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Simultaneously Determination of Fifteen Estrogens and Metabolites in Human Serum by an Efficient Liquid Chromatography-Tandem Mass Spectrometry Method

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Abstract

Determination of female hormones and their metabolites in human serum is one of the most critical steps in human physiological and pathological diagnosis, such as in risk assessment for certain cancers. There are a number of concerns on sample derivatization, method specificity, sensitivity, separation efficiency and long elution time, although a large number of methods using Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) for determination of estrogens and metabolites have been published before. We successfully developed and validated an efficient Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method for determination of fifteen estrogens and metabolites in human serum. The estrogens and metabolites were derivatized with dansyl chloride and analyzed by LC-MS/MS using positive Electrospray Ionization (ESI+) and a Multiple Reaction Monitoring (MRM) mode. A Phenomenex Synergi Hydro-RP 2.5 µm 80 Å column (100 × 2.0 mm) at 60°C was eluted with mobile phases consisting of acetonitrile, methanol, water and formic acid at a flow rate of 0.4 mL/min. All of the fifteen dansyl derivatives of estrogen and metabolites were separated by LC with retention time differences between peaks ≥ 0.2 min. The recoveries of the fifteen estrogens and metabolites from hydrolysis and derivatization were in a range of 74.4-95.6% at 30-801 pg/mL level. The interday accuracies of the dansyl derivatives were in a range of 91.7-109.6% with a precision of 2.7-12.6%RSD. The LC-MS/MS system had linear responses to these derivatives within the calibration range of 12-10980 pg/mL with regression coefficient r2 ≥ 0.9934. The Limits of Quantification (LOQ) of these derivatives were in a range of 5.3-71.1 pg/mL in human serum. In comparison to a typical published method, our method provided a more efficient LC separation using only one-third of the elution time, and a similar or slightly better sensitivity.

Keywords: LC-MS/MS; Estrogens and metabolites; Derivatization; Method development and validation

Abbreviations: GC-MS: Gas Chromatography-Mass Spectrometry; GC-MS/MS: Gas Chromatography- Tandem Mass Spectrometry; LC-MS: Liquid Chromatography-Mass Spectrometry; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; HPLC/ECD: Liquid Chromatography with Electrochemical Detection; MRM: Multiple Reaction Monitoring; ESI^+: Positive Electrospray Ionization; E_2: Estradiol; E_3: Estriol; E_1: Estrone; 16-epiE_3: 16-Epiestriol; 17-epiE_3: 17-Epiestriol; 16α-OHE_1: 6α-Hydroxyestrone; 16-ketoE_2: 16-Ketoestradiol; 2-OHE_2: 2-Hydroxyestradiol; 2-OHE_1: 2-Hydroxyestrone; 4-OHE_1: 4-Hydroxyestrone; 3-$MeOE_1$: 2-Hydroxyestrone-3-methyl ether; 2-$MeOE_2$: 2-Methoxyestradiol; 4-$MeOE_2$: 4-Methoxyestradiol; 2-$MeOE_1$: 2-Methoxyestrone; 4-$MeOE_1$: 4-Methoxyestrone; d_4-E_2: Estradiol-2,4,16,16-d_4; d_3-E_3: Estriol-2,4,17-d_3; d_5-2-OHE_2: 2-hydroxyestradiol-1,4,16,16,17-d5; d_5-2-$MeOE_2$: 2-methoxyestradiol-1,4,16,16,17-d_5; HQC: High Quality Control; LOD: Limit Of Detection; LOQ: Limit Of Quantitation; LQC: Low Quality Control; MQC: Medium Quality Control; Not Detected (ND); RT: Retention Time.

Introduction

Endogenous female hormones are important indicators in human physiology and pathology. Determination of estrogens and metabolites is one of the most critical steps in human physiological and pathological diagnosis, especially in risk assessment of certain cancers. Bioanalytical method development and validation plays an essential role in analyzing female hormones, e.g., estrogens and metabolites in human blood, urine and tissues, because it is very challenge to determine endogenous estrogens and metabolites accurately at extremely low levels, e.g., pg/mL or pmol/L. Varieties of bioanalytical techniques or methodologies have been developed and applied for analyzing estrogens and metabolites, such as radioimmunoassay, Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS), Gas Chromatography-tandem Mass Spectrometry (GC-MS/MS) [1-4], and Liquid Chromatography with Electrochemical Detection (HPLC/ECD) [5]. The method specificity and sensitivity are the major advantages of LC-MS/MS and GC-MS/MS over radioimmunoassay and HPLC/ECD.

The bioanalytical methods developed in recent years focused more on LC-MS/MS and GC-MS/MS techniques, because the earlier studies demonstrated that Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography-Mass Spectrometry (GC-MS) were significantly less sensitive in analyzing estrogens and metabolites than LC-MS/MS and GC-MS/MS [2,6]. It was obvious that those LC-MS/MS methods directly analyzing biological samples of estrogens and metabolites were simple and straightforward [7-11]. However, a

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number of studies demonstrated that the LC-MS/MS methods directly analyzing estrogens and metabolites were significantly less sensitive than those methods analyzing chemically derivatized estrogens and metabolites [1,12-14], because the neutral molecules of estrogens and metabolites might not be effectively ionized under Electrospray Ionization (ESI) or Atmospheric Pressure Chemical Ionization (APCI) modes. Therefore, chemical derivatization became an important sample preparation procedure for estrogens and metabolites before LC-MS/MS analysis.

An ideal derivatization reagent should react with estrogens and metabolites selectively and quantitatively under mild conditions within a short time, and those estrogen derivatives should be stable, and be easily ionized during LC-MS/MS analysis. There were mainly five classes of reagents used for derivatizing estrogens and metabolites, including: 1) sulfonyl chloride, e.g., dansyl chloride, 1,2-dimethylimidazole-4-chloride and pyridine-3-sulfonyl chloride; 4-(1-H-pyrazol-1-yl) benzenesulfonyl chloride [15]; 2) carbonyl chloride or carboxylic acid N-hydroxysuccinimide ester, e.g., picolinoyl chloride [16] and N-methyl-nicotinic acid N-hydroxysuccinimide ester [12]; 3) benzyl bromide, e.g., pentafluorobenzyl bromide [13,17] and 4-nitrobenzyl bromide [18]; 4) fluorobenzene or fluoropyridine, e.g., 2,4-dinitro-5-fluorobenzene analogues [14] and 2-fluoro-1-methyl-2-pyridinium p-toluenesulfonate [19]; and 5) hydrazide, e.g., (Carboxymethyl) trimethylammonium chloride hydrazide (Girard T reagent) [1,20], and p-tolune sulfonhydrazide [21].

In contrast to the sulfonyl chloride, carbonyl chloride, benzyl bromide and fluorobenzene reagents, the hydrazide reagents reacted only with ketolic estrogens and metabolites. They seemed suitable for certain estrogens, but not for determining all the estrogens and metabolites at the same time, because those alcoholic estrogens and metabolites, e.g., estradiol and estriol, were excluded from the related analytical methods [1,20,21]. Pentafluorobenzyl bromide estrogen derivatives were sensitive to both ESI^+ [17] and $APCI^-$ [13,19] modes, and these derivatives had lower Limits of Quantitation (LOQ) values under $APCI^-$ mode than the LOQ values of derivatives of dansyl chloride and 2-fluoro-1-methyl-pyridinium p-toluenesulfonate under ESI^+ mode, because there were less interferences from analogue compounds and the matrix background under $APCI^-$ mode. Nevertheless, the derivatization reaction of estrogens with pentafluorobenzyl bromide was ten times longer than the derivatization reaction with dansyl chloride (30 min vs. 3 min at 6°C) [19]. A study by Higashi et al. indicted that the derivatization reaction of estrogens with 4-nitrobenzene sulfonyl chloride was the most complete and quantitative in comparison to those reactions with 4-nitrobenzoyl chloride, 4-nitrobenzyl bromide, 2,4-dinitro-fluorobenzene. In addition, the reaction with 4-nitrobenzoyl chloride was lack of selectivity, because it could react with both phenolic and alcoholic hydroxyl groups of estrogens at the same time, whereas 4-nitrobenzyl bromide, 2,4-dinitro-fluorobenzene and 4-nitrobenzene sulfonyl chloride reacted with phenolic hydroxyl group only [18]. These results implied that a sulfonyl chloride was a preferred reagent for derivatizing estrogens and metabolites, due to its reaction completeness and selectivity. Further, a sulfonyl chloride reagent containing a basic or preionzed nitrogen atom, e.g., on dansyl molecule or on a pyridine, imidazole, pyrazole or piperazine ring, could significantly enhance the ionization of estrogen derivatives under ESI^+ mode, and increase the detection sensitivity [1,14,15].

Dansyl chloride was a typical sulfonyl chloride reagent used for derivatizing estrogens and metabolites from varieties of matrices, such as river water [19], charcoal-stripped fetal bovine serum [15], mouse plasma and brain [22], human urine [23,24], breast tissue [25], and serum [26]. Since most of the endogenous estrogens and metabolites exist as glucuronide and sulfate conjugates, these conjugates should be hydrolyzed by β-glucuronidase and sulfatase before derivatization. Xu and colleagues published a number of LC-MS/MS methods for determination of fifteen dansylated unconjugated estrogens and metabolites in urine and serum [23-26]. However, these methods had a very long elution time, 100 minutes, which significantly affected the method throughput. In addition, even the 75 min gradient method using a Phenomenex Synergy Hydro-RP 4 µm column (150 × 2.0 mm) was insufficient to separate all the fifteen dansylated estrogens and metabolites.

In this study, we developed an LC-MS/MS method providing a better separation with a significantly shorter elution time (35 min). The method eluted a Phenomenex Synergi Hydro-RP 2.5 µm column (100 × 2.0 mm) at a higher temperature (60°C) using the mobile phases consisting of acetonitrile, methanol, water and formic acid at a faster flow rate (0.4 mL/min). We also attempted to optimize the dansyl derivatization procedures and the detection sensitivity at pg/mL level in human serum. The method was validated using the optimized LC-MS/MS parameters.

Experimental

Reagents

Dichloromethane and formic acid were obtained from EMD Chemical Inc. (Gibbstown, NJ, USA). Methanol and acetonitrile were obtained from Pharmaco (Brookfield, CT, USA). Dansyl chloride (reagent grade) and β-Glucuronidase/sulfatase from Helix pomatia (Type H-2) were purchased from Sigma Chemical co. (St. Louis, MO, USA). Sodium bicarbonate, glacial acetic acid and L-ascorbic acid were purchased from JT Baker (Phillipsburg, NJ, USA). Sodium hydroxide and sodium acetate were purchased from Fisher Scientific (Fairlawn, NJ, USA). Delipidized double charcoal stripped human serum was purchased from Golden West Biologicals (Temecula, CA, USA). Fifteen Estrogens and metabolites, including Estrone (E_1), Estradiol (E_2), estriol (E_3), 16-epiestriol (16-epiE_3), 17-epiestriol (17-epiE_3), 16-ketoestradiol (16-ketoE_2), 16α-hydroxyestrone (16α-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 4-methoxyestrone (4-$MeOE_1$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 4-methoxyestradiol (4-$MeOE_2$), 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1) and 2-hydroxyestradiol (2-OHE_2), were purchased from Steraloids, Inc. (Newport, RI, USA). Deuterium-labeled estrogens and metabolites, including estradiol-2,4,16,16-d_4 (d_4-E_2), estriol-2,4,17-d_3 (d_3-E_3), 2-hydroxyestradiol-1,4,16,16,17-d5 (d_5-2-OHE_2) and 2-methoxyestradiol-1,4,16,16,17-d_5 (d_5-2-$MeOE_2$), were obtained from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). The estrogens, metabolites and the deuterium-labeled analytical standards were ≥ 98% pure.

Instruments

A vortex-mixer (Model: 37600) and a dri-bath (Model: DB-16525) from Thermolyne Corporation (Dubuque, IW, USA) were used for vortex-mixing and heating of the derivatization reaction. A zymark turbovap LV evaporator (Model: ZW700) from Sotax Corp (Horsham, PA, USA) was used for evaporating the solvents from the samples. The LC-MS/MS system consisted of a Shimadzu SIL HTc auto sampler, two Shimadzu LC-10AD VP series pumps, a degasser, a SCL-10 Avp system controller, a CTO-10AS column oven (Columnbia, MD, USA), and an Applied Biosystems/Sciex (Concord, ON, Canada) model API 5000 triple quadrupole mass spectrometer controlled by Analyst software. A Harvard Apparatus (South Nathick, MA, USA) syringe pump with

a 500 µL syringe from Hamilton Co. (Reno, NE, USA) was employed for compound infusion. A Synergi Hydro-RP 2.5 µm 80 Å column, 100 × 2.0 mm, a Kinetex 2.6 µm C_{18} column, 100 × 2.1 mm, and a Synergy Hydro-RP 4 µm 100 Å column, 150 × 2.0 mm, were purchased from Phenomenex (Torrance, CA, USA). An Asentis Express 2.7 µm C_{18} column, 100 × 2.1 mm, was purchased from Sigma-Aldrich/Supelco (St. Louis, MO, USA).

Standard preparation

Stock and working standard solutions: Each stock solution of the estrogens, metabolites or the deuterium-labeled analytic standards was prepared at 80-200 µg/mL by dissolving an accurate weighed standard with methanol containing 0.1% (w/v) L-ascorbic acid in a volumetric flask. The working standard solutions of estrogens and metabolites at 400-4000 ng/mL and the deuterium-labeled standards at 100 ng/mL were prepared by diluting the stock solutions with methanol containing 0.1% (w/v) L-ascorbic acid. The stock and working standard solutions were stored at -20°C and were equilibrated at room temperature before analysis.

Calibration standard and quality control samples: The calibration standards of the fifteen estrogens and metabolites were prepared in a range of 12-10980 pg/mL by sequentially diluting working standard solutions with charcoal stripped human serum containing 0.1% (w/v) L-ascorbic acid to 10 concentration levels. The quality control standards were prepared at four levels: limit of quantization (LOQ, 12-87 pg/mL), low quality control (LQC, 30-210 pg/mL), medium quality control (MQC, 75-801 pg/mL) and high-quality control (HQC, 761-8465 pg/mL) of the estrogens and metabolites. The deuterium-labeled internal standard (100 ng/mL), 20 µL, was added to each of the calibration standard solutions and the quality control solutions.

Sample preparation: The deuterium-labeled internal standard (100 ng/mL), 20 µL, the enzymatic hydrolysis buffer containing 2 mg of L-ascorbic acid, 0.5 mL, the β-glucuronidase/sulfatase solution, 5 µL, and 0.15 M sodium acetate buffer (pH=4.1), 0.5 mL, were added to 0.5 mL of each serum sample. This sample mixture was incubated at 37°C for 20 hours. Then the sample mixture was extracted with 8 mL of dichloromethane for 10 minutes. The aqueous phase was discarded, while the organic phase was transferred into a test tube and was evaporated at 60°C under nitrogen flow to dryness. The dried sample was mixed with 150 µL of 0.1 M sodium bicarbonate buffer (pH=9.0) and 150 µL of dansyl chloride solution (5 mg/mL in acetonitrile) and vortexed for 1 minute. This mixture was transferred into a 400 µL glass insert in a 2 mL HPLC sample vial, and the vial was sealed by an HPLC vial cap. After the vial was heated at 60°C for 15 minutes, it was cooled down to the room temperature, and was analyzed by LC-MS/MS. The same preparation procedures of hydrolysis, extraction and derivatization were used for all of the standard and the serum samples.

Analytical procedures

Method development: The method development was performed using a Phenomenex Synergi Hydro-RP 2.5 µm column, a Phenomenex Kenetex 2.6 µm C_{18} column and a Supelco Asentis Express 2.7 µm C_{18} column. The LC-MS/MS parameters listed in Tables 1 and 2 were evaluated in order to optimize sample derivatization procedures, LC separation efficiency and MS/MS detection sensitivity, e.g., derivatization temperatures and reaction time, column temperature, mobile phases (buffers at difference pHs and different organic phase gradients at different flow rates), injection volume, and MS/MS conditions (gas temperature, voltage, collision energy, etc.). The MS instrument was tuned with the optimized parameters using the dansyl derivatized estrogens and metabolites before method validation.

Method validation: After the LC-MS/MS parameters listed in Tables 1 and 2 were optimized during the method development, the method was validated to confirm the specificity, accuracy, precision, linearity, sensitivity, recovery of sample hydrolysis and derivatization, and sample stability using a Phenomenex Synergi Hydro-RP 2.5 µm column. The four-quality control standard solutions (LOQ, LQC, MQC and HQC) were injected six times each on the first day, and six times on each of the following two days to assess accuracy and precision. The ten calibration standard solutions were injected for evaluating the linearity of each estrogen or metabolite. To evaluate the sample stability, the serum samples were kept on bunch at ambient temperature for 4 hours and were allowed to go through three freeze (-80°C)/thaw (room temperature) cycles in three consecutive days. Then the serum samples were hydrolyzed, derivatized and analyzed by LC-MS/MS. In order to compare our method with a typical published method in sample preparation, LC separation efficiency and MS detection sensitivity, we evaluated the recovery of sample hydrolysis and extraction, and analyzed one set of dansylated estrogens and metabolites at LOQ level using both our method and the published method [26].

Results and Discussion

Method development

The previous published dansyl chloride derivatization procedures were mixing the dried estrogen sample with 50-100 µL of dansyl chloride at 1 mg/mL in acetone, and heating at 60°C for 3 min [19], 5 min [26] or 15 min [15]. In our experiment, the derivatization reaction was evaluated with 150 µL of dansyl chloride at different concentrations, i.e., 1, 3 or 5 mg/mL in acetonitrile; and with different reaction times at 60°C, i.e., 3, 5, 7, 10, 15, 20, 25 or 30 min. The reason of changing solvent from acetone to acetonitrile was that dansyl chloride had a higher solubility in acetonitrile than in acetone. The results indicated that the derivatization reaction was complete enough when the extracted and dried sample from 0.5 mL of serum reacted with 150 µL dansyl chloride (5 mg/mL) at 60°C for 15 minutes. A lower dansyl chloride concentration or a shorter reaction time led to an incomplete derivatization, while a longer reaction time resulted in degradation of the derivatized products, e.g., a darker reaction solution and higher baseline noises during LC-MS/MS analysis.

As sown in Table 1, those mobiles phases, e.g., 25 mM ammonium formate at pH 3.0, 25 mM ammonium acetate at pH 4.7, 0.1% formic acid, and different ratios of methanol, acetonitrile, isopropanol and tetrahydrofuran at different flow rates, e.g., 0.2, 0.3, 0.4 and 0.5 mL/min were assessed to obtain the most efficient separation for the fifteen estrogens and metabolites. The final choice of mobile phases was 0.1% formic acid in water as Mobile phase A, and 0.1% formic acid in a premixed mixture of 85% methanol and 15% acetonitrile (v/v) as Mobile phase B at a flow rate of 0.4 mL/min, because the mobile phases containing ammonium formate, ammonium acetate, isopropanol and tetrahydrofuran, or at the other flow rates, reduced the separation efficiency. The column temperature at 60°C provided a lower column pressure and better separation than at 40-50°C. The injection volume, 20 µL, gave in an appropriate sensitivity for estrogens and metabolites, because a lower injection volume, e.g., 15 µL or less, reduced the analyte signals, while a higher injection volume, e.g., 40-80 µL, elevated baseline noises. A Phenomenex Synergi Hydro-RP 2.5 µm column became the final choice of our method, because it provided a better separation for the fifteen dansylated estrogens than a Phenomenex Kenetex 2.6 µm

Parameter Derivatization	Evaluated settings for development	Optimized settings for validation
Reaction time	3, 5, 7, 10, 15, 20, 25, 30 min	15 min
Dansyl chloride concentration	1, 3, 5 mg/mL	5 mg/mL
HPLC		
Column temperature	40, 50, 60°C	60°C
Mobile phase A	0.1% formic acid in water, pH 2.7 25 mM ammonia formate, pH 3.0 25 mM ammonia acetate, pH 4.7	0.1% formic acid in water, pH 2.7
Mobile phase B	isopropanol: acetonitrile (v/v) methanol: acetonitrile (v/v) tetrahydrofuran: methanol: acetonitrile (v/v) (with 0.1% formic acid) 0, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 75:25, 80:20, 85:15, 90:10, 100	methanol: acetonitrile: formic acid 85:15:0.1(v/v)
HPLC Gradient	many different conditions	0 min 33% A/67% B 30 min 28% A/72% B 30.1-35 min 33% A/67% B
Flow rate	0.2, 0.3, 0.4, 0.5 mL/min	0.4 mL/min
Injection volume	10, 20, 40, 80 µL	20 µL
MS/MS		
Curtain gas	30, 35, 40, 45, 50 psi	40 psi
Gas1	35, 40, 45, 50 psi	50 psi
Gas2	40, 45, 50, 55 psi	50 psi
Ion spray voltage	4000, 4500, 5000, 5500 volts	5500 volts
Gas temperature	400, 450, 500, 550, 600°C	550°C
Collision gas	6, 7, 8, 9, 10, 12 psi	12 psi

Table 1: HPLC-MS/MS parameters for new method development and validation.

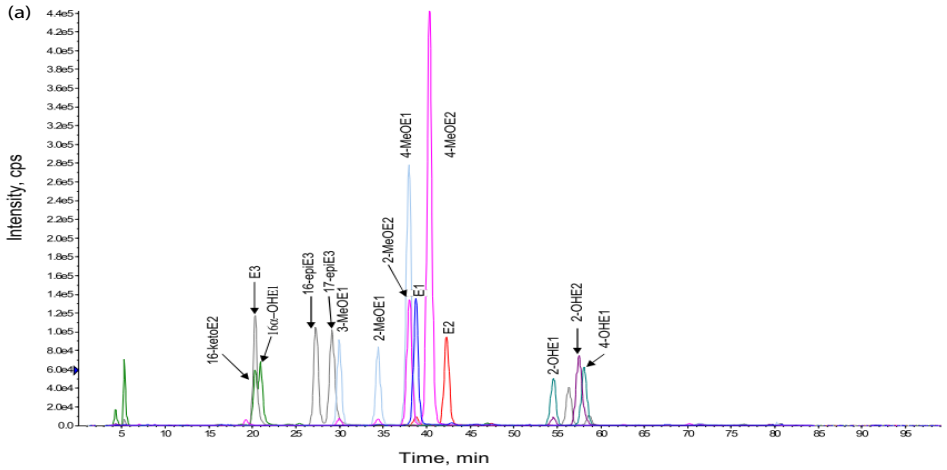
Compound	Molecular weight (g/mol)	Dansyl molecular ion (m/z)	Daughter ion (m/z)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)	Entrance potential (V)
E_1	270.4	504.3	171.1	136	52	8	10
E_2	272.4	506.3	171.2	141	53	10	10
E_3	288.4	522.4	171.1	136	52	10	10
16-epiE_3	288.4	522.4	171.1	136	52	10	10
17-epiE_3	288.4	522.4	171.1	136	52	10	10
16-ketoE_2	286.4	520.3	171.1	136	52	10	10
16α-OHE_1	286.4	520.3	171.1	136	52	10	10
3-$MeOE_1$	300.4	534.3	171.1	136	52	10	10
2-$MeOE_1$	300.4	534.3	171.1	136	52	10	10
4-$MeOE_1$	300.4	534.3	171.1	136	52	10	10
2-$MeOE_2$	302.4	536.3	171.1	136	52	10	10
4-$MeOE_2$	302.4	536.3	171.1	136	52	10	10
2-OHE_1	286.4	753.3	170.1	136	52	10	10
4-OHE_1	286.4	753.3	170.1	136	52	10	10
2-OHE_2	288.4	755.3	170.1	136	52	10	10
d_4-E_2	276.4	510.3	171.1	136	52	10	10
d_3-E_3	291.4	525.3	171.1	136	52	10	10
d_5-2-$MeOE_2$	307.4	541.3	171.1	136	52	10	10
d_5-2-OHE_2	293.4	760.3	170.1	136	52	10	10

Table 2: MRM parameters for MS/MS analysis of estrogens and metabolites.

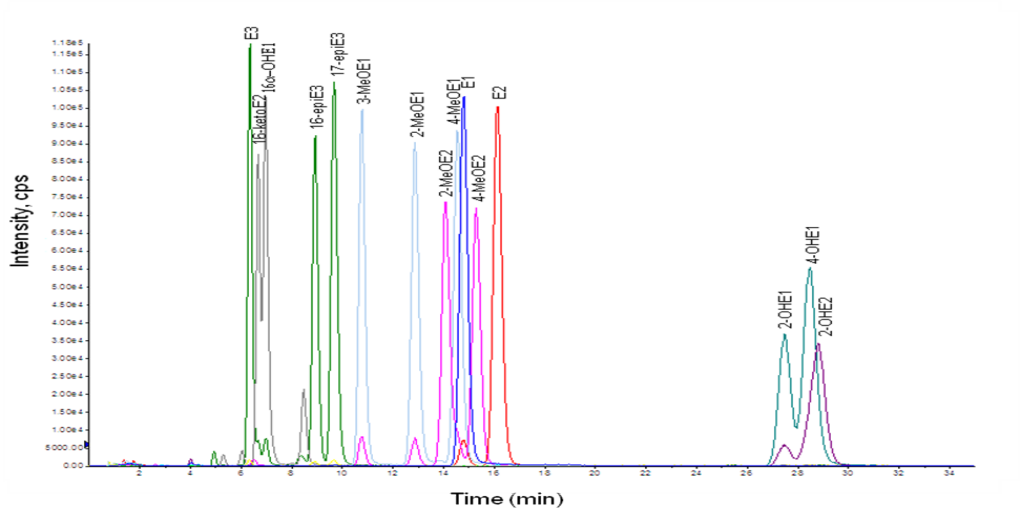
C_{18} column and a Supelco Asentis Express 2.7 µm C_{18} column.

In comparison to the typical publish method [26], our method derivatized the serum sample with dansyl chloride at a higher concentration (5 mg/mL vs. 1 mg/mL) and for a longer time (15 min vs. 3 min), and improved the separation of the fifteen estrogens and metabolites by a smaller particle size column (2.5 µm vs. 4.0 µm) eluted with mobile phases containing water, methanol, acetonitrile and formic acid. The shorter column (100 mm vs. 150 mm), higher column temperature (60°C vs. 40°C) and faster mobile phase flow rate (0.4 mL/mL vs. 0.2 mL/mL) significantly reduced the method run time from 100 minutes to 35 minutes. The typical overlays of MRM chromatographic profiles of dansylated estrogens and metabolites from the published method and from our gradient method are shown in Figure 1a and 1b, respectively. These chromatographic profiles and the retention times of the fifteen estrogens and metabolites listed in Table 3 demonstrated that the two pairs of E_3/16-ketoE_2 and 2-$MeOE_2$/4-$MeOE_1$ peaks were overlapped in the published method, while they were separated by our gradient method with the retention time differences between peaks ≥ 0.2 min.

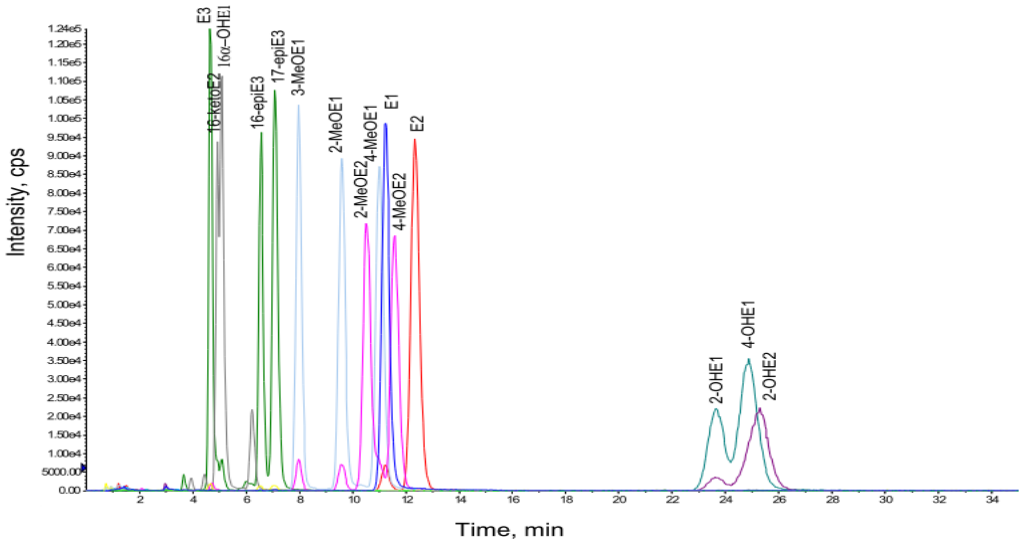
When the Hydro-RP 2.5 µm column was eluted with an isocratic mobile phase consisting of organic phase (methanol/acetonitrile=85/15)/water/formic acid=70/30/0.1 v/v, the separation of the fifteen estrogens and metabolites was similar as that from the gradient method, as shown in Figure 1c. However, the peak heights of three peaks between 23 and 26 minutes were much lower than those from the gradient method, leading to decreased method sensitivity.



a. Chromatographic profiles for the published method by Xu et al. [26].



b. Chromatographic profiles for the new gradient method.



C. Chromatographic profiles for the new isocratic method.

Figure 1: Overlays of typical MRM chromatographic profiles of dansyl derivatives of estrogens metabolites.

Therefore, the gradient method was selected for our method validation.

Method validation

Specificity: As shown in Table 3 and the typical overlay of selected ion chromatograms of fifteen dansylated estrogens and metabolites at HQC level in Figure 1b, most of the estrogens and metabolites were well separated, except that 4-$MeOE_1$/E_1 and 4-OHE_1/2-OHE_2 were partially overlapped. Since dansylated 4-$MeOE_1$, E_1, 4-OHE_1 and 2-OHE_2 had different molecule ions, there were no cross interferences among these compounds in accuracy, precision, linearity and sensitivity. The only significant interference from blank serum was observed for E_3 at the LQC and LOQ levels. These results indicated that the method was specific for these estrogens and metabolites.

So far, the most efficient LC-MS/MS method was reported by Yang et al. which could separate fifteen (out of sixteen) N-methyl nicotinic acid ester derivatized estrogens and metabolites in seven minutes using an Agilent XDB-C18, 50 × 2.1 mm, 1.8 μ column [12]. However, that method had a limit of detection (LOD) within a range of 0.36-2.34 ng/mL, far higher than the LOQ range (5.3-71.1 pg/mL) of our method. The separation efficiencies of other published methods for varieties of derivatized estrogens and metabolites were not so good, although they had comparable sensitivities [13,15-17,26].

Method sensitivity: The sensitivity of our method is presented as LOQ with a signal to noise ratio of 10 to 1. In general, the LOQ values listed in Table 3 are in a range of 5.3-71.1 pg/mL (6.6 pg/mL for E_1, 11.7 pg/mL for E_2 and 5.3 pg/mL for E_3), and are comparable to those LOQ values acquired using the typical published method by Xu et al. (LOQ=8 pg/mL reported in the original article [26]), and to those LOQ values (0.4-10.0 pg/mL) from other published LC-MS/MS and GC-MS/MS methods using varieties of derivatization reagents [1,13,15-17]. Nevertheless, the LOQ values in Table 3 seem having an increasing trend following the retention time. This might be because the longer the retention time, the broader and shorter the peaks, leading to lower detection sensitivities. In addition, the differences in ESI^+ ionization and fragmentation in MS/MS stage for different compounds might also cause the variations in the LOQ values. On the other hand, detection of dansyl derivatives of estrogens and metabolites using ESI^+ mode might be less selective than detection of pentafluorobenzyl estrogen derivatives under $APCI^-$ mode [19], because most of the dansylated estrogens and metabolites had the same daughter ions of m/z 171^+, as shown in Table 2. This suggested that an optimal sulfonyl chloride derivatization reagent should have more specific fragment ions for the derivatized estrogens and metabolites, and the sensitivity should be enhanced more dramatically if the fragment ions contained an isotope(s), e.g., a chlorine or bromine atom(s).

Accuracy: The deuterium labeled internal standards were selected based on the similarities of chemical structure and retention behaviors to the 15 estrogens and metabolites, i.e., d_4-E_2 for E_1 and E_2; d_3-E_3 for E_3, 16-ketoE$_2$, 16α-OHE_1, 16-epiE_3 and 17-epiE_3; d_5-2-$MeOE_2$ for 3-$MeOE_1$, 2-$MeOE_1$, 4-$MeOE_1$, 2-$MeOE_2$, and 4-$MeOE_2$; and d_5-2-OHE_2 for 2-OHE_1, 4-OHE_1 and 2-OHE_2, as shown in Table 2.

The standards, controls and samples at different concentrations were spiked with the same amount of internal standard before hydrolysis and derivatization procedures. Accuracy of this method was determined by analyzing duplicate sample preparations of the estrogens and metabolites at the four quality control levels, HQC, MQC, LQC and LOQ, and the results were compared with the theoretical values. As shown in Tables 4 and 5, the accuracy values at all these levels were within a range of 93.1-112.9% for intraday results, and within a range of 91.7-109.8% for interday results, except those for E_3 at LQC and LOQ levels due to interferences from the blank serum. These results were comparable to those methods reported before [15,26].

Precision: The precision of the method was assessed by evaluating both method precision (intraday precision) and system repeatability (interday precision). The method precision for the estrogens and metabolites was presented by the relative standard deviation of the response of six sample preparations (RSD%, n=6) at the same levels of LOQ, LQC, MQC and HQC on the same day. The relative standard deviations of six sample preparations (RSD%) of estrogens and metabolites on the same day (intraday) were in a range of 1.7-13.2% within the concentration range of 12-8465 pg/mL, as shown in Table 5. Similarly, the relative standard deviations of six sample preparations (RSD%) of the estrogens and metabolites in three consecutive days (interday) were in a range of 2.3-16.9% within the concentration range

Compound	Published method [a]		New Method	
	RT[b] (min)	LOQ (pg/mL)	RT[b] (min)	LOQ (pg/mL)
E_3	20.4[c]	6.5[d]	6.6	5.3[d]
16-ketoE$_2$	20.3[c]	25.2	7.0	13.1
16α-OHE_1	20.9	19.1	7.3	11.9
16-epiE_3	27.3	11.4	9.3	8.5
17-epiE_3	29.1	19.8	10.0	13.6
3-$MeOE_1$	30.0	19.2	11.1	21.0
2-$MeOE_1$	34.5	36.2	13.2	36.8
2-$MeOE_2$	38.0[c]	26.7	14.5	36.0
4-$MeOE_1$	37.9[c]	28.4	14.9	31.8
E_1	38.8	8.7	15.1	6.6
4-$MeOE_2$	40.3	30.2	15.7	38.1
E_2	42.3	11.0	16.5	11.7
2-OHE_1	54.6	74.1	27.8	71.1
4-OHE_1	58.1	66.7	28.8	39.2
2-OHE_2	57.5	57.1	29.3	53.8

[a]Data acquired using the publish method by Xu et al. [26]; [b]Retention time; [c]Overlapped peaks; [d]Calculated based on compound in water

Table 3: Comparison of two HPLC-MS/MS methods in separation and sensitivity.

Compound	LOQ (12-87 pg/mL, n=6)		LQC (30-210 pg/mL, n=6)		MQC (75-801 pg/mL, n=6)		HQC (761-8465 pg/mL, n=6)	
	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%
E_1	101.7	11.3	99.4	8.3	109.6	4.6	105.4	3.2
E_2	93.1	11.3	95.5	7.2	102.7	2.5	109.2	3.3
E_3	N/A*	N/A	N/A	N/A	100.5	6.4	101.5	4.6
16-epiE_3	96.4	13.2	100.1	7.7	101.6	3.3	108.7	6.0
17-epiE_3	103.3	11.7	108.8	8.5	100.6	5.8	108.4	7.4
16-ketoE_2	105.6	10.8	106.0	8.4	104.9	3.3	100.0	6.4
16α-OHE_1	106.6	11.1	110.7	5.9	105.0	5.3	108.4	2.1
3-$MeOE_1$	105.3	12.2	101.8	7.6	101.4	4.0	101.4	4.0
2-$MeOE_1$	101.1	8.5	103.9	6.1	105.5	1.7	104.2	5.7
4-$MeOE_1$	102.6	8.7	98.1	3.8	97.5	3.1	97.5	3.2
2-$MeOE_2$	101.5	8.8	104.6	5.6	107.2	2.6	106.5	2.7
4-$MeOE_2$	108.2	7.3	101.6	6.5	95.7	1.8	94.2	5.1
2-OHE_1	106.4	8.5	105.3	6.5	102.1	10.5	105.0	9.5
4-OHE_1	97.6	10.4	96.7	6.9	106.7	7.6	110.3	7.7
2-OHE_2	98.9	12.1	94.9	9.3	97.9	8.3	112.9	3.3

*Interference from blank serum

Table 4: Accuracy and intraday precision for estrogens and metabolites in serum.

Compound	LOQ (12-87 pg/mL, n=18)		LQC (30-210 pg/mL, n=18)		MQC (75-801 pg/mL, n=18)		HQC (761-8465 pg/mL, n=18)	
	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%	Accuracy%	Precision%
E_1	102.0	16.9	93.8	11.8	103.9	7.4	101.5	6.7
E_2	98.6	9.2	95.4	7.8	102.7	3.6	107.0	3.5
E_3	N/A*	N/A	N/A	N/A	100.2	6.7	97.9	7.3
16-epiE_3	98.9	15.5	98.4	8.0	98.4	7.0	101.9	9.7
17-epiE_3	109.8	15.9	106.1	9.5	97.2	8.0	100.1	9.5
16-ketoE_2	104.5	11.6	109.6	12.6	104.6	4.7	95.2	9.8
16α-OHE_1	102.8	10.4	104.9	8.7	102.7	6.9	98.2	9.8
3-$MeOE_1$	105.7	10.4	103.5	7.3	100.9	3.5	97.1	5.4
2-$MeOE_1$	101.3	8.4	104.5	5.8	104.9	2.7	100.8	7.3
4-$MeOE_1$	102.5	7.0	98.0	3.3	97.0	2.7	95.6	4.6
2-$MeOE_2$	102.3	9.1	105.6	6.5	107.0	2.9	105.6	2.3
4-$MeOE_2$	105.6	8.0	102.9	5.8	96.5	3.1	96.1	5.6
2-OHE_1	103.0	9.8	99.1	9.8	95.9	10.2	95.8	8.7
4-OHE_1	97.2	10.0	91.7	9.0	99.8	11.4	102.2	9.0
2-OHE_2	97.1	8.3	96.5	6.4	96.1	6.7	100.7	10.0

*Interference from blank serum

Table 5: Accuracy and interday precision for estrogens and metabolites in serum.

Compound	Linearity (pg/mL)	r^2	MQC Recovery% (75-801 pg/mL)	LQC Recovery% (30-210 pg/mL)	Serum 1 (pg/mL)	Serum 2 (pg/mL)	Serum 3 (pg/mL)
E_1	12-1486	0.9982	84.9	74.4	233	264	209
E_2	17-1785	0.9963	84.8	75.6	23.1*	24.3*	19.8*
E_3	48-931	0.9953	86.3	76.9	ND	ND	ND
16-epiE_3	12-1429	0.9980	92.1	83.1	ND	ND	ND
17-epiE_3	24-2804	0.9971	88.0	82.0	10.1*	ND	ND
16-ketoE_2	12-1501	0.9951	95.6	79.2	12.8	27.9	25.2
16α-OHE_1	12-1438	0.9960	88.2	78.3	ND	33.9	ND
3-$MeOE_1$	12-1413	0.9964	85.0	83.6	ND	ND	ND
2-$MeOE_1$	25-2844	0.9983	89.0	89.2	3.8*	39.3	45.2
4-$MeOE_1$	65-7082	0.9960	87.5	81.3	29.1	ND	36.4
2-$MeOE_2$	31-3530	0.9976	85.8	80.6	ND	ND	ND
4-$MeOE_2$	84-7341	0.9970	86.4	84.3	ND	ND	ND
2-OHE_1	78-10035	0.9940	86.2	76.5	22.0*	48.9*	33.4*
4-OHE_1	81-10980	0.9934	81.9	79.4	ND	31.3*	30.8*
2-OHE_2	79-9073	0.9950	83.8	82.8	ND	ND	ND

ND: not detected; *The estimated concentration is below the LOQ but above LOD (S/N>3)

Table 6: Linearity, recovery of hydrolysis/derivatization and sample analysis of estrogens and metabolites in human serum.

Compound	Bench top stability [a] (75-801 pg/mL, n=6)		Freeze thaw stability [b] (75-801 pg/mL, n=6)	
	Accuracy%	Precision%	Accuracy%	Precision%
E_1	105.0	3.1	104.6	4.2
E_2	102.4	3.0	101.2	2.3
E_3	90.2	5.1	93.2	5.0
16-epiE_3	87.4	4.9	90.0	6.7
17-epiE_3	86.4	7.0	87.5	9.0
16-ketoE_2	88.7	7.9	92.5	5.6
16α-OHE_1	84.9	8.9	82.3	7.8
3-MeOE_1	96.8	4.4	96.0	2.9
2-MeOE_1	102.8	3.8	103.0	3.8
4-MeOE_1	96.4	5.4	98.0	2.0
2-MeOE_2	106.7	3.3	106.3	2.7
4-MeOE_2	94.1	7.8	97.4	2.9
2-OHE_1	103.5	8.8	115.8	8.4
4-OHE_1	110.0	6.7	118.1	4.8
2-OHE_2	100.0	9.2	109.0	5.4

[a]Sample solution on bunch for 4 hours; [b]After 3 freeze and thaw cycles in three consecutive days

Table 7: Bench top and freeze thaw stability for estrogens and metabolites in serum.

of 12-8465 pg/mL, as shown in Table 5. These results demonstrated that the method had suitable precision and system repeatability within the interested determination ranges.

Linearity and recovery of sample derivatization: The calibration curve of each estrogen compound was constructed by plotting the MS/MS peak ratios of each dansylated estrogen or metabolite vs. the dansylated deuterium labeled internal standard against the sample concentration. The results of weighted least squares linear regression are shown in Table 6. The LC-MS/MS system had linear responses to the fifteen dansylated estrogens and metabolites in the range of 12-10980 pg/mL with regression coefficients $r^2 \geq 0.9934$. Since a large portion of the fifteen estrogens and metabolites exist as glucuronide and sulfate in human body fluids, e.g., serum and urine, the conjugated estrogens and metabolites need to be hydrolyzed with glucuronidase and sulfatase before dansyl derivatization. When we followed glucuronidase/sulfatase hydrolysis procedures from the published method [26], and derivatized the unconjugated fifteen estrogens and metabolites with dansyl chloride under our optimized conditions, the total recovered estrogens and metabolites were within a range of 74.4-95.6% at a concentration range of 30-801 pg/mL, as shown in Table 6. This range of recovery ratio of sample hydrolysis and derivatization was proved to be adequate for sample analysis by the results of accuracy, precision, linearity and sensitivity. In order to demonstrate the suitability of the method for real human serum sample analysis, three batches of unknown human serum samples were analyzed. As shown Table 7, the levels of the determined estrogens and metabolites by our method were close to those from the typical method reported before [26].

Sample stability: The deuterium labeled internal standards were stable, and no chemical and isotope degradation were observed during sample preparation and HPLC-MS/MS analysis. The sample stability was evaluated by allowing the serum samples spiked with estrogens and metabolites to stay at room temperature for four hours, or to go through three freeze/thaw cycles in three consecutive days. Then, these samples underwent hydrolysis, derivatization and LC-MS/MS analysis. The results of accuracy (82.3-118.1%) and precision (2.0-9.0%RSD) demonstrated that the samples were stable during the stability testing, and suitable for sample analysis under the assigned storage conditions, as shown in Table 7.

Conclusions

An efficient LC-MS/MS method was successfully developed and validated for determination of fifteen estrogens and metabolites in human serum. The sample derivatization procedures were optimized, and sample stability was assessed. The method was specific, accurate, precise, sensitive and linear within the calibration ranges. It had a comparable sensitivity to those from the typical published LC-MS/MS methods, while it had a much better LC separation efficiency, i.e., separating all of the fifteen dansylated estrogens and metabolites with a significantly reduced elution time.

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