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An automated derivatization method for anabolic steroids prior to GC-MS/MS analysis

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Abstract

The purpose of this work was to develop an automated method with increased sensitivity and selectivity, with lower detection limits for the analysis of multiple conjugated 3-ketosteroids, using an automated derivatization process and detection by GC/MS. The use of an automation module in the sample preparation instead of the traditional manual sample derivatization procedure for the anabolic steroids screening has been implemented. This technology is used for batches containing more than 30 samples. A triple quadrupole mass spectrometer (GC-MS/MS) instrument, used in MRM mode, coupled to a sample preparation module, which automates sample preparation steps such as addition, extraction, mixing and heating. This automation presents an alternative sample preparation. This procedure has improved the derivatization yield for some anabolic steroid compounds which are part of the regular screening.

Introduction

The detection of anabolic steroids in doping control is carried out using screening procedures which involve manual preparation together with LC/MS or GC/MS techniques, that must achieve the required detection limits for doping control. The detection of 4,9,11-triene and similar structures in urine is a long-standing problem in doping control, which has been solved by applying LC/MS instead of GC/MS. GC/MS analysis requires derivatization to provide volatile compounds. In most of the cases the derivatization yields an improvement in selectivity and sensitivity, however the behaviour of multiple conjugated 3 –ketosteroids makes their detection difficult. This work shows an alternative new automated derivatization process to overcome these problems.

Experimental

Reference materials were obtained from Sigma-Aldrich (St. Louis, MO, USA), NMI (Pymple, Australia), USP (Rockville, MD, USA), Atlantic Pharma (Nantes, France), TRC (Toronto, Canada) , Alltech (State College, PA, USA), Steraloids (Newport, RI, USA), European Pharmacopoeia (Strasbourg, France), AK Scientific (Union City, CA, USA) and Cerilliant (Round Rock , TX, USA). The rest of the reagents and solvents were analytical grade. Three batches of urines were prepared. The first, negative control urines were spiked with standard stock methanolic solution containing all the compounds listed in Table 2, at the detection limits required in the doping control analysis, MRPL. The second and third groups, negative control urines samples were spiked with methyltrienolone, gestrinone, tetrahydrogestrinone and androstantriendione at high concentration levels and at the MRPL concentration levels. The sample preparation procedure includes three steps: enzymatic hydrolysis, liquid-liquid extraction and preparation of TMS derivatives using MSTFA:NH_4I:Dithioerythritol (1:2:4, v:w:w) during 30 minutes at 65°C. All the urine samples were hydrolysed and extracted into an automated liquid-liquid extraction system (Zinsser Lissy GXL System) acquired from Zinsser Analytics (Frankfurt, Germany) specifically designed for the needs of our laboratory. The derivatization step was performed by carrying out two parallel procedures, manual and automated processes. Automated derivatization process was performed in a sample preparation module, 7693A ALS, coupled to an Agilent 7890A

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gas-chromatograph and an Agilent 7000A triple quadruple mass spectrometer. Optimization of the 7693A ALS module prior to its use was required. The optimized parameters are showen in Table 1.

Program step set-points (3-way factorial design)	
Mixing Mode (Uni-directional/ Bi-directional)	Bi-directional
Dwell Time between cycles (sg)	5
Number of cycles	7
Mixing speed (200- 4000 rpm)	4000
Mix cycle time (1-60 sg)	40

Table 1: Optimized sample preparation module parameters.

Compounds	Relative abundance		Compounds	Relative abundance	
	Manual	Automated		Manual	Automated
19-Norandrosterone	0,006	**0,006**	Mesterolone PC	0,004	0,003
19-Noretiocolanolone	0,013	0,012	Metandienone M5	0,004	**0,004**
4-OH-TestosteronePC	0,062	0,061	Metandienone M4 (17-Epimetandienone)	0,005	**0,005**
6-OXO M1 (6α-OH-Androstendione)	0,118	**0,118**	Metandienone M2 (6-OH-Dianabol)	0,006	**0,006**
6-OXO M2 (6α-OH-Testosterone)	0,122	**0,123**	Metandienone M3 (18-Nor)	0,006	**0,006**
6-OXO PC	0,035	**0,038**	Metandienone M1 (Epimetendiol)	0,002	**0,002**
Androstantriendione (ATD)	0,001	**0,004**	Metasterone M1 (3-OH-Metasterone)	0,005	**0,005**
Boldenone M1	0,007	**0,007**	Metenolone M1	0,013	0,012
Boldenone PC	0,014	**0,014**	Metenolone PC	0,010	**0,010**
Aminoglutethimide PC-TrisTMS	0,004	**0,005**	Methyl-1-Testosterone PC	0,001	**0,001**
Bolasterone M1	0,002	**0,002**	5αMethyltestosterona	0,001	**0,001**
Bolasterone PC	0,004	**0,004**	5βMethyltestosterona	0,002	**0,002**
6-OH-Bromantane	0,212	0,211	Mibolerone PC	0,010	**0,010**
Buprenorphine M1 (Norbuprenorphine)	0,002	**0,002**	Morphine	0,025	0,024
Buprenorphine	0,003	**0,003**	Norboletone M2	0,005	**0,005**
Calusterone PC	0,011	0,010	Noretandrolone M1	0,062	0,060
Δ9-Tetrahydrocannabinol	0,041	**0,042**	Noretandrolone M2	0,007	**0,007**
Canrenone PC /Spironolactone	0,056	**0,166**	Oxandrolone M1 (Epioxandrolone)	0,001	**0,001**
Carphedon	0,044	**0,048**	Oxandrolone PC	0,001	**0,001**
Cyclofenil M2	0,111	**0,112**	Oxycodone	0,010	**0,050**
Clenbuterol	0,0004	**0,0004**	Oxymorphone	0,021	**0,021**
Clostebol M1	0,004	**0,004**	Oxymesterone PC	0,021	**0,021**
Codeine	0,059	**0,061**	Pentazocine	0,039	**0,041**
Danazol PC	0,001	**0,001**	p-OH-anfetamine	0,240	0,173
Danazol M2	0,001	**0,001**	Tamoxifen M1	0,017	0,016
Danazol M1 (Etisterone)	0,003	**0,003**	Tibolone M3	0,016	0,015
Oral turinabol M1	0,0003	**0,0003**	Tibolone M2	0,003	0,002
Drostanolone M1	0,011	**0,011**	Tibolone M1	0,005	**0,005**
Stanozololol M3	0,001	**0,001**	Trenbolone M1(Epitrenbolone)	0,002	**0,003**
Stanozololol M2	0,001	**0,001**	Zeranol	0,007	**0,007**
Stanozololol M1	0,0004	**0,0004**	Zeranol M1 (Taleranol)	0,011	**0,011**
Estradienedione M1(9(10)Dehydronandrolone)	0,032	**0,038**	Zilpaterol	0,020	**0,020**
Estradienedione PC	0,013	**0,016**	5αAdiol	0,130	**0,130**
Fluoxymesterone M2	0,012	**0,012**	5βAdiol	0,140	**0,140**
Fluoxymesterone PC	0,012	**0,012**	Androsterone	5,300	5,240
Furazabol M1	0,003	**0,003**	DHEA	0,050	**0,050**
Furazabol PC	0,001	**0,006**	DHT	0,010	**0,010**
6-MAM	0,059	**0,060**	Epitestosterone	0,100	**0,100**
Hydrocodone	0,084	**0,087**	Etiocolanolone	1,630	1,590
Hydromorphone PC	0,026	**0,026**	Testosterone	0,200	**0,200**
Letrozole M1	0,113	**0,115**			
Mesterolone M1	0,017	0,016			

Table 2: Efficiency for over 80 compounds.

Results and Discussion

Analytical results for both methods of derivatization were obtained from three simple experiments. Each batch of samples was divided in two groups which were manually and automatically derivatized prior to GC/MS analysis. Several charts were studied in order to evaluate the sensitivity and stability for multiple conjugated 3-ketosteroids over time. The main observations are shown below:

• In most of the substances the derivatization efficiency remained the same or improved using the automated derivatization. However, the response of Androstantriendione increased 1- to 4-fold. Table 2 shows these results for about 80 compounds.

• A comparison between the traditionally used derivatization process and the automated method for multiple conjugated 3-ketosteroids is shown in Figure 1. These results indicate an instability in the behaviour of TMS products by GC/MS. The best signals were obtained from the automated derivatization since the first analysis on day 1.

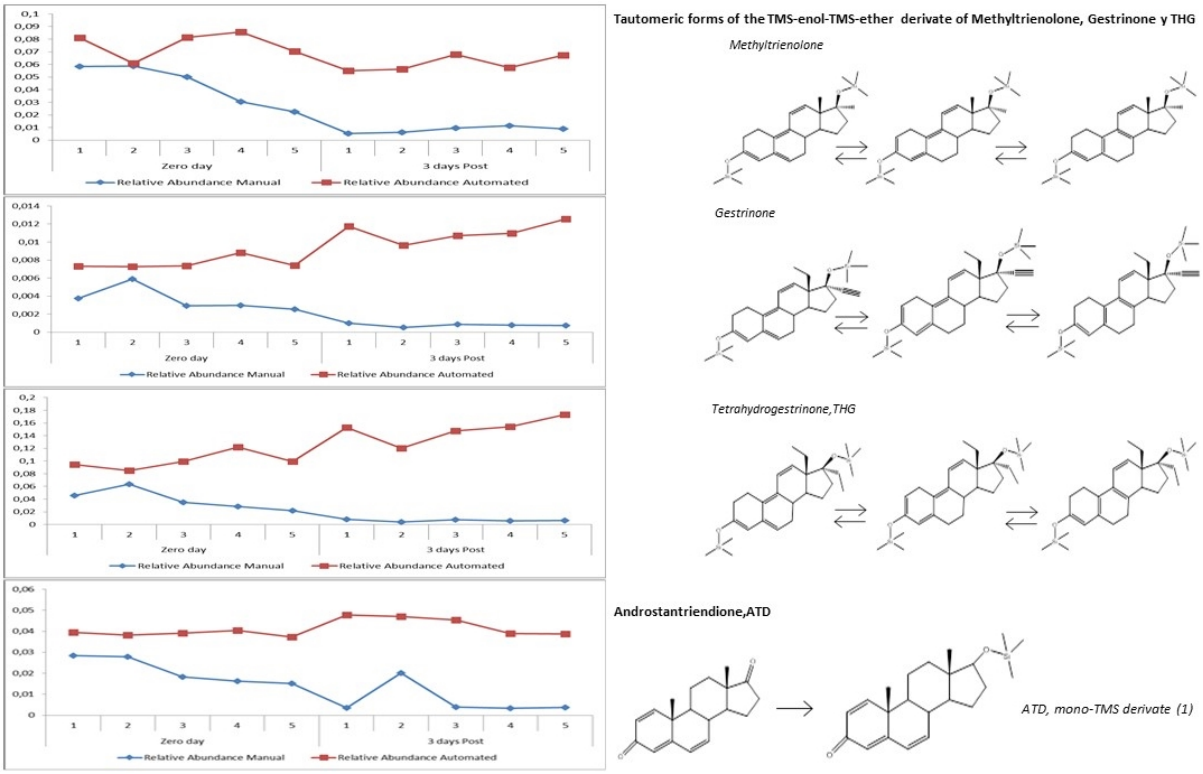

Figure 1: Behaviour of TMS derivatized multiple conjugated 3-ketosteroids over time.

• Figure 2 shows the ability to detect these TMS compounds by GC-MS over time using automated derivatization at their respective MRPLs over time. In the samples manually derivatized, 36 hours later, the detection decreases substantially.

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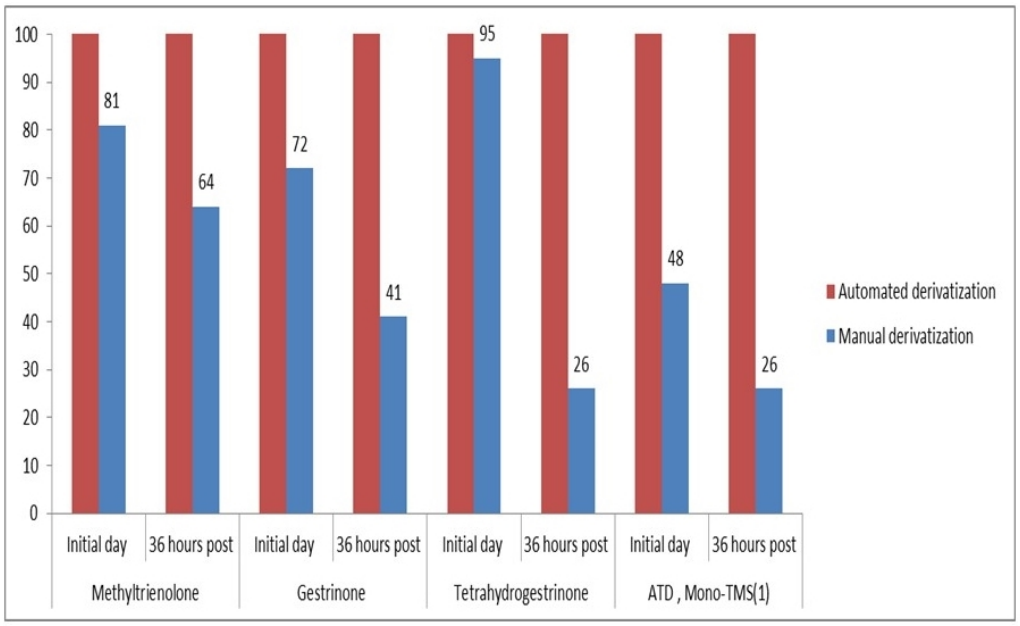

Figure 2: Normalized results at MRPL levels.

Conclusions

Because of the workload of the laboratories, large batches of samples are prepared for analysis daily. Taking into account that the run time analysis is over 25-30 minutes, this means that the preparation process does not affect the samples the same, and some compounds are strongly affected like 4,9,11-trienes and compounds of similar structure. GC-MS/MS with a triple quadruple instrument, is highly selective and sensitive in the MRM mode, coupled to a sample preparation module, which derivatizes and injects sequentially, this avoids downtimes, helps to handle batches containing more than 30 samples, ensures that the treatment of the samples in each batch is exactly the same and achieves the detection limits for doping control requirements for multiple conjugated 3-ketosteroids and improving or holding the derivatization yield for the other anabolic steroids.

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