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Analysis of steroids by on line coupling LC-GC-MS using the TOTAD interface and a fraction collector

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INTRODUCTION

According to the World Antidoping Agency (WADA), the limit of detection for most anabolic steroids must be below 10 ng/mL. In the case of synthetic steroids, the routinely used methods are based in the use of gas chromatography coupled with mass spectrometry (GC-MS). When the potential endogenous steroid misuse should be determined, an analysis by GC hyphenated with combustion isotope ratio mass spectrometry (GC-CIRMS) is mandatory. Attending to the metabolite and the matrix background, to achieve the detection limits required by WADA could be necessary to enhance detection capacities and selectivity of the analytical method. With such aim, some research groups employ off-line consecutive clean-up steps with high performance liquid chromatography (HPLC). The aim of the present work is to achieve these objectives by using the on-line coupling liquid chromatography-gas chromatography with the TOTAD interface, *figure 1 and 2*. The LC fractions containing the analytes are automatically transferred to a fraction collector and then transferred to the GC. This analytical methodology reduces significantly the sample preparations steps, and provides a better signal/noise ratio because cleaner extracts are obtained. Besides all the LC fraction containing the analytes are transferred to the GC so that a much larger volume is injected. The objective of the present work is to develop a sensitive, robust and reliable analytical method of analysis of steroids in urine, which allows reaching the detection limits required to carry out the efficient control of the misuse of anabolic steroids in sports.

EXPERIMENTAL

Steroid metabolites analysed, 11-keto-etiocholanolone, 11β-hydroxy-androsterone, epitestosterone, testosterone,19-norandrosterone, etiocholanolone, androsterone, 5α-andro-stanediol and 5β- androstanediol, were purchased from Steraloids (London, UK), methanolic solutions at concentration levels of 100 mg/l, 1 mg/l and 0,1 mg/l, were prepared and stored

previous to its use at -20ºC. The methanolic solutions were used to spike real and synthetic urinary samples.

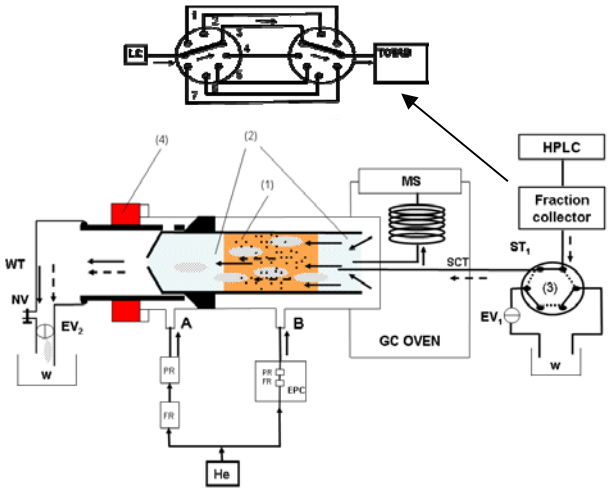

Figure 1; TOTAD interface and fraction collector, where; (1) glass wool, (2) Tenax TA, (EPC) Electronic pressure control with pressure regulator (PR) and flow regulator (FP) Gas flow and Liquid flow (EV1 and 2) Electrovalves. (SCT) Capillary tubing (Transfer line), (W) Waste, (3) Six-port valve, (ST1) Stainless steel tubing, to transfer eluent from LC to six-port valve (WT) Waste tubing Helium flow Solution.

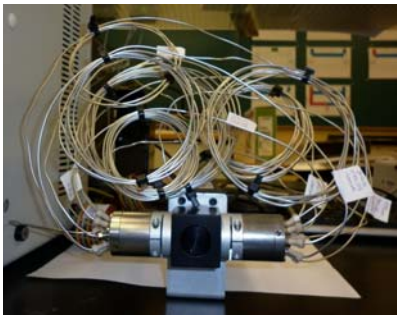

Figure 2. Fraction collector made with two eight-port valves provided with a multiposition microelectric valve

Sample preparation; The sample preparation process consisted of three stages: hydrolysis, extraction and acetylization. 100 µL of phosphate buffer, pH=7, and 50 µL β-glucuronidase from E. Coli were added to 16 mL of sample. The mixture was vortexed for several seconds, covered and heated at 55 ºC for one hour on a heating block The mixture was then allowed to cool at room temperature before extraction. For this, 300 µL carbonate buffer, pH=11, and 5 mL n-pentane were added before shaking at 90 r.p.m. for 5 minutes. The mixture was then placed in a cryogenic bath of ethanol at -30 to -35 ºC until the aqueous fraction was frozen. The organic phase was decanted and dried under a nitrogen stream at 45 ºC. Then 50 µL

pyridine and 50 µL anhydride acetic were added and the mixture was heated at 65 ºC for 30 minutes to obtain the acetyl derivates. After drying at room temperature under a nitrogen stream, the acetyl derivates were regenerated in 400 µL methanol. The sample was now considered ready for analysis by directly coupled LC-GC using the fraction collector.

Instrumentation and conditions of analysis*; The instrumental conditions were:*

- **An HPLC system** (Konik 560) integrated by a ternary pump, a column oven, a 50 µl loop and an UV-Vis detector (200 nm)
- Column: ACE 5 C18, (250 x 4.6mm i.d)
- Column oven: 25ºC.
- Eluent: Water/Acetonitrile (70:30) initially, then changes100% Acetonitrile in 25 min, hold 10 min.
- The flow rate was 1 ml/min during elution and collection of the fraction of interest. During the transfer of the fractions to the GC, the flow rate was changed to 0.1 mL/min.
- **Fraction collector**, to separate the different fractions a collector made with two eight-port valves provided with a multiposition microelectric valve actuator was used, see *figure 1.* TOTAD interface (US patent 6,402,947 B1, exclusive rights assigned to KONIK-Tech, Sant Cugat del Vallés, Barcelona, Spain) with the liner filled with Tenax TA (1 cm length). Temperature adsorption/desorption: 125ºC/325ºC. Desorption time: 5 min
- **A GC-MS system,** integrated by; a Gas chromatograph (Konik 4000B) equipped with an automated TOTAD interface, a capillary column of 30 m length, 0.25 id mm (type 17MS, fil*led with s*ilphenylene siloxane). During the transfer and solvent elimination steps the oven temperature was kept at 60 ºC.Oven temperature: 60ºC; 35ºC/min to 160ºC; 10ºC/min to 270ºC hold 13.8 min; 35ºC/min to 300ºC hold 10 min.
- Quadrupole mass spectrometric detector (MSQ12 Konik), mass spectrometric analysis was performed in EI full-scan mode from 50 to 400 uma. Carrier Gas: Helium at 10 Psig.

RESULTS

Figure 3 shows the LC-UV chromatogram of a methanolic solution of the selected steroids at 100 ng/ml where the six LC fraction to be transferred are indicated. The fraction collector developed allows to store selectively each of the six fractions that contain the analytes. The developed procedure allows obtaining a clean extract (see figure 4). Table 1 gives the Limits of detection (LOD) in ng/mL calculated using a solution of the steroids in methanol and a real urine sample . The LOD was calculated as the amount of product given a signal equal to 5 times the background noise for the Total Ion Chromatogram (TIC) of each compound. The chromatogram was acquired in SCAN mode.

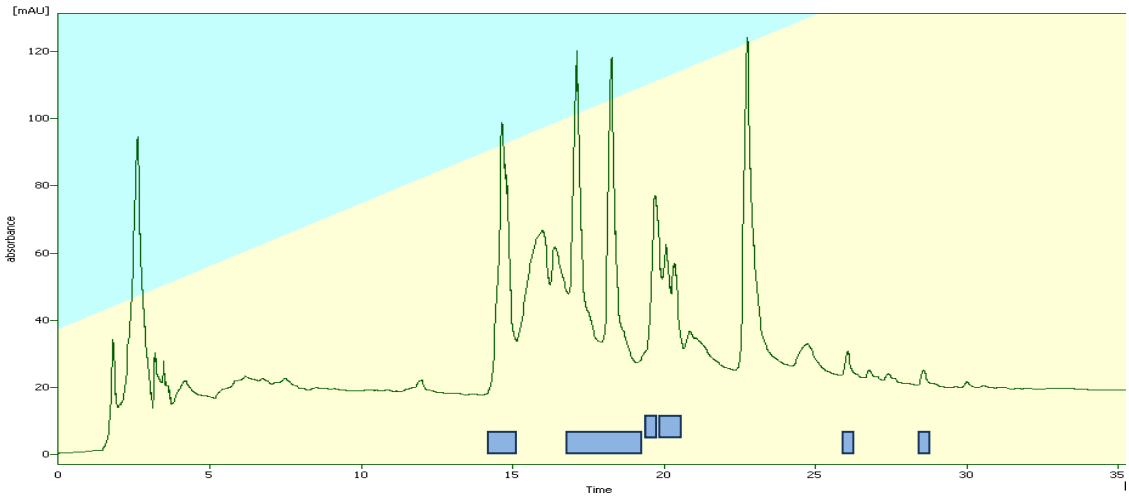

Figure 3. LC-UV chromatogram of a methanolic solution of the steroids at 100 ng/mL. The blue rectangles located above the time axis indicate the LC fractions transferred to the fraction collector.

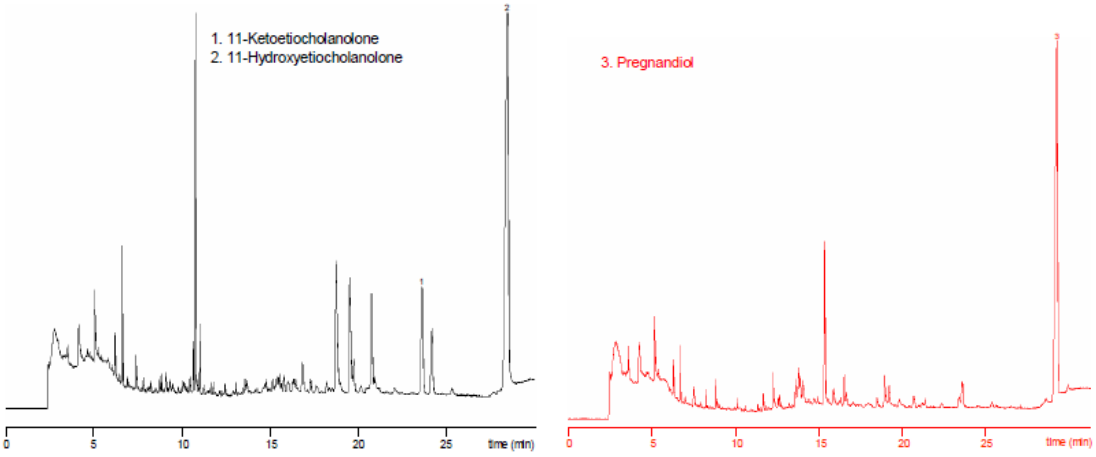

Figure 4. GC-MS chromatograms of two fractions from a real urine sample analyzed by on line RPLC-GC-MS using a fraction collector. The MS acquisition was obtained in full scan mode. The urine was hydrolyzed, extracted, derivatized (as acetate derivates) and dissolved in 400 µl of methanol

CONCLUSION

- The TOTAD interface with the fraction collector is suitable for the automated LC-GC analysis of the six steroid fractions.
- The LODs, acquired in SCAN mode in the GC-MS (quadrupole) system, are all below 10 ng/ml. In the case of 19-Norandrosterone, LOD is below 2 ng/ml.
- The performance of the method when real urine samples were analyzed was not affected.
- Taking into account the requirement of selectivity and sensibility of C-IRMS detector, we are ready to deal with the on-line coupling LC-GC-C-IRMS..

Table 1. Limit of detection LOD (ng/mL) for a selection of compounds calculated from the analysis of; (A) a standard solution of acetate derivates at 100 ng/ml and (B) a real urine sample, which was previously analyzed in the Agencia Estatal Antidopaje laboratory (Madrid) by the routine method.

Compound	**Isolated HPLC Fraction**	**(A) 100 ng/mL standard solution**	**(B) urine sample**	
		LOD (ng/mL)	**ng/mL**	**LOD (ng/mL)**
1- keto-etiocholanolone	1	3,9	119	3,4
11β- hydroxyandrosterone		8,4	760	5,6
Epitestosterone	2	5,7	21	0,77
Testosterone		2,1	34	1,34
19-norandrosterone	3	1,7	---	---
etiocholanolone	4	3,6	860	5,40
androsterone		2,0	3780	5,20
5α- androstanediol	5	4,5	136	1,1
5β-androstanediol		4,5	100	1,1