Stable isotope-coded quaternization for comparative quantification of estrogen metabolites by high-performance liquid chromatography–electrospray ionization mass spectrometry

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ABSTRACT

A fast and sensitive LC–ESI-MS method is described for the comparative quantification of 16 estrogen metabolites based on the derivatization of estrogens with a novel derivatizing reagent, N-methyl-nicotinic acid N-hydroxysuccinimide ester (C1-NA-NHS). The process introduces a quaternary amine to the analytes, making the analytes permanently charged regardless of the pH of the high-performance liquid chromatography (HPLC) mobile phase. This quaternization resulted in a highly efficient separation of 16 estrogen metabolites in 7 min at a detection level below 1 ng/mL. By using a deuterated derivatizing reagent (C1-d_3-NA-NHS), a complete set of deuterated standards was utilized and used as internal standards in a comparative quantification and recovery study, demonstrating acceptable results over a wide concentration range. A pooled breast cancer serum sample was analyzed using the described method, and 15 estrogens were detected in the range of 80–530 pg/mL.

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1. Introduction

Estrogens are part of a group of steroids that are known to stimulate secondary sexual characteristics, along with some other physiological effects such as growth and maturation of long bones. But estrogens may be involved in carcinogenesis as well. Over a century ago, endogenous estrogens were postulated to have a critical role in the development of breast cancer [1]. Evidence has accumulated from studies involving cell cultures, animal models, and epidemiology showing a strong linkage between exposure to estrogens and breast cancer risk [2,3]. Estrogens have also been found to be involved in colon cancer [4] and ovarian cancer, especially when connected to postmenopausal estrogen treatment [5,6].

Estrogens generally appear in tissues and biological fluids at low concentrations. When combined with the complexity of the matrix within which they are found, analysis of estrogens in biological samples is challenging. The recent review by Giese shows that immunoassays, high-performance liquid chromatography (HPLC) with electrochemical or mass spectrometric (MS) detection, and gas chromatography (GC) with electron capture detection (ECD) or MS detection are among the most widely used methods for estrogen analysis [7]. One complication is the structural diversity of estrogens (Fig. 1). Another is the possible conjugation with other species, such as DNA, sulfate and glucuronide [7]. The analytical methodology must deal with this complexity. Immunoassays, such as radioimmunoassay [8], are an important high-throughput tool for estrogen analysis, but fail to measure all the forms of estrogen in a single assay. Even when processed with a separation step, such as HPLC, some methods can only accommodate on a single estrogen [9–12]. Xu et al. developed several LC–MS methods for the simultaneous measurement of endogenous estrogens [13–16], eventually analyzing 15 different estrogens [14,15]. In these separations, multiple estrogens coeluted, and differentiation with mass spectrometry was required to provide resolution of these coeluted analytes.

Another issue that must be addressed is the large number of samples that have to be examined for a meaningful epidemiological analysis. Analytical methods must be relatively fast to meet the throughput needs of such studies. The widely used National Institute of Standard and Technology (NIST) LC–MS/MS method for estradiol requires a 30 min separation, with additional time for sample derivatization [11]. In comparison, a method for simultaneous determination of 15 estrogens developed by Xu et al. requires a 60 min separation time [15] and can be shortened to roughly 10 min with supercritical fluid chromatography [14]. Furthermore,

the concentration of estrogens in physiological samples can be very low, as pointed out earlier, causing analytical difficulties. The circulating estradiol-17β level in postmenopausal women is usually <73 pmol/L, and concentrations ≤18 pmol/L are common [17].

An ultrasensitive chemiluminescent immunoassay for estradiol was reported with detectability of 1.8 pmol/L [17]. Most commercially available estradiol immunoassays have detection limits between 73 and 183 pmol/L [18]. Selected Reaction Monitoring (SRM) in MS-based technique is also a very sensitive analytical tool. With this technique, the limit of detection for estradiol after derivatization was decreased to 1 ng/L (3.7 pmol/L) [11].

Still another challenging aspect is the requirement for derivatization as a tool to improve the detection limit. Electrospray ionization (ESI) is a popular ionization source in LC–MS, but has the shortcoming that sensitivity is analyte-dependent. The structure of estrogens suggests they would not readily protonate during ESI. ESI-MS detection is often facilitated by derivatization with easily protonated reagents, such as p-toluenesulfonhydrazide [13,16] or dansyl chloride [11,14,15]. In contrast, derivatization with an electron-capturing moiety such as o-pentafluorobenzyl [19] or 4-nitrobenzenesulfonyl [20] substantially enhances detection in Electron-Capturing Atmospheric Pressure Chemical Ionization (ECAPCI) mass spectrometry. Similarly, electron capture derivatives have been used in gas chromatography with Electron Capture Detection (GC-ECD) [21] and Electron Capture Mass Spectrometry (GC–EC-MS) [22,23]. These methods usually utilize Electron Impact (EI) ionization and silylating derivatization to increase the volatility and stability of estrogens [24–28].

The demand for high-throughput analysis, such as in the case of epidemiology studies, as mentioned earlier, has resulted in the use of stable isotope-coded internal standards [16,24,27]. A limitation of stable isotope coding is that appropriate heavy isotope labeled standards may not be commercially available. In this case, an available isotope-coded internal standard is used as a structural analogue for the quantification of other analytes whose isotope-coded versions are unavailable. Quantification can suffer when the separation behavior and relative molar response of the instrumentation for the standard and analytes are very different.

The challenges noted above were addressed in these studies through the development of a comparative HPLC–ESI-MS method centering on a novel stable isotope-coding agent that facilitates ionization and quantification, in addition to accelerating HPLC separation. Stable isotope-based internal standard quantification of 16 unique estrogen metabolites was achieved in less than 7 min at a detection level below 1 ng/mL.

2. Materials and methods

2.1. Chemicals and reagents

Estrogen standards (Fig. 1) were purchased from Steraloids Inc. (Newport, RI). Iodomethane, dansyl chloride, β-glucuronidase, anhydrous acetonitrile (ACN), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), nicotinic acid dicyclohexylcarbodiimide (DCC), triethylamine (TEA), N-hydroxysuccinimide, and human serum were obtained from Sigma (St. Louis, MO). HPLC grade ACN, acetone and formic acid were obtained from Mallinkrodt Baker (Phillipsburg, NJ). Iodomethane-d_3 was a product of Cambridge Isotope laboratories (Andover, MA). Double-deionized water was produced by a Millipore Milli-Q gradient A10 system (Bedford, MA).
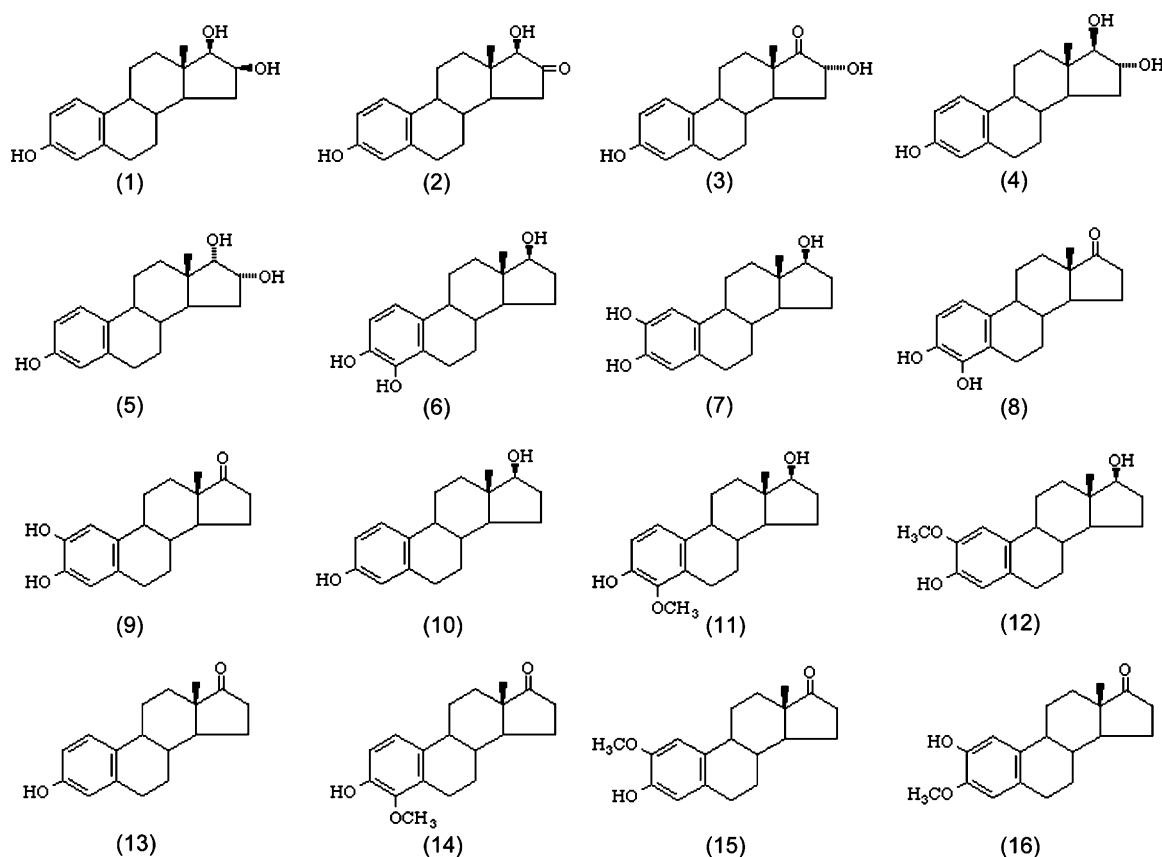

Fig. 1. Structures of the 16 estrogens studied in this work. Compound: (**1**) 16-epiestriol; (**2**) 16-keto-17β-estradiol; (**3**) 16α-hydroxyestrone; (**4**) estriol; (**5**) 17-epiestriol; (**6**) 4-hydroxyestradiol; (**7**) 4-hydroxyestrone; (**8**) 2-hydroxyestradiol; (**9**) 2-hydroxyestrone; (**10**) 17β-estradiol; (**11**) 4-methoxyestradiol; (**12**) 2-methoxyestradiol; (**13**) estrone; (**14**) 4-methoxyestrone; (**15**) 2-methoxyestrone; (**16**) 2-hydroxyestrone-3-methyl ether.
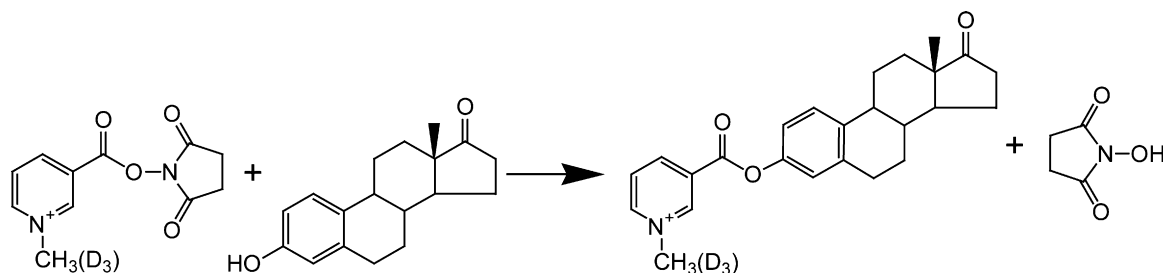
Fig. 2. Representative derivatization reaction of estrogens.

Each estrogen standard was prepared in acetonitrile at 0.2 mg/mL as a stock solution. Standard solutions were prepared by mixing equal amounts of corresponding stock solutions and performing serial dilutions with ACN to the desired concentration.

Derivatizing reagents, N-methyl-nicotinic acid N-hydroxysuccinimide ester (C1-NA-NHS) and N-methyl-d_3-nicotinic acid N-hydroxysuccinimide ester (C1-d_3-NA-NHS), were synthesized as described by Yang et al. [29]. Briefly, nicotinic acid was first esterified by N-hydroxysuccinimide through the activation of DCC to form nicotinic acid N-hydroxysuccinimide ester, which was further quaternized by iodomethane or iodomethane-d_3.

2.2. Derivatization of estrogens

A typical derivatization procedure was as follows: 10 μL of standard solution (12.5 μg/mL each) was added into 37 μL of 50 mg/mL derivatizing reagent, C1-NA-NHS, prepared in dimethyl sulfoxide just before use, followed by the addition of 1 μL TEA. After vortexing the mixture for 30 s, 2 μL of formic acid was added to stop the reaction. For comparative quantification, another aliquot of working solution was simultaneously derivatized by C1-d_3-NA-NHS. These two derivatized solutions were mixed in specific ratios for the analysis. A representative derivatization reaction is shown in Fig. 2.

For comparison, estrogens were also derivatized with dansyl chloride [11]. In this case, 10 μL of standard solution (12.5 μg/mL each) was added into 37 μL of 50 mg/mL dansyl chloride in acetone/0.1 M sodium bicarbonate buffer, pH 10.4 (50:50, v/v), incubated for 5 min at 60 °C and kept at 4 °C for analysis.

2.3. Recovery studies

Two 0.5 mL aliquots of human serum labeled as C (control) and S (spiked) were placed into two 15 mL tubes containing 0.5 mL 0.2 M acetate buffer (pH 4.2), 1 mg of ascorbic acid and 5 μL β-glucuronidase (to release estrogens from glucuronide and sulfate conjugates). Then 8 μL of ACN and 8 μL of standard solution (125 ng/mL each) were added into samples C and S, respectively. After overnight incubation at 37 °C, estrogens in samples C and S were extracted by adding 5 mL of dichloromethane and vortexing the samples for 20 min. The lower layers of samples C and S were removed, dried under nitrogen (~30 min), and derivatized by adding 10 μL of ACN, 37 μL of freshly prepared C1-d_3-NA-NHS (50 mg/mL), 1 μL of TEA, and 2 μL of formic acid after vortexing for 30 s. Ten microliters of standard solutions (500 ng/mL each) were also derivatized by adding 37 μL of freshly prepared C1-NA-NHS (50 mg/mL), followed by 1 μL of TEA and 2 μL of formic acid after 30 s vortexing. A 10 μL of this "light" derivatized standard solution was added to sample S for the recovery studies. Both samples were analyzed by LC–ESI-MS as described below. Recovery was calculated in sample S based on the extracted ion chromatographic peak areas for the pair of deuterated (heavy) and non-deuterated (light) derivatives of estrogens corresponding to their recovered and original spiked amounts, respectively.

2.4. Preparation of breast cancer serum

Two milliliters of serum from patients with breast cancer were placed into a 15 mL tube containing 0.5 mL 0.2 M acetate buffer (pH 4.2), 1 mg of ascorbic acid and 5 μL β-glucuronidase solution. After overnight incubation at 37 °C, the sample was extracted by vortexing for 20 min with 5 mL of dichloromethane. The lower layer was removed, dried under nitrogen, and derivatized by adding 10 μL of ACN, 37 μL of freshly prepared C1-NA-NHS in DMSO (50 mg/mL) and 1 μL of TEA followed by 30 s vortexing. The reaction was stopped with 2 μL of formic acid. Ten microliters of standard solutions (500 ng/mL each) were also derivatized by adding 37 μL of freshly prepared C1-d_3-NA-NHS (50 mg/mL), and 1 μL of TEA followed by vortexing for 30 s and addition of 2 μL of formic acid to stop reaction. Ten microliters of "heavy" labeled standard solution were mixed with 10 μL of "light" labeled human cancer sample solution for the comparative quantification. The mixture was analyzed by the LC–ESI-MS method described below. The concentration factor, 20, was applied for the estimation of estrogen concentration in the original serum. The mixture was analyzed by the LC–ESI-MS method described below. Estrogen concentration (C_E) in serum was estimated according to equation:

$$C_E \, (\text{ng/mL}) = \frac{I_l}{I_h} \times 500 \, (\text{ng/mL}) \times \frac{1}{20}$$

where I_h and I_l represent peak intensities of heavy-labeled standard and light labeled estrogen in serum, 500 ng/mL is concentration of a standard and 20 is concentration factor of serum sample.

2.5. LC–MS

HPLC experiments were performed on an Agilent XDB-C18 column (2.1 mm × 50 mm, 1.8 μm particle size) and an Agilent 1100 Series LC (Agilent Technologies, Wilmington, Delaware). For negative APCI-MS, the mobile phase A consisted of 10 mmol/L ammonium acetate (pH 6.8) containing 5% ACN and mobile phase B was 10 mmol/L ammonium acetate containing 95% ACN. For positive APCI-MS and ESI-MS, the mobile phase A was composed of 5% ACN, 94.95% water and 0.05% formic acid and B was 95% ACN, 4.95% water and 0.05% formic acid. Column elution was achieved in a 30 min linear gradient from 0 to 100% B, at a flow rate of 0.4 mL/min. The injection volume was 5 μL.

MS analysis was carried out on Unique® LC–TOF MS with a high flow ESI and APCI Sources (LECO Corporation, St. Joseph, MI). Operating parameters for positive ESI were as follows: electrospray voltage, 4900 V; desolvation temperature, 370 °C; nebulizer pressure, 400 kPa; interface temperature, 100 °C; nozzle voltage, 230 V; skimmer voltage, 75 V; quad RF voltage, 250 V; quad high

voltage, 76 V; quad low voltage, 32 V; quad exit voltage, 32 V; desolvation gas, 6880 mL/min. The main parameters for negative APCI were: desolvation temperature, 300 °C; nebulizer pressure, 350 kPa; interface temperature, 100 °C; corona current, 5 μA; nozzle voltage, −170 V; skimmer voltage, −57 V; quad RF voltage, 180 V; quad high voltage, −54 V; quad low voltage, −45 V; quad exit voltage, −16 V. Operating conditions for positive APCI were: desolvation temperature, 300 °C; nebulizer pressure, 350 kPa; interface temperature, 100 °C; corona current, 5 μA; nozzle voltage, 170 V; skimmer voltage, 70 V; quad RF voltage, 225 V; quad high voltage, 60 V; quad low voltage, 32 V; quad exit voltage, 29 V. All these parameters were optimized for intensity and resolution using the Agilent tuning solution.

3. Results and discussion

3.1. LC–APCI-MS of underivatized estrogens

Atmospheric pressure chemical ionization (APCI) is being used increasingly for the detection of a wide range of biological substances at low concentrations. In the case of steroids, cholesterols [30], phytoestrol [31], aldosterone [10], and progestogens [32] have been analyzed using APCI-MS or tandem MS in either the positive or negative modes. This suggests that APCI-MS might be useful in the analysis of the estrogens listed in Fig. 1.

Negative APCI-MS was used with 10 mM ammonium acetate (pH 6.3) as the elution buffer to assist the ionization of analytes. From the extracted [M−H]− ion chromatograms for individual analyte injections (data not shown), all of the analytes, except 6 and 7 (which have the same retention time and m/z values) could be separated without deliberate optimization of the separation. Elution order clearly reflected analyte hydrophobicity. Table 1 lists the limits of detection (LOD) at a signal-to-noise ratio of 3 in [M−H]− selected ion monitoring mode. Except for the four catechol estrogens, the LODs were not sufficiently low to be useful for the analysis of biological samples. [M−H]− ions were the most abundant in mass spectra but many other fragment and adduct ions are formed as well (Table 1). Comparatively, catechol estrogens produce fewer fragments, giving relatively higher detection sensitivity. The negative-ion sensitivity problem with APCI-MS has been noted with ethinylestradiol as well (>50 ng/mL) [9].

Similar results were observed using 0.05% formic acid in the mobile phase, and positive APCI-MS mode. For example, the spectrum of estradiol chromatographic peaks showed a large number of ions that have not been previously reported: [M+H]+, [M−H2O+H]+, or [M+CH3CN]+ [32]. These discrepancies indicate that formation of ions in an APCI ion source is instrument dependent. The broad distribution of fragment ions reduces detection sensitivity, diminishing the utility of positive-ion APCI for the quantification of estrogens in biological samples.

3.2. Derivatization of estrogens

As seen in Fig. 1, those endogenous estrogen metabolites [7,15] possess the aromatic steroid core structure with phenol and alcohol hydroxyl groups. These hydroxyl groups are very weakly acidic with ionization of the phenol hydroxyl group being greater than the alcohol. At the pH of the HPLC mobile phase, ionization of ethinylestradiol is theoretically less than 0.001% [9]. Because ionization in the gas phase is directly related to ionization in solution [33], the limited ionization of estrogens in solution implies poor ionization efficiency in ESI-MS. One way to enhance ESI sensitivity is to directly introduce a quaternary amine (permanent positive charge) into analytes. This strategy has been applied to amino acids and peptides [29,34,35], alcohols, phenols and thiols [36,37], sugars [38,39], and carboxylic acids [40]. In a previous study from our group, ESI-MS response to tryptophan was enhanced roughly 26-fold after derivatization with the quaternary amine contain reagent C1-NA-NHS compared to the neutral benzoate derivative [29]. In the terms of reactivity, the phenol hydroxyl group in the estrogens is similar to the amine group in amino acids, suggesting that the amino acid derivatization procedure can be used, with few modifications, for estrogen labeling as well. Problems with the solubility of analytes were addressed by changing to a non-aqueous reaction system in which the estrogens are soluble. Small amounts of water (<10% in volume) in the reaction did not adversely affect the reaction (data not shown). The second modification was implemented in order to stabilize the derivatives by neutralizing the reaction with formic acid after the reaction was completed. Although the reaction is initiated in basic medium, the derivatives are not stable at these conditions. Similar problems have been observed in the case of O-acylation of tyrosine with C1-NA-NHS [29]. In neutral solutions, the derivatives are stable for at least 1 week at 4 °C.

Table 1
Detection limits of underivatized estrogens with LC-negative APCI-MS

Peak number	Estrogens	m/z	Average LOD (μg/mL, n = 5)
1	16-Epiestriol	259.05 (18%), 287.10 (100%)[a], 288 (38%), 317 (10%)	6.60
2	16-Keto-17β-estradiol	285.11 (100%), 299.11 (60%)	4.20
3	16α-Hydroxyestrone	279.09 (10%), 285.12 (100%), 286.08 (30%), 298.98 (10%), 301.14 (15%)	4.80
4	Estriol	259.02 (35%), 285.11 (40%), 287.13 (100%), 301.13 (50%)	15.70
5	17-Epiestriol	259.03 (28%), 261.06 (38%), 285.12 (40%), 287.14 (100%), 301.09 (32%), 303.00 (28%), 305.08 (26%)	11.7
6	4-Hydroxyestradiol	283.06 (40%), 287.14 (100%)	0.03
7	4-Hydroxyestrone	285.11 (100%)	0.05
8	2-Hydroxyestradiol	285.07 (40%), 287.10 (100%), 288.11 (50%)	0.014
9	2-Hydroxyestrone	285.11 (100%)	0.014
10	17β-Estradiol	263 (60%), 271 (100%)	0.143
11	4-Methoxyestradiol	259.03 (60%), 275.00 (100%), 286.10 (70%), 301.16 (75%)	19.2
12	2-Methoxyestradiol	246.06 (50%), 283.07 (90%), 285.06 (80%), 287.01 (100%), 317.11 (90%), 331.13 (50%)	61.5
13	Estrone	259.04 (18%), 269.10 (100%), 285.09 (18%)	37
14	4-Methoxyestrone	248.97 (65%), 261.99 (85%), 283.03 (88%), 299.13 (100%)	8.7
15	2-Methoxyestrone	246.02 (65%), 248.07 (55%), 274.00 (72(%), 284.07 (90%), 299.01 (100%), 331.17 (40%)	32.4
16	2-Hydroxyestrone-3-methyl ether	246.00 (68%), 274.03 (60%), 283.05 (50%), 284.10 (85%), 285.12 (66%), 289.05 (79%), 299.01 (100%), 315.10 (52%)	17.8

[a] Underlined ions were used for detection limit determination.

Under optimized conditions, C1-NA-NHS only reacted with phenol hydroxyl groups, not with alcohol hydroxyls. This property is significant because it increases the analytical selectivity of estrogens in complex biological matrices. Although alcohol hydroxyl groups are partially derivatized at elevated temperature, this is avoided by performing the reaction at room temperature.

3.3. LC–ESI-MS of estrogen derivatives

The estrogens listed in Fig. 1 are very similar in structure, sharing an aromatic steroid backbone, hydroxyl or methoxyl groups at similar positions, and several pairs of positional isomers or enantiomers. An extracted ion chromatogram (Fig. 3A) of the derivatized estrogens shows that except for components 9 and 10, all the other estrogens are well resolved within 7 min. The mass difference between component 9 (2-hydroxyestrone) and component 10 (7β-estradiol) is, however, 14 amu and mass spectrometry easily differentiates between the two. Compared to the separation of underivatized estrogens with ammonium acetate mobile phase, these results suggest that derivatization increased the chromatographic selectivity. Presence of the quaternary amine derivatizing tag on the aromatic ring of estrogens diminishes interaction of that portion of the molecule with the C18 stationary phase. Perhaps this increases interaction with asymmetric positions in the B, C, and D rings of estrogens with the column. It is interesting that derivatization did not change the elution order.

Comparing the reversed phase chromatographic separation of C1-NA-NHS derivatives of estrogens (Fig. 3A) with dansyl derivatized estrogens (Fig. 3B) under identical conditions, significant differences were observed. First, the C1-NA-estrogens eluted much faster than dansyl-estrogens. The total separation time was shortened from roughly 25 to 7 min. This is because the dansyl moiety is more hydrophobic than the quaternary amine-containing C1-NA-NHS group [11,15]. Second, C1-NA-NHS derivatization improved peak shape and increased the separation efficiency. Only one pair of C1-NA-estrogens was not resolved (Fig. 3A), while multiple dansyl-estrogens coeluted (Fig. 3B). This is probably due to the hydrophobic dansyl moiety dominating the interaction of the derivatives with the C18 phase, masking the structural differences of the estrogens.

Differences between the ESI-MS detection sensitivity with C1-NA-estrogens and dansyl-estrogens were small (Fig. 3A and B) except for 2-hydroxyestrone (component 9). A unique characteristic of C1-NA-estrogen spectra was the presence of a single intense quaternary amine molecular ion with no fragmentation or formation of adduct ions (as will be seen in Fig. 4B). Consequently, maximum intensity was gained in selected ion monitoring and quantification was enhanced. This will be advantageous when collision-induced dissociation of the parent ion is used to increase detection selectivity. The m/z values of the derivatives summarized in Table 2 were used in constructing extracted ion chromatograms and determining detection limits. Derivatization decreased the detection limit by one to two orders of magnitude compared with the published detection limits for underivatized estrogens using ESI-MS (e.g. 10 ng/mL versus 0.44 ng/mL for 17β-estradiol, and 5 ng/mL versus 0.36 ng/mL for estrone—Table 2).

3.4. Comparative quantification through isotope labeling

Stable isotope-coded synthetic standards are frequently used in quantitative analysis. Standards identical in structure to analytes of interest behave in analytical systems like the analytes themselves, except for the mass of the standards. When added in known concentrations to a sample, they serve as an internal standard that compensates for inter-analysis variations in sample manipulation. A limitation of this method is that in the analysis of large numbers of metabolites, isotopically coded standards must be synthesized for each molecule. It has been shown in proteomics that one way to deal with this problem is to label large number of peptides through derivatization of primary amines with stable isotope-coded labeling agents. Peptides in one sample are globally coded with a single isotope of the coding agent (light form) while those of a second sample are globally coded with a second isotope (heavy form) [29,40]. After the two samples are mixed, the relative amount of isotopically coded peptides can be determined in a single analysis [41].
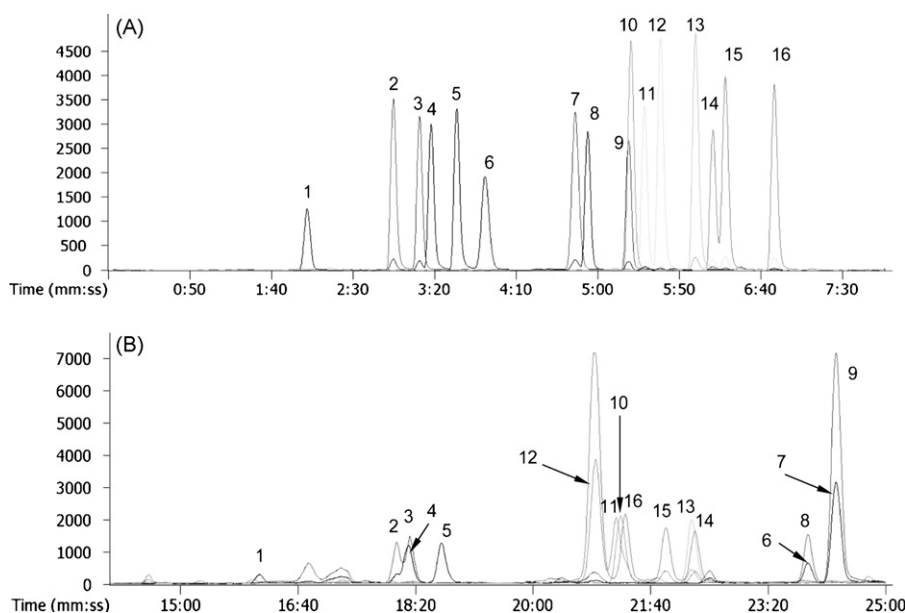

Fig. 3. (A) Extracted ion chromatograms of C1-NA-NHS derivatives of estrogens at 6.25 μg/mL, and (B) extracted ion chromatograms of dansyl-chloride derivatives of estrogens at same concentration by positive ESI-MS. The experimental conditions are described in the text. The peak numbers correspond to the structure numbers in Fig. 1.
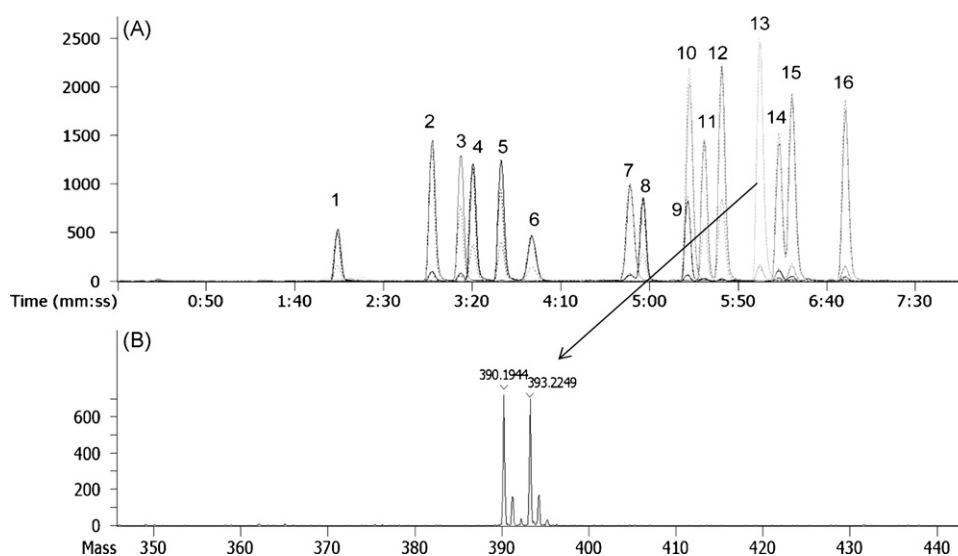
Fig. 4. (A) Extracted ion chromatograms of C1-NA-NHS (solid lines) and C1-NA-NHS-d_3 (dashed lines) derivatives of estrogens and (B) the corresponding spectra of C1-NA-NHS and C1-NA-NHS-d_3 of estrone. The LC–MS conditions were the same as those in Fig. 3.

A similar approach was applied to the analysis of estrogens in this work.

A deuterated version of C1-NA-NHS (C1-d_3-NA-NHS) was synthesized and used for derivatization of a known amount of estrogen, resulting in a set of heavy-labeled estrogen internal standards. It is desirable for isotope-coded internal standards to coelute with the non-deuterated analyte derivatives to minimize quantification errors. A 1:1 mixture of C1-NA-estrogens and C1-d_3-NA-estrogens was examined to determine the degree of analytical similarity. Extracted ion chromatograms of all the derivatives in the mixture and a representative MS spectrum for a single chromatographic peak are shown in Fig. 4A and B, respectively. The two sets of peaks completely overlapped chromatographically. Doublet clusters of ions separated by 3 amu were observed in mass spectra of nearly the same peak intensity. When used in comparative quantification, the stable isotope-coded internal standard method was found to be linear over the concentration ratio ranging from 1:1 to 1:40 for all the estrogens (Table 2) by measuring concentration ratio points at 1:1, 1:5, 1:10, 1:15, 1:20, 1:20, 1:25, 1:30, 1:35, 1:40, 1:50, 1:100.

3.5. Analysis of breast cancer serum

Recovery was studied to examine the extraction efficiency of estrogens from blood samples. Aliquot of pooled human serum (Sigma) was extracted, derivatized and analyzed by procedures described in Section 2. No chromatographic peaks corresponding to ion masses of the 16 estrogen standards were found (using extracted ion chromatograms), indicating that concentrations of estrogens in the pooled serum are under detection limits and serum can be used as a blank for this study. To determine recovery, known amounts of estrogen standards were added to the serum, and the new sample carried through the extraction, drying, and C1-d_3-NA-NHS (heavy) derivatization process. Another aliquot of the standards was directly derivatized with C1-NA-NHS reagent (light form) and added to "heavy" labeled serum. Recovery was determined by comparing the extracted chromatographic peak area of a deuterated (heavy) and non-deuterated (light) derivatives and showed that except for 16α-hydroxyestrone (42.0 ± 0.53% (mean ± S.D., n = 3)) and 17-epiestriol (73.0 ± 0.46% (mean ± S.D., n = 3)), extraction efficiency was >95%. No substances in the human

Table 2
Linearity and dynamical range for comparative quantification and detection limit

Peak number	Derivatized estrogens	m/z		Linearity[a]	Average LOD [ng/mL (pg/injection), n = 5]
		d_3 labeled	Non-d_3 labeled		
1	16-Epiestriol	411.2911	408.2675	$y = 0.9901x + 0.032$; $r^2 = 0.9925$	2.34 (11.71)
2	16-Keto-17β-estradiol	409.2611	406.2359	$y = 0.9243x + 0.013$; $r^2 = 0.9953$	0.39 (1.99)
3	16α-Hydroxyestrone	409.2714	406.2373	$y = 1.1043x + 0.010$; $r^2 = 0.9989$	0.62 (3.12)
4	Estriol	411.2944	408.2596	$y = 0.8445x + 0.052$; $r^2 = 0.9969$	0.69 (3.47)
5	17-Epiestriol	411.2924	408.2642	$y = 0.8582x + 0.044$; $r^2 = 0.9957$	0.46 (2.34)
6	4-Hydroxyestradiol	411.2786	408.255	$y = 0.8376x + 0.047$; $r^2 = 0.9952$	0.75 (3.75)
7	4-Hydroxyestrone	409.2586	406.2433	$y = 0.8411x + 0.060$; $r^2 = 0.9885$	0.56 (2.84)
8	2-Hydroxyestradiol	411.2882	408.258	$y = 1.1139x + 0.019$; $r^2 = 0.9966$	0.69 (3.47)
9	2-Hydroxyestrone	409.2812	406.2363	$y = 0.8512x + 0.054$; $r^2 = 0.9989$	0.75 (3.75)
10	17β-Estradiol	395.2286	392.1844	$y = 0.8421x + 0.016$; $r^2 = 0.9988$	0.44 (2.23)
11	4-Methoxyestradiol	425.3775	422.3347	$y = 0.8921x + 0.033$; $r^2 = 0.9987$	0.48 (2.40)
12	2-Methoxyestradiol	425.3782	422.3412	$y = 0.8656x + 0.041$; $r^2 = 0.9988$	0.37 (1.87)
13	Estrone	393.2098	390.1731	$y = 0.8797x + 0.039$; $r^2 = 0.9928$	0.36 (1.80)
14	4-Methoxyestrone	423.3415	420.3153	$y = 0.8856x + 0.027$; $r^2 = 0.9986$	0.62 (3.12)
15	2-Methoxyestrone	423.3394	420.3125	$y = 0.8934x + 0.021$; $r^2 = 0.9980$	0.46 (2.34)
16	2-Hydroxyestrone-3-methyl ether	423.3481	420.315	$y = 0.9089x + 0.029$; $r^2 = 0.9989$	0.62 (3.12)

[a] x = concentration ratio of non-d_3-labeled to d_3-labeled analytes; y = average extracted ion chromatographic peak intensity ratio of these pair of analytes. Linearity range for all is 1:1–1:40.
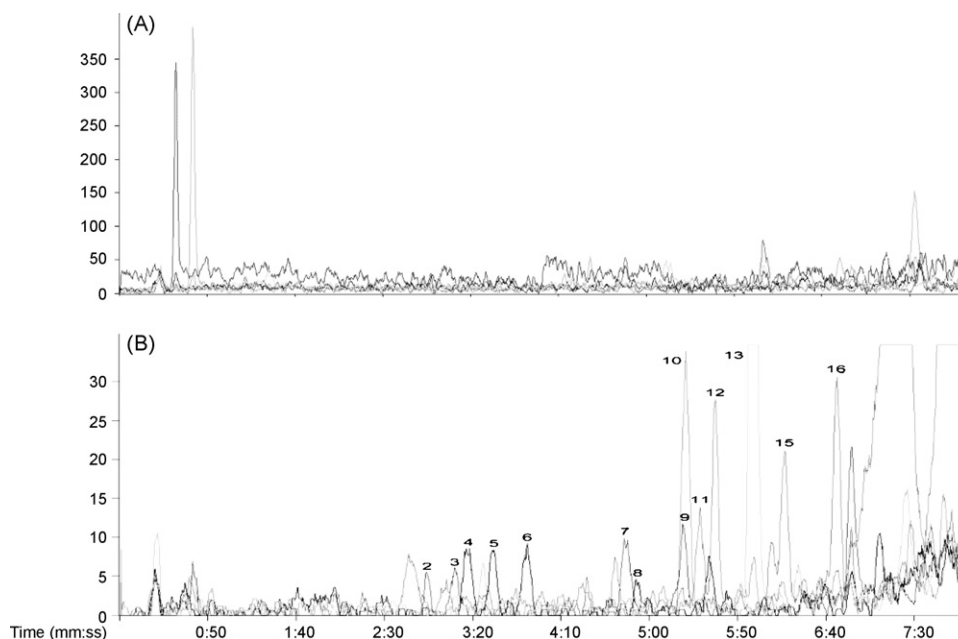
Fig. 5. Extracted ion chromatograms of C1-NA-NHS derivatized (A) pooled healthy human serum and (B) pooled breast cancer serum. The LC–MS conditions were the same as those in Fig. 4 except the injection volume was 15 μL.

serum that interfere with the analysis were found. Understanding the lower recovery for 16α-hydroxyestrone and 17-epiestriol is not straightforward. Based on the current recovery study protocol, the extraction and drying steps, rather than derivatization reaction, affect the recovery measurement. The use of the same mixed standard solution for spiking serum after derivatization with C1-d_3-NA-NHS, and for direct derivatization with C1-NA-NHS is a possible reason for the additional sample error. We speculate that these two estrogens are less stable or less extractable than the others.

This method was used to determine estrogen levels in serum from breast cancer patients, where estrogen concentrations are known to be increased. To analyze breast cancer serum, 2.0 mL of pooled cancer serum and 15 μL injection volume were used. Fig. 5 shows the profile of estrogens in this sample. Except for 16-epiestriol, the other 15 estrogens in the list were identified. Concentrations of estrogens were estimated based on the comparative quantitation protocol. For most of estrogens, the concentrations were in the range of 80–530 pg/mL. An exception is estrone, having a concentration of 1.2 ng/mL, which is much higher than the others. This is reasonable, considering it is the major form of estrogen. Accurate quantification was not attempted because the analyte concentrations in this sample are just slightly over the detection limits.

4. Conclusions

A highly sensitive LC–MS analysis of estrogens at the level needed in life science research highlights a need for chemical derivatization. A novel derivatization reagent introduced in this work, C1-NA-NHS, and the derivatization method benefit LC–ESI-MS analysis of estrogens in several ways: (1) increase detection sensitivity in the positive-ion mode of ESI-MS by one to two orders of magnitude, comparing to underivatized estrogens; (2) reduce hydrophobicity, retention time, and band spreading in reverse phase chromatography, and hence provide a higher analysis speed and higher peak resolution than existing methods; (3) make comparative quantitation and recovery studies more efficient, by allowing multiple sample (or standard) analysis to be performed in a single process by combining the use of isotopic versions of C1-d_3-NA-NHS. While the application of this method to breast cancer serum requires a relative large sample volume (2 mL serum), it could be expected that the combination of this derivatization with a triple quadrupole mass spectrometer will give a more effective approach for detecting cancer-related estrogens.

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