Determination of testosterone : epitestosterone ratio after pentafluorophenyldimethylsilyl-trimethylsilyl derivatisation using gas chromatography-mass spectrometry in equine urine

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Received 15th February 1999, Accepted 9th March 1999

A highly specific method is described for measuring the testosterone : epitestosterone ratio in equine urine by gas chromatography-mass spectrometry (GC-MS) with stable isotope internal standards. The procedure was based on Serdolit Pad-1 resin extraction, enzymatic hydrolysis, and chemical derivatisation prior to instrumental analysis. The mixed derivatives, 3-trimethylsilyl-17-pentafluorophenyldimethylsilyl ether (3-TMS-17-flophemesyl) testosterone and epitestosterone, were found to have excellent analytical properties. The specificity of the derivatisation method exploits a unique feature of steroids: the selective exchange of the alcoholic flophemesyl ether for the trimethylsilyl ether. The sensitivity and specificity of the mixed 3-TMS-17-flophemesyl derivatives allow adequate determinations of testosterone and epitestosterone, even in urine from mares, in 5 ml samples. The repeatability of testosterone and epitestosterone was 6.2 and 5.7%, respectively, and their reproducibility was in the range of 6.4–8.7%.

1. Introduction

Testosterone is the principal endogenous androgenic-anabolic steroid in humans and equines. In human athletes, testosterone is the substance most frequently reported in steroid misuse, and the accepted test for testosterone administration has been the urinary testosterone to epitestosterone (T : E) ratio, a value of > 6 being taken as the hallmark of drug abuse.[1] In contrast to humans, low concentrations of epitestosterone have been detected in normal equine urine. High resolution mass spectrometry (HRMS) or tandem mass spectrometry (MS-MS) coupled with gas chromatography (GC) or high-performance liquid chromatography (HPLC) has been used to identify and determine the T : E ratio by several groups.[2,3]

Many steroids are thermally labile and must be derivatised prior to analysis to avoid decomposition of the compound and to improve its chromatographic performance. Generally, the derivatisation of testosterone and epitestosterone with 3,17-bis-trimethylsilyl ether (TMS) has been the most common approach.[2,4] Possible methods for improving the selective and sensitive detection of steroids in human and equine urine include modifications to: the GC temperature program; the derivatisation method; the type of GC column; and the sample purification.[5–8]

In general, alkyl or aryl compounds with closely bound fluorine atoms are remarkable in that they show little increase in boiling point compared to hydrocarbons containing a similar number of carbon atoms. However, the pentafluorophenyl ring is a strong electron attracting group which is able to influence the mode of fragmentation of steroid derivatives under electron impact in a way which leads to diagnostic mass spectra. The spectra often show marked differences from those of the TMS ethers. The pentafluorophenyldimethylsilyl (abbreviated to flophemesyl for convenience) derivatives generally show a strong molecular ion and provide much more detailed diagnostic information.[9,10]

The objective of this work was to improve the determination of testosterone : epitestosterone ratios in equine urine by using a novel mixed flophemesyl-trimethylsilyl ether derivatisation method. The new method was assessed by GC-MS analyses and comparisons with deuterium labelled testosterone and epitestosterone internal standards were made.

2. Experimental

2.1. Chemicals

Testosterone (4-androsten-17β-ol-3-one) and epitestosterone (4-androsten-17α-ol-3-one) were purchased from Sigma Co. (St. Louis, MO, USA). The 1:1 (v/v) mixture of 16,16,17-$^2H_3$-testosterone (90 ng ml^{-1}) and 16,16,17-$^2H_3$-epitestosterone (15 ng ml^{-1}), an internal standard, was obtained from Cologne Laboratory (Institute of Biochemistry, German Sports University, Germany). *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I), pentafluorophenyldimethyl chlorosilane (flophemesyl chloride) and dithioerythritol (DTE) were purchased from Sigma Co. (St. Louis, MO, USA). Serdolit Pad-1 resin (particle size 0.1–0.2 mm) was supplied by Serva Co. (Heidelberg, Germany) and washed with acetone, methanol and distilled water before use. β-Glucuronidase–arylsulfatase from *Helix pomatia* (aqueous solution stabilized with thiomerosal) was purchased from Boehringer Mannheim Co. (Mannheim, Germany).

2.2. Apparatus

The GC-MS system (Model 5973MSD combined with a Model 6890 plus gas chromatograph, Hewlett-Packard; Avondale, PA, USA) was used in both scan and selected ion monitoring (SIM) modes. The electron energy was 70 eV and the ion source temperature was 230 °C. The gas chromatograph was equipped with a 17 m × 0.2 mm id × 0.11 μm film thickness capillary column coated by cross-linked 5% phenyl methyl silicon fluid

(Hewlett-Packard). The carrier gas was helium at a column head pressure of 121 kPa. The split (1 : 10) method of injection was used. The temperature program was as follows: initial temperature 200 °C (2 min); program rate 10 °C min^{-1} to 250 °C (5 min); 10 °C min^{-1} up to a final temperature of 320 °C, where it was held for 3 min.

2.3. Extraction and derivatisation procedure

An aliquot of urine (5 ml) was taken and internal standard solution (20 μl) was added. An aqueous Serdolit Pad-1 slurry was filled into a Pasteur pipette until a bed height of 1.5 cm was achieved. The column was washed with 3 ml of distilled water, then the mixture was loaded onto a Serdolit Pad-1 resin cartridge. The column was washed with water (5 ml) and n-hexane (5 ml), then eluted with methanol (2 × 1.5 ml) into a test tube; the eluate was evaporated to dryness (40 °C under nitrogen). The residues were redissolved in acetate buffer of pH 5.4 (0.2 mol l^{-1}; 1 ml). In order to hydrolyze the conjugated form, 0.1 ml of enzyme solution was added to the acetate buffer, and the solution was heated at 80 °C for 3 h, then cooled to room temperature and the pH adjusted by adding 20 mg potassium carbonate along with 5 ml n-pentane. The mixture was mechanically shaken (10 min) and centrifuged (2400 rpm, 5 min) and the organic phase was transferred to a test tube. The organic layer was evaporated to dryness in a rotary evaporator. The hydrolyzed buffer solution was extracted two additional times with 5 ml n-pentane to enhance the recovery of this extraction method. The n-pentane fraction was evaporated to dryness. The residue was dried in a vacuum desiccator over P_2O_5–KOH for at least 30 min before the derivatisation procedure.

The flophemesyl chloride solution (50 μl) was added to the residue, and the mixture was allowed to stand at room temperature for 15 min. After the excess reagent had been evaporated under a stream of nitrogen at 70 °C, trimethylsilylating reagent (50 μl, MSTFA–NH_4I–DTE, 1000 : 4 : 2, v/w/w) was added to the residue, and the mixture was heated at 60 °C for 15 min. An aliquot of the flophemesyl-TMS derivatised sample solution was injected into the GC-MS.

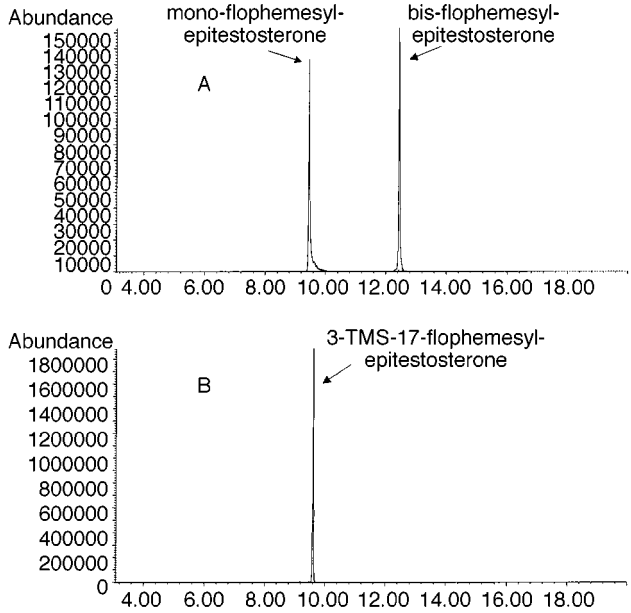

Fig. 1 Total ion chromatogram of epitestosterone after flophemesyl derivatisation procedure (A) and 3-TMS-17-flophemesyl derivatisation procedure (B).

2.4. Evaluation of repeatability and reproducibility

The repeatability of the chromatographic analysis was determined by ten replicate 2 μl injections of a mixture of derivatised standards. The reproducibility for urine samples was examined by several injections of a 2 μl portion of ten derivatised extracts obtained from fortified water samples at 10 and 50 ppb levels.

3. Results

3.1. Establishment of a suitable derivatisation method

Testosterone and epitestosterone as 17-epimers have two ionizable hydrogen atoms. In order to stabilize the compounds and improve the GC properties, an initial effort was made to examine the derivatisation method using flophemesyl chloride. Indeed, the injection of the flophemesyl derivatives of both testosterone and epitestosterone, by GC-MS, showed the presence of two peaks with identical mass spectra in EI mode. Because of mono- and bis-flophemesyl derivatives, we suspected that the reaction at the 3-enol keto position had taken place during the GC injection in the injection port.

In contrast, the efficiency of 3-TMS-17-flophemesyl derivatives was tested by a full-scan spectra of pure steroid standards. The chromatograms did not show any peaks of an unexpected nature, corresponding to derivatised or partially derivatised

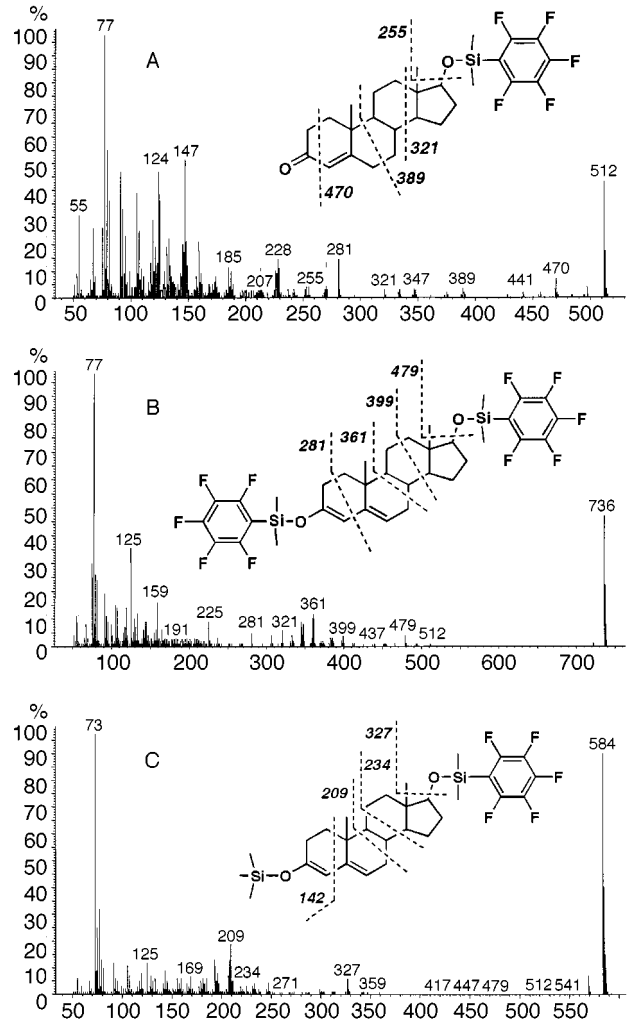

Fig. 2 Scan spectra of 17-flophemesyl-epitestosterone (A), 3,17-bis-flophemesyl-epitestosterone (B) and 3-TMS-17-flophemesyl-epitestosterone (C).

steroids indicating that the derivatisation reaction was complete (Fig. 1).

3.2. Mass spectral analysis

Fig. 2 shows the principal ions detected in the present study. In all the spectra, the compound loss of a methyl group led to M−15 mass units of abundance, less than that of the molecular ion. The flophemesyl derivatives are characterized by 77 u.[10] Peaks at mass units of M−167 $[M-C_6F_5]^+$, M−225 $[M-C_6F_5Si(CH_3)_2]^+$ and M−241 $[M-C_6F_5Si(CH_3)_2O]^+$ also occurred to varying extents in all the spectra. Likewise, occurrence of trimethylsilylation is identified by mass units of M−72 $[M-Si(CH_3)_3]^+$, M−90 $[M-Si(CH_3)_3OH]^+$ and M−105 $[M-Si(CH_3)_3OH-CH_3]^+$, even though their abundances are quite low. In all mass spectra for the flophemesyl derivatisation method, there is a significant peak at 81 u which corresponds to the $[Si(CH_3)F_2]^+$ mass units.

3.3. Stability of the mixed flophemesyl-TMS derivatisation method

The repeatability of the method was evaluated using 1 ppm derivatised mixed standards of testosterone and epitestosterone, and the reproducibility was assessed using extracts fortified with standards of both testosterone and epitestosterone at 10 and 50 ppb levels in water. The peak areas of selected ions (molecular ions) were obtained for testosterone and epitestosterone. They were quantitated by the ratio of the peak area from the spiked sample to that from the corresponding internal standard (16,16,17-$^2H_3$-testosterone) and the absolute values were calculated. The repeatability for testosterone and epitestosterone was 6.2 and 5.7%, respectively, and their reproducibility was in the range of 6.4%–8.7% (Table 1).

3.4. Determination of testosterone : epitestosterone ratio

d_3-Testosterone and d_3-epitestosterone were introduced as internal standards because calibrating the GC-MS system using external standards is difficult due to matrix problems and linearity problems in the GC-MS system.[11] To reduce some of the problems arising from these matrices and instruments, d_3-testosterone and d_3-epitestosterone were introduced as internal standards to obtain standardized conditions in the human doping groups.[12]

Two same-mare urine samples were analyzed by GC-MS after derivatisation using two different methods. In order to determine the T : E ratio, a derivatisation method using a silylating agent was carried out for the formation of 3,17-bis-TMS derivatives in most of the doping groups. Especially in the case of epitestosterone, these derivatives produce peak broadening or are not detected because of very low concentrations in mares and geldings, but 3-TMS-17-flophemesyl derivatives reduce matrix interference and show excellent peak shape (Fig. 3).

Three different urine samples (OC-218, 219 and 220) were analyzed using two different derivatisation methods. All samples were analyzed three times by GC-MS, and the results of the T : E ratio were similar to each other. The results are listed in Table 2.

Table 1 The repeatability and reproducibility of 3-TMS-17-flophemesyl derivatives ($n = 10$)

Substance	Repeatability (RSD,%)	Reproducibility (RSD,%)	
		10 ppb	50 ppb
Testosterone	6.2	7.3	8.7
Epitestosterone	5.7	7.1	6.4

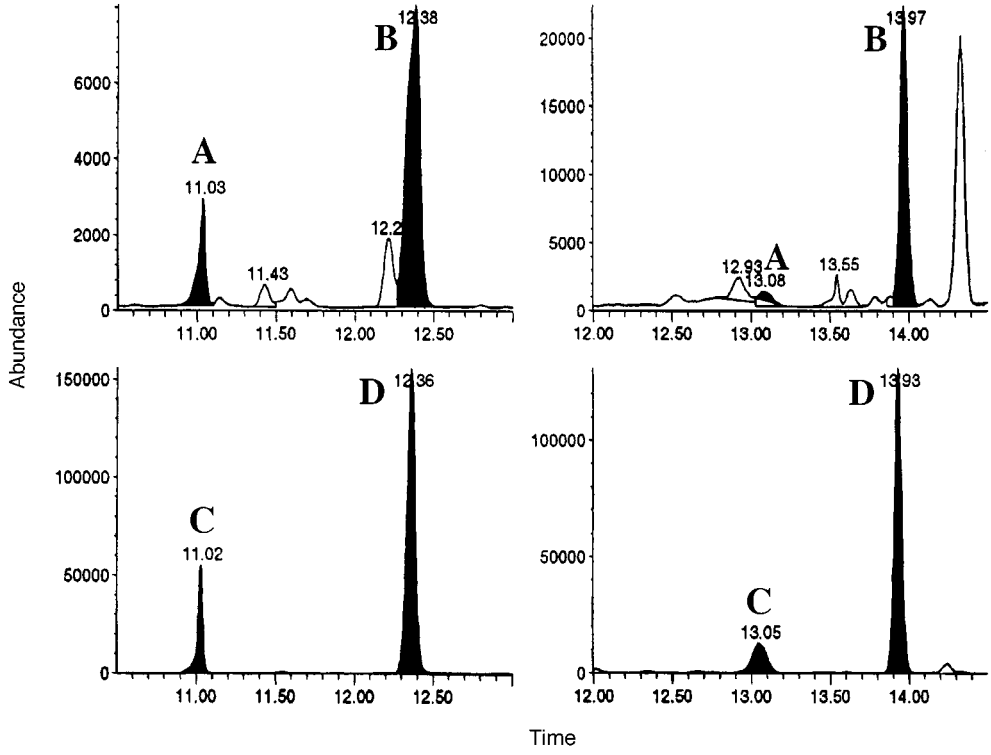

Fig. 3 SIM chromatograms for detection of epitestosterone (A), testosterone (B), d_3-epitestosterone (C) and d_3-testosterone (D) after mixed TMS-flophemesyl (left) and trimethylsilyl (right) derivatisation procedures.

Table 2 Comparison of T : E ratio using two different derivatisation methods

		T : E ratio[a] ($n = 3$)	
Sample	Sex	3,17-bis-TMS	3-TMS-17-flophemesyl
OC-218	Mare	12.4 ± 0.35	11.6 ± 0.33
OC-219	Gelding	8.1 ± 0.28	9.7 ± 0.31
OC-220	Stallion	10.6 ± 0.21	9.9 ± 0.41

[a] T : E ratio calculated by peak area.

4. Discussion

The reactants uncatalyzed by flophemesyl chloride rapidly reacted with unhindered secondary hydroxyl groups to produce silyl ethers. This reagent did not cause the formation of enol ethers and hindered the hydroxyl groups from reacting.[13] Thus we used the silylating agent and flophemesyl chloride for 3-enol keto and 17-hydroxyl groups, respectively.

The derivatisation method of testosterone and epitestosterone to 3,17-bis-TMS derivatives has been the mass (432 u) common approach for determination of the T : E ratio, but the mass increment provided by trimethylsilylation is rather low. Therefore, 3-TMS-17-flophemesyl derivatives have been advocated as a better choice for SIM at the higher mass (584 u) of the molecular ion.

The advantages of using the mixed flophemesyl-TMS derivatisation method include: the ease of reagent removal, without loss of products, by excessive nitrogen gas at high temperature; rapid reaction time; good GC properties; and the formation of intense molecular ions under electron impact mass spectrometry (EI-MS). Moreover, on a theoretical basis, specificity should be improved, because the selective exchange of an alcoholic flophemesyl ether for TMS ether is an exclusive feature of testosterone and epitestosterone.

This study may be the starting point of further studies, which could screen and confirm unambiguously the structure of steroids and diverse applications in chromatographic research.

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Paper 9/01242G