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Improvement in steroid screening for doping control with special emphasis on stanozolol

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Abstract

The Medical Commission of the International Olympic Committee forbids the use of anabolic androgenic steroids and β_2-agonists to improve athletic performance. In this work we have selected examples of anabolic androgenic compounds and their metabolites to evaluate the GC–MS analysis of some trimethylsilyl derivatives. The aim is to set the best GC conditions to improve the detection within the whole range of analyte elution temperatures. The initial column temperature was changed to 105 or 140 °C followed by 40 °C min^{-1} to 200 °C and then 15 °C min^{-1} to 300 °C. Using 140 °C as the initial oven temperature it was possible to obtain narrower initial analyte distributions for the compounds that elutes at the beginning of the chromatogram as clenbuterol, mabuterol, epimethylenediol and norandrosterone, without loss of derivatized metabolites signal. Later, eluting analytes, such as the stanozolol metabolites, furazabol and oxandrolone were not affected. Temperatures below 140 °C, resulted in partial derivatization for some analytes mainly stanozolol related structures. Therefore evaluation of derivatization conditions as occurring in three steps, the vial, vaporization chamber and capillary column, was thoroughly assessed. The new program temperature improves the signal-to-noise ratio for some compounds and shows adequate resolution for endogenous compounds. Some of the difficult key separations necessary for doping control enforcement were also obtained with the proposed method.

Keywords: Doping analysis; Steroids; Stanozolol

1. Introduction

The Medical Commission of the International Olympic Committee (IOC) bans the use of anabolic androgenic steroids and β_2-agonists to improve athletic performance. In 1998, the IOC stated that each IOC laboratory should confirm the presence of five ''key'' anabolic agents at a concentration level of at least 2 ng ml^{-1}. Since that time, there has been an increasing interest in application of advanced detection techniques such as GC–MS–MS and GC–high-resolution (HR) MS for the confirmation of these substances. At present, GC–MS still remains a good choice for the detection of the misuse of anabolic steroids in athletes and animals conducted on an urine sample [1]. Following isolation, steroids are derivatized to yield trimethylsilyl (TMS) derivatives, which are suited for GC–MS analysis.

Most often, the screening procedure is performed

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using MSTFA–NH_4I–ethanetiol (MSTFA=*N*-methyl-*N*-trimethylsilyl trifluoroacetamide) to derivatize the anabolic compounds at 60 °C during 20 min after their isolation from urine. This time is accepted as being enough to complete derivatization of hydroxy and keto groups of anabolic androgenic steroids using a mixture of MSTFA, ammonium iodide and a reducing agent [2]. The TMS derivatives obtained are injected into the column at 180 °C, then programmed at 3 °C min^{-1} to 240 °C and 40 °C min^{-1} to 300 °C. One of the most difficult anabolic to derivatize is stanozolol (Stan; 17α-methyl-17β-hydroxy-5α-androst-2-ene[3, 2-*c*]pyrazole, Fig. 1), first synthesized by Clinton et al. in 1959 [3]. Stan is a relatively old drug (US patent granted in 1962) [4]. Banned by the IOC since 1974, this androgenic has often been abused in sport by athletes [5,6]. It has also been used as a growth promoter in cattle, but as all anabolic steroids it was banned within the European Union [7]. The metabolism of Stan in man was investigated by Schanzer et al. [8] and Ward et al. [9] and others [10,11] and 11 metabolites were characterized. The most relevant for doping control being 3′OH-Stan. In relation to metabolism in animals, 16β-OH-Stan is the main metabolite in horses [12,13] and cattle [14,15].

The stability of Stan and its metabolites are of great concern [6,8,15,16] and as the performance of the GC column diminishes, for example, so does the response of 3′OH-Stan [17]. From the best of our knowledge no systematic research has been successful to establish the factors responsible for Stan's poor chromatographic behavior. Also lacking in usual procedures was the focusing of early eluting anabolics (e.g. mabuterol, clenbuterol), resulting in dramatic reduction of sensitivity [18]. Other low concentration target analytes norandrosterone, epimethendiol and unstable molecules (oxandrolone) are also of concern. Therefore GC–MS analysis of some TMS derivatives of selected anabolic androgenic compounds and their metabolites (Fig. 1) were thoroughly studied. These compounds were selected taking into account their structural characteristics and the fact that most of them, according to IOC legislation, must be detected at ppb levels.

2. Experimental

All procedures were performed under strict quality control guidelines; the laboratory is accredited by ISO.17025.

2.1. Reagents, chemicals and solutions

The following substances: stanozolol, 3′OH-

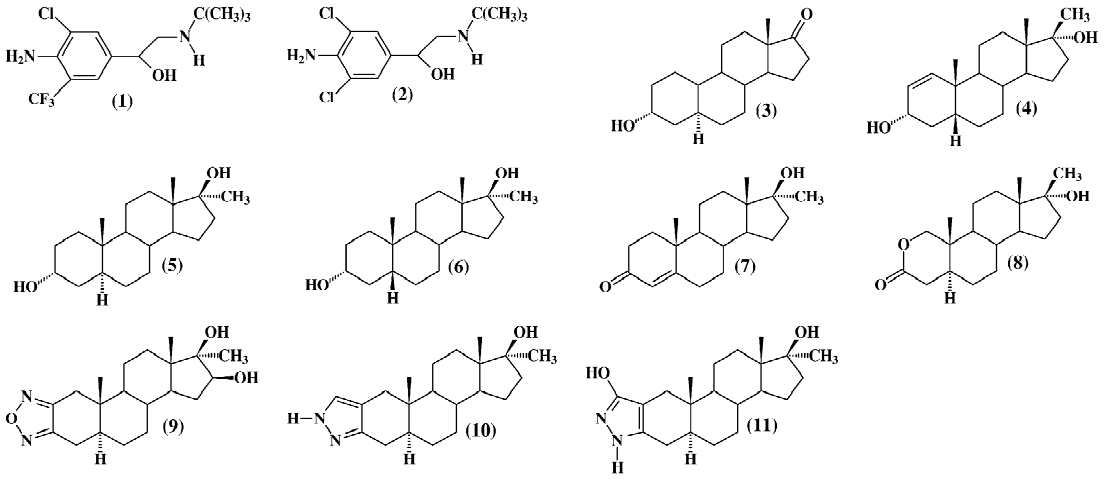

Fig. 1. Structure of the anabolic compounds considered in the GC–MS study. (**1**) Mabuterol, (**2**) clenbuterol, (**3**) norandrosterone, (**4**) epimethendiol, (**5**) methylltestosterone-M1, (**6**) methylltestosterone-M2, (**7**) methyltestosterone, (**8**) oxandrolone, (**9**) 16β-OH-furazabol, (**10**), stanozolol and (**11**) 3′OH-stanozolol. The same numbering was used for the fully derivatized analytes.

stanozolol, mabuterol, clenbuterol, norandrosterone, epimethendiol, 16β-OH-furazabol (16β,17β-dihydroxy-17α-methyl-5α-androstane[2,3-*c*]furan main metabolite, M1), oxandrolone, methyltestosterone metabolites (17α-methyl-5α-androstane-3α,17β-diol, M1 and 17α-methyl-5β-androstane-3α,17β-diol, M2) were a kind of gift from Dr W. Schanzer and H. Geyer from the Institute of Biochemistry, Germany Sports University, Cologne, Germany. Methyltestosterone as an internal standard was bought from Aldrich (Milwaukee, WI, USA). All reagents were analytical grade. MSTFA was purchased from Chem Fabrik (Waldstetten, Germany). NH_4I and ethanethiol from Sigma (St. Louis, MO, USA). Methanol from Tedia (Fairfield, NJ, USA). Stock solutions were prepared in methanol at a concentration of 1000 ng μl^{-1}. These solutions were further diluted to yield appropriate working solutions for the preparation of the calibration standard. The solutions were sealed and frozen at −20 °C until use. Methyltestosterone was used as an internal standard (I.S.), dissolved in methanol at 1000 ng μl^{-1} and diluted to 10 ng μl^{-1}. The sample was prepared once for each derivatization temperature tested and injected into GC–MS twice.

2.2. Derivatization prior to GC–MS analysis

Previous to GC–MS, N,O-TMS and O-TMS derivatives were formed. A 100-μl of 10 ng μl^{-1} of a mixture of the working solution of anabolic compounds and 100 μl of the working solution of I.S. were transferred to a 10-ml glass test tube. The solvent (methanol) was evaporated at 40 °C under a stream of nitrogen (N_2). The dried residues were kept inside desiccators containing P_2O_5–KOH during 20 min. The residue was then dissolved in 100 μl of MSTFA–NH_4I–ethanethiol (1000:2:6, v/w/v) and heated either at 60 or 115 °C.

2.3. Equipment and conditions

A Hewlett-Packard (Palo Alto, CA, USA) gas chromatograph (6890 series) interfaced with a mass-selective detector (5973 series) equipped with a 7673 HP auto-sampler was used. The carrier gas was helium at 1 ml min^{-1}. A HP-1 capillary column (100% methylsiloxane, 17 m×0.20 mm I.D., film thickness 0.11 μm) was used. The injector temperature was 280 °C. Injection mode: splitless (valve closed for 0.80 min). Injection volumes of 1 or 3 μl; constant flow 1 ml min^{-1}. The GC temperature programming rates were as follows: initial column oven temperatures were 105 or 140 °C (held 1 min) then programmed to rise from this initial value up to 200 °C at 40 °C min^{-1}, then to 300 °C at 15 °C min^{-1} (held 3 min). Mass spectrometer operating conditions were as follows: ion source temperature 220 °C; interface temperature: 280 °C; quadrupole temperature: 150 °C; accelerating voltage: 2000 eV; and ionization voltage: 70 eV. Mass spectra were obtained in the scan mode (M_r 50–750). The selected ion monitoring (SIM) mode was employed for analysis of urine. Three diagnostic ions were monitored for each analyte as it is done in our routine procedure. The dwell time was 20 ms.

The transfer line was at 280 °C. A split/splitless deactivated glass single-taper liner from HP (79×7 mm I.D.) (cup 6 mm length×1 mm hole) and an internal volume of 0.9 ml were used. Inside the liner 0.017 mg of deactivated glass wool were well compacted between 23 and 33 mm measured from its top. A retention gap (fused-silica, 3 m×0.3 mm I.D.) was used. The gap was deactivated by flushing with 1,3-diphenyl-1,1,3,3-tetramethyldisilazane (DPTMDS) then heating from 40 °C at 4 °C min^{-1} to 170 °C (held 15 min). The tubing was then rinsed with diethylether followed by methanol. The connection was made by a press-fit connector.

3. Results and discussion

Most often, literature screening procedures have been performed using MSTFA–NH_4I–ethanethiol to derivatize the anabolic compounds at 60 °C during 20 min [2]. The derivatives obtained are injected into GC–MS at 180 °C initial oven temperature (Fig. 2c). In these conditions, the aging of the column is said to reduce the chromatographic behaviour for some compounds, for example, 3′OH-Stan [9,17,7]. Also, for compounds such as mabuterol, terbutaline, salbutamol and clenbuterol that elutes at the beginning of the chromatogram is poor, with strong broadening lowering their S/N ratio, for example for clenbuterol,

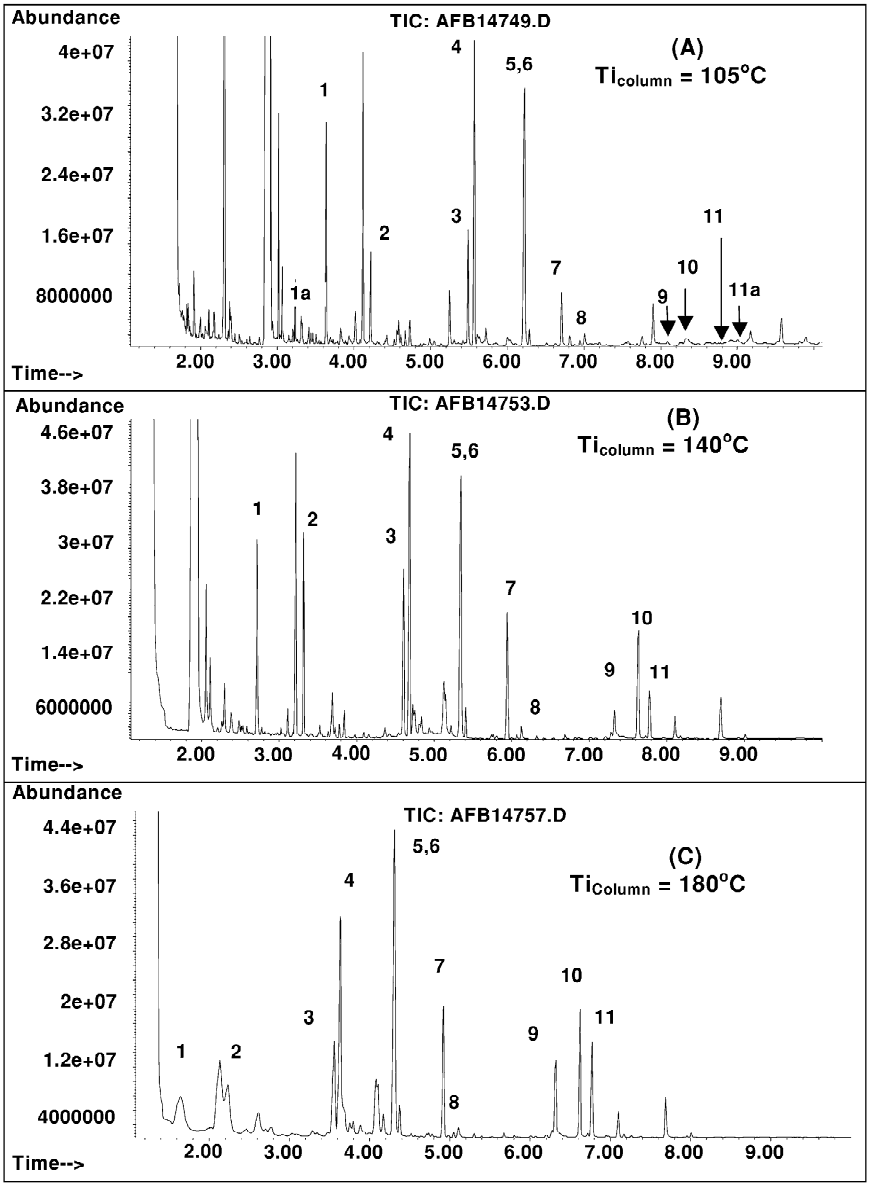

Fig. 2. GC–MS chromatogram. Temperature program from (a) 105 °C, 40 °C min^{-1} to 200 °C and 15 °C min^{-1} to 300 (held 3 min), (b) 140 °C, 40 °C min^{-1} to 200 °C and 15 °C min^{-1} to 300 (held 3 min) and (c) 180 °C, 40 °C min^{-1} to 200 °C and 15 °C min^{-1} to 300 (held 3 min). (**1**) Mabuterol, N-TMS,O-TMS; (**1a**) mabuterol-OTMS; (**2**) clenbuterol, N-TMS,O-TMS, (**3**) norandrosterone-bis-TMS, (**4**) epimethendiol-bis-TMS, (**5,6**) methyltestosterone-M1 and M2-bis-TMS, (**7**) methyltestosterone-bis-TMS (I.S.), (**8**) oxandrolone, (**9**) stanozolol-bis-TMS, (**10**) 16-β-OH-furazabol-bis-TMS, (**11**) 3′OH-Stan-tris-TMS, (**11a**) 3′OH-Stan-bis-TMS.

in our screening, the signal/noise ratio is 3 for the m/z 337 ion at the level of 2 ng ml^{-1}. Recently, the IOC stated that each IOC laboratory should confirm the presence of some key compounds at a concentration level of at least 2 ng ml^{-1} [1]. Therefore, improvements of the GC–low-resolution (LR) MS

conditions would extend the application of this low cost technique to encompass all anabolic residues considered for screening purposes.

In order to improve the detection of the analytes with the aim to increase the signal/noise ratio to obtain lower detection limits, the initial column oven temperature was set below (105 °C) the boiling point of MSTFA (130 °C). Theoretically, this would enhance sample transfer to the capillary column and especially would produce narrower initial analyte distributions (greater S/N), because of their more efficient cold trapping or solvent effect [17].

Using the classical derivatization with MSTFA–NH_4I–ethanethiol [8,19,16] at 60 °C/20 min and injecting the sample at initial column temperature of 105 °C, followed by 40 °C min^{-1} to 200 °C then to

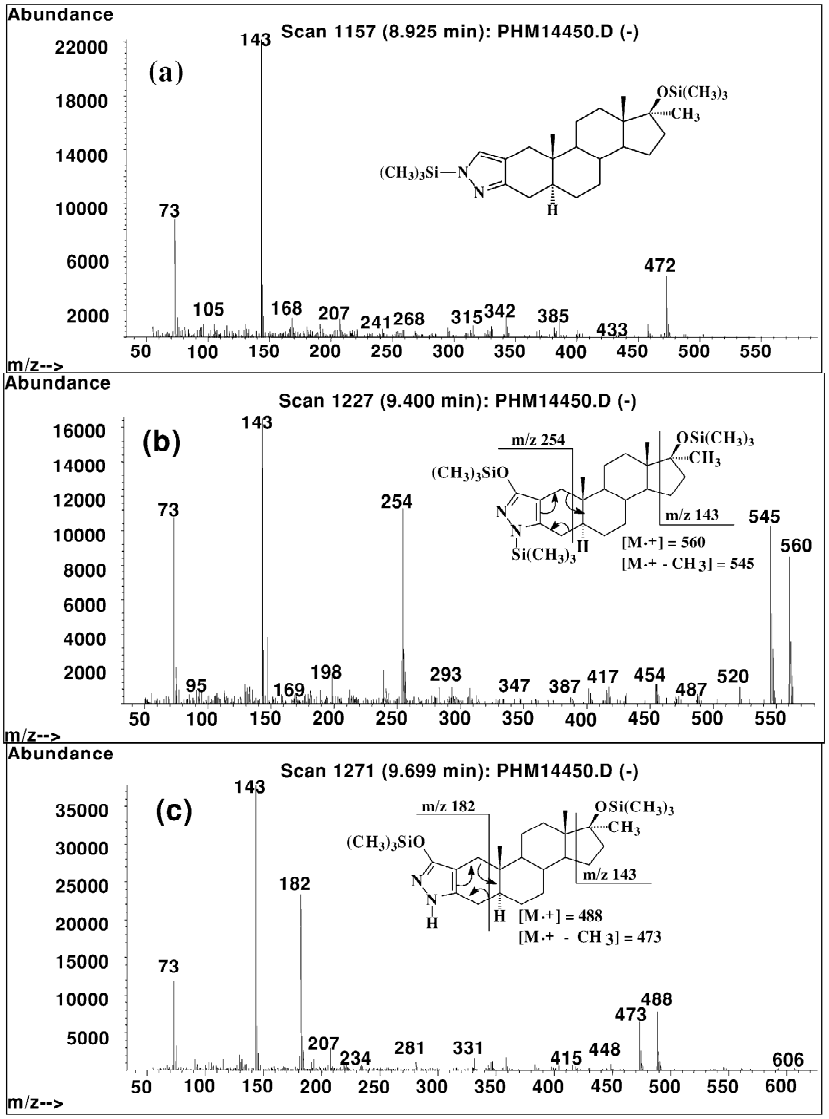

Fig. 3. (a) spectrum of Stan-bis-TMS (**10**), (b) spectrum of 3′OH-Stan-N-TMS,O-bis-TMS (**11**), and c) 3′OH-Stan-N-TMS,O-TMS (**11a**). Temperature program from 105 °C, 40 °C min^{-1} to 200 °C and 15 °C min^{-1} to 300 (held 3 min).

300 °C at 15 °C min^{-1} (held 3 min) some compounds, e.g. mabuterol, stan and their metabolites, were not 100% derivatized. Fig. 2a shows peaks corresponding to partially derivatized compounds, indicating that the derivatization reaction was not complete for these compounds under the conditions used (Figs. 3 and 4).

To test the influence of the derivatization temperature, the reaction mixture was incubated at 115 °C for 20 min. The derivatives were analyzed by GC–LRMS at 105 °C initial oven temperature. The results reported in Table 1 show that the areas of all analytes increase when the derivatization temperature goes from 60 to 115 °C for almost all substances (intermediate values were obtained for all other temperatures evaluated: 80, 100, 105 and 110 °C). The areas of clenbuterol-N-TMS,O-TMS (**2**) increased by 16%; norandrosterone-bis-TMS (**3**), 12%; epimethendiol-bis-TMS, 23% (**4**); methyltestosterone-M1 and M2, 22% (**5,6**); methyltestosterone-bis-TMS, 16% (**7**), furazabol-M1,bis-TMS, 20% (**9**) (see results on Table 1, second line). The exceptions were the smaller areas for oxandrolone (−35%) (**8**) and practically the same value for 3′OH-stan-tris-TMS (−1%) (**11**) (Table 1). The derivatization temperature had major effects on the area of mabuterol-N-TMS,O-TMS, Stan-P-bis-TMS and 3′OH-Stan-bis-TMS, they increased by 76, 66 and 42%, respectively (Table 1, second line). The derivatization behaviour of mabuterol could be explained by the conversion of mabuterol-O-TMS (**1a**) to mabuterol-N-TMS,O-TMS (**1**) at the higher derivatization temperature (115 °C, Figs. 2 and 4) (Table 1). For stanozolol-related structures, it can be advanced that per-derivatization of the remaining (not seen) parent compound and partially derivatized intermediates (only the 3′OH-Stan-bis-TMS (**11a**, Fig. 3) is seen on the chromatographic conditions

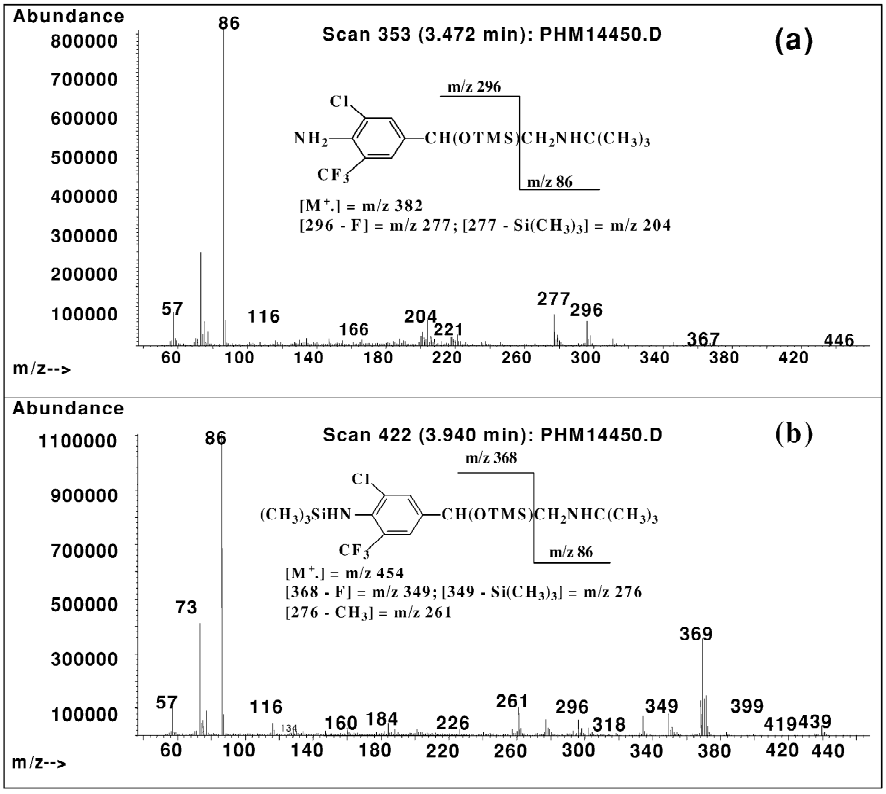

Fig. 4. (a) Spectrum of mabuterol-N-TMS,O-TMS (**1**) and (b) mabuterol-O-TMS (**1a**). Temperature program from 105 °C, 40 °C min^{-1} to 200 °C and 15 °C min^{-1} to 300 (held 3 min).

Table 1
Ratios (%) between the area of analytes[a] as TMS derivatives obtained for derivatization temperature of 115 °C over 60 °C

Oven temperature (°C) (splitless injection)	Derivatization temperature (°C)[c]	Mabuterol,N-TMS,O-TMS	Clenbuterol,N-TMS,O-TMS	Norand, bis-TMS	EMD,bis-TMS	Methyl-M1, M2, bis-TMS	I.S.,bis-TMS	Oxand, bis-TMS	Stan, bis-TMS	Furaz-M1, bis-TMS	3′OH-Stan, tris-TMS	3′OH-Stan, bis-TMS
105 (1 μl)[b]	115/60	76.45	16.16	12.24	23.14	21.67	16.43	−34.63	66.24	20.18	−1.16	41.76
140 (1 μl)[b]	115/60	47.02	18.55	13.30	18.05	16.44	14.58	−35.53	61.66	15.17	36.40	1.01
105 (3 μl)[b]	115/60	7.05	−5.74	−7.22	−2.26	2.22	−6.09	−28.67	101.45	−1.48	7.92	19.15
140 (3 μl)[b]	115/60	0.66	3.96	−2.63	−2.08	−0.52	−1.26	−24.50	28.98	2.24	20.86	43.01

[a] Norand, norandrosterone; EMD, epimethendiol; Methyl-M1, M2, methyltestosterone metabolites 1 and 2, I.S., methyltestosterone; oxand, oxandrolone; Stan, stanozolol; 16-β-OH-furaz, 16-β-OH-furazabol; 3′OH-Stan, 3′OH-stanozolol.
[b] Values in parenthesis correspond to the volume injected.
[c] Derivatization temperature.

Table 2
Ratios (%) between the area of analytes[a] as TMS derivatives obtained for the initial oven temperatures of 140 °C over 105 °C

Oven temperature (°C) (splitless injection)[b]	Derivatization temperature (°C)[c]	Mabuterol,N-TMS,O-TMS	Clenbuterol,N-TMS,O-TMS	Norand, bis-TMS	EMD, bis-TMS	Methyl-M1, M2, bis-TMS	I.S.,bis-TMS	Oxand, bis-TMS	Stan,bis-TMS	Furaz-M1, bis-TMS	3′OH-Stan, tris-TMS	3′OH-Stan, bis-TMS
140/105 (1 μl)	60	45.81	20.43	20.47	18.23	15.93	32.94	−3.37	948.48	10.21	1159.82	−83.24
	115	21.49	22.91	21.61	13.34	10.95	30.83	−4.70	919.63	5.61	1638.44	−87.94
140/105 (3 μl)	60	39.82	82.02	39.81	18.94	12.32	30.84	−7.82	3857.32	7.79	5011.44	−89.80
	115	31.47	100.75	46.74	19.17	9.31	37.57	−2.43	2433.69	11.86	5624.29	−87.76

[a] Norand, norandrosterone; EMD, epimethendiol; Methyl-M1, M2, methyltestosterone metabolites 1 and 2; I.S., methyltestosterone; oxand, oxandrolone; Stan, stanozolol; 16-β-OH-Furaz, 16-β-OH-Furazabol; 3′OH-Stan, 3′OH-stanozolol.
[b] Values in parenthesis are the volume injected.
[c] Derivatization temperature.

used) was also responsible for the enhancement of the 3′OH-Stan-bis-TMS (**11a**) intermediate as well as the fully derivatized 3′OH-Stan-tris-TMS (**11**) and Stan-bis-TMS (**10**). But, the presence of 3′OH-Stan-bis-TMS (**11a**) is indicative that further improvements in derivatization will result in higher areas for the targeted derivatives (see below).

Covey [20] commented that the column could have some influence on the derivatization of resorcyclic lactones (such as the anabolic agent zeranol). Therefore we figured out that maybe increasing the initial column temperature would increase the conversion of all analytes to the fully derivatized products. The same samples derivatized at 60 and 115 °C used to perform the experiments described above were then injected at an initial oven temperature of 140 °C (slightly above the b.p. of MSTFA, see above). Results are shown on Table 1 (third line) and Table 2. For both derivatization temperatures (60 and 115 °C), all compounds showed larger areas at 140 °C initial oven temperature (Table 2, second and third lines) than at 105 °C. Most values were larger by 5–50% (Table 2, second and third lines). Except for oxandrolone-bis-TMS (**8**) (approx. −3%), 3′OH-Stan-bis-TMS (**11a**) (approx. −80%) and the dramatic increase of other stanozolol related structures, such as Stan-bis-TMS (900%) and 3′OH-Stan-tris-TMS (**11**) (1000%) (Table 2, second and third lines).

The same behavior at 140 °C, as shown above for 105 °C, initial oven temperature, was seen between the areas resulting from the derivatization temperatures of 60 and 115 °C (Tables 1 and 2 and Fig. 2b). Actually, the area of mabuterol-N-TMS,O-TMS (**1**) increased a little bit less (47%, Table 1, third line) than before (76%) (Table 2, second line). The other exception, 3′OH-Stan-tris-TMS (**11**), instead of a constant value as for the 105 °C experiment (Table 1, second line), showed an increase in area (36%, Table 1, third line), while 3′OH-Stan-bis-TMS (**11a**) had a small area increase (1.01%, Table 1, third line). These initial results (Fig. 3b) lead to the conclusion, that Stan and its 3′OH metabolite are almost completely derivatized when the initial column oven temperature is at 140 °C. The area enhancement must be due to the presence of partially derivatized compounds that are being fully derivatized inside the column. On the other hand, for mabuterol (**1**) the most important factor is the temperature of derivatization, because at 115 °C mabuterol-O-TMS (**1a**) is almost completely transformed to mabuterol-N-TMS,O-TMS (**1**).

3.1. Volume of injection

To check the influence of the injection process and the initial oven temperature on the analytical results and possible problems of transferring the sample to the column, a higher volume of derivatized sample (3 μl) was injected. The derivatization temperatures tested were: 60 and 115 °C (Tables 1 and 2, 4th and 5th lines).

The sample derivatized at 115 and 60 °C analysed at 140 °C initial oven temperature, injecting 3 μl, showed that the areas of Stan-bis-TMS (**10**) and 3′OH-Stan-tris-TMS (**11**) were 2434 and 5600 percent higher than those observed at 105 °C. For the other compounds the areas were moderately increased by 7%, except for oxandrolone (−7%) and 3′OH-Stan-bis-TMS (−87%).

It was observed that the 3-μl injections showed no losses of the sample vapor produced inside the injection chamber as compared to the results of 1 μl. This result is due, firstly to the characteristic of the MSTFA (b.p. 130 °C and M_r 199). Considering MSTFA as an ideal gas, the volume occupied can be estimated ($PV = nRT$). It is 0.7 ml, compatible with the liner internal volume. The liner type used has a restriction at the top; this type of liner prevents the backflow of vapors from the vaporizing chamber and can reduce substantially the sample loss through the septum purge [17].

Injection with initial column temperature of 140 °C only increased the analysis time by 1 min in relation to the analysis at 180 °C (Figs. 2b,c). On the other hand the decrease in 40 °C is sufficient to promote the reconcentration of the initial band by solvent trapping. Mainly for the more volatile anabolic compounds studied, mabuterol and clenbuterol, this resulted in a dramatic reduction in width, respectively, 3.2 and 4.6 to 0.9 and 1.1 s (Figs. 2b,c). The width of mabuterol and clembuterol are 0.6 and 0.8 s, respectively, at initial column temperature of 105 °C (Fig. 2a).

The increase of quality mainly in the beginning of the chromatogram can be addressed by the number

of theoretical plates (n) of mabuterol and clenbuterol, that for the initial temperature at 180 °C are 360 and 1201 and with the reduction of the initial temperature for 140 °C increases dramatically to 45 307 and 67 698, respectively. But even medium retention time range analytes, e.g. norandrosterone, epimethendiol, showed sharper peaks

4. Real samples

4.1. Fortified sample

Real samples fortified at 2 ng ml^{-1} were prepared according to the method described by Geyer et al. [21] injecting into GC–MS using the initial column

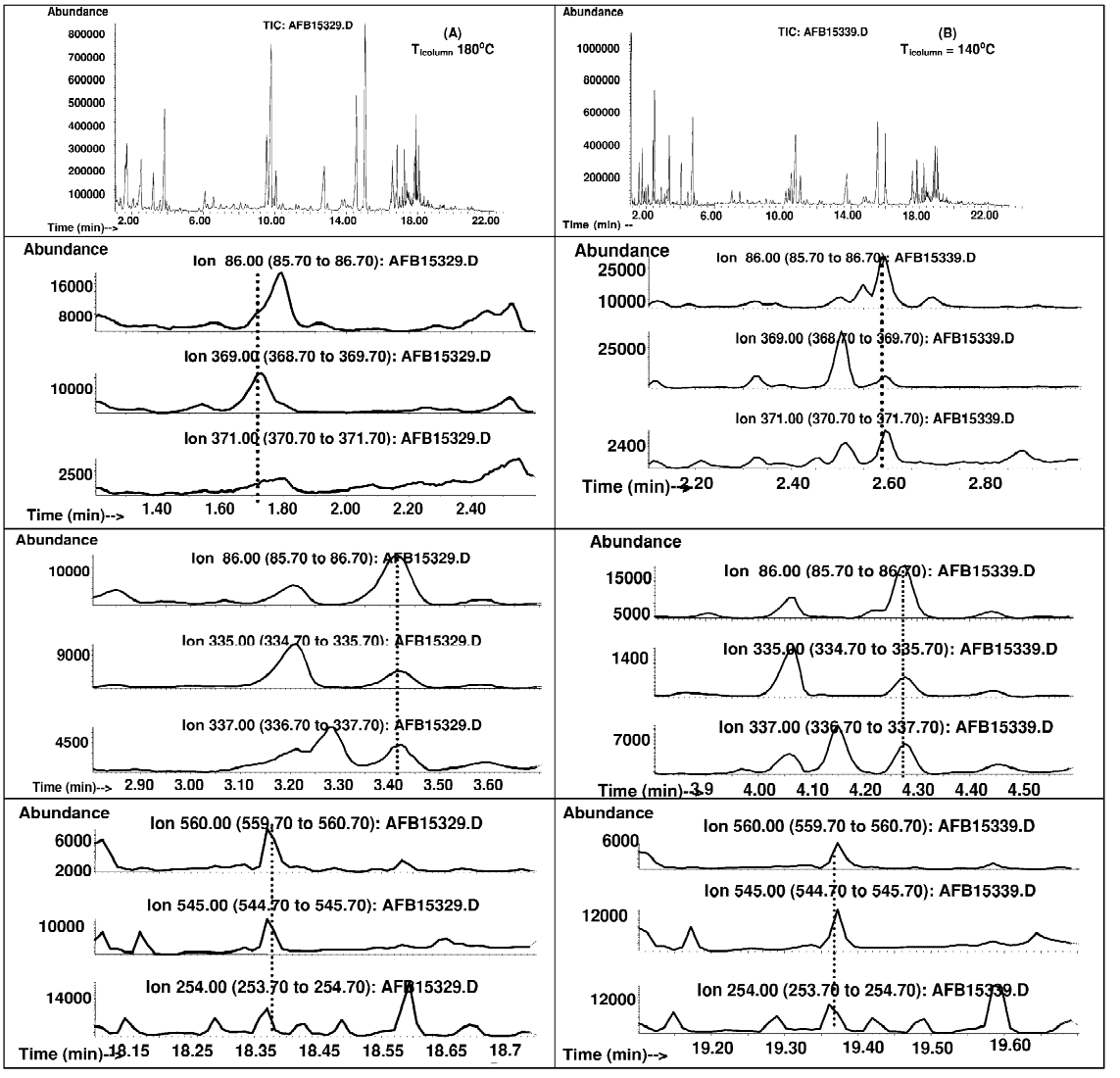

Fig. 5. GC–MS chromatogram of urine spiked with standards at 2 ng ml^{-1}. Temperature program from (a) 180 °C and 3 °C min^{-1} to 240, 40 °C min^{-1} to 300 (held 3 min). (b) 140 °C, 40 °C min^{-1} to 180 °C and 3 °C min^{-1} to 240, 40 °C min^{-1} to 300 (held 3 min). Splitless mode injection (5 m of retention gap is connected to the capillary column).

temperature of 140 °C followed by 40 °C min^{-1} to 180 °C, then to 240 °C at 3 °C min^{-1} (held 0 min), then rising 40 °C min^{-1} to 300 °C (held 3 min). The results showed that with this new program it was possible to obtain narrower initial analyte distributions for the compounds that elute at the beginning of the chromatogram as clenbuterol and mabuterol (Fig. 5). Therefore a better signal/noise ratio. Late eluting analytes, as the stanozolol metabolites, furazabol and oxandrolone were not affected. The S/N ratios for the main compounds observed at 140 °C initial oven temperature were 27, 42, 64, 44, 85, 102 and 31 for mabuterol, clenbuterol, norandrosterone, EMD, methyltestosterone-M1 and M2, 3′OH-Stan, respectively, and using the classical initial oven temperature of 180 °C the S/N ratio for the same compounds were 4, 7, 66, 42, 69, 44 and 26.

Table 3
Comparison of some resolution parameters obtained for some peculiar urines, in relation to experiment 1 ($T_{i\ column}$ = 180 °C) and experiment 2 ($T_{i\ column}$ = 140 °C)

Substance	Resolution	
	Experiment 1	Experiment 2
Androsterone Etiocolanolone	1.00	2.33
5a,3a-Diol 5b,3a-Diol	1.08	1.04
OHA OHE	1.33	2.00
DHEA Epiandrosterone	0.80	0.80
Epitestosterone Methylltestosterone-M2	0.00	0.20
OHA Noretandrolone	2.00	2.78
OHA Testosterone	2.11	5.30
Testosterone Epioxandrolone	0.93	0.58
Epitestosterone 5α-androstanedione	1.86	1.76
Metenolone DHEA	1.14	1.94
Methyltestosterone-M1 Methylltestosterone-M2	1.10	1.10
OHA Bolasterone	1.21	2.44
Norandrosterone EMD	1.57	4.90
TBOH Epitestosterone	1.13	1.34
Boldenone-M1 EMD	0.18	0.33
Fenoterol Protokilol	1.36	2.95

5a,3a-DIOL: 5α-androstan-3α,17β-diol; 5b,3b-DIOL: 5β-androstan-3α,17β-diol; OHA: 11β-OH-Androsterone; OHE: 11β-OH-Etiocholanolone; DHEA: Dehydroepiandrosterone; EMD: Epimethendiol; TBOH: Trenbolone.

4.2. Routine sample

To evaluate the influence of the new temperature program on the separation of endogenous compounds, a few tricky urine samples having high density, microbial presence, disturbing polar substances, vitamins and metabolites of some drugs were selected and analyzed by GC–MS. The resolution between key compounds were calculated (Table 3) and showed that using the new program most compounds, not only those eluted at the beginning of chromatogram (Fig. 6) are well separated.

5. Conclusion

Reduction of initial temperature (T_i) from 180 °C (traditional screening condition) below 140 °C enhanced S/N but showed lower derivatization conversion. Increasing the derivatization temperature resulted in oxandrolone degradation. T_i = 140 °C was a good compromise, with almost 100% derivatization yield specially for stanozolol and its metabolites and excellent peak shape and resolution. Therefore it was demonstrated that additional derivatization inside the capillary column (in the present case, the retention gap) is mandatory and happens above 140 °C.

Detection limits improvements were obtained for the compounds that elute at the beginning of the chromatogram such as mabuterol, clenbuterol, norandrosterone and epimethendiol without changing the

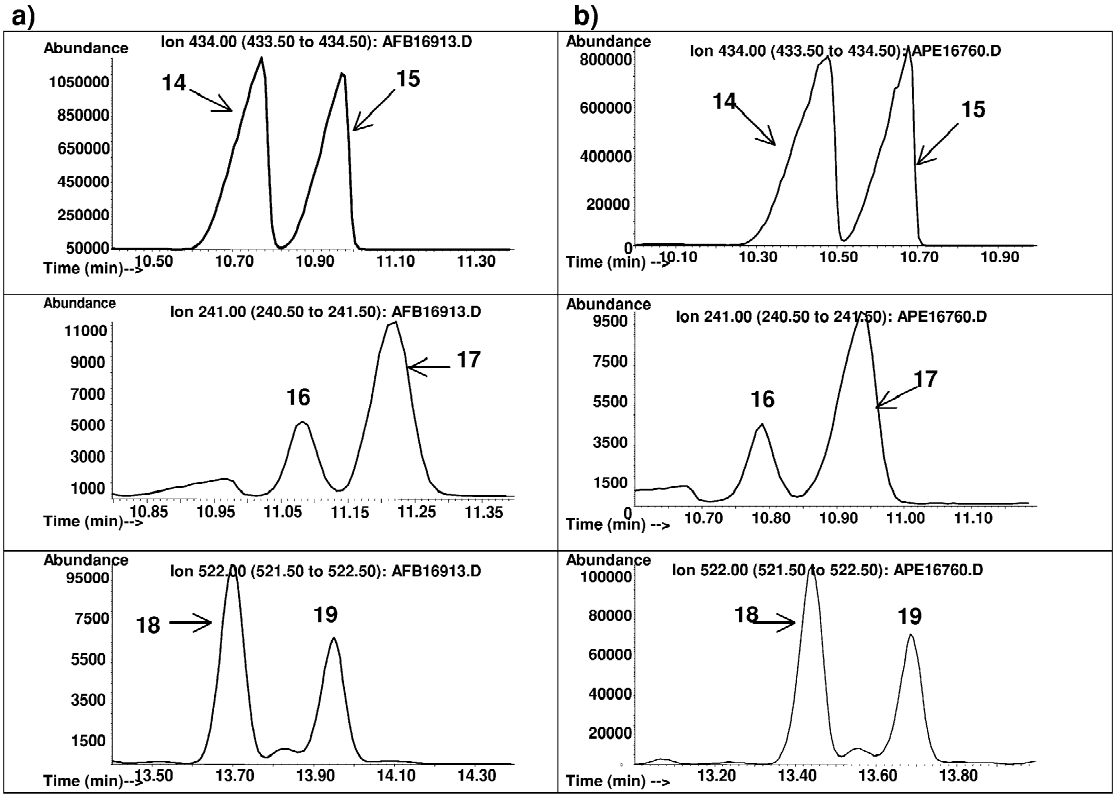

Fig. 6. GC–MS chromatograms illustrating adequate key separations for endogenous steroids. Temperature program from (a) 140 °C, 40 °C min^{-1} to 180 °C and 3 °C min^{-1} to 240 °C and 40 °C min^{-1} to 300 °C (held 3 min). (b) 180 °C and 3 °C min^{-1} to 240 °C and 40 °C min^{-1} to 300 °C (held 3 min) of a urine with a high density: (**14**) androsterone, (**15**) etiocholanolone, (**16**) 3a,5a-Diol, (**17**) 3a,5b-Diol, (**18**) OHA, and (**19**) OHE. Splitless mode injection (5 m of retention gap is connected to the capillary column).

signal/noise ratio for furazabol, stanozolol and their metabolites. All key separations for doping control analysis are reasonably obtained, even for difficult urine samples, like the ones that have medicines, high density and bacterial degradation products.

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