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Ultrasensitive quantification of serum estrogens in postmenopausal women and older men by liquid chromatography–tandem mass spectrometry

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ABSTRACT

An ultrasensitive stable isotope dilution liquid chromatography–tandem mass spectrometry method (LC–MS/MS) was developed and validated for multiplexed quantitative analysis of six unconjugated and conjugated estrogens in human serum. The quantification utilized a new derivatization procedure, which formed analytes as pre-ionized N-methyl pyridinium-3-sulfonyl (NMPS) derivatives. This method required only 0.1 mL of human serum, yet was capable of simultaneously quantifying six estrogens within 20 min. The lower limit of quantitation (LLOQ) for estradiol (E2), 16α-hydroxy (OH)-E2, 4-methoxy (MeO)-E2 and 2-MeO-E2 was 1 fg on column, and was 10 fg on column for 4-OH-E2 and 2-OH-E2. All analytes demonstrated a linear response from 0.5 to 200 pg/mL (5–2000 pg/mL for 4-OH-E2 and 2-OH-E2). Using this validated method, the estrogen levels in human serum samples from 20 female patients and 20 male patients were analyzed and compared. The levels found for unconjugated serum E2 from postmenopausal women (mean 2.7 pg/mL) were very similar to those obtained by highly sensitive gas chromatography–mass spectrometry (GC–MS) methodology. However, the level obtained in serum from older men (mean 9.5 pg/mL) was lower than has been reported previously by both GC–MS and LC–MS procedures. The total (unconjugated + conjugated) 4-MeO-E2 levels were significantly higher in female samples compared with males ($p < 0.05$). The enhanced sensitivity offered by the present method will allow for a more specific analysis of estrogens and their metabolites. Our observations might suggest that the level of total 4-MeO-E2 could be a potential biomarker for breast cancer cases.

1. Introduction

Over the last 7 years, gas chromatography (GC) – and liquid chromatography (LC)–mass spectrometry (MS) – based methodology has become increasingly reliable for the quantification of extremely low concentrations of unconjugated estrogens present in the serum and plasma of postmenopausal women (Table 1) [1–3]. Estrone (E1), 17β-estradiol (E2) and 16α-hydroxy (OH)-E2 are the major unconjugated estrogens that are present in serum (Fig. 1). They are largely bound to protein in the circulation, and their free concentrations are estimated from the steroid hormone binding globulin levels [4–6]. Reliable assays for unconjugated circulating estrogens are important because even though their concentrations are extremely low, increased levels are thought to be an important risk factor for breast [7–10] and endometrial cancer [11–13]. Increased circulating estrogens are also a potential risk factor for prostate cancer in men [14–16], although no systematic studies have been conducted to date to test this possibility. Unfortunately, serum concentrations

Abbreviations: E1, estrone; E2, 17β-estradiol; G, β-glucuronide; GC, gas chromatography; LC, liquid chromatography; HQC, high quality control; LLE, liquid–liquid extraction; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; MS, mass spectrometry; *m/z*, mass to charge ratio; NMPS, N-methyl pyridinium-3-sulfonyl; OH, hydroxy; OMe, methoxy; PS, pyridine-3-sulfonyl; S, sulfate.

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for many of the unconjugated estrogens in postmenopausal women are close to the reported lower limits of quantification (LLOQs), raising concerns that the values could represent an over-estimation of the true values (Table 1). This means that the corresponding sulfate and β-glucuronide conjugates (Fig. 1), which are present in higher concentrations (Table 2), might have some predictive value in determining cancer risk [17–19]. Furthermore, the sulfate conjugates (Fig. 1) could be a potential source of their corresponding unconjugated forms through action of tissue sulfatases [19].

The very low concentrations of circulating estrogens and their metabolites mean that analysis by MS-based methodology is very challenging. Importantly, LC–MS can overcome potential problems of cross-reactivity that usually occur with more sensitive but less specific immunoassay-based methodology [20–22]. Furthermore, MS-based methods make it possible to quantify multiple estrogens in a single analytical run, which allows for more comprehensive analyses to be conducted. Conventional positive ion GC–MS and LC–MS generally have inadequate sensitivity for routine analyses of endogenous estrogens in the serum of postmenopausal women and older men. Therefore, numerous derivatization methods have been developed to improve the sensitivity of both [1]. High sensitivity GC–MS procedures usually employ electron capture negative chemical ionization using pentafluorobenzoyl (PFBO) derivatives [9,12,23–27] (Table 1). The corresponding LC–MS method known as electron capture atmospheric pressure chemical ionization, which uses the pentafluorobenzyl (PFB) derivative [28], is widely used for the quantification of lipids [29] but has found only limited utility for the analysis of estrogens and their metabolites [1].

A second LC–MS approach, which has been used much more widely, involves the use of estrogen derivatives that enhance the electrospray ionization (ESI) signal and therefore improves overall sensitivity during LC–ESI/MS analysis. This approach is exemplified by derivatization of the estrogen phenolic moiety to a dansyl ester [30–33]. The dansyl derivative has been used in a number of studies to quantify unconjugated estrogens [34–38] (Table 1) and conjugated estrogens after hydrolysis with β-glucuronidase/arylsulfatase [34,37–39] (Table 2) in serum samples from postmenopausal women. Alternative approaches to improve ESI signal have included the use of picolinoyl [40] and pyridine-3-sulfonyl [41] derivatives. A third LC–MS approach involves the preparation of pre-ionized (quaternized) derivatives, so that protonation of the estrogen derivative is not required. Therefore, suppression of ionization in the ESI source of the mass spectrometer is minimized. This approach has been reported in studies that utilized the N-methyl-2-pyridyl [42], N-methyl-nicotinyl, and 1-(2,4-dinitro-5-fluorphenyl)-4,4,-dimethylpiperazinyl [43] derivatives attached to the estrogen 3-phenolic moiety. Our group has also used pre-ionized derivatives to improve sensitivity by adding a Girard P (GP) derivative to the 17-oxo moiety of estrone and its metabolites [44] as well as by adding a Girard T (GT) derivative to the 17-oxo-moiety of androgens [45]. However, this approach cannot be employed for the analysis of E2 and it metabolites that lack a 17-oxo-moiety. We now report development of the pre-ionized N-methyl pyridinium-3-sulfonyl (NMPS) derivative (Fig. 2), which provides extremely high sensitivity for LC–ESI/MS/MS analysis of E2 and its metabolites. We demonstrate the utility of this new derivatization for the quantification of estrogens in the serum of postmenopausal women and older men.

2. Experimental

2.1. Reagents and materials

The six estrogens analyzed in this study, E2, 16α-OH-E2, 4-methoxy (MeO)-E2, 2-MeO-E2, 4-hydroxy (OH)-E2 and 2-OH-E2 were purchased from Steraloids Inc. (Newport, RI). [13,14,15,16,17,18-$^{13}C_6$]-E2 ([$^{13}C_6$]-E2), [2,3,4-$^{13}C_3$]-16α-OH-E2 ([$^{13}C_3$]-16α-OH-E2), [13,14,15,16,17,18-$^{13}C_6$]-4-MeO-E2 ([$^{13}C_6$]-4-MeO-E2), [13,14,15,16,17,18-$^{13}C_6$]-2-MeO-E2 ([$^{13}C_6$]-2-MeO-E2), and [13,14,15,16,17,18-$^{13}C_6$]-2-OH-E2 ([$^{13}C_6$]-2-OH-E2) with an isotopic purity of 99% were purchased from Cambridge Isotope Laboratories (Cambridge, MA). [1,4,16,16,17-$^{2}H_5$]-4-OH-E2 ([$^{2}H_5$]-4-OH-E2) with an isotopic purity of 98% was obtained from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). β-glucuronidase/arylsulfatase (*Helix pomatia*) was obtained from Roche (Indianapolis, IN). E2-3-(β-D-glucuronide)-17-sulfate (E2-3G-17S) was obtained from Sigma–Aldrich (St. Louis, MO). Pyridine-3-sulfonyl chloride (97%) was obtained from Matrix Scientific (Columbia, SC). Dry acetonitrile was purchased from Acros Organic (New Jersey, USA). Methyl-*tert.*-butyl-ether (MTBE), iodomethane, methanol, acetone, L-ascorbic acid, formic acid, hydrochloric acid (HCl), sodium chloride, sodium acetate and sodium bicarbonate were obtained from Sigma–Aldrich (Milwaukee, WI). Double charcoal-stripped human serum was obtained from Golden West Biologicals, Inc. (Temecula, CA). All solvents used in this study were HPLC Optima grade unless otherwise noted and were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Clinical study

Twenty postmenopausal women ages 56–65 (mean ± SD, 60.0 ± 2.66) and twenty men ages 51–69 (mean ± SD, 61.6 ± 5.41) were recruited for the study. Menstrual and menopausal status of the women was based on self-report. All participants were healthy and the women were not taking exogenous hormones. The blood collection protocol was approved by the University of Pennsylvania Review Board (Protocol # 800924). After the blood was collected, it was allowed to clot for 1 h at room temperature, serum was separated and aliquots were stored at −80 °C. Serum samples were allowed to thaw at room temperature and aliquots of 0.1 mL were used for the estrogen analyses.

2.3. Preparation of stock solution and working standard solutions

Estrogen standard and internal standard stock solutions were individually prepared in methanol containing 0.1% (w/v) L-ascorbic acid at a concentration of 1 mg/mL then stored at −20 °C. A mixed stock solution of six estrogens or the corresponding internal standards at 1 μg/mL was prepared by adding 10 μL of each estrogen standard stock solution to a 10 mL volumetric flask with methanol containing 0.1% L-ascorbic acid. Working standard solutions of mixed estrogens and working standard solutions of internal standards (1 ng/mL) were prepared by dilutions of the stock solutions with methanol containing 0.1% (w/v) L-ascorbic acid. Stock solution and working solution of catechols were prepared at concentrations 10 times higher than the other estrogens.

2.4. Preparation of calibration standards and quality controls

Charcoal-stripped human serum was used for preparation of calibration standards and quality controls (QCs). Calibration standards were prepared by spiking appropriate amounts of the working standard solution to charcoal-stripped human serum to make the concentrations of 0.5, 1, 2, 5, 10, 20, 50, 100, 200 pg/mL (10 times higher concentration for 4-OH-E2 and 2-OH-E2). The preparation procedures for QC samples at concentrations of 1.5, 75, and 175 pg/mL were the same as that of the calibration standards. 10 μL of working standard solution of internal standards was added to each calibration standard and QC sample.