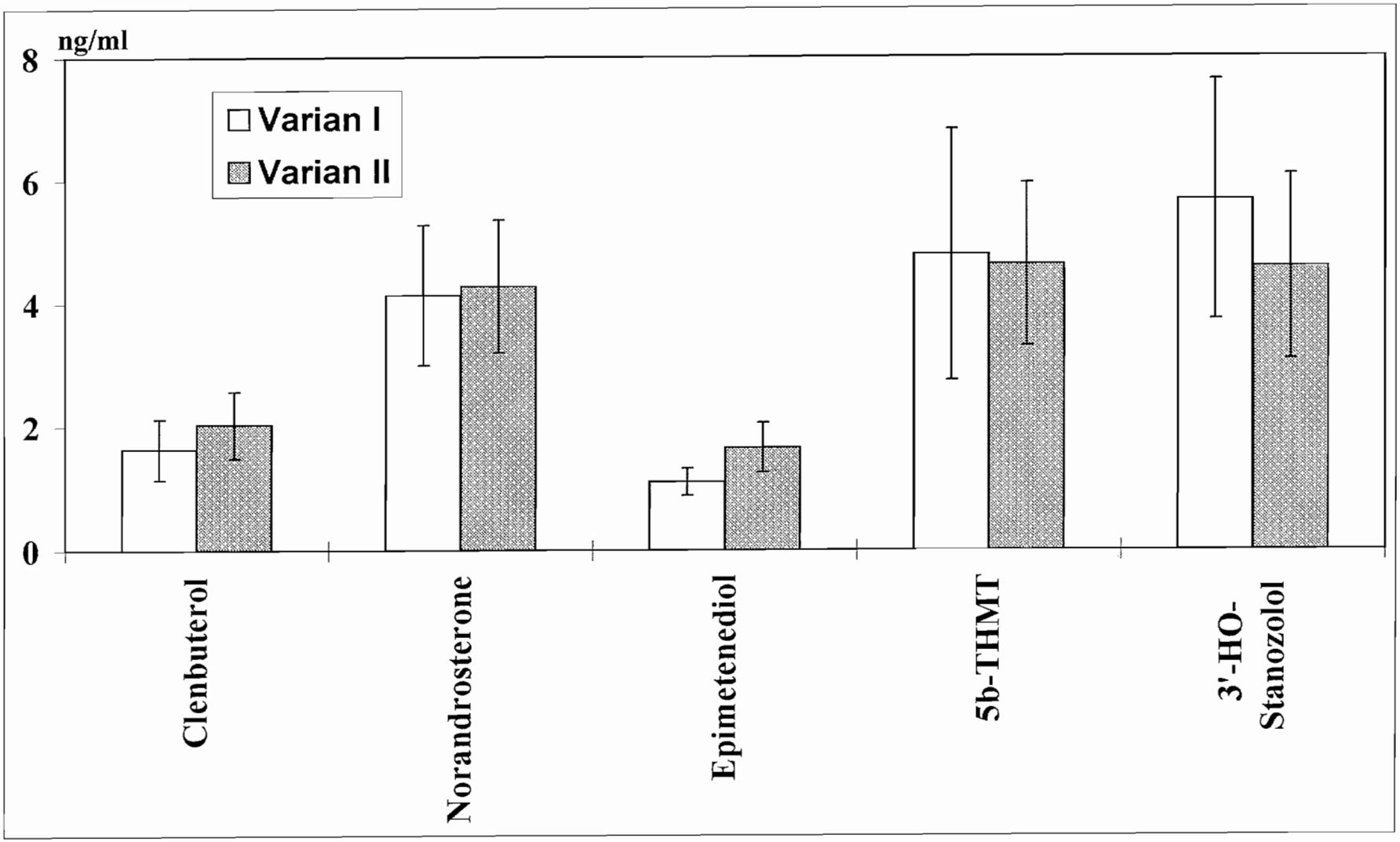

Figure 2: Amounts of the anabolic standards in UPC 2 detected by two Varian instruments (MS/MS) from all sample batches.

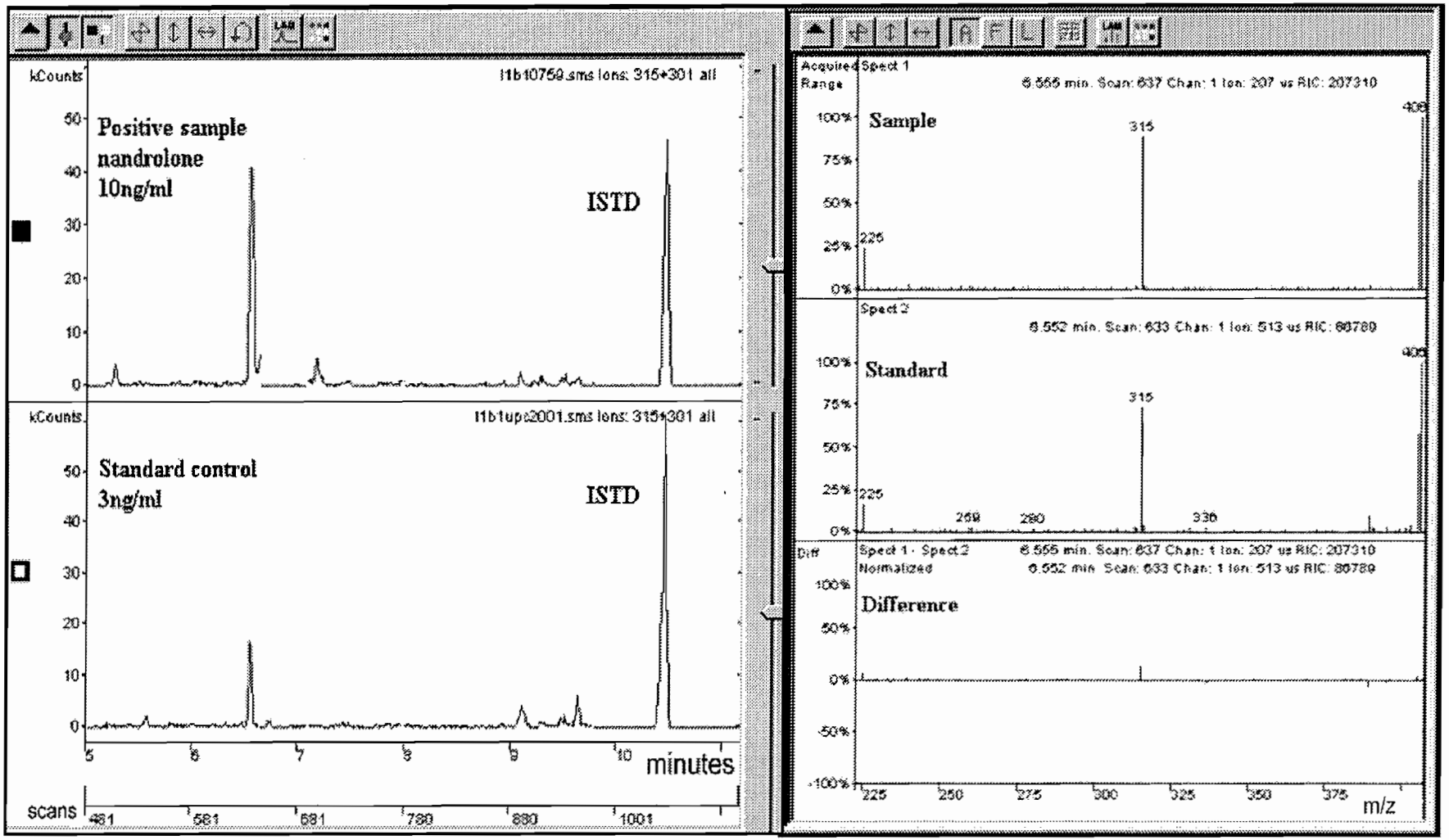

Figure 3: Identification of a norandrosterone positive sample in the MS/MS screening. Comparative mass spectra were analyzed by Saturn View software. Norandrosterone concentration in the sample was quantified by the HR/MS confirmation.

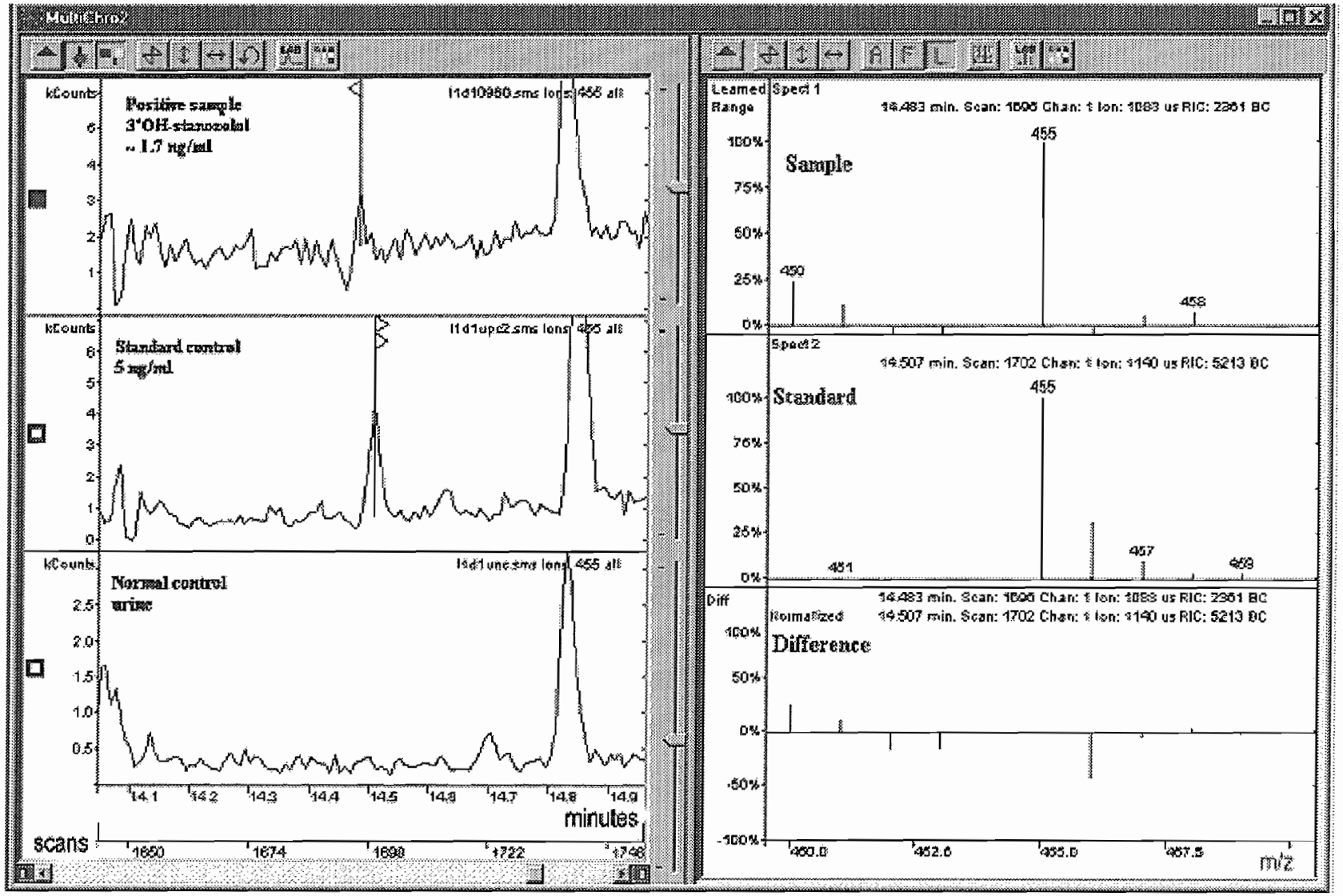

Figure 4: Detection of low concentration 3'-HO-stanozolol in a sample by MS/MS screening. The concentration was subsequently quantified by the MAT95S- HRMS during confirmation.

TABLE 1. MS/MS ion preparation for selective fragmentation of anabolic standards.

Compounds	m/z ion Parent	Daughter	Excitation Amplitude Volts (CID form)
Clenbuterol	335 →	300	0.80 (R)
Nandrolone M1/M2	405 →	315, 225	0.70 (R)
Epimetenediol	358 →	343, 301	80 (NR)
5β - THMT	435 →	345, 255	85 (NR)
Methyltestosterone (ISTD)	446 →	356, 301	0.80 (R)
3'-HO Stanozolol	545 →	455	1.25 (R)

R = CID resonant excitation form
NR = CID non-resonant excitation form

TABLE 2. Reproducibility of the anabolic standard retention times in GC/MS/MS analysis

Instrument	Total batch	MEAN ± S.D.						
		RT ISTD(min)	RRT					
			Clenbuterol	Nandrolone M1	Nandrolone M2	Epimetene diol	5β-THMT	3'-HO-stanozolol
Varian I	**24**	**10.453 ± 0.017**	**0.304 ± 0.005**	**0.626 ± 0.010**	**0.686 ± 0.010**	**0.644 ± 0.010**	**0.829 ± 0.010**	**1.386 ± 0.020**
Varian II	**27**	**10.539 ± 0.050**	**0.315 ± 0.005**	**0.632 ± 0.010**	**0.686 ± 0.010**	**0.650 ± 0.010**	**0.830 ± 0.020**	**1.378 ± 0.050**

RT ISTD = Retention times of the internal standard
RRT = Relative retention times of the anabolic standards

TABLE 3. Comparative Detection of Some Anabolic Steroids from the 13th Asian Games.

Sample Code	Detected Compound	MSD HP 5973 (ng/ml)	MS/MS Varian	HRMS MAT 95S (ng/ml)
# 0518	Norandrosterone	-	+	0.8
# 0759	Norandrosterone	±	+	10
# 0796	3'-HO-stanozolol	+	+	30
# 0812	Norandrosterone	> 600 *	+	ND**
# 0960	3'-HO-stanozolol	-	+	2.1

* from MSD screening ** not determined + detectable - undetectable ± suspicious