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Measurement of sex steroids in murine blood and reproductive tissues by liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

Accurate measurement of sex steroids is essential to evaluate mouse models for human reproductive development and disorders. The recent advent of liquid chromatography–tandem mass spectrometry (LC–MS/MS) assays that match the sensitivity of steroid immunoassay could overcome problems arising from the limited specificity of steroid immunoassay. In this current study we validate a LC–MS/MS assay for the measurement of key sex steroids from murine serum and reproductive tissues. The assay gave excellent dilutional linearity ($r^2 \geq 0.98$) and reproducibility (CV $\leq$ 10% of replicate samples) in serum and reproductive tissues with sensitive quantitation limits; testosterone (T; 2 pg), dihydrotestosterone (DHT; 10 pg), 5α-androstane-3α,17β-diol (3αDiol; 40 pg), 5α-androstane-3β,17β-diol (3βDiol; 40 pg), estradiol (E2; 0.5 pg) and estrone (E1; 0.3 pg). Using 0.1 mL sample, T was the only consistently detectable steroid (detection limit 20 pg/ml) in both male and female mouse serum. In the testis, T and DHT were quantifiable as were both diols at relatively high levels. Prostatic T levels were low and DHT was determined to be the most abundant androgen in this tissue. Uterine and ovarian levels of E2, E1 and T were measurable, with levels varying according to estrous cycle stage. Hence, we demonstrate that this LC–MS/MS method has the sensitivity, specificity and multi-analyte capability to offer accurate steroid profiling in mouse serum and reproductive tissues.

1. Introduction

Steroid hormones are vital for reproductive development, health and hormone-dependent disorders as well as the optimal function of most non-reproductive tissues which express steroidogenic enzymes and steroid receptors. Investigating the pathogenesis of human development or disease is constrained by ethical and practical limitations on clinical research. As a result, mammalian animal models are an indispensable tool for investigating reproductive health, medicine and biology. Among the alternative animal models, the laboratory mouse is the most cost-effective with their unmatched versatility to undergo targeted genetic modification with high fidelity replication of development and/or disease processes involving the highly conserved mammalian reproductive system. Investigating such model systems requires accurate and sensitive measurement of sex steroids in blood and tissues.

Prior to the advent of immunoassay (IA) for steroids in the early 1970s, measurement of sex steroids was largely unavailable as it relied on whole animal bioassays which were laborious, costly and insensitive. Although steroid IA produced a dramatic improvement in sensitivity allowing measurement of steroids to the picogram level, the limitations of specificity were always recognized. Cross-reactivity with structurally related steroids, steroid conjugates and matrix interference required elimination by pre-assay steps of solvent extraction, chromatography and the use of only authentic tritiated steroid tracers. The increasing demand for steroid IA resulted in assay simplification, mostly to allow incorporation of steroid measurements into high throughput immunoassay platforms. This required shedding of the pre-assay steps which safeguarded the specificity of steroid IA. Consequently, it has become evident that steroid IA using unextracted, non-chromatographed samples and the use of bulky non-authentic tracers is susceptible to major limitations in specificity. This has led to apparent method-specific differences in reference ranges, especially at low circulating steroid levels where steroid IA generates unreliable measurements [1–3].

While mass spectrometry (MS)-based measurements of steroids have always remained the gold standard, available MS methods (using gas chromatography) remained insensitive and largely inaccessible as reference methods. This was largely due to the requirement of large amounts of sample and lengthy

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sample preparation steps, thereby limiting throughput. Recently, bench-top liquid chromatography–tandem mass spectrometry (LC–MS/MS) instruments have been developed that overcome historical limitations of MS sensitivity for sex steroid measurement while retaining unmatched, reference level specificity. In addition, LC–MS/MS enables the analysis of multiple analytes from a single sample [4–7], thereby maximizing the information gained from limited amounts of samples. This is especially relevant to material harvested from small animals such as the mouse. Also, it allows for a steroid metabolism profile to be obtained, which is particularly useful when studying steroid dependant and metabolizing tissues, such as the prostate, where significant steroid metabolism occurs [6,8,9] and where novel steroids and metabolism pathways are suggested to be functionally relevant [10–15].

The specificity limitations of steroid IAs developed for use with unprocessed human serum are particularly troublesome when used to measure sex steroids in mouse serum (where a circulating sex hormone binding globulin is absent) or tissues with their different complex matrices. Until recently MS methods were insufficiently sensitive to measure sex steroids directly in mouse samples, although a limited number of LC–MS/MS methods were described that measure steroids in mouse and rat prostate and serum by using derivatized adducts to improve sensitivity [16–19]. As expected, the few available MS-based studies report differences from IA data especially at low blood steroid levels [6,18,20–23]. Therefore, there is a need for the development of methods capable of accurate, precise and direct (non-derivatized) measurement of steroids in mouse serum and tissues. We recently described a novel LC–MS/MS method to measure biologically active sex steroids and their primary metabolites in human serum [24], including both androgens and estrogens; testosterone (T), dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3αDiol), 5α-androstane-3β,17β-diol (3βDiol), estradiol (E2) and estrone (E1) in a single run without derivatization. In this study we report validation of this method for use with murine serum, steroidogenic (ovary, testis) and steroid dependent (prostate, uterus) tissues.

2. Materials and methods

2.1. Materials

T, DHT, 3αDiol and 3βDiol were obtained from the National Measurement Institute (NMI; Sydney, Australia). Deuterium labeled internal standards of these steroids were also from the NMI: testosterone-1,2,3-d_3 (d_3-T), dihydrotestosterone-16,16,17-d_3 (d_3-DHT), 5α-Androstane-3α,17β-diol-16,16,17-d_3 (d_3-3αDiol) and 5α-Androstane-3β,17β-diol-16,16,17-d_3 (d_3-3βDiol). E2 and E1 were from Steraloids (Newport, RI, USA). Deuterium labeled estradiol-2,4,16,16-d_4 (d_4-E2) was from Cambridge Isotope Laboratory (Andover, MA, USA). Tritiated T and E2 used for the thin-layer chromatography (TLC) experiments were obtained from New England Nuclear (Perkin Elmer, MA, USA). HPLC grade methanol and toluene were purchased from Lab-Scan analytical sciences (Dublin, Ireland). Autosampler vials were from Grace Davison (Deerfield, IL, USA). Water of 18-MΩ quality was prepared by a Millipore Milli-Q system (Bedford, MA, USA). Lyophilized BSA (A7906) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Mice

Mice (C3H/HeH X 101/H genetic background) were bred and housed in the ANZAC Research Institute's Molecular Physiology Unit under standard approved conditions with *ad lib* access to feed and water. All procedures were performed under ketamine/xylazine anesthesia as approved by the Animal Welfare Committee of the Sydney South West Area Health Service. To prepare serum, collected blood was allowed to clot at room temperature for 30 min and then centrifuged at 5000 rpm for 5 min. Serum was then stored in plastic tubes at −20 °C until use.

Intact mature males were killed at 8–15 weeks of age to collect blood (by cardiac exsanguination), testes and prostate. The prostate (comprising anterior, ventral and dorsolateral lobes) was removed and dissected free of surrounding fat and connective tissue on ice. Tissues were snap frozen in liquid nitrogen and stored at −80 °C until use. Castration of male mice was performed by bilateral orchidectomy via scrotal incision under anesthesia at 8 weeks of age. These mice were sacrificed 1 week later to allow the collection of blood and prostate tissue. A second group of castrated 8 week old male mice were treated with E2 for 1 week by subdermal placement of a 1 cm silastic implant filled with recrystallized E2 as described previously [25] except that the E2 was diluted 1:1000 by co-crystallization with cholesterol. The estrous cycle of intact mature females was synchronized by adding male bedding to their cages, and staged by vaginal smearing [26]. Ovary, uterus and blood were collected from female mice at both proestrus and diestrus. Proestrus samples were collected at 1600 hrs on the afternoon proestrus was identified by vaginal smearing. Blood from mature hypogonadal (*hpg*) mice [27,28] was collected as described above.

2.3. Sample preparation

Frozen tissue (whole testis, prostate, ovary and uterus) was transferred into 5 mL glass tubes and allowed to thaw. Buffer (0.5% BSA, w/v, 5 mM EDTA in PBS, pH 7.4) was added (500 μL per testis, 250 μL per whole prostate, 300 μL per ovary, 400 μL per uterus) prior to homogenization on ice for 20 s using an IKA T10 basic disperser (IKA Werke, Staufen, Germany) on the highest setting. Homogenates were centrifuged (3000 rpm, 10 min, 4 °C) to separate insoluble debris, and the supernatant was transferred into a fresh 1.5 mL tube and immediately prepared for analysis by LC–MS/MS. Calibration standards and quality control samples were prepared in 4% BSA and aliquots were frozen at −80 °C as previously described [24].

For LC–MS/MS analysis, serum (100 μL made up to 200 μL with PBS) or tissue homogenate (200 μL), along with calibration standards (200 μL) and quality control samples (200 μL) were transferred into clean glass tubes and extracted with 1 mL of hexane:ethyl acetate (3:2 ratio) containing deuterated steroids as internal standards. The concentration of the deuterated species in the organic solvent was 0.32 ng/mL d_3-T and d_3-DHT, 0.15 ng/mL d_4-E2 and d_3-Diols (E1 was quantified using d_4-E2 as the internal standard). Extracted samples were then left to allow phase separation at 4 °C for 1 h before placing them in a −80 °C freezer for 30 min to freeze the lower aqueous layer. The upper organic layer containing extracted target steroids was decanted into a clean glass tube and evaporated overnight at 37 °C. The dried samples were reconstituted in 1.2 mL of 20% methanol in PBS. After thorough mixing samples were transferred into 1.5 mL autosampler vials and 1 mL was injected onto a C8 column for analysis. Steroid levels were calculated as amount per volume assayed for serum and amount per mass of tissue used.

2.4. Tissue and serum steroid levels

Steroid levels in extracts of serum or tissue homogenates were quantified using a stable isotope dilution LC–MS/MS method as described [24]. Briefly, the LC method used an online extraction of the injected sample using a Shimadzu Prominence system (Shimadzu Scientific Instruments, Columbia, MD, USA) with a

Supelcosil LC-8-DB column (Supelco, Bellefonte, PA, USA) followed by gradient elution of the target steroids into an Applied Biosystems API-5000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada). Ionization was performed using atmospheric pressure photoionization (APPI), with toluene as the dopant. Androgens were monitored in the positive mode and estrogens in negative mode using multiple reaction monitoring (MRM). Calibration was performed using internal standardization, with the peak area ratio between the target analyte and its isotopically labeled internal standard used for quantification purposes. Eight-point calibration curves were constructed for each target steroid covering the expected steroid concentrations in serum and tissue. Using 100 μL of calibrant, the amount of steroid loaded onto the column ranged from 2–3200 pg T, 10–3200 pg DHT, 40–1600 pg 3αDiol, 40–1600 pg 3βDiol, 0.5–160 pg E2 and 0.3–160 pg E1. The lowest level used in each case represents the limit of quantification (LOQ) of the assay.

2.5. Validation experiments

Multiple dilutions of tissue homogenate were assayed to assess whether sample matrix interfered with steroid quantification (i.e. dilutional linearity). As the steroid level in the whole tissue extract typically exceeded the capability of the method (i.e. steroid level above the top standard of the calibration curve), the maximum amount of homogenate analyzed represented approximately half a testis, a whole prostate, an ovary or half a uterus. At each dilution, multiple aliquots of homogenate (≥4) were assayed to assess precision. Quantitative recovery was assessed from a spiked female mouse serum pool, which had low endogenous steroid levels, to which known amounts of steroid were added at low (close to the LOQ) and high levels. Recovery was calculated as the amount measured in the sample minus that present endogenously, expressed as a percentage of the amount added. As E2 was difficult to quantify in mouse serum, the ability to measure this estrogen was further tested by assaying serum from castrated male mice treated for 1 week with exogenous E2 (see Section 2.2).

To assess whether steroid metabolism or loss was occurring during sample preparation, radiolabeled T or E2 was added to prostate homogenate and incubated at +4 °C or room temperature then sampled at 0, 30 and 120 min later. After this time, steroids were extracted into hexane:ethyl acetate (3:2, v/v) and analyzed by TLC using dichloromethane:ethyl acetate:methanol (85:13:2) as the mobile phase [29]. Steroid metabolism was assessed by dividing the TLC plate into segments and measuring the radioactivity of each segment. No metabolism was deemed to have occurred when signal was detected in single segment. Steroid loss during sample processing was assessed by comparing the level of radioactivity from homogenates to the level detected from pure radiolabeled standard. No losses were deemed to have occurred if the radioactivity in the homogenate was >90% of that observed from the standard.

2.6. Statistical analysis

Statistical analysis was performed using SPSS (SPSS, Chicago, USA). Data is shown as mean ± standard error of the mean (S.E.M.) or as mean, median and range unless otherwise stated. Non-Gaussian distributions were normalized using a $\log_{10}$ transformation. Data analysis used either two sample *t*-test or analysis of variance (ANOVA) with a post-hoc least significant difference (LSD) test. For all analyses a *p*-value of <0.05 was considered significant. Strength of correlations was assessed by R^2 (coefficient of determination). For those samples where steroid levels were below the LOQ, the data was treated as missing.

Table 1
Recovery of sex steroids from a spike serum pool.

Steroid	Level	[Spiked]	Difference (measured-endogenous)	Recovery (%)
T (ng/mL)	Low	0.10	0.11	110
	High	0.40	0.47	118
DHT (ng/mL)	Low	0.10	0.11	105
	High	0.40	0.38	95
E2 (pg/mL)	Low	10	12	120
	High	40	38	95
E1 (pg/mL)	Low	10	10	100
	High	40	38	95

3. Results

3.1. Serum

T was measured in 100 μL of serum from all intact male and female mice, and was able to be accurately quantified in as little as 25 μL of male mouse serum. Recovery of non-isotopically labeled steroids from a spiked serum pool was between 95 and 120%, both for low and high spiked values (Table 1). For intact wildtype males, serum T displayed wide variability between mice creating a highly skewed distribution (Fig. 1; mean 7.0 ng/mL; median 1.6 ng/mL; range 0.30–39.4 ng/mL). Detectable T levels were also observed from all but one age-matched *hpg* males (mean 0.05 ng/mL, median 0.04 ng/mL, range 0.00–0.13 ng/mL, $p < 0.001$ vs. intact), but at significantly lower concentrations than from wildtype mice. Serum T was undetectable (<0.02 ng/mL) in all samples 1 week after orchidectomy (Fig. 1).

In mature female mice, serum T levels were detectable in all samples at both diestrus (mean 0.10 ng/mL, median 0.06 ng/mL, range 0.02–0.21 ng/mL) and proestrus (mean 0.44 ng/mL, median 0.36 ng/mL, range 0.14–1.42 ng/mL), with a statistically significant difference observed between stages (Fig. 1; $p = 0.003$). At proestrus, serum T levels from intact females were significantly higher than age-matched *hpg* males ($p = 0.001$), but there was no difference at diestrus ($p = 0.129$).

When using 100 μL of mouse serum from male or female mice, all of the other steroids being monitored were below the LOQ of the assay (20 pg/mL T, 100 pg/mL DHT, 400 pg/mL 3αDiol, 400 pg/mL 3βDiol, 5 pg/mL E2 and 3 pg/mL E1), except for serum from the

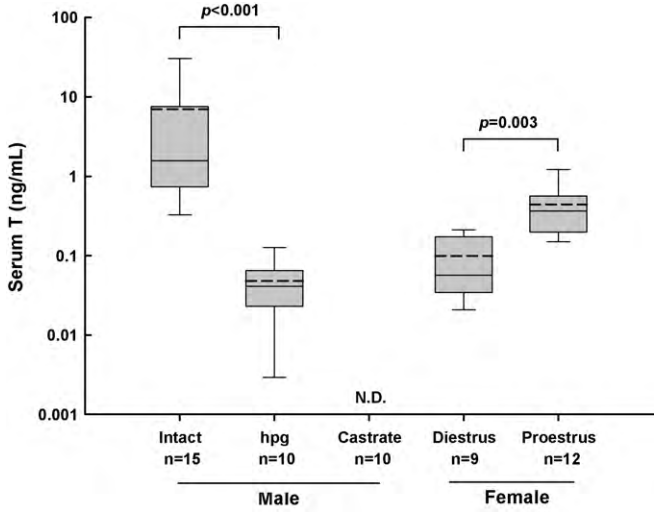

Fig. 1. Boxplots showing T levels from intact, *hpg* and castrated male mice and female mice at two estrus cycle stages. Each box represents the median (solid bar), mean (dotted line), interquartile range and 5th and 95th percentiles. N.D. = not detectable, *hpg* = hypogonadal. Serum T levels are shown on a log scale.

Table 2
Serum sex steroids in various animal species.

Species	Gonadal status	*n*	T (ng/mL)		DHT (ng/mL)		E2 (pg/mL)		E1 (pg/mL)		3αDiol (ng/mL)		3βDiol (ng/mL)	
Baboon	M	4	7.5 ± 2.6	*100*	2.2 ± 1.2	*100*	15 ± 6	*100*	19 ± 7	*100*	<0.4	*0*	<0.4	*0*
	F	5	0.2 ± 0.1	*100*	<0.1	*0*	80 ± 93	*100*	27 ± 18	*100*	<0.4	*0*	<0.4	*0*
Sheep	M	6	2.9 ± 2.7	*100*	<0.1	*0*	<5	*0*	<3	*0*	<0.4	*0*	<0.4	*0*
	F (follicular)	6	<0.02	*0*	<0.1	*0*	<5	*0*	<3	*0*	<0.4	*0*	<0.4	*0*
	F (estrus)	6	<0.02	*0*	<0.1	*0*	<5	*0*	<3	*0*	<0.4	*0*	<0.4	*0*
Horse	F	4	<0.02	*0*	<0.1	*0*	<5	*0*	<3	*0*	<0.4	*0*	<0.4	*0*
Rat	F	4	1.2 ± 0.3	*100*	<0.1	*0*	<5	*0*	<3	*0*	<0.4	*0*	<0.4	*0*

Serum steroid levels represent mean ± standard error of the mean (S.E.M.). Numbers in *italics* indicate the % of samples with quantifiable steroid levels. In those cases where all samples had undetectable levels, the working LOQ for 100 μL of serum is given.

proestrus female. In half of these samples quantifiable levels of 3αDiol were observed (mean 0.9 ng/mL). Also, in one male mouse with exceptionally high serum T levels, DHT and both 3α and 3βDiol were measurable (data not shown).

Although the inability to quantify E2 in these samples was unexpected, it was not due to the performance of the method, as serum E2 was easily quantifiable in all treated castrate male mice ($n = 6$; mean 15 ± 3 pg/mL) and adult male and female humans [24]. In addition, E2 was detected and measured from serum of both male and female baboons but not from horse, sheep or rats (Table 2).

3.2. Tissue steroid measurements

Using radiolabeled steroid as a tracer, no steroid losses or metabolism were observed during homogenization steps. Excellent dilutional linearity of tissue homogenates was observed for testicular T, prostatic DHT, ovarian E2 and uterine E1 (Fig. 2). This observation also held for prostatic T, testicular DHT, 3α and 3βDiol, ovarian E1, E2 and T and uterine E1 and E2 with CVs of 1–10% for replicates of the various dilutions (data not shown). There was sufficient sensitivity to quantify multiple steroids from tissue harvested from an individual mouse, as summarized in Table 3.

3.2.1. Testis

T was detectable in all testis homogenates. DHT, 3αDiol and 3βDiol were also detectable in virtually all specimens, with levels in the low ng/g range (~1% of T levels). The isomeric Diols were more abundant than DHT, and in all cases the 3αDiol levels were greater than 3βDiol. The estrogens (E2 and E1) were not detectable in testis homogenate.

3.2.2. Prostate

DHT was the most abundant steroid and was detectable in all prostate samples from intact mice. T was also detectable in most specimens but at levels ~10% of DHT. 3αDiol was detectable in 17% of prostate samples, 3βDiol, E2 and E1, were not detectable at all. In prostate tissue from castrated male mice DHT was the only steroid

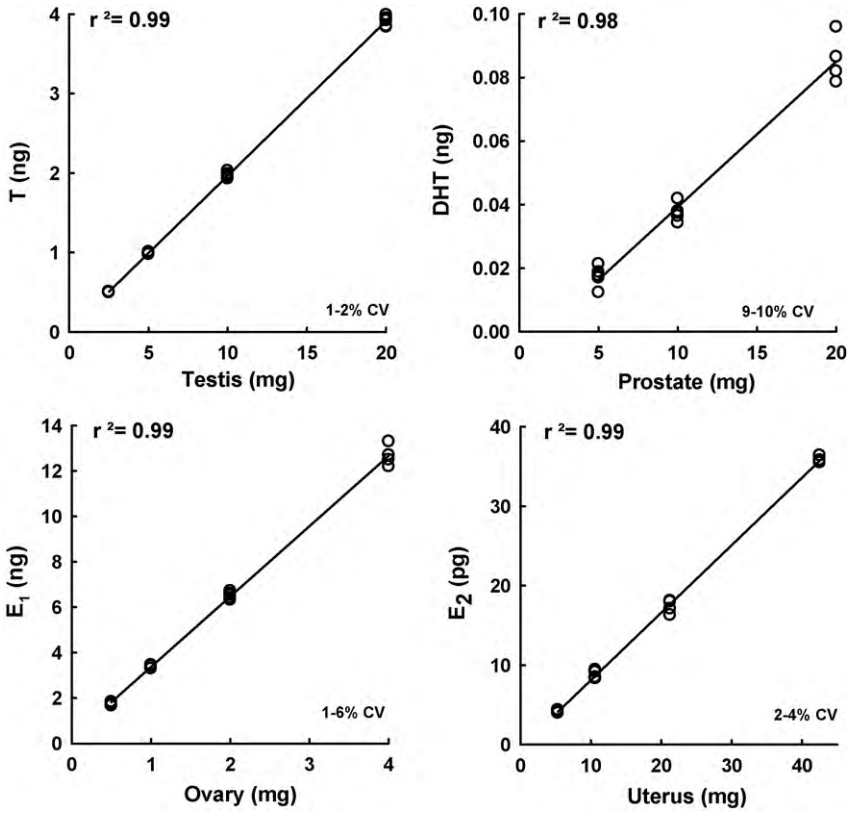

Fig. 2. Dilutional linearity of androgens and estrogens from mouse reproductive tissues. The most abundant target steroid quantified from each tissue was used to assess dilutional linearity (T: Testis; DHT: Prostate; E21: Ovary, E2: Uterus). Replicates (≥4) of each dilution were assayed to assess precision (%CV), with the range for each tissue shown in the bottom right of each plot. The mass of tissue used at each dilution represents less than the mean mass of a whole testis (98 mg), prostate (24 mg), proestrus ovary (7 mg) or proestrus uterus (68 mg).

Table 3
Steroid levels measured from mouse reproductive tissue using LC–MS/MS.

Tissue	Gonadal status	*n*	Tissue weight (mg)	T (ng/g)		DHT (ng/g)		3αDiol (ng/g)		3βDiol (ng/g)		E2 (pg/g)		E1 (pg/g)	
Testis	–	15	98 ± 16	317.0 ± 45.0 174 (40–1903)	*100*	3.4 ± 0.4 1.4 (0.2–23.3)	*100*	8.8 ± 1.6 5.9 (1.5–23.9)	*100*	4.6 ± 0.5 2.0 (0.2–18.4)	*100*	<0.5 pg	*0*	<0.3 pg	*0*
Prostate	Intact	17	24 ± 6	0.7 ± 0.2 0.5 (0.1–2.2)	*71*	5.0 ± 1.0 5.0 (1.9–11.9)	*100*	8.4 ± 0.7 1.2 (1.2–22.8)	*17*	<40 pg	*0*	<0.5 pg	*0*	<0.3 pg	*0*
	Castrate	8	10 ± 2	<2 pg –	*0*	0.4 ± 0.1 0.3 (0.1–1.1)	*100*	<4.0 pg –	*0*	<40 pg –	*0*	<0.5 pg –	*0*	<0.3 pg –	*0*
Ovary	Diestrus	15	7 ± 1	8.4 ± 1.4 7.6 (1.2–18.3)	*100*	<10 pg –	*0*	8.9 ± 1.4 10 (4.4–11.9)	*33*	<40 pg –	*0*	4928 ± 931 4676 (731–11,881)	*100*	2999 ± 710 2847 (61.1–7774)	*93*
	Proestrus	16	5 ± 1	24.0 ± 5.0 16 (0.1–68.0)	*100*	<10 pg –	*0*	10 –	*6*	<40 pg –	*0*	15,311 ± 3398 18,365 (526–43,081)	*100*	3919 ± 881 3136 (54.0–15,863)	*100*
Uterus	Diestrus	15	68 ± 15	<2 pg –	*0*	<10 pg –	*0*	<40 pg –	*0*	<40 pg –	*0*	543 ± 62 556 (135–823)	*100*	38.0 ± 7.0 35 (0.4–84)	*100*
	Proestrus	16	100 ± 18	1.5 ± 08 0.4 (0.1–12.6)	*100*	5.0 ± 3.0 1.0 (0.4–13.7)	*31*	7.4 ± 3.3 2.5 (0.8–22.9)	*63*	4 –	*6*	656 ± 74 665 (85.0–1201)	*100*	36.0 ± 8.0 25.1 (2.6–108)	*100*

Tissue weight given as mean ± standard deviation (S.D.) with median and range given below. Steroid levels represent mean ± standard error of the mean (S.E.M.). Numbers in *italics* indicate the % of samples with quantifiable steroid levels. When <100%, the mean and S.E.M. was calculated using only those samples with quantifiable levels. In those cases where all samples had undetectable levels, the LOQ of that steroid is given (on-column, in pg) and the average weight of the tissue assayed is given on the tissue weight column. If only one sample in a group had quantifiable levels, this value is shown without the S.E.M.

detectable, with levels at ~10% of DHT levels observed from intact mice.

3.2.3. Ovary

T, E2 and E1 were detectable in virtually all ovary homogenates. At proestrus, T and E2 levels were three times higher than at diestrus whereas E1 levels were only increased by about a third. The observed T level increase coincides with the timing of the ovulatory LH surge. 3αDiol was only detectable in a minority of samples at levels close to the LOQ, and the isomeric 3βDiol was not detectable at all.

3.2.4. Uterus

The estrogens were measurable in the uterus homogenate albeit at ~10 times lower for E2 and ~100 times lower for E1 than was observed in the ovary. T was only detectable in proestrus samples at levels ~20 times lower than in the ovary. DHT and 3αDiol were also quantifiable in half of the uterus samples at proestrus but not at diestrus. 3βDiol was only detected in one uterus sample from the mouse with the highest 3αDiol level and relatively high DHT level.

3.3. Steroid correlations

3.3.1. Tissue and serum

Serum T was positively correlated with testis T (Fig. 3A), DHT ($n = 15$, $r^2 = 0.45$, $p = 0.012$) and 3αDiol ($n = 13$, $r^2 = 0.67$, $p = 0.001$). Serum T also showed a strong correlation with prostate T, when detectable ($n = 12$, $r^2 = 0.69$, $p = 0.001$), and DHT (Fig. 3B). No significant correlations were observed between ovarian and uterine steroid concentrations and circulating steroid levels although the correlation between serum T and ovarian T neared statistical significance ($n = 21$, $r^2 = 0.42$, $p = 0.059$).

3.3.2. Within tissue

For the testis, levels of T, DHT and 3αDiol were linearly correlated pairwise with the levels of testicular T and DHT explaining over 60% of the variance ($r^2 > 0.60$) in the other steroids. The strongest correlation observed was between T and DHT (Fig. 3C). Other correlations were observed between DHT and 3αDiol ($n = 13$, $r^2 = 0.70$, $p < 0.001$) and T and 3αDiol ($n = 13$, $r^2 = 0.80$, $p < 0.001$). The only significant correlation observed within the prostate was between T and DHT ($n = 12$, $r^2 = 0.38$, $p = 0.037$).

Ovarian T levels correlated well with E2 (Fig. 3D) and E1 ($n = 30$, $r^2 = 0.19$, $p = 0.014$) with ovarian T explaining 50% of the variance in E2 ($R^2 > 0.50$). Strength of correlation was maintained between T and E2 when samples were categorized by estrus stage (diestrus $n = 15$, $r^2 = 0.54$, $p = 0.002$, proestrus $n = 16$, $r^2 = 0.37$, $p = 0.012$). Also, ovarian E2 and E1 levels correlated strongly with one another ($n = 30$, $r^2 = 0.73$, $p < 0.001$) regardless of estrus stage. This correlation was also observed within uterine tissue, regardless of stage ($n = 31$, $r^2 = 0.48$, $p < 0.001$) and at diestrus ($n = 15$, $r^2 = 0.33$, $p = 0.025$) and proestrus ($n = 16$, $r^2 = 0.67$, $p < 0.001$). No other significant or strong linear steroid correlations were observed within uterine tissue.

4. Discussion

Genetic mouse models provide an indispensable tool to investigate mammalian reproductive physiology and pathology. It is essential to be able to acquire accurate measurements of sex steroids from the very limited serum volumes and tissue samples available from mice. Immunoassays optimized for use with unextracted human serum samples have major limitations at low steroid concentrations and when using murine samples. In this paper we report the validation in murine serum and reproductive tissues of

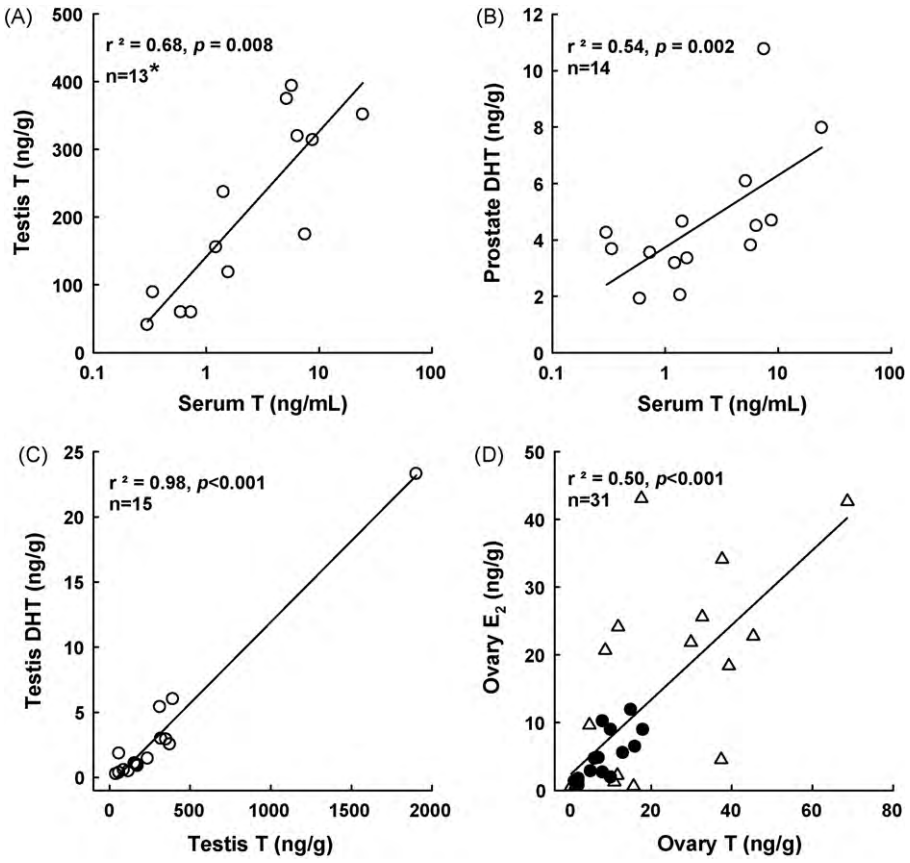

Fig. 3. Plots of key sex steroid correlations within and between mouse serum and tissues. (A) Serum T versus testis T, (B) serum T versus prostate DHT, (C) within testis T versus DHT, (D) within ovary T versus E2. For the ovary correlations; (●) diestrus and (△) proestrus. Serum T levels are shown on a log scale. * One serum T value was very high and was excluded from plot A.

a highly sensitive and specific LC–MS/MS method for the quantification of biologically important sex steroids. The method involves a simple, convenient and low cost liquid–liquid extraction procedure and excludes the need for steroid derivatization prior to assay. We have previously demonstrated the reliability of this method for human serum [24]. The measurements from mouse serum and tissue demonstrated good dilutional linearity, precision and accuracy, allowing accurate measurement of both androgens (T, DHT, 3αDiol and 3βDiol) and estrogens (E2 and E1) from a single sample. There was sufficient sensitivity to allow T measurements from 100 μL of male or female serum and multiple steroids from a single testis, prostate, ovary or uterus per mouse and has good potential for use with other tissues. Furthermore, the results are in accordance with recent studies using mass spectrometry as the detection technique [6,18]. In addition this method could potentially be used for additional steroids to form a more complex profile of steroid metabolism.

One novel observation, which demonstrates the sensitivity of this method, was that serum T was detectable at very low levels in the *hpg* mice (~1% of intact males). These mice have proven complete functional postnatal androgen deficiency [28], and the consistent detection of T contrasts with the undetectable serum levels 1 week after orchidectomy in mature male mice. In the female mouse, the highly sensitive serum T measurement also demonstrated a significant increase of serum T at proestrus, which is consistent with the mid-cycle ovulatory surge of serum T reported in humans [30–33]. As the dominant ovarian follicles are the major source of circulating steroids at ovulation, it is likely the proestrus increase in blood T originates from ovarian T. However, whether this reflects intrafollicular disruption with spillage of T as an E2 precursor or more specific secretion is not clear. The latter possibility is supported by our recent findings that AR-mediated androgen actions are required for normal ovulation in the mouse [26,34]. Within the mouse testis, the abundance of the primary metabolites of DHT, 3α and 3βDiol, and the strong correlation between the levels of T and 3αDiol is consistent with a recent study of the tammar wallaby showing a strong preference for the metabolism of DHT to these products [35].

A striking and unexpected finding was that circulating E2 levels were below the LOQ (5 pg/mL from 100 μL of sample), even in mature, cycling (and presumed fertile) female mice. This was not due to technical limitations of the LC–MS/MS method, as we demonstrated excellent recovery of E2 from a spiked serum pool, even at levels close to the LOQ. In addition, E2 was readily measurable in serum from E2 treated mice as well as from untreated male and female baboon but not male or female sheep, female horse or male rat. This pattern of easily detectable E2 in primates but not other mammalian species does not correspond to the presence of a circulating SHBG, which is absent in rodents but not other mammals [36]. Our findings of such low (undetectable) blood E2 levels are at variance with higher values reported historically by E2 immunoassays [37–44] despite the LC–MS/MS method matching the sensitivity of these assays. This is supported by another MS-based study that showed undetectable serum E2 levels (<5 pg/mL) [45]. Taken together, these findings support the notion that immunoassays grossly overestimate steroid levels, especially at low levels.

An important advantage of MS-based steroid measurement is the multi-analyte capability, thereby allowing multiple steroids to be quantified from a single sample. This provides the unique opportunity to investigate steroid profiles within steroid producing and steroid dependent tissues [14,46–51]. Furthermore,

the multi-analyte capability is highly beneficial as it eliminates the need to use multiple steroid immunoassays and creates new opportunities to study steroid pathways within certain tissues.

A limitation of the present method is the inability to reliably detect the less abundant steroids in serum and tissues. This can potentially be overcome by extracting larger serum volumes or by pooling tissues, both of which are relatively facile steps when using liquid–liquid extraction to prepare samples for analysis. However, pooling tissues would result in the loss of individual mouse measurements. Alternatively, sensitivity could be improved by forming derivatization adducts of the target steroids. This is a common approach, but is not favored for our application as it typically involves lengthy sample preparation procedures and would require additional optimization of the LC–MS/MS method.

In conclusion, we have established a LC–MS/MS assay for analysis of key sex steroids from mouse samples, which involves minimal sample preparation requirements and is capable of high sample throughput. The reliability of the method was evaluated for accuracy, precision and recovery and correlates well with other rodent (rat and mouse) studies using MS-based methods. Overall this method provides an excellent and versatile tool for investigating steroid levels and metabolism in mouse serum and reproductive tissues.

Disclosure statement

The authors have nothing to disclose.

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References

[1] J. Taieb, B. Mathian, F. Millot, M.C. Patricot, E. Mathieu, N. Queyrel, I. Lacroix, C. Somma-Delpero, P. Boudou, Testosterone measured by 10 immunoassays and by isotope-dilution gas chromatography–mass spectrometry in sera from 116 men, women, and children, Clin. Chem. 49 (8) (2003) 1381–1395.
[2] C. Wang, D.H. Catlin, L.M. Demers, B. Starcevic, R.S. Swerdloff, Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography–tandem mass spectrometry, J. Clin. Endocrinol. Metab. 89 (2) (2004) 534–543.
[3] K. Sikaris, R.I. McLachlan, R. Kazlauskas, D. de Kretser, C.A. Holden, D.J. Handelsman, Reproductive hormone reference intervals for healthy fertile young men: evaluation of automated platform assays, J. Clin. Endocrinol. Metab. 90 (11) (2005) 5928–5936.
[4] T. Guo, J. Gu, O.P. Soldin, R.J. Singh, S.J. Soldin, Rapid measurement of estrogens and their metabolites in human serum by liquid chromatography–tandem mass spectrometry without derivatization, Clin. Biochem. 41 (9) (2008) 736–741.
[5] T. Guo, R.L. Taylor, R.J. Singh, S.J. Soldin, Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry, Clin. Chim. Acta 372 (1–2) (2006) 76–82.
[6] J.A. Locke, E.S. Guns, A.A. Lubik, H.H. Adomat, S.C. Hendy, C.A. Wood, S.L. Ettinger, M.E. Gleave, C.C. Nelson, Androgen levels increase by intratumoral de novo steroidogenesis during progression of castration-resistant prostate cancer, Cancer Res. 68 (15) (2008) 6407–6415.
[7] K. Mitamura, T. Nakagawa, K. Shimada, M. Namiki, E. Koh, A. Mizokami, S. Honma, Identification of dehydroepiandrosterone metabolites formed from human prostate homogenate using liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry, J. Chromatogr. A 961 (1) (2002) 97–105.
[8] P.I. Lundmo, A. Sunde, K.J. Tveter, Metabolism of androgens in the seminal vesicles and the different lobes of the prostate in young mature rats, J. Steroid Biochem. 22 (4) (1985) 513–519.
[9] J.H. Abalain, E. Quemener, J.L. Carre, B. Simon, Y. Amet, P. Mangin, H.H. Floch, Metabolism of androgens in human hyperplastic prostate: evidence for a differential localization of the enzymes involved in the metabolism, J. Steroid Biochem. 34 (1–6) (1989) 467–471.
[10] Z. Weihua, R. Lathe, M. Warner, J.A. Gustafsson, An endocrine pathway in the prostate, ERbeta, AR, 5alpha-androstane-3beta,17beta-diol, and CYP7B1, regulates prostate growth, Proc. Natl. Acad. Sci. U.S.A. 99 (21) (2002) 13589–13594.
[11] R. Shibuya, T. Suzuki, Y. Miki, K. Yoshida, T. Moriya, K. Ono, J. Akahira, T. Ishida, H. Hirakawa, D.B. Evans, H. Sasano, Intratumoral concentration of sex steroids and expression of sex steroid-producing enzymes in ductal carcinoma in situ of human breast, Endocr. Relat. Cancer 15 (1) (2008) 113–124.
[12] M. Uemura, K. Tamura, S. Chung, S. Honma, A. Okuyama, Y. Nakamura, H. Nakagawa, Novel 5 alpha-steroid reductase (SRD5A3, type-3) is overexpressed in hormone-refractory prostate cancer, Cancer Sci. 99 (1) (2008) 81–86.
[13] R.B Montgomery, E.A. Mostaghel, R. Vessella, D.L. Hess, T.F. Kalhorn, C.S. Higano, L.D. True, P.S. Nelson, Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth, Cancer Res. 68 (11) (2008) 4447–4454.
[14] H.K. Ghayee, R.J. Auchus, Clinical implications of androgen synthesis via 5alpha-reduced precursors, Endocr. Dev. 13 (2008) 55–66.
[15] V. Luu-The, A. Belanger, F. Labrie, Androgen biosynthetic pathways in the human prostate, Best Pract. Res. Clin. Endocrinol. Metab. 22 (2) (2008) 207–221.
[16] T. Higashi, A. Yamauchi, K. Shimada, E. Koh, A. Mizokami, M. Namiki, Determination of prostatic androgens in 10 mg of tissue using liquid chromatography–tandem mass spectrometry with charged derivatization, Anal. Bioanal. Chem. 382 (4) (2005) 1035–1043.
[17] T. Higashi, T. Nishio, N. Hayashi, K. Shimada, Alternative procedure for charged derivatization to enhance detection responses of steroids in electrospray ionization-MS, Chem. Pharm. Bull. (Tokyo) 55 (4) (2007) 662–665.
[18] B. Kashiwagi, Y. Shibata, Y. Ono, R. Suzuki, S. Honma, K. Suzuki, Changes in testosterone and dihydrotestosterone levels in male rat accessory sex organs, serum, and seminal fluid after castration: establishment of a new highly sensitive simultaneous androgen measurement method, J. Androl. 26 (5) (2005) 586–591.
[19] Z. O'Brien, N. Post, M. Brown, A. Madan, T. Coon, R. Luo, T.A. Kohout, Validation and application of a liquid chromatography–tandem mass spectrometric method for the simultaneous determination of testosterone and dihydrotestosterone in rat prostatic tissue using a 96-well format, J. Chromatogr. (2009).
[20] S.A. Wright, R.C. Douglas, L.N. Thomas, C.B. Lazier, R.S. Rittmaster, Androgen-induced regrowth in the castrated rat ventral prostate: role of 5alpha-reductase, Endocrinology 140 (10) (1999) 4509–4515.
[21] N. Brunner, B. Svenstrup, M. Spang-Thomsen, P. Bennett, A. Nielsen, J. Nielsen, Serum steroid levels in intact and endocrine ablated BALB/c nude mice and their intact littermates, J. Steroid Biochem. 25 (3) (1986) 429–432.
[22] A.M. Flores-Valverde, E.M. Hill, Methodology for profiling the steroid metabolome in animal tissues using ultraperformance liquid chromatography–electrospray-time-of-flight mass spectrometry, Anal. Chem. 80 (22) (2008) 8771–8779.
[23] T. Higashi, A. Nagahama, N. Otomi, K. Shimada, Studies on neurosteroids XIX. Development and validation of liquid chromatography–tandem mass spectrometric method for determination of 5alpha-reduced pregnane-type neurosteroids in rat brain and serum, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 848 (2) (2007) 188–199.
[24] D.T. Harwood, D.J. Handelsman, Development and validation of a sensitive liquid chromatography–tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization, Clin. Chim. Acta (2009).
[25] J.J. Bianco, D.J. Handelsman, J.S. Pedersen, G.P. Risbridger, Direct response of the murine prostate gland and seminal vesicles to estradiol, Endocrinology 143 (12) (2002) 4922–4933.
[26] K.A. Walters, K.J. McTavish, M.G. Seneviratne, M. Jimenez, A.C. McMahon, C.M. Allan, L.A. Salamonsen, D.J. Handelsman, Subfertile female androgen receptor knockout mice exhibit defects in neuroendocrine signaling, intraovarian function, and uterine development but not uterine function, Endocrinology 150 (7) (2009) 3274–3282.
[27] B.M. Cattanach, C.A. Iddon, H.M. Charlton, S.A. Chiappa, G. Fink, Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism, Nature 269 (5626) (1977) 338–340.
[28] J. Singh, C. O'Neill, D.J. Handelsman, Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice, Endocrinology 136 (12) (1995) 5311–5321.
[29] P. Jimenez, R.A. Valdez, M.C. Romano, Metabolism of steroid hormones by *Taenia solium* and *Taenia crassiceps* cysticerci, J. Steroid Biochem. Mol. Biol. 99 (4–5) (2006) 203–208.
[30] H.L. Judd, S.S. Yen, Serum androstenedione and testosterone levels during the menstrual cycle, J. Clin. Endocrinol. Metab. 36 (3) (1973) 475–481.
[31] G.E. Abraham, Ovarian and adrenal contribution to peripheral androgens during the menstrual cycle, J. Clin. Endocrinol. Metab. 39 (2) (1974) 340–346.
[32] G.E. Abraham, Z.H. Chakmakjian, Serum steroid levels during the menstrual cycle in a bilaterally adrenalectomized woman, J. Clin. Endocrinol. Metab. 37 (4) (1973) 581–587.

[33] T. Mushayandebvu, V.D. Castracane, T. Gimpel, T. Adel, N. Santoro, Evidence for diminished midcycle ovarian androgen production in older reproductive aged women, Fertil. Steril. 65 (4) (1996) 721–723.
[34] K.A. Walters, C.M. Allan, M. Jimenez, P.R. Lim, R.A. Davey, J.D. Zajac, P. Illingworth, D.J. Handelsman, Female mice haploinsufficient for an inactivated androgen receptor (AR) exhibit age-dependent defects that resemble the AR null phenotype of dysfunctional late follicle development, ovulation, and fertility, Endocrinology 148 (8) (2007) 3674–3684.
[35] G. Shaw, M.B. Renfree, M.W. Leihy, C.H. Shackleton, E. Roitman, J.D. Wilson, Prostate formation in a marsupial is mediated by the testicular androgen 5 alpha-androstane-3alpha, 17 beta-diol, Proc. Natl. Acad. Sci. 97 (22) (2000) 12256–12259.
[36] P. Philibert, F. Audran, C. Pienkowski, I. Morange, B. Kohler, E. Flori, C. Heinrich, C. Dacou-Voutetakis, M.G. Joseph, A.M. Guedj, H. Journel, A.C. Hecart-Bruna, I. Khotchali, S. Ten, P. Bouchard, F. Paris, C. Sultan, Complete androgen insensitivity syndrome is frequently due to premature stop codons in exon 1 of the androgen receptor gene: an international collaborative report of 13 new mutations, Fertil. Steril. (2009).
[37] K.J. McTavish, M. Jimenez, K.A. Walters, J. Spaliviero, N.P. Groome, A.P. Themmen, J.A. Visser, D.J. Handelsman, C.M. Allan, Rising follicle-stimulating hormone levels with age accelerate female reproductive failure, Endocrinology 148 (9) (2007) 4432–4439.
[38] C.R. Fisher, K.H. Graves, A.F. Parlow, E.R. Simpson, Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene, Proc. Natl. Acad. Sci. U.S.A. 95 (12) (1998) 6965–6970.
[39] V.A. Cameron, D.J. Autelitano, J.J. Evans, L.J. Ellmers, E.A. Espiner, M.G. Nicholls, A.M. Richards, Adrenomedullin expression in rat uterus is correlated with plasma estradiol, Am. J. Physiol. Endocrinol. Metab. 282 (1) (2002) E139–146.
[40] J.E. Fata, V. Chaudhary, R. Khokha, Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17beta-estradiol during the estrous cycle, Biol. Reprod. 65 (3) (2001) 680–688.
[41] S.P. Ng, B.G. Steinetz, S.G. Lasano, J.T. Zelikoff, Hormonal changes accompanying cigarette smoke-induced preterm births in a mouse model, Exp. Biol. Med. (Maywood) 231 (8) (2006) 1403–1409.
[42] M. Haywood, J. Spaliviero, M. Jimemez, N.J. King, D.J. Handelsman, C.M. Allan, Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone, Endocrinology 144 (2) (2003) 509–517.
[43] J.A. Spaliviero, M. Jimenez, C.M. Allan, D.J. Handelsman, Luteinizing hormone receptor-mediated effects on initiation of spermatogenesis in gonadotropin-deficient (hpg) mice are replicated by testosterone, Biol. Reprod. 70 (1) (2004) 32–38.
[44] J. Singh, D.J. Handelsman, Imprinting by neonatal sex steroids on the structure and function of the mature mouse prostate, Biol. Reprod. 61 (1) (1999) 200–208.
[45] C.D. Toran-Allerand, A.A. Tinnikov, R.J. Singh, I.S. Nethrapalli, 17alpha-estradiol: a brain-active estrogen? Endocrinology 146 (9) (2005) 3843–3850.
[46] J.D. Wilson, R.J. Auchus, M.W. Leihy, O.L. Guryev, R.W. Estabrook, S.M. Osborn, G. Shaw, M.B. Renfree, 5alpha-androstane-3alpha, 17beta-diol is formed in tammar wallaby pouch young testes by a pathway involving 5alpha-pregnane-3alpha,17alpha-diol-20-one as a key intermediate, Endocrinology 144 (2) (2003) 575–580.
[47] M. Mahendroo, J.D. Wilson, J.A. Richardson, R.J. Auchus, Steroid 5alpha-reductase 1 promotes 5alpha-androstane-3alpha, 17beta-diol synthesis in immature mouse testes by two pathways, Mol. Cell. Endocrinol. 222 (1–2) (2004) 113–120.
[48] G. Pelletier, V. Luu-The, S. Li, J. Ouellet, F. Labrie, Cellular localization of mRNA expression of enzymes involved in the formation and inactivation of hormonal steroids in the mouse prostate, J. Histochem. Cytochem. 52 (10) (2004) 1351–1356.
[49] H.K. Ghayee, R.J. Auchus, Basic concepts and recent developments in human steroid hormone biosynthesis, Rev. Endocr. Metab. Disord. 8 (4) (2007) 289–300.
[50] G. Pelletier, Expression of steroidogenic enzymes and sex-steroid receptors in human prostate, Best Pract. Res. Clin. Endocrinol. Metab. 22 (2) (2008) 223–228.
[51] D. Mizrachi, R.J. Auchus, Androgens, estrogens, and hydroxysteroid dehydrogenases, Mol. Cell. Endocrinol. 301 (1–2) (2009) 37–42.
[52] A.M. Birrell, A. Hennessy, A. Gillin, J. Horvath, D. Tiller, Reproductive and neonatal outcomes in captive bred baboons (*Papio hamadryas*), J. Med. Primatol. 25 (4) (1996) 287–293.