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New quantification method for estradiol in the prostatic tissues of benign prostatic hyperplasia using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Estrogen is suspected to play a role in the pathogenesis of benign prostatic hyperplasia (BPH) and prostate cancer. To clarify the role of estradiol (E2) in the prostatic tissues (prostatic tissue E2) during the development of prostatic disorders, we developed a new sensitive and specific quantification method for prostatic tissue E2 using liquid chromatography–tandem mass spectrometry (LC–MS/MS). For the solid-phase extraction, E2 was purified by anion-exchange through an Oasis MAX cartridge. In addition, after the formation of 3-pentaflurobenzyl-17β-pyridinium-estradiol derivative (E2-PFBPY), E2-PFBPY was purified by cation-exchange through an Oasis WCX cartridge. These processes in the LC-MS/MS method improved the specificity and sensitivity for prostatic tissue E2 measurement, compared to the radioimmunoassay (RIA) method. The validation tests showed that intra-day and inter-day precisions were both within ±15% (except for 15.5% of the inter-day precision of the lowest concentration), with the accuracy ranging from 88 to 110%. The quantification limit of this assay was 0.15 pg/tube in our method, which was 80-fold more sensitive than that of the RIA method. With the use of our present method, the median E2 levels in the prostatic tissues in patients with BPH ($n = 20$, median age: 71 years) were 12.0 pg/g tissue (95% confidence interval = 9.1–22.6 pg/g tissue). Furthermore, the E2 levels increased significantly with aging. These results showed that our present method would be useful for elucidating the role of prostatic tissue E2 in the development of prostatic disorders with a small amount of tissue samples.

1. Introduction

The role of estrogen in the development of prostatic disorders, including benign prostatic hyperplasia (BPH) and prostate cancer (PCa), is poorly understood. It is known that estrogen can indirectly reduce serum androgen levels via the suppression of hypothalamic–pituitary–gonadal axis [1,2], whereas the direct effects of estrogen on the prostatic tissues are still equivocal. Several reports have revealed that the aromatase enzyme is expressed in both BPH and PCa tissues [3]. Two subtypes of estrogen receptor are also expressed in the prostatic tissues; estrogen receptor α in the stroma and estrogen receptor β predominantly in the epithelium [4,5]. In human BPH tissues, increased accumulation of estradiol (E2) is observed in the nuclei of stromal cells [6], and stromal E2 levels increase with aging [7]. Moreover, in the Noble rat model, the co-administration of E2 and testosterone induces the development of epithelial dysplasia and adenocarcinoma in the dorsolateral prostate [8], whereas the development of E2- and testosterone-related dysplasia is completely prevented by a synthetic estrogen receptor antagonist [9]. These findings evidence the synthesis of estrogen in the prostatic tissues, and suggest that E2 may contribute to the pathogenesis of BPH and PCa in collaboration with the estrogen receptors. Therefore, the measurement of E2 levels in the prostatic tissues is essential for the elucidation of the direct effect of E2 on the development of BPH and PCa.

Despite this, only a few reports have investigated E2 levels in the prostatic tissues [6,7,10,11]. In their methods, the levels of prostatic tissue E2 are measured using radioimmunoassay (RIA), and the validation of their methods has not been performed sufficiently below the level of 10 pg. We had previously developed and validated a precise method for the prostatic tissue E2 measurements by RIA [12]. The sensitivity of our method using RIA was low, and over 1 g of the prostatic tissue was required for a precise analysis.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has recently considered to be a useful method for quantification of steroid hormones because of its accuracy

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and sensitivity [13], but no investigators have performed the precise measurement of prostatic tissue E2 levels using LC–MS/MS. Solid-phase extraction is widely used for the purification of steroid hormone before LC–MS/MS analysis. Oasis MAX cartridges have the ability of anion-exchange, which is one of the retention methods of solid-phase extraction and a selective isolation procedure for acidic compounds such as phenols, including E2 and other estrogens [14–16]. Therefore, an Oasis MAX cartridge might be useful for the specific purification of prostatic tissue E2. Moreover, we had recently developed a new derivatization method for E2 (Fig. 1) [17,18] that might improve the sensitivity of quantification method for prostatic tissue E2 using LC–MS/MS compared to that of the previous RIA method.

In this study, we developed and validated a new determination method for prostatic tissue E2 levels using Oasis MAX extraction, and a new derivatization method for E2 followed by LC–MS/MS analysis. Furthermore, we investigated age-related changes of E2 levels in the prostatic tissues with BPH, to elucidate the role of E2 in the development of BPH.

2. Experimental

2.1. Materials and reagents

Standard E2 was purchased from National Institute of Health Sciences (Tokyo, Japan). [1,2, 3, 4-$^{13}C_4$]-E2 (E2-C_4) was purchased from Hayashi Pure Chemical Ind. Ltd. (Osaka, Japan). Pentaflurobenzyl bromide and 2-fluoro-1-methylpyridinium p-tolensulfonate were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Triethylamine was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Oasis MAX (60 mg, 3 ml) and Oasis WCX (60 mg, 3 ml) cartridges were purchased from Waters Co. (Milford, MA, USA). An InertSep SI cartridge (500 mg, 3 ml) was purchased from GL sciences Inc. (Tokyo, Japan). All solvents were of analytical grade.

2.2. LC–MS/MS

For LC–MS/MS, an API-5000 triple stage quadrupole mass spectrometer equipped with a positive electrospray ionization (ESI) source (Applied Biosystems Inc., Foster City, CA, USA) and a Shimadzu HPLC system (SCL-10Avp system controller, LC-20AD pump, SIL-HTc column oven, CTO-20A auto-sampler; Shimadzu Co. Ltd., Kyoto, Japan) was employed. The column was Xterra MS-C18 column (100 mm × 2.1 mm i.d., 3.5 μm, Waters Co., Milford, MA, USA) and used at 40 °C. The mobile phase consisting of 0.1% HCOOH (Solvent A) and CH_3CN/CH_3OH (50:50) (Solvent B) was used with a gradient elution of A:B = 50:50–40:60 (0.0–3.0 min), 40:60–20:80 (3.0–7.0 min), 0:100 (7.0–9.0 min), and 50:50 (9.0–12.0 min) at a flow rate of 0.4 ml/min. E2 and E2-C_4 were analyzed as their 3-pentaflurobenzyl-17β-pyridiniums (PFBPY derivatives) by ESI-MS/MS. The ESI-MS/MS conditions were as follows: declustering potential, 100 V; spray voltage, 5500 V; collision gas, nitrogen, 6 psi (gas pressure); collision energy, 40 eV; curtain gas, nitrogen, 12 psi (gas pressure); and ion source temperature, 550 °C. The precursor and product ions of PFBPY derivatives were as follows: m/z 544.2 and 339.0 for E2-PFBPY; and m/z 548.2 and 343.2 for E2-C_4-PFBPY.

2.3. Analytical methods of estrogen

2.3.1. Prostatic tissues

Prostatic tissues were obtained from 20 patients to whom open or transurethral surgery for BPH without hormonal therapy had been performed at Gunma University and its affiliate hospitals. The prostatic tissues were excised immediately after collection and stored at −80 °C until analysis. All tissues were obtained in accordance with the requirements and approval of the Committee for Human Ethics and Experimentation at Gunma University, Isesaki Municipal Hospital, and Kiryu Kosei General Hospital. Written informed consent was obtained from all participants.

2.3.2. Sample preparation (Fig. 2)

The frozen prostatic tissues were pulverized in liquid nitrogen to powder, and then homogenized in 10 times volume of distilled water using an Ultra-Turrax homogenizer (IKA, Staufen, Germany) in an ice bath. The homogenized sample (50–100 mg) was transferred to a test tube, and 100 pg of E2-C_4 was added as an internal standard to the homogenized sample, which was then extracted with 3 ml of ethyl acetate. After evaporation, the extract was dissolved in 0.25 ml of CH_3OH, diluted with 1 ml of distilled water, and

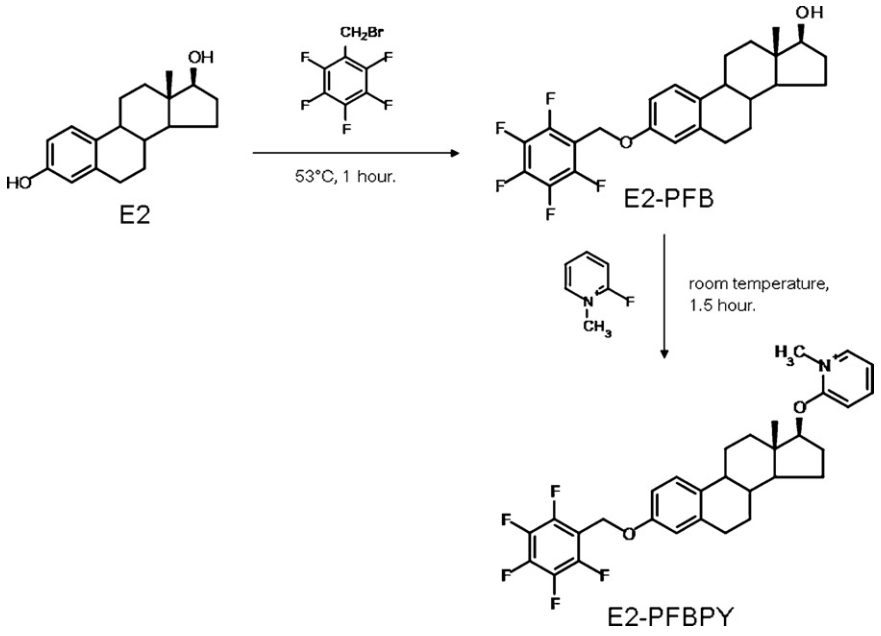

Fig. 1. Derivatization of E2.

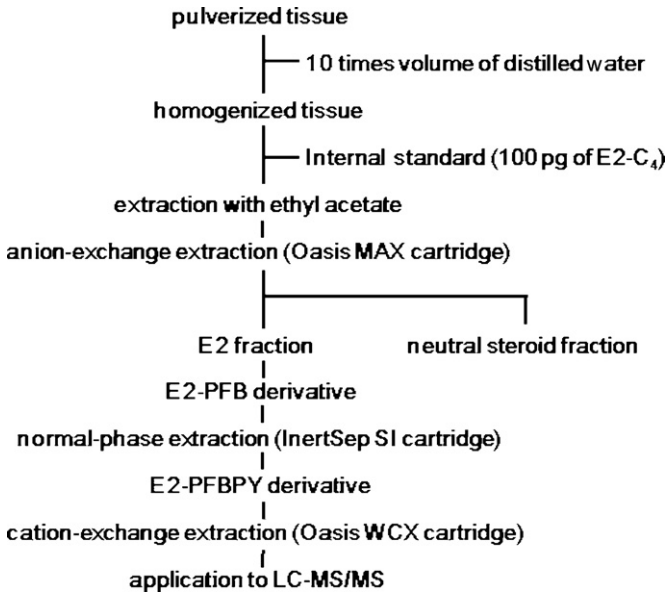

Fig. 2. Flow sheet of the analytical method.

then applied to an Oasis MAX cartridge which had been successively conditioned with 3 ml of CH_3OH, 3 ml of distilled water, 1 ml of 0.2 M NaOH, and 3 ml of distilled water. The cartridge was washed with 3 ml of CH_3OH and 2 ml of HCOOH/50% CH_3OH (2:98), and then E2 was eluted with 3 ml of HCOOH/80% CH_3CN (2:98). After evaporation, the residue was subjected to derivatization described below.

2.3.3. Derivatization of E2 with pentaflurobenzyl bromide and 2-fluoro-1-methylpyridinium p-tolensulfonate

E2 was dried perfectly using a vacuum desiccator at 40 °C for 1 h. The dried E2 was reacted with 0.1 ml of pentaflurobenzyl bromide/CH_3CN (1:40) and 50 μl of 0.8% KOH at 53 °C for 1 h using a thermostat, and then 3-pentafluorobenzyl-estradiol (E2-PFB) was dried under a steam of nitrogen. The residue was dissolved in 0.25 ml of ethyl acetate, diluted with 1 ml of hexane, and then applied to an InertSep SI cartridge, which had been successively conditioned with 3 ml of ethyl acetate and 3 ml of hexane. The cartridge was washed with 1 ml of hexane and 2.5 ml of ethyl acetate/hexane (10:90), and then E2-PFB was eluted with 2.5 ml of ethyl acetate/hexane (50:50). E2-PFB was evaporated to dryness using a centrifugal evaporator, and then dried perfectly using a vacuum desiccator at 40 °C for 1 h. E2-PFB was then reacted with 0.2 ml of mixed solution (20 mg of 2-fluoro-1-methylpyridinium p-tolensulfonate in 1 ml of dichloromethane) and 30 μl of triethylamine/dichloromethane (1:10) at room temperature for 1.5 h. After reaction, the sample was dried under a stream of nitrogen. E2-PFBPY was dissolved in 0.5 ml of CH_3OH and diluted with 0.5 ml of distilled water, and then the mixture was applied to an Oasis WCX cartridge which had been successively conditioned with 3 ml of CH_3CN, 3 ml of CH_3OH and 3 ml of distilled water. The cartridge was washed successively with 1 ml of 0.3% ammonia solution, 3 ml of CH_3CN and 2 ml of HCOOH/50% CH_3OH (2:98), and then E2-PFBPY was eluted with 2 ml of HCOOH/80% CH_3CN (2:98). After evaporation, the residue was dissolved in 0.1 ml of the mobile phase, and 20 μl of the solution was subjected to an LC–MS/MS analysis, as described above.

2.3.4. Calibration of standard curves

Seven concentrations of standard E2 (0.15–100 pg/tube) were put into each tube. Subsequently, 100 pg of E2-C_4 was added to each standard tube as internal standards. E2 was isolated from each solution, and then E2 was derivatized as described above and subjected to an LC–MS/MS analysis. The calibration curve was constructed by plotting the peak area ratio (analyte steroid/internal standard) against the amount of analyte steroid.

2.4. Validation of the analytical method

2.4.1. Assay accuracy

The prostatic tissue samples (9 mg) collected from 5 individuals, which were spiked with (spiked samples) or without (un-spiked samples) E2, were prepared. An internal standard was added to each sample, and the resulting sample was then measured as described above. The assay accuracy was evaluated by analyzing the recovery rate, which was defined as $F_s/F_0 \times 100\%$, where F_s and F_0 were the measured amounts of E2 in the spiked samples and those of un-spiked samples and the spiked amount, respectively. The spiked amounts of E2 were 0.5 pg and 10 pg.

2.4.2. Assay precision

The prostatic tissues (3 mg), which were spiked with various amounts of E2, were used for the determination of the assay precision. Internal standard was added to each sample, and the resulting sample was then measured as described above. The intra-day precision was evaluated by measuring the resulting samples ($n = 5$) on the same day. The inter-day precision was evaluated by measuring the resulting samples for 3 days ($n = 5$, each day). The recovery rate was also measured as described above. The spiked amounts of E2 were 0.15 pg, 0.25 pg, 5 pg, and 50 pg.

2.5. Statistical analysis

The correlation between the prostatic tissue E2 levels and age in patients with BPH was analyzed using Spearman rank correlation coefficients. E2 levels below the quantification limit were set equal to the lower limit of quantification for statistical analysis; which might have introduced bias in favor of not finding a significant difference. All data were analyzed using SPSS software v.11.0 (SPSS, Inc., Chicago, IL). Tests were two-sided, and P values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Recovery of E2 during purification with Oasis MAX and Oasis WCX cartridges

After conditioning an Oasis MAX cartridge with NaOH solution, E2 was retained to the solid-phase, whereas other neutral steroids (e.g., testosterone) were recovered from the liquid-phase that passed though the solid-phase (Fig. 3). The mean recovery rates of E2 and testosterone were 83.4% ($n = 2$) and 98.6% ($n = 2$), respectively. After conditioning an Oasis WCX cartridge with ammonia solution, E2-PFBPY was retained to the solid-phase. The mean recovery rate of E2-PFBPY from an Oasis WCX cartridge was 88% ($n = 2$).

3.2. Development of method of quantitative analysis

3.2.1. LC–MS/MS

The molecular cation was only observed at m/z 544.2 for E2-PFBPY as a base peak (Fig. 4a). Collision of the molecular ion of E2-PFBPY (Fig. 4b) gave the characteristic product ions at m/z 339.0 (ring A moiety of E2-PFBPY which cleaved ring C moiety), m/z 181.0 (the pentaflurobenzyl moiety), and m/z 110.0 (the pyridinium moiety). MRM transition of m/z 544.2 to m/z 339.0 was chosen by means

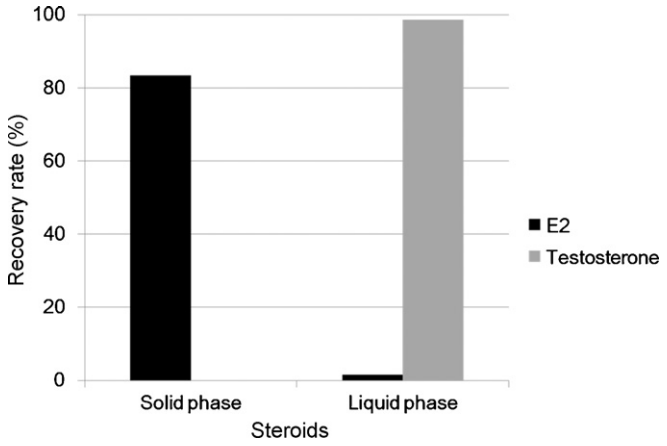

Fig. 3. Recovery of E2 and testosterone from the solid-phase and the liquid-phase that passed through an Oasis MAX cartridge.

of providing the highest signal to the noise ratio and lowest background peak, whereas the highest ESI responses were obtained by using MRM transition of *m*/*z* 544.2 to *m*/*z* 110.0. The typical MRM chromatogram of E2 was shown in Fig. 5. The resolution of the peak demonstrated no interfering peak.

3.2.2. Calibration curve and limit of quantification

Standard calibration curve for E2 was linear, and coefficient of correlation was beyond 0.999. Regression analysis of five standard calibration curves showed a mean (SD) slope of 0.0042 (0.0001) and a mean y intercept of 0.00028 pg (0.00013 pg) within the range of 0.15–100 pg/tube. Relative error ($n = 5$) and coefficient of variation ($n = 5$) at the lowest level on the standard curve were −6.7% and 13.4%, respectively. Signal to the noise ratio at the lowest level on the standard curve was more than 10. The limit of quantification was defined as the lowest level on the standard curve of the analyte with an acceptable accuracy (relative error < ±20%) and precision (coefficient of variation < ±20%), and with signal to the noise ratio of not less than 5. According to this definition, the lower limit of quantification for E2 was 0.15 pg/tube.

3.3. Assay accuracy and precision

The assay accuracy for E2 was defined as the recovery rate described above. Mean (SD) recoveries in E2 assays were 104.1% (9.3%) for 0.5 pg of spiked amount and 98.6% (5.1%) for 10 pg (Table 1). All the assay accuracies ranged from 85 to 115%. The intra-day assay precision (relative standard deviation, RSD) and the inter-day assay precision (RSD) for E2 were 1.3–6.9% and 3.1–15.5%, respectively (Table 2). All the assay precisions were within ±15%, except for 15.5% of the inter-day assay precision at the lowest spiked concentration.

3.4. The E2 levels in BPH

Twenty patients at 58–82 years of age (median, 71 years) underwent open or transurethral surgery for BPH. The median prostatic tissue E2 levels in patients with BPH were 12.0 pg/g tissue (95% confidence interval = 9.1–22.6 pg/g tissue). The prostatic tissue E2 levels increased significantly with aging (rs = 0.51, $P = 0.02$; Fig. 6).

4. Discussion

In this study, we developed a sensitive and specific analytical method for quantifying E2 in the prostatic tissue by using LC–MS/MS for the first time. Several reports have suggested that local E2 may contribute to the pathogenesis of BPH and PCa [19,20], but only a few groups have reported the details of prostatic tissue E2 [6,7,10–12]. All of these previous reports have determined prostatic tissue E2 levels by RIA methods. However, it is well known that the dose–response curves using RIA data are non-linear, and consequently various curve-fitting methods have been used for the determination by RIA [21,22]. Despite use of various curve-fitting methods, only a segment of the standard curve is linear. Thus, low levels of the steroid hormones are usually determined at a portion of the calibration curve where the variance is large. These findings have been previously confirmed by various reports [23–25]. Therefore, measurement of prostatic tissue E2 levels by RIA may lead to false results, since the prostatic tissue E2 levels are known to be very low [12]. In contrast, the calibration curves of LC–MS/MS are linear, and consequently LC–MS/MS avoids the bias at low levels of E2 within the range of the calibration curves. Hsing et al. report the low reproducibility of the RIA method as compared to the LC–MS/MS

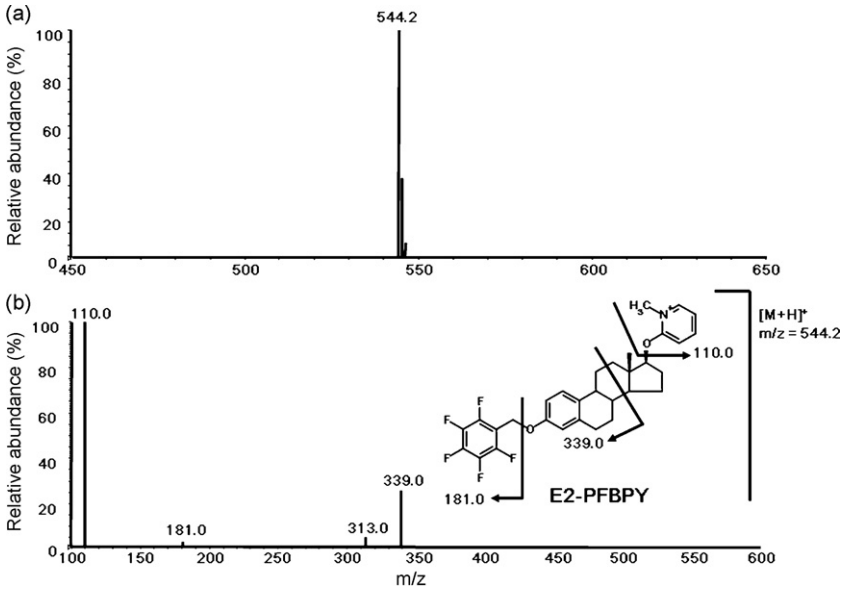

Fig. 4. Mass spectra of (a) E2-PFBPY and (b) its product ion.

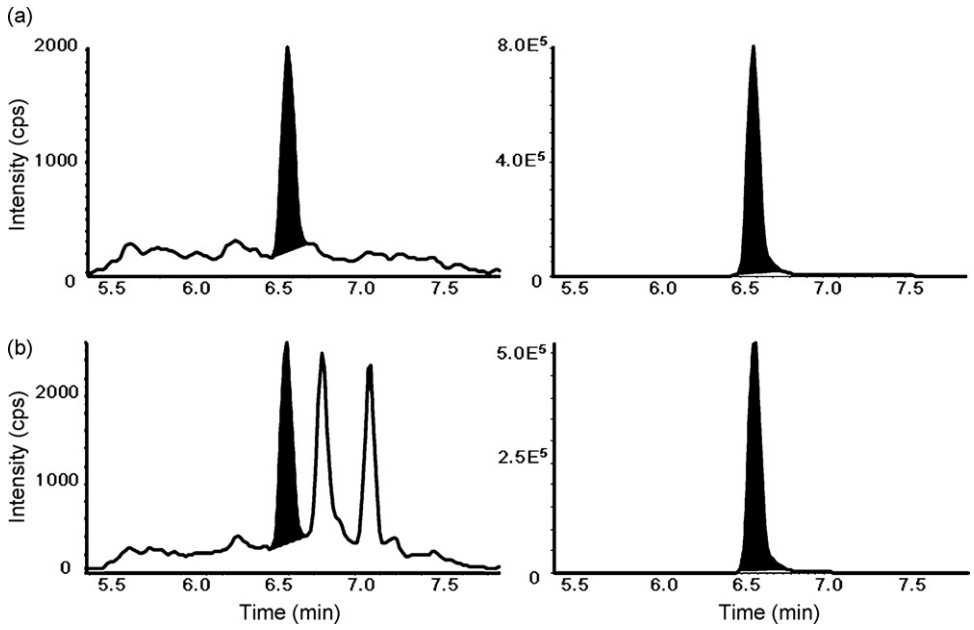

Fig. 5. Typical LC–MS/MS chromatograms of PFBPY derivatives obtained from (a) standard E2 (0.5 pg) + internal standard and (b) prostatic tissue (100 mg) + internal standard.

method for quantifying serum E2 [13]. Nelson et al. report that RIA and LC–MS/MS give different measurement results for serum E2 below the level of 13.5 pg/ml [26]. These findings demonstrate the usefulness of LC–MS/MS method for the determination of low levels of prostatic tissue steroid hormones, such as E2. Furthermore, when using LC–MS/MS method, the tandem MS can increase specificity of analytical steroid hormones, since the molecular ion is cleaved twice and there is little chance for contamination by impurities (Fig. 4).

The derivatization of steroid hormones is essential for improving the detection sensitivity of LC–MS/MS due to the low ionization of steroid hormones [16]. We had previously measured the serum E2 levels by LC–MS/MS using reversed-phase extraction and derivatization procedure to form E2-PFBPY, and the quantification was

Table 1
Assay accuracy.

Patient	Matrices	Spiked (pg)	Measured (pg)	Expected (pg)[a]	Recovery rate (%)[b]
1		0	Not detected	–	–
		0.5	0.52	0.50	104.0
		10.0	9.76	10.00	97.6
2		0	0.13	–	–
		0.5	0.54	0.63	85.7
		10.0	10.61	10.13	104.7
3		0	0.09	–	–
	Prostatic tissue (9 mg)	0.5	0.55	0.59	93.2
		10.0	9.21	10.09	91.3
4		0	0.18	–	–
		0.5	0.75	0.68	110.3
		10.0	9.90	10.18	97.2
5		0	Not detected	–	–
		0.5	0.44	0.50	88.0
		10.0	9.98	10.50	99.8

[a] Values were obtained by addition of spiked amount and measured amount of un-spiked sample.
[b] Recovery rate: measured value/expected value × 100.

Table 2
Intra-day and inter-day assay precision.

Matrices	Spiked (pg)	Intra-day assay			Inter-day assay		
		Measured (pg)[a]	RSD (pg)[b]	Recovery rate (pg)[c]	Measured (pg)[a]	RSD (pg)[b]	Recovery rate (%)[c]
Prostatic tissue (3 mg)	0	0.15 ± 0.02	–	–	0.11 ± 0.05	–	–
	0.15	0.30 ± 0.02	6.9	100.0	0.25 ± 0.04	15.5	97.4
	0.25	0.41 ± 0.02	4.4	102.5	0.36 ± 0.05	12.5	99.3
	5.00	5.06 ± 0.15	2.9	98.3	4.84 ± 0.30	6.3	94.7
	50.00	50.24 ± 0.65	1.3	100.2	49.34 ± 1.51	3.1	98.5

[a] Mean ± SD for the intra-day ($n = 5$) and inter-day (3 days, $n = 5$ for each day) assay variation tests.
[b] Relative standard deviation.
[c] Recovery rate: measured value/expected value × 100. Expected values were obtained by addition of spiked amount and measured amount of un-spiked sample for each case.

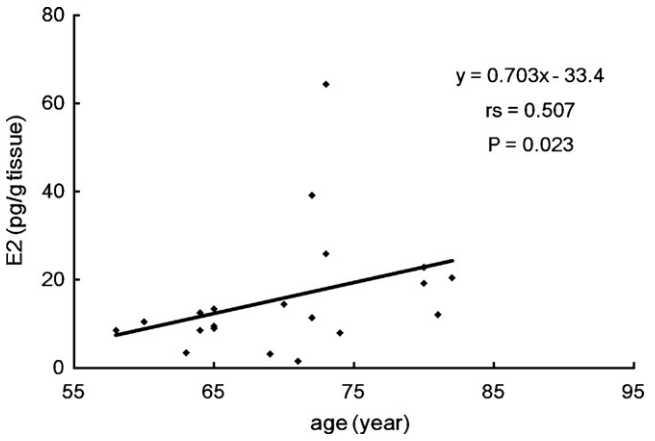

Fig. 6. Relation of the prostatic tissue E2 with ages.

possible at the 0.1 pg/ml levels (un-published date). We had also previously measured the tissue E2 levels by the same method, but the inadequate purification resulted in low sensitivity of E2 (data not shown). Thus, in this study, we improved the purification method of E2.

Oasis MAX cartridges are made of divinylbenzene-*N*-vinylpyrrolidone copolymer backbone, on which quaternary amine groups are covalently bonded [15]. Due to the presence of these quaternary amine groups, Oasis MAX cartridges can retain acidic compounds, such as E2, through anion-exchange, which is a subtype of ion-exchanges [14]. Thus, after conditioning an Oasis MAX cartridge with NaOH solution, E2 was dissociated from proton and consequently could be efficiently retained to the cartridge, whereas other steroids could not be retained (Fig. 2). Cation-exchange is also a subtype of ion-exchanges used for retention on the solid-phase [27]. Oasis WCX cartridges are made of divinylbenzene-*N*-vinylpyrrolidone copolymer backbone, on which carboxylic acid groups are covalently bonded. Due to the presence of these carboxylic acid groups, Oasis WCX cartridges can retain strong basic compounds such as quaternary amine. Interestingly, E2 has changed to have a basic feature after the formation of E2-PFBPY because of the presence of quaternary amine on pyridinium. Thus, after conditioning an Oasis WCX cartridge with ammonia solution, proton in the cartridge was dissociated from carboxylic acid and consequently E2-PFBPY could be efficiently retained to the cartridge.

These purification methods using solid-phase extraction and derivatization procedure to form E2-PFBPY improved the specificity and sensitivity of prostatic tissue E2 by LC–MS/MS. Our validation studies demonstrated that both intra-day and inter-day precisions were less than 20% and yielded mean E2 recoveries of 104% with calibration curve linearity of 0.999. Moreover, we confirmed a good linearity for each prostatic tissue ($n = 5$, 10–100 mg) in the evaluation of the additivity of prostatic tissues (data not shown). The quantification limit (0.15 pg/tube) for the prostatic tissue E2 in our present study by LC–MS/MS was 80-fold more sensitive than that of our previous method by RIA [12], and our new method enabled the quantification of prostatic tissue E2 with only a small amount of tissue sample (16 mg). Our results on the quantification of prostatic tissue E2 in BPH generally resembled those in the previous reports, except for a study by Belis et al. [6,10,12]. In the RIA method by Belis et al., 500 mg of prostatic tissues are used for the measurement of tissue E2 level, and the validation of this method has not been performed below the level of 20 pg. Thus, the measurements of prostatic tissue E2 below the levels of 40 pg/g tissue may lead to false results, which may be the reason why their results are higher than those of other reports.

As the incidence of BPH increases with aging, age-related changes in the endocrine environment in the prostatic tissue must be associated with the pathogenesis of BPH. Our present results showed the prostatic tissue E2 levels increased significantly with aging, which were consistent with the results of Krieg et al. [7]. They report that age-related increase of E2 levels in the prostatic stroma and age-related decrease of 5-alpha dihydrotestosterone (DHT) in the prostatic epithelium lead to the dramatic increase of the ratio of estrogen to androgen in the prostatic stroma with age. We had also previously reported that the ratio of E2 to DHT level in the prostatic tissue increased with age and showed a significant positive correlation with the proportion of the prostatic stroma [12]. These findings suggest that the prostatic tissue E2 has a synergistic role with prostatic tissue DHT in the pathogenesis of BPH. Furthermore, in contrast with the age-related increase of the prostatic tissue E2, the serum E2 levels remained unchanged with aging [28]. This discrepancy suggests the increase of E2 synthesis in the prostatic tissues with age, but no further reports are available about the changes of aromatase expression and activity in BPH with aging. Therefore aromatase in BPH should be investigated in the future to elucidate the relationship between aging and development of BPH. Moreover, Hiramatsu et al. report that aromatase expression and activity are identified in both BPH and PCa tissues [3]. Ellem et al. report that aromatase expression is observed in PCa cell lines and microdissected PCa cells [29]. Tsuchiya et al. report that the aromatase gene polymorphism may be a prognostic predictor of metastatic PCa [30]. These reports suggest that estrogen has a significant role in the development of PCa, as well as BPH. Therefore, measurement of estrogen levels in the prostatic tissue is necessary for elucidating the mechanism of both BPH and PCa development. Furthermore, our sensitive method would provide reliable results about the prostatic tissue E2 levels with a small amount of tissue sample, such as a biopsy sample, and consequently would be a powerful tool for future studies on the role of prostatic tissue E2 in development of BPH and PCa.

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