Research Article

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Analysis of non-ketoic steroids 17α-methylepithiostanol and desoxymethyltestosterone in dietary supplements

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Dietary supplements containing 17α-methyl-2,3-epithio-5α-androstane-17β-ol (17α-methylepithiostanol), which is a 17-methylated analogue of epithiostanol or a prodrug of desoxymethyltestosterone (17α-methyl-5α-androst-2-en-17β-ol), have recently appeared on the Internet. 17α-Methylepithiostanol and desoxymethyltestosterone are classified as prohibited substances on the World Anti-Doping Agency (WADA) list. Two preparations, EPISTANE™ and P-PLEX™, were obtained from the Internet so that their contents could be investigated. This study involved gas chromatography/mass spectrometry (GC/MS) analysis after trimethylsilyl (TMS) derivatization, liquid chromatography/mass spectrometry (LC/MS) in atmospheric pressure photoionization (APPI) mode and nuclear magnetic resonance (NMR) spectroscopy. Analysis using LC/MS in APPI mode would be a useful tool for detecting heat-labile and non-polar steroids.

Although the labelling of EPISTANE™ indicates that it contains 17α-methyl-2α, 3α-epithio-5α-androstane-17β-ol only, 17α-methyl-2β,3β-epithio-5α-androstane-17β-ol and desoxymethyltestosterone were identified in the supplement. The results showed that P-PLEX™ contained desoxymethyltestosterone and its isomer 17α-methyl-5α-androst-3-en-17β-ol. Urine samples can be screened after EPISTANE™ or P-PLEX™ administration using the normal screening procedure for anabolic steroids with GC/MS.

Keywords: doping; desoxymethyltestosterone; 17α-methylepithiostanol; supplement; atmospheric pressure photospray ionization; mass spectrometry

Introduction

Dietary supplements can be contaminated with stimulants or anabolic steroids that are not declared on their labels. Health concerns over the effects of these supplements continue to surface.[1] The use of such dietary supplements by elite athletes may result in inadvertent doping. In contrast, the use of designer steroids sometimes occurs to avoid the target doping test. In 2003, an anabolic steroid, norbolethone, which is not produced by pharmaceutical companies, was detected in a doping control urine sample taken in 2002.[2] The tetrahydrogestrinone (THG) scandal was brought to light by Catlin *et al*. in 2004.[3] Tetrahydrogestrinone is a chemically modified steroid – a so-called 'designer steroid' – synthesized from gestrinone to avoid target doping analysis. In sports doping testing, gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) have become powerful tools to identify steroids in dietary supplements.[4–6] Several research groups have identified steroids in dietary supplements, such as androst-4-ene-3,6,17-trione (6-OXO),[7,8] 17α-methyl-5α-androst-2-en-17β-ol (desoxymethyltestosterone, Madol),[9] 1-androgens (1-testosterone, 1-androstenediol *etc.*),[10] 6α-methylandrostenedione[11] or androsta-1,4,6-triene-3,17-dione.[12] In 2008, adverse analytical findings of methyltrienolone (17α-methyl-estra-4,9,11-trien-17β-ol-3-one), which was developed in the 1960s,[13] were reported by several anti-doping control laboratories prior to the Beijing Olympic Games.[14,15] These steroids are not new but have never been approved for human use and there is little detailed information about them, such as information about their side effects.[11,12,16] In 2009, 17β-hydroxyandrostano[3,2-c]isoxazole and 17β-hydroxyandrostano[2,3-d]isoxazole were identified in a supplement labelled with the wrong steric composition.[17]

These non-licensed and undeveloped toxic steroids tend to be marketed on the Internet. Diel *et al*. reported that desoxymethyltestosterone is a powerful anabolic steroid with serious toxic side effects.[16] Moreover, desoxymethyltestosterone is expressly listed as a prohibited steroid defined in section S1 on the WADA Prohibited list.[18] Nevertheless, dietary supplements containing desoxymethyltestosterone are still legally available on the Internet. In 2008, Eenoo *et al*. reported that the supplement 'Hemaguno' possibly contained desoxymethyltestosterone(17α-methyl-5α-androst-2-en-17β-ol) and 17α-methyl-5α-androstan-17b-ol (the reduced steroid of desoxymethyltestosterone). His research group was using GC/MS method.[19] As shown in Table 1, many dietary supplements that are available on the Internet contain '17α-methylepithiostanol' and 'desoxymethyltestosterone', if the labels are correct (Table 1). The aim of this study was to investigate whether the content of two typical dietary supplements, 'EPISTANE™' and 'P-PLEX™', was correct.

The steroids were characterized by GC/MS, LC/MS and NMR. Although LC/MS using electrospray ionization (ESI) in steroid analysis is a powerful tool, the method has failed to reliably ionize neutral compounds or non-ketoic steroid desoxymethyltestosterone.[20] Robb *et al*. reported that atmospheric pressure photoionization

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Table 1. Dietary supplements containing 17α-methyl-2α,3α-epithio-5α-androstane-17β-ol and desoxymethyltestosterone

Declared steroid	Brand name	Manufacture
17α-methyl-2α,3α-epitio-5α-androstane-17β-ol	EPISTANE	Innovative Body Enhancement LLC
	HAVOC	Recomp Performanc Nutrition Inc.
	Epithin-E	Generic Labz
	Methyl-E	Engineered Sports Technology
	HEMAGUNO	Spectra Force Research
	OXODROL PRO	IDS
	KRYPTONITE	Super Hero
	Epidrol	Hardcore Formulations
	Hemobolin-250	Pharma Resources
	Epi-Max	Anabolic Formulation
	Epiotren	Intense Nutraceuticals
	CynergE	BCS Labs.
	E-Stane	Competitive Edge Labs
	Supremacy	Maximus Labs
	JACKED	Generation X Labs
	Spawn	Myogenix
Desoxymethyltestosterone	P-PLEX	Competitive Edge Labs
	P-MAX	Growth Labs
	Phera-Plex	Anabolic Xtreme
	Ergo Max	Anabolic Xtreme
	Phera-MAX	Generic Labz
	Phera-Bol	Juggernaut Nutrition
	Phera-50	Generic Edge Technologies

(APPI) is especially superior to atmospheric pressure chemical ionization (APCI) or ESI in ionization less polar compounds.[21] This paper also describes the advantage of detecting non-ketoic steroids using LC/time-of flight mass spectrometry (LC/TOFMS) in APPI mode.

Experimental

Chemicals and reagents

All reagents were of analytical or HPLC grade. Acetonitrile (99.8%, CH_3CN), ethanol (99.5%), methanol (99.8%, CH_3OH), toluene (99.5%), diethyl ether (99.5%), acetic acid (99.7%, CH_3COOH), potassium carbonate (99.5%, K_2CO_3), sodium sulfate (99%, Na_2SO_4), sodium hydroxide (96%, NaOH), disodium hydrogen phosphate (99%, Na_2HPO_4) and sodium dihydrogenphosphate dihydrate (99–102%, $NaH_2PO_4{\cdot}2H_2O$) were purchased from Kokusan Chemical Co., Ltd (Tokyo, Japan). Hydrochloric acid (35–37%, HCl) was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). β-Glucuronidase from *E. Coli* K12 (140 U/mL) was from Roche Diagnostics GmbH (Mannheim, Germany). Supelco Amberlite XAD-2 (300 mg/3 mL) was purchased from Sigma (St Louis, MO, US). Ultra-pure water was prepared using a Milli-Q Ultra pure system (Millipore, Bedford, MA, US). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was from Macherey-Nagel (Düren, Germany). Iodotrimethylsilan (97%, TMS-iodo) was from Sigma-Aldrich, Inc. (St Louis, MO, US). Dithioerythritol (DTE) was from Nacalai Tesque, Inc. (Kyoto, Japan).

17α-Methyltestosterone was purchased from Sigma-Aldrich, Inc. (St Louis, MO, US). The reference standard of desoxymethyltestosterone (containing its Δ-3,4 isomer) was a kind gift from the WADA-accredited doping control laboratory in Montreal, Canada (Laboratoire de contrôle du dopage INRS – Institut Armand-Frappier).

Dietary supplements

Two dietary supplements, EPISTANE™ (Lot I05284; Innovative Body Enhancement L.L.C., www.ibe-technology.com) and P-PLEX™ (Lot 080107; Competitive Edge Labs, L.L.C., Phoenix, AZ, USA), were obtained on the Internet. EPISTANE™ was labelled 'ingredients: 2,3a-epithio-17a-methyletioallocholan-17b-ol: 10 mg'. P-PLEX™ was labelled: 'ingredients: 17a-methyl-etioallocholan-2-ene-17b-ol 15 mg'.

Supplement analysis

The powders in 10 capsules (average mass: 0.34 g/capsule for EPISTANE™, 0.24 g/capsule for P-PLEX™) were suspended in 15 mL methanol. After shaking for 5 min and centrifuging for 5 min at 1000 *g*, the methanolic layer was separated using a new glass tube. The aliquot was evaporated to dryness under a nitrogen (N_2) stream. Six mL of 0.2 M NaOH were added to the residue and the analytes were extracted with 5 mL diethylether, followed by washing with 0.2 M HCl and distilled water. The ether layer was evaporated to dryness under an N_2 stream. For GC/MS analysis, the extracted analytes were dissolved in 50 µL MSTFA/TMS-iodo/DTE (10 mL/20 µL/20 mg) and heated at 60 °C for 15 min. For LC/MS analysis, the extracted analytes were dissolved in 100 µL CH_3COOH (1%)/CH_3CN (90 : 10, v/v).

Mass spectrometry

GC/MS analysis

The GC/MS system was an Agilent 6890N/5973 inert mass selective detector equipped with an Ultra-1 capillary column, length 17 m, 0.25 mm internal diameter, 0.11 µm film thickness (Agilent Technologies, Palo Alto, California, US). The GC temperature was set at 180 °C (hold 1 min) and was increased by 3 °C/min to 229 °C, and then by 40 °C/min to 300 °C (hold 2 min). The injection volume was 2 µL in split mode (11 : 1). The carrier gas was helium at 12 psi. The injector temperature was set at 280 °C. The interface temperature was set at 300 °C. Electron ionization (EI) was accomplished at 70 eV. EI source temperature was set at 230 °C. For cyclic scan analysis, the mass range varied from *m/z* 50 to 500 and the scan rate was 2.3/s. The instrument was calibrated prior to analysis using perfluorotributylamine (PFTBA).

LC/APPI-TOFMS analysis

The LC/TOFMS system was an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a QSTAR XL MS/MS system (Applied Biosystems, California, US). The analytical column was a Supelco Discovery C_{18} (4.0 mm × 50 mm), and the mobile phases were 1% CH_3COOH (mobile phase A) and CH_3CN (mobile phase B). The column oven temperature was 25 °C and the flow rate was 0.25 mL/min.

A gradient elution was as follows: 65% A, linear to 10% A in 10 min, followed by an increase to 65% A in 0.1 min. Finally, the column was equilibrated for 5 min, and the total runtime was

16 min. Ionization was accomplished using APPI in positive mode. The nebulizing temperature was 400 °C and the ion source voltage was set at 1300 V. Declustering potential and focusing potential were set at 50 V and 250 V, respectively. N_2 gas was employed as nebulizer gas (3.94 L/min) and auxiliary gas (2.00 L/min). Toluene was employed as dopant (20 µL/min). The TOF mass range varied from *m/z* 140 to 600. The mass resolution was 10 000. For product ion scan analysis using collision-induced dissociation (CID), the precursor ions were *m/z* 321 (collision energy: 35 eV, collision gas: N_2) and 271 (collision energy: 30 eV, collision gas: N_2), respectively. Maintenance was performed by manufacturer once every six months, and the calibration sample was polypropylene glycol (PPG). Daily calibration was performed prior to analysis using caesium iodide (CsI) and sex hormone inhibitor (iPD1) in accordance with the manufacture's instruction.

Nuclear magentic resonance spectroscopy

For NMR analysis, the supplement extracts were dissolved in 700 µL chloroform-d_1 ($CDCl_3$). ^{1}H-NMR and ^{13}C-NMR were measured on an Inova-500 (Varian Inc., Palo Alto, California, US) using $CDCl_3$ as a solvent and tetramethylsilane as an internal standard. ^{1}H-NMR and ^{13}C-NMR were operated at 500 MHz using an acquisition time of 10 s (16 repetitions) and at 125 MHz using an acquisition time of 3 s (3200 repetitions), respectively. All NMR measurements were performed in 5 mm NMR sample tubes at 25 °C. Chemical shifts were expressed in δ-values, which are given relative to tetramethylsilane signal as an internal standard.

Analysis of urine sample after administration of supplements

Two spot urine samples were obtained from healthy male volunteers who reported taking these supplements regularly. In accordance with the Ethics Guidelines for Clinical Studies (issued by the Ministry of Health, Labour and Welfare, Japan), approval was obtained from the Planning and Coordination Department, Mitsubishi Chemical Medience Corporation on human investigation (approval No. 2009-B012). Two male volunteers (subject A: 51 years old and subject B: 40 years old) were informed in advance of the details of the study and written consent was obtained. Based on their information, the urine sample obtained from subject A was collected 12 hours after the previous ingestion of two capsules of EPISTANE™, and the urine sample obtained from subject B was 10 hours after the previous ingestion of two capsules of P-PLEX™. No other medicines or supplements were taken during sample collection. Urine samples were prepared according to our standard operating procedure based on a steroid-screening method described by several researchers.[22,23] After centrifugation, 3 mL urine sample and 50 µL internal standard in ethanol (10 µg/mL 17α-methyltestosterone) were applied to a solid-phase extraction cartridge (XAD-2, 300 mg/3 mL). After washing with 2 mL distilled water, the analyte was eluted with 2 mL CH_3OH. The extract was evaporated to dryness under an N_2 stream at 50 °C. The residue was added to 1 mL 0.2 M phosphate buffer (pH 7.0) followed by the addition of 25 µL β-glucuronidase from *E. coli* K12 solution and the mixture was heated at 50 °C for 60 min. After the addition of 7% K_2CO_3 solution, target steroids were extracted with 5 mL diethylether. The organic layer was evaporated to dryness under N_2 stream at 60 °C, and then the dried residue was dissolved in 50 µL MSTFA/TMS-iodo/DTE (10 mL/20 µL/20 mg) and was heated at 60 °C for 15 min. A volume of 2 µL mixture was injected into the GC/MS system. For GC/MS in selected ion monitoring (SIM) analysis, the monitoring ions of dihydro dihydroxy-desoxymethyltestosterone-tris-TMS were *m/z* 143, 269 and 523, and each dwell time was set at 10 ms.

Results and Discussion

GC/MS analysis

EPISTANE™

The main peak (peak-1 of Figure 1) and the minor peak (peak-2 of Figure 1) were detected in EPISTANE™ extract by GC/EI-MS. The retention time of peak-1, peak-2 and 17α-methyltestosterone-bis-TMS as internal standard were 8.0 min, 16.8 min and 15.3 min, respectively. The typically observed ions of peak-1 were at *m/z* 360 (M^+), 345 ($M^+ - CH_3\cdot$), 270 (M^+ − TMSOH), and 143. The typically observed ions of peak-2 were at m/z 392 (M^+), 377 ($M^+ - CH_3\cdot$), 360 (M^+ − S), 345 (M^+ − S − $CH_3\cdot$) and 143 (Figure 2). The most abundant ion at *m/z* 143 was a characteristic D-ring fragment ion generated from TMS ether of a 17-methylated-17-hydroxysteroid.[5] The result of peak-1 agreed with that of the reference standard of desoxymethyltestosterone (Figure 2). Peak-2 was suggested to be 17α-methylepithiostanol-mono-TMS. The absolute intensity of peak-2 was 25% of that of peak-1 (total ion chromatogram). No 17α-methyl-5α-androst-3-ene-17β-ol-mono-TMS (Δ-3,4 isomer of desoxymethyltestosterone) was detected. In addition, Eenoo *et al*. reported that 17α-methyl-5α-androstan-17β-ol mono-TMS (*m/z* 362) could be detected close to the peak of desoxymethyltestosterone-mono-TMS in supplement 'Hemaguno'™;[19] however, it could not be detected in the extract of EPISTANE™.

P-PLEX™

In the P-PLEX™ extract, desoxymethyltestosterone-mono-TMS (peak-3 of Figure 1) was detected as the main peak and its Δ-3,4 isomer 17α-methyl-5α-androst-3-ene-17β-ol-mono-TMS (peak-4 of Figure 1) was detected as the minor peak by GC/EI-MS. The retention times of peak-3, peak-4 and 17α-methyltestosterone-bis-TMS as internal standard were 8.0 min, 8.1 min and 15.3 min, respectively. The typical observed ions of peak-3 were at *m/z* 360 (M^+), 345 ($M^+ - CH_3\cdot$), 270 (M^+ − TMSOH) and 143. The typical observed ions of peak-4 were at *m/z* 360 (M^+), 345 ($M^+ - CH_3\cdot$), 270 (M^+ − TMSOH), and 143 (D-ring fragment). The absolute intensity of peak-4 was 19% of that of peak-3 (total ion chromatogram). The result agreed with that of the reference standard, and the presence of Δ-3, 4 isomer was suggested to be a by-product synthesized from 5α-androst-2-en-17-one or epiandrosterone.[9,24]

LC/TOFMS in APPI mode

EPISTANE™

The elemental composition was confirmed by high resolution and high accuracy mass spectrometry using a QSTAR XL TOFMS system in APPI mode. Two peaks were detected in the EPISTANE™ extract (Figure 3). The retention time of peak-5, peak-6 and 17α-methyltestosterone as an internal standard were 9.5 min, 11.5 min and 4.9 min, respectively. The peak-5 was suggested to be a protonated 17α-methylepithiostanol ($C_{20}H_{33}OS$). As shown in Figure 4, the typical observed ions of peak-5 were at *m/z* 321 (MH^+), 303 ($MH^+ - H_2O$) and 269 ($MH^+ - H_2O - H_2S$). The most abundant ion was neutral loss of water at *m/z* 303.

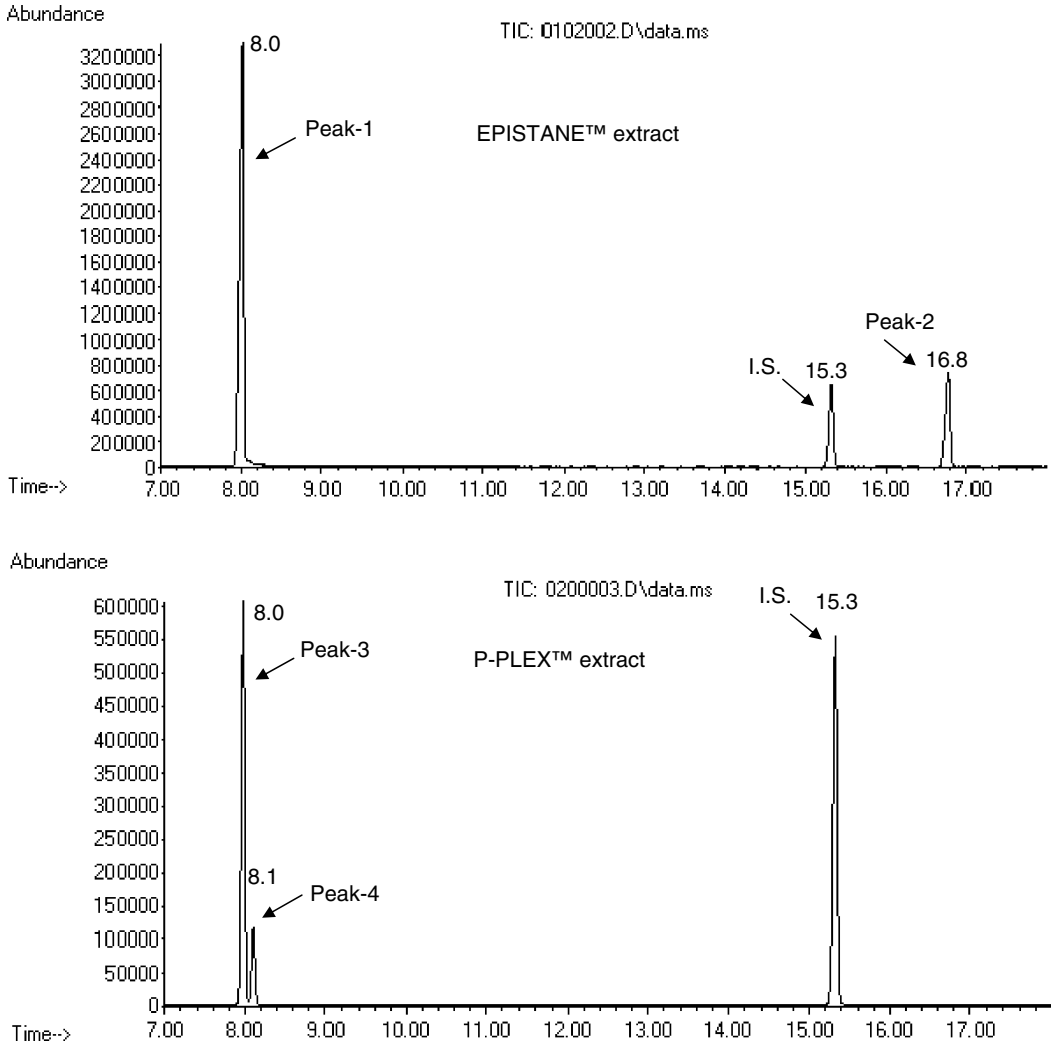


Figure 1. Total ion chromatograms of EPISTANE™ extract (*upper*) and P-PLEX™ extract (*lower*) by GC/EI-MS after TMS derivatization.

The accurate mass and elemental composition of peak-5 were determined ($C_{20}H_{33}OS$, 321.2253, theoretical mass: 321.2252, error: 0.31 ppm). Also, desoxymethyltestosterone (peak-6 of Figure 3) were successfully detected by APPI mode. As shown in Figure 4, the typical observed ions of peak-6 were at *m/z* 271 ($MH^+ - H_2O$) and the protonated ion could not be observed. The accurate mass and elemental composition of peak-6 ($MH^+ - H_2O$) were determined ($C_{20}H_{31}$, 271.2427, theoretical mass: 271.2426, error: 0.37 ppm). The results of peak-6 agreed with those of the reference standard of desoxymethyltestosterone. The absolute intensity of peak-6 was 20% that of peak-5 (TIC chromatograms). The product ion spectra of the extract in the CID experiment are shown in Figure 5. Product ions generated from ion *m/z* 321 (MH^+ of 17α-methylepithiostanol) were also observed at *m/z* 303 ($MH^+ - H_2O$) and 269 ($MH^+ - H_2O - H_2S$). The product ions generated from ion *m/z* 271 ($MH^+ - H_2O$ of desoxymethyltestosterone) were observed at *m/z* 215 (A,B,C-ring fragment) and several product ions separated by methylene units (14 Da) were observed, and the results agreed with those of reference standard of desoxymethyltestosterone. As mentioned above, the most abundant peak on GC/MS analysis was desoxymethyltestosterone; however, the LC/MS result differed from the GC/MS result. We also reported that epithiostanol (2α,3α-epithio-5α-androstane-17β-ol) is pyrolized to 5α-androst-2-en-17β-ol during GC/MS analysis;[25] therefore, our interpretation is that thiosteroid (peak-5) might be pyrolized to desoxymethyltestosterone by dethionylation during derivatization or on the GC/MS injection port (Scheme 1).

P-PLEX™

The elemental composition was confirmed by high-resolution and high-accuracy mass spectrometry using a QSTAR XL TOFMS system in APPI mode. As shown in Figure 3, desoxymethyltestosterone was successfully detected in APPI mode as in EPISTANE™ (peak-7). The results of peak-7 agreed with those of the reference standard of desoxymethyltestosterone (data were not shown). The GC/MS results showed the Δ-3,4 isomer; however, no other peaks were found. This was probably because of insufficient separation on the LC column in this study.

Nuclear magnetic resonance spectroscopy

EPISTANE™

Nuclear magnetic resonance analysis was conducted to investigate the steric isomer of the A-ring episulfide. Comprehensive investigation of ^{1}H-NMR spectra of steroidal episulfides has been

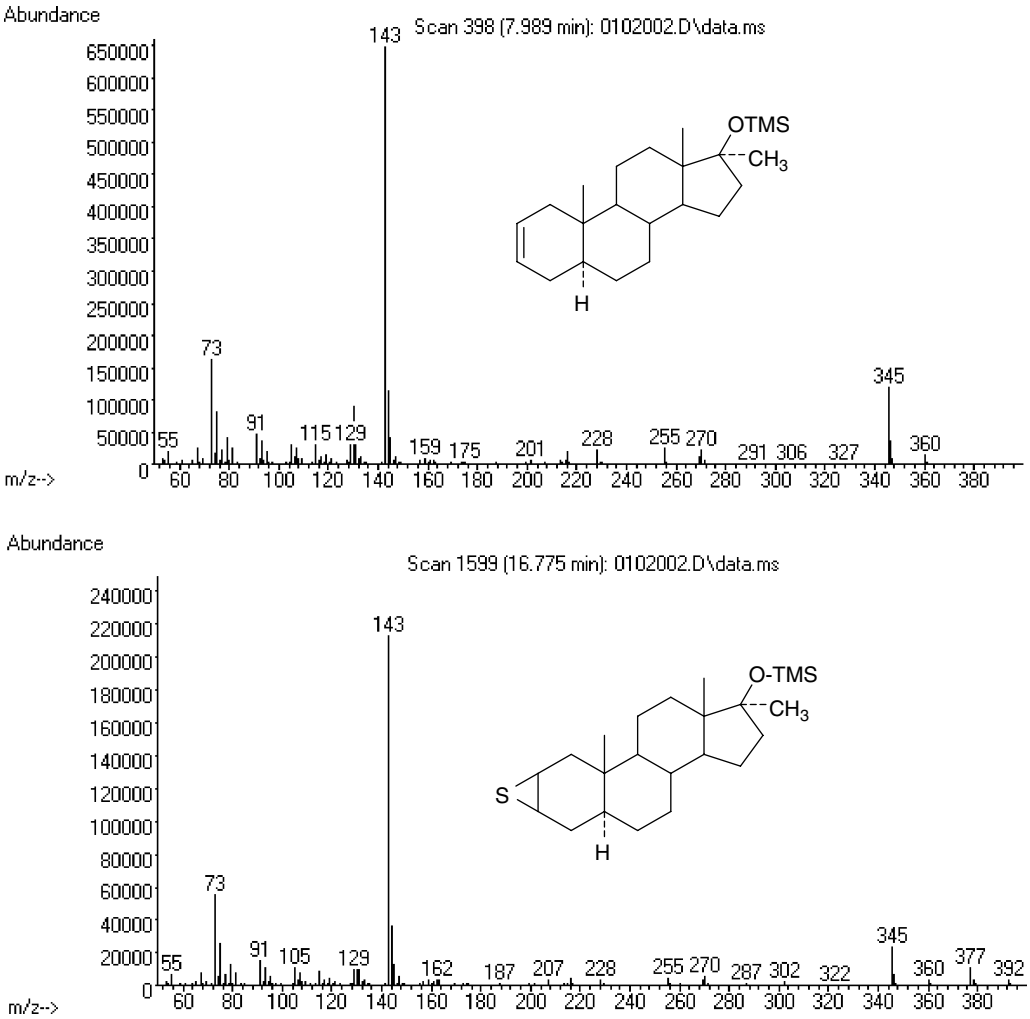
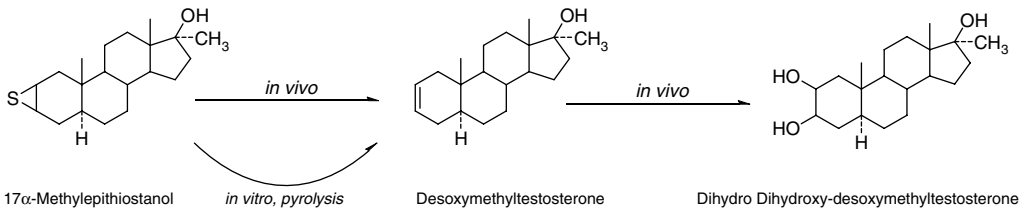


Figure 2. GC/EI-MS spectra of EPISTANE™ extract *(upper: desoxymethyltestosterone-mono-TMS, lower: 17a-methylepithiostanol-mono-TMS)*.



Scheme 1. Proposed pathway of pyrolysis and metabolism of 17α-methylepithiostanol.

reported.[26] A mulitiplet signal at δ 3.2 ppm was observed and the splitting patterns were similar to the 2β,3β-isomer of episulfides. This appeared to be an episulfide in the 2β,3β-position but it was not identified and remained unclear. Tori and Komeno reported that a bulkier substituent exerts stronger steric γ- and δ-effects and appears to be useful in ^{13}C-NMR spectroscopy, and differences in chemical shift on the ^{13}C-NMR were observed between epimeric pairs of steroidal A-ring episulfides.[27] The ^{13}C-NMR data generated from the EPISTANE™ extract were compared with the values obtained by Tori and Komeno, who examined 2α,3α-epithio-5α-androstan-17β-yl acetate and its 2β,3β-isomer.[27] As shown in Table 2, the steric γ-effect was found to be produced on carbons bearing an axial hydrogen atom by introducing episulfide rings *cis* to the hydrogen (C-5 atom). This γ-effect resulted in chemical shift of +7.8 ppm compared with that of 2α,3α-isomer. A long-range δ-effect upon C-19 and C-9 resonances was also observed. These δ-effects resulted in chemical shifts of +2.7 ppm and +2.3 ppm compared with those of 2α,3α-isomer. Therefore, the signals at δ 43.2 ppm, δ 56.3 ppm and δ 14.9 ppm generated from the extract were assigned to C-5, C-9 and C-19, respectively. The steric isomer was proposed to be a 2β,3β-isomer. In contrast, the label of EPISTANE™ clearly states '2α,3α-isomer'. The full structure of these differences should be further elucidated using the synthesized reference standards of 17α-methyl-2α,3α-epithio-5α-androstan-17β-ol and 17α-methyl-2β,3β-epithio-5α-androstan-17β-ol.[28] The full NMR data and assignment data are not shown here because no authentic reference standard was available. Also, three 3H singlet signals at δ 0.77 ppm (10-CH$_3$),

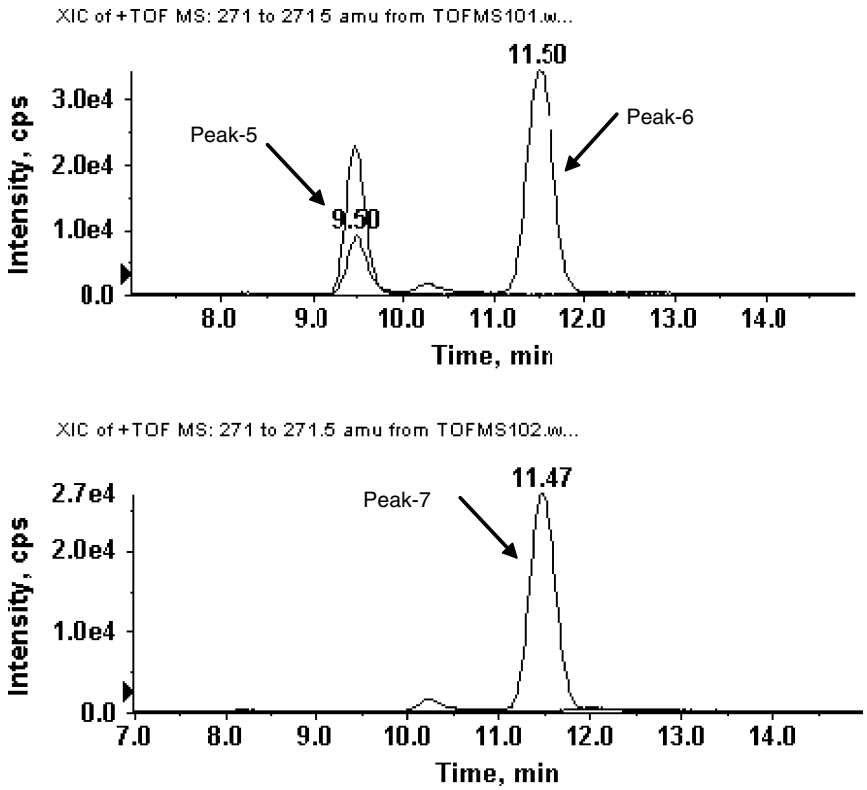


Figure 3. APPI-TOF mass chromatograms (*m/z* 271 and *m/z* 371) of EPISTANE™ extract (*upper*) and P-PLEX™ extract (*lower*) *(peak-5: 17a-methylepithiostanol, peak-6 and-7: desoxymethyltestosterone).*

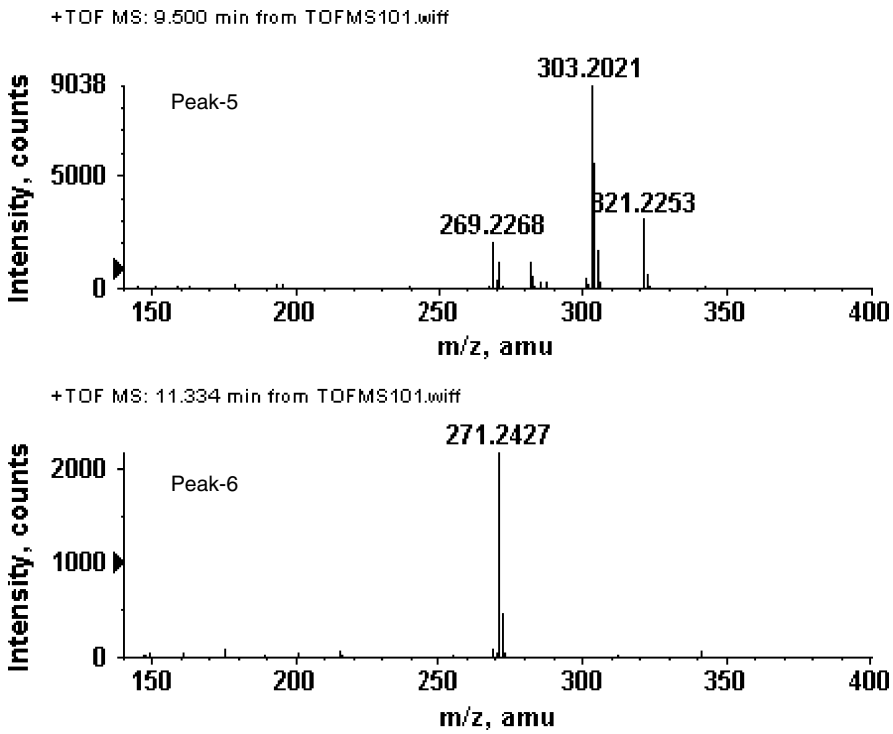


Figure 4. APPI-TOF mass spectra of EPISTANE™ extract *(upper: 17a-methylepithiostanol, lower: desoxymethyltestosterone).*

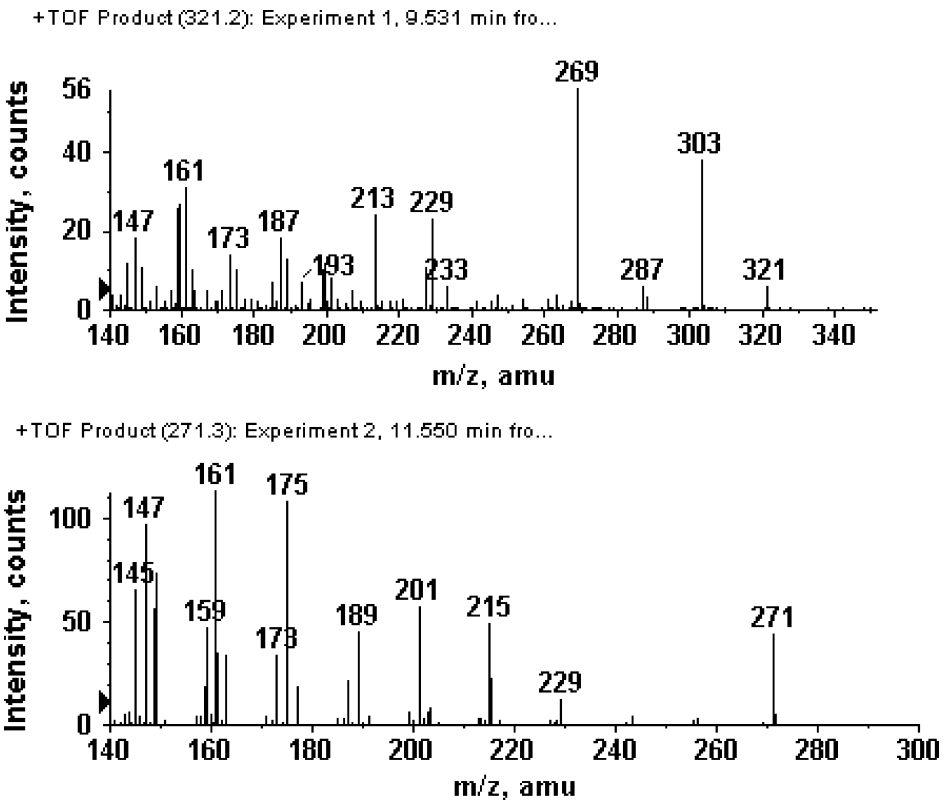
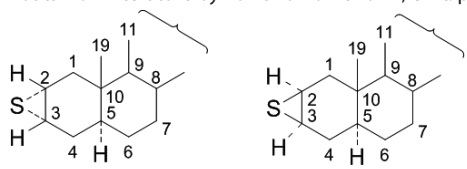


Figure 5. APPI-MS/MS spectra of EPISTANE™ extract *(upper: 17a-methylepithiostanol, lower: desoxymethyltestosterone)*.

Table 2. ^{13}C-NMR assignment of epithiosteroid in EPISTANE™ extract

Compounds	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-19
2α,3α-epitio-5α-androstane-17β-yl acetate[a]	40.3	34.6	37.6	30.5	35.4	28.1	31.0	35.0	53.6	35.2	20.2	12.6
2β,3β-epitio-5α-androstane-17β-yl acetate[a]	39.3	36.8	35.2	30.1	*43.0*	28.3	31.3	34.6	*56.1*	34.6	20.3	*14.8*
extract of EPISTANE™	39.5	37.0	35.7	30.2	*43.2*	28.3	31.6	35.5	*56.3*	34.7	20.4	*14.9*

[a] data from literature by Tori and Komeno[27], Unit: ppm



0.86 ppm (13-CH_3), 1.21 ppm (17α-CH_3) and a multiplet signals at δ 5.6 ppm (C2,C3 olefinic protons) were observed in ^{1}H-NMR analysis. In the ^{13}C-NMR spectrum, the set of signals at δ 125.89 ppm and δ 125.87 ppm was assigned to a Δ-2,3 olefinic carbon. The results agreed with those of desoxymethyltestosterone.[9]

P-PLEX™

Nuclear magnetic resonance data showed that the extract contains desoxymethyltestosterone and its Δ-3,4 isomer. In the ^{1}H-NMR spectrum, three 3H singlet signals at δ 0.77 ppm (10-CH_3), 0.86 ppm (13-CH_3), 1.21 ppm (17α-CH_3) and a multiplet signal at δ 5.6 ppm (C2,C3 olefinic protons) were observed. Similar 3H singlet signals at δ 0.79 ppm (10-CH_3), 0.87 ppm (13-CH_3), 1.22 ppm (17α-CH_3) and a multiplet signal at δ 5.3 ppm (C3,C4 olefinic protons) were also observed. In the ^{13}C-NMR spectrum, the set of signals at δ 125.89 ppm and δ 125.87 ppm was assigned to a Δ-2,3 olefinic carbon and that of signals at δ 131.34 ppm and δ 125.5 ppm was assigned to a Δ-3,4 olefinic carbon. The results agreed with the value obtained by Sekera *et al.*,[9] who examined desoxymethyltestosterone and its Δ-3,4 isomer.

Analysis of administration study urine sample

Two spot urine samples were analysed according to our routine steroid screening by GC/MS in SIM mode. The metabolism of desoxymethyltestosterone in humans consists of reduction of the double bond and hydroxylation. Eight metabolites have been reported.[29] The metabolite of desoxymethyltestosterone (dihydro dihydroxy-desoxymethyltestosterone) in tris-TMS form could

be detected in both urine samples, of EPISTANE™ and P-PLEX™ (retention time = 16.0 min). The parent steroid desoxymethyltestosterone could not be detected in either urine sample. The parent steroid, 17α-methylepithiostanol, could not be detected in the EPISTANE™-administration study urine by GC/MS and LC/TOFMS in APPI mode. It is known that epithiostanol is metabolized to olefin steroid (5α-androst-2-en-17β-ol) by oxygenation and dethionylation;[25,30] it is therefore suggested that 17α-methylepithiostanol, which is a 17-methylated analogue of epithiostanol, might be metabolized to olefin steroid desoxymethyltestosterone (Scheme 1). In EPISTANE™-administration study urine it remained unclear whether the origin of dihydro dihydroxy-desoxymethyltestosterone in urine was the metabolite of 17α-methylepithiostanol or the original desoxymethyltestosterone because EPISTANE™ contains not only 17α-methylepithiostanol but also desoxymethyltestosterone.

Conclusions

The steric composition of steroids in the supplements was characterized by GC/MS, LC/MS and NMR. The use of LC/MS in APPI mode was considered a helpful tool for detecting heat-labile thiosteroids with low proton affinity. Nuclear magnetic resonance spectroscopy was also useful for the characterization of the steric composition of episulfide. The label of EPISTANE™ indicated that it contained 17α-methyl-2α, 3α-epithio-5α-androstane-17β-ol only; however, the results showed that the non-labelled steroid desoxymethyltestosterone was also included. The supplement P-PLEX™ contained both desoxymethyltestosterone and its Δ-3,4 isomer, and the bulk or the route of synthesis might be the same as in previous literature.[9,24] On the other hand, the Δ-3,4 isomer of desoxymethyltestosterone could not be detected in EPISTANE™. This suggests that the origin of desoxymethyltestosterone in EPISTANE™ may differ from that of P-PLEX™. The overall results also showed that EPISTANE™ contained 17α-methylepithiostanol; however, its steric isomer was suspected to be the 2β,3β-isomer. For the final identification, EPSITANE™ should be further compared with the reference standard of 17α-methyl-2α,3α-epithio-5α-androstane-17β-ol. Anti-doping laboratories can reveal desoxymethyltestosterone abuse as detecting a urinary metabolite of desoxymethyltestosterone by GC/MS; however, if metabolites of desoxymethyltestosterone are detected, the possibility of metabolites of 17α-methylepithiostanol should be a concern. The bulk materials of desoxymethyltestosterone and 17α-methyl-2α,3α-epithio-5α-androstane-17β-ol are available from Xianju Green Leaf Pharmaceutical Factory (Shanghai, China, http://www.greenleafpharm.com/, accessed 22 June 2009) via the Internet. Dietary supplement suppliers probably obtain anabolic steroids from such companies. Therefore, further investigation of bulk steroids should be conducted.

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