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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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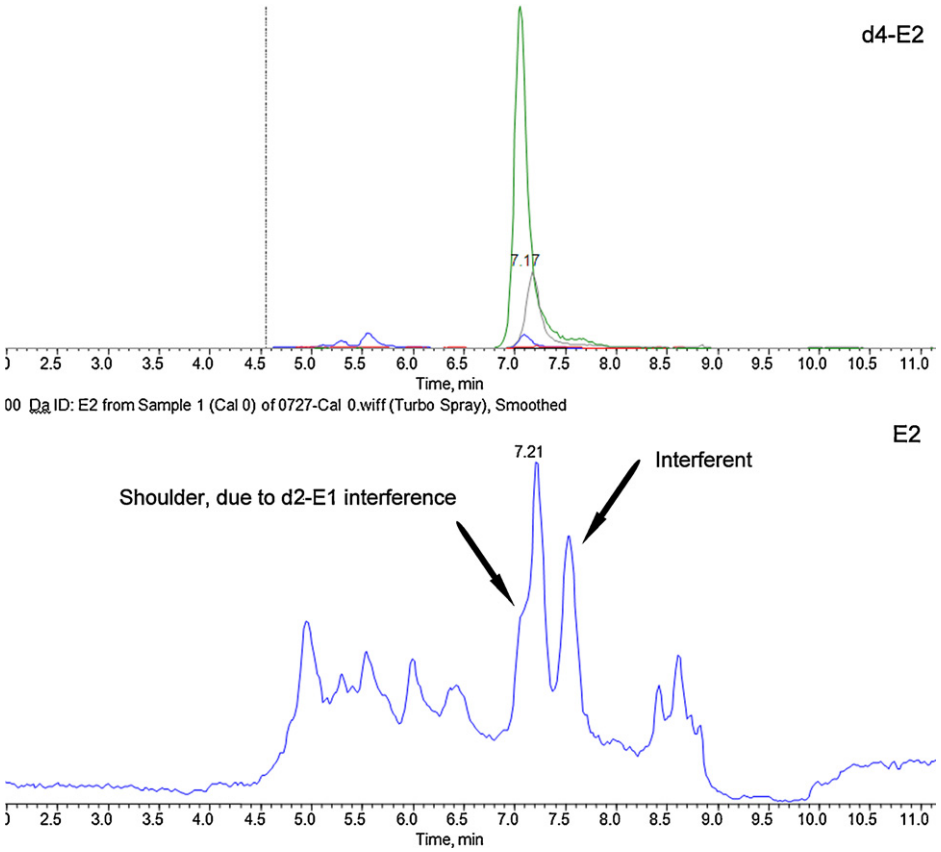

Fig. 1. Extracted ion chromatogram for E2 in a sample at low concentration. The peak appearing at 7.5 min (isobaric interference) is chromatographically separated meanwhile the shoulder on the left side could be an issue and was eventually shown to be a d2-E1 impurity of the d4-E1 internal standard.

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).

Table 1
Recoveries obtained by spiking low-concentration serum samples.

E2	Measured pg/mL (pmol/L)	Recovery (%)	E1	Measured pg/mL (pmol/L)	Recovery (%)
Sample A	3.5 (12.8)		Sample D	19.1 (70.7)	
A + 5 pg (18 pmol)	8.30 (30.5)	97.6	A + 5 pg (18 pmol)	25.0 (92.5)	103.7
A + 10 pg (37 pmol)	13.0 (47.7)	96.3	A + 10 pg (37 pmol)	30.5 (113)	104.8
Sample B	3.1 (11.4)		Sample E	21.5 (79.6)	
B + 5 pg (18 pmol)	7.7 (28.3)	95.1	B + 5 pg (18 pmol)	27.0 (100)	101.9
B + 10 pg (37 pmol)	13.0 (47.7)	99.2	B + 10 pg (37 pmol)	31.5 (117)	100.0
Sample C	25.4 (93.2)		Sample F	51.5 (191)	
C + 5 pg (18 pmol)	30.7 (113)	102.8	C + 20 pg (74 pmol)	71.7 (265)	100.2
C + 10 pg (37 pmol)	35.7 (131)	104.9	C + 50 pg (185 pmol)	103.2 (379)	101.6
C + 50 pg (184 pmol)	75.7 (278)	103.8			

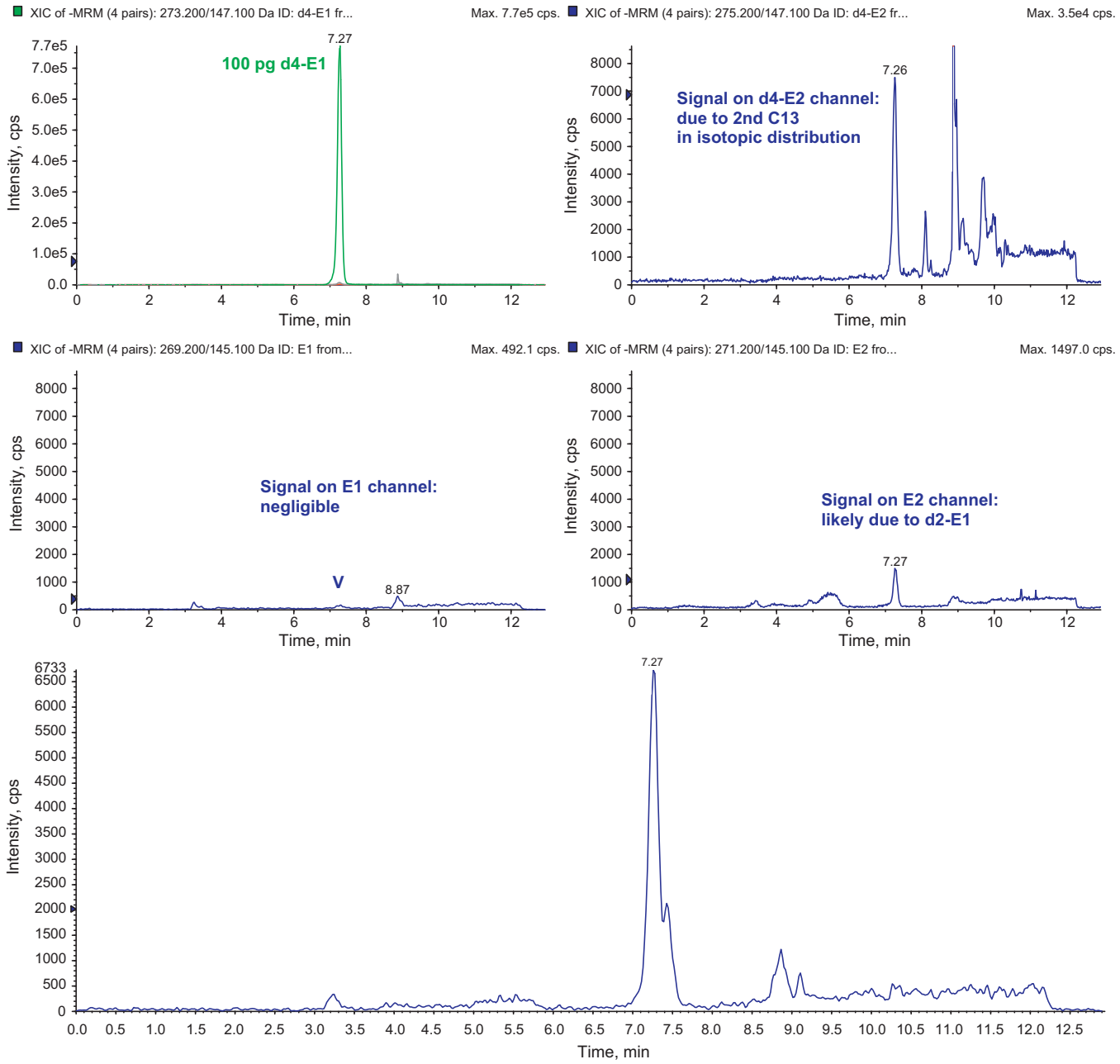

Fig. 2. Traces for documenting the interferences generated by d4-E1. Upper panel, clockwise: signal generated by injection of 100 pg of d4-E1, signal generated on d4-E2 channel as expected from the C-isotopic distribution, signal generated on E2 channel likely due to d2-E1 as impurity, signal generated on E1 channel. Lower panel: trace on the E2 channel generated by a mixture containing d4-E1 at high concentration and E2 at very low concentration.

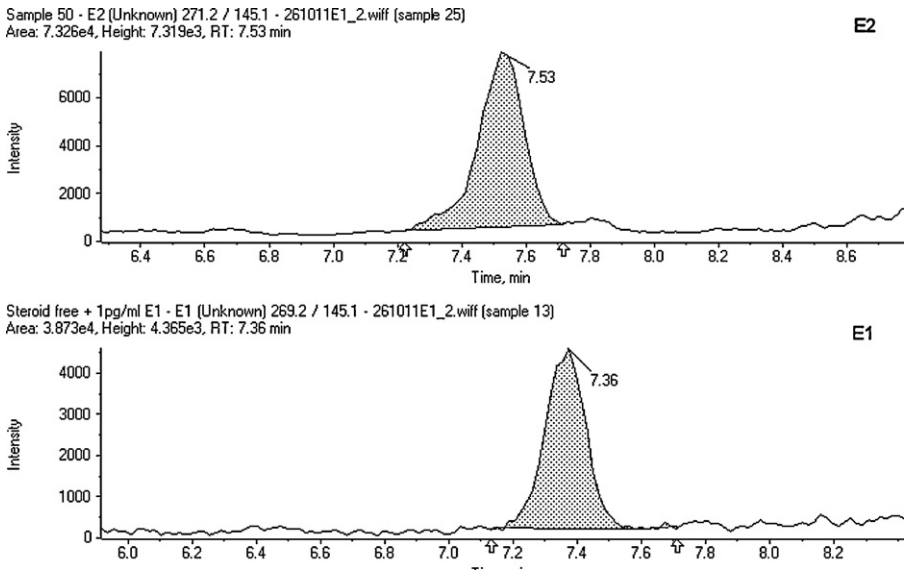

Fig. 3. Low serum sample XIC chromatograms as integrated by MultiQuant software for E2 and E1: Top: E2 7 pg/mL (18 pmol/L), Bottom: E1 1.4 pg/mL (5.2 pmol/L).

- With the valve activation, the "Separation" pump performs a gradient from 48% eluent B to 58% in 4 minutes and then to 70% in 1 min. The gradient is completed by a further minute at 90% and 3 minutes at 100% eluent B.
- With the switching-back of the valve, C8 column is flushed for 1 min by 650 μL/min of 100% eluent A as delivered by the "Separation" pump meanwhile the guard column is reconditioned by the "Loading" pump with 80% of eluent A at 1.5 mL/min.

Measurements are accomplished by the tandem mass spectrometer running in multiple reaction monitoring (MRM) mode by exploiting transitions *m/z* 269/145 for the for E1, 271/145 for E2, 273/147 for d4-E1 and 275/147 for d4-E2, with a declustering potential (DP) of −150 V and a collision energy (CE) of −55 eV for all the analytes.

In the final version, d4-E1 transition has been omitted. Data processing was performed through MultiQuant version 2.0.2.

3. Results and discussion

3.1. Chromatography

One of the challenges in measuring most of the steroids by LC–MS is their inherent poor sensitivity accountable to a low polarity of these molecules. In this frame, estradiol has demonstrated to provide better sensitivity when measured in negative ion mode with the ESI source, and APCI ionization has shown to give a lower yield. For willing to pursue the approach in skipping any derivatization, all the actions must be taken in order both to improve the achievable sensitivity and to minimize any possible adverse effect on the ionization process like the well-known Ion Suppression effect. Relying on some experiences made on homologous molecules, we have observed a benefit in promoting the estrogen ionization by adding some fluoride ions. The rationale of the mechanism behind this sensitivity improvement (80% for estradiol and 35% for estrone, as seen in Fig. 5) is still under speculation, however the optimum fluoride concentration has been found to be around 0.2 mM, as a trade-off between a range where a low concentration impacts the process yield, and a too high concentration of fluoride ions starts to hamper analyte ionization as due to Ion Suppression effect generated by the massive presence of negatively charged ions like the fluoride itself.

With the 2D-LC strategy as above described, both analytes eluted at around 7.2 min. Analyte losses and carry-over were consistently mitigated by either the cortisol spiking and by the two dimensional set-up which allows to inject the extract reconstituted in a fully organic solution without affecting the chromatographic retention.

The expected matrix isobaric components were sufficiently segregated from the analytes. Nonetheless during the method development it was noted for ultra low E2 samples that, in addition to a small peak following the E2 peak in some samples which could be chromatographically resolved, there was a constant frontal shoulder in the E2 peak, visible only at these very low concentrations (see Fig. 1). Through experimentation it was elucidated that, since the method was set up by using deuterated d4 internal standards for both E1 and E2, this shoulder was due to an impurity of the d4-E1 internal standard (Fig. 2). As the fragmentation ions are similar to E2, apparently d2-E1 variant, as impurity of d4-E1 is present in low concentration and interferes on the E2 channel. As the method was developed to be able to measure very low concentrations, even such a small impurity is a potential confounding factor (as illustrated by Fig. 1). Although it is a constant confounding factor for integration and therefore does not alter calculation results for most samples, peak shape and integration would suffer for very low samples. A small interference of the d4-E1 internal standard was also visible in the d4-E2 internal standard channel but was as such irrelevant versus the much bigger internal standard area. The decision was taken to calculate both E1 and E2 versus d4-E2, in order to guarantee optimal performance and integration at all levels (an example of the peak shape after elimination of d4-E1 is shown in Fig. 3). Kushnir et al. reported a similar problem with d5-E2 in their dansyl chloride derivatization based method [14].

3.2. Method performance

The finalized method exhibits excellent sample recoveries (as summarized in Table 1) both for E2 and E1. Carry-over was evaluated using a 4× High, 2× Low, High, Low, High, Low sample sequence, and a carry-over below 0.2% was observed both for E2 and E1. 1/*x* weighted linear regression is used for curve fitting. Recoveries on standards (0–2000 pg/mL, 10 points, triplicates) range from 97.3 to 113.0% for E1 and from 98.1 to 106.6% for E2. Inter assay

Table 2
CV measurements on serum samples in pg/mL (pmol/L).

	LOQ	Intra-assay		Inter-assay
E2				
n	7	7	8	8
Mean	0.28 (1.03)	1.13 (4.15)	18.9 (69.4)	21.1 (77.4)
SD	0.05 (0.18)	0.11 (0.40)	0.67 (2.46)	0.84 (3.08)
%CV	18.3	9.8	3.7	4.0
E1				
n	7	7	8	8
Mean	0.49 (1.81)	1.60 (5.92)	24.0 (88.8)	25.1 (92.9)
SD	0.06 (0.22)	0.10 (0.37)	1.62 (5.99)	1.91 (7.07)
%CV	12.1	6.3	6.7	7.6

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. CV measurements are summarized in Table 2.

Using very low serum pools and spiked steroid free serum, LOQ (<20%CV, $N >= 6$) could be ascertained at 0.3 pg/mL (1.1 pmol/L) for E2 and well below 0.5 pg/mL (1.9 pmol/L) for E1 (12% CV at this level). Typical chromatograms of very low serum samples are shown in Fig. 3.

These LOQ's were determined using the current development based on 500 μL serum. In an experiment using only 100 μL of serum (which may be appropriate if very limited sample is available) a CV of 12.2% for E2 at 5 pg/mL (18 pmol/L) and a CV of 10.3% for E1 at 7 pg/mL (26 pmol/L) was obtained. Thus when using only 100 μL of serum instead of 500 μL, LOQ will still remain well below 5 pg/mL (18 pmol/L), important for studies on newborn children with limited sample availability.

3.3. Discussion and comparison with routine assays

Middle and Kane [11] observed in their review of estradiol assays major differences in trueness between most currently used estradiol assays. To make matters worse most manufacturers also try to compensate for inherent aspecificity issues by manipulating calibration curves to give lower results in the low range, yielding improbable recoveries. These routinely used assays were deemed unfit for most clinical applications requiring low estradiol measurements based on EQC sample results. To assess the severity of the problem not only up to this level but also for very low real patient samples such as seen in pre-pubertal children and postmenopausal or amenorrheic women we compared the 2D-LC–MS/MS method to a routine method (Modular E170 ECLIA estradiol assay by Roche Diagnostics). This method was among the better assays in the review in terms of trueness and

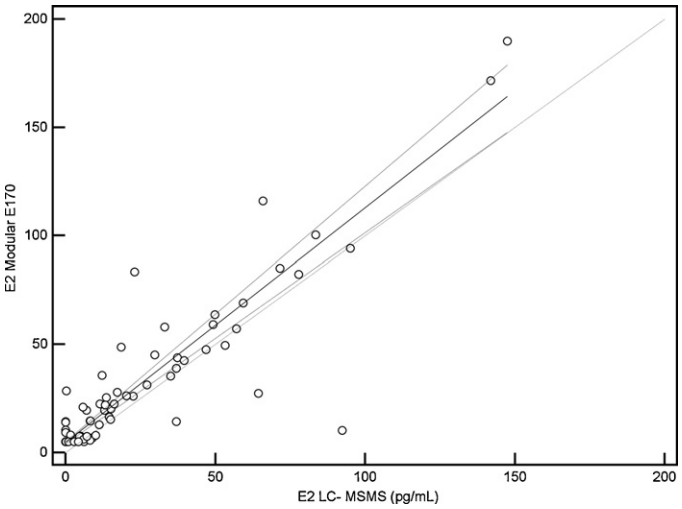

Fig. 4. E2 (Modular E170 Roche versus 2D-LCMSMS) (samples below 200 pg/mL) (734 pmol/L) (Passing Bablock).

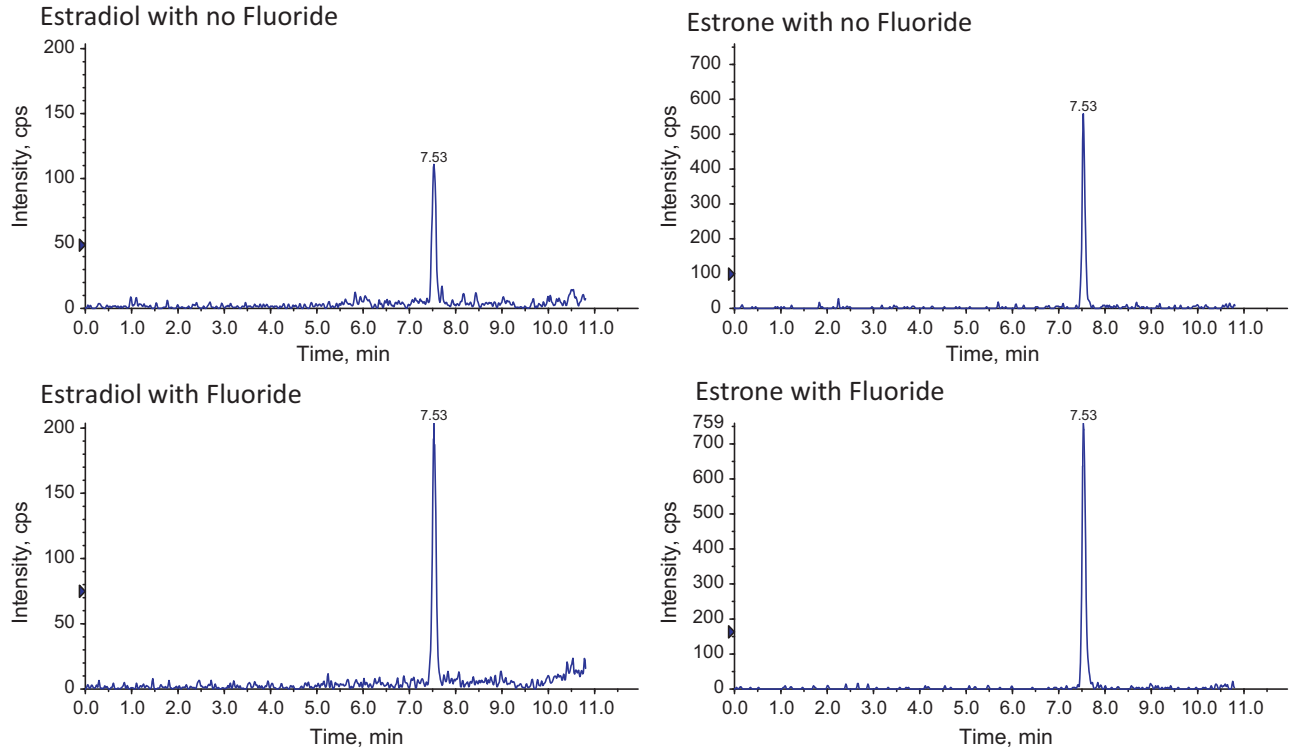

Fig. 5. XIC traces for estradiol (left panels) and estrone (right panels) injected as standards with the 2nd-dimension chromatographic eluent containing no (upper panels) and 0.2 mM Fluoride (lower panels).

variability versus ID-GCMS and is one of the most widely used immunoassays. Deming regression shows a regression equation of E170 = 1.68 + 1.18 LC–MS/MS (95% CI 1.04–1.32) (n = 75). Even if overall correlation and scatter are acceptable for clinical interpretation of normal female samples (which confirms the earlier ID-GCMS results), below 100 pg/mL (370 pmol/L) specificity problems become increasingly apparent. Below 20 pg/mL (74 pmol/L) accuracy rapidly deteriorates and ends completely near 10 pg/mL (37 pmol/L) therefore excluding any accurate use for pediatric or postmenopausal studies (Fig. 4). For estrone which was compared to the Bio-line RIA a different but equally serious problem was observed (Deming regression: RIA = 29.8 + 0.46 × LC–MS/MS, 95% CI 0.31–0.60) (n = 29). A substantial intercept of 30 pg/mL (110 pmol/L) and an obviously narrow dynamic range seriously limit the practical usability of the immunoassay and are one further illustration of the need for ultra sensitive LC–MS/MS measurements to enable accurate clinical assumptions.

4. Conclusions

The primary goal of this endeavour was to devise a robust, accurate and ultrasensitive determination of E2 and E1, convenient for large-scale studies and avoiding unwanted derivatization steps as current commercial direct estrogen assays are unusable for these samples. The finalized setup, although employing sophisticated plumbing, has proven that measuring serum values well below 0.5 pg/mL (1.9 pmol/L) with excellent CV's, needed for pediatric, pre-pubertal and postmenopausal studies can be very reliably achieved on a daily basis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.02.034.

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