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Separation of dansylated 17 β -estradiol, 17 α -estradiol and estrone on a single HPLC column for simultaneous quantitation by LC-MS/MS

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Abstract

We show here that baseline separation of dansylated estrone, 17 β -estradiol and 17 α -estradiol can be done, contrary to previous reports, within a short run time on a single RP-LC analytical column packed with particles bonded with phenyl-hexyl stationary phase. The chromatographic method coupled with isotope-dilution tandem MS offers a simple assay enabling the simultaneous analysis of these analytes. The method employs ¹³C-labeled estrogens as internal standards to eliminate potential matrix effects arising from the use of deuterated estrogens. The assay also offers adequate accuracy and sensitivity to be useful for biological samples. The practical applicability of the validated method is demonstrated by the quantitative analyses of *in vivo* samples obtained from rats treated with Premarin®.

Keywords

Estrogens; Phenyl-hexyl stationary phase; LC-MS/MS; Dansylation; ¹³C-labeled internal standards

Introduction

17 α -estradiol (α -E2, Fig. 1) is the C17-epimer of the well-known principal human estrogen, 17 β -estradiol (β -E2, Fig. 1). This estrogen occurs naturally in ungulates [1]; therefore, its conjugated form is a constituent of the complex mixture of equine estrogens isolated from the urine of pregnant horses and widely prescribed for the treatment of menopausal symptoms (marketed as, e.g., Premarin®) [2]. The transient endogenous formation and subsequent putative role of α -E2 in immature rats and adult rats after gonadectomy have been proposed [3]. A potential precursor steroid for endogenous α -E2 is estrone (E1, Fig. 1) that is also formed from β -E2 *via* 17 β -hydroxysteroid dehydrogenase [4]. Since α -E2 has appreciable binding to the nuclear estrogen receptors [3,5], its administration results in, among others, significant uterotrophic effect [6,7]. This typical yet unwanted peripheral estrogenic response [8] is comparable to that of β -E2 [6] and, in part, could be attributed to the local formation of β -E2 from α -E2. Altogether, due to the potential role of α -E2 in

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rodents lacking a significant endogenous β -E2 source [3] and metabolic interplays among E1, β -E2 and α -E2, routine analytical methods capable of identifying and simultaneously measuring these estrogens from biological samples are needed. These assays would allow to enhance our understanding of the pharmacological responses elicited by these steroids as well as to decipher their potential contributions to the observed pharmacological effects.

Determination of estrogens both from environmental and biological samples is routinely done by chromatography-coupled MS methods [9,10,11]. In most environmental analyses, the availability of large sample sizes and subsequent sample enrichment eliminates the need for derivatization [10,12]. In bioanalytical tests, however, sample sizes are usually limited and/or the analytes are present in low concentration. Therefore, appropriate derivatization to increase assay sensitivity is usually needed [9,11,13], although a sensitive two-dimensional (2D) approach for clinical serum E1 and β -E2 quantitation without derivatization has recently been reported [14]. With GC, silylation is one of the most popular choices [15,16], while dansylation (Dns) [17,18] is the widely applied derivatization of the phenolic A-ring, when HPLC is utilized for estrogen analysis.

We have previously developed a sensitive GC–MS/MS-based method for the simultaneous quantitation of E1, β -E2 and α -E2 from biological matrices upon derivatization with N-(trimethylsilyl)imidazole [16]. While an excellent separation of the silylated analytes can be achieved by GC, the tedious sample preparation and long run times compared to those of HPLC [16,19,20] may limit the overall usefulness of the otherwise powerful GC technique. At the same time, LC–MS-based assays focusing on these estrogens do not exhibit adequate sensitivity and assay throughput to be practically useful for their simultaneous analysis from biological matrices [3,17,21]. In particular, an alkylsilica stationary phase (Phenomenex Synergi Max-RP) resulted in the co-elution of Dns-E1 and Dns- α -E2 in a study that aimed at gaining quantitative proof-of-concept data on the endogenous formation of α -E2 in various rat tissues [3]. Since “cross-talk” effects [22,23] may have also occurred among the SRM channels used for monitoring the co-eluting compounds, the quality of analytical data could have been affected. To improve the reliability of this assay, a recent report unsuccessfully screened a wide variety of RP columns to enable chromatographic separation of these derivatized estrogens on a single column [21]. When a 2D-LC approach was adopted in which separation was performed first on an amide-C18 column and, then, the effluent was injected by heart-cutting onto the second and third octadecylsilica columns optimized for high-performance separation (“Shim-pack”) and operated in series, only an impractically long analysis time (~45 min) allowed for a complete separation of the dansylated analytes. Moreover, this assay also lacked the necessary sensitivity to be useful for the simultaneous MS/MS quantitation of these estrogens from biological matrices. While other 2D-LC approaches employed, with or without derivatization, significantly shortened analysis times, they suffered from inadequate chromatographic separation when these particular estrogens were targeted [17,24].

The goal of the present study was to develop a simple, accurate and reliable LC–MS/MS method utilizing only a single RP column for the simultaneous analysis of E1, α -E2 and β -E2 from biological samples and thereby addressing the shortcomings of previously reported methods [3,21]. In addition, since assays developed for estrogen quantification employ deuterated estrogens as internal standards (ISs) [3,17,18] that might produce chromatographic isotope effects [25,26], we also introduced and evaluated the use of ^{13}C -labeled ISs instead [9,26,27]. We show that baseline separation of the dansylated analytes can be achieved with a straightforward 1D-LC system and the practical applicability of the validated assay is demonstrated *via* the simultaneous quantitation of E1, α -E2, and β -E2 in the serum and brain of Premarin®-treated rats.

Materials and methods

Chemicals and reagents

Estrone (E1), 17 α -estradiol (α -E2), and 17 β -estradiol (β -E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Estrone- d_4 (E1- d_4), 17 α -estradiol- d_2 (α -E2- d_2), and 17 β -estradiol- d_5 (β -E2- d_5) with an isotopic purity of 98% were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). ^{13}C -labeled estrone (E1- $^{13}\text{C}_6$) and 17 β -estradiol (β -E2- $^{13}\text{C}_6$) with an isotopic purity of 99% were supplied by Cambridge Isotope Laboratories (Andover, MA, USA). Premarin® for intravenous injection was obtained from Wyeth/Pfizer (Philadelphia, PA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents used for LC-MS/MS measurements were of HPLC grade and obtained from Fisher Scientific (Atlanta, GA, USA). Charcoal-stripped human serum was ordered from Innovative Research (Novi, MI, USA).

Preparation of calibration and quality control samples

Stock solutions of the analytes and their ISs were prepared in acetonitrile at 1 mg/mL concentration for each analyte (E1, α -E2 and β -E2). These solutions were further diluted with acetonitrile to obtain a series of working standards with concentrations of 1.25, 2.5, 5.0, 10, 20, and 40 ng/mL for each estrogen, respectively. The working IS solution of E1- $^{13}\text{C}_6$, α -E2- d_2 , and β -E2- $^{13}\text{C}_6$ containing 10 pg/ μL labeled estrogens was prepared similarly. All standards were stored in a refrigerator at approximately -20°C . Calibration samples were prepared by mixing equal volume (10 μL) of working standards and working IS solution (100 pg IS) into 200 μL of charcoal-stripped human serum. Accordingly, in the serum calibration samples each estrogen was present at 62, 125, 250, 500, 1000, and 2000 pg/mL concentration, while the concentration of ISs was set at 500 pg/mL (100 pg/sample) resulting in 0.125:1, 0.25:1, 0.5:1, 1:1, 2:1 and 4:1 analyte to IS ratios, respectively. Quality control (QC) samples were prepared analogously using 180, 900, and 1800 pg/mL (low, middle, and high levels according to FDA Guidelines [28]) concentrations for each estrogen, and 500 pg/mL for each IS. Estrogens were extracted from the serum (200 μL) with ethyl acetate. The organic layer was separated by centrifugation at 2500 rpm for 10 min and evaporated under a nitrogen stream at room temperature. The residue was derivatized by adding 30 μL of 1 mg/mL dansyl chloride (Dns-Cl) solution in acetonitrile and 20 μL of aqueous sodium bicarbonate (100 mM, pH 10.5) [17]. The samples were vortexed and kept in a heating block at 60°C for 10 min. Thereafter, the samples were centrifuged at 14,500 rpm for 3 min, transferred to autosampler vials, sealed, and assayed by LC-MS/MS.

Chromatographic separation of estrogen dansyl derivatives

Two different RPLC columns from Phenomenex (Torrance, CA, USA) were used in the study: a Synergi Max-RP (PSM-RP) column (150 mm $\times$ 2.0 mm i.d., 4 μm particle size) [3] and a Kinetex Phenyl-Hexyl (PK-PH) column (50 mm $\times$ 2.1 mm i.d., 2.6 μm “core-shell” particle size). The eluent was mixed from (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). The columns were operated at 30°C with a flow rate of 0.4 mL/min. Separation factors (α) were evaluated under isocratic conditions. The eluent composition was set to 75% (v/v) B for the PSM-RP column and 62% (v/v) B for the PK-PH column, respectively, to achieve the same retention factor ($k = 6$) [29] for the first eluting compound, Dns- β -E2. Gradient separation of the dansylated analytes were done by an eluent composition initially set to 50% (v/v) B and, then, linearly increased to 100% B by varying the gradient time (t_G) to achieve identical gradient retention factors (k^*) in the optimal range ($0.5 < k^* < 20$) for both columns [29]. Accordingly, t_G set at 1.3, 2.7, 4.0 and 8.0 min on the PK-PH column resulted in the same k^* (2.3, 4.6, 7.0, and 14.0, respectively) as t_G set at 4.0, 8.0, 12.0 and 24.0 min for the PSM-RP column.

LC-MS/MS analysis

A TSQ Quantum Ultra triple-quadrupole instrument operated in positive ion mode with a heated-electrospray ionization (H-ESI) probe (Thermo Electron Corporation, Trace Chemical Analysis, Austin, TX, USA) was employed. Gradient separations were carried out using a Surveyor LC system (Thermo). The PK-PH column was operated at 0.4 mL/min flow rate and with the following gradient program: 50% B to 87% B in 6 min, then ramped to 100% B in 0.2 min and held for 1.8 min, finally the column was equilibrated with 50% B for 3 min. The autosampler injection volume was 5 μ L and the tray temperature was maintained at 18 °C. H-ESI spray voltage, H-ESI temperature, and capillary temperature were maintained at 3.5 kV, 350 °C, and 325 °C, respectively. Nitrogen sheath gas and auxiliary gas flow rates were 30 and 20 arbitrary unit (corresponding to approximately 0.45 and 6.0 L/min according to the manufacturer's specification), respectively. Collision-induced dissociation was performed with argon at 1.5 mTorr pressure. Selected reaction monitoring (SRM) with unit mass resolution for the precursor and product ions was used for quantitation of steroids. Five SRM transitions were monitored with dwell time and collision energy set at 15 ms and 35 V, respectively. Dns- α -E2 and Dns- β -E2 were monitored at the SRM transition of m/z 506 $\rightarrow$ 171. SRM transitions of m/z 504 $\rightarrow$ 171, 508 $\rightarrow$ 171, 510 $\rightarrow$ 171, and 512 $\rightarrow$ 171 were set up for Dns-E1, Dns- α -E2- d_2 , Dns-E1- 13 C₆, and Dns- β -E2- 13 C₆, respectively. Data acquisition and processing were controlled by the XCalibur software (version 2.1) of the instrument.

Estimation of isotope effects of stable isotope-labeled estrogens

Samples containing E1, β -E2 and either their corresponding deuterated- or 13 C-labeled IS were used for these studies. The concentrations of the analytes and their ISs were 2 or 10 ng/mL. The injected quantities of the dansylated estrogens were sufficiently small enough (10 or 50 pg) to approach infinite-dilution in the mobile phase [30]. The SRMs for Dns-E1- d_4 and Dns- β -E2- d_5 were m/z 508 $\rightarrow$ 171 and m/z 511 $\rightarrow$ 171, respectively. Other LC-MS/MS parameters were identical to those described in the previous subsection. The single isotope effect (% *IE*) was calculated as $100[(k_U / k_L)^{1/n} - 1]$, where k_U and k_L are the retention factors for the unlabeled and labeled analyte pairs, respectively, and n denotes the number of isotope-labeled atoms [30].

Assay Validation

The assay developed for the simultaneous quantitation of E1, α -E2, and β -E2 was validated in accordance with the US FDA Guidance [28] using 200 μ L of serum. Limits of detection (LODs) were calculated according to the International Conference on Harmonization Guidelines [31] based on the standard deviation of the y -intercepts and the slope of regression lines in the range of the LOD. LOD confidence intervals were determined according to a recent report [32]. The calculated LODs were also verified experimentally by the analysis of QC samples spiked with the analytes at their LOD levels. Assay selectivity and "cross-talk" effects were assessed by analyzing serum samples with and without the addition of individual ISs or analytes, respectively [22,23]. Assay calibration was performed by isotope dilution using six different analyte to IS molar ratios (0.125:1, 0.25:1, 0.5:1, 1:1, 2:1, and 4:1) [16,33,34]. Calibration curves were obtained using linear regression without weighting. The linearity of the assay was assessed daily on three consecutive days using six concentrations of E1, α -E2, and β -E2 ranging from 62 (lower limit of quantitation, LLOQ [28]) to 2000 pg/mL while the IS concentration was kept constant at 500 pg/mL for each estrogen. For the estimation of precision and accuracy, QC samples were prepared daily at three concentration levels [28]: low QC within 3-times of LLOQ (180 pg/mL), medium QC at 900 pg/mL, and high QC at 1800 pg/mL. The samples were run in quintuplicate during a 3-day interval. Precision describing the uncertainty of measurements for the same sample was expressed in % coefficient of variation (CV %). Accuracy, indicating the extent of

agreement between measured (c_m) and nominal concentrations (c_n) was estimated at three concentration levels (180, 900, and 1800 pg/mL) [28]. Percentage accuracy was calculated as $[(c_m - c_n)/c_n] \times 100$ [16,25]. Recovery and matrix effects were determined at three concentration levels (180, 900, and 1800 pg/mL, $n = 9$). Percentile recovery (%RE) for each estrogen was estimated as the ratio of analyte/IS peak area ratio spiked before extraction to analyte/IS spiked postextraction multiplied by 100. Percentile matrix effect (%ME) was estimated as the ratio of analyte/IS response ratio of QC solutions post-spiked into serum to neat, unextracted QC solutions multiplied by 100 ($n = 9$) [35].

***In vivo* samples**

All animal procedures were approved by the Animal Care and Use Committees of University of North Texas Health Science Center. Ovariectomized (OVX) Sprague-Dawley rats (350 g) were purchased from Harlan Laboratories (Indianapolis, IN, USA). Animals ($n = 5$) were treated with Premarin® (1 mg/kg body weight, i.v.) reconstituted in 200 μ L saline. At various time points, animals were euthanized with intraperitoneal administration of 60 mg/kg ketamine (100 mg/mL) and 10 mg/kg xylazine (20 mg/mL), and blood was collected by cardiac puncture. The blood was clotted on ice and spun at 3000 rpm for 20 min to obtain serum samples. Tissue homogenates in pH 7.4 phosphate buffer were prepared as reported earlier [36]. Extraction of estrogens from the serum (200 μ L) and tissues (20% w/v homogenates) were carried out analogously to those of calibration and QC samples. The amount of each internal standard was 100 pg/sample.

Results and discussion

LC separation of dansylated estrogens

Separation of Dns-E1 and Dns- α -E2 using a robust 1D-LC method is a critical aspect of the present study, since previous attempts [3,21] have failed to achieve acceptable chromatographic resolution of these compounds on a variety of RP columns. Even more sophisticated 2D-LC approaches focusing on these estrogens have shown serious limitations in terms of assay sensitivity, separation and analysis time [17,21,24]. Here, we evaluated a Phenomenex Kinetex Phenyl-Hexyl (PK-PH) column as a possible solution for a rapid yet adequate 1D-LC separation of the analytes and also compared its performance to that of Phenomenex Synergi Max-RP (PSM-RP). The latter column has densely bonded C12-phase and has previously produced co-elution of DnsE1 and Dns- α -E2 under the applied conditions [3]. Apart from differences in the stationary phase-chemistry, we also aimed at taking advantage of the PK-PH “core-shell” particles to afford additional increase in chromatographic resolution [37]. In order to test column selectivity, isocratic separations of the dansylated estrogens were performed with mobile phase compositions (75% B for PSM-RP and 62% B for PK-PH column, respectively) that afforded $k = 6$ retention factor, which is within the recommended optimal range [29] for the first eluting compound, Dns- β -E2, on both columns. These conditions resulted in similar separation factors (α) for the Dns- α -E2/ β -E2 pair on both columns, whereas α for the derivatized E1/ α -E2 peak pair was significantly higher on the PK-PH column, as shown in Table 1.

Since LC-MS/MS methods for the simultaneous analysis of dansylated derivatives of E1, α -E2 and β -E2 in complex biological samples employ gradient elution, we also tested these two columns under various gradient times, thus, gradient slopes (Separation of the derivatized estrogens on both columns is demonstrated in a video animation available in the Electronic Supplementary Material). To exclude resolution differences due to different column geometries, we plotted the estimated resolution (R_s) of the critical Dns-E1/Dns- α -E2 peak pair against $\log k^*$ and showed that the core-shell PK-PH column provided larger R_s values under all experimental conditions applied (Fig. 2a). This finding indicated that the

improved resolution was due to the introduction of both the new stationary phase and the core-shell packing material with smaller particle size. Therefore, the PK-PH column allowed for shorter analysis times without sacrificing chromatographic resolution (Fig. 2b). Specifically, baseline separation ($R_s > 1.5$) of Dns-E1 and Dns- α -E2 was achieved within 2.5 min, and only 2.7 min was needed to reach $R_s > 2.0$ [29]. Although the PSM-RP column also produced $R_s > 2.0$, it required significantly longer run time than the PK-PH column (11.3 min vs. 2.7 min, Fig. 2b), which would reduce the assay throughput by about 4-times. Consequently, the latter column was chosen for assay validation. As shown in Fig. 3, the gradient HPLC method allowed for an excellent separation of all three derivatized estrogens ($R_s = 5.0 \pm 0.3$ and $R_s = 2.4 \pm 0.2$ between Dns-E1/Dns- α -E2 and Dns- α -E2/Dns- β -E2, respectively) within 5 min, contradicting thereby previous studies that were unsuccessful in the separation of Dns-E1 and Dns- α -E2 on a single RP column to a satisfactory extent [3,21].

Selection of isotope-labeled internal standards for quantitation

Routinely used deuterated ISs frequently have slightly different retention times (t_R) than their corresponding analytes. This chromatographic isotope effect may interfere with the accuracy of quantitation [9,26]. However, both E1 and β -E2 are now commercially available with $^{13}\text{C}_6$ -labeling. Nevertheless, the in-house or custom synthesis of α -E2- $^{13}\text{C}_6$ would be prohibitively expensive even from E1- $^{13}\text{C}_6$. It is noteworthy that in the first report about the quantitation of α -E2 in the presence of E1 and β -E2 in rat tissues [3] no deuterated α -E2 IS was used. This was most probably due to the fact that the commercially available α -E2- d_2 is isobaric with E1- d_4 and, also, co-eluting compounds (i.e., Dns-E1 and Dns- α -E2) could not be distinguished through SRM MS/MS on the triple-quadrupole instrument used. On the other hand, Nguyen *et al.* [21] mistakenly claimed the use of tritiated α -E2 as IS in the above study [3], when in fact [^3H] α -E2 was used in receptor binding assays.

The advantages of the $^{13}\text{C}_6$ -labeled estrogens are the 6 Da mass difference and identical retention time with their corresponding analytes [9]. Accordingly, we did not observe t_R shift between the analytes and their ^{13}C -labeled ISs and, therefore, the %IEs for E1- $^{13}\text{C}_6$ and β -E2- $^{13}\text{C}_6$ were practically zero; $0.007 \pm 0.009\%$ and $0.001 \pm 0.002\%$, respectively. On the other hand, the deuterated ISs showed chromatographic isotope effects with %IE of $0.09 \pm 0.03\%$ ($\Delta t_{R(E1/E1-d4)} = 0.014 \pm 0.005$ min) and $0.18 \pm 0.01\%$ ($\Delta t_{R(\beta-E2/\beta-E2-d5)} = 0.032 \pm 0.002$ min) for E1- d_4 and β -E2- d_5 , respectively. The t_R shift between Dns- α -E2 and its deuterated IS was 0.010 ± 0.003 min, corresponding to $0.11 \pm 0.08\%$ IE. Collectively, these results confirm previous reports [9,26,27] on the benefits of ^{13}C -labeled ISs for eliminating potential matrix effect arising from the differential suppression or enhancement of ionization.

Assay validation

The LC-MS/MS method for the simultaneous measurement of dansylated derivatives of E1, α -E2, and β -E2 on the selected PK-PH column was validated over 3 days using calibration samples prepared daily upon extracting merely 200 μL of serum samples. The calibration curves were linear in the concentration range of 62 to 2000 pg/mL with correlation coefficients higher than 0.99. The deviation from the nominal concentrations of all the calibration standards were within the acceptance limit values ($\pm 15\%$ or $\pm 20\%$ for LLOQ) according to the FDA Guidelines [28]. In Table 2, we summarized the intra- and inter-day precision and accuracy results obtained for the QC samples. These values for all three estrogens were within the acceptable range [28] at all concentration levels. Recoveries and matrix effects for each analyte were assessed at three QC concentration levels. As shown in Table 3, excellent recoveries ranging from 98 to 107% were obtained. Matrix effects [35] were negligible as the estimated %ME values were 98-105%, 100-108% and 98-100% for

Dns-E1, Dns- α -E2 and Dns- β -E2, respectively. LODs were 3 ± 2 , 3 ± 2 , and 4 ± 1 pg/mL in serum for E1, α -E2, and β -E2, respectively. It is noteworthy that our assay afforded a 7–10-fold increase in sensitivity compared to that of a recent report [21] using a 2D-LC setup and extra-long analysis time. We also did not find “cross-talk” interferences [22,23] between the SRM transition channels (data not shown). Altogether, these results confirm that the assay presented here is accurate and precise for the simultaneous monitoring of E1, α -E2, and β -E2 upon dansylation. Since charcoal-stripped human serum was applied as a matrix during method validation, the assay is primarily proposed for human studies under regulatory environment. However, the application of ^{13}C -labeled ISs that co-elute with their corresponding analytes eliminates the need of matrix matching [27]. This argument and the limited availability of authentic estrogen-free rat tissues also prompted us to apply this assay for preclinical animal experiments.

Application of the validated assay for in vivo samples

Once validated, the method was applied for the detection and quantitation of E1, α -E2, and β -E2 after a single i.v. administration of 1 mg/kg Premarin® to OVX rats. Although Premarin® is a complex mixture of various conjugated equine estrogens [2], our interest is focused on the analysis of these estrogens in the serum and various organs of rodents used as models for estrogen-responsive diseases in our laboratory. As a typical sample, we show here that all three estrogens could be detected in the serum 30 min after drug administration (Fig. 4). Expectedly, E1 was present at the highest concentration, 1892 ± 73 pg/mL (mean $\pm$ SEM, $n = 5$), since E1-sulfate is the major constituent of Premarin® [2]. The corresponding α -E2 and β -E2 concentrations were 82 ± 13 pg/mL and 1134 ± 75 pg/mL, respectively (mean $\pm$ SEM, $n = 5$). A representative extracted ion chromatogram (EIC) of a dansylated serum extract of a treated animal is shown in Fig. 4. The assay was also used for the simultaneous quantitation of these estrogens in the brain which is a proposed locus for the endogenous formation of α -E2 in adult gonadectomized rodents [3]. However, we could not detect α -E2 in the brain of control OVX rats neither in these studies nor in unrelated experiments (data not shown). At the same time, Premarin® treatment not only produced significant serum estrogen levels (Fig. 4), but E1, α -E2 and β -E2 were also present at 3554 ± 109 pg/g, 237 ± 25 pg/g and 633 ± 25 pg/g (mean $\pm$ SEM, $n = 5$), respectively, in the corresponding brain samples 30 min after drug treatment (Fig. S1 in the Electronic Supplementary Material). A complete pharmacokinetic studies in rats treated with the Premarin® will be reported elsewhere.

Conclusions

The main finding of this study is that complete separation of dansylated derivatives of E1, α -E2, and β -E2 can be accomplished, contrary to previous reports, on a single RP-column within a short run time. We demonstrated that the simple assay presented here offers adequate accuracy and sensitivity. By employing ^{13}C -labeled estrogens as internal standards, potential matrix effects arising from the use of deuterium-labeling can also be avoided. Collectively, the validated assay is suitable for the simultaneous and sensitive quantitative analysis of E1, α -E2, and β -E2 in biological samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical structure of 17 α -estradiol (α -E2), 17 β -estradiol (β -E2) and estrone (E1).

Fig. 2.

Resolution (R_s) between Dns-E1 and Dns-α-E2 as a function of $\log k^*$ (a) and analysis time (b) on a Phenomenex Kinetex Phenyl-Hexyl (◆) and a Phenomenex Synergi Max-RP (■) analytical column. Analysis time (t) denotes the retention time of the latest-eluting analyte of interest, Dns-E1, in the assay. Error bars represent standard deviation ($n = 6$). Cross-hatched area shows the unacceptable range of resolution ($R_s < 1.5$).

Fig. 3.

Chromatographic separation of Dns-E1, Dns- α -E2, and Dns- β -E2 (10 pg of each injected on column) on a Phenomenex Kinetex Phenyl-Hexyl (50 mm $\times$ 2.1 mm i.d, 2.6 μ m “core-shell” particle size) column. LC-MS/MS analysis was performed with a mobile phase consisting of (A) 0.1% formic acid in water (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). Gradient profile used was 50-87% B in 6 min.

Fig. 4. Representative SRM chromatograms of serum extract from a Premarin-treated OVX rat. Serum concentrations of 1892 ± 73 , 82 ± 13 and 1134 ± 75 pg/mL (mean $\pm$ SEM, $n = 5$) were determined for E1, α -E2 and β -E2, respectively. Blood was collected 30 min after drug administration (1 mg/kg, i.v.).

Table 1

Separation factor (α) values of dansylated estrogens obtained by isocratic separation ($k = 6$ for Dns- β -E2) on two analytical columns

	$\alpha_{\text{Dns-E1/Dns-}\alpha\text{-E2}}$ (mean $\pm$ SD) ^a	$\alpha_{\text{Dns-}\alpha\text{-E2/Dns-}\beta\text{-E2}}$ (mean $\pm$ SD) ^a
PSM-RP ^b	1.076 $\pm$ 0.002	1.155 $\pm$ 0.002
PK-PH ^c	1.319 $\pm$ 0.002	1.133 $\pm$ 0.001

^a
 $n = 3$

^b PSM-RP denotes Phenomenex Synergi Max-RP column

^c PK-PH denotes Phenomenex Kinetex Phenyl-Hexyl column

Table 2

Precision and accuracy of the assay developed for the simultaneous measurement of E1, α -E2, and β -E2 in human serum^a

Intra-assay (days) ^b	QC concentration (pg/mL)	E1		α -E2		β -E2	
		Precision (CV %)	Accuracy (%)	Precision (CV %)	Accuracy (%)	Precision (CV %)	Accuracy (%)
1	180	8.8	0.9	11.4	6.5	14.4	-9.6
	900	4.2	-0.1	3.7	1.6	3.9	2.1
	1800	4.3	0.9	7.9	-6.9	6.8	1.1
2	180	5.3	13.7	6.2	10.8	12.9	6.6
	900	7.8	4.5	6.5	6.8	10.8	-1.0
	1800	8.0	1.6	3.0	-3.1	5.3	0.2
3	180	6.3	14.1	10.1	12.9	12.3	1.4
	900	5.0	0.9	6.6	8.2	7.4	5.6
	1800	6.3	-0.8	4.1	-2.8	6.4	-1.1
inter-assay ^c	180	15.0	9.4	10.4	10.1	14.4	-0.6
	900	14.6	1.8	7.0	5.5	9.0	2.2
	1800	14.4	0.6	7.1	-4.2	6.0	0.1

^aPrecision denotes uncertainty of series of measurements expressed in % coefficient of variation (CV %). Accuracy, indicating the extent of agreement between measured (c_M) and nominal (c_N) concentrations, was calculated as $[(c_M - c_N)/c_N] \times 100$.

^b $n = 5$

^c $n = 15$

Table 3

Recoveries and matrix effects of quality control (QC) samples

Nominal conc. (pg/mL)	E1		α-E2		β-E2	
	RE^a (%) $\pm$ SD	ME^b (%) $\pm$	SD RE^a (%) $\pm$ SD	ME^b (%) $\pm$ SD	RE^a (%) $\pm$ SD	ME^b (%) $\pm$ SD
180	98.5 $\pm$ 6.8	100.5 $\pm$ 4.0	93.3 $\pm$ 4.5	108.0 $\pm$ 12.6	105.8 $\pm$ 0.9	99.9 $\pm$ 12.2
900	101.3 $\pm$ 3.0	105.3 $\pm$ 4.1	102.4 $\pm$ 0.9	99.7 $\pm$ 0.6	103.2 $\pm$ 4.8	100.5 $\pm$ 4.0
1800	101.1 $\pm$ 4.9	98.1 $\pm$ 5.5	107.1 $\pm$ 10.6	102.5 $\pm$ 3.2	100.9 $\pm$ 7.5	98.0 $\pm$ 3.4

^a Recovery was calculated as the ratio of analyte/internal standard response ratio spiked before extraction to the response ratio spiked post extraction multiplied by 100 ($n = 9$).

^b Matrix effect was determined by taking the ratio of the analyte/internal standard response ratio spiked post extraction to the response ratio of the neat analyte and internal standard multiplied by 100 ($n = 9$). A value of $> 100\%$ indicates ionization enhancement and a value of $< 100\%$ indicates suppression of ionization.