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GC-MS STUDIES OF 16-ANDROSTENES AND OTHER C_{19} STEROIDS IN HUMAN SEMEN

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Summary—Human semen was examined for the presence of 16-androstenols, 16-androstenones and androgens. Extracts were analysed by gas chromatography-mass spectrometry after derivatization of steroids under study. In a qualitative study, 5α-androst-16-en-3α- and 3β-ols, 5,16-androstadien-3β-ol and 5α-androstan-3β-ol were detected in a semen pool **A**. Hydroxyl groups were converted to tert-butyldimethylsilyl ethers, the ions selected for monitoring being $[M-57]^+$, consistent with loss of the tert-butyl group. For a more detailed quantitative study, a second semen pool **B** was used. In this case, all hydroxyl groups were converted to trimethylsilyl ethers, while oxo groups were not derivatized. As with semen pool **A**, separation of steroids was achieved using capillary gas chromatography with appropriate temperature programming. Quantification was carried out by mass spectrometry using selected ion monitoring of two significant ions and appropriate internal standards. The following steroids were identified at the concentrations indicated: 5α-androst-16-en-3α- and 3β-ols and 5,16-androstadien-3β-ol (concentration range, 0.5–0.7 ng/ml). 5α-Androst-16-en-3-one and 4,16-androstadien-3-one were also present at levels of 0.7–0.9 ng/ml. Two androgens, testosterone and 5α-dihydrotestosterone were found at concentrations of 0.5 and 0.3 ng/ml, respectively. These data, showing the presence of 16-androstenes and androgens in human semen, appear to be consistent with testicular formation of these steroids. The possible significance of the odorous 16-androstenes is discussed.

INTRODUCTION

The gas chromatographic (GC) and mass spectral (MS) behaviour of a number of 16-androstenes, saturated analogues and various derivatives of these steroids have been published previously by us [1]. Our detailed study was considered to be necessary so that suitable significant ions for selected ion monitoring (SIM) and appropriate internal standards could be chosen. During the past four decades, 16-androstenes have been quantified in urine [2–4], peripheral blood plasma [5, 6], saliva [7, 8] and in axillary secretions from men and women [5, 9–12]. In each case, marked sex differences were noted, and, in male pig castrates, the quantities of the various 16-androstenes measured were considerably lower than those in the intact animals [6]. This is consistent with a testicular source for these steroids, a finding amply confirmed by biosynthetic studies, both *in vivo* and *in vitro* [13], and by analytical studies[14] of porcine testis tissue.

Earlier work [15] had shown that human testis could form 16-androstenes, and recent studies have confirmed and extended these findings [16–18]. In view of the testicular origin of these steroids and of their possible involvement as human pheromones [19], we have utilized GC-MS with SIM for their measurement in human semen. Androgens such as testosterone and 5α-DHT, already known to be present [20, 21], have been measured simultaneously with the 16-androstenes, for comparison.

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Abbreviations: Testosterone, 17β-hydroxy-4-androsten-3-one; epi-testosterone, 17α-hydroxy-4-androsten-3-one; 5α-DHT, 17β-hydroxy-5α-androstan-3-one; tert-butyldimethylsilyl ethers, TBDMS ethers; trimethylsilyl ethers, TMS ethers; *O*-methyl oxime, MO; pentafluorobenzyl oxime, PFB oxime; gas chromatography-mass spectrometry, GC-MS.

MATERIALS AND METHODS

Steroids

Authentic steroids and chemicals used for derivatization of steroids were obtained from sources noted previously [1]. The GC-MS behaviour of testosterone, epi-testosterone, 5α-DHT and 19-nortestosterone and some derivatives were examined using the method described for 16-androstenes and saturated analogues [1]. Standard curves were set up in duplicate over the range 0–50 ng using 0, 5, 10, 20 and 50 ng of each analyte, together with 10 ng each of 5β-androstan-3β-ol (for 16-androstenols), 5α-androstan-3-one (for 16-androstenones) and 19-nortestosterone (for the androgens). The ratios of areas of steroids to that of the appropriate internal standard were plotted against the quantity of steroids of interest. In each case, the values for particular points did not differ by more than 5%.

Semen

Human semen was collected mostly from men who presented at the Infertility Clinic, Guy's Hospital. The majority of the donors had sperm counts $>50 \times 10^6$/ml. In pilot experiments, quantification of steroids was attempted in extracts from single semen samples (volume 2–4.5 ml) but the volume of semen proved to be too small for accurate quantification by SIM. It was decided, therefore, to pool semen samples, and two such collections were made: **A**, a pooled sample (25 ml) obtained from 11 men and **B**, a pooled sample (35 ml) obtained from 14 men. No internal standards were added to **A** since this pool was used to ensure that the steroids of interest were present at concentrations measurable by SIM. Having demonstrated the presence of these steroids by this experiment (see Results), 5β-androstan-3β-ol, 5α-androstanone and 19-nortestosterone (10 ng each) were added as internal standards before extraction of the larger semen volume **B**.

Extraction

In both cases, **A** and **B**, extraction was carried out with ethyl acetate (HPLC Grade, Rathburn Chemicals Ltd., Walkerburn, Scotland) (2 × 50 ml, then 4 × 25 ml). The combined extracts were evaporated to a small volume (1–2 ml) using a Buchler Vortex evaporator (Gallenkamp, London, England) at temperatures not exceeding 25°C, so as to minimize possible losses of volatile 16-androstenes [22]. These extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The extract was then dissolved in 0.5 ml of ethyl acetate and applied to the top of a two-tier column: the upper part consisted of a glass Pasteur pipette (containing anhydrous Na_2SO_4) the tip of which was tightly fitted into a Sep-Pak Florisil cartridge [Millipore (UK) Ltd., Watford, England] [23]. Further volumes of ethyl acetate (4 × 0.5 ml) were used to transfer as much as possible of the steroid extracts to the Florisil column. This was then washed with ethyl acetate (3 × 3 ml) and the eluate collected and evaporated to dryness. The residue was purified by redissolving in the mobile phase (methanol–dichloromethane, 95:5, v/v) (30 μl) and 20 μl of this subjected to HPLC using a Model RR/035 HPLC system (HPLC Technology Ltd.), equipped with a Spherisorb 5 ODS column (25 cm × 4.6 mm, i.d.). For elution of steroids, the mobile phase was used at a flow rate of 0.9 ml/min. Earlier pilot studies with 5α-[5α,6α-$^3H_2$]androst-16-en-3-one (Isocommerz, Kontor, Dresden; sp. act. 28 Ci/mmol) had shown that this was eluted between 5–9 min [24]. To ensure that all the 16-androstenes were collected, fractions were collected for 3–11 min pooled and carefully evaporated to dryness as described above. It should be emphasised that by using this method, we did not wish to separate the steroids but only to remove contamination. The residue was redissolved in ethyl acetate (0.5 ml) and this solution was transferred to silanized glass vials and the solvent removed at 20°C using a Savant Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY).

Derivatization

Steroids extracted from semen pool **A**, to which no internal standards were added, were derivatized as described previously [1] forming PFB oximes with oxo groups and tert-butyldimethylsilyl (TBDMS) ethers with hydroxyl groups. Although these latter derivatives gave intense $[M-57]^+$ ions, which arose by loss of the tert-butyl group, they were, in our hands, difficult to prepare quantitatively. Consequently, when examining semen pool **B**, to which internal standards had been added, trimethylsilyl (TMS) ethers were prepared [1], leaving oxo groups, if present, underivatized. For these experiments the GC oven temperature was programmed at a rate which differed from the standard one (see Table 1), and was designed to maximize the separation of the mono-func-

Table 1. GC retention times and principal mass spectral ions of some C_{19} steroids, 19-nortestosterone and some derivatives

Steroid	Derivative	Retention time (min)	Base Peak as% of tic[a]	Principal mass spectral ions m/z[b]
Testosterone	None	12.522	7.3	288(81)[M]$^+$; 246(50); 228(19); 203(27).
	3-oxo,17β-TMS ether	12.705	6.9	360(100)[M]$^+$; 345(53)[M-15]$^+$; 270(62)[M-90]$^+$; 226(41); 147(27); 129(67).
	3-MO,17β-TMS ether	13.002	6.8	389(100)[M]$^+$; 358(20)[M-31]$^+$; 343(5)[M-31-15]$^+$; 254(8); 268(24).
	3-PFBO,17β-TMS ether	17.607; 17.802[c]	5.2	555(28)[M]$^+$; 358(58)[M-197]$^+$; 268(55); 195(61).
Epi-testosterone	None	12.666	5.0	288(90)[M]$^+$; 246(40); 228(61); 203(35).
	3-oxo,17α-TMS ether	12.239	6.0	360(100)[M]$^+$; 345(26)[M-15]$^+$; 270(75)[M-90]$^+$; 226(50).
	3-MO,17α-TMS ether	12.534	7.5	389(100)[M]$^+$; 358(24)[M-131]$^+$; 343(3)[M-31-15]$^+$; 284(11); 268(49).
	3-PFBO,17α-TMS ether	17.127;17.518[c]	6.0	555(11)[M]$^+$; 358(26)[M-197]$^+$; 268(49); 195(80).
5α-DHT	None	11.56	8.7	290(92)[M]$^+$; 257(12); 231(100); 199(15).
	3-oxo,17β-TMS ether	12.198	7.5	362(22)[M]$^+$; 347(80)[M-15]$^+$; 272(86)[M-90]$^+$; 257(83)[M-15-90]$^+$; 231(14); 161(17); 149(27).
	3-MO,17β-TMS ether	12.705; 12.791[c]	6.8	391(84)[M]$^+$; 360(27)[M-131]$^+$; 346(4); 286(57); 270(29).
	3-PFBO,17β-TMS ether	17.323; 17.549[c]	9.9	558(2)[M]$^+$; 467(10); 452(12); 360(10)[M-197]$^+$; 270(19).
19-Nortestosterone	None	12.027	10.2	274(100)[M]$^+$; 256(16); 215(24); 197(10).
	3-oxo,17β-TMS ether	12.264	6.0	346(98)[M]$^+$; 331(57)[M-15]$^+$; 256(93)[M-90]$^+$; 215(41); 160(37); 129(10).
	3-MO,17β-TMS ether	12.581	10.4	375(100)[M]$^+$; 344(16)[M-31]$^+$; 285(9); 254(27).
	3-PFBO,17β-TMS ether	17.28; 17.68[c]	6.0	541(28)[M]$^+$; 344(46)[M-197]$^+$; 270(53); 254(50); 195(46).

For GC, the standard temperature was used, *viz*: 120 up to 300°C at a ramp of 8°C/min.
[a]Where two peaks are recorded in previous column only value for the first peak is recorded.
[b]Abundance is given as percentage of base peak (not always listed).
[c]*Syn*- and *anti*-forms.

tional 16-androstenes, *viz*: 150 to 220°C at 2°C/min then to 310°C at 50°C/min.

GC-MS

GC-MS was carried out using two different instruments. Steroids extracted from semen pool **A** were converted to the TBDMS ethers as above and subjected to GC using a Pye Unicam 204 gas chromatograph fitted with a fused silica column (25 m × 0.33 mm i.d., coated with OV-1) which was directly interfaced with the mass spectrometer source [23]. Helium carrier gas flow rate was 1 ml/min. Samples were injected 'on-column' in *n*-decane at 175°C and the column temperature-programmed to provide retention times (RTs) of steroid derivatives between 7–8 min [23]. Mass spectra were determined using a VG Analytical Ltd, (Manchester, England) model 305 mass spectrometer-2025 data system. The source conditions employed were, El ionization at 40 eV and a trap current of 500 μA.

Steroids extracted from semen pool **B** were examined using an HP 5890 GC (Hewlett-Packard, Palo Alto, CA) fitted with a HP-1 cross-linked methyl silicone gum (12 m × 0.2 mm × 0.33 μm film thickness) column with helium carrier gas at a flow rate of 1 ml/min, passing the end of the GC column directly into the ion source of an HP 5970 Series Mass Selective Detector. Production of reconstructed ion chromatograms and selected ion monitoring were under the control of the computer system supplied with the HP 5970.

RESULTS AND DISCUSSION

Choice of internal standard for quantification of 16-androstenes

In earlier experiments [8, 10], 5α-androstan-3β-ol was utilized as internal standard for SIM of 16-androstenes in axillary hair and axillary secretions because this compound satisfied the necessary criteria in that it did not occur endogenously in these tissues and was resolved from the 16-androstenols of interest, yet having a similar RT [1]. In the present study using semen pool **A**, however, it appeared that 5α-androstan-3β-ol or some other compound with similar GC and MS characteristics occurs in human semen. It was necessary, therefore to find an alternative internal standard. Of the isomers of this compound available commercially, 5β-androstan-3β-ol was shown to be satisfactory in this respect for the 16-androstenols, except that no significant $[M]^+$ ion was seen in the mass spectrum of the TMS ether derivative, the base peak being m/z 258

Table 2. Standard curves obtained from SIM analysis of reference steroids

Steroid derivative	Ion monitored	A	B	r^a
5α-Androst-16-en-3α-ol (TMS)	346[M]$^+$	32.69	3.30	0.9998
5,16-Androstadien-3β-ol (TMS)	344[M]$^+$	34.23	2.91	0.9998
5α-Androst-16-en-3β-ol (TMS)	346[M]$^+$	32.53	2.54	0.9995
5α-Androst-16-en-3-one	272[M]$^+$	20.17	10.63	0.9999
4,16-Androstadien-3-one	270[M]$^+$	25.63	8.55	0.9986
5α-Dihydrotestosterone (TMS)	347[M-15]$^+$	16.34	4.95	0.9984
Testosterone (TMS)	360[M]$^+$	14.72	5.84	0.9994

[a]Calibration lines, determined by linear regression analysis are expressed as $y = Ax + B$, where y = area ratio of steroid peak to its internal standard, 5β-androstan-3β-ol (TMS), 5α-androstanone or 19-nortestosterone (TMS), x = amount of steroid in sample (ng), A = slope, B = intercept, and r = correlation coefficient. Oxo groups were not derivatized.

[M-90]$^+$, which arose by loss of the trimethyl silanol group from the molecular ion [1]. 5α-Androstan-3-one was shown to be a suitable standard for the 16-androsten-3-ones [1].

GC characteristics

Characteristic mass spectral ions of 16-androstenes derivatives have been reported previously [1]. The GC RTs and mass spectral characteristics of three C_{19} steroids, together with the internal standard 19-nortestosterone, are summarized in Table 1. Testosterone, epitestosterone, 5α-DHT and their derivatives were generally resolved from each other by the conditions used. In only one case, 5α-DHT-3-methyl oxime-17β-TMS ether, were the *syn*- and *anti*- forms of the methoximes resolved, but the two isomers of the pentafluorobenzyl (PFB) oximes of all steroids examined were readily separable. 19-Nortestosterone proved to be an acceptable internal standard for quantification of the steroids in Table 1 because it does not occur endogenously in human semen and its derivatives had RTs similar, though not identical, to those of the steroids of interest.

Principal mass spectral ions

Inspection of the data in Table 1 shows that many of the steroids examined, whether derivatized or not, show a molecular ion which occurs either in appreciable abundance or as the base peak. Notable exceptions to this, are the PFB oximes-TMS ethers which give a [M]$^+$ ion of low abundance with, in some cases, an abundant [M-197]$^+$ ion, which arises as a result of the loss of the PFB oxime group from the molecular ion. The 3-oxo-17-TMS ether derivatives generally showed an abundant [M]$^+$ ion (although for 5α-DHT-TMS ether, the [M]$^+$ ion intensity is only 22% of the base peak). Three other prominent ions were noted in several instances, *viz*: [M-15]$^+$, [M-90]$^+$ and [M-15-90]$^+$ (but not shown in Table 1), consistent with the loss of a methyl group and/or trimethylsilanol group, respectively. The [M]$^+$ ion, together with two of these three possible ions, were found to be useful in SIM (Table 4).

Characteristics of calibration lines for 16-androstenes and androgens

The data obtained are summarized in Table 2. In some cases, e.g. 5α-androst-16-en-3-one and 4,16-androstadien-3-one, the intercepts for the standard lines were significantly different from zero. However, the concentrations of all the androst-16-enes were above the lowest standard (5 ng). The correlation coefficients of all lines were close to 1.000, indicating that the standard error of the slope was close to zero.

Qualitative and quantitative analysis of steroids in the human semen pools A and B, respectively

Table 3 summarizes the GC RTs of several putative 16-androstenes and 5α-androstan-3β-ol (as TBDMS ethers) in semen pool **A**, to which no internal standard had been added. SIM was carried out, monitoring ions at m/z 329, 331 and 333 for 5,16-androstadienol, 3α(β)-16-androstenols and the saturated steroid 5α-androstan-3β-ol. These ions all arise from loss from the molecular ion of the tert-butyl group from the TBDMS ethers [1]. Comparison of the RTs of steroids extracted from this semen pool with those of the corresponding authentic steroids provided some evidence for identification of these steroids in pool **A**.

Table 3. Preliminary identification of C_{19} steroids in human semen pool A by comprison of retention times of isolated steroids to those of authentic steroids

Steroid	GC retention time: Isolated steroid[a]	GC retention time: Standard steroid[a]	Ion monitored
5α-Androst-16-en-3α-ol	420	420	331[M-57]$^+$
5,16-Androstadien-3β-ol	462	462	329[M-57]$^+$
5α-Androst-16-en-3β-ol	463	464	331[M-57]$^+$
5α-Androstan-3β-ol	482	482	333[M-57]$^+$

[a]Steroids analysed by GC as TBDMS (tert-butyldimethylsilyl) ether derivatives.

Table 4. Quantification of C_{19} steroids in human semen

Steroid derivative	Relative retention time[a] Isolated steroid	Standard steroid	Ions monitored[b]	Quantity (ng/ml)
5α-Androst-16-en-3α-ol (TMS)	0.959	0.959	346[M]$^+$; 241[M-90-15]$^+$	0.7
5,16-Androstadien-3β-ol (TMS)	1.043	1.044	344[M]$^+$; 254[M-90]$^+$	0.6
5α-Androst-16-en-3β-ol (TMS)	1.048	1.049	346[M]$^+$; 241[M-90-15]$^+$	0.5
5α-Androstan-3β-ol (TMS)	1.090	1.091	333[M-15]$^+$; 258[M-90]$^+$	0.1
5β-Androstan-3β-ol (TMS)	1.000	1.000	258[M-90]$^+$243[M-90-15]$^+$	IS
5α-Androst-16-en-3-one	0.967	0.968	272[M]$^+$; 257[M-15]$^+$	0.7
4,16-Androstadien-3-one	1.055	1.058	270[M]$^+$; 255[M-15]$^+$	0.9
5α-Androstan-3-one	1.028	1.029	274[M]$^+$; 202	IS
5α-Dihydrotestosterone (TMS)	1.761	1.765	347[M-15]$^+$; 257[M-15-90]$^+$	0.3
Testosterone (TMS)	1.927	1.931	360[M]$^+$; 270[M-90]$^+$	0.5
19-Nortestosterone (TMS)	1.795	1.797	346[M]$^+$; 256[M-90]$^+$	IS

The ketonic groups were underivatized; IS = internal standard.
[a]Retention times relative to that of 5β-androstan-3β-ol (TMS) (15.485 min = 1.000).
[b]The first ion listed was used for quantitation. The second ion was used for further confirmation of identification by ensuring that the retention times of both ions were identical.

Table 4 summarizes the RTs on GC, the ions monitored and the quantities found in human semen pool **B** of 16-androstenes, 5α-androstan-3β-ol and two other C_{19} steroids. Figures 1–3 show reconstructed ion chromatograms for the various steroids of interest and these data provide further evidence to support the presence of several 16-androstenes at concentrations in the range 0.5–0.9 ng/ml, of a trace of 5α-androstan-3β-ol (0.1 ng/ml) and of 5α-DHT and testosterone (0.3 and 0.5 ng/ml, respectively). On the assumption that the internal standard ion fragment is not contaminated, the concentrations given in Table 4 represent the maximum amounts of the steroids examined. In all probability the actual concentrations may be lower since, as shown in Fig. 2(b), the ion at *m/z* 272 does not occur as a symmetrical peak. In view of the very low concentrations of the steroids examined and of the extreme difficulty in isolating and purifying them from a complex biological matrix at such low concentrations, we believe that it may be necessary to exercise caution in the interpretation of the quantitative

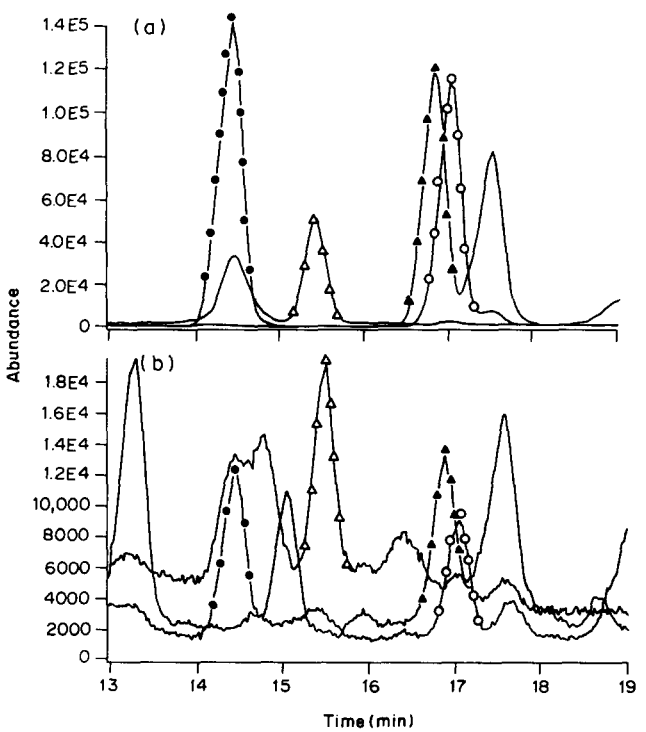

Fig. 1. SIM of TMS ethers of (a) authentic steroids: 5α-androst-16-en-3α-ol (●), and -3β-ol (○) at *m/z* 346 ([M]$^+$), 5,16-androstadien-3β-ol (▲) at *m/z* 344 ([M]$^+$) and 5β-androstan-3β-ol (△) at *m/z* 258 ([M-90]$^+$), internal standard; and (b) Steroids in extracts from semen pool **B**. The ketonic functions were underivatized.

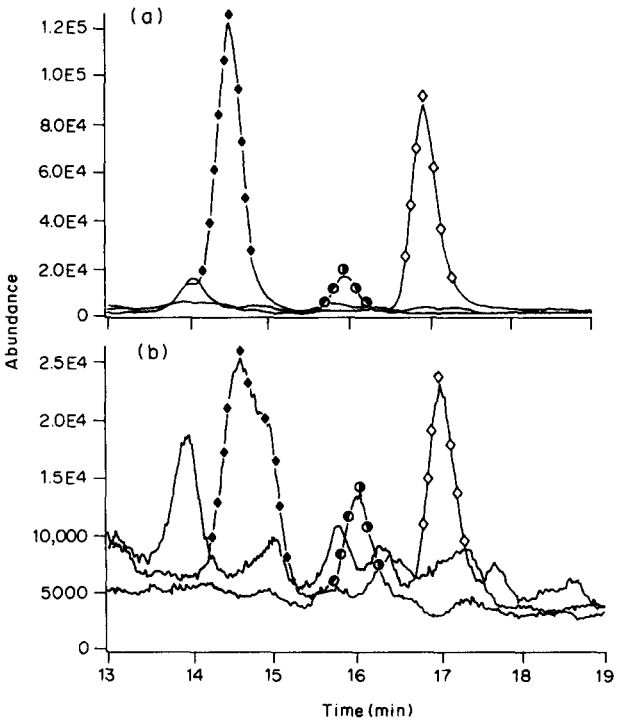

Fig. 2. SIM of (a) underivatized authentic steroids: 5α-androst-16-en-3-one (◆) at m/z 272 ($[M]^+$), 4,16-androstandien-3-one (◇) at m/z 270 ($[M]^+$) and 5α-androstan-3-one (◑ at m/z 274 ($[M]^+$), internal standard; and (b) steroids in extracts from semen pool **B**.

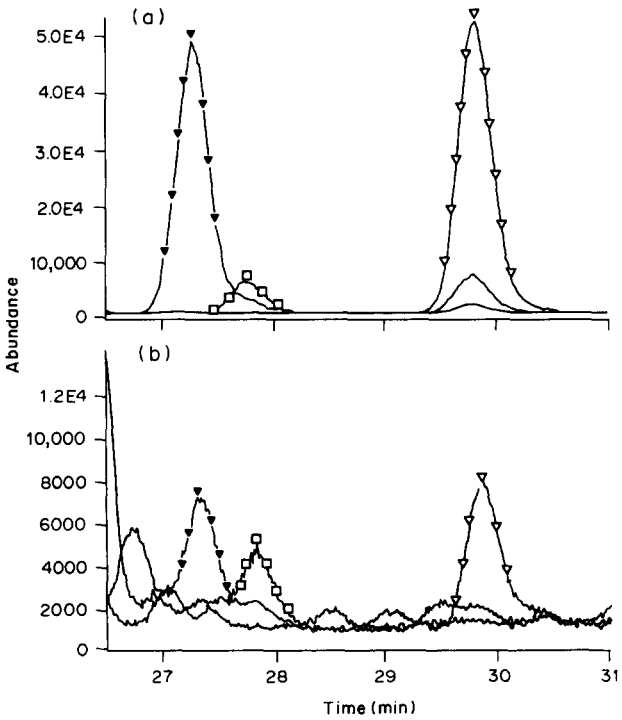

Fig. 3. SIM of TMS ethers of (a) authentic steroids: 5α-DHT (▼) at m/z 347 ($[M-15]^+$), testosterone (▽) at m/z 360 ($[M]^+$) and 19-nortestosterone (□) at m/z 346 ($[M]^+$), internal standard; and (b) steroids in extracts from semen pool **B**. The ketonic functions were underivatized.

data. Indeed, this view is supported by the results of Reiffsteck *et al.* [21], who obtained a lower value for testosterone in human seminal plasma. These studies demonstrate the value of stable isotope dilution MS in the measurement of very low concentrations of steroids. Unfortunately, as deuterated 16-androstenes are not available commercially, it was necessary to use non-isotopic internal standards.

The presence of the 16-androstenes and androgens in semen seems to be consistent with the testicular origin of these steroids in humans. For testosterone, this is well-documented, e.g. [15], while there is accumulating evidence for the testicular biosynthesis of 5,16-androstadienol and other 16-androstenes [15–18]. There are earlier reports of androgens in human semen [20] but, to our knowledge, the suggested occurrence of the steroid, tentatively identified as 5α-androstan-3β-ol in pool A, has not been reported before and its significance is entirely unknown.

Present evidence suggests that some of the odorous 16-androstenes may have important pheromonal effects in humans [19, 25]. For example, exposure to the odour of 5α-androst-16-en-3α-ol appears to cause alteration of judgement in women [26], while the odour of 5α-androst-16-en-3-one has been shown to affect choice of location in the presence of other people [27]. In the context of the present work, the effects of the odour of male axillary sweat extract in causing changes in menstrual cycle length in human female volunteers may be relevant [28]. Although the active principle(s) in the sweat extracts is not yet firmly established, the odorous 16-androstenes present [9–12] may be candidates. However, whether these steroids, now quantified in human semen, could be absorbed through the vaginal wall by analogy with the absorption of substances through the buccal or nasal epithelium [29], is not known at present, but in view of the seemingly important effects in humans of some of these steroids, these possibilities warrant further investigation.

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