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The analysis of estrone and 17β-estradiol by stir bar sorptive extraction–thermal desorption–gas chromatography/mass spectrometry: Application to urine samples after oral administration of conjugated equine estrogens

Adriana Stopforth, Ben V. Burger, Andrew M. Crouch, Pat Sandra *

Department of Chemistry, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

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Abstract

The development of a sensitive and solvent-free method for the measurement of estrone (E_1) and 17β-estradiol (17β-E_2) in human urine samples is described. The deconjugated estrogens were derivatized *in situ* with acetic acid anhydride and the derivatives were extracted directly from the aqueous samples using stir bar sorptive extraction (SBSE). The compounds containing a secondary alcohol function are further derivatized by headspace acylation prior to thermal desorption and gas chromatography/mass spectrometry (GC/MS). A number of experimental parameters, including salt addition, temperature and time, were optimized to increase the recovery of E_1 and 17β-E_2 by SBSE. The derivatization reactions were also optimized to obtain the highest yields of the acylated estrogens. Detection limits of 0.02 and 0.03 ng mL^{-1} were obtained for E_1 and 17β-E_2, respectively. The method was applied to determine the effect of conjugated equine estrogen intake on the excretion of E_1 and 17β-E_2 in human urine samples. Increased levels of the endogenous estrogens were detected after administering a standard dose of Premarin to a female volunteer. Routine monitoring of estrogen levels is recommended to avoid a high urinary excretion of E_1 and 17β-E_2, nowadays enlisted as endocrine disrupting chemicals (EDCs), during hormone replacement therapy.

Keywords: Stir bar sorptive extraction; Gas chromatography/mass spectrometry; Estrone; 17β-Estradiol; Conjugated equine estrogens; Urine samples

1. Introduction

The accurate and sensitive measurement of various estrogenic hormones is becoming more and more important due to an increased understanding of the significance of these steroids in health and disease. Not only are the estrogens essential for maintenance of the female reproductive system, they are also important regulators of growth and bone metabolism [1]. Measurement of the main biologically active estrogens, namely estrone (E_1) and 17β-estradiol (17β-E_2) in blood and urine can be used to identify inborn errors of steroid metabolism; to monitor hormone replacement therapy; and to detect early puberty [2–4]. Highly sensitive assays are also required to study the role of these hormones in Alzheimer's disease and breast cancer [5,6].

Evidence that estrogens may promote breast and ovarian cancer has recently received considerable attention. Epidemiological studies have indicated that women exposed to high serum and urinary levels of the estrogens are at increased risk of developing cancer [7,8]. Postmenopausal women who receive hormone replacement therapy (HRT) are also at risk, especially when the combined estrogen–progestogen formulations are used [9]. HRT preparations that contain endogenous estrogens or a mixture of the conjugated equine estrogens are frequently prescribed to treat menopausal symptoms such as hot flashes and excessive sweating. The most frequently used drug is a formulation obtained from pregnant mares' urine called Premarin (Wyeth) [9]. It has been estimated that during 1998 more than 46 million prescriptions for Premarin were issued in the United States alone [10]. The high use of this and other types of estrogen formulations (including the oral contraceptives) are regarded as

* Corresponding author at: Research Institute for Chromatography, Kennedypark 26, B-8500 Kortrijk, Belgium. Tel.: +32 56 204031; fax: +32 56 204859.
E-mail address: pat.sandra@richrom.com (P. Sandra).

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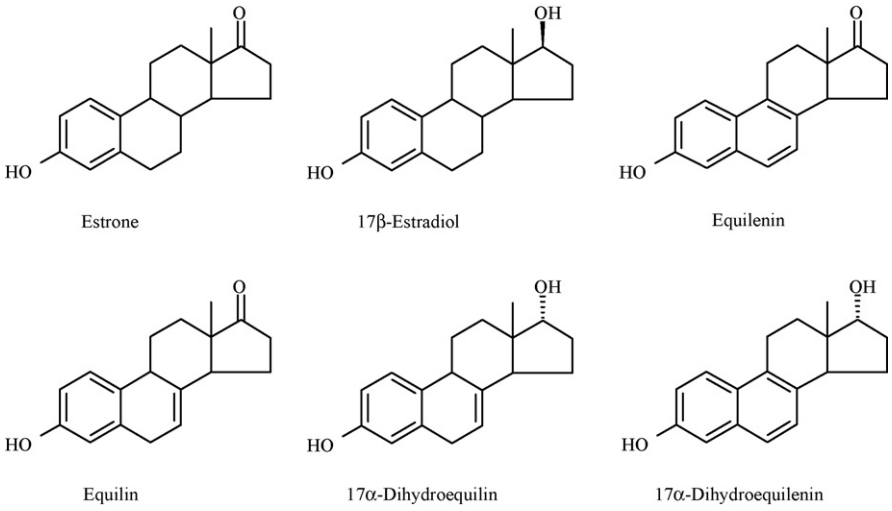

Fig. 1. Chemical structures of the human and equine estrogens analyzed by SBSE–HD–TD–GC/MS.

possible sources for levels of estrogenic chemicals found in the discharges of sewage-treatment plants (STP) [11].

STP effluents have been shown to be estrogenic to fish [12]. Therefore, there has been growing concern over the release of various endocrine disrupting chemicals into the aquatic environment. The estrogenic component of domestic STP effluents has been shown to consist mainly of E_1, 17β-E_2 and the synthetic contraceptive steroid 17α-ethinylestradiol (EE) [13,14]. Under normal circumstances, non-pregnant premenopausal women excrete approximately 7.4 μg of urinary E_1 and 3.1 μg of urinary 17β-E_2 per day [15]. These values are considerably lower than the quantities excreted by most postmenopausal women who receive various formulations of HRT [16]. It has been shown that serum and urine estrogen levels in postmenopausal women who receive estradiol supplementation for example, are far greater than those obtained for their premenopausal counterparts [16,17]. Standard HRT doses and/or dose guidelines may be too high for a lot of women, therefore frequent monitoring of estrogen levels during HRT has been recommended [10,17].

In the clinical setting, urinary estrogen levels are frequently determined by means of biological assay, including enzyme immunoassay (EIA) and radioimmunoassay (RIA). These methods are often selected because of their affordability, ease of implementation, and high throughput which make them amenable to large scale investigations [18]. However, a number of limitations such as cross-reactivity owing to a wide range of structurally similar compounds, and poor inter-laboratory reproducibility caused by batch-to-batch variation of the antibodies, may complicate the interpretation of results [19–21].

Gas chromatography in combination with mass spectrometry (GC/MS) has addressed many of the shortcomings associated with immunoassays. The technique is highly specific and accurate, and has been used as a reference method to validate EIA [19,21] or RIA methods [20,21]. Unfortunately, the routine application of GC/MS for the detection of the estrogens has been hampered by the need to perform extensive sample preparation, which in turn has led to rather slow turnaround times. One of the ways to improve sample throughput, is to combine the extraction, concentration and purification of the compounds in a single step. Several investigators have already reported a significant reduction in analysis time by using solventless sample preparation techniques such as stir bar sorptive extraction (SBSE) [22,24] and solid phase microextraction (SPME) [23,24].

One of the main advantages of using sorptive extraction methods is the ability to analyze organic compounds, such as the estrogens, directly from an aqueous sample. The compounds are enriched by a polymeric phase that is coated onto a solid support, such as a glass stir bar (i.e. SBSE) [25] or an optical silica fiber (i.e. SPME) [26]. Affinity of the estrogens for the polymer layer can be enhanced by optimizing the stirring speed, temperature, pH and ionic strength of the sample solution. Furthermore, the phenolic hydroxyl groups of the estrogens can be derivatized *in situ* with acetic acid anhydride to increase the affinity of the compounds for the polymeric phase coating [27,28]. The aliphatic hydroxyl groups on the other hand, are more difficult to derivatize and this step can only be accomplished after the compounds have been extracted from the sample. Two headspace derivatization methods for SBSE have recently been described to improve the gas chromatographic properties of hydroxyl containing compounds that cannot be derivatized in aqueous solution. The first approach involves the in-tube silylation of the extracted compounds by BSTFA [29], whereas the second approach is based on the formation of the acetate derivatives of the extracted compounds by exposing the stir bars to acetic acid anhydride vapors in modified headspace vials [30].

In this investigation, the development of a new method for the analysis of E_1 and 17β-E_2 in human urine samples is described. The method is based on the *in situ* derivatization of the estrogens with acetic acid anhydride; extraction of the derivatives by aqueous SBSE; and final exposure of the stir bars to acetic acid anhydride vapors before thermal desorption and GC/MS. The method has been applied to determine the effect of conjugated equine estrogens intake (i.e. Premarin) on the excretion of E_1 and 17β-E_2 in human urine samples.

2. Experimental

2.1. Materials, standard solutions and urine samples

Estrone (E_1), 17β-estradiol (17β-E_2) and equilin (Eq) were purchased from Sigma–Aldrich (Johannesburg, South Africa). Equilenin (Eqn), used as internal standard, was supplied as a 200 μg/2 mL standard solution in acetonitrile by Riedel-de Haën (Sigma–Aldrich, Johannesburg, South Africa). 17α-Dihydroequilin (17α-Eq) and 17α-dihydroequilenin (17α-Eqn) were obtained from Steraloids (Newport, RI, USA). The chemical structures of the compounds are shown in Fig. 1. Concentrated solutions of the individual estrogens (80 μg mL^{-1}) and Eqn (20 μg mL^{-1}) were prepared in methanol. From these solutions, a combined working solution was prepared for E_1 and 17β-E_2 at a concentration of 1 μg mL^{-1}. A separate solution was prepared for the internal standard (Eqn) at the same concentration. All solutions were stored at −20 °C until used.

Premarin tablets (0.625 mg; Wyeth, Johannesburg, South Africa) were purchased from a local pharmacy. A solution of the conjugated equine estrogens (62.5 μg mL^{-1}) in methanol was prepared according to a modification of the procedure described by Seibert and Poole [31]. The outer coating of one tablet was removed using a piece of damp, lint-free tissue paper until the shellac layer was exposed. The tablet was crushed to a powder using a mortar and pestle. The conjugated equine estrogens were extracted by vortexing with two 6 mL portions of methanol. The fractions were combined, evaporated under nitrogen, and reconstituted in 10 mL methanol.

Sodium hydroxide pellets (NaOH), β-glucuronidase/sulfatase from Helix Pomatia (Type H-2), anhydrous sodium carbonate (Na_2CO_3) and pyridine were obtained from Sigma–Aldrich (Johannesburg, South Africa). Acetic acid anhydride and dichloromethane were supplied by Merck (Darmstadt, Germany). Ammonium sulfate ($(NH_4)_2SO_4$) and vitamin C were obtained from Fluka (Sigma–Aldrich, Johannesburg, South Africa). The 15 mL screw cap vials were from Supelco (Sigma–Aldrich, Johannesburg, South Africa) and the headspace derivatization vials [30] were prepared from 2 mL autosampler vials that were obtained from Agilent Technologies (Chemetrix, Johannesburg, South Africa). A 10-position magnetic stirrer combined with a convection oven was designed and built by J. Blom and colleagues (Department of Mechanical Engineering, University of Stellenbosch). Twister stir bars (10 mm × 0.5 mm × 0.5 mm d_f; PDMS) were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany). The stir bars were pre-conditioned by sonication in a 1:1 mixture of dichloromethane:methanol for 5 min after which they were heated at 280 °C for 10 min under a nitrogen flow of 50 mL min^{-1}.

First morning urine samples were obtained from two postmenopausal women aged 54 and 57 years, respectively. At the time of the study, the 57-year-old woman received a formulation of conjugated equine estrogens as a hormone replacement (i.e. 0.625 mg Premarin per day). Control urine samples were obtained from three non-pregnant, premenopausal women and two male volunteers that were collected at random between 9 and 12 h. All urine samples were stored at −25 °C prior to analysis. Urine creatinine levels were determined by Pathcare (Cape Town, South Africa).

2.2. Enzymatic hydrolysis, SBSE and derivatization procedure

After allowing the urine samples to thaw to room temperature, 1 mL aliquots were transferred to 15 mL screw cap vials containing 2 mL of a buffer solution (pH 4.6) that consisted of 1 M $(NH_4)_2SO_4$, 5.7 mM vitamin C and 2 mM NaOH. Twenty microliters of a crude solution of Helix Pomatia was added to the sample mixtures and the vials were incubated at 40 °C for 16 h [32]. The samples were spiked with 2 ng mL^{-1} of the internal standard (Eqn), and 500 mg Na_2CO_3 and 50 μL pyridine were added. The vials were vortexed gently until the salt was dissolved. Using a stop-watch, the *in situ* derivatization step was performed in a fume hood as follows. After adding 500 μL of acetic acid anhydride, 6 s elapsed before the open vials were vortexed for 5 s; another 9 s elapsed until the samples were vortexed for a further 20 s. Three milliliters of the derivatized samples were transferred to clean 15 mL vials prior to performing SBSE.

A conditioned stir bar was placed in each vial and the samples were stirred at 1100 rpm for 60 min using a home-built multiposition magnetic stirrer/oven that was heated to 40 °C. After SBSE extraction, the stir bars were removed from the sample vials, washed with distilled water and dried with tissue paper. A second derivatization step was performed by placing the stir bars inside 2 mL headspace vials as previously described [30] and 20 μL acetic acid anhydride and 20 μL pyridine were added. The vials were incubated at 80 °C for 30 min to form the acetate derivatives of the extracted compounds. Urine samples that were obtained from two postmenopausal women and water samples spiked with different concentrations of the Premarin solution (Section 2.1) were prepared as described above, except that the internal standard Eqn was not added.

2.3. Thermal desorption–gas chromatography/mass spectrometry (TD–GC/MS)

TD–GC/MS analyses of the derivatized estrogens were performed with an Agilent 6890 gas chromatograph that was equipped with a TDS 2 thermodesorption system and a TDS A autosampler (Gerstel). Thermal desorption of the compounds was accomplished in the solvent-venting mode using helium at a flow rate of 100 mL min^{-1}. The temperature of the TDS 2 was ramped from 50 to 150 °C (held for 1 min), and from 150 to 300 °C (held for 10 min) at a rate of 60 °C min^{-1}. The split-valve was closed after 2 min. The desorbed compounds were transferred through a heated transfer line (320 °C) to a programmable vaporization (PTV) inlet (CIS 4, Gerstel) that was cooled to 10 °C using liquid nitrogen. The sample was injected by increasing the PTV inlet temperature to 300 °C (held for 5 min) at a rate of 12 °C s^{-1}. The splitless time was 2.5 min. Chromatographic separation of the derivatives was

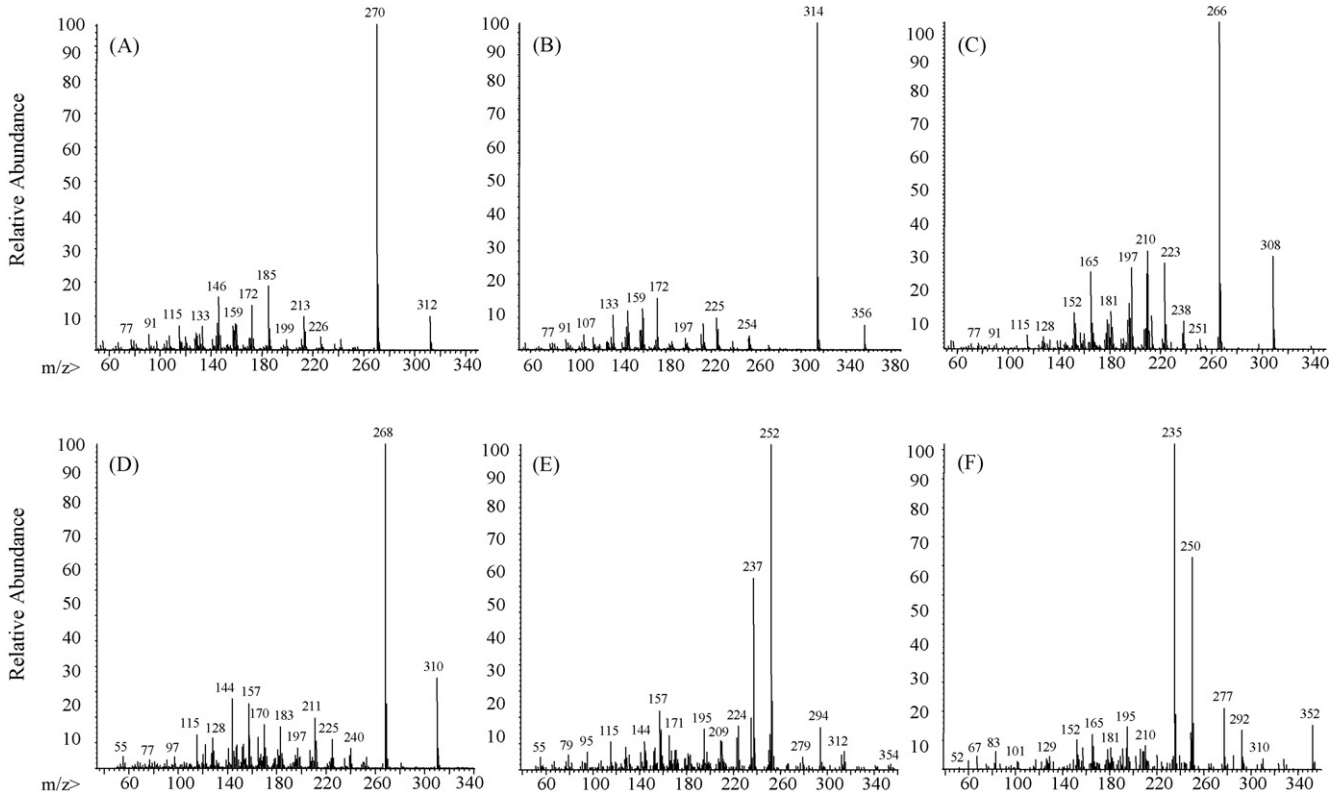

Fig. 2. Electron impact mass spectra of the acetate derivatives of (A) estrone (E_1); (B) 17β-estradiol (17β-E_2); (C) equilenin (Eqn); (D) equilin (Eq); (E) 17α-dihydroequilin (17α-Eq); (F) 17α-dihydroequilenin (17α-Eqn).

performed on an HP5MS capillary column (30 mL × 0.25 mm i.d. × 0.25 μm d_f; Agilent) using helium at a flow rate of 1.25 mL min^{-1}. The oven temperature was programmed from 70 °C (held for 2 min) to 220 °C at 10 °C min^{-1}, and from 220 to 300 °C (held for 2 min) at 3.2 °C min^{-1}. The total run time was 44 min.

The gas chromatograph was interfaced with a 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA) that was operated in the full scan and selected ion monitoring (SIM) modes. The GC/MS interface, ion source and quadruple temperatures were maintained at 280, 230 and 150 °C, respectively. Mass spectra of the acetate derivatives were recorded in the electron impact mode by scanning over a mass range of 50–550 amu (ionization voltage 70 eV). For SIM, two to three ions were selected from each spectrum to detect trace amounts of the compounds in human urine. The monitored ions included the base peak and one or two other target ions of each derivative, i.e.: E_1 (*m/z* 270; 312), 17β-E_2 (*m/z* 225; 314), Eq (*m/z* 268; 310), Eqn (*m/z* 266; 308), 17α-Eq (*m/z* 237; 252; 294) and 17α-Eqn (*m/z* 235; 250; 277). The underlined values are the base peaks of the acetate derivatives.

3. Results and discussion

3.1. Mass spectrometry

The mass spectra of the derivatized estrogens are shown in Fig. 2. Pure water samples, spiked at a concentration of 0.48 μg mL^{-1} of each estrogen were analyzed as described in Section 2. The most intense fragment ions for the acetate derivatives of E_1 and 17β-E_2 (Fig. 2A and B) were formed by the loss of [CH_2=C=O] from the C_3-acetyl groups of the compounds. The loss of [CH_3COOH] from the base peak of 17β-E_2 (*m/z* 314) and subsequent fragmentation of the D rings in both compounds, resulted in complex spectra containing several low intensity fragment ions [33]. Similar fragmentation patterns to that of E_1 acetate were observed for the equine estrogens, namely Eq and Eqn (Fig. 2C and D). The ions at *m/z* 268 and 266 were formed by the loss of [CH_2=C=O] from the molecular ions of Eq acetate (*m/z* 310) and Eqn acetate (*m/z* 308). Further decompositions corresponded to that of E_1 acetate as reported previously [34].

The mass spectra of the acetate derivatives of 17α-Eq and 17α-Eqn differed significantly from those obtained for the simple estrogens (Fig. 2E and F). Ions corresponding to the loss of [CH_2=C=O] and [CH_3COOH] from the C_3- and C_{17}-acetyl substituents were present in the spectra of both compounds, namely *m/z* 312 and 294 for 17α-Eq and *m/z* 310 and 292 for 17α-Eqn. The base peak (*m/z* 252) of the diacetate derivative of 17α-Eq was formed by the loss of 42 mass units from the ion at *m/z* 294. An additional loss of 57 mass units from this ion (i.e. *m/z* 294) resulted in the formation of the ion at *m/z* 237. The fragmentation of 17α-Eqn appeared to follow the same pattern, except that a complete reversal in the intensity of the ions at *m/z* 235 and 250 was demonstrated. A summary of the monitored ions is presented in Table 1.

Table 1
Summary of the ions monitored by GC/MS in SIM mode

Compound (abbreviation)	M[a]	Monitored ions
1. Estrone (E_1)	312	*m/z* 270[b], 312
2. 17β-Estradiol (17β-E_2)	356	*m/z* 225, 314
3. Equilin (Eq)	310	*m/z* 268, 310
4. Equilenin (Eqn)	308	*m/z* 266, 308
5. 17α-Dihydroequilin (17α-Eq)	354	*m/z* 237, 252, 294
6. 17α-Dihydroequilenin (17α-Eqn)	352	*m/z* 235, 250, 277

[a] Molecular mass of the acetate derivatives.
[b] Underlined values are the base peaks of each derivative.

3.2. Method optimization

A number of experimental conditions that affect the sensitivity of the SBSE–headspace derivatization (HD)–TD–GC/MS procedure were optimized to improve the recovery of the estrogens from the urine samples. Prior to performing SBSE, the deconjugated estrogens were derivatized in the aqueous sample to enhance the extraction of the compounds by the stir bar coating. The aqueous derivatization step is performed with acetic acid anhydride in the presence of Na_2CO_3 and pyridine within a few seconds. During the reaction, the polar phenolic hydroxyl groups of the estrogens are replaced with less polar acetate groups, thus increasing the affinity of the compounds for the non-polar, polydimethylsiloxane coating of the stir bar. The efficiency of the reaction was optimized by adding different amounts of acetic acid anhydride and Na_2CO_3 to the samples.

Urine samples that were obtained from two male volunteers were spiked with 2 ng mL^{-1} of E_1, 17β-E_2 and Eqn. The samples were analyzed as described in Section 2. Firstly, the amount of Na_2CO_3 used was optimized by adding different quantities of the carbonate (i.e. 200–600 mg) and a fixed volume of the reagent to the samples (i.e. 0.5 mL acetic acid anhydride). Thereafter, the amount of reagent used was optimized by adding different volumes of acetic acid anhydride to the samples (i.e. 0.2–0.6 mL), while keeping the amount of Na_2CO_3 constant (i.e. 500 mg). In both experiments the estrogen derivatives were extracted at 50 °C during 60 min. Fig. 3 shows the results of the mean values for $n = 3$. The R.S.D.s were less than 5% for all points.

The peak areas of the derivatives increased gradually and reached a maximum after 500 mg of Na_2CO_3 and 0.5 mL of acetic acid anhydride (0.4 mL for 17β-E_2) were added to the samples. Further increases in the volume of the reagent and the quantity of the catalyst resulted in decreased amounts of the compounds being extracted. As well as improving the efficiency of the derivatization reaction, the amount of acetic acid anhydride and Na_2CO_3 added to the samples also influenced the pH and ionic strength of the solutions. It is known that to achieve optimal extractions by SBSE, the compounds should be present in their neutral form [35]. Since the estrogens are ionizable compounds, the pH and ionic strength of the sample will affect the overall amount of estrogens extracted by the stir bar. Under the optimized derivatization conditions, a final pH of ca. 5.5 was obtained prior to the extraction of the estrogen derivatives. The efficiency of the extraction at different pH levels was not investigated because of the buffering effect produced by the reagent and

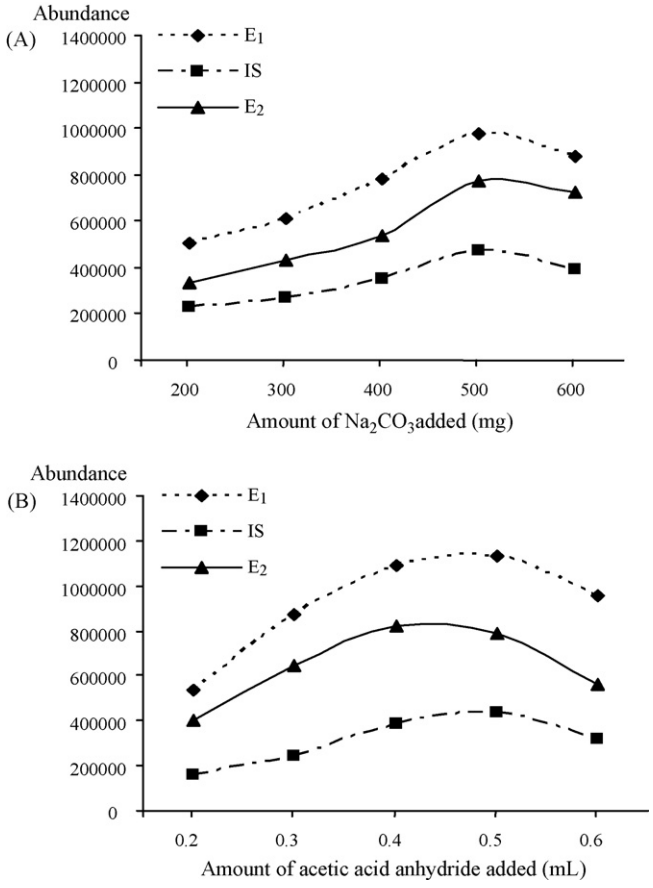

Fig. 3. The amount of Na_2CO_3 (A) and acetic acid anhydride (B) added to prepare the monoacetate derivatives of E_1, 17β-E_2 and the IS. One milliliter urine samples were spiked with 2 ng mL^{-1} of each compound and were analyzed by SBSE–TD–GC/MS. Conditions are given in Section 2.

carbonate. However, Fig. 3A demonstrates that by increasing the ionic strength of the sample solution, increased amounts of the estrogens are recovered by the stir bar. Therefore, an amount of 500 mg Na_2CO_3 and a volume of 0.5 mL acetic acid anhydride were selected as optimal to prepare the monoacetate derivatives of the estrogens prior to extraction by SBSE.

Further improvements in the recovery of the estrogens were accomplished by optimizing the temperature and the time of extraction. By increasing the temperature of the sample solution, the diffusion coefficients of the analytes will increase, but at the same time their partition coefficients may decrease [35]. Therefore, the optimal extraction temperature for E_1, 17β-E_2 and Eqn was determined by performing a series of experiments at 40, 50, 60, 70 and 80 °C, while the extraction time was held constant at 60 min. The recovery of the estrogens decreased slightly at extraction temperatures of 60 °C and higher. The initial peak areas of E_1 and Eqn remained unchanged between the temperature interval 40–50 °C, whereas the peak areas of 17β-E_2 increased slightly up to 50 °C and then leveled off between 50 and 60 °C. To select the most appropriate extraction temperature for the compounds, the reproducibility of the method was evaluated at 40 and 50 °C, respectively. The reproducibility ($n = 4$) for E_1 and 17β-E_2 improved when a lower extraction temperature was used (i.e. 2.3% for E_1 and 3.3% for 17β-E_2 at 40 °C

versus 3.9% for E_1 and 6.2% for 17β-E_2 at 50 °C). Therefore, 40 °C was chosen as the optimal extraction temperature for the compounds. Thereafter, the extraction-time profiles of E_1, 17β-E_2 and Eqn were obtained by stirring the samples for various lengths of time which ranged from 30 to 120 min. Sixty minutes was sufficient to achieve equilibrium extraction for the studied estrogens.

Lastly, the headspace acylation of 17β-E_2 was optimized by exposing the stir bars to acetic acid anhydride and pyridine vapors for 30 min at temperatures ranging from 60 to 90 °C. The peak areas obtained for 17β-E_2 remained relatively unchanged within the selected temperature range. To determine the most efficient headspace derivatization temperature, the reproducibility of the method was evaluated at 70 and 80 °C, respectively. Reproducibility ($n = 4$) for 17β-E_2 improved significantly when a higher headspace derivatization temperature was used (i.e. 1.3% for E_1 and 6.3% for 17β-E_2 at 70 °C versus 1.3% for E_1 and 3.5% for 17β-E_2 at 80 °C). Therefore, 80 °C was selected as the optimal temperature to derivatize the extracted compounds. The optimum time required to form the diacetate derivative of 17β-E_2 was investigated by exposing the stir bars to acetic acid anhydride and pyridine vapors for various lengths of time, i.e. 15–60 min. An optimal yield was obtained after 30 min.

3.3. Quantification of E_1 and 17β-E_2 in human urine samples

The efficiency of the optimized SBSE–TD–HD–GC/MS method was demonstrated by measuring trace levels of E_1 and 17β-E_2 in urine samples that were obtained from three healthy, non-pregnant, premenopausal women. The levels were determined by the standard addition method in samples that were spiked at two concentration levels, namely 2 and 4 ng mL^{-1} of each compound. A fixed amount of the internal standard (Eqn) was added (i.e. 2 ng mL^{-1}) prior to analyzing the spiked and non-spiked samples. The slopes of the curves were obtained by plotting the peak area ratios of E_1 (*m/z* 270) and 17β-E_2 (*m/z* 314) corrected for the IS (*m/z* 266) against the concentration of the analytes. The correlation coefficients (r^2) of both compounds ranged between 0.995 and 0.999. To determine the original steroid concentrations, the linear curves were extrapolated to the negative axis, whereby levels of 3.6, 4.4 and 2.9 ng mL^{-1} were obtained for E_1 and 1.1, 1.4 and 0.96 ng mL^{-1} were obtained for 17β-E_2. The levels were corrected for the amount of creatinine measured in each sample as shown in Table 2. The limits of detection for the method were calculated at a signal to noise (S/N) level of 3 and were 0.02 ng mL^{-1} for E_1 and 0.03 ng mL^{-1} for 17β-E_2. The limits of quantitation were calculated at a S/N of 10 and were 0.05 and 0.1 ng mL^{-1} for E_1 and 17β-E_2, respectively. The precision of the SBSE method was estimated from the relative standard deviation of 12 replicate analyses (i.e. 4 replicates that were analyzed on 3 different days). The intra-day repeatability was 1.8% for E_1 and 4.2% for 17β-E_2, whereas the inter-day repeatability was 1.6, 1.8 and 1.9% for E_1 and 2.7, 3.9 and 4.5% for 17β-E_2.

3.4. Increased urinary excretion of E_1 and 17β-E_2 following the oral administration of Premarin

The estrogenic components of Premarin were identified by analyzing a pure water sample spiked at a concentration of 0.19 μg mL^{-1} of the conjugated equine estrogens (Section 2.1). Accurate identification of the steroids was made by comparing the GC/MS scan results of each compound with the retention times and mass spectra of the corresponding standards. All the samples were analyzed by SBSE–TD–GC/MS as described in Section 2. SIM chromatograms of the target compounds, namely E_1, Eq, Eqn, 17β-E_2, 17α-Eq and 17α-Eqn were obtained by analyzing a pure water sample spiked at a concentration of 31.3 ng mL^{-1} of the Premarin solution. Fig. 4A-i illustrates that a number of the estrogens co-eluted under the present gas chromatographic conditions, i.e. E_1 (*m/z* 270) co-eluted with Eq (*m/z* 268), and 17β-E_2 (*m/z* 314) co-eluted with 17α-Eq (*m/z* 252). However, it was possible to detect the individual compounds by using the extracted-ion SIM chromatograms of each estrogen as shown in Fig. 4A-ii and -iii.

Significant differences were observed between the GC/MS profiles of Premarin and those obtained for the postmenopausal urine samples. Fig. 4B-i shows that the peaks identified in sample A (i.e. peaks 1–4) were also detected in a urine sample obtained from a 57-year-old postmenopausal woman who received a preparation containing conjugated equine estrogens (i.e. 0.625 mg Premarin per day). A significant amount of E_1 was excreted by this volunteer, whereas only trace amounts of Eq and 17α-Eq were detected in the sample (Fig. 4B-ii and -iii). The main components of Premarin have been identified as the sulfate conjugates of E_1 (50–60%), Eq (20–30%) and 17α-Eq (14–20%) [36]. Possible metabolic pathways for the equine estrogens have previously been reported by Bhavnani et al. [37]. These authors demonstrated that the equine estrogens, including Eq and 17α-Eq are extensively metabolized

Table 2
Levels of urinary E_1 and 17β-E_2 obtained for three healthy, premenopausal women

No.	Age	r^2[a]	E_1 (ng mL^{-1})	E_1[b]	r^2[a]	17β-E_2 (ng mL^{-1})	17β-E_2[b]
1	20	0.995	3.6	2.2	0.999	1.1	0.68
2	23	0.999	4.4	2.6	0.998	1.4	0.84
3	20	0.998	2.9	2.2	0.999	0.96	0.72

One milliliter urine samples spiked with 2 ng mL^{-1} of the IS (Eqn) were analyzed by SBSE–TD–GC/MS. Quantification was performed by the standard addition method.

[a] Correlation coefficients.

[b] Levels corrected for creatinine content (ng mg creatinine^{-1}).

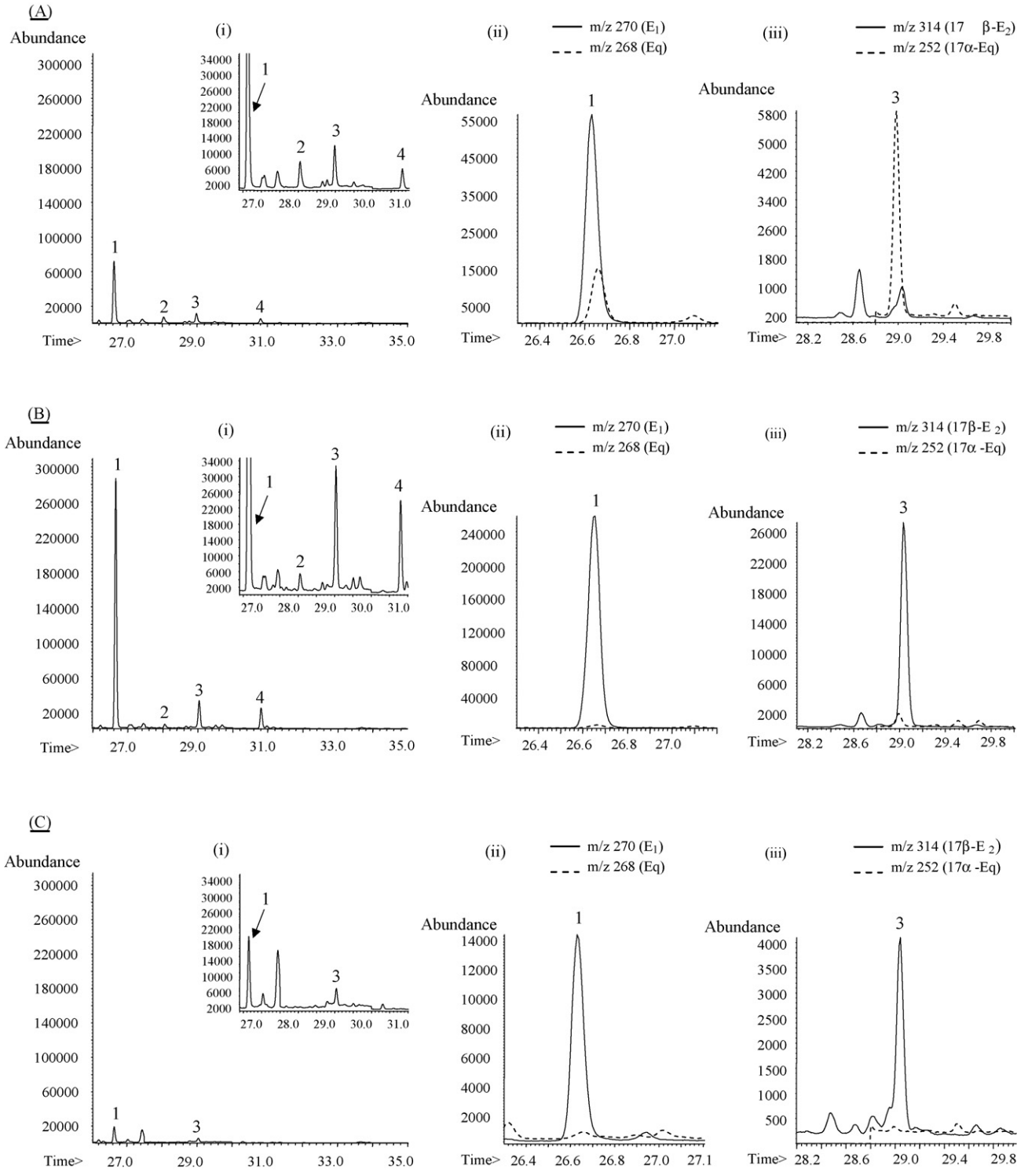

Fig. 4. SIM chromatograms of (A) a water sample spiked with 31.3 ng mL^{-1} of the Premarin solution; (B) the non-spiked urine sample of a postmenopausal woman who received Premarin; and (C) a postmenopausal urine sample without Premarin. The peak identities are (1) E_1 and Eq; (2) Eqn; (3) 17β-E_2 and 17α-Eq; and (4) 17α-Eqn. Additional chromatograms represent (i) magnified sections of the corresponding chromatograms; (ii) extracted-ion SIM chromatograms of peak 1; and (iii) extracted-ion SIM chromatograms of peak 3. Conditions are given in Section 2.

in vivo [38,39]. Given that low quantities of Eq and 17α-Eq are excreted in human urine, it is expected that these estrogens will have a negligible impact on the quantification of E_1 and 17β-E_2 during conjugated equine estrogen supplementation.

Reference levels for E_1 and 17β-E_2 were obtained by analyzing a urine sample that was donated by a 54-year-old postmenopausal woman who did not receive any form of HRT (Fig. 4C-i). This volunteer excreted much lower levels of the endogenous estrogens as compared to the postmenopausal

woman who received daily estrogen supplementation (Fig. 4B-i). To investigate this finding further, the results were compared against normal levels of the estrogens found in healthy premenopausal women. Fig. 5 demonstrates that the excretion of E_1 and 17β-E_2 in a urine sample of a 23-year-old premenopausal woman (A; E_1: 2.6 ng mg creatinine^{-1}, 17β-E_2: 0.84 ng mg creatinine^{-1}) were substantially lower than the levels detected in a 57-year-old postmenopausal woman who received a formulation containing conjugated equine estrogens (B; E_1: 56.9 ng mg creatinine^{-1}, 17β-E_2: 11.6 ng mg creatinine^{-1}).

Similar findings to those observed in this study have been reported by other investigators. Tepper et al. demonstrated that approximately 57% of postmenopausal women who received 2 mg of an oral estradiol formulation, experienced serum estrogen levels five times in excess of what was required to manage their menopausal symptoms [17]. They concluded that it may be inappropriate to treat all women with the same steroid dosage. In a similar study, Friel et al. reported that after studying hundreds of women who received various formulations of HRT, they noticed that some women excreted higher quantities of E_1 and 17β-E_2 as compared to those seen in healthy premenopausal women [16]. Both studies concluded that currently marketed pharmaceutical preparations contain doses of the estrogens in excess of what are required to manage the symptoms of a large number of postmenopausal women. Doses containing as little as 0.3 mg day^{-1} of the conjugated equine estrogens have been shown to be as effective in controlling menopausal symptoms as the standard doses that are currently prescribed (i.e. 0.625 mg day^{-1}) [10,40]. Estrogen replacement therapy is based on fixed-dose regimens, which are infrequently monitored during long periods of treatment [17]. The short-term risks of exposure to high estrogen levels are still unknown [41], although recent findings from a large scale clinical trial provided convincing evidence that the long-term use of HRT is associated with an increased risk of breast and endometrial cancer [42].

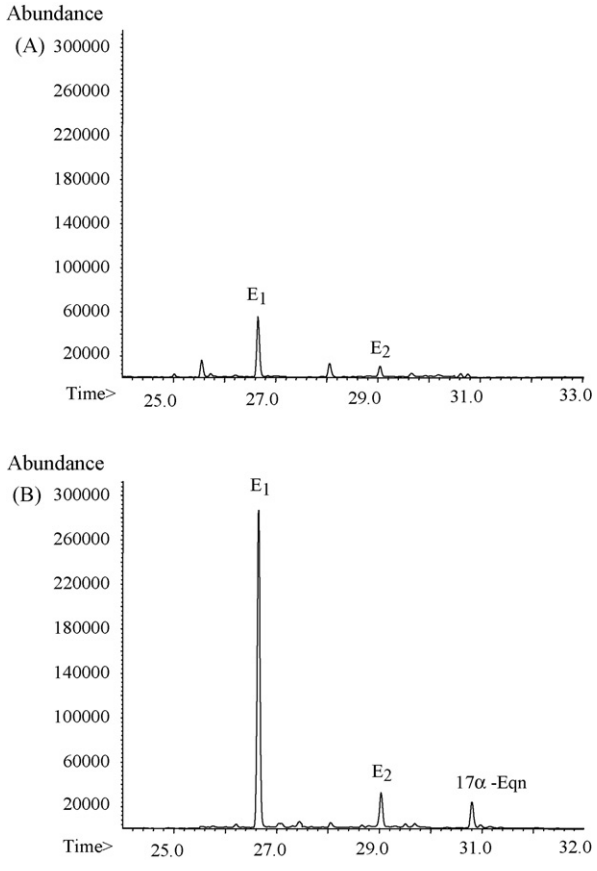

Fig. 5. SIM chromatograms of (A) a 1 mL urine sample of a 23-year-old premenopausal woman (E_1: 2.6 ng mg creatinine^{-1}; 17β-E_2: 0.84 ng mg creatinine^{-1}); and (B) a 1 mL urine sample of a 57-year-old postmenopausal woman who received Premarin (E_1: 56.9 ng mg creatinine^{-1}; 17β-E_2: 11.6 ng mg creatinine^{-1}). The extraction, derivatization and chromatographic conditions are described in Section 2.

Another important consideration is the fact that surplus estrogens will be excreted into domestic waste removal systems. It is currently estimated that 20–50% of women in the western world use some form of HRT (i.e. those aged 45–70 years) [43]. In view of the frequent use of these products and the fact that standard HRT doses may be too high for many women, it is likely that the use of HRT formulations will make a significant contribution to the levels of estrogens found in STP effluents. The presence of these chemicals in wastewater discharges have been attributed to the widespread intersexuality detected in fish [44], although the overall threat to fish populations from this source is still unknown [45]. In light of the potential health risks to women who use various formulations of HRT, as well as the potential harmful effects of these compounds in the environment, it seems reasonable to recommend that urinary estrogen levels be frequently monitored during HRT. Future work should aim to establish the usefulness of these measurements in determining the dose–response relationships of HRT preparations.

4. Conclusion

Regulatory authorities have become increasingly concerned about the presence of estrogenic chemicals in the aquatic environment. Possible sources for these compounds in wastewater discharges have been attributed to the frequent use of pharmaceutical products that may contain endogenous, chemically modified or conjugated equine estrogens. Estrogen replacement therapy is currently based on fixed-dose regimens that are seldom tailored to meet individual requirements. Therefore, excessive amounts of the estrogens may be excreted by postmenopausal women who receive conventional doses of various HRT preparations. In the present study it was demonstrated that increased amounts of E_1 and 17β-E_2 were excreted by a postmenopausal volunteer who received a standard oral dose of the conjugated equine estrogens (i.e. 0.625 mg day^{-1}). Urine levels of the estrogens were determined by aqueous SBSE that was combined with two derivatization steps to form the mono- and diacetate derivatives of the extracted compounds. The derivatives were thermally desorbed and analyzed on-line by GC/MS. The sensitivity of the method was improved by optimizing a number of experimental conditions which enhanced the recovery of the compounds by the stir bar coating. Detection limits of 0.02 and 0.03 ng mL^{-1} were obtained for E_1 and 17β-E_2, respectively. The developed SBSE procedure is highly sensitive

and easy to perform, which are important considerations for the routine clinical monitoring of urinary estrogen levels by GC/MS.

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