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Liquid chromatography–mass spectrometric assay of androstenediol in prostatic tissue: Influence of androgen deprivation therapy on its level

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ABSTRACT

Androstenediol (Adiol, androst-5-ene-3β,17β-diol) is suspected of being an endogenous proliferation agent of prostate cancer (PCa) even after androgen deprivation therapy (ADT). A liquid chromatography–electron capture atmospheric pressure chemical ionization–mass spectrometric (LC–ECAPCI–MS) method for the determination of Adiol in prostatic tissue was developed and validated for evaluating the influence of ADT on the prostatic Adiol level. After derivatization of Adiol with 4-nitrobenzoyl chloride, the detection response of the derivative was increased 150 times more than that of intact Adiol. The LC–MS method was specific and reliable for the measurement of a trace amount of Adiol in 30 mg of tissue. The clinical study using the developed method showed that the prostatic Adiol level was not changed by ADT. That is, the prostatic Adiol levels of PCa patients with ADT ($n = 12$), benign prostate hypertrophy patients without ADT ($n = 8$) and bladder cancer patients (without prostatic disease) ($n = 6$) were 0.83 ± 0.28, 0.62 ± 0.31 and 0.71 ± 0.28 ng g^{-1} tissue, respectively, and there was no significant difference between these groups.

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1. Introduction

Prostate cancer (PCa) is the most common malignancy in aged males in the United States and is also sharply increasing in Japan. Because PCa depends initially on androgens, primarily 5α-dihydrotestosterone (DHT), androgen deprivation therapies [ADT, usually a combination of luteinizing hormone-releasing hormone (LH-RH) agonists and antiandrogens] often are the first choice of several therapeutic procedures for PCa [1,2]. However, PCa eventually recurs during ADT despite castrate levels of serum androgens. Little is known about the mechanisms behind the transition of the disease to an androgen-independent stage. One of the probable causes is the mutation of the androgen receptor (AR) [3], which leads to an anti-androgen withdrawal phenomenon; in patients with PCa who manifest disease progression during ADT, discontinuance of antiandrogen treatment might result in prostate-specific antigen decline, often associated with clinical improvement. Another possibility exists that other steroids whose production cannot be blocked by LH-RH agonists are involved in the growth stimulation of DHT-independent prostate tumors [4].

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Androstenediol (Adiol, androst-5-ene-3β,17β-diol) derived from dehydroepiandrostenone (DHEA) is convertible into testosterone (T) and further metabolized into DHT. Mitamura et al. reported that Adiol is the major metabolite of DHEA in human prostate homogenate in vitro [5]. It has been demonstrated that Adiol is a strong activator of the AR in several cell lines [1,6], and some anti-androgens fail to block completely the androgenic activity of Adiol [6]. These findings indicate that Adiol may cause the proliferation of PCa even after ADT. However, the influence of ADT on the Adiol level in the prostate is poorly understood.

Liquid chromatography–mass spectrometry (LC–MS) has been recently used for prostatic androgen analysis due to its specificity and versatility [1,2,7,8]. However, Adiol has a rather low response using either electrospray ionization or atmospheric pressure chemical ionization (APCI) due to their low proton-affinitive properties, which leads to a lack of sensitivity. Electron capture APCI (ECAPCI)–MS using a commercial APCI interface operating in the negative-ion mode is a highly sensitive technique for electron-affinitive compounds [9–12]. We have demonstrated that LC–ECAPCI–MS coupled with the introduction of a nitrobenzene moiety enables the analysis of trace levels of neuroactive steroids [11] and estrogens [12] in various biological samples.

With this background information, in the present paper, a new LC–ECAPCI–MS method combined with derivatization for the determination of prostatic Adiol is described. Using the method, the influence of ADT on the Adiol level in the prostate is also examined.

2. Experimental

2.1. Materials and reagents

Adiol was synthesized by reducing DHEA with $NaBH_4$. [7,7,16,16,17α-$^2H_5$]-Adiol (IS) and [7,7,17α-$^2H_3$]-Adiol [D_3-Adiol, which was used for the determination of the recovery rate during the pretreatment (Section 2.7) and the influence of the prostate components on derivatization efficiency (Section 2.8)] were prepared from [7,7-$^2H_2$]-DHEA [13] in our laboratories according to known methods [14]. Both of the deuterium-labeled Adiols showed single peaks in their LC-positive APCI–MS chromatograms (chemical purities: more than 99%) and their isotopic purities were as follows; IS (99.5% $^2H_5$, 0% $^2H_4$, 0.3% $^2H_3$, 0.2% $^2H_2$, 0% $^2H_1$ and 0% $^2H_0$) and D_3-Adiol (99.0% $^2H_3$, 1.0% $^2H_2$, 0% $^2H_1$ and 0% $^2H_0$). Stock solutions of each steroid were prepared as 100 μg ml^{-1} solutions in ethanol. Subsequent dilutions were carried out with ethanol to prepare 1, 2, 5 and 10 ng ml^{-1} solutions. 4-Nitrobenzoyl chloride (NBC) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively washed with ethyl acetate (2 ml), methanol (2 ml) and water (2 ml) prior to use. All other reagents and solvents were of analytical grade.

2.2. LC–MS(–MS)

LC–MS(–MS) was performed using a ThermoQuest LCQ (San Jose, CA) liquid chromatograph–ion trap–mass spectrometer connected to a Jasco PU-980 (Tokyo) chromatograph. A YMC-Pack Pro C18 RS (5 μm, 150 mm × 4.6 mm i.d.; YMC, Kyoto, Japan) column was used at 40 °C. Methanol–water (100:1, v/v) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. Adiol and IS were analyzed as their 3,17-bis-nitrobenzoates (NB-derivatives, Fig. 1) by APCI–MS–MS [selected reaction monitoring (SRM)] in the negative-ion mode. Helium was used as the collision gas, and the relative collision energy was set at 20%. The sheath gas flow rate was set at 60 units, and the capillary voltage and tube lens offset were −10 and −15 V, respectively. The heated capillary temperature and vaporizer temperature were 200 and 425 °C, respectively. The precursor and monitoring ions of the NB-derivatives were as follows: Adiol-NB, *m/z* 588.2 and 588.2; IS-NB, *m/z* 593.2 and 593.2.

2.3. Calibration curve for Adiol

Standard solutions of Adiol (10, 20, 50, or 100 pg) and IS (200 pg) placed in a tube were derivatized as described below and

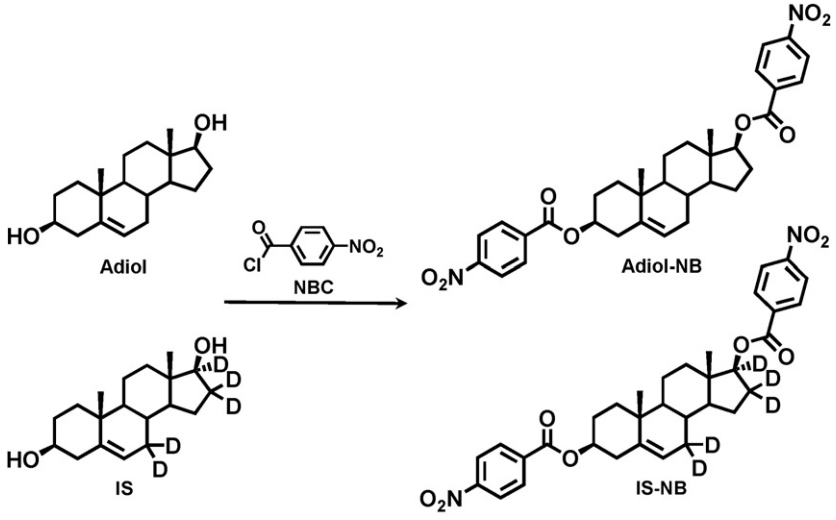

Fig. 1 – Structures of Adiol, IS and their derivatized forms with NBC. D = deuterium (^2H).

subjected to LC–MS–MS, and the calibration curves were constructed by plotting the peak area ratios (Adiol/IS) against the amounts of Adiol (pg).

2.4. Prostatic tissue

Prostatic tissues were obtained from 12 PCa patients with neoadjuvant hormone therapy (combination of LH-RH agonist and anti-androgen drug) for 3–6 months before radical prostatectomy, 8 benign prostate hypertrophy (BPH) patients without any hormone therapy and 6 bladder cancer (BCa) patients (radical cystoprostatectomy for BCa) and stored at −20 °C prior to use. Some of the BCa patients had received chemotherapy with platinum preparations but no patient had received hormone therapy. All the patients gave informed consent at Kanazawa University Hospital (Kanazawa, Japan).

2.5. Pretreatment procedure for analysis of Adiol in human prostate

Prostatic tissue was minced by scissors and crushed by a glass homogenizer on ice. IS (200 pg) was added to the crushed tissue (30 mg) and further homogenized in methanol–water (3:7, v/v, 0.2 ml) using a glass homogenizer. The homogenate was heated at 60 °C for 30 min and centrifuged at 1500 × *g* (4 °C, 5 min). The supernatant was saved and the precipitate was suspended with methanol–water (3:7, v/v, 0.2 ml) and heated at 60 °C for 30 min. After centrifugation at 1500 × *g* (4 °C, 5 min), the supernatants were combined (this solution is called the prostate extract). The prostate extract was added to acetonitrile (1 ml), vortex-mixed for 30 s and centrifuged at 1500 × *g* (4 °C, 5 min). The supernatant was diluted with water (3 ml) and purified using a Strata-X cartridge. After successive washing with water (2 ml), methanol–water (7:3, v/v, 2 ml) and hexane (1 ml), the steroids were eluted with ethyl acetate (1 ml). After evaporation, the residue was dissolved in hexane–isopropanol (19:1, v/v, 40 μl) containing progesterone (20 ng) and subjected to high-performance liquid chromatography (HPLC) [Hitachi L-7110 chromatograph (Tokyo) with Shimadzu SPD-10A UV detector; column, Develosil (3 μm, 100 mm × 4.6 mm i.d., Nomura Chemical, Seto, Japan); mobile phase, hexane–isopropanol (19:1, v/v); flow rate, 1.0 ml min^{-1}; column temperature, 40 °C and detection, 240 nm]. Because Adiol and IS were eluted just behind progesterone, which shows an intense absorption at UV 240 nm (retention time, 4.6 min), the reproducible collection of the fraction containing Adiol and IS (retention time, 5.1–6.4 min) could be done by use of the peak of progesterone as a mark. After evaporation, the residue was subjected to derivatization with NBC as described below.

2.6. Derivatization reaction

The calibration curve samples or prostate samples were dried and added with reagent, NBC (20 μg) in benzene (40 μl) and catalyst, quinuclidine (20 μg) in benzene (10 μl). The mixture was kept at 80 °C for 10 min, the additional reagent and catalyst (20 μg each) were then added, and the entire mixture was further kept at 80 °C for 30 min. After addition of ethanol (30 μl) to decompose the excess reagent, the solvents were evaporated and the resulting residue was dissolved in ethanol (30 μl), 10 μl of which was subjected to LC–MS–MS.

2.7. Recovery rate of Adiol during pretreatment procedure

Because prostate tissue in which Adiol was not detected could not be obtained, the recovery rate of Adiol during the pretreatment procedure was determined using the tissue spiked with D_3-Adiol. The ethanolic solution of D_3-Adiol (50 pg in 10 μl) or ethanol (10 μl, control sample) was added to the prostatic tissue (30 mg), and the resulting samples were pretreated. D_3-Adiol (50 pg) was then added to only the control sample, and IS (200 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS–MS. The contaminant of D_3-Adiol in IS and the isotopic ion of D_3-Adiol might have some influence on the peak areas of D_3-Adiol (the analyte for the recovery determination) and IS, respectively. However, the influence of the contaminant on the measurement of D_3-Adiol was not observed due to the extremely high isotope purity of IS. The influence of the isotopic ion at *m*/*z* 593 of D_3-Adiol on the peak area of IS could be also neglected (less than 1%). Based on these results, the recovery rate was calculated from the peak area ratios (D_3-Adiol/IS) of the spiked and control samples without any correction.

2.8. Influence of prostate components on derivatization efficiency

D_3-Adiol (50 pg) was derivatized for preparation of a control sample (this sample was derivatized without prostate components). D_3-Adiol (50 pg) was added to the pretreated prostate sample and then derivatized (test sample, derivatization was carried out with prostate components). After addition of the derivatized IS (200 pg of IS was derivatized beforehand) to both the control and test samples, they were subjected to LC–MS–MS. The influence of prostate components on derivatization efficiency was evaluated from the peak area ratios (D_3-Adiol/IS) of the test and standard samples.

2.9. Assay accuracy (matrix effect and analytical recovery)

The matrix effect was examined by comparing the slope of the calibration curve constructed as described above and that of a curve prepared by adding Adiol (10, 20, 50 and 100 pg) to prostatic tissue (30 mg) (matrix sample). The matrix samples were prepared using five different tissues.

The analytical recovery was determined as follows. Ethanol (10 μl, unspiked sample) or an ethanolic solution of Adiol (30 pg in 10 μl, spiked sample) was added to the prostatic tissue (30 mg) (the spiked concentrations of Adiol was 0 or 1.0 ng g^{-1} tissue, respectively). After the addition of IS (200 pg), the resulting samples were pretreated, derivatized and analyzed by LC–MS–MS. The analytical recovery of Adiol was defined as $F/(F_0 + 1.0) \times 100\%$, where F and F_0 are the Adiol concentrations in the spiked and unspiked samples (0.64 or 1.12 ng g^{-1} tissue, see Table 1), respectively.

Table 1 – Assay precision and accuracy

	BPH tissue			PCa tissue		
	Measured[a] ($ng\,g^{-1}$ tissue)	Expected[b] ($ng\,g^{-1}$ tissue)	Precision/ recovery (%)	Measured ($ng\,g^{-1}$ tissue)	Expected ($ng\,g^{-1}$ tissue)	Precision/ recovery (%)
Intact (intra-assay)	0.63 ± 0.044	–	R.S.D.[c]: 7.0	1.03 ± 0.083	–	R.S.D.: 8.1
Intact (inter-assay)	0.64 ± 0.048	–	R.S.D.: 7.5	1.12 ± 0.057	–	R.S.D.: 5.1
Spiked sample[d]	1.56	1.64	Recovery[e]: 95.1	2.26	2.12	Recovery: 106.5

[a] Mean ± S.D. ($n = 5$) for the intra- and inter-assay variation tests and mean of duplicate assays for the analytical recovery test.
[b] Expected values were calculated based on the values obtained in the inter-assay variation test.
[c] S.D./mean × 100.
[d] Adiol (30 pg) was spiked into the prostatic tissue (30 mg) and then analyzed.
[e] Analytical recovery: measured value/expected value × 100.

2.10. *Assay precision*

The intra-assay precision was assessed by determining two prostate samples at different concentration levels ($n = 5$ for each sample) on a day. The inter-assay precision was assessed by determining these samples over 5 days. The precision was determined as the relative standard deviation (R.S.D., %).

2.11. *Determination of prostatic DHT*

The DHT level in PCa or BPH tissue was determined by the previously developed method [7].

3. Results and discussion

3.1. *LC–ECAPCI–MS(–MS) of NB derivatives*

For the APCI–MS operating in the negative-ion mode, the NB-derivatives of Adiol and IS provided only their molecular anions $[M]^-$ at *m/z* 588.2 and 593.2, respectively (Fig. 2a and b). This demonstrated that the derivatives underwent electron capture in the APCI source. The limit of detection [signal to noise ratio (S/N) = 5] of the derivatized Adiol (6.8 fmol per injection) was 150 times greater than that of intact Adiol [1.0 pmol (300 pg) per injection] analyzed by positive APCI–MS.

The derivatization rate of nanogram amounts of Adiol with NBC was almost quantitative, because after derivatization of Adiol (10 ng), intact steroid was not detected in LC–positive APCI–MS [the minimum detectable amount of intact Adiol was 300 pg (3% of the initial amount)]. Next, 10 ng and 50 pg of Adiol were individually derivatized and then dissolved in 6 ml and 30 μl of ethanol, respectively. Ten microliters of the respective solutions were subjected to LC–MS. As a result, the peak areas obtained from both the solutions were practically equal. This result demonstrates that derivatization rate is quantitative even when picogram amounts of Adiol are derivatized.

The use of the SRM mode may allow for the discrimination and quantification of Adiol from prostate components without the need for a long chromatographic separation, due to its high specificity. Based on this, MS–MS analysis of the derivative was also examined, where $[M]^-$ was used as the precursor ion. However $[M]^-$ of the derivative was very stable, and therefore, no characteristic product ion was formed in MS–MS (Fig. 2c). Based on this result, the following SRM mode was employed

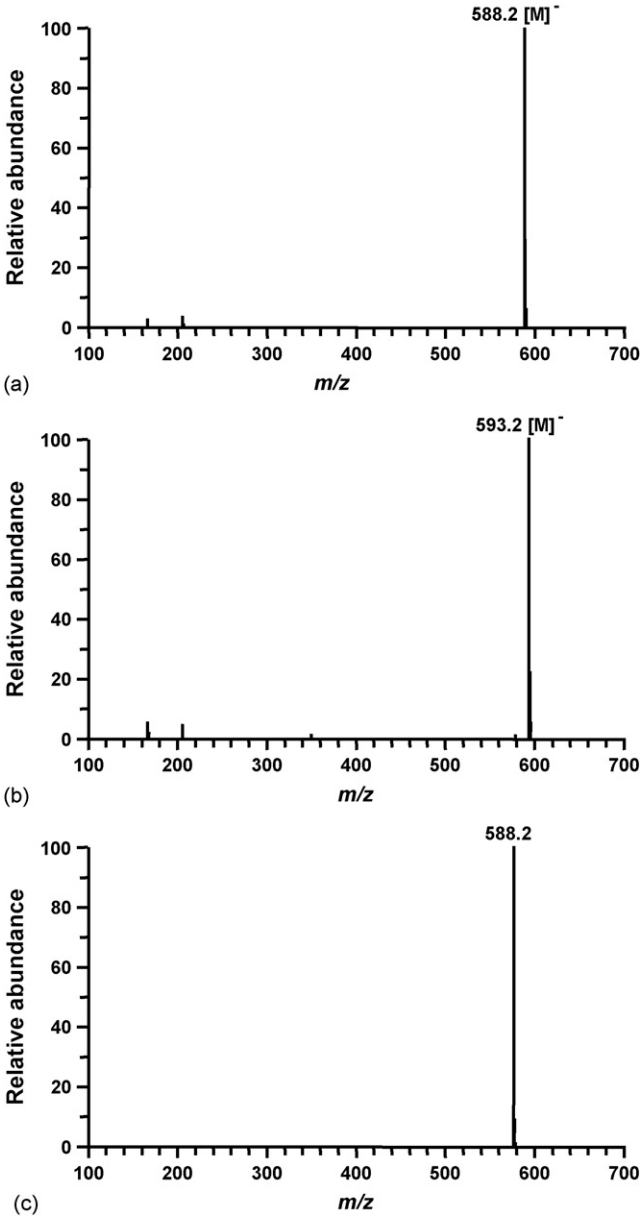

Fig. 2 – Mass spectra of derivatized (a) Adiol, (b) IS and (c) product ion mass spectrum of derivatized Adiol.

for the determination of Adiol in the prostatic tissue; after collision of $[M]^-$ with 20% relative collision energy, the residual same ion was monitored, by which the noise ions derived from the prostate components were reduced while comparatively maintaining the intensity of the monitoring ion.

3.2. Pretreatment of prostate tissue

The extraction of Adiol from the prostatic tissues was performed by the previously developed method [7] with a slight modification. The prostate extract was purified using a Strata-X cartridge and normal-phase HPLC. The Adiol fraction was then treated with excess NBC. The recovery rate [mean ± standard deviation (S.D.), five different tissues] of Adiol during the extraction and purification steps was 61.7 ± 2.6%. The above pretreatment procedure was useful to remove endogenous components that interfere with the derivatization reaction, so that the derivatization efficiency of the prostate sample was almost equal to that of the standard sample (96.1 ± 2.4%, mean ± S.D., $n = 3$).

Typical chromatograms of the prostate samples obtained from patients with PCa, BPH and BCa are shown in Fig. 3. The peaks corresponding to the derivatized Adiol and IS were clearly observed at 5.0 and 4.9 min, respectively. This slight difference in their retention times is due to isotope effect.

3.3. Calibration curve and limit of quantitation

The calibration curve was constructed by plotting the peak area ratio (Adiol/IS, y) versus the amount (pg) of Adiol per tube (x) using the standard solutions. The regression line obtained from the combination of five standard curves was $y = 0.00516x + 0.00209$ with a correlation coefficient (r^2) of 0.999 within the range of 10–100 pg per tube. The R.S.D. value of the slope was 1.0%.

To determine the extent to which the prostate matrix affects the quantification, the slope of the above calibration curve was compared to that of the curve prepared with the matrix sample. As a result, the slope of the latter was 0.00524 ± 0.00015 (mean ± S.D., $n = 5$, R.S.D. 2.9%), which was practically identical to the slope of the curve constructed with standard solutions. This result clearly revealed that the prostate matrix did not affect the calibration curve. Based on this result and the fact that it is impossible to obtain Adiol-free prostatic tissue, the calibration curve was constructed using the standard solutions in the following studies. The applicability of this curve to the prostatic Adiol assay was also examined in the analytical recovery test that will be discussed later.

Because Adiol-free prostatic tissue was not available, the limit of quantitation of this assay could not be determined. However, at least 0.35 ± 0.05 ng g^{-1} tissue (mean ± S.D., $n = 5$) of prostatic Adiol could be determined with acceptable reproducibility (R.S.D. 15.0%) and S/N value (more than 6).

3.4. Assay precision and accuracy

The intra-assay ($n = 5$) R.S.D. values were less than 8.1% and good inter-assay ($n = 5$) R.S.D. values (less than 7.5%) were also obtained, as shown in Table 1.

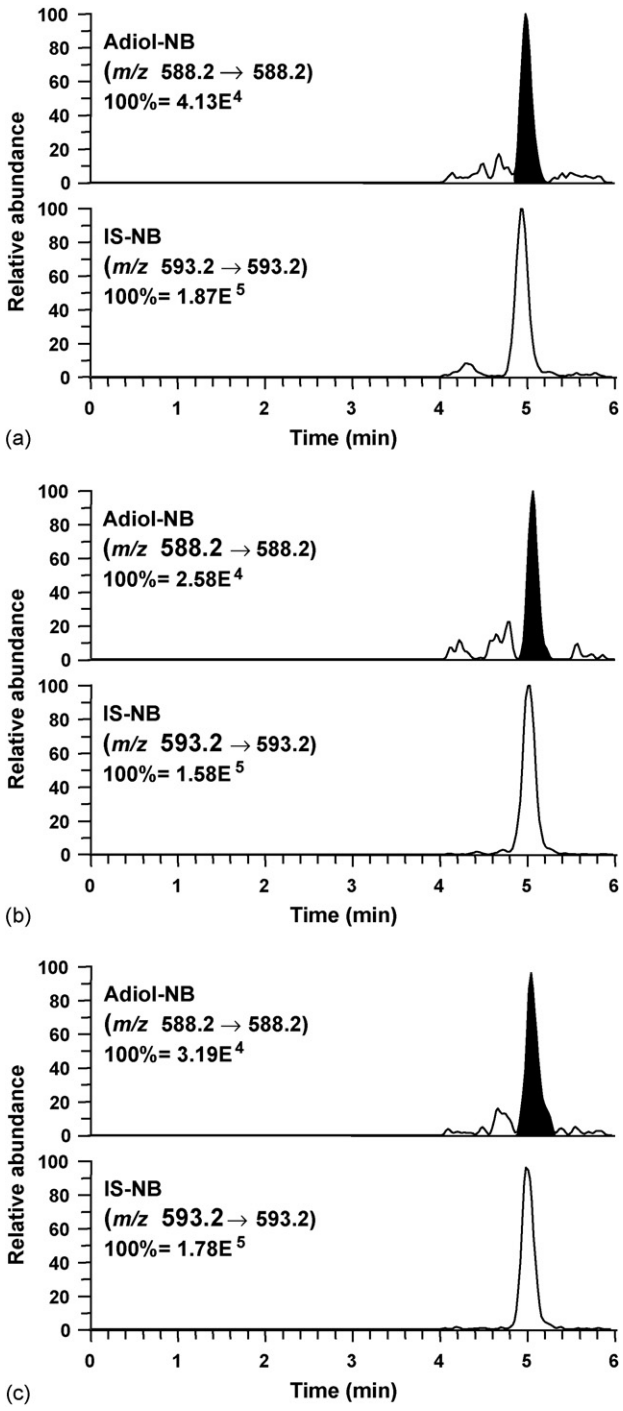

Fig. 3 – Representative chromatograms obtained from prostatic tissue of (a) PCa, (b) BPH and (c) BCa patients. Adiol concentrations were (a) 1.00, (b) 0.64 and (c) 1.08 ng g^{-1} tissue, respectively.

The prostatic tissues with the addition of a known amount of Adiol were pretreated and analyzed in order to examine the analytical recovery, and a satisfactory recovery was obtained (Table 1). Although the calibration curve was constructed using the standard solutions of Adiol in the present study as mentioned above, this result demonstrates that the prostatic Adiol can be accurately determined using the curve.

Table 2 – Prostatic Adiol and DHT levels

	Adiol		DHT	
	Mean ± S.D. (ng g^{-1} tissue)	Range (ng g^{-1} tissue)	Mean ± S.D. (ng g^{-1} tissue)	Range (ng g^{-1} tissue)
PCa (n = 12)	0.83 ± 0.28	0.48–1.45	Not detected	–
BPH (n = 8)	0.62 ± 0.31	0.26[a]–1.22	4.95 ± 1.39	3.10–7.41
BCa (n = 6)	0.71 ± 0.28	0.35–1.08	Not examined	–

[a] When the Adiol level was below 0.35 ng g^{-1} tissue, 50 mg of tissue was used for its determination.

These data indicate that the present method is highly reproducible and accurate.

3.5. *Influence of ADT on prostatic Adiol level*

The influence of ADT on the Adiol level in the prostate was examined using the developed method. The prostatic Adiol levels in the patients with PCa, BPH and BCa are summarized in Table 2. There was no significant difference in the level between the three groups. This result demonstrates that Adiol is present in PCa tissue after ADT at the same levels of tissues without hormone treatment and contrasts with the prostatic DHT level, which is significantly reduced by ADT (Table 2). As mentioned in Section 1, several reports proved that Adiol is a strong activator of the AR in PCa cells without being metabolized to T or DHT [1,6] and that some anti-androgens fail to block completely the androgenic activity of Adiol [6]. From our results and the above findings, Adiol is strongly suspected of being a proliferation agent of PCa even after ADT. Therefore, the development of new therapeutic approaches that block Adiol's androgenic action is worth investigating. Regarding this, Miyamoto et al. [15,16] recently reported new steroidal anti-androgens, which can block the Adiol-induced AR transactivation.

4. Conclusion

In this study, we developed the LC–MS method for the determination of Adiol in prostatic tissue after converting Adiol to a highly detectable derivative in ECAPCI–MS. The method was specific and reproducible, and enabled the analysis of a trace amount of Adiol using a small amount of sample.

The clinical study using the developed method found that reduction of the prostatic Adiol level by ADT was not observed. This result and the in vitro experimental data previously reported [1,6] strongly suggest that Adiol may be one of the agents that advance PCa even when DHT is exhausted by ADT.

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