Purification of urine samples to improve detection limit of anabolic agents

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Received: 31.3.2006
Revised: 8.8.2006
Accepted: 30.8.2006

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ABSTRACT

Objective: To investigate recovery percentage of clenbuterol, nandrolone, stanozolol, and epimetendiol by two different solid phase extraction procedures viz. XAD2 (polystyrene divinylbenzene) and SPE columns (C18, Samprep, Rankem) so as to improve detection limit of anabolic steroids.

Materials and Methods: The urine samples were spiked with different concentrations of drugs. The preliminary work was done with six samples, each of clenbuterol, nandrolone, and epimetendiol at 1, 2, 5, and 10 ng/mL and of stanozolol at 5,10, 20, and 40 ng/mL that were processed and injected into high resolution mass spectrometer. Later the study was limited to clenbuterol and epimetendiol at 2, 5, and 10 ng/mL concentrations. The data were analysed by comparing XAD2 and SPE column values.

Results: The results show that the recovery percentage of nandrolone and stanozolol at various concentrations did not signify any difference between the two columns. However, there was a significant increase in the recovery of clenbuterol at 2 ng/mL (P<0.002), 5 ng/mL (P<0.001), and 10 ng/mL (P<0.001) concentrations, where as for epimetendiol there was significant increase in the recovery at 2, 5, and 10 ng/mL (P<0.01) with SPE column compared to XAD2 column.

Conclusion: It is possible to enhance the detection limit of clenbuterol and epimetendiol by SPE column compared to XAD2 column. This procedure may be used for confirmation of suspicious samples found in routine testing.

KEY WORDS: Anabolic steroid, detection limit, mass spectrometry, SPE column, urine test, XAD2 extraction.

Introduction

The International Olympic Committee (IOC) and World Anti-Doping Agency (WADA) have banned the use of anabolic androgenic steroids (AAS) in sports in 1976 and 2002, respectively. To impose the ban, urine samples of sport persons are tested for anabolic steroids and their metabolites by Gas Chromatography–Mass Spectrometer (GC-MS) and high-resolution mass spectrometer (HRMS).[1] The samples are extracted for unconjugated and conjugated steroids with varying chemical properties. After extraction, the steroids are derivatized to yield trimethyl silyl derivatives for detection by GC/MS and HRMS. Use of HRMS for AAS has improved the detection limit of few steroids viz nandrolone, stanozolol, epimetendiol and clenbuterol. Approximately 50 AAS fall in the WADA banned list.[2] The detection limit for all anabolic steroids is 10 ng/mL except clenbuterol, nandrolone, stanozolol, methyl testosterone, and epimetendiol that are to be detected at 2 ng/mL in urine samples. These four drugs are to be detected in lower concentration compared to rest of anabolic steroids (detection limit is 10 ng/mL); hence, sample purification is required to facilitate detection at 2 ng/mL. Sample preparation also needs improvement to improve the detection limit of these steroids apart from improved instrumentation. Improvement of sample preparation may be achieved by the use of various solid phase extraction procedures,[3-5] HPLC clean up[6,7] or Immunoaffinity Chromatography (IAC) gels.[8-11] The objective of present study was to investigate the recovery percentage of clenbuterol, nandrolone, stanozolol, and epimetendiol by two simple solid phase extraction procedures, using XAD2 and SPE columns.

Materials and Methods

Reference standards and chemicals

The reference standards of clostebol and clenbuterol were

purchased from Sigma-Aldrich (St Louis, MO, USA) and of nandrolone, stanozolol, epimetendiol from National Analytical Research Laboratory (National Measurement Institute, Sydney, Australia). The derivatizing reagent N-methyl-N-Trimethysilyltrifluoroacetamide (MSTFA), iodo-TMS and Dithioerythritol were purchased from Sigma-Aldrich (St Louis, MO, USA). The hydrolyzing enzyme β-glucuronidase, *E. coli* is purchased from Roche, Germany. All other reagents and chemicals such as methanol, diethyl ether, and anhydrous sodium sulphate were of analytical grade or HPLC grade.

Two types of solid phase extraction columns were used. The Amberlite XAD2 was purchased from Sigma-Aldrich (St Louis, MO, USA) and C-18 Samprep; 100 mg/3 mL from Rankem, India was purchased.

Extraction procedure

The samples were processed by XAD2 extraction[12] and SPE column.

XAD2 extraction: About 2 mL of centrifuged urine was passed into pre-prepared XAD2 column and 50 mL of clostebol (conc. 2.5 ng/mL) was added as the internal standard. The column was eluted with 2.5 mL of methanol and the eluent dried under nitrogen evaporator at 60°C. To this was added 1 mL of 0.2 M-phosphate buffer pH-7.0 and 50 μL of β-glucuronidase (*E. coli*) and the mixture was incubated at 60°C for 60 minutes. The hydrolysate was alkalinized by adding 250 μL of 7% potassium carbonate solution to adjust the pH of the mixture between 9 and 10. About 5 mL of distilled diethyl ether was added and mixed by moderate horizontal mixing in the shaker for 10 minutes. The ether layer was transferred to a labeled, glass test tube and the ether evaporated at 60°C under the nitrogen evaporator. After evaporation of diethyl ether the tube was placed for 10-15 minutes in a dessicator containing P_2O_5, to remove traces of moisture if any.

SPE extraction: SPE columns were conditioned with 2 mL of methanol and equal amount of distilled water. The procedure followed was similar to that of XAD2 extraction.

Derivatization procedure

The dried residue was dissolved in 50 μL of MSTFA/iodo-TMS/dithioerythritol (1000/2/2:v/v/w) mixture. The mixture was incubated at 60°C for 15 min and then transferred into 200 μL conical glass vials. Nearly 2 mL was injected into HRMS for analysis.

Instrumentation and conditions

HRMS was used for evaluation of concentration of the samples. The parameters of gas chromatograph [Table 1] and Mass Spectrometer [Table 2] are mentioned.

Results

Limit of detection (LOD)

Limit of detection was defined as the lowest level at which a compound could be identified in urine samples, with diagnostic ions present with a signal to noise (S/N) ratio greater than 3. A single diagnostic ion 405.2640, 546.3493, 335.0690, and 448.3193 was used to determine the LOD of nandrolone, stanozolol, clenbuterol, and epimetendiol in urine sample, respectively, by HRMS. The

Table 1

Instrumental parameters of gas chromatograph

Instrument	Agilent 6890 GC, 7679 automatic liquid sampler
Injection mode	Automatic split, split ratio 11:1
Injection volume	2 µL
Injector port temperature	290°C
Carrier flow	120 kpa helium at 180°C (constant pressure)
Oven program	180°C for 1 min, 229°C at 3°C/min, 300°C at 40°C/min, final hold for 3 min
Column	Ultra 1, fused silica, 0.2 mm I.D. × 17 m length column, film thickness: 0.11 µm

Table 2

Instrumental parameters of high resolution mass spectrometer

Instrument	JEOL JMS-700 M Satation
Tune	Auto tune
Tune peak width	0.5 amu
Acquisition mode	Selective ion monitoring
Ion current	300 µA
Ionizing energy	70 eV
Resolution	10000
Workstation	Unix workstation

Table 3

Mean recovery of Nandrolone in spiked urine samples with XAD2 and SPE columns

Spiked concentration	*Concentration recovered Mean ± SE (ng/mL)*		*P value*
*(ng/mL)**	*XAD2*	*SPE*	
1	0.3087 ± 0.4039	0.6708 ± 0.1627	0.075
2	1.0882 ± 0.1275	1.3326 ± 0.1289	0.026
5	3.0686 ± 0.3650	2.8080 ± 0.3322	0.615
10	6.3979 ± 0.5388	7.2279 ± 0.6567	0.070

**n*=6 samples for each concentration

Table 4

Mean recovery of stanozolol in spiked urine samples with XAD2 and SPE columns

Spiked concentration	*Concentration recovered Mean ± SE (ng/mL)*		*P-value*
*(ng/ml)** n=6	*XAD2*	*SPE*	
5	4.2462 ± 0.4382	3.6081 ± 0.3135	0.345
10	5.3439 ± 0.4636	6.5573 ± 0.4967	0.226
20	11.9424 ± 0.6465	4.1893 ± 0.3705	0.000
40	23.6920 ± 1.8582	13.0167 ± 1.2055	0.000

**n* is the number of samples for each concentration

LODs of clenbuterol and epimetendiol as required by WADA is 2 ng/mL.[2]

Recovery, accuracy, and precision

The preliminary work was done with six spiked urine samples of each of clenbuterol, nandrolone, and epimetendiol at 1, 2, 5, and 10 ng/mL and stanozolol at 5, 10, 20, and 40 ng/mL and were analyzed with HRMS. The results showed that for nandrolone and stanozolol the drug recovery at various concentrations (upto 10 ng/mL) was not different between two columns [Tables 3 and 4]. At 20 and 40 ng/mL, recovery of stanozolol was significantly more ($P<0.00$) with XAD2 columns.

In a later study the extraction accuracy of the sample preparation procedure with three concentrations of clenbuterol and epimetendiol was undertaken ($n=10$) and processed with XAD2 and SPE columns.

Clenbuterol. The percentage recovery was 30.45, 46.63, and 41.60% with XAD2 and 72.09, 72.16, and 89.24% with SPE columns [Table 5]. The recovery percentage was significantly higher with SPE column extraction. The significance levels were

Figure 1: Total ion chromatogram of clenbuterol and epimetendiol of SPE columns

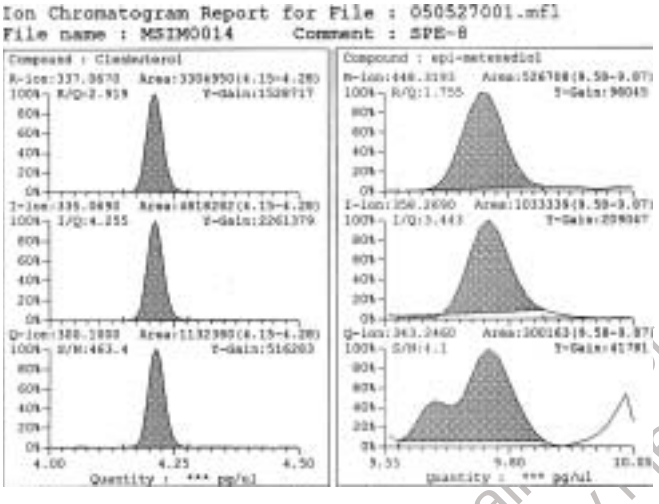

Figure 2: Total ion chromatogram of clenbuterol and epimetendiol of XAD2 columns

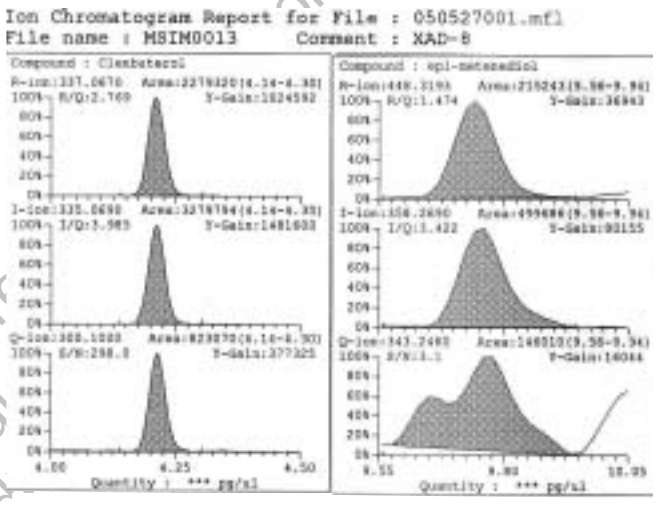

Table 5

Recovery % of clenbuterol and epimetendiol in spiked urine samples with XAD2 and SPE columns

*Spiked concentration (ng/mL)*n=10*	*Recovery percentage (Mean ± SE)*			
	Clenbuterol		*Epimetendiol*	
	XAD2	*SPE*	*XAD2*	*SPE*
2	30.455 ± 0.097	72.095 ± 0.180	105.00 ± 0.294	136.87 ± 0.351
5	46.934 ± 0.325	72.164 ± 0.222	69.69 ± 0.585	104.69 ± 0.468
10	41.606 ± 0.732	89.245 ± 0.661	66.63 ± 1.082	99.65 ± 1.228

*n is the number of samples for each concentration

Table 6

Mean recovery of clenbuterol and epimetendiol in spiked urine samples with XAD2 and SPE columns

*Spiked concentration (ng/mL)*n=10*	*Within-day spiked mean ± SE (ng/mL) of recovered drug*			
	Clenbuterol		*Epimetendiol*	
	XAD2	*SPE*	*XAD2*	*SPE*
2	0.6091 ± 0.09	1.4419 ± 0.18	2.13 ± 0.29	3.41 ± 0.35
5	2.3467 ± 0.32	3.6082 ± 0.22	3.48 ± 0.58	5.23 ± 0.46
10	4.1606 ± 0.73	8.9245 ± 0.66	6.66 ± 1.08	9.96 ± 1.22

*n is the number of samples for each concentration

$P<0.002$ (2 ng/mL), $P<0.001$ (5 and 10 ng/mL).

Epimetendiol. The recovery percentage for 2, 5, and 10 ng/mL with XAD2 were 105, 69.69, 66.63, and 136.87, 104.69, and 99.65% with SPE [Table 5]. The recovery percentage was significantly higher with SPE column extraction. The significance level were, $P<0.015$ (2 ng/mL), $P<0.010$ (5 ng/mL) and $P<0.014$ (10 ng/mL).

The recovery of clenbuterol and epimetendiol at 2, 5, and 10 ng/mL were calculated individually for XAD2 and SPE columns [Table 6]. The total ion chromatogram of clenbuterol and epimetendiol with both XAD2 and SPE columns is shown in Figures 1 and 2.

Discussion

The results of the present study reveal that the extraction procedure for nandrolone involving XAD2 column, is good and there is no additional advantage using SPE column. With both types of columns nandrolone recovery was comparable. However, there is no available literature on comparison of these two types of columns for nandrolone recovery, though both types of columns have been studied separately.[4,5]

The recovery of stanozolol at 5 and 10 ng/mL was comparable in both types of columns, whereas recovery was significantly more ($P<0.000$) at 20 and 40 ng/mL with XAD2 extraction. However, improvement in recovery of Stanozolol at higher concentration (20 and 40 ng/mL) is not of much value because it is required to improve detection at lower concentration (2 and 5 ng/mL). The purification techniques for Stanozolol reported are Immunoaffinity chromatography (IAC)[9] and use of Oasis cartridges.

There was a significant increase in the recovery of clenbuterol and epimetendiol at different concentrations, which necessitates the need to do confirmation testing with C 18 SPE columns. Though use of C-18 Sep-pak columns for extraction of clenbuterol has been reported.[13] There is no study comparing recovery percentage of two types of extraction. Further use of SPE columns for epimetendiol extraction has not been reported in literature so far. The purification techniques used for epimetendiol are based on HPLC clean up[6] and IAC gels.[6]

Conclusion

Based on the present work it is concluded that recovery of Clenbuterol and Epimetendiol has improved with SPE columns compared to existing XAD2 columns at various concentrations. Hence, it can be used for confirmation of samples found positive for clenbuterol and epimetendiol.

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