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COMPARATIVE ANALYTICAL TECHNIQUES FOR THE IDENTIFICATION OF SOME ANDROGENIC ANABOLIC STEROIDS (GC/ MS – LC/MS/MS)

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INTRODUCTION

Due to some structural particularities, the following androgenic anabolic steroids: trenbolone, tetrahydrogestrinone (THG), formebolone and oxandrolone are difficult to be identified in the screening 4 for combined fractions [1].

Based on data found in the literature [2,3,4], we decided to establish the procedure of the sample preparation and the identification of these androgenic anabolic steroids by two analytical techniques (GC/MS and LC/MS/MS) [5,6].

The purpose of this work consists in establishing the adequate parameters of their detection by LC/MS/MS and in finding the derivatization procedure and the optimal conditions for the identification of these compounds by GC/MS.

MATERIAL AND METHODS

Reference substances

17β-trenbolone was purchased from AGAL-NARL (Australia) and 2-hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one (formebolone metabolite), epioxandrolone, tetrahydrogestrinone (THG) and methyltestosterone were obtained from the Cologne Doping Laboratory. Iodine was purchased from Janssen Chimica (Belgium) and N-methyl-N-trimethylsilyltrifluoroacetamide, methyl iodide and the other reagents from Merck (Germany).

Sample preparation

The urine samples were spiked at 6 different levels with each of the androgenic anabolic steroids studied. Final concentrations were 50; 20; 10; 5; 2.5 and 1.25ng/ml, respectively.

The sample preparation was performed by the liquid-liquid extraction procedure [7]. For LC/MS/MS, the samples were dissolved in 100µl mixture methanol:water 4:1 and 10µl were injected [8,9]; for GC/MS, the samples were dried in vacuo, derivatized with 50µl derivatization mixture and 3µl were injected.

Simultaneously, in order to improve the limits of detection, the samples were also prepared by extraction on XAD-2 resin, followed by the separation of the free fraction from the conjugated fraction. The formebolone metabolite and the epioxandrolone were analyzed from the free fraction. The 17β-trenbolone and the THG were analyzed, after enzymatic hydrolysis, from the conjugated fraction. In the case of 17β-trenbolone and THG the samples were spiked after the removal of the free fraction.

Derivatization

The following mixtures and derivatization conditions were tested:

- MSTFA/TMS-Imidazole 100:2 (v:v), 80^0C, 60min [6];
- MSTFA/CH_3I 1000:4 (v:v), 60^0C, 20min;
- MSTFA/I_2 1000:3 (v:w), rt, 90min [10,11].

Analytical parameters

GC/MS parameters

- Instrument: Agilent 6890N/5973N;
- Column: CP-SIL 5CB (methyl silicone), 17m length, i.d. 0.25mm, film thickness 0.12µm;
- Carrier gas: Helium 0,8mL/min;
- Injector: 300^0C, 1:10 split;
- Interface: 300^0C;
- Temperature program: 160°C, 2min., 10°C/min, 300°C, 4 min;
- MS: EI mode, 70eV electron energy.

LC/MS/MS parameters

- Instrument: Varian 1200L;
- Column: OmniSpher 3 C18, 2.0x100mm, particle size 3µm;
- Eluents: A = 5mM ammonium acetate in water with 0,1% acid acetic,
  B = methanol;
- Flow: 0,25ml/min;
- Gradient A: 0.5min 70%, 0.5min 70%→50%, 15min 50%→30%, 1min 30%, 5min 70%;
- Interface: APCI 400^0C; positive ionization mode;
- Collision gas: Argon, 1.5mTorr;
- Detector: 1800V (high gain).

RESULTS AND DISCUSSION

The formebolone and oxandrolone metabolites are hard to be identified in the screening 4 for combined fractions due to interferences from the biological matrix. Therefore, for these compounds it is preferred the free fraction separation procedure and the derivatization of the extract with MSTFA/Imidazole.

But this derivatization method didn't lead to satisfactory results for 17β-trenbolone, which belongs to the estra-4,9,11-trien-3-one class of compounds, compounds known to have a difficult derivatization. We tried then the derivatization with MSTFA/CH_3I and also the derivatization with MSTFA/I_2. The first derivatization method gave unsatisfactory results, but the MSTFA/I_2 derivatization method performed very good and resulted in a 2.5ng/mL limit of detection for 17β-trenbolone (table 1). Figure 1 shows the chromatogram and the EI-mass spectrum of the 17β-trenbolone derivatized with MSTFA/I_2. But this derivatization method didn't lead to satisfactory results for the formebolone and oxandrolone metabolites.

For 17β-trenbolone and epioxandrolone the results obtained by LC/MS/MS and GC/MS, for the samples prepared by the liquid-liquid extraction procedure, are similar. For the formebolone metabolite the LC/MS/MS technique resulted in a lower limit of detection than the GC/MS technique. The best results obtained by LC/MS/MS technique were for THG: 1.25 ng/ml limit of detection.

The XAD-2 extraction procedure improved the performance of the GC/MS technique, resulting in the limits of detection from the last column of table 1. For the LC/MS/MS technique, the XAD-2 extraction procedure only improved the limit of detection for the formebolone metabolite.

The analysis results are shown in table 1 (for GC/MS) and in table 2 (for LC/MS/MS).

The establishing of the validation parameters is to be presented in a future work.

Table 1. GC/MS results

Compound	RT, min	RRT	m/z (abundance)	LOD, ng/ml [1)]	
				LLE	SPE
Formebolone M	14.09	1.235	143 (100%), 367 (61.8%), 382 (47.4%), 562 (24.8%)	20	10
17β-Trenbolone	13.13	1.150	442 (100%), 380 (50.0%), 524 (7.9%), 539 (7.0%)	10	2.5
Epioxandrolone	10.85	0.953	143 (100%), 308 (31.1%), 321 (22.7%), 363 (19.0%)	5	1.25
SI-MT	11.42	1.000	301, 446	-	-

[1)] Limit of detection (S/N>3 for the least intense diagnostic ion)

Table 2. LC/MS/MS results

Compound	RT, min	RRT	Parent ion	Daughter ion (abundance)	LOD, ng/ml [1)]	
					LLE	SPE
Formebolone M	7.8	0.52	347.2	281 (100%), 147 (62%), 173 (33%)	10	5
17β-Trenbolone	11.0	0.73	271.2	199 (100%), 107 (25%), 253 (101%)	10	10
Epioxandrolone	16.5	1.09	324.2[2)]	289 (100%), 135 (20%), 107 (15%)	5	5
THG	17.0	1.13	313.2	241 (100%), 159 (78%), 295 (147%)	1.25	1.25
SI-MT	15.1	1.00	303	109	-	-

[1)] Limit of detection (S/N>3 for two diagnostic transitions)
[2)] Ammonium adduct

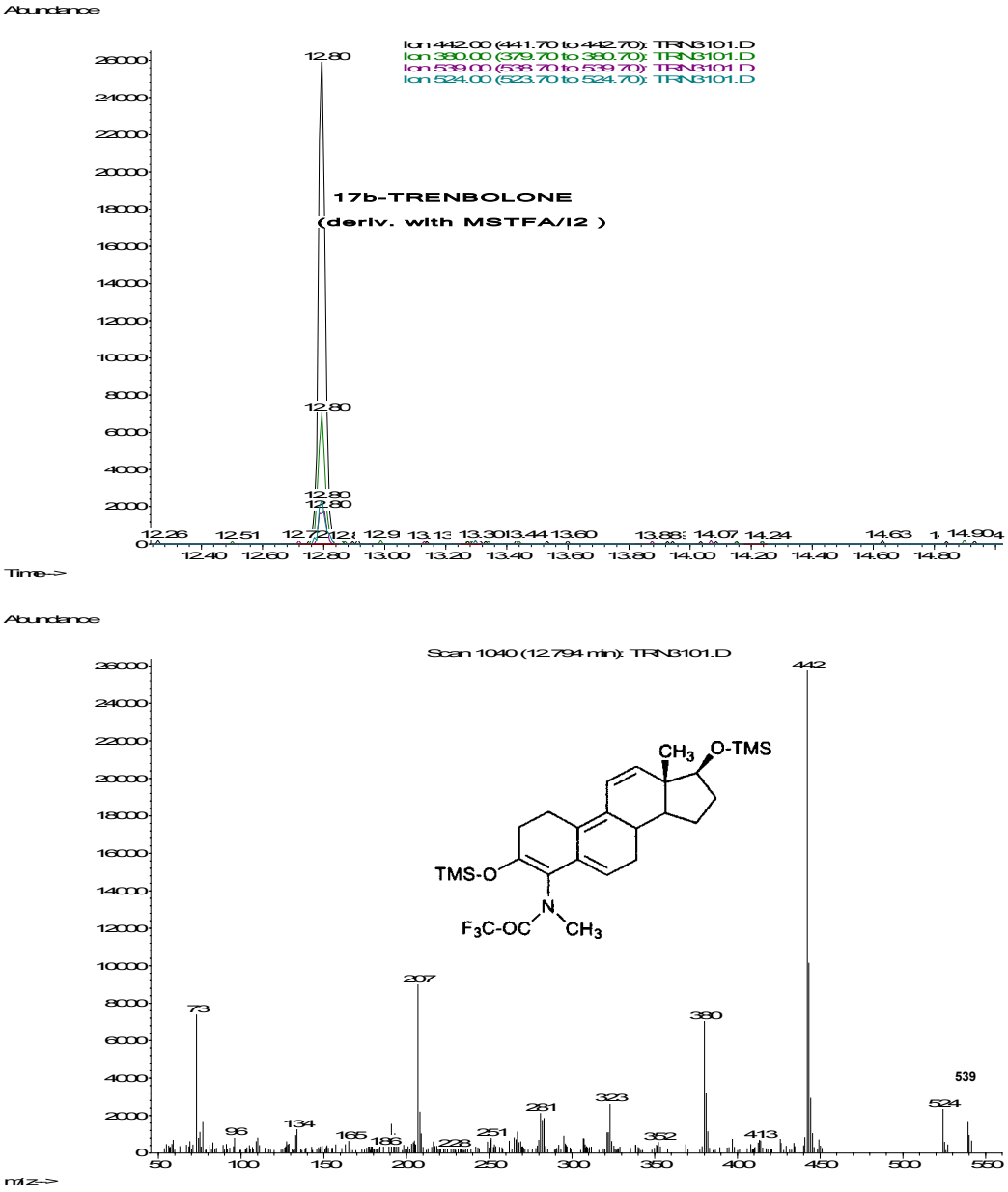

Figure 1. Chromatogram and EI-mass spectrum of the 17β-trenbolone derivatized with MSTFA/I_2

CONCLUSIONS

- The results obtained recommend the use of the LC/MS/MS technique for the screening of formebolone, oxandrolone, trenbolone and THG, the limits of detection being ≤ 10ng/ml (WADA MRPL for androgenic anabolic steroids).
- Furthermore, the LC/MS/MS technique is more suitable for the screening procedure than the GC/MS technique since it doesn't require derivatization.
- A common derivatization procedure for all the studied steroids was not found. For a good identification at low concentrations a specific derivatization is required: MSTFA/TMS-Imidazole for formebolone and oxandrolone and MSTFA/I_2 for trenbolone.
- The clean-up of the samples by extraction on XAD-2 followed by the separation of the free fraction from the conjugated fraction, together with the use of specific derivatizations allowed the decreasing of the limits of detection obtained by the GC/MS technique.

REFERENCES

1. Grosse, J.; Thieme, R.; Lang, R.; Mueller, R.K. In: *Recent Advances in Doping Analyses (7)*, Sport und Buch Strauß, Köln (1999), 143-152.
2. Marcos, J.; Pascual, J.A.; De La Torre, X.; Segura, J. In: *Recent Advances in Doping Analyses (9)*, Sport und Buch Strauß, Köln (2001), 99-106.
3. Geyer, H.; Bredehöft, M.; Mareck, U., Parr, M.K.; Reinhart, U.; Schänzer, W. In: *Recent Advances in Doping Analyses (11)*, Sport und Buch Strauß, Köln (2003), 77-84.
4. Schanzer, W.; Donike, M., *Analytica Chimica Acta*, 275 (1993) 23-48.
5. Marques, M.A.S.; Geyer, H.; Schänzer, W.; Opfermann, G.; Pereira, H.M.G., De Aquino Neto, F.R. In: *Recent Advances in Doping Analyses (11)*, Sport und Buch Strauß, Köln (2003), 277-282.
6. Donike, M.; Geyer, H.; Gotzmann, A.; Kraft, M.; Mandel, F.; Nolteernsting, E.; Opfermann, G.; Sigmund, G.; Schanzer, W.; Zimmermann,J. In: *Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport, Florence, 1987*, International Athletic Foundation, Monte Carlo (1988), 53-80.
7. Mareck, U.; Thevis, M.; Guddat, S.; Gotzmann, A.; Bredehöft, M.; Geyer, H.; Schänzer, W. In: *Recent Advances in Doping Analyses (12)*, Sport und Buch Strauß, Köln (2004), 65-68.
8. Catlin, D.H.; Sekera, M.H.; Ahrens, B.D.; Stancevic, B.; Chang, Yu-Chen; Hatton, C.K., *Rapid Communications in mass spectrometry*,18 (2004) 1245-1249.
9. Huynh, T.; Trout, G.J.; Kazlaukas, R. In: *Recent Advances in Doping Analyses (11)*, Sport und Buch Strauß, Köln (2003), 271-277.
10. Sterk, S.S.; Ohlrich, D.; Christensen, F.; Le, N.B.; Blokland, M.H.; Schwillens, P.L.W.J.; Van Ginkel, L.A.; Stephany, R.W. See http://www.euroresidue.nl/ER_IV/Contributions%20I-Z/Sterk%201-35-1041.pdf.
11. Maume, D.; Le Bizec, B.; Marchand, P.; Montrade, M.P.; Andre, F., *The analyst*, 123 (1998) 2645-2648.