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Identification of dehydroepiandrosterone metabolites formed from human prostate homogenate using liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry

Kuniko Mitamura[a], Takashi Nakagawa[a], Kazutake Shimada[a,*], Mikio Namiki[b], Eitetsu Koh[b], Atsushi Mizokami[b], Seijiro Honma[c]

[a]*Faculty of Pharmaceutical Sciences, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-0934, Japan*
[b]*Department of Urology, School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan*
[c]*Teikoku Hormone Mfg. Co., Ltd., 1604 Shimo-Sakunobe, Takatsuku, Kawasaki 213-8522, Japan*

Abstract

The identification of the in vitro metabolites of dehydroepiandrosterone formed from human prostate homogenate was investigated by hyphenated techniques using the stable-isotope dilution method. A mixture of dehydroepiandrosterone and [$^2H_4$]dehydroepiandrosterone was incubated with hypertrophied human prostate tissue homogenate in the presence of NAD, NADH and NADPH. The metabolites were extracted with AcOEt–hexane, purified by solid-phase extraction, and then analyzed by LC–atmospheric pressure chemical ionization MS and/or GC–MS. Androst-5-ene-3β,17β-diol (major product), androst-4-ene-3,17-dione, testosterone, 5α-dihydrotestosterone, androsterone, and 7α-hydroxydehydroepiandrosterone were identified in comparison with authentic samples based on their chromatographic behavior and mass spectra. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Androsterones; Steroids; Dehydroepiandrosterone; Testosterones

1. Introduction

During the last decade, prostate cancer has become a commonly diagnosed malignancy in males. It is well known that prostate cancer is an androgen-dependent cancer, the growth of which is promoted by androgens [1]. The major circulating androgen in the blood of males is testosterone (T) secreted from the testis, and converted into the potent androgen 5α-dihydrotestosterone (DHT) in the prostate. In humans, the adrenal glands secrete a large amount of the inactive precursor steroid dehydroepiandrosterone (DHEA). It is known that DHEA is converted into androst-4-ene-3,17-dione (AD) or androst-5-ene-3β,17β-diol (A-diol) and then into T, and further metabolized into the potent androgen DHT in peripheral tissues, including the prostate. DHT is further metabolized to androsterone (A), as shown in Fig. 1 [1–4]. Therefore, a combination therapy of surgical or medical castration using luteinizing hormone-releasing hormone agonist with antiandrogen, the so-called ''total androgen blockade'' therapy, has been used as a treatment for this disease. However, subsequent to this therapy, prostate cancer recurs and progresses to a terminal stage despite reduced circulating T. Although the mechanism of recurrence of the cancer, which is androgen independent, still remains unclear, it was found recently that A-diol,

*Corresponding author. Tel./fax: +81-76-234-4459.
E-mail address: shimada@dbs.p.kanazawa-u.ac.jp (K. Shimada).

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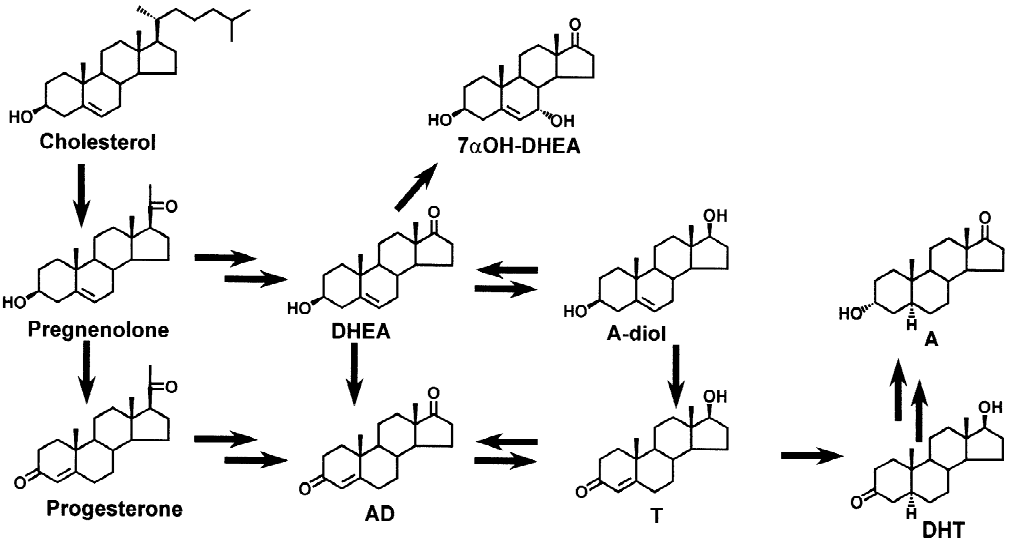

Fig. 1. Biosynthetic pathway of androgens.

without conversion to T, can activate androgen receptor target genes and that some antiandrogens fails to block completely the androgenic activity of A-diol in human prostate cancer cells [5,6]. These data show that it is important to clarify the metabolites of DHEA, especially A-diol, in the human prostate. Although the in vitro metabolites of DHEA formed with mammalian prostate homogenate have been investigated using HPLC or GC [7–9], the obtained metabolites, such as A-diol, AD, T and DHT, were identified only by their t_R values in comparison with those of authentic samples, and therefore ambiguity still remains as to their structures.

In this study, the in vitro metabolites of DHEA formed from human prostate homogenate were investigated by stable-isotope dilution LC–atmospheric pressure chemical ionization MS (LC–APCI-MS) and GC–MS to confirm the structures of these metabolites.

2. Experimental

2.1. Materials and reagents

DHEA, NAD, NADPH and NADH were purchased from Sigma–Aldrich Japan (Tokyo, Japan). AD, T, DHT, A and *O*-methylhydroxylamine hydrochloride were purchased from Tokyo Kasei Kogyo (Tokyo). [2,2,4,6-$^2H_4$]DHEA [10,11], A-diol, 7α-, 7β-hydroxydehydroepiandrosterone (7αOH-, 7βOH-DHEA) [12] were synthesized in our laboratories. 7-Oxodehydroepiandrosterone (7-oxoDHEA) and the other authentic androgens were purchased from Steraloids (Newport, RI, USA). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Wako (Osaka, Japan). Bond Elut C_{18} cartridges (500 mg) were obtained from Varian (Harbor, CA, USA).

2.2. Incubation and extraction

The prostate tissues were obtained from patients with prostatic hypertrophy, who gave informed consent, at Kanazawa University Hospital. The tissues (27.8 g) were crushed under liquid nitrogen, homogenized in 0.1 *M* Tris–HCl buffer (pH 7.4) (110 ml) and then centrifuged at 400 *g* at 4 °C for 5 min. The obtained supernatant (100 ml) was mixed with substrate {DHEA (1 mg) and [$^2H_4$]DHEA (1 mg) in 2 ml of EtOH} and cofactors [NADH (70 mg), NADPH (70 mg), NAD (70 mg)], and incubated at 37 °C for 2 h in air. The homogenate of rat prostate (Wistar, male, 10 weeks) (SLC, Hamamatsu, Japan) in 0.1 *M* Tris–HCl buffer (pH 7.4) was

deactivated with heat (80 °C, 5 min) and then used as a deactivated enzyme source.

Substrate and its metabolites were extracted with hexane–AcOEt (1:4) from the incubation mixture. After successive washings with 5% aqueous $NaHCO_3$ and water, the organic layer was dried over Na_2SO_4 and evaporated in vacuo. The obtained residue was dissolved in methanol (3 ml) and diluted with water (7 ml), and the resulting solution was divided and applied to 10 cartridges of Bond Elut C_{18}. After washing with water (1 ml), the eluates with 70% MeCN (2.5 ml) were combined and evaporated under a nitrogen gas stream.

2.3. LC–MS analysis

LC–MS was performed on a LCQ liquid chromatograph–ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) connected to a PU-980 chromatograph (Jasco, Tokyo), and APCI (positive-ion mode) was used. The ionization conditions were as follows. Ion source current, 5 μA; vaporizer temperature, 575 °C; capillary temperature, 240 °C; capillary voltage, 15 V; sheath gas flow-rate, 80 units; tube lens offset voltage, 15 V; monitoring ions, m/z 200–350 (total-ion monitoring mode). The chromatographic conditions were as follows: column, YMC-Pack ODS-AQ (5 μm, 150×6.0 mm I.D.) (YMC, Kyoto, Japan); mobile phase, MeOH–water (13:7); flow-rate, 1.0 ml/min; temperature, 40 °C.

2.4. Preparative HPLC

HPLC was performed on a LC-6A chromatograph (Shimadzu, Kyoto) equipped with a J'sphere ODS-H80 (4 μm, 150×4.6 mm I.D.) (YMC) column and an SPD-10A UV detector (220 nm) (Shimadzu). The flow-rate and the temperature were set at 1.0 ml/min and 40 °C, respectively. MeOH–water (3:2 or 4:3) was used as the mobile phase.

2.5. GC–MS analysis

O-Methylhydroxylamine hydrochloride in pyridine (2%, 0.2 ml) was added to the obtained fraction and the mixture was heated at 60 °C for 1 h. The entire solution was extracted with AcOEt and the organic layer was successively washed with 5% aqueous HCl and water, and the solvent was then evaporated

Table 1
LC–MS data for the authentic androgens[a]

Compound	M_r	t_R (min)	Ion (m/z)
7βOH-DHEA	304	5.6	287 $[M+H-H_2O]^+$ (50)[b], 269 $[M+H-2H_2O]^+$ (100)
7-OxoDHEA	302	6.0	303 $[M+H]^+$ (20), 285 $[M+H-H_2O]^+$ (100)
7αOH-DHEA	304	6.1	287 $[M+H-H_2O]^+$ (50), 269 $[M+H-2H_2O]^+$ (100)
11-Oxoetiocholanolone	304	9.9	287 $[M+H-H_2O]^+$ (100)
11β-Hydroxyetiocholanolone	306	10.3	271 $[M+H-2H_2O]^+$ (100), 253 $[M+H-3H_2O]^+$ (30)
AD	286	11.5	287 $[M+H]^+$ (100)
T	288	14.2	289 $[M+H]^+$ (100)
A-diol	290	14.8	273 $[M+H-H_2O]^+$ (95), 255 $[M+H-2H_2O]^+$ (100)
DHEA	288	15.7	271 $[M+H-H_2O]^+$ (100), 253 $[M+H-2H_2O]^+$ (50)
5α-Androstane-3β,17β-diol	292	18.1	275 $[M+H-H_2O]^+$ (25), 257 $[M+H-2H_2O]^+$ (100)
Epiandrosterone	290	18.9	273 $[M+H-H_2O]^+$ (80), 255 $[M+H-2H_2O]^+$ (100)
Epietiocholanolone	290	19.4	273 $[M+H-H_2O]^+$ (100), 255 $[M+H-2H_2O]^+$ (60)
DHT	290	21.1	273 $[M+H-H_2O]^+$ (100), 255 $[M+H-2H_2O]^+$ (40)
Etiocholanolone	290	26.1	273 $[M+H-H_2O]^+$ (100), 255 $[M+H-2H_2O]^+$ (60)
5α-Androstane-3α,17β-diol	292	27.4	257 $[M+H-2H_2O]^+$ (100)
A	290	29.1	273 $[M+H-H_2O]^+$ (100), 255 $[M+H-2H_2O]^+$ (45)

[a] The conditions are cited in the Experimental section.
[b] Relative abundance.

under a nitrogen gas stream and dried in vacuo. BSTFA (20 μl) was added to the obtained residue and the mixture was heated at 60 °C for 30 min. The solvent was evaporated under a nitrogen gas stream and dried in vacuo. The residue was dissolved in hexane and an aliquot was applied to a GC–MS system.

GC–MS was performed on a GCQ gas chromatograph–ion trap mass spectrometer (ThermoQuest) equipped with a 5% diphenyl–95% dimethylpolysiloxane capillary column (Rtx-5MS, 30 m × 0.25 mm I.D., 0.25 μm d_f) (Restex, Bellefonte, PA, USA), and used with the following conditions: ionization method, electron ionization (70 eV); carrier gas, helium; linear flow-rate, 40 cm/s; transfer line temperature, 275 °C; ion source temperature, 200 °C. The column oven temperature was set at 60 °C for 4 min, ramped to 280 °C at 40 °C/min and held at 280 °C for 10 min. The injector temperature was set at 60 °C for 1 min, ramped to 280 °C at 180 °C/min and held at 280 °C for 17 min. In the case of GC–MS–MS analysis of the methyloxime–trimethylsilyl ether (MO–TMS) derivatives of DHT, the precursor ion was selected at *m/z* 286 $[M-TMSOH-CH_3]^+$, and the collision energy was set at 1.0 V. Assignment of the used monitoring ion (*m/z* 254) was not done.

3. Results

3.1. LC–MS analysis

In the first place, our effort was directed to

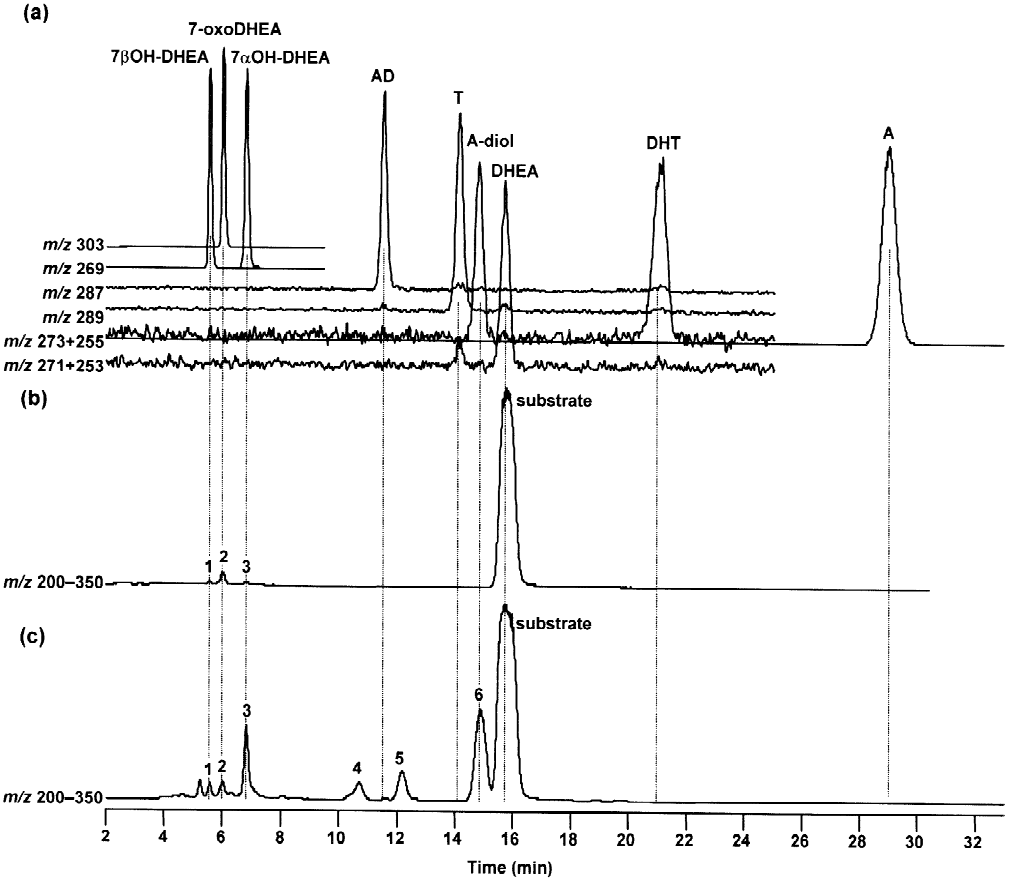

Fig. 2. Typical LC–MS chromatograms of the extract from the incubation mixture. (a) Authentic samples, (b) extract from incubation mixture with heated rat prostate homogenate (deactivated enzyme), (c) extract from incubation mixture with human prostate homogenate.

identify the metabolites using LC–APCI-MS without any derivatization. The t_R values and the predominant ions of authentic potential metabolites are listed in Table 1.

The incubation mixture with homogenate of human prostate or heated rat prostate (deactivated enzyme) was pre-treated as described in the Experimental section and then subjected to LC–APCI-MS analysis. Chromatograms obtained in total-ion (m/z 200–350) detection mode are shown in Fig. 2b and c, together with those of authentic samples (Fig. 2a). As a consequence, three peaks (1–3) together with that corresponding to the substrate (DHEA/[$^2H_4$]DHEA; m/z 271/275=20/11) were detected in the chromatogram of the extract from the incubation mixture using the deactivated enzyme (Fig. 2b). Peaks 1–3 were identified as 7βOH-DHEA (1), 7-oxoDHEA (2) and 7αOH-DHEA (3) by comparison with authentic samples based on the chromatographic behavior and the mass spectra with twin peaks which have 4 mass unit differences depending on the substrate used (DHEA/[$^2H_4$]DHEA). Although DHEA is enzymatically hydroxylated at the C-7 position in the human body [13,14], it is well known that DHEA is oxidized non-enzymatically at the C-7 position (allyl position), so these peaks may arise from the allylic oxidation of the substrate with atmospheric oxygen and the cofactors used. On the contrary, six peaks (1–6) together with that corresponding to the substrate were detected in the chromatogram of the extract from the incubation mixture using human prostate homogenate (Fig. 2c). Peak 3 increased compared with peaks 1–3 obtained from the incubation mixture using the deactivated enzyme. It is recognized that an enzyme that catalyzes the 7α-hydroxylation of DHEA is present in the prostate gland [9]. These data show that 7α-hydroxylation also occurs enzymatically in the incubation mixture using human prostate. The mass spectra of peaks 4 and 5 show twin ions (m/z 255/259, 273/277 and 253/257, 271/275, respectively) which indicates that the compounds arose from the substrate used, but we could not find the corresponding androgen having the same t_R as shown in Table 1. The major product (peak 6) had the same t_R as the A-diol of an authentic sample. Furthermore, the mass spectrum of peak 6 gave the predominant twin ions at m/z 255/259 and 273/277, which were identified as the dehydrated ions of A-diol/[$^2H_4$]A-diol (Fig. 3). All these data confirm the existence of A-diol in the incubation mixture.

Although the peaks corresponding to AD and T were not observed in the total-ion chromatogram, these were detected in the mass chromatograms monitored with their protonated molecular ions at m/z 287 and 289, respectively (Fig. 4a and b). The mass spectra of the peaks corresponding to AD and T showed predominantly the ions corresponding to the protonated molecular ions, together with ions 1–3 mass units higher, depending on the substrate

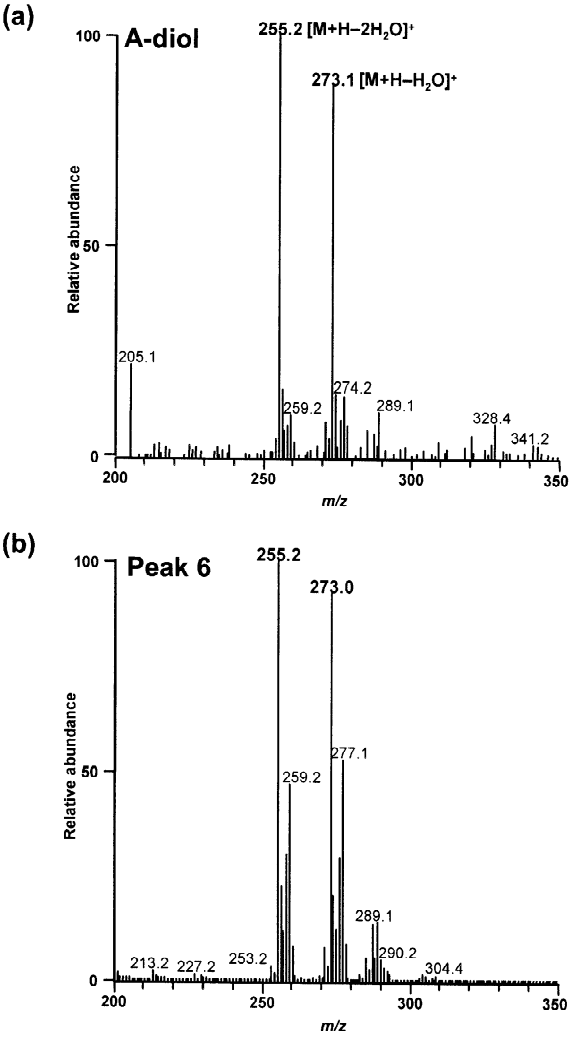

Fig. 3. Mass spectra of A-diol. (a) Authentic sample, (b) extract from incubation mixture (peak 6).

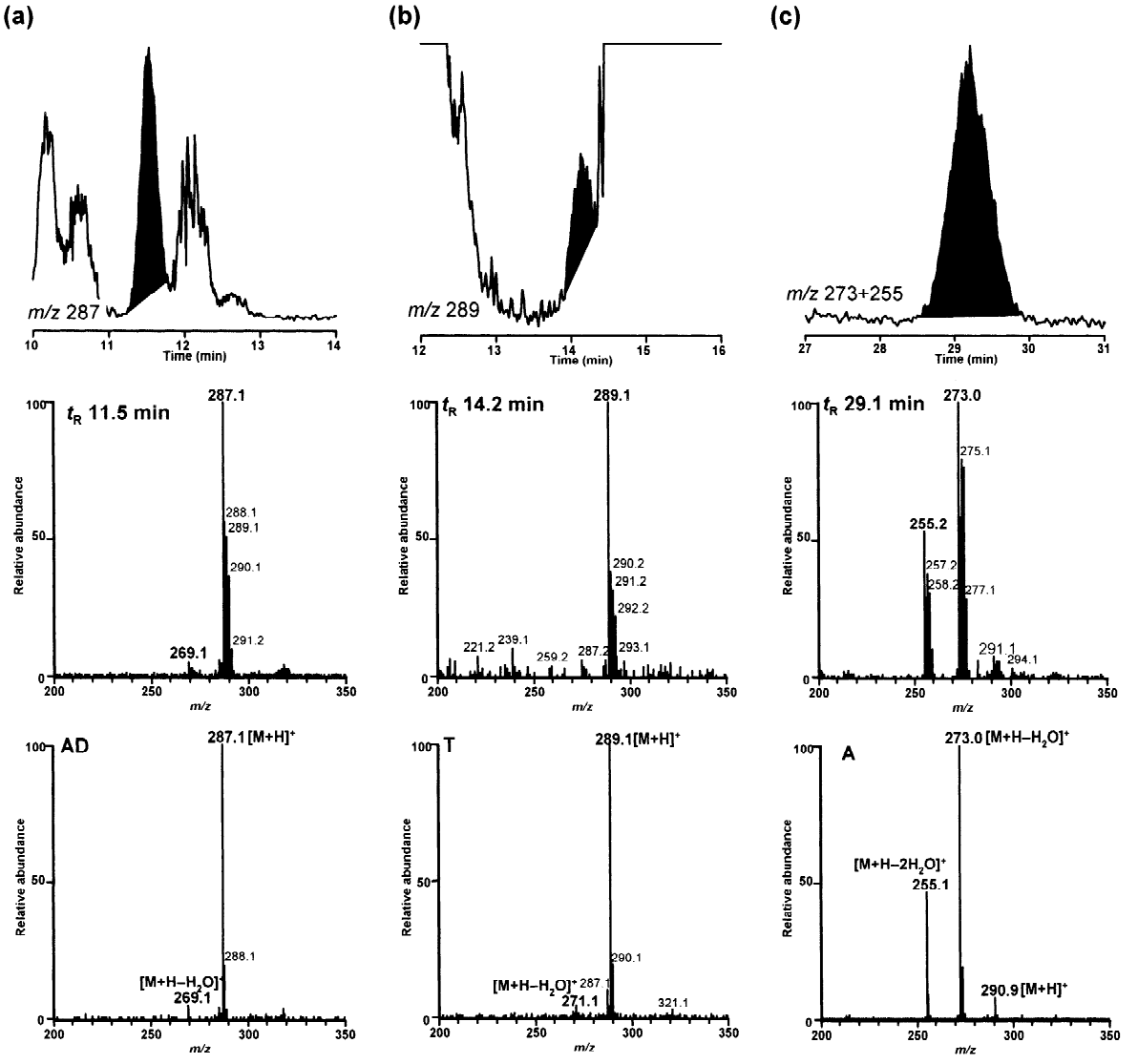

Fig. 4. LC–MS chromatograms (top), mass spectra of the peaks corresponding to AD, T and A (middle) and those of authentic samples (bottom). (a) AD, (b) T, (c) A.

used. The peak corresponding to A was also detected in the mass chromatogram monitored as the sum of *m*/*z* 273 and 255 (Fig. 4c). The mass spectrum of the peak showed ions also resulting from the deuterated substrate used. Although DHT is a potent androgen in the prostate, it was not detected in this LC–MS analysis.

3.2. Preparative HPLC and GC–MS analysis

In order to confirm the existence of DHT and characterize the unknown peaks (peaks 4 and 5), the extract from the incubation mixture was subjected to preparative HPLC with UV detection (220 nm) using methanol–water (3:2) as the mobile phase. The

peaks corresponding to peak 4, AD, peak 5, T and A-diol were detected at t_R 8.0, 9.3, 10.1, 11.1 and 12.9 min, respectively, and the respective fraction ($t_R \pm 0.5$–1 min) was collected. The fractions eluted at 4.0–6.0, 19.3–21.2 and 21.2–30.0 min contained 7αOH-DHEA, DHT and A, respectively. The fraction eluted at 2.0–4.0 min was further purified with methanol–water (4:3) as the mobile phase and gave eluates corresponding to 7βOH-DHEA (t_R 4.0–4.7 min) and 7-oxoDHEA (t_R 4.7–5.5 min), which were collected. After evaporation of the solvent, the residue obtained was subjected to derivatization with *O*-methylhydroxylamine–BSTFA and then to GC–MS analysis. The t_R values and the predominant ions of authentic androgens are listed in Table 2. The production of DHT was confirmed by GC–MS–MS using m/z 286 as the precursor ion and m/z 254 as the monitoring ion. The MO–TMS derivative of the fraction corresponding to DHT gave two peaks resulting from *E*- and *Z*-isomers, which was the same as that of the authentic DHT (Fig. 5a and b). The mass spectra of the MO–TMS derivative of DHT obtained from the incubation mixture showed ions also resulting from the deuterated substrate used.

The obtained fractions corresponding to peaks 4 and 5 were further separated into several peaks by HPLC using a MeCN–water system as mobile phase. Therefore, it is difficult to characterize these products at this time.

The fractions corresponding to A-diol, AD, T, 7αOH-DHEA, 7βOH-DHEA, 7-oxoDHEA and A were also identified using GC–MS and comparison with authentic samples.

4. Discussion

We have confirmed A-diol, AD, T, DHT, 7αOH-DHEA and A as the metabolites of DHEA by using stable-isotope dilution LC–MS and/or GC–MS. The stable-isotope dilution method was used to obtain information concerning the origin of the detected androgens or the metabolized position of a steroid

Table 2
GC–MS data for the authentic androgens[a]

Compound	Derivatization	M_r	t_R (min)	Ion (m/z)
7βOH-DHEA	MO–bisTMS	477	13.12	477 $[M]^+$ (2)[b], 387 $[M-TMSOH]^+$ (100), 356 $[M-TMSOH-OCH_3]^+$ (10)
7-OxoDHEA	bisMO–TMS	432	14.30	432 $[M]^+$ (40), 401 $[M-OCH_3]^+$ (90), 386 $[M-OCH_3-CH_3]^+$ (100)
7αOH-DHEA	MO–bisTMS	477	12.38	477 $[M]^+$ (2), 387 $[M-TMSOH]^+$ (100), 356 $[M-TMSOH-OCH_3]^+$ (10)
AD	bisMO	344	13.02, 13.05[c]	344 $[M]^+$ (65), 313 $[M-OCH_3]^+$ (100)
T	MO–TMS	389	12.92, 12.97	389 $[M]^+$ (100), 358 $[M-OCH_3]^+$ (40), 268 $[M-TMSOH-OCH_3]^+$ (60)
A-diol	bisTMS	434	12.28	434 $[M]^+$ (25), 344 $[M-TMSOH]^+$ (100), 254 $[M-2TMSOH]^+$ (85)
DHEA	MO–TMS	389	12.37	389 $[M]^+$ (5), 358 $[M-OCH_3]^+$ (70), 268 $[M-TMSOH-OCH_3]^+$ (100)
DHT	MO–TMS	391	12.65, 12.73	391 $[M]^+$ (85), 360 $[M-OCH_3]^+$ (50), 286 $[M-TMSOH-CH_3]^+$ (100), 254[d] (100)
A	MO–TMS	391	11.92	391 $[M]^+$ (2), 360 $[M-OCH_3]^+$ (50), 270 $[M-TMSOH-OCH_3]^+$ (100)

[a] The conditions are described in the Experimental section.
[b] Relative abundance.
[c] Two peaks resulting from *E*- and *Z*-isomers were detected.
[d] Product ion of MS–MS (precursor ion, m/z 286).

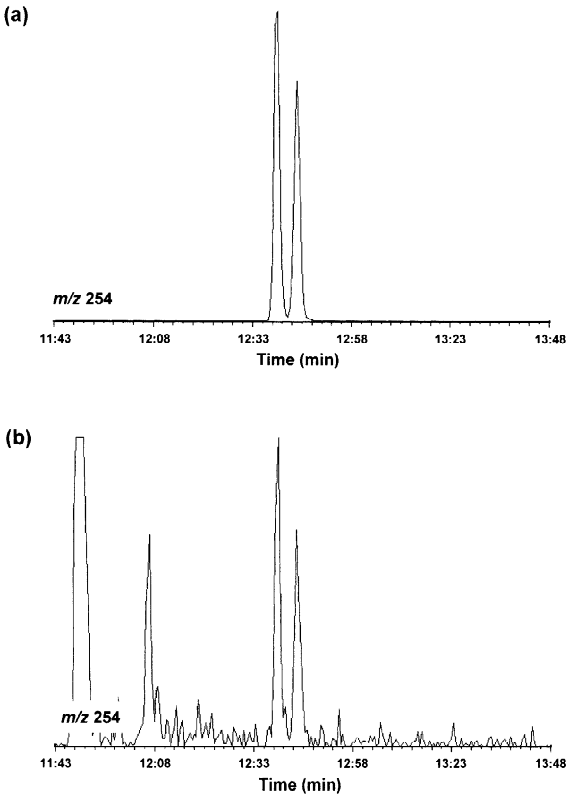

Fig. 5. GC–MS–MS chromatograms of DHT as the MO–TMS derivative. (a) Authentic sample, (b) fraction obtained corresponding to DHT.

skeleton. A-diol and 7αOH-DHEA gave corresponding twin ions in their LC–MS spectra, which indicates that they originate from the substrate used (DHEA/[$^2H_4$]DHEA). On the contrary, in the case of the metabolites where conversion occurred at or near the deuterated position (C-2, -4 or -6), such as AD, T, DHT and A, 1–3 mass units higher ions together with the normal ions were observed in the mass spectra depending on the deuterated substrate used. DHT was detected only in the GC–MS chromatogram, which may be due to the low sensitivity of LC–MS compared with that of GC–MS in measuring androgens in biological fluids [3,15,16]. The existence of DHT in the incubation medium supports the hypothesis proposed by Labrie et al. [17] that DHEA is converted into T and then DHT in the prostate, which is responsible for the recurrence of the cancer.

The other unknown products (peaks 4 and 5) could not be identified and further purification is necessary to obtain additional structural information. We could not detect etiocholanolone or its derivatives with the 5β-configuration, which indicates that 5β-reductase is not present in the prostate.

It should be noted that A-diol was the major metabolite of DHEA in human prostate. It has been demonstrated that A-diol has activity for prostate and breast cancer cell growth [18,19] and androgen receptor transcription [5]. This might raise significant interest in the prostate or serum concentration of A-diol in a patient with prostate cancer which is expected to contribute to the elucidation of the mechanism of recurrence of prostate cancer and to the development of new and better treatments.

Acknowledgements

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