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High sensitive analysis of steroids in doping control using gas chromatography/time-of-flight mass-spectrometry

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The method of high sensitive gas chromatographic/time-of-flight mass-spectrometric (GC/TOF-MS) analysis of steroids was developed. Low-resolution TOF-MS instrument (with fast spectral acquisition rate) was used. This method is based on the formation of the silyl derivatives of steroids; exchange of the reagent mixture (pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)) for tert-butylmethylether; offline large sample volume injection of this solution based on sorption concentration of the respective derivatives from the vapour-gas mixture flow formed from the solution and inert gas flows; and entire analytes solvent-free concentrate transfer into the injector of the gas chromatograph. Detection limits for 100 μl sample solution volume were 0.5–2 pg/μl (depending on the component). Application of TOF-MS model 'TruTOF' (Leco, St Joseph, MO, USA) coupled with gas chromatograph and ChromaTOF software (Leco, St Joseph, MO, USA) allowed extraction of the full mass spectra and resolving coeluted peaks. Due to use of the proposed method (10 μl sample aliquot) and GC/TOF-MS, two times more steroid-like compounds were registered in the urine extract in comparison with the injection of 1 μl of the same sample solution.

Keywords: gas chromatography; low-resolution time-of-flight mass-spectrometry; offline large sample volume injection; doping control; steroids

Introduction

A lot of anabolic androgenic steroids and their metabolites are prohibited by the World Anti-Doping Agency (WADA).[1] At present, the number of steroids used by athletes has increased and new substances are constantly being developed (designer steroids) that are difficult to detect.

Gas chromatography/mass-spectrometry (GC/MS) with quadrupole mass analyzer operated in selected ion monitoring (SIM) mode is the main analytical technique used for the routine screening of urine samples (for steroids) in doping control today. However possibilities of retrospective analysis and detection of designer steroids are restricted because target detection of a limited number of known compounds is performed and only a few selected ions are registered in the SIM mode.

Some recent papers dealt with methods which can be used for detecting designer steroids. Georgakopoulos *et al*.[2] used a high resolution time-of-flight mass-spectrometry (TOF-MS) combined with a high performance liquid chromatography (HPLC) and GC. Nielen *et al*.[3] used HPLC with androgen bioassay and quadrupole-TOF-MS detection. Silva *et al*.[4] discussed the applicability of the two-dimensional GC with a low resolution TOF-MS (with fast spectral acquisition rate) in doping control and possibility of application of this technique for detection of the new drugs and their metabolites. Additional opportunities were shown by Virus *et al*.[5], who used HPLC coupled to orbitrap mass spectrometry.

Moreover, standard procedure[6] consists of carrying out the derivatization reaction of the steroid concentrate with 50 μl of silylating reagent (usually *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)) and the analysis of only a small part of the reaction mixture. To decrease the limits of detection (LOD) and contamination of the instrument due to the silylating reagent, it is desirable to exchange this reagent for an inert solvent. We decided to investigate such opportunity using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as reagent. Only some authors have used BSTFA and fulfilled its exchange for such solvent after the reaction was complete[7,8] but only 1 μl of the solution was analyzed.

Registration of athlete steroid profiles is very important today. It is possible to suggest that it would be more reliable if more steroid-like compounds were registered. Decreasing the steroid detection limits is also important for the prevention of doping use far before the competition takes place. Therefore, new approaches are required for a high sensitive analysis of target compounds, identification of designer steroids, and investigation of steroids profiles for athletes.

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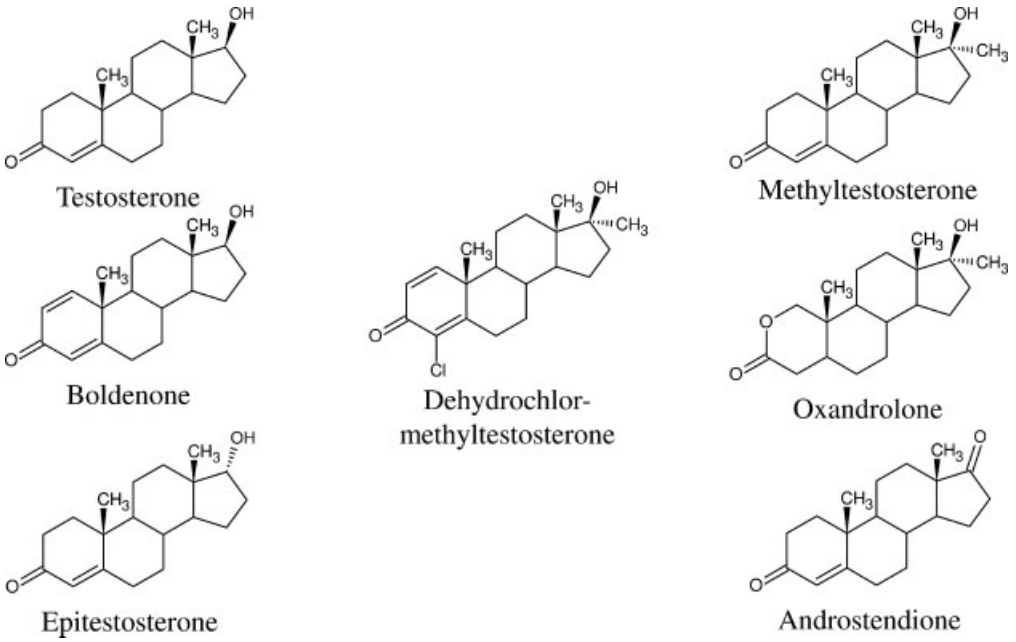

Figure 1. The structures of the studied steroids.

In our preliminary publication[9] we have shown the possibility of the reagent exchange in regard to three steroids for a highly volatile inert solvent – *tert*-butylmethylether (TBME), offline large sample injection into gas chromatograph, and GC/MS analysis of the entire analyte concentrate. The mass-spectrometer with quadrupole analyzer was used in that case.

The goal of this work was to investigate the possibilities to decrease the concentration detection limits for the set of steroids, to increase the number of registered steroid-like compounds in urine samples, and to increase their identification reliability. In order to solve above-mentioned problems we have investigated possibility of the reagent mixture exchange for volatile inert solvent (TBME), offline large sample injection of the analytes solution, and GC/low-resolution TOF-MS (with fast spectral acquisition rate) analysis of the entire analytes derivatives concentrate.

Experimental

Reagents and chemicals

Methanol and TBME were HPLC-grade from Scharlau (Barcelona, Spain). Pyridine was purchased from Aldrich (Milwaukee, WI, USA). Testosterone, epitestosterone, methyltestosterone, dehydrochlormethyltestosterone, boldenone, oxandrolone, and androstendione were supplied from LGC Standards (formerly LGC Promochem, Wesel, Germany). The β-glucuronidase preparation (from *Escherichia coli*) was purchased from Roche (Mannheim, Germany). Analytical grade potassium carbonate, sodium hydrogen carbonate, and diethyl ether were obtained from Merck (Darmstadt, Germany). The derivatization reagent BSTFA with 1% trimethylchlorosilane was bought from Regis Technologies (Morton Grove, IL, USA).

Standard solutions

Stock solutions of the respective steroids were prepared in methanol at 10 and 100 ng μl^{-1}. Working solutions and model mixtures of steroids were prepared from the stock solutions and stored in the dark at 4 °C.

Concentration of steroids derivatives in the model mixture was selected so that the intensities of corresponding peaks (on mass chromatogram) were approximately equal.

The structures of the studied steroids are shown in Figure 1.

Sample pretreatment

The extraction and purification of urine samples was carried out in accordance with the typical procedure used in the doping control.[5] Briefly, enzymatic hydrolysis of 3 ml urine was performed by adding β-glucuronidase from *E. Coli* (pH 7.0, 1 h, 57 °C). Then 100 mg of potassium hydrocarbonate and potassium carbonate (2 : 1) was added. The steroids were extracted with 5 ml of diethyl ether. After centrifugation, the organic layer was separated and evaporated to dryness. After that 50 µL of methanol was added to the dried residue and the solution was delivered from Moscow Anti-Doping Center to the Chemistry Department of MSU.

Derivatization

Before derivatization, the methanol solution of urine extract and respective target steroids was evaporated to dryness. The dried residue was derivatized using 20 µL pyridine and 40 µL BSTFA mixture for 1 h at 80 °C. The reaction mixture was again dried under nitrogen flow and 50–250 µL of TBME were added in case of a model solution (depending on required concentration of final solution) and 50 µL of TBME for the real urine sample. In case of large sample injection of model mixtures solutions, additional dilution was performed.

GC-MS

GC-MS analysis was carried out using Leco 'TruTOF' mass-spectrometer (Leco, St Joseph, MO, USA), coupled with Agilent

'7890A' gas chromatograph (Agilent Technologies, Palo Alto, CA, USA).

Compounds were separated on a fused-silica capillary column (Restek RTX-1 ms, 30 m × 0.32 mm × 0.25 μm). Splitless/split injection mode (splitless time 30s) and offline large sample injection (which will be discussed below) was used. The sample volume of organic solution directly injected into the GC was 1 μl. The column oven temperature was maintained at 40 °C for 1 min after injection then programmed at 50 °C min^{-1} rate to 177 °C (1 min hold) then programmed at 5 °C min^{-1} rate to 250 (0 min) and programmed at 10 °C min^{-1} rate to 320 (0 min). Injector temperature was 280 °C. Temperature of transfer line was 300 °C. Helium (99.9999%) was used as carrier gas at a flow rate of 1.5 ml min^{-1}.

Electron ionization mode was used. Ion source temperature was 250 °C. Electron energy was 70 eV. Mass spectra were scanned over the range m/z = 50–700 a.m.u., acquisition rate was 20 spectra s^{-1}.

ChromaTOF software v. 4.20 from Leco (St Joseph, MO, USA) was used for automatic processing of GC/MS data.

Offline large sample volume injection

Method of offline large sample volume injection was developed by us in general and its comprehensive description can be found in the literature.[9–11] It is based on sorption concentration from gas-vapour mixture flow formed due to mixing of organic solution (extract) and inert gas flows in a quartz capillary (quartz liner) containing small quantity of the sorbent (quartz wool). The concentration is carried out outside of the analytical system (offline). The flow rates of the organic solution and inert gas were chosen to be sufficient to ensure formation of gas-vapour mixture in it and to exclude liquid drops formation at the needle outlet. Organic solution flow was formed using manual injection. Optimum flow rates of organic TBME solution of steroid derivatives and inert gas flow were chosen by our research. Sample volume of TBME solution in case of the real urine sample was 10 μl and for model mixture −100 μl. The quartz liner is put into a stainless steel cartridge which can be connected by the needle to the GC injector. Entire analytes concentrate is transferred into GC injector due to thermal desorption in a helium flow. Optimal conditions of thermal desorption (temperature, helium flow rate and time) were chosen by our experiments.

Table 1. LOD for studied steroids derivatives registered for injection of 1 μl organic solution in splitless/split mode and for offline large sample injection (100 μl)

Investigated substance	LOD, pg/μl (splitless/split injection of 1 μl)	LOD, pg/μl (offline large sample injection of 100 μl)
TMS-epitestosterone	200	1
TMS-testosterone	200	2
TMS-methyltestosterone	60	0.5
TMS-dehydrochlormethyltestosterone	200	1
TMS-boldenone	100	1
TMS-oxandrolone	60	0.5
Androstendione	300	2

* LODs were calculated for S/N = 5 from respective mass chromatograms.

Results and Discussion

In the beginning of our work we have investigated the possibility to exchange the reagent mixture used for the derivatization reaction for an inert highly volatile solvent (TBME), and the stability of the investigated steroid derivatives in TBME solution over time. As the result the experiment has proved the possibility of such an exchange and that the respective derivatives were stable in the solution for at least 5 days.

The solution of this problem has allowed to us to investigate the optimal conditions necessary for the offline large sample volume injection of seven steroid derivatives into GS/TOF-MS. This investigation has included optimization of thermal desorption conditions as well sorption conditions for isolation of these derivatives from vapour-gas flow formed from organic extract and inert gas flows.

Thermal desorption was investigated by applying 1 μl of model mixture of steroid derivatives on a quartz wool of the liner.

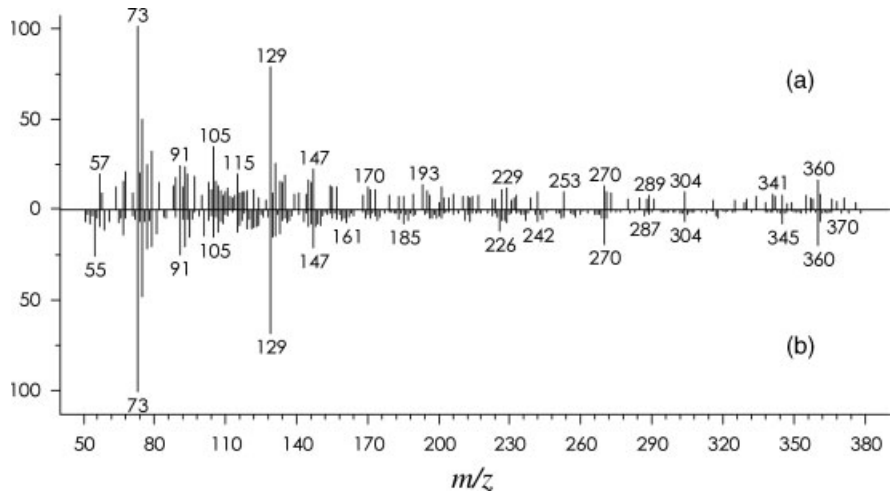

Figure 2. Mass spectrum of TMS-testosterone registered for LOD 0.2 ng/μl (a) and for 4 ng/μl (b). Splitless/split injection mode was used.

Optimal conditions included: temperature −250 °C; helium flow rate −5.0 ml min^{-1}; time of thermal desorption −10 min.

It was shown that optimal conditions for such concentration of steroid derivatives from 100 µl of their solution in TBME were: inert gas flow rate −60 ml min^{-1}; solution flow rate −50 µl min^{-1}.

For determination of completeness of concentration of model steroid derivatives from TBME solution, we have compared respective derivatives peak areas registered for 100 µl and 1 µl of sample aliquots (concentration of the analytes in the former solution was one hundred times less than in the latter one). It meant that the analytes transfer into GC/MS in case of the offline large sample volume injection was quantitative when respective peak areas were approximately equal.

Our research resulted in the development of a new high sensitive method for the set of seven steroids analysis in organic solution. It was based on the following processes: evaporation of the solution to dryness, derivatization, exchange of reagent mixture for the inert volatile organic solution, the analytes isolation due to sorption from vapour/inert gas mixture formed from the organic solution and inert gas flows, transfer of the entire analyte concentrate using thermal desorption into GC injector, and GC/TOF-MS analysis of this concentrate.

Within the framework of the experiment, concentration LOD was defined as the concentration of the analyte for which signal to noise ratio was equal to 5. LODs for all investigated compounds are presented in the Table 1. As it is seen from Table 1, offline large sample injection (100 µl) allows to decrease the LOD of respective steroid derivatives about 100 times compared to standard splitless/split injection of 1 µl. LODs were calculated using mass chromatograms for the most abundant peak of the respective steroid derivatives mass spectra. Full mass spectra were obtained for the concentration corresponding to LOD that extended applicability of TOF-MS for doping control as full mass spectra were registered for the analyte traces. For example, mass spectra of testosterone TMS-derivative registered for LOD (0.2 ng µl^{-1}) and for 4 ng µl^{-1} in case of standard splitless/split injection are presented in Figure 2.

The last step of our research was the investigation of the number and composition of steroid-like compounds in a real urine sample registered using GC/TOF-MS when 1 µl and 10 µl of TBME solution were injected. In case of 1 µl sample, 20 steroid-like compounds were registered in the urine sample using Leco ChromaTOF software (Table 2; St Joseph, MO, USA). The offline large sample volume injection (10 µl) allowed registering in addition 20 steroid-like compounds. The identification was carried out using NIST 05 mass spectra library (reversed match more than 700). Some components were identified using NIST library as the same compounds. Our results are preliminary. We are keeping in mind for the future to identify these compounds more reliably using standard solutions of the supposed compounds for registration respective retention times and full mass spectra. We don't have respective standard solutions just now. The minimal concentrations (calculated using peak area) of registered steroid-like compounds in urine were about 4 ng/ml in the case of injection of 1 µl and about 0.5 ng/ml in the case of offline large sample injection (10 µl).

Table 2. The steroid-like compounds registered in urine sample using GC/TOF-MS, ChromaTOF software and NIST mass spectra library

R_t**, min	Name of substances identified using NIST library***
13,41*	5-androstene-3β,17β-diol, bis TMS
13,69	5α-androst-16-en-3β-ol, TMS
14,40*	5α-androst-16-en-3β-ol, TMS
14,84*	5α-androst-16-en-3β-ol, TMS
15,78*	5-androstene-3β,17β-diol, bis TMS
17,00*	5β-androstan-3α,17β-diol, bis TMS
17,03*	5α-androstan-17-one-3α-ol, TMS
17,30	5-androstene-17-one-3β-ol, TMS
17,77	5α-androstan-17-one-3α-ol, TMS
17,81*	Androstan-3α-ol-17-one
18,00	5β-androstan-17-one-3β-ol, TMS
18,19*	Androstan-3,17-dione
18,40*	5β-androstan-11,17-dione-3α-ol
18,70*	5-androstene-17-one-3β-ol, TMS
18,88	5β-androstan-3α,17β-diol, bis TMS
19,08	5β-androstan-11,17-dione-3α-ol, TMS
19,22	5-androstene-3β,17β-diol, bis TMS
19,40	4-androstene-3-one-17α-ol, TMS
19,63*	4-androstene-3-one-17α-ol, TMS
19,83	5-androstene-17-one-3β,16α-diol, bis TMS
20,17	5α-androstan-17-one-11β-hydroxy-3α-(trimethylsilyloxy)
20,20	4-androstene-3-one-17β-ol, TMS
20,65	5α-androstan-17-one-3α, 11β-diol, bis TMS
20,86	5β-androstan-17-one-3α, 11β-diol, bis TMS
21,03*	5β-androstan-3α,16α-diol, bis TMS
21,83*	5-androstene-3β,16α,17α-triol, tris TMS
21,95*	5α-pregnan-20-one-3α,16α-diol, bis TMS
22,64	5-pregnene-3β,16α,20α-triol, tris TMS
22,98*	5β-pregnan-17-ol, 3α,20-bis(trimethylsilyloxy)
23,20	5β-pregnan-17-ol, 3α,20-bis(trimethylsilyloxy)
23,76	4-pregnene-17-ol, 3β,20-bis(trimethylsilyloxy)
24,34	5-pregnene-20-one, 3β,21-bis(trimethylsilyloxy)
24,71*	5β-pregnan-11-one-3α,20,21-triol, tris TMS
24,75	Cholesterol, TMS
25,00*	5β-pregnan-11-one-17α-hydroxy-3α,20-bis(trimethylsilyloxy)
25,43	5β-pregnan-11-one-17α-hydroxy-3α,20-bis(trimethylsilyloxy)
25,60	5β-pregnan-11-one-3α,17α,20-triol, tris TMS
26,10*	β-sitosterol, TMS
26,18*	Stigmasta-5,24(28)-dien-3β-ol, TMS
26,30*	4-pregnene-3,11-dione-17β,20,21-triol, tris TMS

* these components were registered only in the case of offline large sample injection (10 µl).
** retention times are presented for offline large sample injection (10 µl).
*** some different components were identified using NIST library as the same substances; respective retention times (R_t) of which are the following: (13,41; 15,78; 19,22); (13,69; 14,40;14,84); (17,00; 18,88); (17,03; 17,77); (17,30; 18,70); (19,40; 19,63); (22,98; 23,20); (25,00; 25,43).

Conclusions

The research resulted in the development of high sensitive method for steroid derivatives analysis. The method was based on reagent mixture exchange for volatile solvent (TBME), isolation of analytes derivatives from large organic solution sample (10 or 100 µl) after formation of vapour-gas mixture from organic solution and inert gas flows due to sorption from this mixture and GC/TOF-MS entire analytes concentrate analysis. The possibility to decrease concentration LOD about 100 times was demonstrated within the experiment.

Application of this new method to urine analysis (organic solution sample, 10 µl) has allowed registering two times more steroid-like compound derivatives in comparison with splitless/split analysis of 1 µl of the same sample solution.

Offline large sample injection means that sample preparation is performed outside an instrument and only analytes are transferred into the gas chromatograph (without reagent mixture and solvent). It should be noted that this approach can be used to carry out routine analysis (retrospective analysis is possible) and find out new unknown doping compounds (so-called designer drugs). It is more reliable due to full mass spectra are registered when GC/TOF-MS is used and more components are registered due to the algorithm of automatic peak finding and extracting pure mass spectra.

The method can be useful for confirmation of the results of the doping control received on preliminary (screening) stage due to use of full scan mass spectra for ng-pg quantities of the analytes and recognition of them on the level less than required by WADA.

Acknowledgement

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