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Simultaneous quantification of four native estrogen hormones at trace levels in human cerebrospinal fluid using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Estrogens are known to exhibit neuroprotective effects on the brain. Their importance in this regard and in others has been emphasized in many recent studies, which increases the need to develop reliable analytical methods for the measurement of estrogen hormones. A heart-cutting two-dimensional liquid chromatography separation method coupled with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) has been developed for simultaneous measurement of four estrogens, including estriol (E3), estrone (E1), 17β-estradiol (17β-E2), and 17α-estradiol (17α-E2), in human cerebrospinal fluid (CSF). The method was based on liquid–liquid extraction and derivatization of estrogens with dansyl chloride to enhance the sensitivity of ESI-based detection in conjunction with tandem mass spectrometry. Dansylated estriol and estrone were separated in the first dimension by an amide-C18 column, while dansylated 17β- and 17α-estradiol were resolved on the second dimension by two C18 columns (175 mm total length) connected in series. This is the first report of a method for simultaneous quantification of all four endogenous estrogen compounds in their dansylated form. The detection limits for E1, 17α-E2, 17β-E2, and E3 were 19, 35, 26, and 61 pg/mL, respectively. Due to matrix effects, validation and calibration was carried out in charcoal-stripped CSF. The precision and accuracy were more than 86% for the two E2 compounds and 79% for E1 and E3 while the extraction recovery ranged from 91% to 104%. The method was applied to measure estrogens obtained in a clinical setting, from the CSF of ischemic trauma patients. While 17β-estradiol was present at a significant level in the CSF of some samples, other estrogens were present at lower levels or were undetectable.

1. Introduction

Estrogens are best known for their role as sex hormones. They are not only important components of the reproductive system and bone health, but they also play a crucial role in neuroprotective processes [1,2]. It has been demonstrated that estrogens, used in hormone therapy, can prevent and decrease the incidence of stroke-related mortality in postmenopausal women [3,4]. The beneficial effects of estrogens in pre- and post-ischemic treatment have been demonstrated in a number of laboratory studies [5–8]; 17β-estradiol (17β-E2) and its less bio-active metabolite, estrone (E1), have been the endogenous estrogens of greatest interest. The administration of 17β-E2 has been reported to reduce mortality rates and increase survival from ischemic injury [6,8–10], whereas 17α-estradiol (17α-E2) has been considered to be less active due to weaker binding affinity to estrogen receptors [11]. However, recent studies have indicated that 17α-estradiol not only has strong neuroprotective activity, but also is locally synthesized in the brain [5,12]. These findings support the notion that all native estrogen hormones may have beneficial effects in the brain, particularly for the treatment of stroke and traumatic brain injuries. Thus, development of analytical methods that allow simultaneous measurement of all native estrogens (E1, 17β-E2, 17α-E2, and estriol (E3)) is important for supporting future research to investigate and further define these physiological effects.

Research on the neuroprotective effects of estrogen has been primarily performed on rodents [5,8,13] due to limited availability of samples such as brain tissues, cerebral blood, or cerebrospinal fluid (CSF) from humans. Among these, CSF is the most accessible. CSF is mainly produced by the brain and contains components that reflect the physiological state of the central nervous system [14,15].

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Understandably, obtaining significant quantities of CSF for method development and analysis can still be difficult.

Trace level quantification of small molecules in biological samples is challenging. Most biological matrices contain a wide variety of abundant species that can interfere with separation and detection of analytes of interest. Native, unmodified estrogen compounds represent an analyte class that can be present in low quantities in many biological matrices and do not exhibit appreciably high absorption properties or mass spectrometric ionization efficiencies to allow for facile detection without some form of preconcentration [16,17]. Radioimmunoassay is a highly sensitive method currently used for estrogen quantification in plasma (measurable in the pg/mL concentration range). However, this method requires use of radioactive materials, and artifacts have been reported to occur due to nonspecific binding of radioactive labels [9,18]. Moreover, method reproducibility has been questionable in inter-laboratory assays [19]. It is worthy to note that the College of American Pathologists has recommended that radioimmunoassays be improved with respect to analytical accuracy [9], and that no such assay has ever been reported validated for analysis of estrogens from CSF. As an alternative, mass spectrometry (MS) is a good candidate for estrogen detection, especially when it is coupled with gas chromatography (GC) or high performance liquid chromatography (LC) separations [20]. Although detection limits of low pg/mL could be achieved using GC–MS, the simultaneous measurement of all four native estrogens have not been reported [21–24]. The substantial growth of LC–MS for the past decades has increased numbers of its applications in the field. The sensitivity of LC–MS can be further enhanced to low pg/mL detection limits when chemical derivatization is employed in conjunction with the use of an electrospray ionization source [25–28], despite the additional sample preparation steps required. The introduction of protonated or charged moieties to poorly ionizable estrogens in liquid phase dramatically increases their detection sensitivity using ESI-MS. Among several derivatization reagents, dansyl chloride is the most popular reagent [9–11,29,30] and has been originally used for the fluorometric detection of estrogens [31]. However, dansylated E1 and 17α-E2 have not previously been chromatographically resolved on standard reversed phase columns [11] (Fig. 3), resulting in co-elution of the two compounds. Additionally, their commercial deuterated standards are isobaric, making their analysis by stable isotope dilution and reversed phase LC–MS more difficult. Unless customized isotope standards are used, such as in the work reported by Toran-Allerand et al. [11], complete separation of these compounds is necessary. As researchers work to achieve lower and lower detection limits, the use of derivatization reagents will remain an attractive approach to do so.

In this work, we present the development and validation of a sensitive method, which involves liquid–liquid extraction, derivatization with dansyl chloride, and LC–MS/MS, to simultaneously quantify four native estrogens (E1, 17α-E2, 17β-E2, and E3) in human CSF. To address difficulties associated with chromatographic resolution of all four dansylated estrogens in reversed phase mode, a heart-cutting two-dimensional (2D) column set-up has been developed that is amenable for use with standard LC–MS instruments. Whereas comprehensive 2D LC instruments often require additional pumps, solvent reservoirs, switching valves, and degassing systems [32], this method can be used with only the addition of a single six-port valve to a standard binary gradient LC system. The heart-cutting approach requires initial separation of the sample on a commercial amide-C18 (a polar-embedded C18 phase), followed by separation of a critical pair on a commercial C18 phase. The method has been fully validated according to accepted bioanalytical method validation procedures [33]. From 1 mL of CSF, detection limits in the parts-per-trillion concentration range for each of the four estrogens are reported with satisfactory accuracy, precision, and recovery. To the best of our knowledge, this is the first reported method for separation of all four endogenous estrogens in their dansylated form in samples of CSF, which were collected from patients admitted to the hospital following traumatic brain injury.

2. Experimental

2.1. Chemicals and reagents

All estrogens and dansyl chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Internal standards consisting of estrone-2,4,16,16-d4 (E1-d4) and 17β-estradiol-16,16,17-d3 (17β-E2-d3) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Estriol-2,4-d2 (E3-d2) and 17α-estradiol-2,4-d2 (17α-E2-d2) were purchased from CDN isotopes (Quebec, Canada). Sodium hydrogen carbonate, sodium hydroxide, and dextran-coated charcoal were obtained from Sigma–Aldrich (St. Louis, MO, USA). Formic acid, acetone, and methanol, as well as HPLC–MS grade acetonitrile (ACN) and water, were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Preparation of charcoal-stripped cerebrospinal fluid

Dextran-coated charcoal was used to strip free hormones from CSF for method validation and calibration. An amount of 2 g of dextran-coated charcoal was added to 100 mL of CSF. The mixture was shaken overnight at 4 °C, followed by centrifugation at 2000 × *g* according to manufacturer guidelines. The CSF supernatant was filtered through a 13 mm syringe filter (0.2 μm PTFE membrane).

2.3. Preparation of stock solutions, calibration standards, and quality control samples

Stock solutions of estrogens and deuterated estrogens were prepared by accurately weighing and dissolving approximately 1.0 mg of each compound in 1.000 mL of methanol. Working standard solutions were prepared at 10 and 1 ng/mL by diluting the stock solutions with methanol. The calibration curve samples were prepared in duplicate by adding an appropriate amount of working standard solutions to 1 mL aliquots of charcoal-stripped CSF (CSCSF) to obtain standards of 20, 30, 50, 100, 200, 250, and 300 pg/mL. Quality control samples were prepared in quintuplicate at three concentration levels (70, 110, and 220 pg/mL). Each of the four stable isotopically labeled internal standards was spiked in all prepared and unknown samples at 125 pg/mL concentration, prior to processing.

2.4. Sample preparation

A volume of 2.000 mL of ethyl acetate was added to 1.000 mL of each CSF sample. The mixture was vortexed for 30 s, shaken for 15 min, and then centrifuged for 20 min at 2000 × *g*. Ninety percent (1.800 mL) of the organic supernatant was transferred into a 5-mL polypropylene centrifuge tube. The extraction was repeated a second time and the supernatants were combined. A total of 3.600 mL of supernatant was evaporated until dryness under a nitrogen stream at room temperature. The residue was reconstituted with 50.0 μL of sodium bicarbonate buffer (100 mM, pH 10.14) and then dansylated by adding 50.0 μL of dansyl chloride (1 mg/mL in acetone). The samples were incubated for 5 min at 60 °C, loaded into autosampler vials with glass inserts, and then transferred to the autosampler for LC–MS/MS analysis.

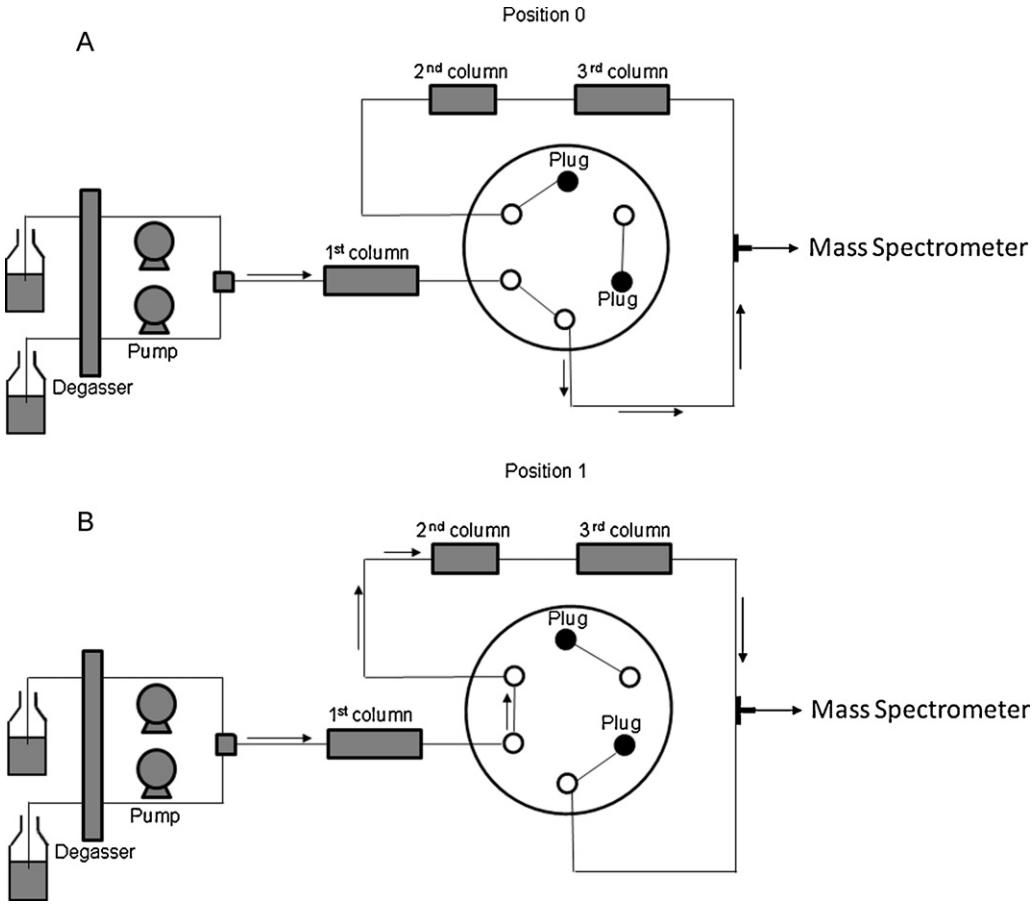

Fig. 1. Schematic flow diagram of heart-cutting 2D-LC-MS system. (A) Position 0 and (B) Position 1 refer to divert valve settings.

2.5. LOD, recovery, and matrix effect

Method validation, according to FDA guidelines [33], was performed in charcoal-stripped CSF (CSCSF). Samples used for limit of detection were prepared in septuplicate at a concentration of 70 pg/mL. The accuracy and precision were determined by analyzing the quality control samples, in quintuplicate, at low, medium, and high concentrations. The recovery was evaluated by comparing the response of the analytes added to CSF before and after extraction. The relative slopes of calibration curves in CSCSF vs. no matrix were used to evaluate the presence of matrix effects in the experiment.

2.6. LC–MS/MS conditions

The LC system (Thermo-Fisher Scientific, Inc., San Jose, CA, USA) was equipped with a Surveyor Autosampler and a Surveyor MS binary pump. The chromatographic system was arranged with the use of three analytical columns, including a Varian (Paolo Alto, CA, USA) Polaris Amide-C18 (2×100 mm, 3 μm, 200 Å) and two Shimadzu (Tokyo, Japan) Shim-pack XR-ODS columns (2×100 mm and 2×75 mm, 2.2 μm, 120 Å). This heart-cutting two-dimensional (2D) chromatographic configuration, shown schematically in Fig. 1, was arranged to allow for heart-cutting to transfer sample components from the first to the second dimension using the built-in divert valve (2-position-6-port switching valve, VICI cheminert) on a LCQ Deca XP mass spectrometer (Thermo-Fisher Scientific, Inc., San Jose, CA, USA). The separation was performed with an elution gradient of mobile phase A (H_2O/ACN/formic acid:95/5/0.1) and mobile phase B (ACN/H_2O/formic acid:95/5/0.1). The LCQ was operated in the positive ionization mode with a conventional electrospray source. The separation period (45 min) was divided into three segments. Single reaction monitoring (SRM) MS/MS (parent $[M+H]^+ \rightarrow m/z$ 171 transition) was used in conjunction with two scan events for the analytes within each segment. The time course of the method, including mobile phase compositions, flow rates, valve settings, and segmentation is presented in Fig. 2. A detailed overview of instrument settings in each segment is given in Table 1. Data analysis was performed using Thermo Xcalibur (version 2.0) software.

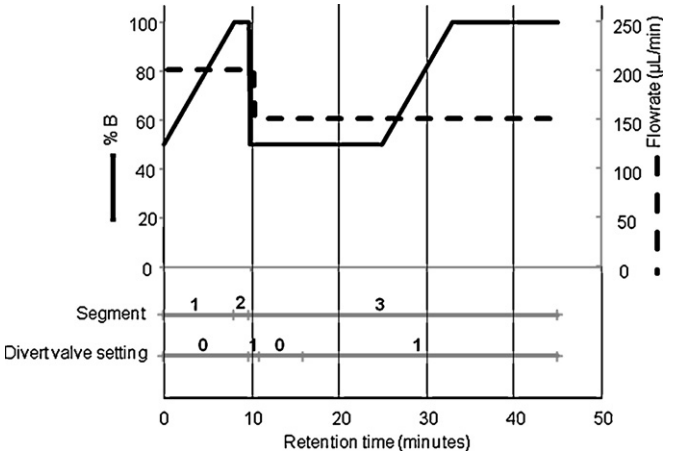

Fig. 2. Flow rate, mobile phase, segment, and divert valve setting sequence event program for heart-cutting 2D-LC-MS system.

Table 1
Optimized instrumental settings for each segment of the heart-cutting 2D-LC-MS method.

Analytical condition	Segment 1 (0–8 min)	Segment 2 (8–9.7 min)	Segment 3 (9.7–45 min)
Column	Polaris amide C18	Polaris amide C18	Shim-pack C18 XR-ODS
Mobile phase composition: (A) H_2O/ACN/formic acid (95/5/0.1) (B) ACN/H2O/formic acid (95/5/0.1)	0–8 min: 50–100%B	8–9.7 min: 100%B	9.9–25 min: 50%B 25–33 min: 50–100%B 33–45 min: 100%B
Flowrate (μL/min)	200	200	150
Valve position	0	0	9.7–11 min: 1 11–16 min: 0 16–45 min: 1
MS scan event	MS/MS	MS/MS	MS/MS
Event 1			
Monitored compound	Dansylated E3	Dansylated E1	Dansylated 17β- and 17α-E2
Parent mass (*m/z*)	522	504	506
Isolation width (*m/z*)	1	1	1
Collision energy (%)	42	42	42
SRM range (*m/z*)	170.5–171.5	170.5–171.5	170.5–171.5
Event 2			
Monitored compound	Dansylated E3-d2	Dansylated E1-d4	Dansylated 17β-E2-d3Dansylated 17α-E2-d2
Parent mass (*m/z*)	524	508	508.5
Isolation width (*m/z*)	1	1	2
Collision energy (%)	42	42	42
SRM range (*m/z*)	170.5–171.5	170.5–171.5	170.5–171.5

3. Results and discussion

3.1. Method development

Although dansylation has been demonstrated to enhance the sensitivity during LC–MS in determination of estrogens, the inability to chromatographically resolve all four native estrogens in their dansylated form has been a significant limitation. This derivatization approach has been implemented in many recent publications to analyze estrogens in water [27] or different biological samples such as plasma, serum, or mouse brain [9–11,29,30]; however, only two dansylated estrogens, E1 and 17β-E2, were analyzed in most of the cases [9,10,30]. These studies featured separations using reversed phase stationary phases including C12 [9,10] or phenyl [30] bonded phases. In our experience, dansylated E3 always elutes ahead of the other dansylated estogens and can be easily separated. However, dansylated E1 and 17α-E2 are co-eluted when standard C18 (Fig. 3A) stationary phases are used (several different phases were tested; data not shown). In addition, their commercially available deuterated forms, E1-d4 and 17α-E2-d2, are isobaric, which makes quantitative analysis relative to isotopically labeled internal standards difficult in this situation. Toran-Allerand et al. [11] have reported the measurement of dansylated E1, 17α- and 17β-E2 using customized isotopically labeled internal standards, but the peaks of dansylated E1 and 17α-E2 were still unresolved. Although the detection limits were in the low pg/mL range in their work, the descriptions of specific values for detection limits for 17α- and 17β-E2 were a little ambiguous.

After screening several stationary phases consisting of C18, C6-phenyl, amide-C18, and modified cyclodextran phases, we determined that dansylated E1 and 17α-E2 could be resolved on the Polaris Amide-C18 column. Unfortunately, dansylated 17β- and 17α-E2 co-eluted (Fig. 3B) in this set-up, and since these compounds are also isobaric, the use of the Polaris Amide-C18 alone was not a viable option for simultaneous separation and quantification of all four estrogens. To address these problems, a heart-cutting 2D-LC arrangement was conceived, consisting of a binary pump, 3 columns, and a 2-position-6-port valve (Fig. 1). The Polaris Amide-C18 column was used as the first dimension and two Shim-pack XR-ODS columns (175 mm total length) were used as the second dimension. To obtain baseline resolution of the critical pair, two C18 columns connected in series were required.

Samples were initially injected onto the amide-C18 column by the autosampler with the valve in position 0. All three columns were initially equilibrated with at least five column volumes of 50% B. In the first segment, dansylated E3 and E1 were separated on Polaris Amide-C18 column by a linear gradient elution from 50% to 100% B and detected by tandem MS. Dansylated E3 was eluted first at approximately 94% B and dansylated E1 was eluted later at 100% B. Dansylated 17β- and 17α-E2 coeluted last. The critical pair was diverted to the Shim-pack XR-ODS columns by switching the valve to position 1 for a period of 1.7 min (segment 2). The diverted fraction was minimized to the smallest volume possible to limit the amount of high organic solvent mobile phase that was introduced into the second dimension, while simultaneously ensuring that any variation in retention for the critical pair could be accommodated. After the fraction was diverted, the valve was again switched to allow re-equilibration of the first dimension and eliminate the high organic content mobile phase (segment 3). After sufficient re-equilibration (5 min at 150 μL/min), all of the columns were again placed in line by switching the valve back to position 1. Dansylated 17β- and 17α-E2 were resolved with a linear gradient elution from 50% to 100% B (Fig. 3C). The analysis was carried out in positive ionization SRM MS/MS mode. Different events were used to isolate the parent ion masses (for each estrogen and its related internal standard) (Table 1) prior to fragmentation. Each parent ion was monitored for its transition to *m/z* 171, signifying the common loss of the desulfonated dansyl moiety from each of the dansylated analytes. After optimization, the LC–MS/MS method, including baseline resolution of dansylated 17β- and 17α-E2, was able to be completed in 45 min.

3.2. Method validation

The method was validated in CSCSF to determine the specificity, linearity, limit of detection (LOD), accuracy, precision, and extraction recovery. The specificity of the method was demonstrated by analyzing different pooled CSCSF samples. No interference was observed at the analyte retention and *m/z*. The cross-talk was examined by analyzing different samples with the addition of estrogen standards or deuterated internal standards. No cross-talk was detected between different MS events within a segment. The calibration range was established by analyzing standards ranging in concentration from 50 to 300 pg/mL for E3 and from 20 to 300 pg/mL for E1, 17β- and 17α-E2. The coefficient of correlation

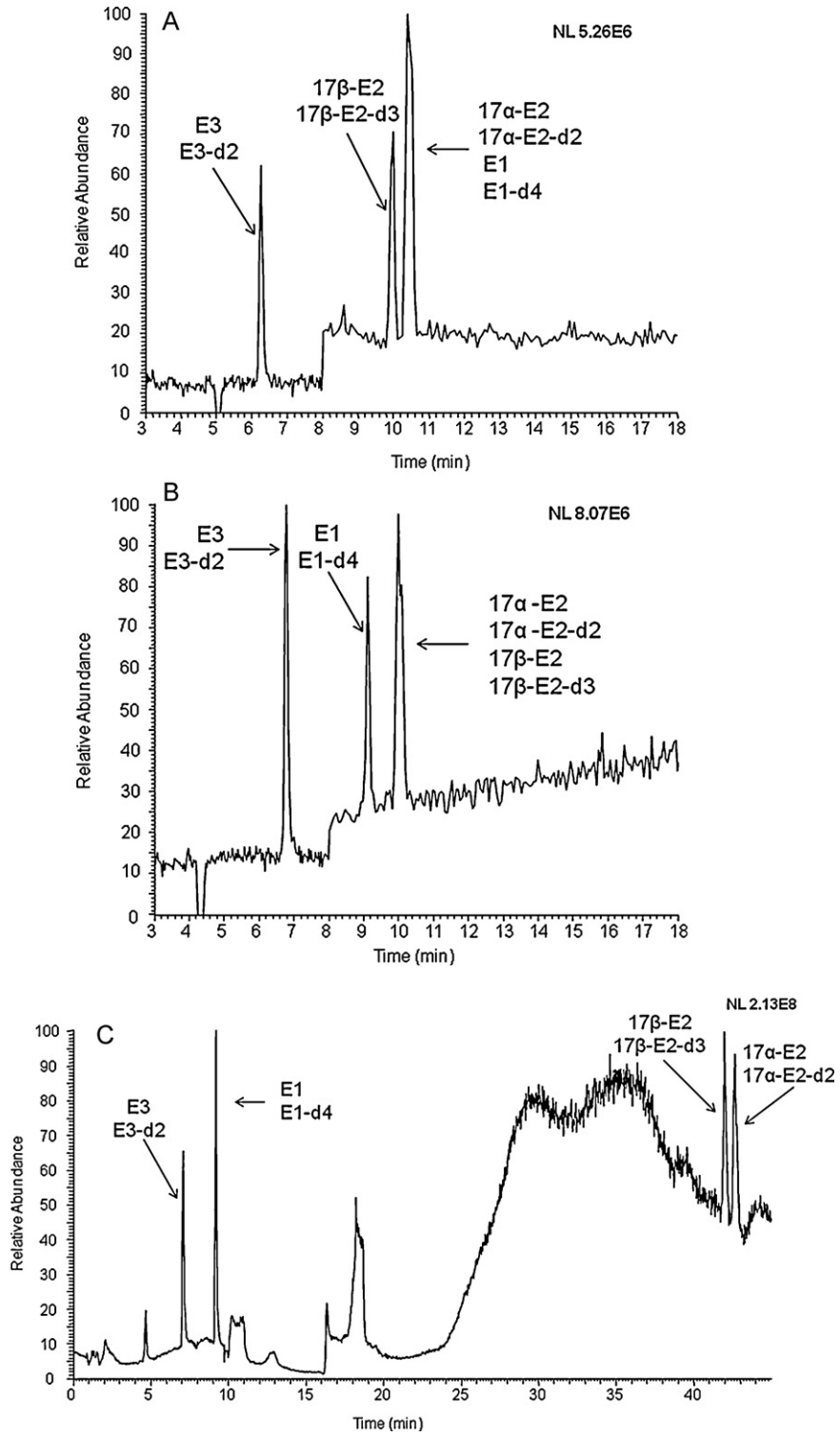

Fig. 3. Ion chromatograms of the four estrogen analysis using (A) Shim-pack XR-ODS column, (B) Polaris Amide-C18 column, and (C) heart-cutting 2D-LC-MS system (the base line variation from minute 16 to 40 was due to the valve switching and mobile phase gradient). The analyses were carried out in SIM mode according to the settings in different segments using 1 ng/mL of standard solution.

(R^2) in each case was determined to be greater than 0.996. The detection limits for E1, 17α-E2, 17β-E2, and E3 were 19, 35, 26, and 61 pg/mL, respectively. These detection limits refer to the use of maximum 1 mL of CSF per sample for processing and analysis. The higher LOD of E3 may be the result of coeluting interferences from the matrix, which slightly suppress its ionization efficiency. The target values for mean accuracy and precision of quality control samples at each concentration level were less than 21% of the expected concentration for E3 and E1 and less than 14% for 17α- and 17β-E2. An extraction recovery of 91–104% was determined by comparing the response signal before and after the extraction at low, medium, and high concentration level quality control samples.

Although the detection limits were higher than those from other reported methods [9–11,30], they can be improved using a more

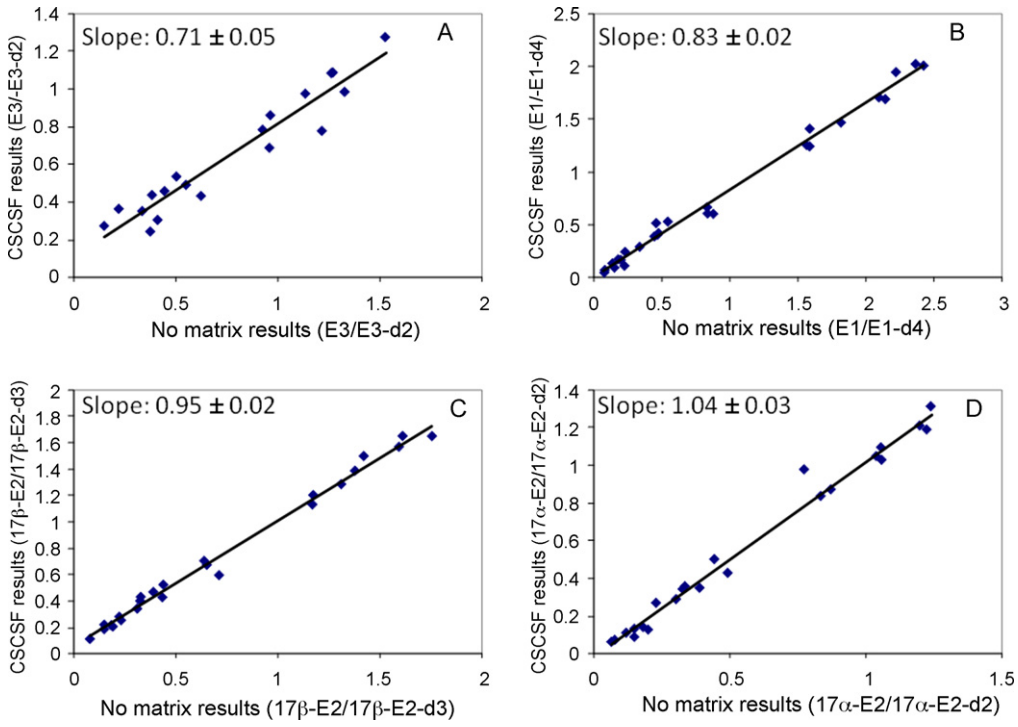

Fig. 4. Evaluation of matrix effects using regression lines for (A) E3, (B) E1, (C) 17β-E2, and (D) 17α-E2.

sensitive mass spectrometer. As indicated in the study of Diaz-Cruz [20], a triple-quadrupole mass spectrometer can provide five times to one order of magnitude lower detection limits than a single-quadrupole. A newer version of triple-quadrupole can also give better detection limits than older units. Kushnir et al. [30] have reported the limits of quantification of E1 and 17β-E2 in human serum as low as 1 pg/mL in positive ESI mode using dansyl chloride derivatization and 2D-LC–MS system equipped with an API 4000 triple quadrupole mass spectrometer (ABI-Sciex). However, similar limits of quantification were also achieved in negative ESI mode without derivatization using 1D-LC coupled to an API 5000 triple quadrupole mass spectrometer [34]. Our work has been performed on an ion trap instrument which is not ideal to obtain the most sensitive quantitative determinations. However, our configuration to reliably and reproducibly separate and quantify all four native estrogens in their derivatized form, and our focus on CSF as a new and important matrix for investigating neuroprotective effects of estrogens, are novel aspects. The method is also highly amenable to being improved in future work, with better-suited instrumentation, to achieve even lower detection limits.

3.3. Matrix effects

Since dansyl chloride was used in excess for the derivatization reaction, interference from other side reaction products was judged to be significant. The coefficient of variation for 10 replicate injections of a 1 ng/mL estrogen standard solution was less than 14% for the four estrogens without the biological matrix of CSCSF. Matrix effects were investigated by comparing the results of calibration performed in the absence of matrix with that from a CSCSF matrix, an accepted approach [35,36]. The results obtained from calibration in CSCSF matrix were normalized to those in no matrix to establish a regression line (Fig. 4). The experiment was performed three times on separate days. The slopes of the regression lines for the data were 0.71, 0.83, 0.95, and 1.04 for E3, E1, 17β- and 17α-E2, respectively, each with relative standard deviation ≤7%. In the ideal case, a slope with a value of unity in such plots indicates the absence of matrix effects. The deviation of the slope to a value less than unity indicates ion suppression, whereas a slope greater than unity indicates ion enhancement in the presence of the matrix. A significant matrix effect was observed for E3 and E1 (29% and 17% reduction in signal, respectively, in the presence of CSCSF), but was negligible for 17β- and 17α-E2. The absence of a matrix effect for 17β- and 17α-E2 can be attributed to their transfer and separation in the second dimension of the heart-cutting 2D-LC method. Late elution and 2D-separation of these estrogens are speculated to reduce the interference from other matrix compounds.

3.4. Application

The method was applied to measure endogenous estrogens in the CSF of ischemic trauma patients. CSF was collected from brain-injured patients with a surgically placed ventriculostomy. From these traumatic brain injury patients, approximately 10 mL of CSF was collected from the buretrol under sterile conditions. After collection of the CSF, the samples were immediately placed on ice and centrifuged at 4 °C for 10 min. The supernatant was snap frozen in the liquid nitrogen and the samples were stored at −80 °C until analyzed. Three pooled samples from different patients were separately collected and analyzed. The results are given in Table 2 and LC–MS/MS data collected for sample 1 is shown in Fig. 5. 17β-E2 was detected in a significant amount in two of the samples. No 17α-E2 was observed in any of the samples. E1 and E3 were present at low concentration or lower than LOD in one or more of the three samples tested. Further research is underway to measure estro-

Table 2
Estrogen content in samples of CSF from human ischemic trauma patients.

Samples	E3 (pg/mL)	E1 (pg/mL)	17β-E2 (pg/mL)	17α-E2 (pg/mL)
1	<LOD	61 ± 2[a]	184 ± 9	ND
2	ND	<LOD	ND	ND
3	ND	ND	208 ± 34	ND

[a] ≤LOQ.

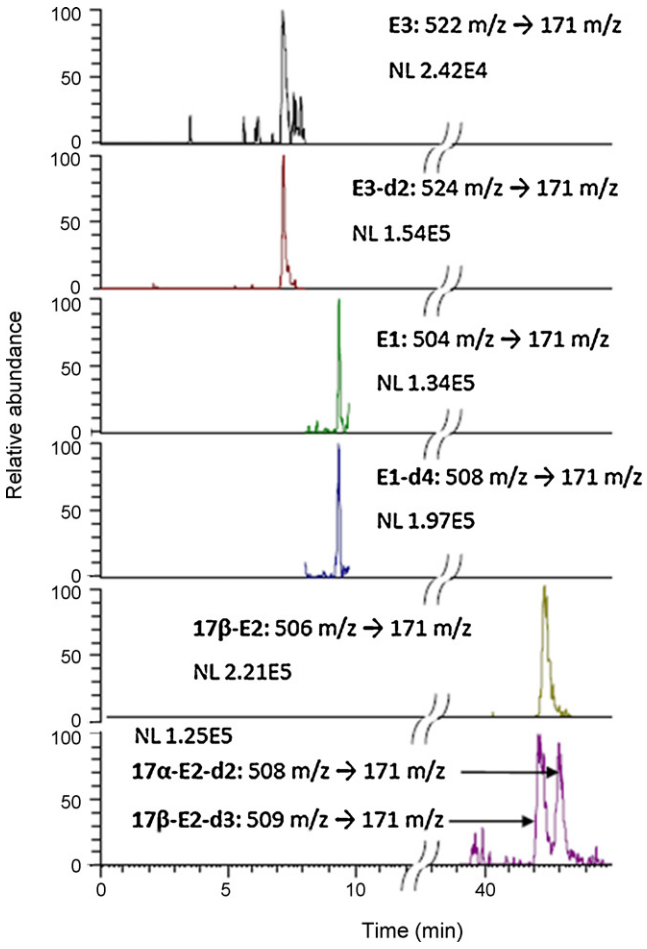

Fig. 5. Extracted ion chromatograms for the four dansylated estrogens and their internal standards in the CSF of an ischemic trauma patient (Sample 1 from Table 2).

gens in CSF of ischemic trauma patients in connection with a phase two clinical trial. Samples of patients who have been administered estrogens (as Premarin®) or a placebo intravenously will be quantitatively evaluated by the LC–MS/MS method to support trials at Parkland hospital in Dallas, TX.

4. Conclusions

A heart-cutting 2D LC–MS/MS system for simultaneous measurement of the native estrogen hormones in human CSF has been developed and validated. The system provides a sufficient resolution for the four estrogens to allow for their simultaneous quantitative determination at ultra-trace level concentrations. Although the run time for each trial is fairly long (45 min), this method is amenable for use with a standard binary gradient LC system hyphenated to tandem MS. Analysis time can be reduced to less than 20 min with the addition of another binary pump to eliminate the mid-run re-equilibration in the third segment of the method. Furthermore, lower detection limits are likely to be achieved with the use of a triple quadrupole or other similar mass spectrometer systems.

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