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Measurement of Estradiol in Human Serum by LC-MS/MS Using a Novel Estrogen-Specific Derivatization Reagent

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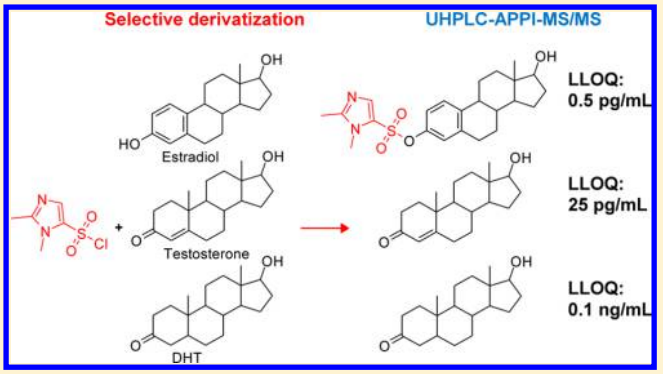
Supporting Information

ABSTRACT: A liquid chromatography−tandem mass spectrometry (LC-MS/MS) method is described that employs a novel derivatization reagent for the measurement of serum estradiol (E2), with simultaneous analysis of underivatized testosterone (T) and dihydrotestosterone (DHT). The main advantage of the new derivatization reagent 1,2-dimethylimidazole-5-sulfonyl chloride is its analyte-specific fragmentation that enables monitoring of confirmatory mass transitions with high sensitivity. The reaction mixture can be analyzed without additional purification steps using a 9.5 min gradient run, and sensitive detection is achieved with a triple quadrupole mass spectrometer using atmospheric pressure photoionization. Method validation was performed with human serum samples, including a comparison with a standard LC-MS/MS method using 120 samples from a clinical study, and analysis of certified E2 serum reference materials BCR-576, BCR-577, and BCR-578. The lower limits of quantification for E2, T, and DHT were 0.5 pg/mL, 25 pg/mL, and 0.10 ng/mL, respectively, from a 200-μL sample. Validation results indicated good accuracy and agreement with established, conventional LC-MS/MS assays, demonstrating suitability for analysis of samples containing E2 in the low pg/mL range, such as serum from men, children, and postmenopausal women.



Measurement of 17β-estradiol (E2) in the clinical setting has been performed by various immunoassays for decades,[1] but growing evidence of sensitivity issues and method-specific bias of automated, unextracted (direct) immunoassays[2−6] has increased interest in methods based on mass spectrometry, particularly liquid chromatography−tandem mass spectrometry (LC-MS/MS). However, despite the potentially higher selectivity of LC-MS methods, it has been difficult to achieve the sensitivity required for the analysis of specimens with very low E2 levels, such as serum from children, men, postmenopausal women, and women receiving aromatase inhibitors for breast cancer treatment.[7,8] Yet, the clinical importance of measuring E2 can be high in these populations, requiring methods capable of accurately and precisely determining <5 pg/mL E2 concentrations in routine clinical specimens.[5]

A major factor that limits the sensitivity of the methods is the inherently weak ionization efficiency of E2. While ionization of E2 can be achieved with all the common commercially available ion sources (electrospray, atmospheric pressure chemical ionization, atmospheric pressure photoionization),[9] several methods have been described that employ chemical derivatization of E2 with structures having higher gas phase proton affinity or a permanent charge.[10−17] One particularly common reagent for E2 derivatization is dansyl chloride, a traditional fluorescent label for amino acids and proteins.[18−22] An advantage of dansyl derivatization of E2 is that it can be performed in an aqueous reaction solvent under mild conditions, which also makes it possible to inject the reaction mixture directly into a reversed phase column.[18] Moreover, sulfonyl chloride reagents such as dansyl are typically not reactive with the 17-hydroxyl group of E2, which limits the number of reaction products and detectable ion species, greatly simplifying the analysis. This also enables selective derivatization of estrogens, as other steroids without phenolic hydroxyl groups are not reactive with dansyl.[23] However, a notable drawback of this method is related to the collisional fragmentation of dansyl-E2 ion, where the charge is almost completely retained by the dansyl moiety.[18] Although this contributes to high signal intensity, it also prevents monitoring of additional, confirmatory mass transitions, increasing the susceptibility of the method to interferences from other compounds in the sample that react with dansyl chloride and cannot be distinguished from dansyl-E2. The only alternative sulfonyl chloride reagent described for the LC-MS/MS analysis of E2 with fragmentation that has been shown to enable sensitive monitoring of confirmatory mass transitions is pyridine-3-sulfonyl chloride (PS).[24]

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In this study, we aimed to improve the sensitivity of our existing LC-MS/MS method[25] to enable reliable measurement of sub-5 pg/mL E2 concentrations in human serum. To achieve this, we set out to develop a derivatization method that would allow monitoring of confirmatory mass transitions for E2, while retaining the ability of the original method to measure key androgens testosterone (T) and dihydrotestosterone (DHT). Herein, we report the development and validation of this method, which is based on selective derivatization of E2 with a novel commercially available sulfonyl chloride reagent that provides increased sensitivity over the previously reported PS.

MATERIALS AND METHODS

Chemicals and reagents. A certified 1 mg/mL E2 solution in acetonitrile was purchased from Cerillant. Certified reference materials for E2 (BCR-576, BCR-577, BCR-578) were obtained from European Commission's Institute for Reference Materials and Measurements. Internal standard (ISTD) of E2 (17β-estradiol-2,4,16,16-d4) was from Cambridge Isotope Laboratories. T, DHT, and their ISTDs (testosterone-1,2,3-d3; DHT-16,16,17-d3) were from Australian National Measurement Institute (NMI). Steroids for interference testing were from Steraloids (estrone, etiocholanolone, epietiocholanolone, 5-androstenediol, epiandrosterone, androstanedione, etiocholanedione, dehydroandrosterone) and NMI (androsterone, dehydroepiandrosterone, epitestosterone). Derivatization reagents PS and 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS; Aldrich CDS016647) were from Matrix Scientific and Sigma-Aldrich, respectively. Acetone was of analytical reagent grade from Ajax Finechem, HPLC-grade toluene and premium-grade methyl *tert*-butyl ether (MTBE) were from RCI LabScan. Methanol was of LC-MS grade (Sigma-Aldrich) and water from a Milli-Q Advantage system. Serum and plasma used for the calibration, QC and validation samples were pooled human serum and plasma. Activated charcoal (Norit A) was from BDH Chemicals. BSA was purchased from Calbiochem and PBS from Medicago. Autosampler vials were of extreme recovery type from Agilent Technologies.

Preparation of calibration standards. Stock solutions of T and DHT (1.2 mg/mL) were prepared in methanol. E2 stock solution (1 mg/mL) was a certified spiking solution from Cerillant. Each stock solution was diluted to 1 μg/mL with methanol and mixed in a 20 mL volumetric flask with water to form a working solution with 52.5% methanol content and following analyte concentrations: T: 500 ng/mL, DHT: 500 ng/mL, E2:25 ng/mL. Calibration samples were prepared by spiking 2904 μL of charcoal stripped plasma (CSP) with 96 μL of working solution and diluting with unspiked CSP to obtain a series of 11 calibration samples with concentrations 0.01−16 ng/mL for T and DHT, and 0.5−800 pg/mL for E2. QC samples were prepared similarly (T, DHT: 0.025, 0.2, 1.6, 8 ng/mL; E2:1.25, 10, 80, 400 pg/mL). CSP was prepared by mixing plasma with activated charcoal (50 mg/mL), stirring overnight at room temperature, centrifuging and filtering with 0.45 and 0.22 μm poly(ether sulfone) filters.

ISTD stock solutions were prepared in methanol and diluted with 20% (v/v) methanol:water to obtain a working solution (d3-T: 5 ng/mL, d3-DHT: 6.25 ng/mL, d4-E2:0.25 ng/mL).

Serum samples and preparation. Serum samples for method comparison were drawn from frozen sera stored from the ongoing Concord Health and Aging in Men Project (CHAMP) study of men over the age of 70 years, approved by the Human Research Ethics Committee (Concord Hospital) of the Sydney Local Health District within NHMRC guidelines for human experimentation, which comply with the Helsinki Declaration.[26] Samples (200 μL) were prepared in 12 × 75 mm borosilicate glass tubes by adding 50 μL of ISTD working solution, briefly mixed, equilibrated for 15 min at 4 °C, and extracted with 1 mL of MTBE. Organic extracts were transferred into vials, evaporated to dryness and reconstituted in 30 μL of sodium bicarbonate (50 mM, pH 10.5) and then 20 μL of 1 mg/mL DMIS in acetone. Vials were capped, heated at 60 °C for 15 min, and mixed shortly before analysis. Calibrants, QCs, and blanks were prepared in the same manner.

LC-MS/MS conditions. The HPLC system was Shimadzu Nexera with a Phenomenex Kinetex Phenyl-Hexyl column (100 × 2.1 mm; 1.7 μm). Column temperature was 50 °C and flow rate 0.38 mL/min with the following gradient (A: water, B: methanol): 0 min: 30% B, 0.01−5.75 min: from 55% to 70% B, 5.76−7.00 min: 100% B, 7.01−9.50 min: 30% B. Eluent was diverted to waste at 0−3.3 and 7.0−9.5 min. Injection volume was 25 μL and the autosampler temperature 15 °C. An AB SCIEX API 5000 triple quad mass spectrometer was used with PhotoSpray (APPI) ion source in positive polarity. Toluene was used as dopant, delivered at 0.038 mL/min. Ion source, curtain, and collision gas was nitrogen. Multiple reaction monitoring was employed with both quadrupoles at unit resolution. Two mass transitions were monitored for each analyte as follows (first product ion quantitative, second confirmatory): T: 289 → 109 + 253, d3-T: 292 → 109 + 256, DHT: 289 → 187 + 205, d3-DHT: 292 → 190 + 208, E2:431 → 96 + 367, d4-E2:435 → 96 + 371. Additional instrument parameters can be found in the Supporting Information.

Data analysis. Data acquisition and processing was performed with Analyst 1.6.2 (AB SCIEX). Peak area ratios of analyte and ISTD quantifier transitions were calculated as a function of analyte concentration. Calibration curves and lower limits of quantification (LLOQs) were according to the FDA guidance.[27] Selectivity was monitored by calculating the response ratio of the quantifier and confirmatory ions (QI/CI ratio) for both analytes and their ISTDs, and comparing the QI/CI ratio of the calibrants to that of the unknown samples. Statistical analyses were performed with Analyse-it 3.90.5.

Method validation. Precision of the method was determined by replicate analyses of unaltered pooled samples representing different concentrations. Two sets of pooled serum (male and pediatric) were prepared, from which six replicate samples were analyzed on 4 days. To expand the concentration range in the male and pediatric serum pools, we prepared two additional sample pools (serum and plasma), from which five replicate samples were analyzed on 3 days.

Accuracy of the E2 measurement was evaluated by analyzing certified serum reference materials BCR-576 (31 pg/mL, 0.114 nM), BCR-577 (188 pg/mL, 0.69 nM), and BCR-578 (365 pg/mL, 1.34 nM), and calculating the mean percent bias from the specified concentrations.

In addition, we analyzed 120 individual patient serum samples with low expected E2 levels and compared with the concentrations assigned by our previously published assay[25] (with modifications described in Supporting Information). Moreover, recovery studies were performed by spiking a pool of plasma with working standard solution and diluting with unspiked plasma to 0.4, 0.8, 1.6, and 4 ng/mL for T and DHT and 20, 40, 80, and 200 pg/mL for E2. Five replicate samples per pool were analyzed and recoveries were calculated using the

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equation: [(mean final concentration - mean initial concentration)/added concentration].

Extraction recovery was studied by preparing two sets of samples from male and pediatric serum pools; one as described above and the other by replacing the ISTD working solution with 20% (v/v) methanol, extracting normally and spiking with the ISTD solution before evaporation and derivatization. Extraction recovery was calculated by comparing the mean ISTD peak areas of pre- and postextraction spiked samples. To evaluate matrix effect (ME), the postextraction spiked serum samples were compared with a set of evaporated and derivatized neat solutions according to the equation ME(%) = B/A × 100, where A is the mean ISTD peak area in neat samples, and B the mean ISTD peak area in the postextraction spiked serum (n = 6 for all the samples). In addition, slopes of the calibration curves in CSP and in 4% (w/v) BSA in PBS were compared.

Postpreparative sample stability was determined by analyzing two sets of five replicate plasma samples, one immediately after its preparation and the other after storage for 17 h in the autosampler. Reinjection reproducibility was studied by analyzing a set of 5 replicate plasma samples immediately and again after 12 h. Mean concentration of the stored samples were compared with that of the freshly analyzed set.

Carry-over was examined by injecting a solvent blank after the highest calibrant in six runs and comparing the analyte peak areas in the blank with that of the calibrant. Selectivity was determined by analyzing neat solutions of structurally similar, potentially interfering steroids. Lack of ISTD interferences in the sample matrix were assured by analyzing unspiked CSP, serum, and plasma samples. In addition, QI/CI response ratios of analytes and ISTDs in 149 patient serum samples were compared with those of calibrators from the same batch. Interference was assumed if the QI/CI ratios differed more than 30%.

RESULTS AND DISCUSSION

Derivatization. Our experiments with various derivatization reagents suggested sulfonyl chlorides such as dansyl and PS (Figure 1) to be most practical, due to their selective reactivity with phenolic hydroxyl groups and compatibility with aqueous reaction solutions. Different sulfonyl chlorides are commercially available as building blocks for organic synthesis, which led us to experiment with DMIS. A structural isomer of DMIS (1,2-dimethylimidazole-4-sulfonyl chloride) has been described earlier as a derivatization agent for 1-hydroxypyrene and other phenolic compounds,[28−30] but when combined with E2, its fragmentation was reported to be nonspecific, resulting almost entirely in ions originating from the dimethylimidazole moiety.[24] In contrast, the isomer we employed in the present study appears to undergo a different dissociation process, resulting in charged fragments m/z 272 and m/z 367 that both contain E2 (Figure 2). We propose that the ion m/z 367 is formed by the loss of SO_2 from the protonated DMIS-E2 with rearrangement to $[C_{23}H_{31}N_2O_2]^+$. The corresponding ion for d4-E2 would be m/z 371, which was also observed. These fragments are analogous to those proposed for PS derivatives of E2.[24] The ions m/z 272 and m/z 96 have been described earlier and are most likely the radical E2 cation and a protonated dimethylimidazole moiety, respectively.[28] The ion m/z 161 is assigned to SO_2-containing dimethylimidazole $[C_5H_9N_2O_2S_1]^+$ as also described earlier.[30]

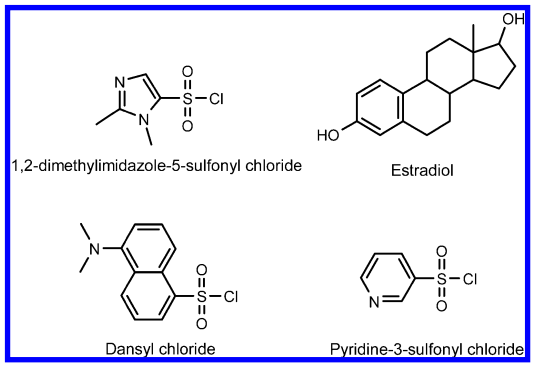

Figure 1. Chemical structures of the novel derivatization reagent (1,2-dimethylimidazole-5-sulfonyl chloride), estradiol, and two previously reported sulfonyl chloride reagents.

The greatest sensitivity for the derivatized E2 was achieved using mass transitions m/z 431 → 367 and m/z 431 → 96 that had an intensity ratio of approximately 2:10. Due to the very low background of the first transition, both could be used throughout the concentration range of the method. For quantification, the transitions were summed for increased signal-to-noise (S/N) ratio.

The derivatization reaction was not sensitive to temperature or time. Heating at 60 °C for 10−15 or 50−60 min (n = 6) had no significant effect on the measured mean plasma E2 concentration (P = 0.68). Moreover, heating at 60 °C for 10−15 min (n = 6) or keeping the samples at room temperature resulted in the same concentrations (P = 0.77).

When compared with PS using identical reaction conditions and chromatography with individually optimized detection parameters, DMIS provided a stronger response. The peak areas (n = 5) of the two most intense transitions of DMIS-E2 were 6.3- and 2.5-fold greater than those of PS-E2 (m/z 414 → 272, m/z 414 → 350) (both P < 0.001). The corresponding S/N ratios of DMIS-E2 were also 3.0- and 1.6-fold greater (P = 0.002 and P = 0.016, respectively). Summing the transitions increased the response and S/N ratio of both DMIS-E2 and PS-E2. However, DMIS-E2 still exhibited 5.0 times greater peak area and 2.5 times greater S/N ratio (both P < 0.001). Thus, derivatization of E2 with DMIS was found to result in a significant sensitivity advantage over PS, without losing the ability to monitor analyte-specific transitions.

Since DMIS was inactive toward the 17-hydroxyl group of E2, we assumed that steroids without phenolic hydroxyls would be unaffected by the reaction. This was indeed the case and using APPI in positive polarity enabled detection of both the derivatized E2 and the underivatized androgens, making it possible to analyze them in a single run without polarity switching.[25] In addition to the suitability of APPI for the analysis of T and DHT in positive polarity, we have found it to be less susceptible to matrix effects and background interferences than ESI.[9] However, when using standard solutions without biological background, we were able to achieve similar sensitivity and linear range for DMIS-E2 using any of the common ionization techniques (ESI, APCI, APPI) (results in the Supporting Information).

The ion source conditions and the MRM transition for T could be in large part transferred to the developed method from our existing T assay. However, for the analysis of DHT, a new MRM transition was developed. With the significantly lower ion source temperature required for DMIS-E2, we found

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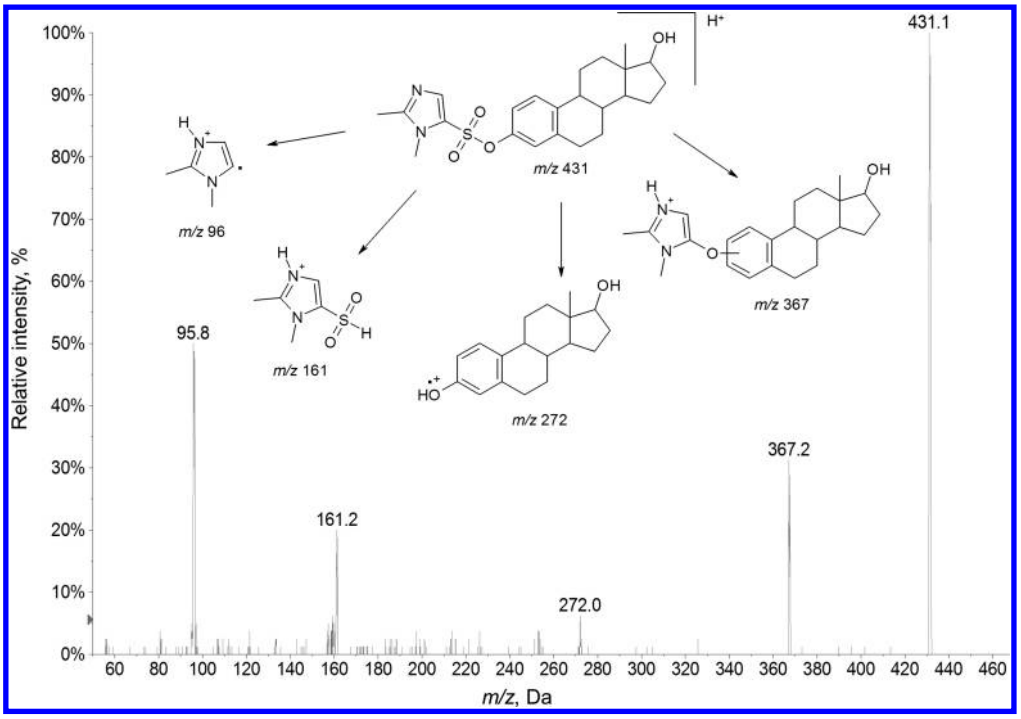

Figure 2. Product ion spectrum of derivatized E2 with proposed structures. Sum spectrum from a 33−41 eV collision energy ramp.

Table 2. Precision of the Developed LC-MS/MS Assay for the Analysis of Estradiol, Testosterone, and DHT in Unaltered Human Male and Pediatric Serum

	Male serum[a]				CV, %	
	Day 1	Day 2	Day 3	Day 4	Within-run	Between-run
Estradiol, pg/mL	17.1 ± 0.7	17.1 ± 0.9	17.3 ± 1.8	16.5 ± 1.2	3.6	6.1
Testosterone, ng/mL	3.01 ± 0.10	2.93 ± 0.08	2.72 ± 0.29	3.01 ± 0.24	2.7	6.9
DHT, ng/mL	0.31 ± 0.02	0.32 ± 0.02	0.36 ± 0.04	0.27 ± 0.07	5.9	11.9
	Pediatric serum[a]				CV, %	
	Day 1	Day 2	Day 3	Day 4	Within-run	Between-run
Estradiol, pg/mL	5.6 ± 0.2	5.6 ± 0.2	6.5 ± 0.8	5.6 ± 0.3	3.3	9.7
Testosterone, ng/mL	0.48 ± 0.02	0.47 ± 0.03	0.46 ± 0.07	0.49 ± 0.03	3.1	7.7
DHT, ng/mL	<LLOQ	<LLOQ	<LLOQ	<LLOQ		

[a]Measured mean values ±95% confidence intervals (n = 6); <LLOQ: concentration below the lower limit of quantification.

the most intense ion for DHT to be m/z 289 instead of the commonly observed m/z 291 $[M + H]^+$ or m/z 273 $[M - H_2O + H]^+$ that we used in our existing DHT assay. This type of ion, assigned $[M - 1]^+$, has been previously observed for some classes of compounds using APPI, APCI, and MALDI[31−33] and may be a result of hydride abstraction or a loss of hydrogen from $[M + H]^+$. Although $[M - 1]^+$ is not well-known as a precursor ion in quantitative LC-MS/MS, by using it for DHT, we were able to achieve similar sensitivity as with our existing assay, despite being restricted to lower source temperature (390 °C instead of 500 °C).

Measurement of T and DHT was not affected by the derivatization of E2. Two sets of plasma samples (n = 5) processed with and without DMIS in the reaction mixture had comparable T and DHT concentrations (mean ±95% CI): T 2.87 ± 0.11 and 2.91 ± 0.11 ng/mL (P = 0.54); DHT 0.70 ± 0.03 and 0.76 ± 0.13 (P = 0.19), respectively. Moreover, the reaction did not add complexity to the original method, as brief heating of the vials was the only additional sample preparation step needed and could be equally achieved at room temperature. Using autosampler vials with low residual volume as reaction vessels enabled injecting the 50-μL samples twice, allowing reanalysis in the case of instrument interruptions.

Calibration curves and sensitivity. The best linear curve fit was achieved using a weighted ($1/y^2$) regression. The LLOQs in a 200-μL sample were 0.5 pg/mL (1.8 pM) for E2 (CV 15.6%, accuracy 114%), 25 pg/mL (87 pM) for T (CV 16.5%, accuracy 100%), and 0.10 ng/mL (0.35 nM) for DHT (CV 2.2%, 96%). The upper limits of quantification were 0.8 ng/mL for E2, 16 ng/mL for T, and 8 ng/mL for DHT with 11, 10, and 7 calibration levels, respectively. The mean (SD) curve equations from 10 assays were 16.8(2.6)x + 0.02(0.02) for E2, 0.77(0.12)x + 0.02(0.01) for T, and 0.68(0.10)x + 0.10(0.06) for DHT with respective correlation coefficients being 0.998(0.001), 0.995(0.002), and 0.995(0.005).

Precision and accuracy. Within-run and between-run precision values for the male and pediatric serum samples are shown in Table 2. Results from the two additional sets of samples with higher concentrations were as follows (within-run CV, between-run CV): serum E2:22.1 pg/mL (4.7%, 10.2%), serum T: 4.78 ng/mL (2.3%, 10.4%), serum DHT: 0.39 ng/mL (7.7%, 22.5%); plasma E2:11.3 pg/mL (1.9%, 7.5%), plasma T:

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2.53 ng/mL (4.0%, 8.8%), plasma DHT: 0.49 ng/mL (4.3%, 9.9%). For the spiked recovery samples, maximum CVs at all concentration levels were below 5.2%. The E2 reference samples BCR-576 (n = 8), BCR-577 (n = 5), and BCR-578 (n = 4) had mean bias of −1.9% (95% CI: −4.2 to 0.3), −7.6% (95% CI: −8.2 to −6.9), and −8.2% (95% CI: −13.3 to −3.1), respectively, with CVs between 0.5% and 3.5%. Spiked samples had mean accuracies of 96% (E2), 103% (T), and 101% (DHT).

Extraction recovery and matrix effect. Extraction recoveries were 81−92% for all the analytes, with CVs between 4.1% and 10.3%. Spiking experiments suggested slight ion suppression for DHT (ME 85−90%), whereas T was not affected by the sample background (ME 98−103%). Ion enhancement was observed for E2 (ME 123−126%), which may also be the effect of the serum background on the derivatization reaction rather than on ionization. The slopes of the calibration curves prepared in CSP and 4% BSA solution were comparable at 15.5x and 14.7x (E2), 0.676x and 0.662x (T), and 0.726x and 0.711x (DHT), indicating lack of notable matrix effects.

Stability and carry-over. Samples were stable in the autosampler for at least 17 h at 15 °C (run time for 100 samples). Mean concentrations were between 95% and 102% of freshly analyzed samples, with CVs less than 6.5%. Reinjection reproducibility was also good with mean concentrations of the second injection within 1% of those of the first, with CVs less than 10.6%. Carryover in blank injections after the highest calibrant was insignificant at 0.02−0.09% for all the analytes.

Selectivity. None of the structurally related steroids studied interfered with the analytes, being either chromatographically separated or with different mass transitions. ISTD mass transtions were without interferences from the sample matrix. No traces of analytes were observed in derivatized ISTD working solution, indicating stability of the isotope labels during the reaction. When monitoring the QI/CI ratios of analytes and ISTDs in a set of 149 serum samples, all except three of the samples (1 DHT, 2 E2) exhibited QI/CI ratios outside the set ±30% criteria. The concentrations in these samples were close to LLOQs, and thus the deviation is more likely due to too small CI peaks rather than a noteworthy selectivity issue.

Application. In the set of 120 patient serum samples used for the method comparison, 105 had E2 concentration above LLOQ of the developed method, with the median E2 concentration (quartiles 1 and 3) of 10.9 (3.92, 19.7) pg/mL. Analysis of certified serum reference samples resulted in acceptable accuracy, demonstrating good performance of the method at higher E2 levels (31−365 pg/mL). Concentrations of T and DHT were 1.67 (0.11, 3.78) ng/mL and 0.28 (0.18, 0.42) ng/mL with 117 and 76 of the samples above LLOQs, respectively. Representative chromatograms of serum samples are shown in Figure 3.

Comparison of the new and existing assay methods was performed using serum samples where results were above LLOQ for both methods (81 samples for E2, 115 samples for T, and 58 samples for DHT). Passing−Bablok regression analyses and Bland−Altman plots are shown in Figure 4. The results indicated lack of a statistically significant difference between the E2 assays, yielding a regression slope of 1.10 (95% CI: 0.99 to 1.20) and an intercept of 1.19 (95% CI: −0.26 to 2.70). A similar result was obtained for T (slope 0.99, 95% CI: 0.97 to 1.02), with only a minor systematic difference suggested

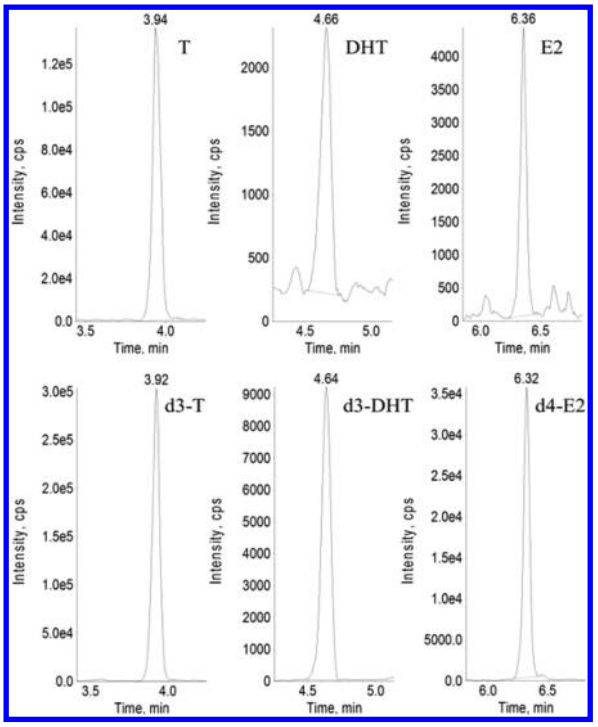

Figure 3. Serum MRM chromatograms of testosterone (T), dihydrotestosterone (DHT), and DMIS-derivatized estradiol (E2), with corresponding internal standards. Sample concentrations: T 0.48 ng/mL (1.67 nM); DHT 0.31 ng/mL (1.07 nM); E2 5.6 pg/mL (20.6 pM).

by the intercept of 0.009 (95% CI: 0.002 to 0.015). Regression analysis for DHT resulted in a slope of 1.07 (95% CI: 0.88 to 1.4) with an intercept of −0.03 (95% CI: −0.14 to 0.03), showing agreement between the methods but also considerably higher variability than that for E2 and T. The median percent bias values for E2, T, and DHT were 17.1% (95% CI: 12.9 to 22.1), 2.0% (95% CI: −1.3 to 6.3), and −2.5% (95% CI: −10.3 to 4.3), respectively. Overall, the results indicate agreement between the new and standard LC-MS/MS assays, except for E2 at the 5−10 pg/mL range of the standard method, where some samples show relatively large positive bias (see the log-scaled regression and Bland−Altman plots in Figure 4). The difference observed for these samples is most likely a result of low-level background interferences in the serum that contribute to the MRM peak of E2 used in the standard method (m/z 271 → 145).[9] As the measured concentrations were close to the LLOQ of the standard method (5 pg/mL), reliable monitoring of a qualifier MRM transition was not possible, leaving the origins of the observed E2 response uncertain. This highlights the value of high-intensity qualifier ions that can be monitored throughout the concentration range of the method, such as the secondary product ion for DMIS-E2 (m/z 431 → 367). In the present case, removing the samples with >100% difference (n = 5, Figure 4) produced an E2 regression with a slope of 1.14 (95% CI: 1.04 to 1.27) and an intercept of 0.46 (95% CI: −1.63 to 1.61). The mean difference between the assays after removing the five outlying samples was reduced to 12.9% (95% CI: 6.4% to 19.3%), which we consider an acceptable result for a comparison against a routine assay that is not a reference measurement protocol.

Based on the Passing−Bablok regression and the Bland−Altman difference analysis, there was no systematic bias

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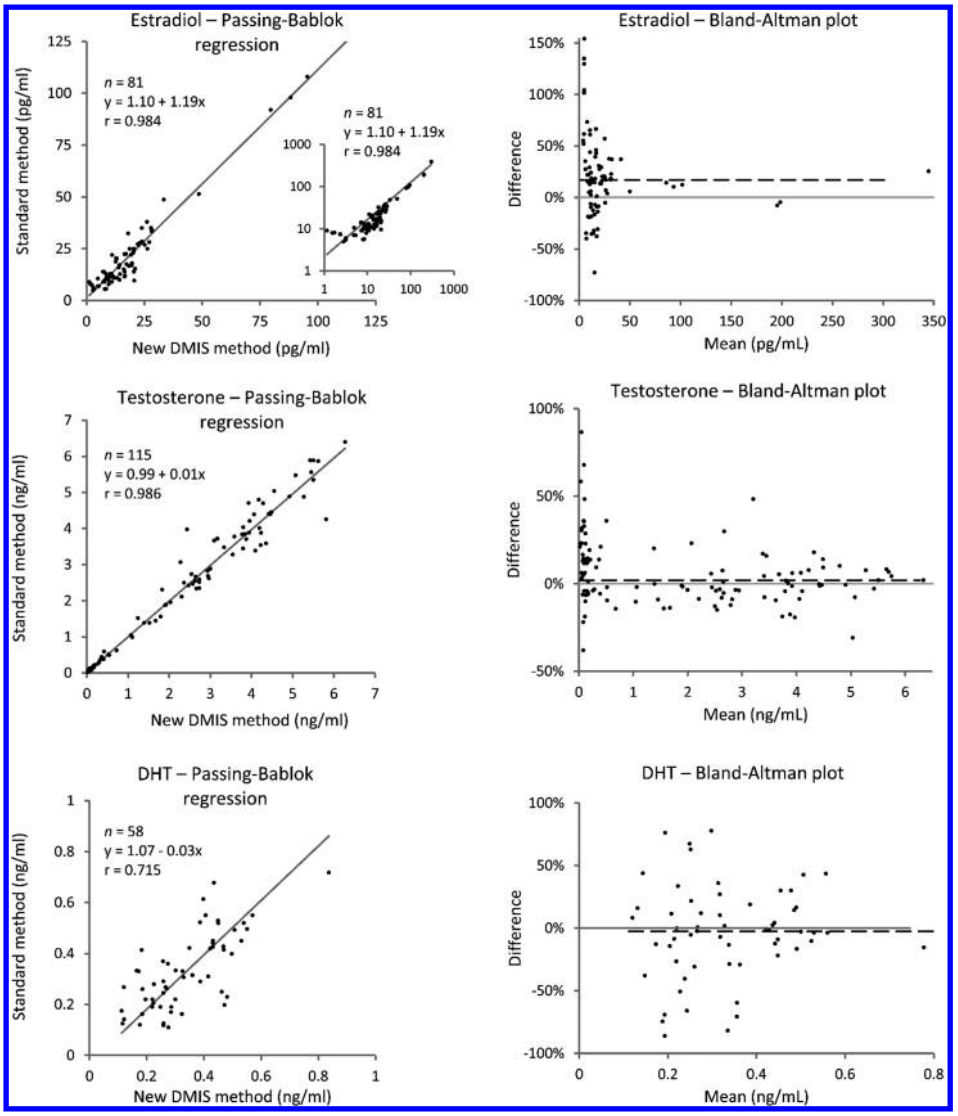

Figure 4. Comparison of the developed method (New DMIS method) with an established LC-MS/MS method that does not include derivatization (Standard method) using patient serum samples. The dashed lines in the Bland–Altman plots represent median difference, with difference calculated as C(Standard method) − C(New DMIS method)/C(Mean), where C is the measured analyte concentration. The inset in the regression plot for estradiol shows the data in logarithmic scale; see text for details.

between the DHT assays, although quantitation was affected by the narrow concentration range and variability in the data. It is clear that even if DHT can be measured with the developed method, its utility is limited to samples with relatively high concentrations. Nevertheless, our results indicate that using the ion m/z 289 as a precursor enables achieving assay sensitivity that is similar to that of a method based on conventional MRMs. Further characterization of the potential of this ion for DHT analysis would be needed, but this was beyond the scope of the study.

By employing DMIS derivatization, we were able to achieve a 10-fold increase in sensitivity for E2 over our existing method. The significance of the additional sensitivity obtained is demonstrated by the results of the method comparison: 29 of the 120 samples had E2 concentrations between 0.5 and 5 pg/mL, being below the LLOQ of our standard method but measurable with the developed assay. However, 15 of the samples remained unmeasurable with concentrations below 0.5 pg/mL, indicating the need for even higher assay sensitivity. This could potentially be achieved with larger sample volumes, additional solvent extractions, larger injection volumes and precolumn concentration, or by employing a mass spectrometer with higher sensitivity. Our preliminary experiments with a more sensitive instrument (QTRAP 6500, AB SCIEX) have resulted in LLOQ of 0.13 pg/mL (0.46 pM) for E2 by using the same method and standards as described here. However, obtaining a calibration matrix free from trace levels of E2 is challenging, so that for accurate analysis of serum E2 at sub-picomolar levels it may be necessary to consider alternative calibration techniques.[34,35]

CONCLUSIONS

In the present study, our aim was to develop an ultrasensitive method for the analysis of E2 in human serum by using a derivatization reagent with analyte-specific fragmentation, while preserving the ability to measure T and DHT. We achieved this by employing a novel, commercially available derivatization reagent 1,2-dimethylimidazole-5-sulfonyl chloride (DMIS). The derivatization reaction is simple and similar to the well-known dansyl chloride, enabling selective derivatization of the phenolic

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hydroxyl group of E2. However, unlike dansyl chloride, it also allows monitoring of E2-specific confirmatory mass transitions through a wide concentration range for improved confidence on method selectivity during routine use. Derivatized E2 can be ionized efficiently with APPI, enabling simultaneous analysis of the androgens without polarity switching or mobile phase additives. In comparison with another known derivatization reagent with analyte-specific fragmentation (pyridine-3-sulfonyl chloride), DMIS provides a significant sensitivity advantage. Based on the validation results and application of the method to human serum samples from a clinical study, this method appears to be suitable for convenient measurement of androgens and estrogens within a single run in pediatric, male, and postmenopausal female serum samples over a wider concentration range than previously possible.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01042.

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Notes

The authors declare no competing financial interest.

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