APPLIED ORGANOMETALLIC CHEMISTRY, VOL. 11, 205–212 (1996)

GC–SIM/MS Profiling of Urinary Steroids as their Per-trimethylsilyl Derivatives

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Complete GC–SIM/MS separation and identification of 19 steroids (including several steroid isomers) in a male urine sample, as their per-trimethylsilyl ethers, is described. Trimethylsilylation of their functional groups (hydroxyl and carbonyl) was achieved by a rapid, simplified and one-step derivatization method, using *N*-methyl-*N*-trimethylsilyl-2,2,2,-trifluoroacetamide as the silylating agent and the solvent as well, along with catalytic amounts of trimethylsilyl iodide and dithioerythritol. Furthermore, a GC/MS method for the analysis of a synthetic mixture of underivatized urinary steroids is proposed.

Keywords: urinary steroids; per-TMS derivatives; GC-SIM/MS profiling

INTRODUCTION

In view of the very low concentrations of the diagnostically important steroids included in the complex urine matrix, and the extreme difficulty in isolating and purifying them at such low concentrations, the combined gas chromatography–selected ion monitoring/mass spectrometry (GC–SIM/MS) technique is widely accepted as the most accurate and specific method for their analysis and quantitative interpretation, using reliable reference values.[1–5] The GC/MS technique is also characterized by the advantageous potential of determining a total steroid profile in contrast to radioimmunoassay, where only a single steroid can be measured at a time.

It should be stressed, however, that the specificity of GC/MS, particularly in urinary steroids analysis, is closely connected with the resolution capacity of the capillary column and that of the mass spectrometer, and optimization of all the stages of the analytical procedure including extraction, hydrolysis, derivatization of the urine sample. Therefore, as GC–SIM/MS is rapidly becoming a valuable tool and a standard feature in clinical laboratories, much scientific interest is focused on data processing and automation–optimization of the time-consuming analytical procedure.

Due to the thermal instability and low volatility of the diagnostically important urinary steroids, their derivatization has become essential for their GC/MS analysis. Consequently, the choice of suitable derivatives as much as the simplicity of the derivatization method is of primary importance for their GC/MS analysis. The most common derivatives used for urinary steroids are trimethylsilyl (TMS)[6, 7] ethers, t-butyldimethylsilyl (TBDMS)[8] ethers or heptafluorobutyrates (HFB)[9] for the derivatization of their hydroxyl groups, and methyloximes[10, 11] for their carbonyl ones. However, all the above-mentioned approaches suggest prolonged reaction periods, with complex conditions, multistep procedures and intensive work-up.[12]

N-Methyl-*N*-trimethylsilyl-2,2,2-trifluoroacetamide (MSTFA), as a silylating mixture with trimethylsilyl iodide (TMSI), was first used by Donike[13] in doping analysis, with excellent results. Recently, the above silylating mixture, modified by *in situ* formation of TMSI as the result of the catalytic action of the added ammonium iodide (NH_4I) on MSTFA, has been used for the reductive silylation of a number of hydroxyquinones, in both their hydroxyl and carbonyl groups.[14]

We report here our results concerning complete GC–SIM/MS separation and identification of 19 steroids—including several steroid isomers—in a male urine sample as their per-TMS

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CCC 0268–2605/97/030205–08 $17.50

Received 9 November 1995
Accepted 16 January 1996

ethers, with well-formed peak shapes due to their increased relative retention times (RRTs) and highly diagnostic MS features. Trimethylsilylation of all their hydroxyl and carbonyl groups was achieved by a rapid and simplified per-TMS derivatization method, using MSTFA as the silylating agent and the solvent as well, along with TMSI and dithioerythritol (DTE). Furthermore, a GC/MS method for the analysis of an underivatized mixture of urinary steroids is proposed.

EXPERIMENTAL

Chemicals and reagents

MSTFA, TMSI and DTE were obtained from Fluka and were analytical-reagent grade. *Escherichia coli* β-glucoronidase was obtained from Boehringer, Mannheim and stored at +4 °C. Steroids were obtained from Sigma and were analytical-reagent grade. All other chemicals and solvents were analytical-reagent grade and were used without further purification.

Sample preparation

A 2 ml portion of the urine of a healthy male volunteer in the laboratory was added to an Amberlite XAD-2 column. The column consisted of a Pasteur pipette and was closed with a glass pearl with a bed height of 2 cm. The mixture in the column was washed with 2 ml of twice-distilled water and eluted with 2 ml of methanol. The methanol eluate was then evaporated to dryness and the residue was dissolved in 1 ml of 0.2 M sodium phosphate buffer (pH 7.5). Then 50 µl of β-glucuronidase from *E. coli* was added to the buffer solution and the mixture was hydrolyzed for 1 h at 50 °C. Use of β-glucuronidase instead of β-glucuronidase-arylsulfatase was indicated by the fact that most of urinary steroids are β-glucuronide and not sulfate conjugates. The solution was alkalized with 250 ml of 7% potassium carbonate solution to pH 9–10 and the steroids were extracted with 5 ml of diethyl ether on a mechanical shaker for 5 min. After centrifugation the ethereal layer was transferred to a glass tube and evaporated to dryness under vacuum.

Derivatization

The dry residue was treated with 100 µl of a mixture of MSTFA/TMSI/DTE [1000:2:4 (v:w:w)] and heated for 20 min at 60 °C.

Gas chromatography–mass spectrometry

Analysis of the per-TMS-derivatized mixture of urinary steroids was obtained with a Hewlett–Packard 5890 gas chromatograph interfaced with a Hewlett–Packard 5980 MSD quadrupole mass spectrometer. The samples were introduced by split injection with a split ratio of 1/10 at 250 °C on an Ultra-1 capillary column (25 m $\times$ 0.22 mm i.d.) with 0.11 µm thin film and dimethylpolysiloxane coating. The column was initially set at 180 °C for 1 min and the temperature was then raised at 8 °C min^{-1} to 220 °C, at 3 °C min^{-1} to 250 °C and 14 °C min^{-1} to 280 °C. Helium was used as a carrier gas with a pressure of 5 psi (pounds per square inch).

Analysis of the underivatized mixture of urinary steroids was obtained with the previously mentioned gas chromatograph interfaced with an HP 5989 mass spectrometer. The samples were introduced on column at 50 °C. The column was set initially at 50 °C for 1 min and the temperature was then raised at 20 °C min^{-1} to 280 °C, which was maintained for 20 min. Helium was used as a carrier gas with a pressure program: initially 2 psi, rising at 0.3 psi min^{-1} to 7 psi. The injector was set initially at 50 °C for 1 min and was then raised at 150 °C min^{-1} to 300 °C.

The mass spectrometer, in both analyses, was set to scan from 90 to 600 amu per nominal second with an ionizing voltage of 70 eV.

RESULTS AND DISCUSSION

Per-TMS derivatization

The main objectives of a derivatization procedure include simplicity and brevity, minimization of by-product and co-product formation, and improved GC/MS characteristics of the derivatives.

Per-TMS silylation of urinary steroids was found to fulfil the above-mentioned criteria by the quantitative formation of their TMS ethers, in only 20 min, as verified by the absence of the corresponding peaks in their total ion chromato-

gram or any retained compounds, which would contaminate the column. The tightly sealed derivatization apparatus prevented any steroid derivative loss and no isomer formation was observed. Additionally, it is of particular interest in working with such low concentrations that by the proposed derivatization method no work-up is demanded, since MSTFA is a powerful solvent for both the underivatized and derivatized steroid forms and its by-product *N*-methyl-2,2,2-trifluoroacetamide (MTFA, m.p. 52–53 °C) is a highly volatile compound.[15]

TMSI is a powerful silylating agent.[16] It readily silylates both carbonyl and hydroxyl groups to the corresponding enol silyl and silyl ethers respectively. MSTFA is also a powerful silylating agent for functional groups possessing an active proton site.[17] Therefore, silylation of **1**–**19** is carried out by TMSI, probably as depicted in Scheme 1. MSTFA interacts with HI by-product to regenerate consumed TMSI. Thus, decomposition of TMSI on standing is prevented.[18] DTE also stabilizes TMSI.[13]

Per-TMS ethers of urinary steroids were found to be thermally stable and give fine, sharp peaks and sensitivity in their GC analysis. Trimethylsilylation of carbonyl groups, besides that of hydroxyl groups, increased even further their high molecular masses and shifted the monitored ions to a cleaner background in their GC–SIM/MS analysis. Furthermore, as highly volatile products, they kept the capillary column clean so that it was suitable for repeated use; this is one of the most desirable features in clinical routine analysis.

GC–SIM/MS characteristics

Per-TMS ethers of urinary steroids were identified by their retention times, characteristic ion chromatograms and complete mass spectra, which were compared with those of authentic standards.

The GC relative retention times (RRTs) and mass-spectral characteristics of the urinary steroids identified as per-TMS ethers, and the ions monitored, are summarized in Table 1.

Inspection of the data in Table 1 shows that most of the mass spectra of the TMS ethers of steroids identified reveal a molecular ion which occurs as the base peak or in appreciable abundance. Notable exceptions to this are TMS ethers of 5α-androstane-3α,17β-diol (**3**), 5β-androstane-3α,17β-diol (**4**), 5-androstenediol (**7**), pregnanediol (**16**) and pregnanetriol (**17**). Mass spectra of TMS ethers of isomers **3** and **4** are characterized by an abundant $[M-195]$ ion at m/z 241 which was monitored. The molecular ion of the per-TMS ether of **7**, although of low abundance, characterizes its mass spectrum; thus, it was selected for ion monitoring. Regarding the TMS ethers of **16** and **17**, the characteristic ions at m/z 269 and 255 were monitored, which are attributed to $[M-195]$ and $[M-249]$ ions. The TMS ethers of **1**–**19**, that are characterized by the same ion, i.e. **1**, **2** and **7**, were easily separated by their quite different RRTs leading to well-defined peaks (Table 1;

Table 1 GC retention times and principal mass-spectral ions of per-TMS ethers of steroids identified in a male urine sample

Compd no.	Steroid trivial name	Steroid systematic name	Derivative	RRT[a]	Mol. ion (% rel. int.[b])	Ion monitored	Principal ions, *m/z* (% rel. int.[b])
1	Androsterone	5α-Androstan-3α-ol-17-one	3α,17-DiTMS	0.831	434(100)	434	435(30), *434(100)*, 420(29), 419(65)
2	Etiocholanolone	5β-Androstan-3α-ol-17-one	3α,17-DiTMS	0.839	434(100)	434	435(32), *434(100)*, 420(26), 419(68)
3	5α-Androstane-3α,17β-diol	5α-Androstane-3α,17β-diol	3α,17β-DiTMS	0.860	436(31)	241	436(31), 346(57), 256(87), 241(80), 215(46)
4	5β-Androstane-3α,17β-diol	5β-Androstane-3α,17β-diol	3α,17β-DiTMS	0.866	436(30)	241	436(30), 346(51), 256(85), 241(78), 215(43)
5	11-Ketoandrosterone	5α-Androstan-3α-ol-11,17-dione	3α,11,17-TriTMS	0.913	505(100)	505	506(48), *505(100)*, 415(32)
6	Dehydroepiandrosterone	Androst-5-en-3β-ol-17-one	3β,17-DiTMS	0.917	432(100)	432	433(36), *432(100)*, 417(55), 327(23)
7	5-Androstenediol	Androst-5-ene-3β,17β-diol	3β,17β-DiTMS	0.930	434(12)	434	434(12), 344(15), 220(24), 215(20)
8	Epitestosterone	Androst-4-en-17α-ol-3-one	17β,3-DiTMS	0.946	432(100)	432	433(36),*432(100)*, 418(6)
9	Estrone	Estra-1,3,5[10]-trien-3-ol-17-one	3,17-DiTMS	0.957	414(100)	414	415(35), *414(100)*, 399(38), 309(12), 231(11)
10	4-Androstenedione	Androst-4-ene-3,17-dione	3,17-DiTMS	0.979	430(100)	430	431(35), *430(100)*, 415(80)
11	Estradiol	Estra-1,3,5[10]-trien-3,17β-diol	3,17β-DiTMS	0.984	416(100)	416	417(38), *416(100)*, 401(6), 285(43), 231(11)
12	Testosterone	Androst-4-en-17β-ol-3-one	3,17β-DiTMS	1.000	432(100)	432	433(39), *432(100)*, 418(8)
13	11β-Hydroxyandrosterone	5α-Androstane-3α,11β-diol-17-one	3α,11β,17-TriTMS	1.004	522(100)	522	523(48), *522(100)*, 417(51), 327(48), 168(60)
14	11β-Hydroxyetiocholanolone	5β-Androstane-3α,11β-diol-17-one	3α,11β,17-TriTMS	1.022	522(100)	522	523(45), *522(100)*, 417(53), 327(50), 168(52)
15	Pregnanediol	5β-Pregnane-3α,20α-diol	3α,20α-DiTMS	1.130	464(—)	269	449(2), 347(6), 284(6), 269(5), *117(100)*

Table 1 Continued

Compd no.	Steroid trivial name	Steroid systematic name	Derivative	RRT[a]	Mol. ion (% rel. int.[b])	Ion monitored	Principal ions, *m/z* (% rel. int.[b])
16	Pregnanetriol	5β-Pregnane-3α,17α,20α-triol	3α,17α,20α-TriTMS	1.156	554(—)	255	436(40), *435(100)*, 345(6), 255(23)
17	Estriol	Estra-1,3,5[10]-triene-3,16α,17β-triol	3,16α,17β-TriTMS	1.224	504(100)	504	*504(100)*, 386(52), 345(85), 311(64), 147(53)
18	Progesterone	Pregn-4-en-3,20-dione	3,20-DiTMS	1.228	458(100)	458	459(43), *458(100)*, 445(54), 370(9)
19	17α-Hydroxyprogesterone	Pregn-4-en-17α-ol-3,20-dione	3,17α,20-TriTMS	1.320	546(100)	546	*546(100)*, 316(79), 301(44), 147(38)

[a] Relative to testosterone retention times.
[b] Abundance is given as a percentage of base peak.

Fig. 1).

One of the major goals of the derivatization method described and the GC–SIM/MS scheme used was the effective separation of the isomeric steroids included. Figure 1 shows fine GC separation of the isomeric steroids **6**, **8**, **12** and **3**, **4** and **1**, **2**, by selected ion monitoring of their characteristic fragment ions. Steroids **13** and **14** were also completely separated by molecular ion monitoring at *m*/*z* 522.

GC/MS method for analysis of underivatized steroids

As previously mentioned, derivatization of urinary steroids became necessary because of their

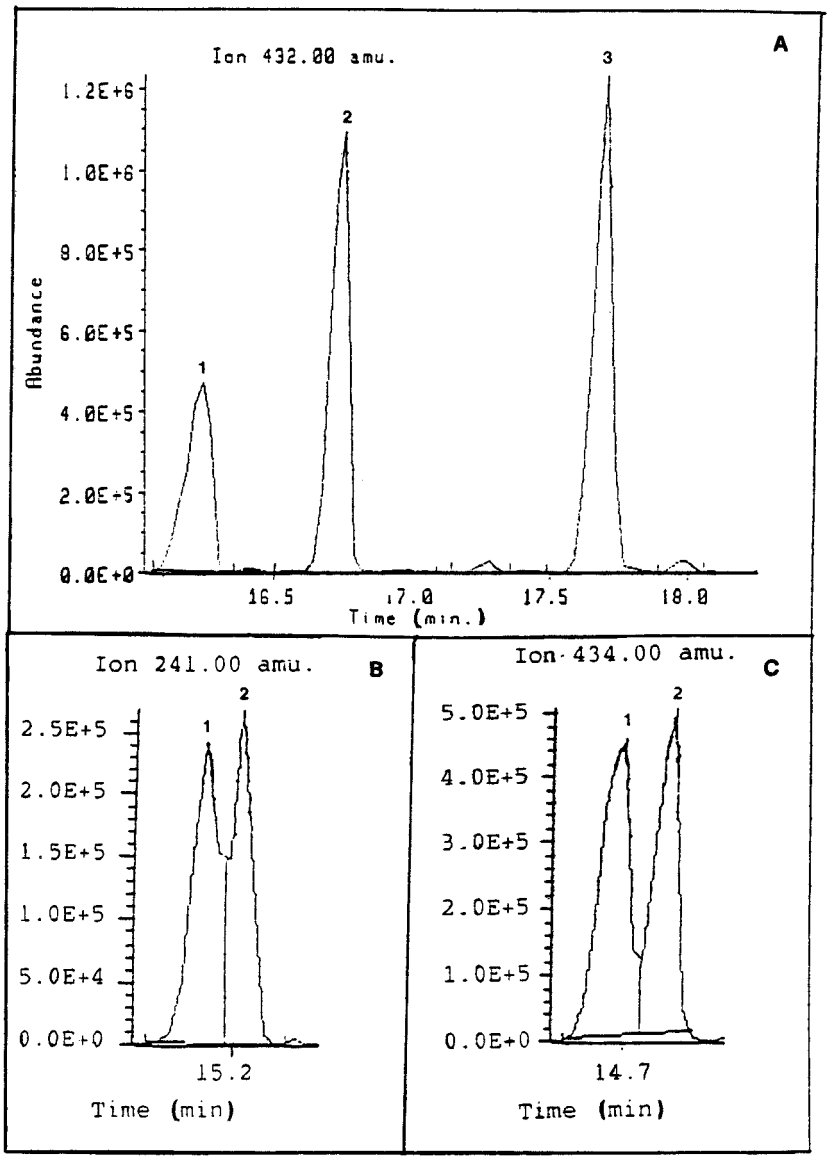

Figure 1 GC separation of isomeric urinary steroids as their per-TMS ethers, by selected ion monitoring of their characteristic fragment ions. (A) Peak 1, dehydroepiandrosterone; 2, epitestosterone; 3, testosterone; *m*/*z* 432. (B) Peak 1, 5α-androstane-3α,17β-diol; 2, 5β-androstane-3α,17β-diol; *m*/*z* 241. (C) Peak 1, androsterone; 2, etiocholanolone; *m*/*z* 434. For conditions, see the Experimental section.

thermal instability during GC/MS analysis. However, the technique of on-column injection of the sample protects a thermally unstable mixture from decomposition, by keeping it at a

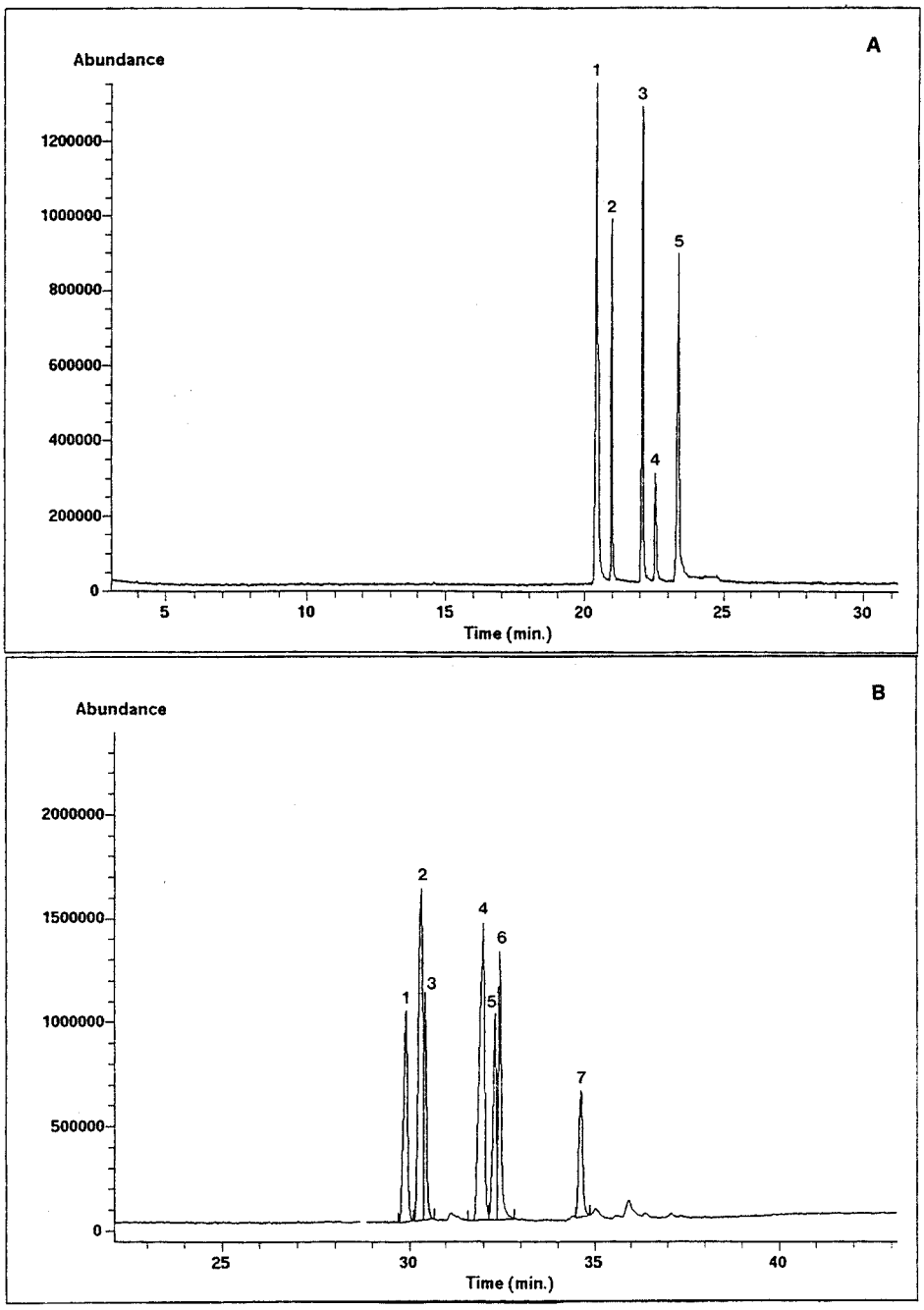

Figure 2 Total ion chromatograms of underivatized mixtures of urinary steroids. For conditions, see the Experimental section. (A) Peak 1, estrone, estradiol; 2, methyltestosterone; 3, progesterone; 4, estriol; 5, 17-hydroxyprogesterone. (B) Peak 1, androsterone; 2, dehydroepiandrosterone; 3, 5-androstenediol; 4, testosterone; 5, methyltestosterone; 6, pregnanediol; 7, pregnanetriol.

low temperature at the beginning of its passage in the column. Moreover, use of a suitable temperature program that allows a gradual, slow increase of column temperature, combined with a relatively mild program for carrier gas pressure in GC/MS analysis, may prevent thermal decomposition and non-appearance of a number of peaks during elution of a sensitive sample, and lead to a successful separation of the sample components.

In order to simplify the analytical procedure and minimize its length, we analyzed underivatized synthetic mixtures of urinary steroids by on-column injection, using the GC/MS method described in the Experimental section. The proposed method was the result of our efforts to determine the optimum combination of column temperature and helium pressure programs. As shown in Fig. 2, the proposed method led to well-defined, sharp peaks for most steroids included in the mixtures. However, the method failed to separate estrone and estradiol, which were co-eluted in the same sharp peak.

The re-usability of the column was studied by repeated analyses of underivatized urine extracts, in order to determine the application of the proposed method to routine clinical analysis. We therefore conclude that the above-mentioned method provides a convenient determination of total urinary steroid profiles. However, their nonvolatility and consequent partial retention on the column limit its use in routine analysis due to gradual deactivation and final column contamination, a problem that is overcome by derivatization. Thus, it seems to be valuable only for special analyses such as particularly complicated mixtures.

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