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Yoo, Hong

Napoli, Joseph

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Quantification of Dehydroepiandrosterone, 17β-Estradiol, Testosterone, and Their Sulfates in Mouse Tissues by LC-MS/MS

Hong Sik Yoo, **Joseph L. Napoli***

Program in Metabolic Biology, Department of Nutritional Sciences and Toxicology, University of California–Berkeley, Berkeley, California 94720, United States

Abstract

We report a high-performance, liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) assay to quantify without derivatizaton dehyroepiandrosterone (DHEA), 17β-estradiol (E2), testosterone (T), and their sulfates in serum and tissues. This assay functions well with multiple adipose depots, a previously unattained analysis. To delipidate and facilitate recovery, tissues were homogenized in acetonitrile, and the homogenate was frozen. The supernatant was evaporated, resuspended in an aqueous acetate buffer, and extracted with hexane to separate free (unconjugated) from sulfated steroids. Sulfated steroids in the aqueous medium were then hydrolyzed with sulfatase and extracted with hexane. Each extract was analyzed separately. HPLC resolution combined with the sensitivity and specificity of MS/MS allowed quantification of DHEA, E2, and T with 10, 10, and 5 fmol lower limits of quantification and linear ranges to 1 pmol. Application of the method to mouse serum and tissues reveals ranges of DHEA, E2, and T and their sulfates, and tissue-specific differences in steroid profile, especially white versus brown adipose. In addition, marginal decreases of T in all tissues and considerable increases in DHEA in male iWAT and eWAT in response to a high-fat diet further strengthen the inference regarding the role of steroid metabolism in adipogenesis. This assay permits detailed studies of interactions between adiposity and sex steroids in serum and tissues, including adipose.

Graphical Abstract

*Corresponding Author: jna@berkeley.edu. Phone: 510-642-5202.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

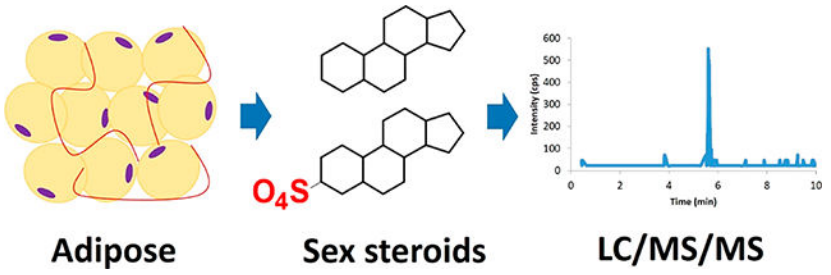
Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b03759.

Sulfatase activity test and correlation between T and T-S (PDF)



Steroid hormones are classified into five major groups: progestens, glucocorticoids, mineralocorticoids, androgens, and estrogens. All are biosynthesized from cholesterol. Since the signaling nature and therapeutic potential of steroids began to be appreciated, dating back to the early 20th century, several quantification methods have been developed, such as competitive ELISA,[1] radioimmunoassay,[2] GC/electron capture detection,[3] and GC/mass spectrometry.[4] With advances in LC/MS, there has been a growing trend toward simultaneous measurement of multiple steroids,[5,6] necessitated by inter-connected metabolic nets, which can be complementary or contrasting. The application of quantitative analytical methods, however, has been limited predominantly to urine and serum.[7,8] Sensitive assays applicable to tissues have been elusive, because of difficulties in maintaining sensitivity for diverse steroids, while minimizing matrix effects and interfering peaks.

Sex steroids correlate with adiposity. Dehydroepiandrosterone (DHEA) therapy induces statistically significant decreases in visceral and subcutaneous pads by 13 cm^2 for each fat pad.[9] In men, muscle size and strength are androgen dose-dependent, whereas fat mass is estrogen-dependent.[10] Free testosterone (T) levels are ~50% higher in obese women compared to nonobese women.[11] In rats, estradiol (E2) activates thermogenesis in brown adipose tissue (BAT).[12] Adipose tissues serve as steroid reservoirs and sites of steroid metabolism, which affect systemic levels. In human abdominal subcutaneous fat, 17β-hydroxysteroid dehydrogenase (HSD) activity correlates inversely with levels of DHEA and DHEA-sulfate.[13] Human breast adipose catalyzes conversion of androstenedione into testosterone, estrone, and estradiol.[14] Hence, sex steroids influence lipid biology in a context-dependent manner.

Apart from free (unconjugated) steroids, sex steroids also occur conjugated with sulfate. Sulfotransferases catalyze sulfo-conjugation using 3′-phospho-adenosine-5′-phosphosulfate as cosubstrate, while steroid sulfatases (STS) liberate steroids from their sulfates, increasing tissue concentrations. Interestingly, steroid sulfation affects intermediary metabolism. High-fat diet (HFD) induced metabolic phenotypes were exacerbated when human STS was overexpressed in adipose of male mice, possibly through increasing androgens.[15] In female mice, however, overexpression of STS ameliorated steatosis caused by a HFD, possibly through increasing estrogen. In contrast, liver STS overexpression reduced obesity and type 2 diabetes in both male and female mice by affecting estrogen and androgen concentrations, respectively.[16]

These data reveal a compelling relationship between sex steroids and adiposity, signifying the importance of quantifying multiple steroids in energy regulating tissues. Here we report

an assay based on high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) to quantify T, DHEA, E2 and their sulfates in serum, liver, BAT, inguinal white adipose tissue (iWAT), epididymal white adipose tissue (eWAT), and parametrial white adipose tissue (pmWAT) to facilitate study of steroid functions. This assay is the first HPLC-MS/MS method to quantify steroids in adipose tissues and should contribute to the study of steroid functions in energy balance and adipose biology.

EXPERIMENTAL SECTION

Materials

Optima LC/MS grade methanol, acetonitrile, water, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Sulfatase (Type H-1, from *Helix pomatia*) was purchased from Sigma-Aldrich (St. Louis, MO). Steroid standards DHEA, E2, T, DHEA-d5, estradiol-d5, and testosterone-d3 were purchased from Sigma-Aldrich. Steroid standards were prepared on the day of use.

Animals and Tissues

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were fed an AIN93G diet with 4 IU/g vitamin A (retinyl palmitate) for more than 10 generations before analyses. A group of mice (6–8 weeks old) was fed a HFD beginning 1 week prior to euthanasia. At the onset of HFD feeding, female mice were transferred to cages in which males had been housed to initiate their estrus cycle. Serum, liver, eWAT or pmWAT, iWAT, and BAT samples were snap-frozen in liquid nitrogen immediately after harvest. Samples were stored at −80 °C until assay. Animal experiments were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Protocols were approved by the UC-Berkeley Animal Care and Use Committee.

Extraction of Free Steroids

Tissue samples (~100 mg each) were placed into 2 mL round-bottom Eppendorf tubes with a metal bead, along with 1.5 mL of acetonitrile and a 20 μL mixture of internal standards, including 100 nM DHEA-d5, 50 nM E2-d5, 50 nM T-d3 (IS mix) prepared in acetonitrile; then the samples were homogenized with a TissueLyser II (Qiagen, Germantown, MD) at 30/sec for 30 s. The homogenate was stored for 30 min at −20 °C and then centrifuged for 5 min at 12000 × *g* and at 4 °C. The supernatant was transferred to a glass round-bottom tube and evaporated under a N_2 stream. The residue was resuspended in 2 mL of 0.2 M sodium acetate buffer (pH 5.0) and extracted with 10 mL of hexane. The mixture was centrifuged for 2 min at 1200 × *g*, and the upper hexane layer was transferred to a new glass tube and evaporated under nitrogen with gentle heating at 25–30 °C in a water bath (Model N-EVAP 112, Organomation Associates, Berlin, MA). The residue was reconstituted in 40 μL of methanol with vortexing. To remove a small amount of white precipitate, the mixture was spun for 20 s at 1200 × *g*. The supernatant was transferred into an autosampler vial insert with care not to include any solid material. A total of 1 μL was injected for LC-APCI-MS/MS analysis of unconjugated steroids.

Extraction of Conjugated Steroids

Conjugated steroids were extracted from the remaining aqueous phase after extraction of free steroids, and residual hexane was removed by pipetting and a stream of N_2. Sulfatase (100 μL of 10 mg/mL in the acetate buffer) and 20 μL of IS mix were added to the aqueous phase. The reaction was incubated overnight at 37 °C. Hydrolyzed steroids were extracted with 10 mL of hexane. The hexane layer was evaporated under a stream of N_2. The residue was reconstituted in 40 μL of methanol.

HPLC

DHEA, E2, and T were separated via reverse-phase chromatography with an Agilent 1290 system (Santa Clara, CA, U.S.A.) equipped with a binary pump, column compartment, and autosampler. The column compartment was maintained at 40 °C: samples were kept in the autosampler at 10 °C. Separation was achieved with an analytical Ascentis Express RP-Amide column (100 × 2.1 mm, 2.7 μm, Sigma Aldrich) at a flow rate of 0.4 mL/min. Mobile phases were (A) 0.1% formic acid in water; (B) 0.1% formic acid in methanol. The following gradient was applied over a run time of 25 min: 0 to 2 min, 50% B; 2 to 8 min, 50 to 95% B; 8 to 20 min, holding at 95% B; 20 to 23.5 min, 95 to 50% B; 23.5 to 25 min, back to 50% B and re-equilibrating for 1.5 min.

MS/MS

Analytes were detected with a Sciex API-4000 triple-quadrupole mass spectrometer in positive atmospheric pressure chemical ionization (APCI) mode. Analyst version 1.6 software controlled the instrument, which was operated in the multiple reaction monitoring (MRM) mode. Mass transitions to produce optimum sensitivity were determined by injecting 1 pmol standards as described in Table 1. Optimized MS variables were: curtain gas, 10 psig; collision gas, 7 psig; ion source gas 1, 70 psig; nebulizer current, 3 μA; source temperature, 350 °C; declustering potential, 55 V; entrance potential, 10 V; collision exit potential, 5 V. Optimized collision energy values were 45, 20, and 25 eV for T, DHEA, and E2, respectively.

Calibration Curve/Accuracy/LOQ/LOD

Calibration curves were generated by serial dilutions of DHEA, E2, and T standard mixture (3 repeats). Accuracy was determined by dividing the area under the curve (AUC) of standards by the slopes of calibration curves at 3 different concentrations (10/100/1,000 fmol). The lower limit of quantification (LOQ) and the lower limit of detection were defined as signal-to-noise ratios of 10 and 3, respectively.

Precision/Recovery

To determine coefficients of variation and recovery, ~1.2 g of pooled samples (male liver, eWAT, iWAT, or BAT), or serum (1.2 mL) were homogenized in 18 mL of acetonitrile. Then, 240 μL of IS mixture was added and the resulting sample was divided into 12 aliquots, 6 of which were assayed immediately for intra-assay variation. The other 6 aliquots were stored at −80 °C and assayed individually over 6 consecutive days to determine interassay variation. Recovery was calculated using the 6 intra-assay samples per tissue type by

comparing AUCs of DHEA-d5, E2-d5, and T-d5 measured in tissues with amounts quantified in pure IS in methanol, which was not extracted.

Statistics

Results are expressed as mean ± SEM, unless noted otherwise. Statistical analyses were made by two-tailed unpaired *t* tests using GraphPad Prism 7 (San Diego, CA). To prevent distortion of comparisons by missing data, values below the LOD were plotted using the highest amount below LOD (1, 1, and 2 fmol, respectively, for T, DHEA, and E2) normalized by average tissue weight (or volume). *p*-Values <0.05 were accepted as statistically significant.

RESULTS AND DISCUSSION

Extraction

Acetonitrile was chosen as homogenizing solvent versus methanol and several combinations of methanol/isopropanol. Although methanol extracted most steroids efficiently from serum, liver, and BAT, E2, and E2-d5 were recovered poorly from white adipose. This probably was because E2 fractionated into the fat phase, based on the log of its octanol/water partition coefficient (K_{ow} = 4.01), which is much higher than those of T (K_{ow} = 3.32) and DHEA (K_{ow} = 3.23).[17] Homogenization in acetonitrile followed by freezing separated E2 from precipitated lipids. To maximize extraction efficiency, acetonitrile was replaced with acetate buffer (pH 5.0), from which free steroids were extracted efficiently into the hexane phase, whereas sulfated steroids were retained in the aqueous phase. No steroids were detected in the second hexane phase when samples were extracted with hexane twice (data not shown). We diluted DHEA-S and E2-S standards in acetate buffer and extracted them to test for unintended hydrolysis. T-S standards are not available. DHEA and E2 signals were not detected up to 200 pmol (data not shown). Sulfatase activity was tested by comparing a hydrolyzed DHEA-S standard with a free DHEA standard, which showed 83–111% recovery (Table S1) and a linear (r^2 = 0.9986) concentration–response (Figure S1).

Previous reports applied a combination of methanol and water (7:3) or dimethoxymethane (4:1) to separate steroids from lipids.[13,18–20] Bélanger et al., used a GC/MS although it was not reported how it was validated. The other three studies detected steroids using RIA. These studies did not report recovery of E2 from white adipose tissue. Contemporary mass spectrometry is coupled usually with reverse phase LC because normal phase solvents can ignite in the MS source. It is critical to remove as much lipid impurities as possible to prevent compromising reverse-phase columns. Our extraction method efficiently removed lipids and recovered steroids of interest.

LC-MS/MS

A water/methanol mixture with 0.1% formic acid was superior for signal sensitivity compared to a water/acetonitrile combination. Retention times with this methanol-based phase were stable (±0.01 min) during a given analysis. For MS analysis, APCI in positive mode was used because we found it was better suited for analysis of these highly lipid-soluble steroids. In APCI, a nebulized sample is ionized by corona discharge, which

provides relatively strong ionization, suitable for compounds that do not readily form ions in solution, whereas ESI produces ions through ion evaporation that has greater sensitivity with polar analytes. Fragmentation and MS/MS ion transitions for steroids were optimized for maximum sensitivity. The fragmentation patterns of ionized ($[M + H]^+$) or ionized and dehydrated ($[M + H - H_2O]^+$) steroids are shown in Table 1.

Performance

Representative standard curves for each analyte using LC-MS/MS had linear dynamic ranges from 2 to 1000 fmol (Figure 1). The best-fit curves were similar, even at the low ends of the linear working ranges. LOQs of T, DHEA, and E2 were 5, 10, and 10 fmol, respectively (Figure 2). Accuracy values ranged between 95–102%, except for 10 fmol DHEA (88%; Table 2).

Average recovery was 96%; coefficients of variation were 20% or lower (Table 3). The mobile phase gradient and running time were optimized to separate interfering peaks from analytes (Figure 3).

Application

T was detected in male serum, eWAT, iWAT, and BAT, but not in liver (not shown) nor in female tissues (Figure 4). The biological variation in T levels was considerable, with a 50-fold difference in serum of mice fed the LFD, but only a 9-fold range in mice fed the HFD. Levels of free and sulfated T tended to be higher in serum of the LFD-fed group than in the HFD-fed group, but differences were not statistically significant. HFD-fed males, however, had greater numbers of mice with serum-conjugated T below the LOD. In contrast to serum, tissue levels of T and T-S were all above the LOD. T-S levels correlated with free T levels ($r^2 = 0.76$, $p < 0.0001$) and did not seem to vary with dietary fat (Figure S2). Quantified values of T were not affected by the nature of normalization (tissue weight vs protein amount; Figure 4E,F).

Free and conjugated DHEA were detected in serum, liver, BAT, and pmWAT (Figure 5A–D). At least 50% of females had serum free and conjugated DHEA below the LOD. This was also true for the liver. Female BAT DHEA-S levels were markedly, but not significantly, higher than free DHEA (Figure 5C). DHEA-S was not detected in pmWAT, although free DHEA values were mostly above the LOD (Figure 5D). In males, free DHEA was detected only in iWAT and eWAT of HFD-fed mice (Figure 5E,F), which suggests a role for DHEA in early adipogenesis. Free and conjugated E2 were detected only in serum and BAT of females (Figure 6A,B). E2-S levels were significantly higher than free E2 in both dietary conditions. Feeding a HFD did not affect free E2 levels in serum and BAT.

The HFD experiment was done to investigate the early effects of high fat on mice in puberty. Juveniles may be particularly prone to adverse effects of HFD, as evidenced by the observation that the rate of weight gain increases until 10–12 weeks of age.[21] We chose 1 week HFD exposure, postulating that steroid metabolism contributes to early adipocyte hyperplasia. This timing was based on the preadipocyte proliferation rate maximizing 1 week after initiating a HFD, but decreasing to baseline level thereafter, even as fat mass keeps increasing by hypertrophy.[22]

COMPARISON WITH PREVIOUS ASSAYS

To the best of our knowledge, the current assay is the only LC-MS/MS method that reports quantification of steroids in adipose tissues (Table 4). Sensitivity is high compared to other assays that also do not rely on derivatization, despite the difficulty of extracting steroids from adipose. Given the rarity of tissue data, it is worth noting that we have preliminary data from female mouse bone marrow samples in which DHEA, DHEA-S, and E2-S have been detected with values greater than the LOQ.

CONCLUSION

The present study reports a straightforward and effective extraction for steroids from diverse tissues, including adipose, coupled with LC-MS/MS. Quantification of steroids in mouse tissues exposed for one week to a HFD revealed novel findings: (1) free DHEA was detected only in the HFD-fed group of male iWAT and eWAT; (2) sulfated DHEA and E2 were higher than free steroids in female BAT, in contrast to iWAT and eWAT; (3) circulating E2-S was higher than E2, whereas circulating DHEA-S and T-S were lower than their unconjugated counterparts; (4) the HFD tended to marginally, but not statistically significantly, lower T levels in all tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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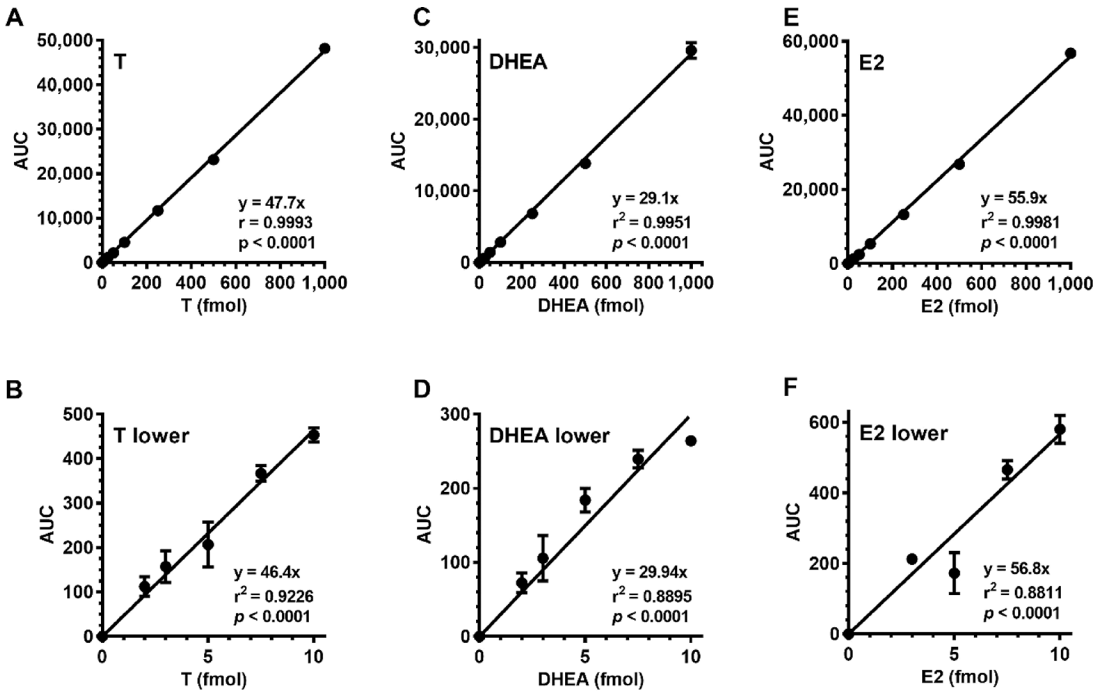

Figure 1.

Representative calibration curves for (A, B) T; (C, D) DHEA; (E, F) E2. The y-axis shows the area of the peak (AUC) generated by the MS transition of each steroid monitored by MS/MS. The *x*-axis shows the molar amount analyzed. Data did not deviate from linearity ($r^2 > 0.995$) over the tested ranges. Panels B, D, and F are enhancements of the lower ranges of A, C, and E, respectively. Each had slopes in the lower ranges consistent with the slopes over the entire ranges. Data were fit by linear regression analysis. Each point represents three replicates.

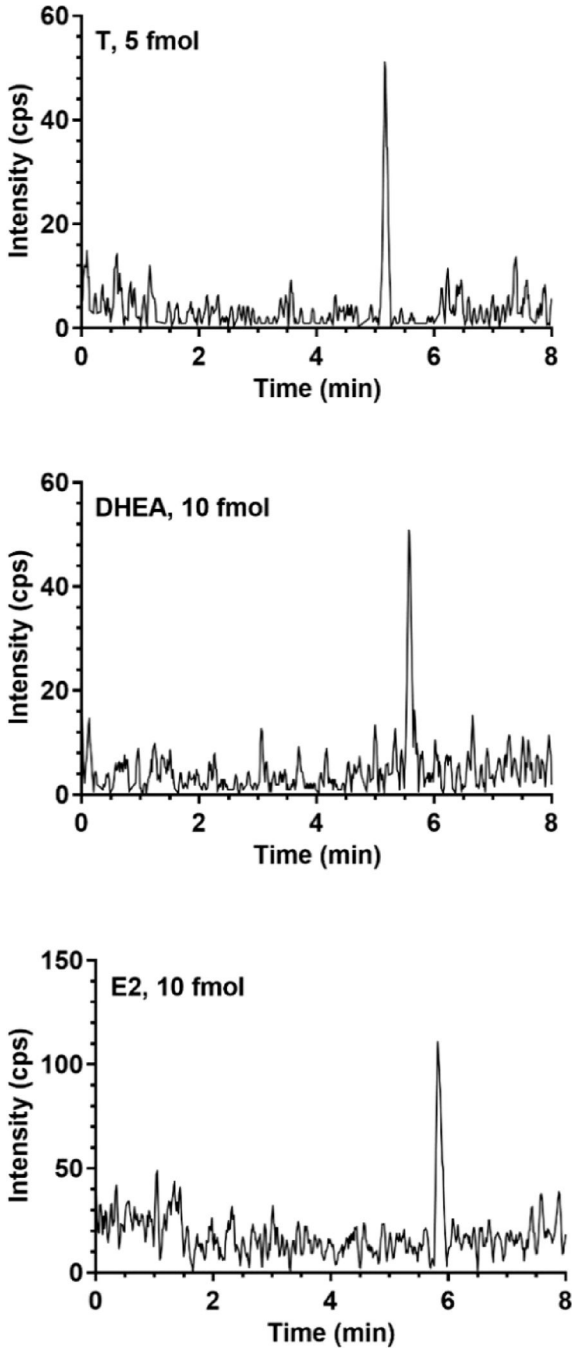

Figure 2.
Lower limit of quantification (LOQ) for each analyte.

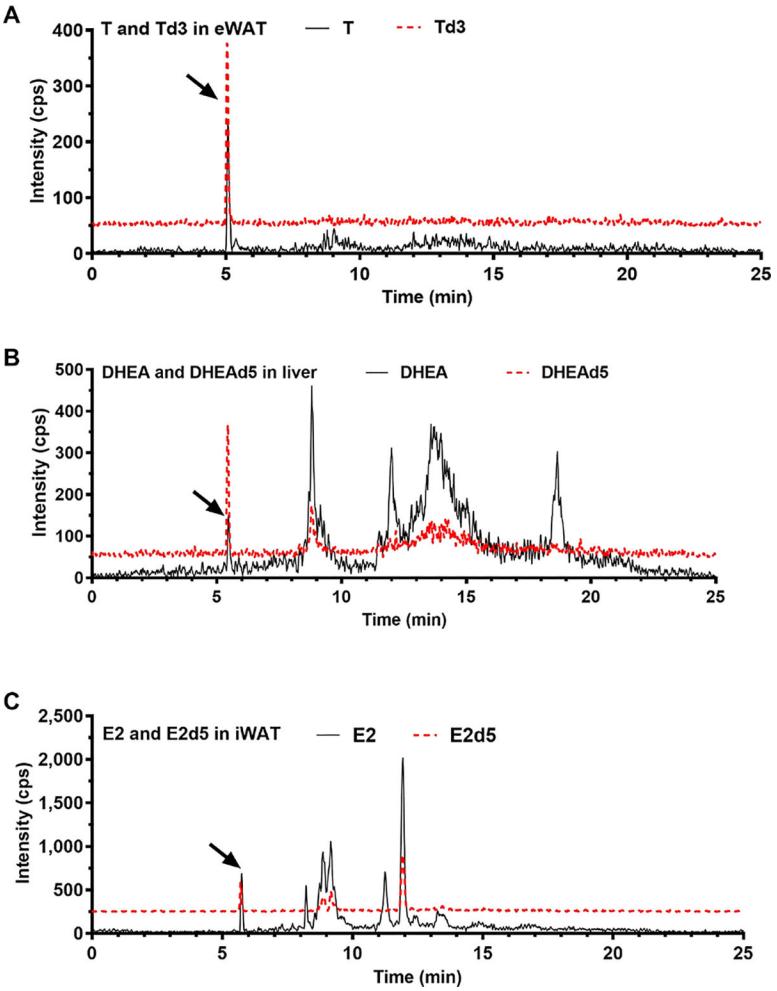

Figure 3.

Representative MRM chromatograms of (A) T extracted from eWAT; (B) DHEA extracted from liver; and (C) E2 extracted from iWAT. Baselines of internal standards (red) of Td3, DHEAd5, and E2d5 were offset by 50, 50, and 250 cps, respectively, to enhance clarity.

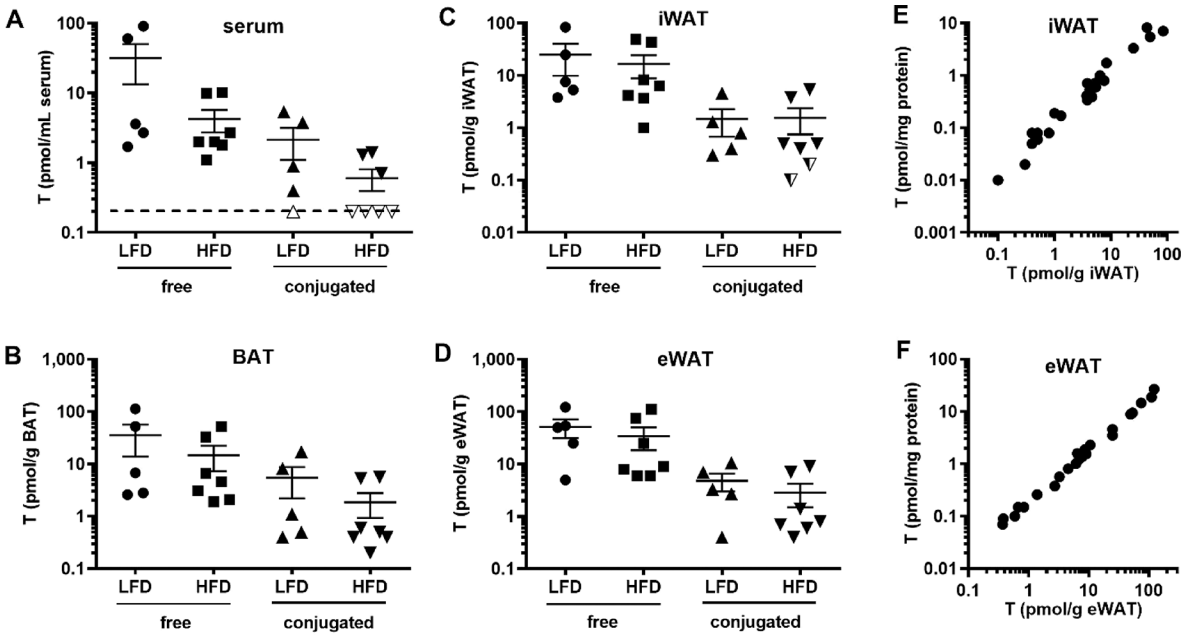

Figure 4.

Free and conjugated T levels in (A) serum; (B) BAT; (C) iWAT; and (D) eWAT from male mice fed a low-fat diet vs a HFD for 1 week. Normalization to protein amount was compared to normalization with tissue weight in (E) iWAT and (F) eWAT. $N = 5$ (LFD) and 7 (HFD).

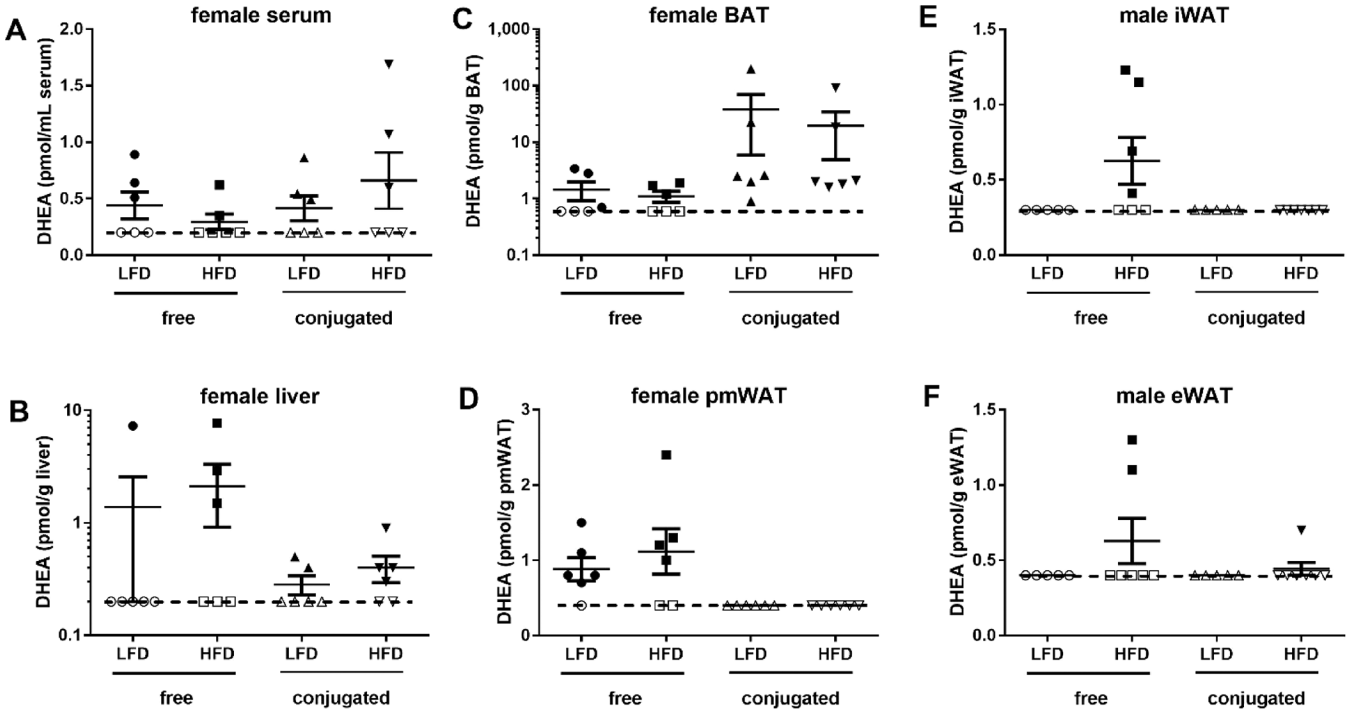

Figure 5.

Free and conjugated DHEA levels in (A) serum; (B) liver; (C) BAT; (D) pmWAT (females); (E) iWAT; (F) eWAT (males; means ± SEM, $n = 6$, females; $n = 5$, male LFD; $n = 7$, male HFD). Filled shapes, half-filled shapes, and open shapes indicate values over LOQ, values between LOQ and LOD, and values below LOD, respectively.

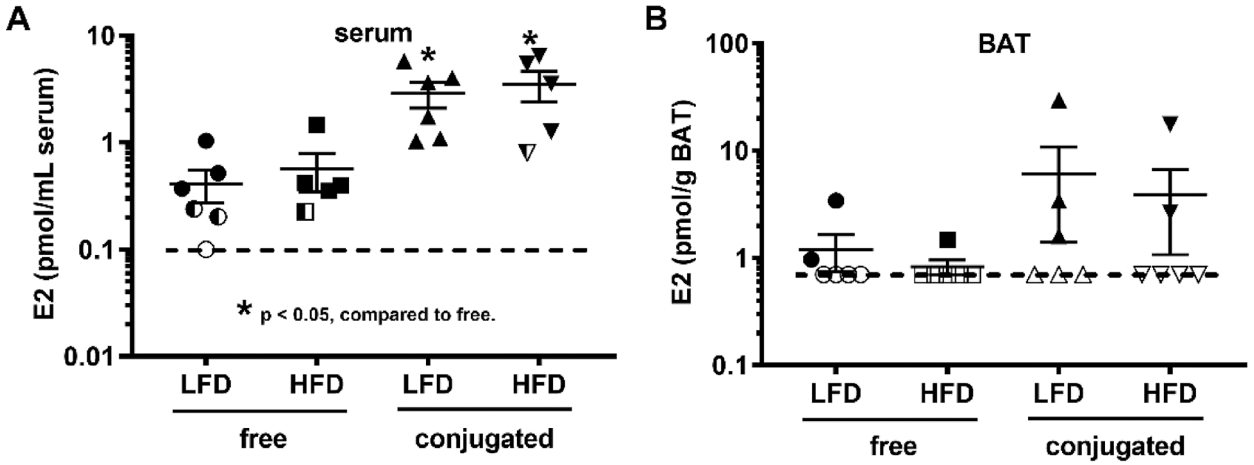

Figure 6.

Free and conjugated E2 levels in serum (A) and BAT (B) of females (n = 6 per group). Filled shapes, half-filled shapes, and open shapes indicate values over LOQ, values between LOQ and LOD, and values below LOD, respectively.

Table 1.

Mass Transitions and Collision Energies for Each Analyte

		mass transition		
analyte	**MW (g/mol)**	**Q1 (*m/z*)**	**Q3 (*m/z*)**	**CE (eV)**
T	288.42	289.3 $[M + H]^+$	97.3	45
T-d3	291.44	292.4 $[M + H]^+$	97.1	45
DHEA	288.42	271.2 $[M + H - H_2O]^+$	213.1	20
DHEA-d5	293.46	276.4 $[M + H - H_2O]^+$	218.5	20
E2	272.38	255.3 $[M + H - H_2O]^+$	159.2	25
E2-d5	277.41	260.3 $[M + H - H_2O]^+$	161.3	25

Table 2.

Accuracies Calculated at Three Different Concentrations[a]

	amount (fmol)		
standard	**10**	**100**	**1000**
T	99 ± 13%	96 ± 2%	101 ± 2%
DHEA	88 ± 13%	98 ± 4%	102 ± 7%
E2	102 ± 21%	95 ± 7%	102 ± 3%

[a]All values are mean ± SD, 3 repeats.

Table 3.

Recovery and Precision[a]

			CV (%)	
tissue	**IS**	**recovery (%)**	**intra-assay**	**interassay**
liver	T-d3	102 ± 13	13	5
	DHEA-d5	96 ± 13	14	12
	E2-d5	98 ± 12	12	16
eWAT	T-d3	99 ± 10	10	7
	DHEA-d5	100 ± 20	20	10
	E2-d5	98 ± 6	6	14
iWAT	T-d3	98 ± 15	15	18
	DHEA-d5	102 ± 16	16	14
	E2-d5	88 ± 14	16	17
BAT	T-d3	99 ± 10	10	12
	DHEA-d5	96 ± 18	19	13
	E2-d5	92 ± 14	16	7
serum	T-d3	103 ± 8	8	5
	DHEA-d5	114 ± 8	7	9
	E2-d5	94 ± 7	8	4

[a]All values are mean ± SD, 6 repeats.

Table 4.

Comparison of Current Assay with Published Sex Steroid Assays[a]

reference	method	derivative	analytes	LOD (fmol)	LOQ (fmol)	intra-assay CV (%)	linear dynamic range	tissues	species
current	LC/APCI/MS2	-	DHEA, E2, T	2, 3, 2	5, 10, 5	<20	to 1000 fmol	serum, liver, adipose	mouse
Denver et al., 2019[7]	LC/MS^2	MPPZ	E2 and 7 others	0.77	1.6	7.6	to 1595 fmol	plasma	human
Lee et al., 2016[23]	LC/MS^2	-	DHEA, E2, T,9 others		35, 14, 73	5.1, 4.0, 7.5	to 1000, 100, 50 ng/mL	serum	human
Matysik et al., 2017[24]	LC/MS/HRMS	-	T, 7 others		4	1−11.5		serum	human
McCulloch et al., 2017[6]	LC/FF/APPI/MS^2	-	E2, T, 5 others	3.7, 0.7				serum	human
Schofield et al., 2017[25]	LC/MS^2	-	E2, T		2.4, 4.6	<20	6−600 pg/mL, 1−1170 ng/dL	serum	human
Star-Weinstock et al., 2012[26]	LC/ESI/MS^2	quaternary aminooxy	T		0.7	<15	1−5000 pg/mL	serum	human
Ankarberg-Lindgren et al., 2018[27]	GC/MS^2	PFBBr, PFBHA, PFPA	E2, T, 3 others	0.05, 2.7		<9		serum	human
Gaikwad, 2013[28]	LC/MS^2	-	DHEA, E2, T, and 98 others	87, 9, 9		<19.7, <19.7, <8.8	to 20 ng/μL	breast	human

[a]Blank spaces indicate that data are not available.