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Measurement of estradiol, estrone, and testosterone in postmenopausal human serum by isotope dilution liquid chromatography tandem mass spectrometry without derivatization

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ABSTRACT

Background: A high-throughput, sensitive, specific, mass spectrometry-based method for quantitating estrone (E1), estradiol (E2), and testosterone (T) in postmenopausal human serum has been developed for clinical research. The method consumes 100 μl human serum for each measurement (triplicates consume 300 μl) and does not require derivatization. We adapted a commercially available 96-well plate for sample preparation, extraction, and introduction into the mass spectrometer on a single platform.
Methods: Steroid extraction from serum samples and mass spectrometer operational parameters were optimized for analysis of estradiol and subsequently applied to other analytes. In addition to determining the limit of detection (LOD) and limit of quantitation (LOQ) from standard curves, a serum LOQ (sLOQ) was determined by addition of known steroid quantities to serum samples. Mass spectrometric method quantitative data were compared to results using a state-of-the-art ELISA (enzyme-linked immunosorbent assay) using stored serum samples from menopausal women.
Results: The LOD, LOQ, sLOQ was (0.1 pg, 0.3 pg, 1 pg/ml) for estrone, (0.3 pg, 1 pg, 3 pg/ml) for estradiol, and (0.3 pg, 1 pg, 30 pg/ml) for testosterone, respectively. Mass spectrometry accurately determined concentrations of E2 that could not be quantified by immunochemical methods. E1 concentrations measured by mass spectrometry were in all cases significantly lower than the ELISA measurements, suggesting immunoreactive contaminants in serum may interfere with ELISA. The testosterone measurements broadly agreed with each other in that both techniques could differentiate between low, medium and high serum levels.
Conclusions: We have developed and validated a scalable, sensitive assay for trace quantitation of E1, E2 and T in human serum samples in a single assay using sample preparation method and stable isotope dilution mass spectrometry.

1. Introduction

The estrogens (estrone E1, estradiol E2, and estriol E3), testosterone (T), and progesterone (P4) are steroid hormones with numerous, characterized functions in adults, where the steroid concentrations are relatively abundant and can be routinely measured [1]. The biology of steroids at lower concentrations is less understood, primarily because the methods to quantitate steroids in low abundance are insufficiently accurate, specific, sensitive, or reproducible [2,3]. Peri- or postmenopausal women and geriatric men constitute two growing populations whose quality of life can likely be improved by assessment of levels of the estrogens,

Abbreviations: E1, estrone; E2, estradiol; T, testosterone; μl, microliter; LOD, limit of detection; LOQ, limit of quantitation; sLOQ, serum limit of quantitation; ELISA, enzyme-linked immunosorbent assay; pg, picogram; ml, milliliter; E3, estriol; P4, progesterone (pregn-4-ene-3,20-dione); LC, liquid chromatography; MS/MS, tandem mass spectrometry; MS, mass spectrometry; MRM, multiple reaction monitoring; ε, extinction coefficient; *m/z*, mass to charge ratio; RCF, relative centrifugal force; s/n, signal to noise ratio; CoV, coefficient of variation; NIST, National Institutes of Standards; HPLC, high performance liquid chromatography.

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testosterone and perhaps progesterone, below current reliable detection limits [4]. The menopausal transition can negatively impact women's lives both psychologically (irritability, anxiety, insomnia, mood imbalance, and depression [4,5] and physically (hot flashes, metabolic syndrome and osteoporosis [4,6]. Elderly men also experience a decline in their physical stature and cognitive function, which are thought to be caused by an age-related decrease in testosterone production [7]. Similar issues related to accurate sex steroid measurement complicate pediatric diagnosis and therapy for abnormalities of pubertal maturation, because the cardinal sex steroids are often at or below currently available detection limits in these individuals [2].

For hormone measurement at relatively low circulating concentrations, traditional immunoassays such as ELISAs (enzyme-linked immunosorbent assay) suffer from nonspecific antibody interactions, inconsistent reproducibility, inadequate sensitivity, and require separate assays for each compound of interest [8]. Alternately, a stable isotope dilution mass spectrometry based method directly analyzes the steroid of interest for unambiguous identification and quantitation of multiple hormones in a single sample. The stable isotope standard normalizes for sample loss during preparative steps, indicates an elution time to compensate for any chromatographic drift and can highlight the presence of isobaric contaminants with identical mass transitions by comparing the ratio of several mass transitions [9]. Furthermore, this method is compatible with time-course studies with frequent, small volume, blood sampling and with studies that address the low steroid concentration biology.

We sought to develop a mass spectrometry-based method to quantitate the sex steroids E1, E2, E3, P4 and T primarily for postmenopausal clinical studies and secondarily for elderly men and children. The elderly populations have low concentrations of the above mentioned sex steroids while the sample volume drawn from children is small. A volume and sensitivity constraint of 100 µl and 1 pg/ml for each steroid was established as a goal for method development. We chose a non-derivatized approach because derivatization introduces an additional source of variation, increases sample preparation time and cost, and requires multiple chemical derivatives if the compounds of interest have different functional groups. Moreover, it has been documented that derivatization conditions can hydrolyze some estrogens resulting in erroneous measurements [8,10]. We also sought to implement a reproducible sample preparation method amenable to the considerable number of samples utilized in clinical studies.

While there are a number of excellent mass spectrometry based assays, none were sufficient to overcome the constraints listed above. A sampling of the literature for current E2 mass spectrometry based quantitative methods reveals current methods are quite sensitive but require chemical derivatization [11,12], larger sample volume requirement [13–15], or sample preparation methods not amenable to the number of samples in clinical studies.

We present herein the development of a readily scalable sample preparation technique along with a sensitive and reproducible stable isotope dilution tandem mass spectrometry based method to quantitate E2, E1, E3, and T in postmenopausal serum without the use of derivatization reagents. In addition to the four steroids listed above, we also analyzed P4 but excluded it from our final assay because of an unidentified, isobaric contaminant with identical mass transitions to those used for quantitating P4. Our assay consumes little serum (100 µl per measurement) and utilizes a commercially available 96-well plate.

2. Materials and methods

2.1. Reagents and solvents

Estrone, 17-β-estradiol, estriol, progesterone, testosterone, methanol, acetonitrile, isopropyl alcohol, and sodium hydroxide were of the highest grade commercially available from Sigma–Aldrich (St. Louis, MO). The following stable isotopes were purchased from Cambridge Isotope Laboratories (Andover, MA): [D_4]-estradiol (2, 4, 16, 16, 95–97 atom% [D_4]), [D_4]-estriol (2, 4, 16, 17, 98 atom% [D_4]), [D_4]-estrone (2, 4, 16, 16, 97 atom% [D_4]), and [D_9]-progesterone (2, 2, 4, 6, 6, 17A, 21, 21, 21, 98 atom% [D_9]). [$^{13}C_3$]-Testosterone (2, 3, 4, 99 atom% [$^{13}C_3$]) was purchased from Cerilliant (Round Rock, TX). Stable isotope structures listed in Supplementary Table 1.

2.2. LC–MS/MS

Two Shimadzu UFLCXR 50326 LC-20AD pumps (Kyoto, Japan) and a Leap Technologies PAL HTC-xt Sample Handler (Carrboro, North Carolina) were coupled to an AB Sciex Triple Quad QTRAP 5500 ESI-LC–MS/MS mass spectrometer (Framingham, MA) for all

Table 1
Mass spectrometry method characteristics.

Analyte	Mass transitions (quantifier/qualifier) *m/z*	LC tR (min)[c]	Ext. rec. (%)[d]	LOD (pg)[e]	LOQ (pg)[e]	Within assay var. (%)[f]	Bet. assay var. (%)[f]	Serum LOQ (sLOQ) (pg/ml)[g,i]	CoV at sLOQ (%)
E3[a]	287.2 → 171.1/145.1	4.7	96	1.0	1.0	6.5	3.0	30.0	5.9
[D_4]-E3	291.2 → 173.1/147.1								
E1[a]	269.2 → 145.1/183.1	7.1	86	0.1	0.3	6.9	4.5	1.0	4.3
[D_4]-E1	273.2 → 147.1/187.1								
E2[a]	271.2 → 183.1/145.1	7.3	96	0.3	1.0	8.2	3.0	3.0	3.4
[D_4]-E2	275.2 → 187.1/147.1								
T[b]	289.2 → 97.1/109.1	7.6	82	0.3	1.0	7.2	3.5	30.0	8.5
[$^{13}C_3$]-T	292.2 → 100.1/112.1								
P4[b]	315.2 → 97.1/109.1	9.0	50	0.3	1.0	7.3	2.9	N/A[h]	N/A[h]
[D_9]-P4	324.2 → 100.1/113.1								

[a] Estrone, estradiol, and estriol are analyzed as negative ions.
[b] Testosterone and progesterone are analyzed as positive ions.
[c] Liquid chromatography retention time.
[d] Extraction recovery.
[e] Limit of detection (LOD) and quantitation (LOQ) calculated from standard curve (methanol solvent).
[f] Calculated from standard curves (within assay variability $n = 5$, between assay variability $n = 3$).
[g] LOQ and variability calculated from addition of standards to unaltered serum samples ($n = 3$).
[h] Cannot determine due to chemical interference (see Fig. 1).
[i] To convert E3 (pg/ml) to pM, multiply by 3.47, E2: 3.67, E1: 3.72, T: 3.47, P4: 3.18.