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Development and application of electrospray-active derivatization reagents for hydroxysteroids

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Abstract

New derivatization reagents, 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ) and 4-(4-methyl-1-piperazyl)-3-nitrobenzoyl azide (APZ), were developed for the liquid chromatography–electrospray ionization-mass spectrometric (LC–ESI-MS) determination of steroids having a hydroxy group. PPZ reacted with a phenolic hydroxy group in estrogens. After quaternarization of the PPZ-estrogens with methyl iodide, the resulting derivatives provided more than a 2000-fold higher sensitivity compared to the intact estrogens. After derivatization of steroids having an alcoholic hydroxy group (5-ene-steroids or 5α-reduced steroids) with APZ followed by methylation, their detection responses increased more than 500 times. These derivatization procedures coupled with LC–ESI-MS/MS were successfully used for the determination of estrogens in the serum and prostatic 5α-dihydrotestosterone.
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Keywords: Derivatization reagent; LC–ESI-MS/MS; Steroid; Hydroxy group; Sensitivity

1. Introduction

A specific and sensitive method for the characterization and determination of steroids in biological fluids and tissues is useful for the elucidation of the nature, diagnosis and treatment of diseases. Because of the close structural similarity, the metabolic versatility and their occurrence at low concentrations (nanomolar and even picomolar) in body fluids and tissues, the development of reliable analytical methods of steroids is a challenging subject for analytical chemists. Numerous methods have been described to analyze steroids, such as immunoassay, receptor binding assay, high-performance liquid chromatography (HPLC) and gas chromatography (GC)–mass spectrometry (MS) [1,2], but all of these approaches have both advantages and disadvantages.

Liquid chromatography (LC) coupled with atmospheric pressure ionization-MS, especially electrospray ionization (ESI)-MS is now being widely used for steroid analysis due to its specificity, versatility and simultaneous multi-analyte quantification capability [3,4]. Because the ionization process occurs in the liquid-phase during ESI, the pre-formation of ions is very important in the ESI detection mode. Therefore, the glucuronidated and sulfated conjugates of steroids are excellent substrates for detection by ESI-MS, while neutral steroids, except for those having the 3-oxo-4-ene-structure (Δ^4-steroids), are inherently difficult to ionize in ESI due to their low proton-affinitive or acidic properties. Δ^4-Steroids, such as testosterone, can be detected at the low picogram level by the positive ESI-MS in the selected ion monitoring (SIM) or selected reaction monitoring (SRM) mode, because they are more basic than most neutral steroids due to the charge delocalization in the protonated form and can provide some characteristic A-ring product ions in MS/MS [5,6]. However, the ionization efficiencies of biologically important steroids, such as estrogens, 5-ene-steroids and 5α-reduced steroids, are relatively low for ESI; conventional LC–ESI-MS(/MS) sometimes does not demonstrate the required sensitivity for the trace analysis of these steroids. To improve the detectabilities of these poorly ionizable steroids, many derivatization procedures have been proposed [7]. Among these procedures, the introduction

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of permanently charged moieties (*i.e.*, charged derivatization) is the most effective and has been successfully used for the ESI-MS analyses of various poorly ionizable steroids [8–11]. For example, Girard reagent P (GP) [8] and 2-hydrazino-1-methylpyridine (HMP) [10] are the ESI-active derivatization reagents which react with the oxo-group of steroids, and the resulting derivatives can readily provide an intense molecular cation, $[M]^+$, in the positive ESI-MS. An alternative functional group that most steroids have is the hydroxy group, but we can find only one example of charged derivatization for the hydroxy group. 2-Fluoro-1-methylpyridine (FMP) has been used as the derivatization reagent for alcohols [12], but a complicated purification step is necessary to remove excess reagent prior to the LC–MS analysis [13]. Thus, the derivatization reagent for alcohols that is currently in use is not satisfactory for practical applications.

Based on background information, we designed and synthesized some new derivatization reagents that easily react with the phenolic (estrogens) or alcoholic (5-ene-steroids and 5α-reduced steroids) hydroxy group of steroids and can significantly enhance their detection responses. The application of the developed derivatization methods to biological sample analyses is also described.

2. Experimental

2.1. Chemicals and reagents

Estrone (E_1), estradiol (E_2), dehydroepiandrosterone (DHEA), pregnenolone (PREG) and 5α-dihydrotestosterone (DHT) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Allopregnanolone (AP) was purchased from Steraloids (Newport, RI, USA). Stock solutions of each steroid were prepared as 100 μg/mL in ethanol. Subsequent dilutions were carried out with ethanol to prepare 0.5, 1, 2, 5, 10, 20 and 50 ng/mL solutions. [2,4,6,6,9-$^2H_5$]-E_1 (D_5-E_1) [14] and *N*,*N*-diethyl-2,4-dinitro-5-fluoroaniline (FDNDEA) [15] were synthesized in our laboratory by the known methods. [16,16,17α-$^2H_3$]-DHT (D_3-DHT) [16] was donated by Teikoku Hormone Medical Research Center (Kawasaki, Japan). A Strata-X cartridge (60 mg adsorbent; Phenomenex, Torrance, CA, USA) was successively activated by 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 an Applied Biosystems API 2000 triple quadrupole mass spectrometer (Foster City, CA, USA) interfaced to a Shimadzu LC-10AT chromatograph (Kyoto, Japan). A receiver (Anest Iwata SAT-36-100, Yokohama, Japan) was equipped between the nitrogen generator and the mass spectrometer for the large and stable supply of nitrogen. A YMC-Pack C8 column (5 μm, 150 mm × 2 mm i.d.; YMC, Kyoto) and a YMC-Pack Pro C18 RS column (5 μm, 150 mm × 2 mm i.d.; YMC) were used for the analyses of the estrogens and other steroids, respectively, at the flow rate of 0.2 mL/min and 40 °C. Intact steroids and their derivatives were analyzed by ESI-MS in the positive-ion mode and the conditions were as follows: declustering potential; 30 V (intact steroids) or 80 V (derivatized steroids), focusing potential; 350 V (intact steroids) or 250 V (derivatized steroids), entrance potential; 10 V, ion spray voltage; 5 kV, curtain gas; 45 psi, ion source gas 1; 80 psi, ion source gas 2; 80 psi, turbo gas temperature; 500 °C and interface heater; on. Nitrogen gas was used as the collision gas in the SRM mode. The data were collected and quantified using Applied Biosystems Analyst software (Version 1.3.1).

2.3. Syntheses of derivatization reagents

Melting points were measured using a Yanagimoto melting point apparatus (Kyoto) without correction. The ^1H NMR spectra were obtained using a JEOL JNM-EX-270 (270 MHz) spectrometer (Tokyo) and tetramethylsilane was used as the internal standard. The electron ionization (EI) mass spectra were determined using a JEOL JMS-SX-102A spectrometer (Tokyo).

2.3.1. N′-(5-Fluoro-2,4-dinitrophenyl)-N,N-dimethyl-1,2-ethanediamine (PED)

N,*N*-Dimethylethylenediamine (27 μL, 0.3 mmol) was added to a solution of 1,5-difluoro-2,4-dinitrobenzene (100 mg, 0.5 mmol) in acetonitrile (1 mL). The mixture was stirred at room temperature for 30 min. The solvent was evaporated and the residue was chromatographed on silica gel (column; 120 mm × 12 mm i.d., mobile phase; chloroform–methanol (20:1 to 8:1, v/v)) to give a yellow solid, which was then recrystallized from 2-propanol to give PED as yellow needles (30.5 mg, mp 131–132 °C). ^1H NMR in $CDCl_3$ (ppm), 2.32 (s, 6H), 2.67 (t, $J = 6$ Hz, 2H), 3.34 (q, $J = 6$ Hz, 2H), 6.56 (d, $J = 15$ Hz, 1H), 9.00 (brs, 1H), 9.15 (d, $J = 9$ Hz, 1H). EI-MS: *m*/*z* 272 ($M^{+\bullet}$, 90%), *m*/*z* 58 (100%).

2.3.2. 1-(2,4-Dinitro-5-fluorophenyl)-4-methylpiperazine (PPZ)

N-Methylpiperazine (28 μL, 0.3 mmol) was added to a solution of 1,5-difluoro-2,4-dinitrobenzene (100 mg, 0.5 mmol) in acetonitrile (1 mL). The mixture was stirred at room temperature for 30 min. The solvent was evaporated and the residue was chromatographed on silica gel (column; 120 × 12 mm i.d., mobile phase; chloroform–methanol (20:1, v/v)) to give a yellow solid, which was then recrystallized from hexane to give PPZ as yellow needles (32.3 mg, mp 78–79 °C). ^1H NMR in $CDCl_3$ (ppm), 2.37 (s, 3H), 2.57 (t, $J = 6$ Hz, 4H), 3.29 (t, $J = 6$ Hz, 4H), 6.80 (d, $J = 15$ Hz, 1H), 8.71 (d, $J = 9$ Hz, 1H). EI-MS: *m*/*z* 284 ($M^{+\bullet}$, 100%).

2.3.3. 1-(2,4-Dinitro-5-fluorophenyl)-4,4-dimethylpiperazinium iodide (MPPZ)

Methyl iodide (100 μL, 1.6 mmol) was added to a solution of PPZ (10 mg, 0.04 mmol) in acetone (200 μL). The mixture was stirred at 60 °C for 30 min. Excess reagent and solvent were evaporated and the residue was dried to give a yellow solid

(10.6 mg, mp 189–192 °C). ^{1}H NMR in CD_3OD (ppm), 2.33 (s, 6H), 2.55 (t, *J* = 6 Hz, 4H), 3.30 (t, *J* = 6 Hz, 4H), 7.20 (s, 1H), 8.55 (d, 1H). EI-MS: *m/z* 318 ($M^{+\bullet}$, 80%), *m/z* 112 (100%).

2.3.4. 4-(4-Methyl-1-piperazyl)-3-nitrobenzoyl azide (APZ)

3,4-Dihydro-2H-pyran (DHP, 2 mL, 21.9 mmol) was added to a solution of 4-fluoro-3-nitrobenzoic acid (200 mg, 1.1 mmol) and pyridinium *p*-toluenesulfonate (PPTS, 10.5 mg, 0.04 mmol) in chloroform (5 mL). The mixture was stirred at room temperature for 4 h. The reaction mixture was diluted with ethyl acetate and then washed with saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 followed by concentration to give the tetrahydropyranyl ester.

The ester was added to a solution of *N*-methylpiperazine (1.2 mL, 10.8 mmol) in acetonitrile (6 mL) and stirred at room temperature for 15 min. After evaporation of the solvent, a yellow oil was obtained. Hydrochloric acid (6 M, 0.25 mL) was added to a solution of the yellow oil in ether (4 mL) at 0 °C and stirred for 10 min at the same temperature. The desired product precipitated as the hydrochloride salt was collected by filtration, washed with ether and then dried to give a colorless solid [3-nitro-4-(4-methyl-1-piperazyl) benzoic acid] (211 mg, mp 165–167 °C). ^{1}H NMR in CD_3OD (ppm), 2.37 (s, 3H), 2.60 (t, *J* = 6 Hz, 4H), 3.24 (t, *J* = 6 Hz, 4H), 7.00 (d, *J* = 8 Hz, 1H), 7.22 (m, 1H), 7.96 (dd, *J* = 2.7, 9 Hz, 1H), 8.35 (d, *J* = 3 Hz, 1H). EI-MS: *m/z* 265 ($M^{+\bullet}$, 100%), *m/z* 232 (80%).

Triethylamine (200 μL, 1.5 mmol) was added to a solution of 3-nitro-4-(4-methyl-1-piperazyl)benzoic acid (150 mg, 0.5 mmol) and diphenylphosphoryl azide (DPPA, 300 μL, 1.4 mmol) in acetonitrile (3 mL), and the mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with ethyl acetate and then washed with saturated $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and concentrated. The residue was chromatographed on silica gel (column; 200 × 12 mm i.d., mobile phase; chloroform–methanol (20:1 to 8:1, v/v)) to give APZ as yellow needles (130 mg, mp 90–91 °C). ^{1}H NMR in $CDCl_3$ (ppm), 2.37 (s, 3H), 2.60 (t, *J* = 6 Hz, 4H), 3.24 (t, *J* = 6 Hz, 4H), 7.00 (d, *J* = 8 Hz, 1H), 7.22 (m, 1H), 7.96 (dd, *J* = 2.7, 9 Hz, 1H), 8.35 (d, *J* = 3 Hz, 1H). EI-MS: *m/z* 290 ($M^{+\bullet}$, 100%).

2.4. Derivatization of estrogens with FDNDEA, PED or PPZ

To a solution of estrogen in acetone (40 μL), a solution of FDNDEA, PED or PPZ (10 μg) in acetone (10 μL) and 1 M $NaHCO_3$ (10 μL) were added, and the mixture was then incubated at 60 °C for 1 h. The reaction mixture was diluted with 50% methanol (500 μL) and passed through a Strata-X cartridge for desalting. After washing with water (2 mL), the derivatized estrogen was eluted with ethyl acetate (1 mL). After evaporation of the solvent, the derivative was quaternarized with methyl iodide (Section 2.6) or dissolved in methanol–10 mM ammonium formate (1:1, v/v), an aliquot of which was subjected to LC–MS(/MS).

2.5. Derivatization of 5-ene-steroids or 5α-reduced steroids with APZ

To a solution of 5-ene-steroids or the 5α-reduced steroid in benzene (40 μL), a solution of APZ (40 μg) in benzene (40 μL) was added, and the mixture was incubated at 80 °C for 30 min. After the further addition of the reagent (40 μg in benzene 40 μL), the entire mixture was further incubated at 80 °C for 30 min. After the addition of ethanol (500 μL), the solvents were evaporated. The residue was treated with methyl iodide as described below.

2.6. Quaternarization of PED-, PPZ- and APZ-derivatives

To the PED-, PPZ- or APZ-derivative prepared as described above, methyl iodide (100 μL) was added. The mixture was incubated at 60 °C for 30 min, and then excess reagent was evaporated off. The methylated PED-, PPZ- or APZ-derivative (MPED-, MPPZ- or MAPZ-derivative, respectively) was dissolved in methanol–10 mM ammonium formate (1:1, v/v), an aliquot of which was subjected to LC–MS(/MS).

2.7. Derivatization of DHEA with FMP

DHEA (100 pg) was reacted with FMP and then purified according to the known method [13]. The obtained FMP-derivative was dissolved in methanol–water containing 0.05% formic acid (1:1, v/v), an aliquot of which was subjected to LC–MS.

2.8. Effect of derivatization for detection responses

The effect of the derivatization for the detection responses was evaluated by the limit of detection (LOD, the amount of intact steroids or derivatives per injection giving a signal to noise ratio (S/N) of 5). The most abundant ions of respective steroids were monitored in the SIM mode. The steroids (10, 20 or 100 pg) were converted to their FDNDEA-, PED-, PPZ-, MPED-, MPPZ- or MAPZ-derivatives as described above. These derivatives were dissolved in methanol–10 mM ammonium formate (1:1, v/v, 100 μL) and subjected to LC–MS. By stepwise decreasing the injection volume of the resulting solution, the amount of derivative giving an S/N of 5 was determined. The LODs of the intact steroids were determined using solutions of 100 ng/mL in the same way.

2.9. Determination of estrogens in pregnant women serum

Serum was obtained from healthy pregnant women during the first, second or third trimester; they gave informed consent at Kyoto Prefectural University of Medicine (Kyoto). Ten microliters (second and third trimester) or 20 μL (first trimester) sample of the serum was added to acetonitrile (100 μL) containing D_5-E_1 (100 pg), vortex mixed for 30 s and centrifuged at 1500 × *g* (4 °C, 5 min). The supernatant was diluted with water (400 μL) and purified using a Strata-

X cartridge. After successive washing with water (2 mL) and 30% methanol (2 mL), estrogens were eluted with ethyl acetate (1 mL). After evaporation, the residue was subjected to derivatization with PPZ followed by methylation. The resulting derivative was dissolved in methanol–10 mM ammonium formate (1:1, v/v, 30 μL), 10 μL of which was subjected to LC–MS/MS. The operating LC–MS/MS conditions were as follows: mobile phase, acetonitrile–methanol–10 mM ammonium formate (4:1:5, v/v/v); collision energy, 70 eV; collision cell exit potential, 10 V; precursor and product ions, E_1-MPPZ, *m/z* 549.3 and 502.4, E_2-MPPZ, *m/z* 551.3 and 504.3, D_5-E_1-MPPZ, *m/z* 554.3 and 506.4. The other conditions were the same as those described in Section 2.2.

2.10. Calibration curves for estrogens

Healthy male serum, in which estrogens were not detected by the present method, was used as the blank serum. The blank serum (20 μL) was spiked with E_1 and E_2 (10, 20, 50, 100, 200 and 500 pg each, corresponding to 0.5, 1.0, 2.5, 5.0, 10, 25 ng/mL) and D_5-E_1 (100 pg), which was then pretreated, and derivatized and subjected to LC–MS/MS. The calibration curves were constructed by plotting the peak area ratio (E_1/D_5-E_1 and E_2/D_5-E_1, *y*) versus the concentration of E_1 and E_2 (*x*, ng/ml).

2.11. Recoveries of E_1, E_2 and D_5-E_1 during pretreatment

The spiked sample was prepared by the addition of E_1 and E_2 (5 ng each) to the blank serum (1.0 mL), in which endogenous estrogens were not detected by the present method. The spiked sample and the blank serum (10 μL) were independently pretreated. E_1 and E_2 (50 pg each) were then added to only the blank sample and D_5-E_1 (100 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS/MS. The recoveries of estrogens during pretreatment were calculated from the peak area ratios (E_1/D_5-E_1 or E_2/D_5-E_1) of the spiked and blank sample.

The blank serum (10 μL) was added to methanol (100 μL) containing D_5-E_1 (100 pg; spiked sample) or without D_5-E_1 (blank sample), and the resulting samples were pretreated. D_5-E_1 (100 pg) was then added to only the blank sample and E_1 (100 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS/MS. The recovery of D_5-E_1 was calculated from the peak area ratios (D_5-E_1/E_1) of the spiked and blank sample.

2.12. Determination of DHT in human prostate

Prostatic tissues were obtained from patients with benign prostatic hyperplasia (BPH) without any hormonal treatment, and prostate cancer (PCa) with androgen deprivation therapy (ADT, a combination of luteinizing hormone-releasing hormone agonists and anti-androgen) and stored −20 °C prior to use. All the patients gave informed consent at Kanazawa University Hospital (Kanazawa, Japan).

The tissue was minced by scissors and mashed by a glass homogenizer on ice. Ten milligrams of the mashed tissue was further homogenized in 30% methanol (150 μL) containing D_3-DHT (200 pg). 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 30% methanol (150 μL) and heated at 60 °C for 30 min. After centrifugation at 1500 × *g* (4 °C, 5 min), the supernatants were combined and added to acetonitrile (750 μL). After a vortex-mix (30 s) and centrifugation 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), 70% methanol (2 mL) and hexane (1 mL), the steroids were eluted with ethyl acetate (1 mL). After evaporation, the residue was subjected to the derivatization with APZ followed by methylation. The resulting derivative was dissolved in methanol–10 mM ammonium formate (1:1, v/v, 30 μL), 10 μL of which was subjected to LC–MS/MS. The operating LC–MS/MS conditions were as follows: mobile phase, acetonitrile–10 mM ammonium formate (7:8, v/v); collision energy, 30 eV; collision cell exit potential, 10 V; precursor and product ions, DHT-MAPZ, *m/z* 567.2 and 567.2, D_3-DHT-MAPZ, *m/z* 570.2 and 570.2. The other conditions were the same as those described in Section 2.2.

2.13. Calibration curve for DHT

A PCa tissue (10 mg), in which DHT was remarkably reduced due to ADT and not detected by the present method, was used as the blank tissue. The blank tissue was spiked with DHT (10, 20, 50, 100 and 200 pg; corresponding to 1.0, 2.0, 5.0, 10 and 20 ng/g tissue) and D_3-DHT (200 pg), which was then pretreated, derivatized and subjected to LC–MS/MS. The calibration curve was constructed by plotting the peak area ratio (DHT/D_3-DHT, *y*) versus the concentration of DHT (*x*, ng/g tissue).

2.14. Recoveries of DHT and D_3-DHT during pretreatment

An ethanolic solution of DHT (50 pg in 10 μL; spiked sample) or EtOH (10 μL; blank sample) was added to the PCa tissue (10 mg), in which DHT was not detected by the present method, and the resulting samples were pretreated. DHT (50 pg) was then added only to the blank sample and D_3-DHT (200 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS/MS. The recovery of DHT was calculated from the peak area ratio (DHT/D_3-DHT) of the spiked and blank sample.

An ethanolic solution of D_3-DHT (200 pg in 10 μL; spiked sample) or EtOH (10 μL; blank sample) was added to the PCa tissue (10 mg) and the resulting samples were pretreated. D_3-DHT (200 pg) was then added only to the blank sample and DHT (200 pg) was added to both samples. After derivatization, the samples were subjected to LC–MS/MS. The recovery of D_3-DHT was calculated from the peak area ratio (D_3-DHT/DHT) of the spiked and blank sample.

3. Results and discussion

3.1. Derivatization reagents for estrogens

Estrogens typically have a phenolic hydroxy group on the A-ring, and therefore, this functional group can be used to introduce the ESI-responsive moieties. It is known that Sanger's reagent (*i.e.*, 2,4-dinitrofluorobenzene) and its analogues easily react with a phenolic hydroxy group under alkaline conditions, but not with an alcoholic hydroxy group [17]. This property is a significant advantage for increasing the specificity in the trace analysis of estrogens in complex biological matrices, because endogenous components having a phenolic hydroxy group are limited. Recently, a Sanger's reagent analogue, FDNDEA, was introduced for the LC–ESI-MS quantification of amino acids [15]. This derivatization procedure enhanced the detection responses of amino acids due to the introduction of the easily ionizable diethylamino group and may also be applicable to the estrogen analysis. However, because this tertiary amino group directly bounded with the dinitrobenzene moiety and cannot be quarternarized, the sensitivity enhancement was limited. If the quarternarization is possible, the effect of the derivatization may be much greater.

To quarternarize the amino group in the Sanger's reagent analogue, it should be located far from the dinitrobenzene moiety. In this regard, 1,5-difluoro-2,4-dinitrobenzene was the ideal starting material for the synthesis of a new reagent, because it has two reactive fluorine atoms; one of which is usable for the reaction with estrogens and the other is utilizable for the introduction of the amino group that is able to be quarternarized. That is, when *N*,*N*-dimethylethylenediamine or *N*-methylpiperazine was reacted with 1,5-difluoro-2,4-dinitrobenzene, the desirable reagents, PED and PPZ, respectively, could be readily prepared (Fig. 1a).

PED and PPZ reacted with estrogens in the presence of $NaHCO_3$ within 1 h, as did FDNDEA (Fig. 1b). Incidentally, when stronger base, such as NaOH or Na_2CO_3, was used, PED and PPZ were partially decomposed. Moreover, a higher reaction temperature also increased the decomposition of the reagents, which lowered the derivatization yield. A careful examination on derivatization condition has proven that the condition described in Section 2.4 is most suitable. The derivatization rate of nanogram amounts of estrogens with PED or PPZ was almost quantitative, because when E_1 (10 ng) was derivatized, the intact steroid was not detected by LC–ESI-MS (the minimum detectable amount of intact E_1 was 350 pg; 3.5% of the

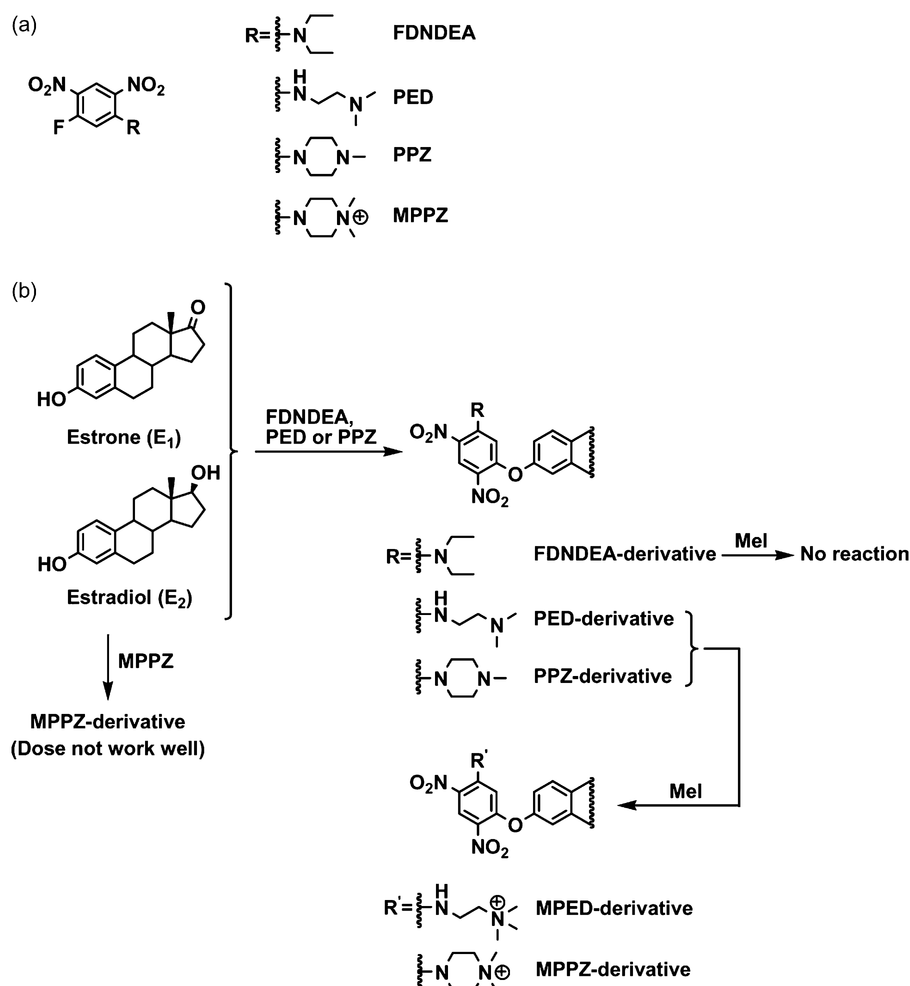

Fig. 1. Derivatization reagents (a) and derivatization procedures (b) for ESI-MS of estrogens.

initial amount). Next, 30 ng and 30 pg of E_1 were individually derivatized and then dissolved in 30 mL and 30 μL of methanol–10 mM ammonium formate (1:1, v/v), respectively. Ten microliters of the respective solutions were subjected to LC–MS. As a result, the peak areas obtained from both solutions were almost equal. This result demonstrates that the derivatization rate is quantitative even when picogram amounts of estrogens are derivatized with PED or PPZ.

We also synthesized MPPZ as the already-charged derivatization reagent (Fig. 1a), but it only slightly reacted with the estrogens (Fig. 1b), although we did not have any plausible explanation for this phenomenon. Therefore, estrogens were converted to their charged derivatives through the PED- or PPZ-derivatives as shown in Fig. 1b. That is, the PED- and PPZ-derivatives of the estrogens were quaternarized with methyl iodide to form the respective MPED- and MPPZ-derivatives. This reaction also quantitatively proceeded in the PED- and PPZ-derivatives, because after the treatment of PED-E_1 or PPZ-E_1 (200 fmol) with methyl iodide, the unreacted PED-E_1 or PPZ-E_1 was not detected by LC–ESI-MS (the minimum detectable amount of the derivative was 6.0 or 2.6 fmol, respectively; 3.0 or 1.3% of initial amount, respectively). Incidentally, the MPED- and MPPZ-derivatives were stable for at least 1 month at 4 °C.

3.2. Effects of derivatization for the detection responses in estrogens

For the ESI-MS operating in the positive-ion mode, the FDNDEA-, PED- and PPZ-derivatives of the estrogens provided their protonated molecules, $[M+H]^+$, as the most abundant ions (Table 1). On the contrary, the MPED- and MPPZ-derivatives gave only their molecular cations, $[M]^+$ (the mass spectra of E_1- and E_2-MPPZ are shown in Fig. 2a and b, respectively).

The effects of the respective derivatization procedures for the detection response were evaluated by the LODs, in which the mobile phases were adjusted so that the t_R values of the respective derivatives were around 6 min. Because intact estrogens are hard to ionize in ESI-MS, their sensitivities are very low (E_1, 1300 fmol and E_2, 2200 fmol). The PED- and PPZ-derivatizations increased the sensitivities 220–860-fold over the intact estrogens and produce good results compared to the FDNDEA-derivatization (180–240-fold). Quaternarization of the PED- and PPZ-derivatives further increased the detection responses. In particular, the LODs of the MPPZ-derivatives were impressive; an injection of 0.55 fmol of the derivatives (equivalent to 0.15 pg of E_1 or E_2) was readily detected, and this derivatization provided a three orders of magnitude increase in the sensitivity of the estrogens.

3.3. Determination of estrogens in pregnant woman serum

To prove the utility of the conversion of estrogens to the MPPZ-derivatives in biological sample analyses, the determination of E_1 and E_2 in pregnant woman serum, which is clinically important for the diagnosis of the fetoplacental function, was performed. The collision-induced dissociation of the MPPZ-derivative was first carried out to select a product ion for the SRM determination (Fig. 2). When the molecular cations were fragmented with a 70 eV energy, the product ions at m/z 502.4 $[M–47]^+$, 504.3 $[M–47]^+$ and 506.4 $[M–48]^+$ were obtained from E_1-, E_2-, D_5-E_1-MPPZ, respectively. Although these product ions could not be identified (probably $[M–NO_2–H (\text{or } ^2H)]^+$), they were specific ions of the MPPZ-derivatives and had a satisfactory intensity. Based on these results, the transitions of m/z 549.3 → 502.4 (E_1-MPPZ), m/z 551.3 → 504.3 (E_2-MPPZ) and m/z 554.3 → 506.4 (D_5-E_1-MPPZ) were monitored for the determination of the serum estrogens.

Pregnant woman serum was pretreated as described in Section 2.9. The recovery rates of E_1, E_2 and D_5-E_1 during the pretreatment were 80.2 ± 6.2, 78.4 ± 4.9 and 77.1 ± 7.1% (mean ± S.D., n = 5), respectively. The chromatograms shown in Fig. 3a were obtained from the serum of a healthy male subject, in which estrogens were not detected. Fig. 3a reveals that there was no interfering peak derived from the endogenous components and the derivatization reagent at the elution position of the derivatized E_1 and E_2. Fig. 3b shows the chromatograms obtained from a pregnant woman (21 weeks of pregnancy), in which the peaks corresponding to the derivatized E_1 and E_2 were

Table 1
ESI-MS data of estrogens and their derivatives

Compound (mw)	Most abundant ion (m/z)	Mobile phase[a] (t_R (min))	LOD (fmol)	Increasing sensitivity[b]
E_1 (intact) (270)	271.2 $[M+H]^+$	2:1 (5.9)	1300 (350)[c]	1
E_1-FDNDEA (507)	508.3 $[M+H]^+$	6:1 (5.9)	7.4 (2.0)	180
E_1-PED (522)	523.3 $[M+H]^+$	6:1 (6.4)	6.0 (1.6)	220
E_1-PPZ (534)	535.3 $[M+H]^+$	5:1 (5.6)	2.6 (0.7)	500
E_1-MPED (537)	537.3 $[M]^+$	2:1 (5.8)	2.2 (0.6)	580
E_1-MPPZ (549)	549.3 $[M]^+$	2:1 (5.6)	0.55 (0.15)	2300
E_2 (intact) (272)	273.3 $[M+H]^+$	2:1 (5.7)	2200 (600)	1
E_2-FDNDEA (509)	510.4 $[M+H]^+$	7:1 (5.6)	9.2 (2.5)	240
E_2-PED (524)	525.3 $[M+H]^+$	11:2 (5.7)	5.9 (1.6)	380
E_2-PPZ (536)	537.3 $[M+H]^+$	5:1 (6.0)	2.6 (0.7)	860
E_2-MPED (539)	539.3 $[M]^+$	20:9 (6.0)	1.8 (0.5)	1200
E_2-MPPZ (551)	551.1 $[M]^+$	2:1 (6.1)	0.55 (0.15)	4000

[a] The proportion (v/v) of methanol–10 mM ammonium formate.
[b] The sensitivities of intact steroids are taken as 1.
[c] The values in parentheses are amounts (pg) converted into intact steroids.

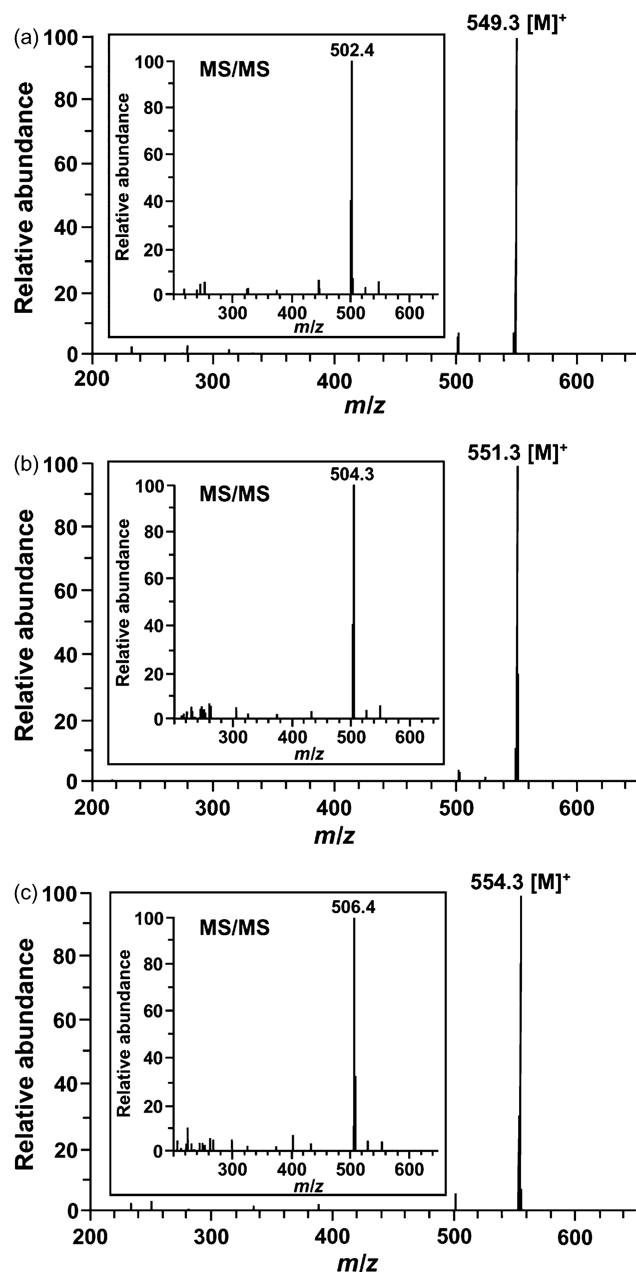

Fig. 2. ESI-MS and ESI-MS/MS spectra (insets) of E_1-MPPZ (a), E_2-MPPZ (b) and D_5-E_1-MPPZ (c). One hundred nanograms of E_1, E_2 and D_5-E_1 were converted to their MPPZ-derivatives and then dissolved in methanol–10 mM ammonium formate (1:1, v/v, 100 μL), 3 μL of which was injected into the LC–MS(/MS). The MS/MS spectra were recorded by the collisional activation of $[M]^+$ with a 70 eV collision energy.

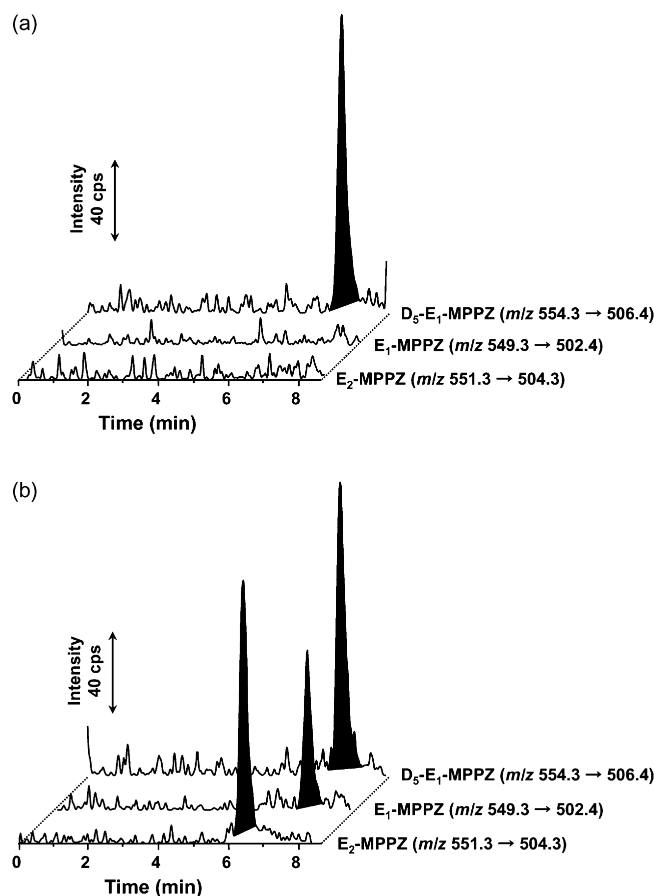

Fig. 3. Chromatograms of derivatized E_1, E_2 and IS from the serum of a healthy male subject (a) and a pregnant woman (21 weeks) (b). The measured concentrations of E_1 and E_2 in the pregnant woman serum were 1.84 and 3.43 ng/mL, respectively.

clearly observed at 7.2 and 6.5 min, respectively. The regression lines showed a good linearity within the range of 0.5–25 ng/mL (E_1, $y = 0.315x + 0.010$ with the correlation coefficient (r) of 0.998; E_2, $y = 0.401x + 0.008$ with r of 0.999). Reproducibility of this assay was evaluated using the serum of pregnant women during first, second and third trimesters (Table 2). All the coefficient of variation (CV) values were below 5.0%. These results show that the present method enables the reproducible determination of estrogens in the serum of a pregnant woman using a small amount of sample within a 9 min chromatographic run time. The estrogen levels obtained by the present method were coincided with those in the previous report [18].

3.4. Derivatization reagent for 5-ene-steroids and 5α-reduced steroids

It has been reported that some 5-ene-steroids and 5α-reduced steroids are related to diseases that frequently develop with age. For example, DHEA is now referred to as a neuroactive steroid and is involved in dementia [19] and a high level of DHT is also well known as the pathogenesis of prostate diseases [20]. As already described, the ESI-sensitivities of the 5-ene-steroids and 5α-reduced steroids are extremely poor due to their low proton-affinity. Therefore, a practical derivatization procedure is indispensable in order to detect trace amounts of these steroids in biological matrices. Because they have one or more alcoholic hydroxy group(s), this functional group is available for introducing an ESI-responsive moiety. The carbonyl halide has been conventionally used as the reacting group of a derivatization reagent for alcohols. To complete the derivatization reaction with the carbonyl halide reagent, however, a base is required as the proton scavenger, and in most cases, it forms an organic solvent insoluble salt, which requires a time-consuming post-treatment procedure. On the contrary, the carbonyl azide can

Table 2
Reproducibility in determination of estrogens in pregnant women serum

	E_1		E_2	
	Mean ± S.D. (ng/mL, $n = 5$)	CV (%)	Mean ± S.D. (ng/mL, $n = 5$)	CV (%)
First trimester (8 weeks)	<0.5	–	0.89 ± 0.04	4.1
Second trimester (21 weeks)	1.84 ± 0.08	2.8	3.43 ± 0.12	3.6
Third trimester (34 weeks)	10.99 ± 0.46	4.2	21.94 ± 0.58	2.6

readily react with alcohols by heating even in the absence of a base.

For the ESI-responsive (permanently charged) moiety, we chose the *N*,*N*-dimethylpiperazine group, which can be prepared from the *N*-methylpiperazine group by the treatment with methyl iodide, based on the results from the estrogen analysis study (Section 3.2). Using this information, we designed and synthesized the carbonyl azide derivative, APZ, as the new derivatization reagent for the 5-ene-steroids and 5α-reduced steroids. APZ was synthesized from 4-fluoro-3-nitrobenzoic acid as the starting material as shown in Fig. 4a. This reagent is stable for at least 3 months at −20 °C. APZ reacted with various steroids within 1 h at 80 °C, regardless of the positions of the hydroxy group in the steroid molecules, and the derivatives could be converted into the corresponding MAPZ-derivatives by methyl iodide (Fig. 4b). We confirmed that the conversion of DHT to its MAPZ-derivative quantitatively proceeded by a method similar to that used in the estrogen study (Section 3.1). Although the conversion rates in AP, DHEA and PREG were not determined, they are strongly anticipated to be quantitative, because their hydroxy groups at the 3-position are more reactive than the hydroxy group at the 17-position of DHT. We also confirmed that the MAPZ-derivatives were stable for at least 3 months, when they were stored at 4 °C.

3.5. Effects of derivatization for the detection responses in 5-ene-steroids and 5α-reduced steroids

Intact steroids provided their dehydrated protonated molecules, $[M+H–H_2O]^+$, (DHEA and PREG) or protonated molecules, $[M+H]^+$, (DHT and AP) as the most abundant ions in the positive ESI-MS (Table 3). In contrast, all the MAPZ-derivatives provided only their $[M]^+$. The LODs of the intact steroids and their MAPZ-derivatives were examined in a manner similar to that done for the estrogens. The derivatization provided a 500–2000-fold higher sensitivity compared to the intact steroids and enabled the detection of steroids in the low femtomolar range.

The proposed procedure was compared to the FMP-derivatization that is currently in use for the ESI-MS of hydroxysteroids [12,13] using DHEA as a model compound (Table 3). The LOD of DHEA-MAPZ was about 6-fold lower than that of DHEA-FMP. As described in Section 1, the FMP-derivatization requires complicated purification steps for removal of the excess reagent prior to the LC–MS analysis [13]. In contrast, the MAPZ-derivatives can be analyzed without reagent removal. Thus, our method is superior to the FMP-derivatization based on the sensitivity and handling after derivatization.

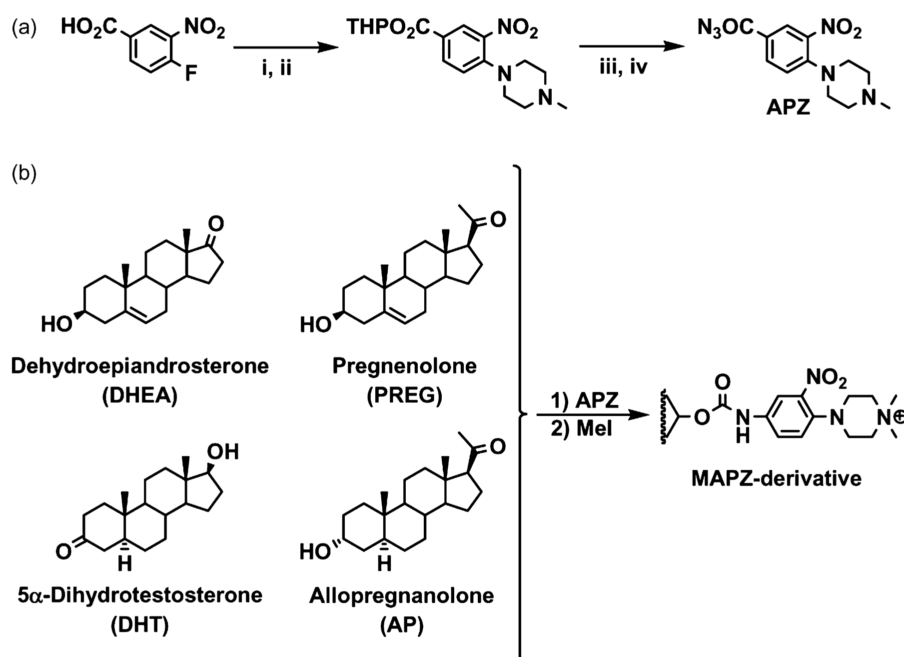

Fig. 4. Synthesis of APZ (a) and conversion of 5-ene-steroids or 5α-reduced steroids to their MAPZ-derivatives (b). (i) DHP, PPTS, chloroform; (ii) *N*-methylpiperazine, acetonitrile; (iii) 6 M HCl, ether; (iv) DPPA, triethylamine, acetonitrile.

Table 3
ESI-MS data of 5-ene-steroids/5α-reduced steroids and their derivatives

Compound (mw)	Most abundant ion (*m*/*z*)	Mobile phase[a] (t_R (min))	LOD (fmol)	Increasing sensitivity[b]
DHEA (intact) (288)	271.2 $[M+H–H_2O]^+$	6:1 (6.3)	2700 (800)[c]	1
DHEA-MAPZ (565)	565.3 $[M]^+$	3:5 (5.9)	1.4 (0.4)	2000
DHEA-FMP (380)[d]	380.2 $[M]^+$	3:4 (6.0)	8.7 (2.5)	320
PREG (intact) (316)	299.2 $[M+H–H_2O]^+$	10:3 (6.4)	800 (250)	1
PREG-MAPZ (593)	593.2 $[M]^+$	5:2 (5.6)	1.3 (0.4)	630
DHT (intact) (291)	292.2 $[M+H]^+$	3:1 (6.0)	690 (200)	1
DHT-MAPZ (567)	567.2 $[M]^+$	5:2 (6.4)	1.0 (0.3)	670
AP (intact) (318)	319.2 $[M+H]^+$	9:2 (6.2)	310 (100)	1
AP-MAPZ (595)	595.2 $[M]^+$	7:2 (6.2)	0.6 (0.2)	500

[a] The proportions (v/v) of methanol–10 mM ammonium formate (intact steroids and MAPZ-derivatives) and methanol–water containing 0.05% formic acid (DHEA-FMP).
[b] The sensitivities of intact steroids are taken as 1.
[c] The values in parentheses are amounts (pg) converted into intact steroids.
[d] J'sphere ODS H-80 column (4 μm, 150 mm × 2 mm i.d.; YMC) was used.

3.6. Determination of DHT in the human prostate

The development of BPH and PCa is dependent on androgens, primarily DHT [19]. Therefore, ADT is often the first choice of several therapeutic procedures for prostate diseases. Thus, the prostatic DHT seems to be a good index for the follow-up of patients affected by prostatic diseases under ADT. For the evaluation of the effects of the therapy in the clinical field, a sensitive method that enables the measurement of prostatic DHT with a small volume of sample (*ca*. 10 mg) collected by a biopsy needle is required.

Based on these requirements, the above developed derivatization procedure was applied to the determination of DHT in the human prostate. We first examined the MS/MS of the MAPZ-derivative of DHT, where $[M]^+$ was used as the precursor ion. $[M]^+$ of the derivative was rather stable, and therefore, the ion was detected without being fragmented even by the addition of a 30 eV collision energy. Although the product ion was observed at *m*/*z* 295.0 when a 55 eV of collision energy was applied, its intensity was not satisfactory. Indeed, the S/N of the prostatic DHT in a BPH tissue was 10 when it was measured by the SRM ($[M]^+ \rightarrow$ residual $[M]^+$) with 30 eV collision energy, while it fell to 6 in the SRM ($[M]^+ \rightarrow$ *m*/*z* 295.0) with 55 eV collision energy. From these results, the determination of prostatic DHT was done with the former SRM mode. By this technique, it was expected that the noise ions derived from the prostate components were reduced with comparatively maintaining the intensity of the monitoring ion.

The prostate extract was pretreated as described in Section 2.12. The recovery rates of DHT and D_3-DHT during the pretreatment were 74.8 ± 5.8 and 73.6 ± 4.2% (mean ± S.D., $n = 5$), respectively. The chromatograms shown in Fig. 5a were obtained from the tissue of a PCa patient with ADT in which DHT was not detected. Fig. 5b shows the chromatograms obtained from a BPH patient without ADT, and the peak corresponding to DHT was clearly observed at 5.8 min. The regression line for DHT showed a good linearity in the range of 1.0–20 ng/g tissue; $y = 0.081x + 0.041$ with $r = 0.998$. The level of DHT in the prostate of the BPH patient was determined to be

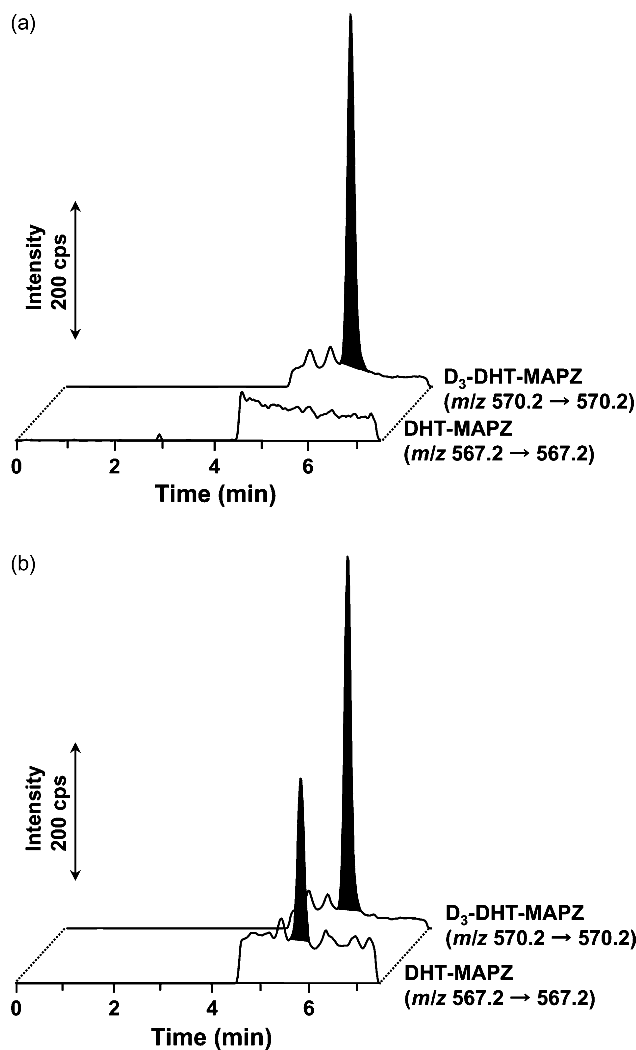

Fig. 5. Chromatograms of derivatized DHT and D_3-DHT from the prostatic tissues of a PCa patient with ADT (a) and a BPH patient without ADT (b). The measured concentration of DHT in the BPH tissue was 5.64 ng/g tissue.

5.64 ± 0.26 ng/g tissue (mean ± standard deviation (S.D.), five replicates) with an intra-assay CV of 4.5%. Thus, the present method is useful for evaluating the effect of ADT.

4. Conclusion

The new derivatization reagents, PPZ and APZ, were developed for the LC–ESI-MS analysis of estrogens and 5-ene-steroids/5α-reduced steroids, respectively. These reagents readily reacted with picogram amounts of steroids, and the derivatives were converted into the charged forms. These procedures remarkably enhanced the detection responses of these steroids in the positive ESI-MS and their LODs were in the low femtomolar range. The MPPZ- and MAPZ-derivatization procedures were then applied for the determination of the serum estrogens and prostatic DHT, respectively. These clinical studies revealed that the proposed derivatization procedures coupled with LC–ESI-MS/MS are able to reproducibly quantify the trace amount of steroids in complex biological matrices. The developed derivatization procedures are also applicable not only to steroid analysis, but also to the analysis of various compounds having a hydroxy group.

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