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W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

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S. Karkeni, S. Bouabdallah, H. Trabelsi, K. Bouzouita

Quantification at low Concentration of 19-Norandrosterone in Human Urine by GC\MS\MS\MS and GC\HRMS. A Comparative Study

Laboratoire National de contrôle des Médicaments et de dépistage du dopage, 11 bis rue jebel Lakhdar, 1006 Bab sâadoun,Tunis-Tunisie.

1-INTRODUCTION

In our laboratory all the screening of anabolic steroids at low detection levels is carried out by low-resolution Quadruple gas chromatography mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS).However, it is known that 19-norandrosterone (19-NA) is banned by WADA/IOC if the concentration exceeds 2 ng/ml [1]. Therefore, it is necessary to establish a quantitative method for the determination of the concentration. The first estimation of 19-NA was done by HRMS screening by comparison of the signal of the suspect sample which probably contains the substance and the positive quality control sample which has a concentration of 2 ng/ml in urine (2 ml). A result is positive, if the concentration of 19-NA in urine is higher than 2 ng/ml. Characterized by a short oven temperature program and a high sensitivity, this GC/HRMS method was validated and used for the quantitation of 19-NA.

Recently published methods [2] reported the use of a tandem mass spectrometer for the identification and quantitation of drugs in a biological matrix. Thus our interest was to evaluate the advantages/disadvantages of the ion trap MS^3-mode for the quantitation of the 19-NA which have more sensitivity and selectivity than GC/MS^2 mode compared with the HRMS.

2-EXPERIMENTAL

Sample preparation

After addition of internal standard 17-α–methyltestosterone (50 µg/ml) to a 2 ml urine sample, 200 µl of phosphate buffer (pH 6 –6.5) were added and the urine was hydrolyzed with 40 µl of beta-glucoronidase (1h, 55° C). The urine was adjusted to pH 9 with potassium carbonate buffer and extracted with 5 ml of n-pentane [3]. The tube was shaken in a mechanic agitator for 20 min . The urinary extract was centrifuged at 2500 rpm for 5 min to separate the organic layer from the aqueous. The organic phase was then evaporated to dryness under a nitrogen stream.

Derivatization of urine extract

The dry steroid residue was derivatized with 50 µl of MSTFA/NH_4I/dithioerythritol and heated for 30 min at 65°C [4], divided in two vials. Two µl of the solution were simultaneously injected into the gas chromatography/mass spectrometer (GC/MS/MS/MS) and GC/HRMS analyzer.

Instrumental conditions [5-7]

1- GC/HRMS

Instrument	Micromass Autospec-Ultima
Column:	
- Brand	Hewlett Packard
- Type	HP-1
- Length	25 m
- Inner Diameter	0.2 mm
- Film Thickness	0.11 µm
Flow Parameters:	
- Carrier gas	Helium
- Flow rate of carrier gas (cst flow)	1ml/min
Injection parameters:	
- Injection mode	Split
- ratio	1:10
- Injection volume	1µl
- Injector temperature	280 °C
Oven temperature program	
- Initial temperature	180°C
- Initial time	0 .85 min
- Rate1	15°C/min
- Final temperature	270 °C
- Second time	0 min
- Rate 2	50°C/min
- Final temperature	325 °C
- Final time	2.05 min
Mass Spectrometric parameters	
- Ionization mode	EI
- Acquisition mode	SIR
- Resolution	≈ 10000
- Trap current	300 µA
- Electron impact	36 eV

2- GC/MS/MS/MS

Instrument	VarianSaturn 2000
Column:	
- Brand	Hewlett Packard
- Type	HP-1
- Length	17 m
- Inner Diameter	0.2 mm
- Film Thickness	0.11 µm
Flow Parameters:	
- Carrier gas	Helium
- Flow rate of carrier gas	1.1 ml/min
- Head pressure (cst pressure)	21.5 psi
Injection parameters:	
- Injection mode	Split
- ratio	1:10
- Injection volume	2 µl
- Injector temperature	280 °C
Oven temperature program	
- Initial temperature	170°C
- Initial time	0 min
- Rate1	3°C/min
- Final temperature	230 °C
- Second time	0 min
- Rate 2	40°C/min
- Final temperature	310 °C
- Final time	3 min
Mass Spectrometric parameters	
- Ionization mode	EI
- Acquisition mode	MS^3
- Interface temperature	300°C
- Ion trap temperature	200°C
- Manifold temperature	40°C
- Target	5000
- Multiplier voltage	Autotune
- Filament	80µA
Methyl testosterone mass spectrometric conditions	
- precursor ion	m/z 446
- PI fragmentation mode	CID
- CID type	resonant
- CID time	20 msec
- CID amplitude	0.49 V
- Excitation storage level	m/z 98
Nandrolone M1 mass spectrometric conditions	
- **precursor ion 1**	m/z 420
- PI fragmentation mode	CID
- CID type	resonant
- CID time	20 msec
- CID amplitude	0.48 V
- Excitation storage level	m/z 138.9
- Daughter ion	m/z 405
- **precursor ion 2**	m/z 405
- PI fragmentation mode	CID
- CID type	Resonant
- CID time	20 msec
- CID amplitude	0.48 V
- Excitation storage level	m/z 134
- Daughter ions	m/z 225, 315

3-RESULTS

Calibration, recovery and reproducibility.

Linearity was tested in the concentration range 1-10 ng/ml. For the determination of linearity, a standard calibration curve of at least 6 points was used. A blank urine sample was also analyzed to confirm the absence of interferences, the graphs were constructed by plotting the peak area ratio and fitted to the equation y= bx + a by least squares regression [4]. The diagnostic ion m/z 315 is the target for quantitation ion in GC/MS/MS/MS and m/z = 405.2645 for GC/HRMS. The acceptance criteria for correlation coefficient were not less than 0.995. The obtained results are summarized in ***Table 1.***

Recovery was calculated using GC/MS^3 at three different concentrations (2 – 5 – 10 ng/ml) using spiked human urine with six replications per concentration during three days. The recovery is in the range of 75.1% - 93.69%.

The reproducibility of these two methods was determined by analyzing three spiked urines at three concentrations (1,2,3 ng/ml) for ten days. The obtained results of RSD were 10.39% and 13.47% for the GC/MS/MS/MS and the GC/HRMS respectively. The lowest concentration was to meet the following acceptance criteria; no more than 20%.

Detection limits

The limits of detection at signal to noise ratio (S/N = 3) obtained by GC/MS3 and GC/HRMS methods were 0.3 ng /ml and 0.1 ng/ml respectively.

The limit of quantitation (LOQ) with a signal to noise of 10:1 was estimated to be 1 ng/ml and 0.5 ng/ml for GC/MS^3 and GC/HRMS respectively.

Table 1. Linearity parameters

Parameters	**GC/MS3**			**GC/HRMS**		
Linearity:						
- **Range (ng/ml)**	1 – 10			1 – 10		
- **Intercept**	$+ 2\ 10^{-5}$			$-7\ 10^{-5}$		
- **Slope**	0.001			0.01		
- **r**	0.9998			0.9995		
Recovery						
- **Concentrations (ng /ml)**	2	5	10			
- **Mean (%)**	75.1	91.87	93.69			
- **RSD (%)**	5.52	2.06	1.99			
Reproducibility						
- **Concentration (ng/ml)**	1	2	3	1	2	3
- **RSD**	10.39	11.56	12.99	8.11	6.82	13.96
LOD (ng/ml)	0.3			0.1		
LOQ (ng/ml)	1			0.5		

4-Conclusion

The GC/HRMS and GC/MS/MS/MS methods were validated and applied to the determination of the main metabolite of nandrolone (19-norandrosterone). The obtained results show (Table 2.) that the two procedures are in fair agreement and are suitable for the analysis of this metabolite in human urine. However, it appears that GC/HRMS method is more convenient for major events according to the short oven program applied in comparison with GC/MS/MS/MS, which is less sensitive, and more time consuming.

Table 2. Determination of the 19-norandrosterone in positive human urines

	GC/MS/MS/MS	*SD*	*RSD%*	*GC/HRMS*	*SD*	*RSD%*
Sample1	4.10	0.2	4.88	4.17	0.09	2.18
Sample2	3.93	0.11	2.89	4.08	0.06	1.68
Sample3	8.96	0.39	4.39	8.51	0.2	2.38

5-Bibliography

1- The World Anti-Doping Agency (2003): International standard for laboratories, version 3.0 June 2003. http://www.wada-ama.org/

2- L.Amendola, C.Clamonici, F.Rossi, F.Botre: Determination of clenbuterol in human urine by GC-MS-MS-MS: Confirmation analysis in antidoping control. *J Chrom B*, 773 (2002)7-16.

3- U.Mareck-Engelke, H.Geyer, W.Schänzer: 19-Norandrosterone - Criteria for the decision making process. In: Schänzer,W., Geyer, H., Gotzmann A., Mareck-Engelke,U. (eds.) *Recent Advances in Doping Analysis (6),* Sport und Buch Strauß, Köln 1998, 119-124.

4- ISO/IEC/EN 17025, General requirements for the competence of calibration and testing laboratories, 1999.

5- K.K.Murray: Internet resources for mass spectrometry, *J Mass Spectrom*, 34, (1999) 1-9.

6- E.de Hoffmann: Tandem mass spectrometry: a primer, *J Mass Spectrom*, 13, (1996) 129-137.

7- A.K.Shukla, J.H.Futrell: Tandem mass spectrometry: dissociation of ions by collosional activation, *J Mass Spectrometry*, 35 (2000) 1069-1090.

8- J.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. *J Chrom B*, 704 (1997) 129-141.

9- T.Kusamaran, X.de la Torre, R.de la Torre B.Rasmussen, P.Wilairat, T.Anukarahanonta: Monitoring of low concentration anabolic steroids in urine samples from the 13th Asian games by ion-trap GC/MS/MS. In: Schänzer,W., Geyer, H., Gotzmann A., Mareck-Engelke,U. (eds.) *Recent Advances in Doping Analysis (7)*, Sport und Buch Strauß, Köln 1999, 255-256.

10- G.Trout, J.Murby, R.Kazlauskas: High resolution mass spectrometry in the antipodes. In: Schänzer,W., Geyer, H., Gotzmann A., Mareck-Engelke,U. (eds.) *Recent Advances in Doping Analysis (6)*, Sport und Buch Strauß, Köln 1998, 269-276.