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Development of a highly sensitive method for the quantification of estrone and estradiol in serum by liquid chromatography tandem mass spectrometry without derivatization

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ABSTRACT

Measurement of estrone (E1) and estradiol (E2) values <1 pg/mL (3.7 pmol/L) is necessary for postmenopausal, pediatric and male serum samples. Until now this was rarely reached and only through derivatization which can present problems for estradiol. A very sensitive LC–MS/MS method was developed avoiding derivatization, convenient for large-scale studies. The desired sensitivity and specificity were achieved using ESI negative mode, LLE and a 2D chromatography consisting of a trapping column and a second dimension reverse-phase C8 analytical column. A mixture of an aqueous solution of ammonium fluoride at 0.2 mM and methanol was used on the analytical column to further increase the sensitivity. Serum LOQ was <0.5 pg/mL (1.9 pmol/L) for E2 and E1 and recoveries ranged from 95 to 105%. No carry-over was detectable. Inter assay CV's were 4.0% at 21 pg/mL (77 pmol/L) for E2, 7.6% at 25 pg/mL (93 pmol/L) for E1. Comparison with commercial direct estrogen assays (Roche Diagnostics E170 for E2, Bioline RIA for E1) exposed analytical unsuitability (due to a combined lack of sensitivity and specificity) for the assay of male, postmenopausal or pediatric samples.

1. Introduction

Both for clinical research and potential clinical applications, there is considerable interest for reliable and practical measurement of low estrogen serum concentrations of estrone (E1) and estradiol (E2). Particularly in postmenopausal women (<30 pg/mL or 110 pmol/L), in amenorrheic premenopausal women (e.g. in anorexia nervosa and other forms of hypothalamic amenorrhea), in prepubertal children or in patients treated with inhibitors of aromatase [1–4]. Postmenopausal E2 serum levels have been associated with breast cancer, osteoporosis and fracture risk [1,5,6]. Estrogens also play an important physiological role in men, who present at adult age with moderately low serum E2 levels (mean levels around 18 pg/mL or 67 pmol/L) [7]. Male serum E2 levels have been associated with parameters of skeletal health such as rate of bone loss and fracture risk in the elderly [8,9].

Unfortunately the most widely used techniques for E2 measurement, direct serum immunoassays, do not perform well in the lower range such as seen in postmenopausal women due to lack of sensitivity and specificity. For concentrations below 20 pg/mL (74 pmol/L) correlation with concentrations measured with a reference GC–MS/MS method is very poor [6,10,11]. Although indirect immunoassays with extraction and chromatographic separation steps (e.g. on LH20 gel column) perform better, they have largely been abandoned because they require the use of radioactive isotopes and large amounts of serum (up to 2 mL).

Highly sensitive bioassays for E2 have been proposed [1,2,6], but their use is limited to research settings in a limited number of specialized labs. Presently, mass spectroscopy-based methods (GC–MS/MS; LC–MS/MS) are the methods of choice for steroid hormone assays where GC based methods suffer from requiring large sample volumes and long run times limiting practical usage [12].

To increase sensitivity for estrogen measurement on LC–MS/MS, reported methods often resorted to derivatization [13–17]. However derivatization based methods are less preferable for estradiol measurement because PH and temperature changes can potentially influence hydrolysis of the conjugated estrogens resulting in falsely high measurements [18,19]. In addition specificity can be compromised [24,25] and the more lengthy and delicate sample preparation is less suitable for large-scale studies. Some attempts have been made to design methods without derivatization [19–22] but these were until now not sensitive enough for the analysis of the very low serum concentrations typically seen in children

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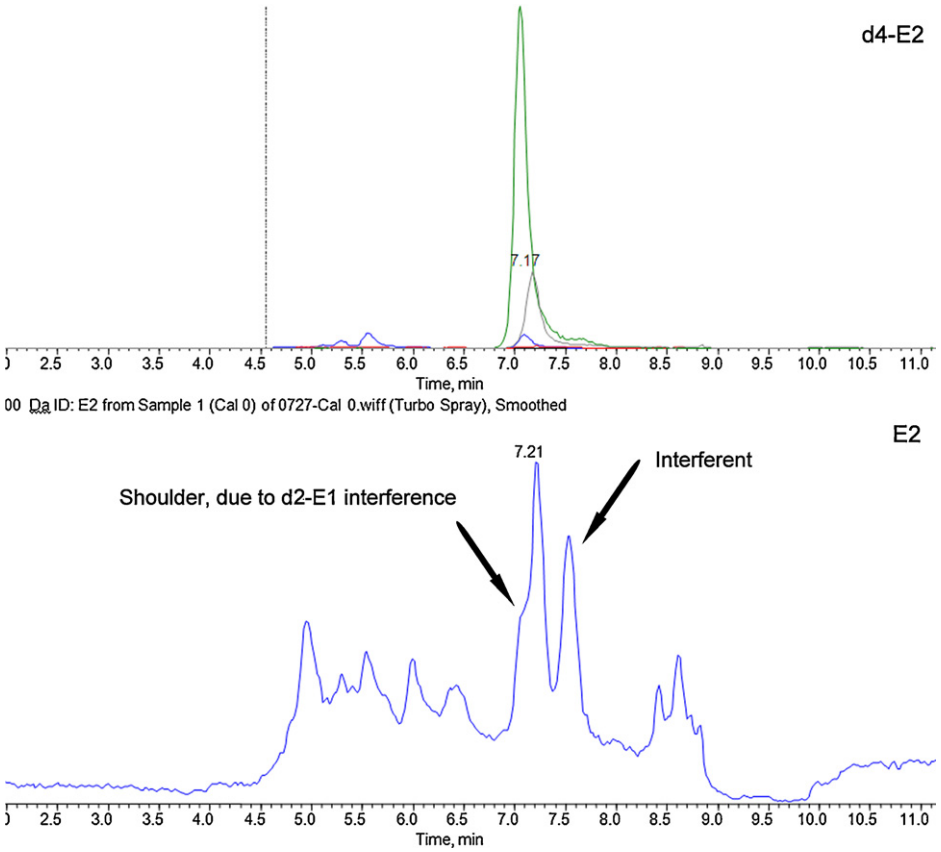

Fig. 1. Extracted ion chromatogram for E2 in a sample at low concentration. The peak appearing at 7.5 min (isobaric interference) is chromatographically separated meanwhile the shoulder on the left side could be an issue and was eventually shown to be a d2-E1 impurity of the d4-E1 internal standard.

or postmenopausal women [23]. We describe here a very sensitive assay with LOQ well below 0.5 pg/mL (1.9 pmol/L) for E2 and E1 with serum extraction followed by direct measurement on 2D-LC–MS/MS without need for derivatization.

2. Materials and methods

17beta estradiol (E2) and estrone (E1) were obtained from Sigma–Aldrich, 17beta estradiol-*d4* (d4-E2) and estrone-*d4* (d4-E1) were purchased from CDN Isotopes, Inc. All standards and internal standards were dissolved in methanol. Methanol, water and acetonitrile were LC–MS grade from BioSolve BV (Varkenswaard, The Netherlands).

As for comparison with routine assays, 17β-estradiol was measured by electrochemiluminescence immunoassay 'ECLIA' on a Modular E170 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). Estrone was measured by RIA (Bio-line, Brussels, Belgium).

For measurement of E2 and E1 by LC–MS/MS an AB Sciex 5500 triple-quadrupole mass spectrometer (AB Sciex, Toronto Canada) was used, coupled with an electrospray ionization (ESI) probe on the Turbo-V source and operated in negative ion mode. The liquid chromatography system for 2D-LC operation consisted of a Shimadzu system leveraging four Pump modules LC20AD UFLC and an Autosampler SIL20AC (Shimadzu Scientific Instruments, Columbia, MD, USA). As for a first dimension, sample loading and cleaning were carried out on a Supelco Supelguard LC-8-DB (3.0 mm × 20 mm) trapping column (Supelco, St. Louis, MO, USA) meanwhile the chromatographic separation as for the second dimension was performed on a reverse-phase C8 analytical column (Supelco LC-8-DB, 3.3 cm × 2.1 mm, 3 μm particle size). Both columns were kept at room temperature and the built-in switching valve of the 5500 mass spectrometer was used for column switching.

Serum samples used were anonymous leftovers from routine analysis, collected and used in accordance with local ethical committee guidelines. For LOQ, recovery and linearity studies, very low content samples and spiked charcoal stripped serum samples were used. For blanks both methanol and extractions containing only internal standard were utilized.

Samples were extracted with 2.5 mL of 9:1 hexane–ethylacetate mixture on 500 μL of serum after the addition of 25 μL of cortisol (6 μg/mL (1.65 nmol/L) in methanol) and 25 μL E2-d4 (10 ng/mL in methanol (37 nmol/L)). After mixing for 3 min, samples were frozen and decanted with supernatant collection. With a second extraction, supernatants were combined, dried, washed with 0.5 mL of 9:1 hexane–ethylacetate and dried again to be reconstituted in a final solution of 125 μL methanol of which 100 μL are injected.

The two dimension-liquid chromatographic process is articulated through the following steps.

- Upon the injection, the sample is cleaned through the guard column with an aqueous solution containing 80% water (eluent A) and 20% of a mixture methanol–acetonitrile (1:1, eluent B) and delivered by two pump modules (the "Loading" pump) at 1.5 mL/min for 3 min.
- With the activation of the valve at 3 min, the guard column is connected to the C8 column in forward mode and both columns are flushed by 400 μL/min of an eluent consisting in 52% of an aqueous solution of ammonium fluoride at 0.2 mM (eluent A) and 48% of methanol (eluent B) supplied by the second pair of pump modules (the "Separation" pump).