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# Stable isotope dilution high-performance liquid chromatography–electrospray ionization mass spectrometry method for endogenous 2- and 4-hydroxyestrogens in human urine

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## Abstract

A sensitive, precise and accurate stable isotope dilution high-performance liquid chromatography–electrospray ionization mass spectrometry method has been developed for measuring endogenous 2- and 4-hydroxyestrogens, the main catechol estrogens in human urine. Compared to the published methods using gas chromatography–mass spectrometry, this approach simplifies sample preparation and increases the throughput of analysis. The unique part of our method is the use of a simple and rapid derivatization step that forms a hydrazone at the C-17 carbonyl group of catechol estrogens. This derivatization step has greatly enhanced method sensitivity as well as HPLC separability of 2- and 4-hydroxyestrogens. Standard curves were linear over a 100-fold calibration range with correlation coefficients for the linear regression curves typically greater than 0.996. The lower limit of quantitation for each catechol estrogen is 1 ng per 10-ml urine sample, with an accuracy of 97–99% and overall precision, including the hydrolysis, extraction and derivatization steps, of 1–3% for samples prepared concurrently and 2–11% for samples prepared in several batches. This method is adequate for measuring the low endogenous levels of catechol estrogens in urine from postmenopausal women.

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**Keywords:** Stable isotope dilution LC–MS; Hormonal carcinogenesis; Hydroxyestrogens; *p*-Toluenesulfonylhydrazide

## 1. Introduction

A critical role for endogenous estrogen in the development of breast cancer has been postulated for more than a century, ever since Beatson demonstrated that oophorectomy induced tumor remission in human breast cancer [1]. Substantial evidence supports a causal relationship between risk of human

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breast cancer and levels of endogenous estrogen [2]. Increased risk has been reported in women with high serum and urine estrogens [3,4], as well as in those women exposed to increased estrogen levels over time as a result of late menopause, early onset of menstruation and/or postmenopausal obesity [5]. A key mechanism in estrogen-related breast cancer may be the metabolic activation of estrogens to genotoxic metabolites called catechol estrogens [6,7], mainly 2-hydroxyestrone and 4-hydroxyestrone in humans (Fig. 1). Electrophilic quinone products of these catechol estrogens can react with DNA to form both stable and depurinating adducts known to generate mutations and cell transformation that can initiate cancers [7,8]. It is conceivable that quantitative measurement of endogenous catechol estrogens may play an important role in elucidating the mechanism of breast carcinogenesis and in estimating the risk of breast cancer in individual women.

Current methods for measuring endogenous catechol estrogens most often involve radioimmunoassay (RIA) [9–12], enzyme immunoassay (EIA) [13,14], high-performance liquid chromatography (HPLC) with electrochemical detection [15–17], and stable isotope dilution gas chromatography–mass spectrometry (GC–MS) [18]. RIA and EIA suffer from relatively poor specificity due to the cross-reactivity of antibodies [19–22]. HPLC with electrochemical detection has been used for catechol estrogen analysis in hamsters treated with catechol estrogens [23] and in pregnant women, whose estrogen levels are elevated at least 10-fold [15–17]. Stable isotope dilution GC–MS is both sensitive and specific, and has been successfully used not only for urine sam-

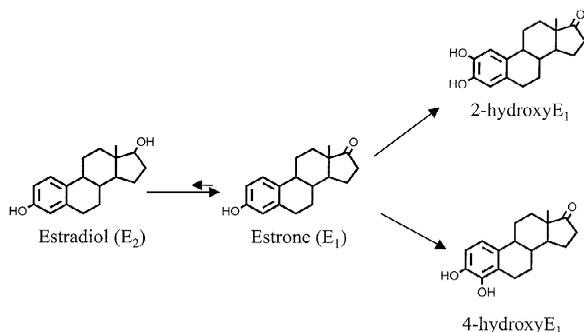


Fig. 1. Formation of the main endogenous catechol estrogens in humans.

ples from non-pregnant premenopausal women but also for urine from postmenopausal women, in which catechol estrogens are substantially reduced [18,24]. However, the stable isotope dilution GC–MS method requires extensive and laborious sample preparation, including three  $C_{18}$  solid-phase extractions, six ion-exchange column separations, four liquid–liquid extractions, and two derivatization procedures for each urine sample [18,24]. Although liquid chromatography–mass spectrometry (LC–MS) has been used in *in vitro* and *in vivo* pharmacological studies of catechol estrogens in rat brains [25,26], the sensitivity of LC–MS with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) is not adequate for the endogenous levels of catechol estrogens in women [27]. In this report, we present a stable isotope dilution HPLC–ESI–MS method with a simple and rapid derivatization step that greatly improves method sensitivity and HPLC separability of catechol estrogens and makes LC–MS a much more competitive method for human endogenous catechol estrogen analysis.

## 2. Experimental

### 2.1. Chemicals and reagents

Catechol estrogens (CE), 2-hydroxyestrone (2-hydroxy $E_1$ ) and 4-hydroxyestrone (4-hydroxy $E_1$ ) (Fig.

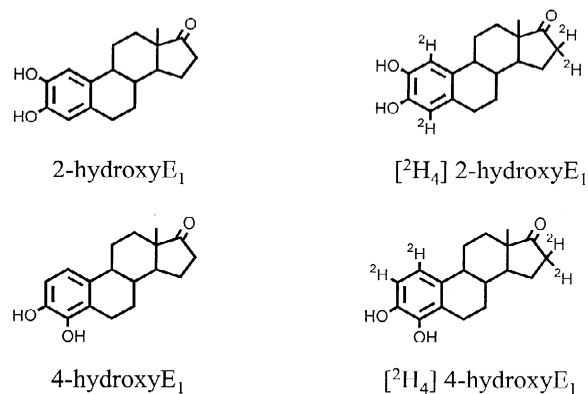


Fig. 2. Chemical structures of 2-hydroxyestrone and 4-hydroxyestrone (CE), and  $[^2H_4]$ 2-hydroxyestrone and  $[^2H_4]$ 4-hydroxyestrone (d-CE).

2), were obtained from Steraloids (Newport, RI, USA). Deuterium-labeled catechol estrogens (d-CE), [ $^2\text{H}_4$ ]2-hydroxyestrone and [ $^2\text{H}_4$ ]4-hydroxyestrone (Fig. 2), were purchased from C/D/N Isotopes (Pointe-Claire, Canada). All CE and d-CE analytical standards have chemical and isotopic purity  $\geq 98\%$ , respectively, as reported by the manufacturers, and were used without further purifications. *p*-Toluenesulfonhydrazide (TSH) was purchased from Aldrich (Milwaukee, WI, USA). Methanol (HPLC grade) and formic acid (reagent grade) were obtained from EM Science (Gibbstown, NJ, USA), and water (HPLC grade) was obtained from Mallinckrodt Baker (Paris, KY, USA). Glacial acetic acid (HPLC grade), L-ascorbic acid (reagent grade), boric acid (reagent grade), sodium hydrogencarbonate (reagent grade) and sodium hydroxide (reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA), and sodium acetate (reagent grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA).  $\beta$ -Glucuronidase/sulfatase from *Helix pomatia* (Type H-2) and QAE Sephadex A-25 were obtained from Sigma (St. Louis, MO, USA). All glassware including Pasteur pipettes was silanized with Aqua-Sil (Pierce, Rockford, IL, USA). QAE-Sephadex gels in acetate and borate forms were prepared as previously described [18].

## 2.2. Urine sample collection

Twenty-four-hour urine samples were collected in 3-l bottles containing 3 g ascorbic acid, to prevent oxidation, from two healthy non-pregnant premenopausal women (aged 34 and 38 years) and two healthy postmenopausal women (aged 58 and 60 years; 5+ years past last menstrual cycle). None of the women was taking exogenous estrogens. For the two premenopausal women, samples were collected during the midfollicular (days 8–9 of menstrual cycle) and midluteal phases (6 days before the anticipated menses) of the menstrual cycle. Immediately after the urine collection was completed, urine volume was recorded and sodium azide, to prevent bacterial growth, was added to achieve a 0.1% (w/v) concentration. One half of the 24-h urine from each of two postmenopausal women was mixed to prepare a pooled postmenopausal urine, and the remaining two halves were non-pooled postmenopausal urines.

Similarly, pooled and non-pooled premenopausal urines during either midfollicular or midluteal phase were prepared. Aliquots of both pooled and non-pooled urines were stored at  $-80^\circ\text{C}$  until analysis.

## 2.3. Preparation of stock and working standard solutions

Stock solutions of CE and d-CE were each prepared at  $80\ \mu\text{g ml}^{-1}$  by addition of 2 mg catechol estrogen powders to a volumetric flask and diluting to 25 ml with 100% methanol. These solutions were stored at  $-20^\circ\text{C}$  until needed to prepare working standard solutions. During each day of analysis, working standards of CE and d-CE were prepared by serial dilutions of stock solutions with 100% methanol. In our analyses, d-CE working standard solution was prepared at  $800\ \text{ng ml}^{-1}$ , while CE working standard solutions were prepared at 800 and  $50\ \text{ng ml}^{-1}$ .

## 2.4. Preparation of calibration standards

Since CE are naturally present at various levels in all human urine samples including those from males, use of CE-spiked urine to generate calibration curves was impractical. Therefore, non-biological matrix calibration standards were prepared by combining  $50\ \mu\text{l}$  of the d-CE working internal standard solution ( $40\ \text{ng d-CE}$ ) with various volumes of either CE working standard solution, which typically ranged from 0.5 to  $64\ \text{ng CE}$ .

## 2.5. Urinary CE hydrolysis and extraction procedure

To a 10-ml aliquot of urine sample,  $50\ \mu\text{l}$  of the d-CE working internal standard solution ( $40\ \text{ng d-CE}$ ) was added, followed by 10 ml of freshly prepared enzymatic hydrolysis buffer containing 50 mg of L-ascorbic acid,  $100\ \mu\text{l}$  of  $\beta$ -glucuronidase/sulfatase from *Helix pomatia* (Type H-2) and 10 ml of 0.15 M sodium acetate buffer (pH 4.1), as previously described [18]. The sample was incubated overnight at  $37^\circ\text{C}$ . After hydrolysis, the sample was applied to a primed Bond Elut LRC  $\text{C}_{18}$  column (Chrom Tech, Apple Valley, MN, USA) and washed with 5 ml of water. CE and d-CE were eluted with 3

ml of methanol and further purified on QAE-Sephadex in acetate and borate forms, respectively, as described by Fotsis and Adlercreutz [18].

### 2.6. Derivatization procedure

The fraction containing both CE and d-CE was evaporated to dryness under nitrogen gas (Reacti-Vap III, Pierce) and derivatized to form the CE and d-CE *p*-toluenesulfonylhydrazones (CE-TSH and d-CE-TSH, respectively) (Fig. 3) by reaction with 400  $\mu$ g *p*-toluenesulfonylhydrazide in 200  $\mu$ l methanol and heating at 60 °C (Reacti-Therm III Heating Module, Pierce) for 30 min. Calibration standard mixtures were derivatized in the same way. After derivatization, urine samples and calibration standards were evaporated to dryness under nitrogen and redissolved in 100  $\mu$ l methanol for LC–MS analysis.

### 2.7. HPLC–MS

LC–MS analysis was performed on a Finnigan LCQ DECA ion trap mass spectrometer with a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA) controlled by Xcalibur software. Liquid chromatography was carried out on a reversed-phase Luna C<sub>18</sub>(2) column (150×2.0 mm, 3  $\mu$ m; Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol as solvent A and water with 0.1% (v/v) formic acid as solvent B. The LC flow-rate of 200  $\mu$ l/min was used for both ESI and APCI modes. Sensitivity was such that only 5  $\mu$ l of each 100- $\mu$ l sample was injected by the autosampler for analysis. The entire chromatography effluent was passed into the mass spectrometer interface for subsequent detection.

For the analysis of CE-TSH and d-CE-TSH, a

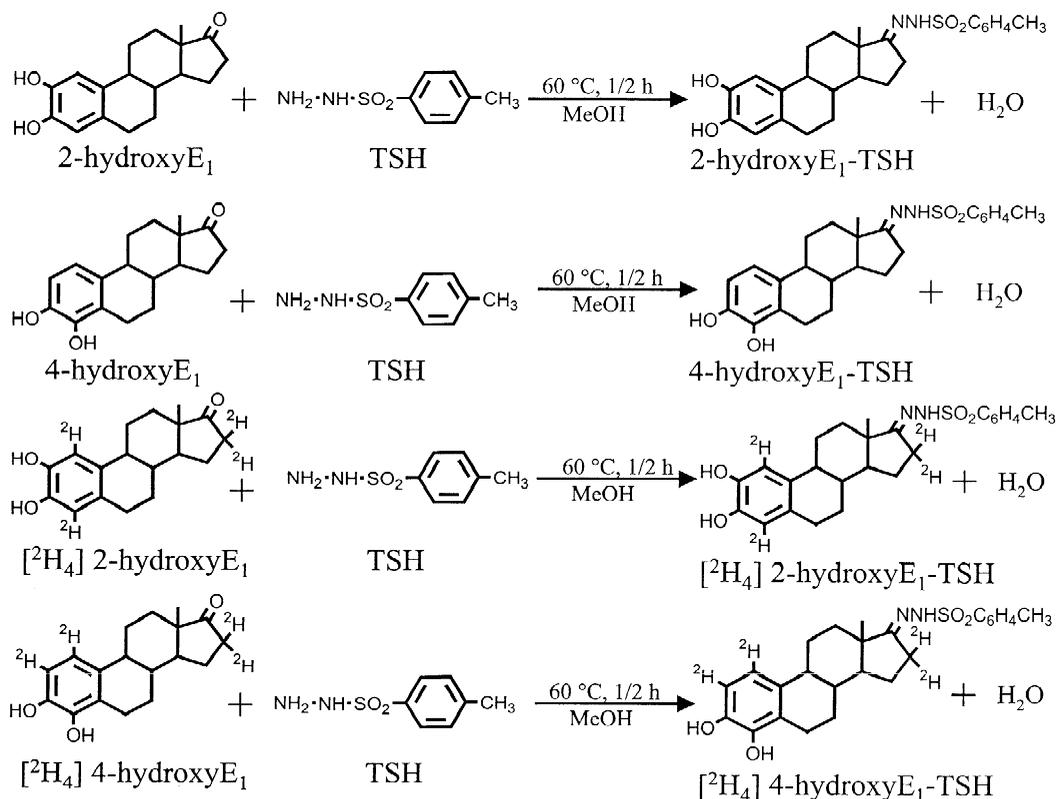


Fig. 3. TSH derivatization of CE and d-CE. TSH=*p*-Toluenesulfonylhydrazide.

linear gradient of A–B changing from 60:40 to 75:25 in 15 min was employed. After changing back from 75:25 to 60:40 in 2 min, the mobile phase composition A–B stayed at 60:40 for 8 min before the next injection. The ESI positive ion mode was used as follows: ion source voltage, 5 kV; heated capillary temperature, 250 °C; capillary voltage, 15 V; sheath gas flow-rate, 70 units; auxiliary gas flow-rate, 15 units; tube lens offset, 50 V. MS full scan mode was employed for characterizing mass spectra of CE-TSH and d-CE-TSH (Fig. 4). MS selected ion monitoring (SIM) mode was used for the quantitative analysis. The protonated molecules  $[\text{MH}^+]$  of CE-TSH and d-CE-TSH were monitored with SIM ranges set at  $m/z$  453.5–456.5 and  $m/z$  457.0–461.0, respectively. The less abundant natriated molecules  $[\text{MNa}^+]$ , about 15–20% of  $[\text{MH}^+]$ , were used as the second ion pairs for confirming the analyte identification.

For the purpose of comparison, the LC–MS performance of CE and d-CE without TSH derivatization was also examined. A linear gradient of A–B changing from 40:60 to 60:40 in 10 min was employed, and then held at 60:40 for an additional 10 min. After changing back from 60:40 to 40:60 in 2 min, the mobile phase composition A–B stayed at 40:60 for 8 min before the next injection. The APCI positive ion mode was used as follows: ion source current, 10  $\mu\text{A}$ ; vaporizer temperature, 450 °C; heated capillary temperature, 175 °C; capillary voltage, 15 V; sheath gas flow-rate, 80 units; tube lens offset, 30 V. MS full scan mode was employed for characterizing the mass spectra of CE and d-CE (Fig. 5). MS SIM mode was used for the analysis of calibration standards without TSH derivatization. The protonated molecules  $[\text{MH}^+]$  of CE and d-CE were monitored with SIM ranges set at  $m/z$  285.5–288.5 and  $m/z$  289.0–293.0, respectively.

### 2.8. Quantitation of CE

CE-TSH/d-CE-TSH area ratios were determined for the SIM chromatographic peaks using Xcalibur software. Calibration curves were constructed by plotting CE-TSH/d-CE-TSH peak area ratios obtained from calibration standards versus CE concentrations and fitting these data using linear regression. CE concentrations in urine samples were then interpolated using this linear function. For d-CE-

TSH, significant exchange loss of deuterium from the  $[\text{}^2\text{H}_4]$  species was noted (Fig. 4C and D) during sample processing and/or analysis. Therefore, the summed intensity of all three observable  $[\text{MH}^+]$  ions in the d-CE-TSH cluster, rather than that of the  $[\text{}^2\text{H}_4]$  species alone, was used as the reference for quantitation.

### 2.9. Absolute recovery of CE after hydrolysis and extraction procedure

To one set of six 10-ml aliquots of the pooled postmenopausal urine, 50  $\mu\text{l}$  of the d-CE working internal standard solution (40 ng d-CE) was added, followed by the hydrolysis and extraction procedure described above. A second set of six 10-ml aliquots of the pooled postmenopausal urine was treated identically, except that the d-CE was added after the hydrolysis and extraction procedure instead at the beginning. Both sets of samples were then derivatized and analyzed in consecutive LC–MS analyses. The absolute recovery of CE after the hydrolysis and extraction procedure was calculated by dividing the CE-TSH/d-CE-TSH peak area ratio from a sample of the second set with that from a comparable sample of the first set, and then calculating the mean of the six values.

### 2.10. Accuracy and precision of the urinary CE analysis

To assess accuracy and intra-batch precision, 50  $\mu\text{l}$  of the d-CE working internal standard solution (40 ng d-CE) was added to each of 18 10-ml aliquots of the pooled postmenopausal urine. Then, identical known amounts of CE (0, 8 or 30 ng, respectively) were added to each of six urine aliquots. All the urine samples were hydrolyzed, extracted, derivatized, and analyzed as described above. The endogenous CE concentration for the pooled postmenopausal urine was determined as the mean of the measured values from the six blank samples. This baseline CE concentration was then subtracted from the values determined for CE spiked urine samples to assess method accuracy and intra-batch precision. In addition, duplicate aliquots of the pooled urines from both postmenopausal and premenopausal midluteal

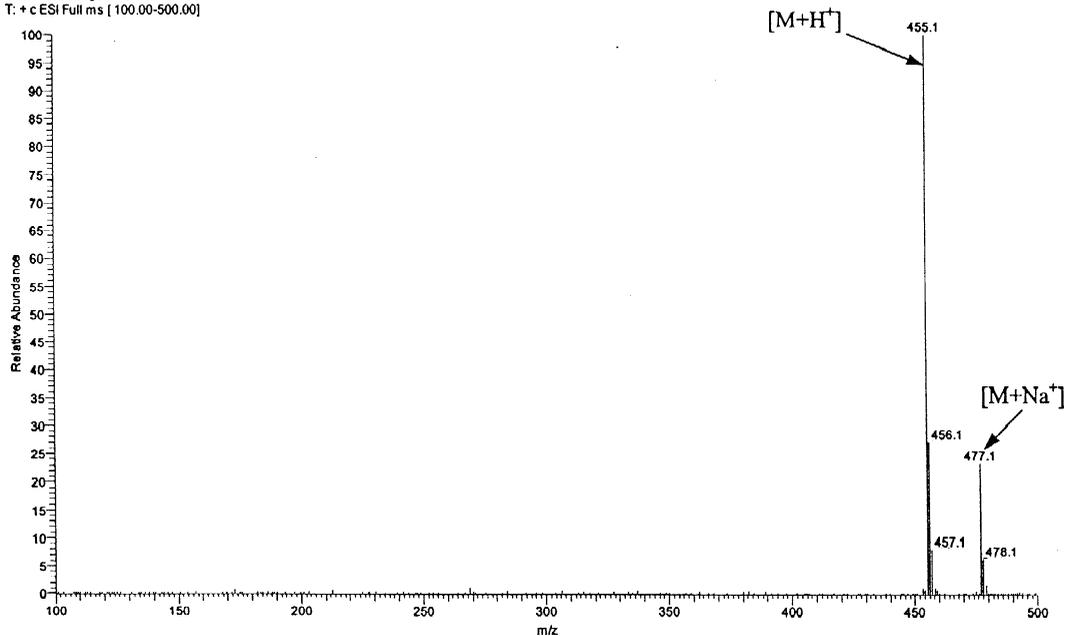
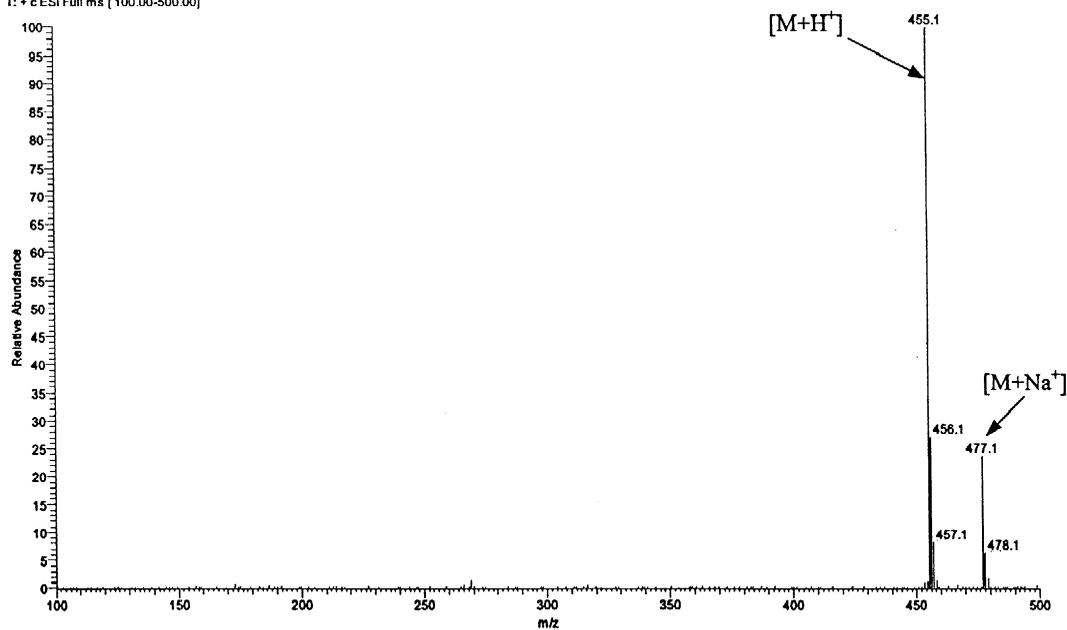
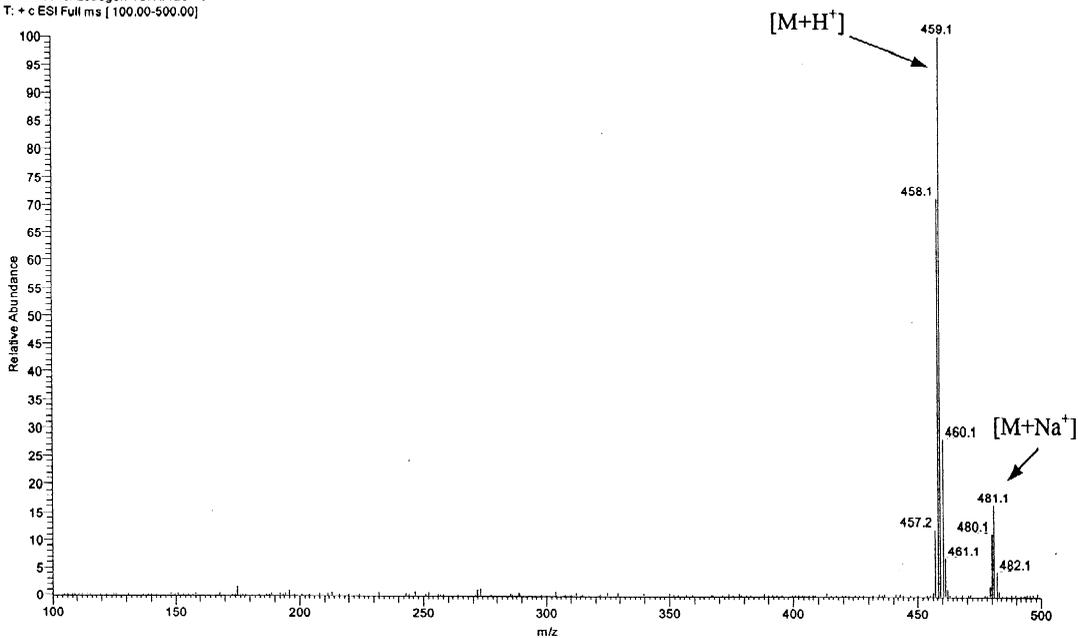
**A) 2-hydroxyestrone-TSH (MW=454)**Catechol Estrogen-TSH #132-168  
T: + c ESI Full ms [ 100.00-500.00]**B) 4-hydroxyestrone-TSH (MW=454)**Catechol Estrogen-TSH #287-338  
T: + c ESI Full ms [ 100.00-500.00]

Fig. 4. Electrospray ionization mass spectra of CE-TSH (A, B) and d-CE-TSH (C, D).

**C) [<sup>2</sup>H<sub>4</sub>] 2-hydroxyestrone-TSH (MW=458)**

d4-Catechol Estrogen-TSH #123-151  
T: + c ESI Full ms [ 100.00-500.00]



**D) [<sup>2</sup>H<sub>4</sub>] 4-hydroxyestrone-TSH (MW=458)**

d4-Catechol Estrogen-TSH #273-316  
T: + c ESI Full ms [ 100.00-500.00]

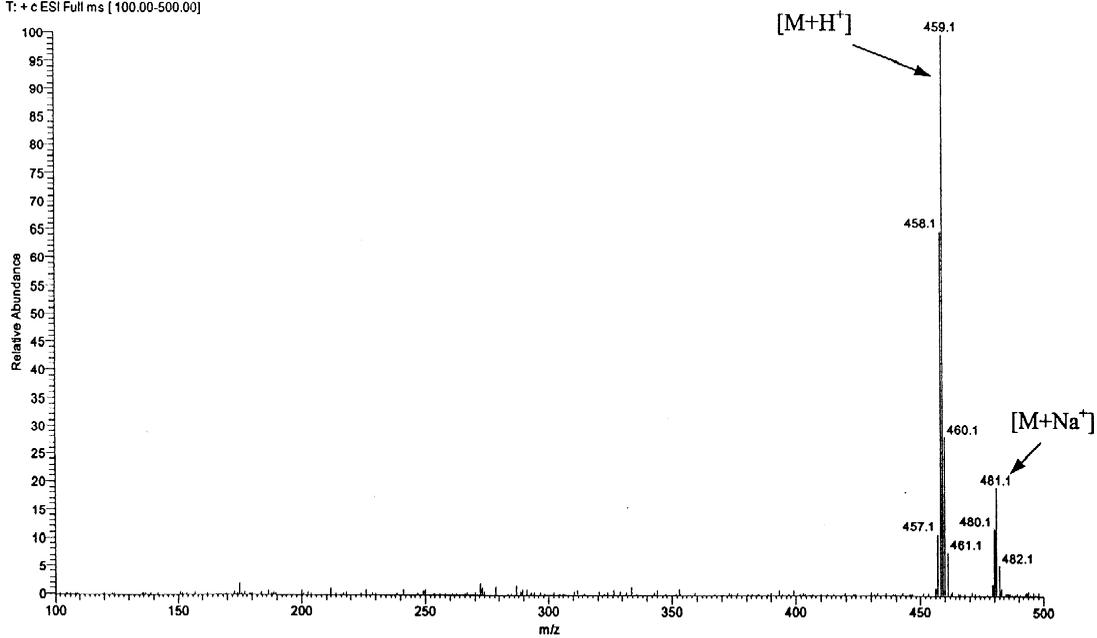


Fig. 4. (continued)

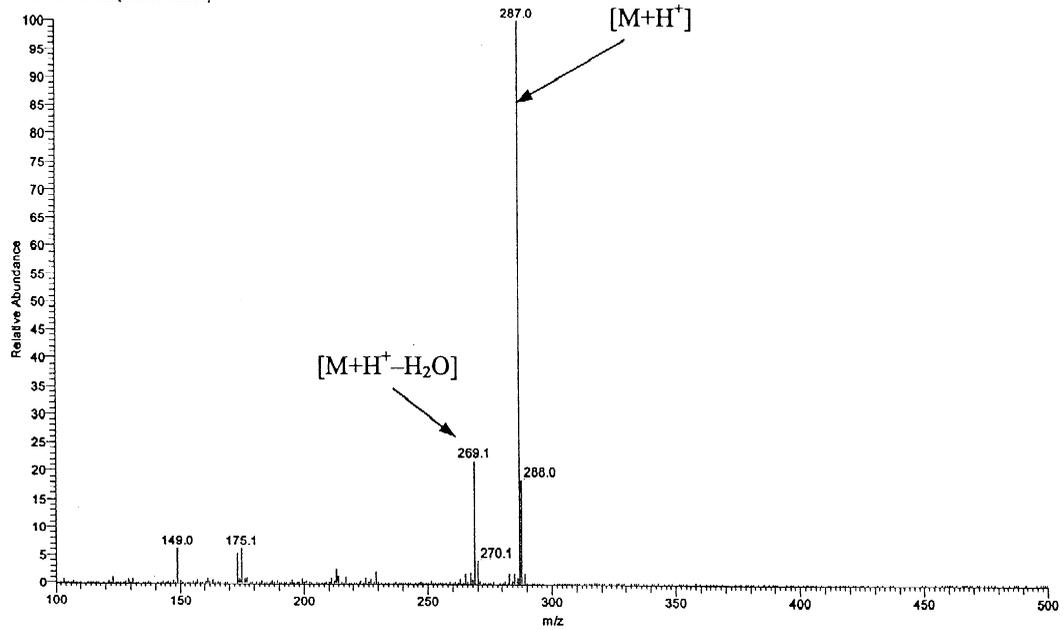
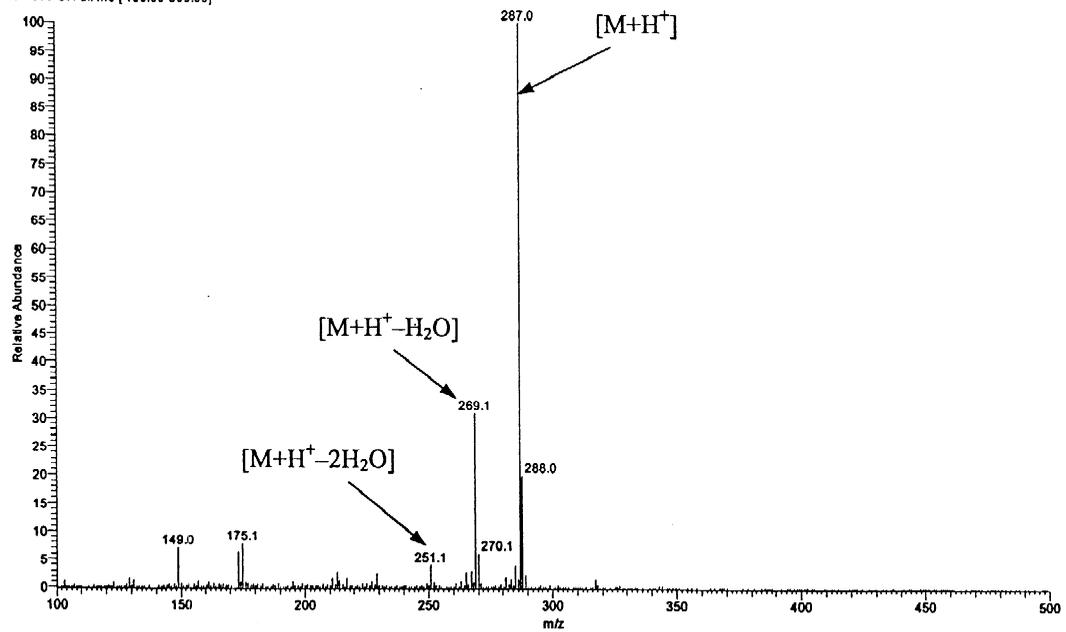
**A) 2-hydroxyestrone (MW=286)**Catechol Estrogens #1459-1486  
T: + c APCI Full ms [ 100.00-500.00]**B) 4-hydroxyestrone (MW=286)**Catechol Estrogens #1503-1537  
T: + c APCI Full ms [ 100.00-500.00]

Fig. 5. Atmospheric pressure chemical ionization mass spectra of CE (A, B) and d-CE (C, D).

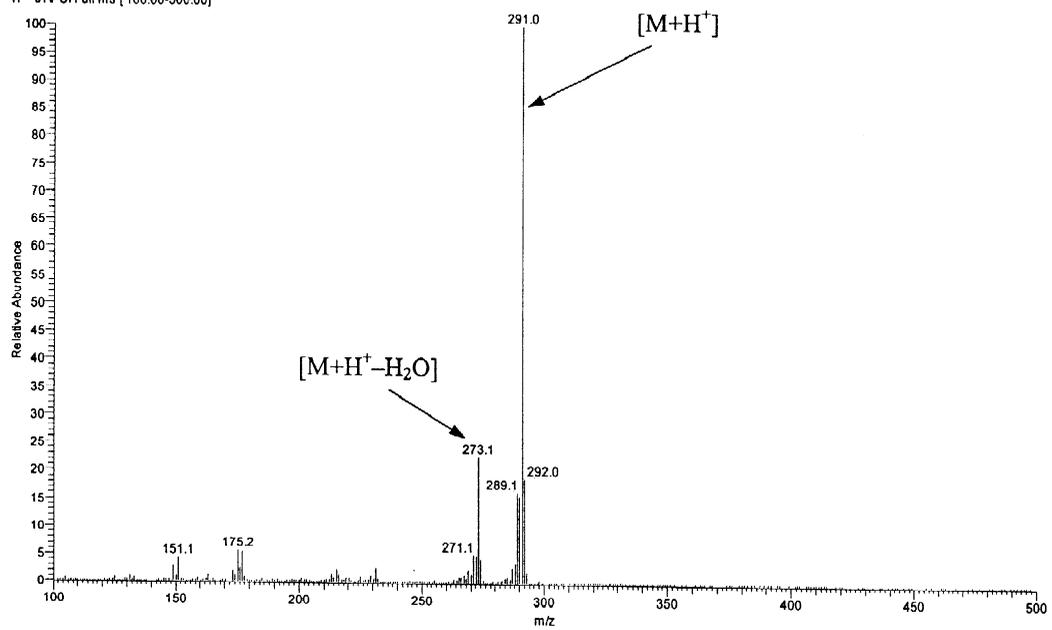
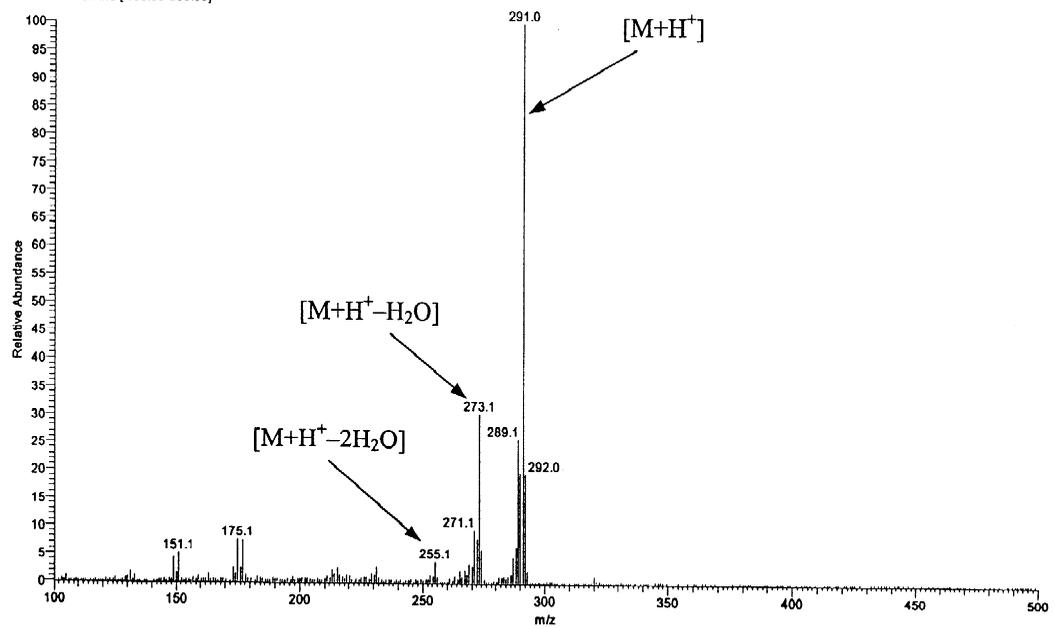
**C) [<sup>2</sup>H<sub>4</sub>] 2-hydroxyestrone (MW=290)**d4-Catechol Estrogens #1441-1455  
T: + c APCI Full ms [100.00-500.00]**D) [<sup>2</sup>H<sub>4</sub>] 4-hydroxyestrone (MW=290)**d4-Catechol Estrogens #1479-1501  
T: + c APCI Full ms [100.00-500.00]

Fig. 5. (continued)

phase women were hydrolyzed, extracted, derivatized, and analyzed in four different batches to further assess the inter-batch precision of the urinary CE analysis.

### 3. Results and discussion

#### 3.1. ESI and APCI mass spectra

The ESI mass spectra for CE-TSH and d-CE-TSH are presented in Fig. 4. These spectra are characterized by intense protonated molecule  $[\text{MH}^+]$  and less abundant natriated molecule  $[\text{MNa}^+]$ , about 15–20% of  $[\text{MH}^+]$ , for both CE-TSH and d-CE-TSH. In contrast to CE-TSH, the mass spectra of d-CE-TSH (Fig. 4C and D) showed an isotopic contribution from three different isotopomers, i.e.,  $[\text{}^2\text{H}_4]$  ( $m/z$  459),  $[\text{}^2\text{H}_3\text{H}]$  ( $m/z$  458), and  $[\text{}^2\text{H}_2\text{H}_2]$  ( $m/z$  457) indicating that deuterium loss through back exchange with protium had occurred during sample analysis. To compensate for this, the sum of the  $[\text{}^2\text{H}_4]$  ( $m/z$  459),  $[\text{}^2\text{H}_3\text{H}]$  ( $m/z$  458), and  $[\text{}^2\text{H}_2\text{H}_2]$  ( $m/z$  457) intensities was employed for d-CE-TSH during quantitative analysis, as previously described by Adlercreutz et al. [28].

Since the sensitivity of LC–MS analysis for CE and d-CE without derivatization is poor during ESI, the APCI mode was chosen for their analysis. The APCI mass spectra for CE and d-CE without derivatization are shown in Fig. 5. In addition to protonated molecule  $[\text{MH}^+]$ , the spectra of  $[\text{MH}^+ - \text{H}_2\text{O}]$ ,  $[\text{MH}^+ - 2\text{H}_2\text{O}]$  and steroid ring fragments were also observed for both CE and d-CE. Compared with ESI mass spectra of d-CE-TSH, APCI mass spectra of d-CE (Fig. 5C and D) showed a different pattern of isotopic contribution from  $[\text{}^2\text{H}_3\text{H}]$  ( $m/z$  290) and  $[\text{}^2\text{H}_2\text{H}_2]$  ( $m/z$  289), which suggested that the extent of deuterium loss might be associated with hydrazone formation or ionization conditions.

#### 3.2. Importance of TSH derivatization in CE analysis

A key element to our success is the use of TSH derivatization in CE analysis, and its importance is shown in Fig. 6. First, it improved the peak separation and shortened the chromatography time. With-

in 11 min, baseline separation of 2-hydroxy $\text{E}_1$ -TSH and 4-hydroxy $\text{E}_1$ -TSH was achieved with a difference in retention times of more than 1 min, whereas underivatized 2-hydroxy $\text{E}_1$  and 4-hydroxy $\text{E}_1$  did not begin eluting until 15 min after injection and were still not fully separated, with a difference in retention times of less than 0.6 min, on the same  $\text{C}_{18}$  column. Second, TSH derivatization improved HPLC column retention of the analytes. Therefore, a higher mobile phase methanol composition could be employed for chromatography of CE-TSH and d-CE-TSH, which improved the efficiency of the ESI process and enhanced method sensitivity, compared with CE and d-CE without derivatization. Third, CE- and d-CE-TSH derivatization resulted in stable and intense protonated molecule  $[\text{MH}^+]$  with no fragmentation during their ionization process (Fig. 4), which contributed to the improved sensitivity. Finally, the hydrazone in CE-TSH and d-CE-TSH has greater proton affinity than the ketone in CE and d-CE. This has greatly enhanced the method sensitivity during ESI positive ion mode.

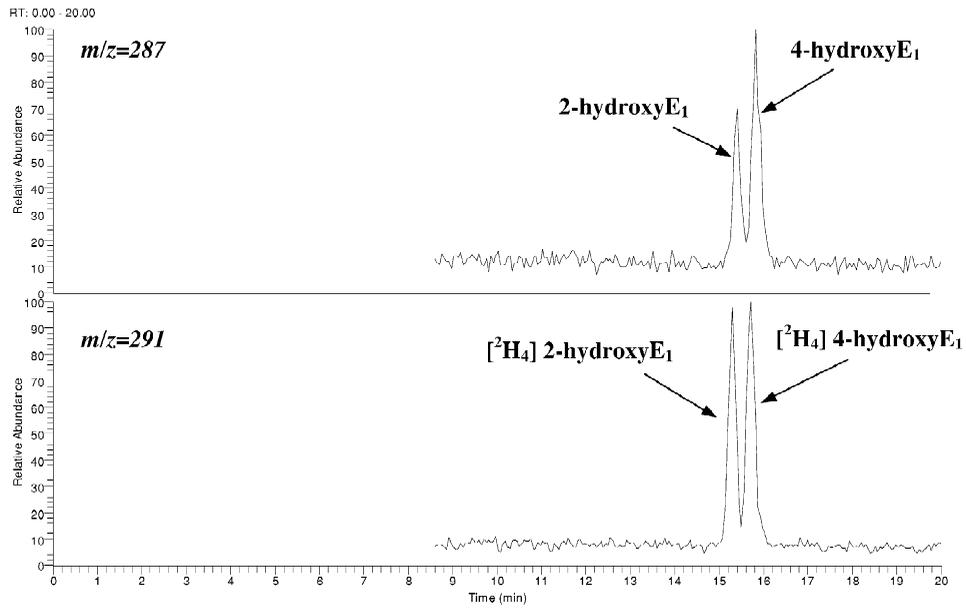
#### 3.3. Chromatographic SIM profiles of CE-TSH and d-CE-TSH in standards and pooled human urine

Even though sample preparation in this method is substantially simplified, compared with the published stable isotope dilution GC–MS method [18], we found it is adequate for quantitative analysis of endogenous CE in postmenopausal urine. The HPLC–ESI–MS SIM chromatographic profiles for a 0-ng working standard, a 1-ng working standard, and a blank postmenopausal urine sample are shown in Fig. 7. Using a simple methanol–water reversed-phase HPLC linear gradient, 2-hydroxy $\text{E}_1$ -TSH and 4-hydroxy $\text{E}_1$ -TSH were eluted from the  $\text{C}_{18}$  column in about 9.5 and 10.6 min, respectively, with symmetrical peak shapes. CE-TSH was readily detected and quantified, with no interference, even at the low endogenous levels in postmenopausal urine (Fig. 7C).

#### 3.4. Standard curve and limit of quantitation

Standard curves were linear over a 100-fold calibration range (0.5–64 ng CE/sample) with correlation coefficients for the linear regression curves

**A) 16-ng working standard without derivatization (800 pg CE and 2 ng d-CE on column)**



**B) 16-ng working standard with derivatization (800 pg CE and 2 ng d-CE on column)**

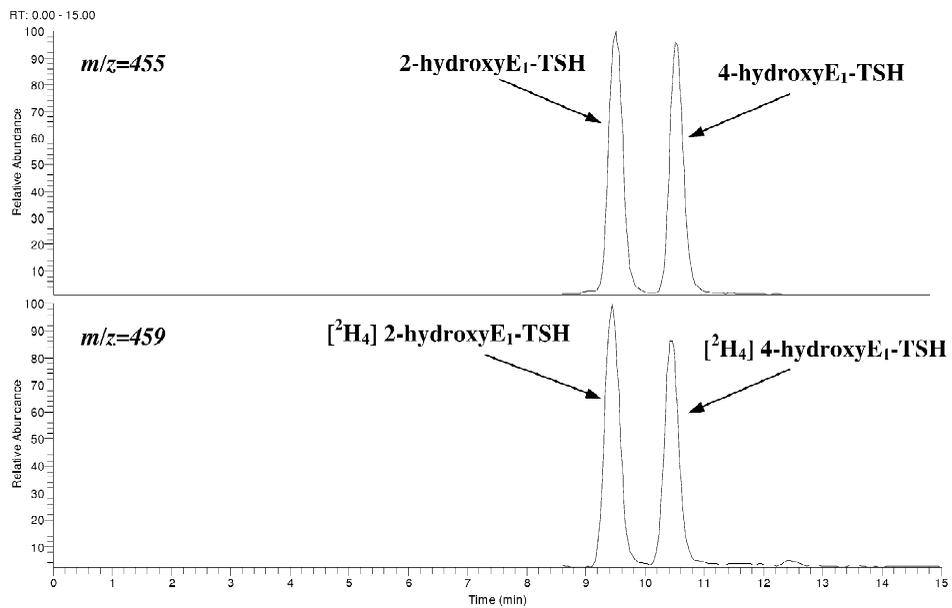
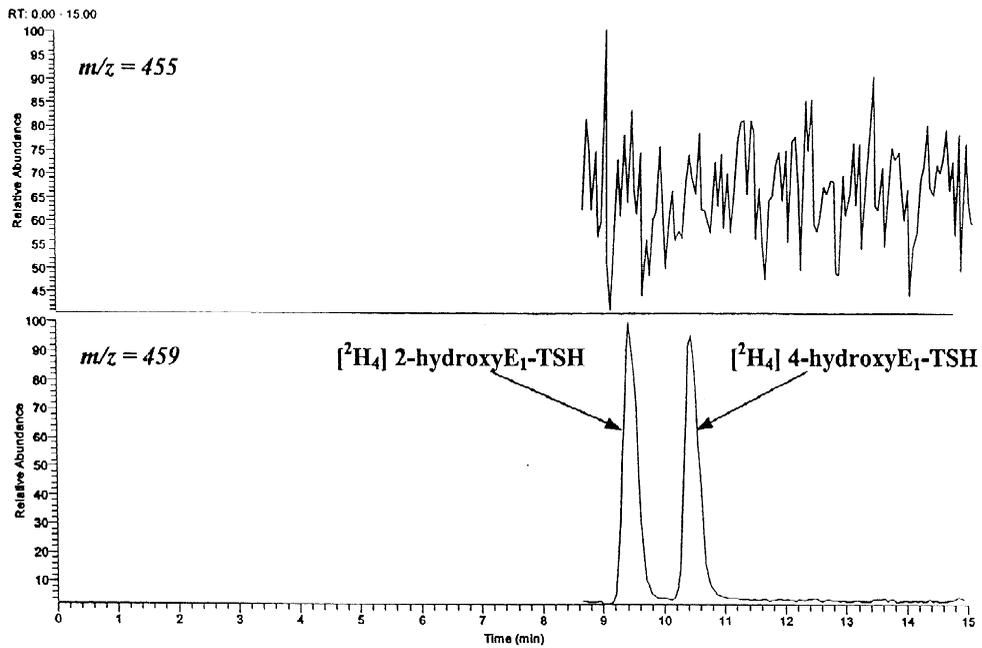


Fig. 6. Comparison of chromatographic SIM profiles of CE and d-CE with and without TSH derivatization.

**A) 0-ng working standard (0 CE and 2 ng d-CE on column)**



**B) 1-ng working standard (50 pg CE and 2 ng d-CE on column)**

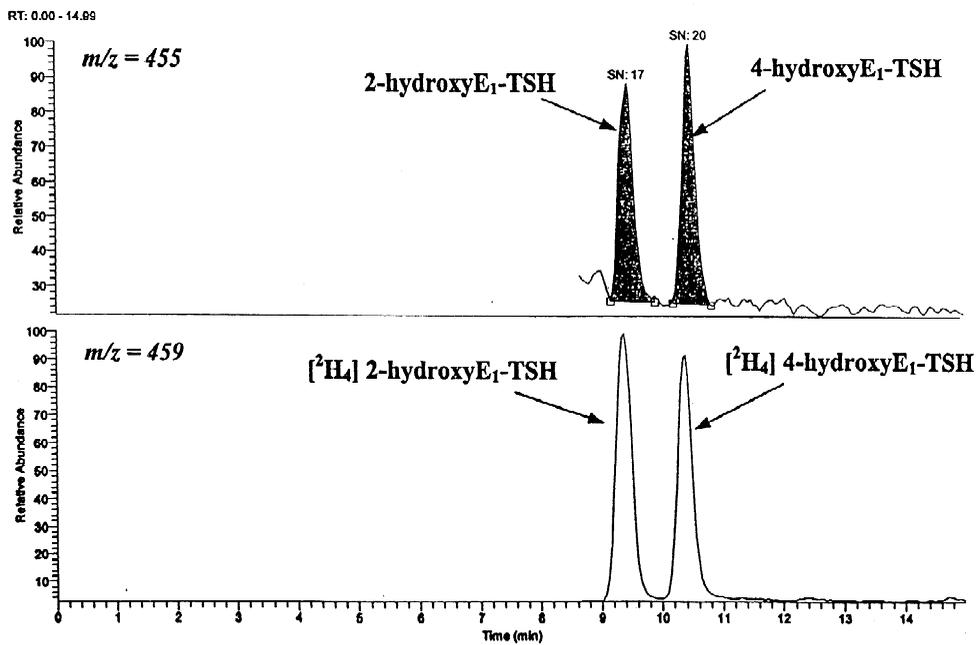


Fig. 7. Chromatographic SIM profiles of CE-TSH and d-CE-TSH corresponding to a 0-ng working standard (A); 1-ng working standard (B); and blank postmenopausal urine (only d-CE added) (C).

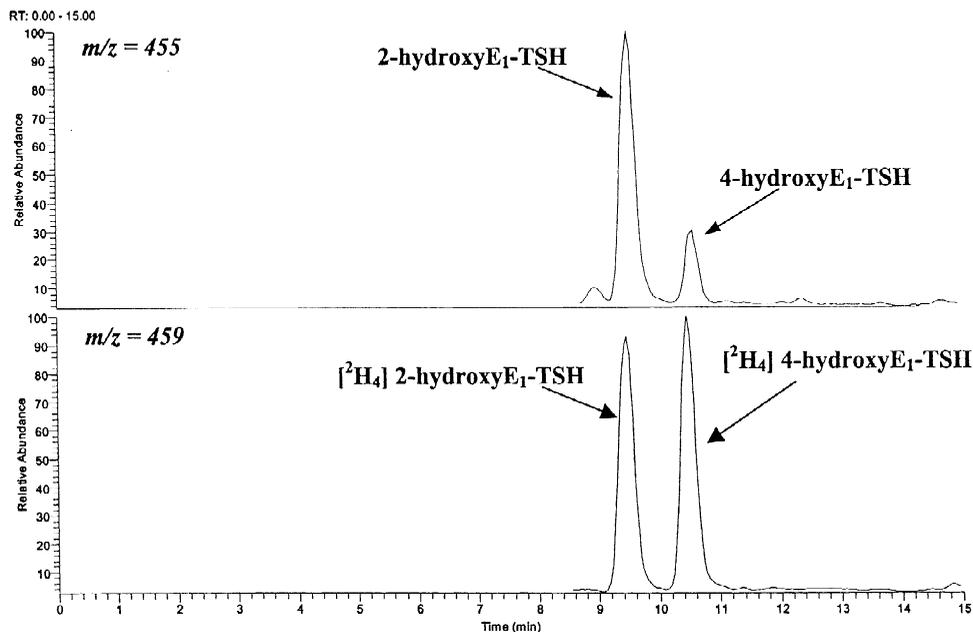
**C) Postmenopausal urine (endogenous CE and 2 ng d-CE on column)**

Fig. 7. (continued)

typically greater than 0.996. Replicate ( $n=6$ ) injections of a 1-ng working standard, representing 50 pg on column, resulted in relative standard deviations (RSDs) of SIM peak area ratios for 2-hydroxyE<sub>1</sub>-TSH/[<sup>2</sup>H<sub>4</sub>]2-hydroxyE<sub>1</sub>-TSH and 4-hydroxyE<sub>1</sub>-TSH/[<sup>2</sup>H<sub>4</sub>]4-hydroxyE<sub>1</sub>-TSH of 1.0 and 1.6%, respectively. The signal-to-noise ( $S/N$ ) ratios obtained for the 1-ng working standard, representing 50 pg on column, were typically greater than 15 (Fig. 7B), which provided an adequate lower limit of quantitation for endogenous CE analyses in urines from postmenopausal women.

### 3.5. Absolute recovery of CE after hydrolysis and extraction procedure

The absolute recovery of CE after the hydrolysis and extraction procedure was determined by comparing SIM chromatographic peak area ratios of CE-TSH/d-CE-TSH in pooled urine from postmenopausal women that had been spiked with d-CE before and after the hydrolysis and extraction pro-

cedure. Mean absolute recoveries were  $82.4 \pm 2.9$  and  $81.5 \pm 2.5\%$ , respectively, for 2-hydroxyE<sub>1</sub> and 4-hydroxyE<sub>1</sub>.

### 3.6. Accuracy and precision of the urinary CE analysis

Accuracy, intra- and inter-batch precision data for the stable isotope dilution HPLC–ESI-MS SIM analysis of human urine samples are presented in Tables 1 and 2. The analysis of six 10-ml aliquots of the pooled postmenopausal urine generated a mean concentration for endogenous 2-hydroxyE<sub>1</sub> and 4-hydroxyE<sub>1</sub> of 9.64 and 1.40 ng/10 ml, respectively (Table 1). Subtraction of these baseline values from the mean concentrations of six identical postmenopausal urine aliquots to which 8 or 30 ng of CE had been added led to the estimates of accuracy, which was 98.76 and 97.06% for 2-hydroxyE<sub>1</sub> and 98.01 and 98.99% for 4-hydroxyE<sub>1</sub>, respectively (Table 1). The intra-batch precision, as estimated by the RSDs from six replicate analyses at each level,

Table 1

Accuracy and intra-batch precision of urinary CE analyses, including hydrolysis, extraction, and derivatization steps<sup>a</sup>

	Postmenopausal urine		Postmenopausal urine+8 ng CE		Postmenopausal urine+30 ng CE	
	2-HydroxyE <sub>1</sub>	4-HydroxyE <sub>1</sub>	2-HydroxyE <sub>1</sub>	4-HydroxyE <sub>1</sub>	2-HydroxyE <sub>1</sub>	4-HydroxyE <sub>1</sub>
Mean ( <i>n</i> =6)	9.64	1.40	17.54	9.24	38.76	31.10
SD ( <i>n</i> =6)	0.31	0.07	0.29	0.18	1.15	0.33
Accuracy (%)	N/A	N/A	98.76	98.01	97.06	98.99
Precision (%)	3.25	4.74	1.65	1.94	2.96	1.05

<sup>a</sup> The mean is expressed in units of ng/10 ml urine. N/A=Not applicable.

ranged from 1.64 to 3.25% for 2-hydroxyE<sub>1</sub> and 1.05 to 4.73% for 4-hydroxyE<sub>1</sub>, respectively (Table 1). The inter-batch precision estimated by the RSDs for four independent batch analyses of pooled postmenopausal and premenopausal midluteal urine samples were 2.36 and 2.37% for 2-hydroxyE<sub>1</sub> and 4.44 and 10.68% for 4-hydroxyE<sub>1</sub>, respectively (Table 2).

### 3.7. Application to pre- and postmenopausal urine samples

The usefulness of this method was also demonstrated in the analyses of endogenous CE in the non-pooled urine samples from two postmenopausal women and two premenopausal women during mid-follicular and midluteal phases as described in the experimental section. Duplicate 10-ml aliquots from each 24-h urine sample were hydrolyzed, extracted, derivatized, and analyzed to determine CE concentration. When this information was combined with the associated 24-h urine volume, it provided estimates of 24-h urinary CE excretion in each of the postmenopausal women and premenopausal women during midfollicular and midluteal phases (Fig. 8).

Our data correspond well with those previously reported [18,24,29].

## 4. Conclusion

A sensitive, precise and accurate stable isotope dilution HPLC–ESI-MS method for measuring endogenous CE in human urine has been developed. Compared to the published methods using GC–MS, this approach simplifies sample preparation and increases the throughput of analysis. The unique part of our method is the use of a simple and rapid derivatization step that forms *p*-toluenesulfonylhydrazone for CE and d-CE. This derivatization step greatly enhances method sensitivity as well as HPLC separability of the 2- and 4-hydroxyE<sub>1</sub>. Standard curves were linear over a 100-fold calibration range (0.5–64 ng CE/sample) with correlation coefficients for the linear regression curves typically greater than 0.996. The lower limit of quantitation for each CE is 1 ng per 10-ml urine sample, with accuracy of 97–99% and overall precision, including the necessary preparation and derivatization steps, of 1–3%

Table 2

Inter-batch precision of urinary CE analyses, including hydrolysis, extraction, and derivatization steps<sup>a</sup>

	Postmenopausal urine		Premenopausal mid-luteal urine	
	2-HydroxyE <sub>1</sub>	4-HydroxyE <sub>1</sub>	2-HydroxyE <sub>1</sub>	4-HydroxyE <sub>1</sub>
Mean ( <i>n</i> =4)	9.78	1.34	32.46	3.95
SD ( <i>n</i> =4)	0.23	0.06	0.77	0.42
Precision (%)	2.36	4.44	2.37	10.68

<sup>a</sup> The mean is expressed in units of ng/10 ml urine.

### Urinary Endogenous CE Excretion in Women (nmol/24h)

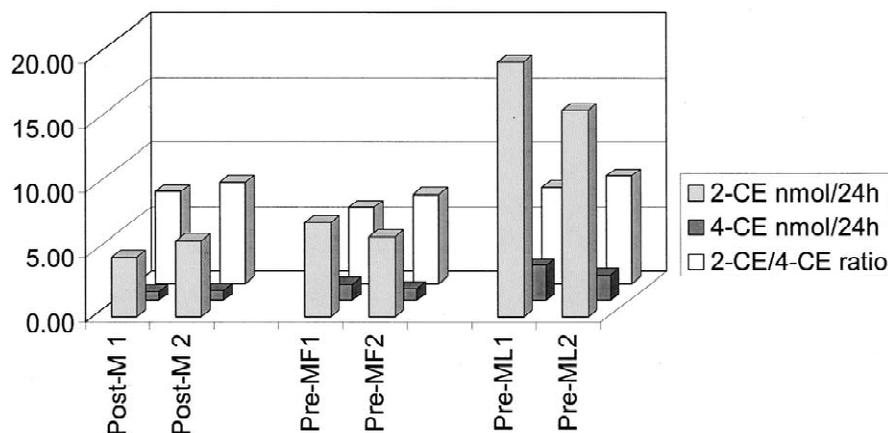


Fig. 8. Urinary endogenous CE excretion (nmol/24-h) in postmenopausal women (Post-M) and premenopausal women during midfollicular (Pre-MF) and midluteal (Pre-ML) phases. 2-CE=2-hydroxyestrone; 4-CE=4-hydroxyestrone.

for samples prepared concurrently and 2–11% for samples prepared in several batches. This method is adequate for measuring the low endogenous levels of 2- and 4-hydroxyE<sub>1</sub> in urine from postmenopausal women.

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