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Analysis of testosterone and dihydrotestosterone in mouse tissues by liquid chromatography-electrospray tandem mass spectrometry

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Abstract

A novel method was established for simultaneous quantitation of testosterone (T) and dihydrotestosterone (DHT) in murine tissue and serum samples. Endogenous T and DHT, together with the internal standards, 17 α -methyl-T and 17 α -methyl-DHT, were extracted from tissues, and then derivatized by reaction with 2-hydrazino-4-(trifluoromethyl)-pyrimidine (HTP). Analysis by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) resulted in production spectra of HTP derivatives of both T and DHT that showed analyte-specific fragmentations; the latter fragmentations were characterized by use of high-resolution Orbitrap MS/MS. These specific fragmentations enabled quantitation of T and DHT in the multiple-reaction monitoring (MRM) mode. The method was validated with charcoal-stripped serum as the matrix; the LLOQ was 0.10 ng/ml for T and 0.50 ng/ml for DHT. The method was then used for determination of serum and tissue levels of T and DHT in transgenic mice carrying a hypomorphic NADPH-cytochrome P450 reductase gene (Cpr-low mice). Remarkably, ovarian T levels in Cpr-low mice were found to be 25-fold higher than those in wild-type mice, a finding that at least partly explains the female infertility seen in the Cpr-low mice. In conclusion, our method provides excellent sensitivity and selectivity for determination of endogenous levels of T and DHT in mouse tissues.

Keywords

LC-MS; steroids; testosterone; dihydrotestosterone; metabolism; cytochrome P450; mice

Introduction

Testosterone (T) is the primary circulating androgen in both males and females [1;2]. Abnormal levels of T have been associated with many disease conditions, including hypogonadism in

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males [3], and excessive androgen syndrome [4] and androgen insufficiency in females [1;5]. 5 α -Dihydrotestosterone (DHT), a biologically more active form of androgen than T, is mainly produced by the action of steroid 5 α -reductases (SRD5A1/2) on T in androgen-target tissues. DHT plays critical roles in male development [2]. Furthermore, DHT is implicated in many pathological processes, including benign prostatic hyperplasia [6]. An abnormal ratio of DHT to T, as a result of genetic defects in the conversion of T to DHT, could cause male pseudohermaphroditism [7]. The ratio of DHT to T is also a valuable clinical measure for evaluation of the efficacies of 5 α -reductase inhibitors [8;9] and androgen replacement therapy [10;11]. Therefore, the capability to simultaneously determine levels of T and DHT is desirable, both in the diagnosis of sex steroid hormone-related diseases, and in the monitoring of the effects of androgen replacement therapy.

Radioimmunoassay (RIA) is commonly used for determination of serum levels of T and DHT [12;13;14;15]. However, an increasing number of studies have shown that, due to inherent limitations in specificity, RIA methods are unsuitable for determination of the very low levels of T present in females and children, or in hypogonadal men [16;17;18;19]. Gas chromatography-mass spectrometry (GC-MS) provides excellent specificity and sensitivity for determination of neutral steroids [17;20;21]. However, methods based on this instrumentation usually require extra steps for sample preparation and clean-up, and problems with thermal stability of the steroid derivatives are often encountered. The determination of DHT is even more challenging than that of T, given the fact that DHT is present at much lower levels in most biological samples [13;14;15;22;23].

In recent years, several LC-MS-based methods, all of which use chemical derivatization to increase the detection sensitivity, were developed for simultaneous determination of T and DHT *in vivo* [24;25;26;27;28;29;30]. A number of reagents, including 2-hydrozino-1-methylpyridine (HMP) [24;25;26], 2-fluoro-1-methylpyridinium-p-touenesulfonate (FMP) [28;29], and hydroxylamine [27], produce derivatives that enhance sensitivity, for LC-MS determination of T and DHT in various biological samples. However, most of these reagents, including HMP, 2,3-pyridinedicarboxylic anhydride, and hydroxylamine, form two isomeric (*cis* and *trans*) products with either T or DHT [25;27;30]. The chromatographic separation of these isomers of the T and DHT derivatives not only complicates quantitative analysis, but also causes increased susceptibility to interferences by endogenous or exogenous compounds. Moreover, ions derived from the derivatization reagent moieties, but not analyte-specific ions, dominate the production spectra of HMP derivatives, thus compromising the selectivity of the method [24;25;26]. The hydroxylamine derivative of T provides structurally relevant MS/MS transitions for the quantitation of T, but not for DHT [27]. The FMP derivatives of T and DHT each elute as a single peak under optimized LC conditions; however, the reaction of FMP with T and DHT has a low efficiency, thus requiring an excessive amount of FMP. Consequently, additional clean-up steps are required for FMP derivatives, before they can be analyzed by LC-MS [25;28;29].

In the present study, a novel derivatization method, which uses as little as 10 μ g of a commercially available reagent, 2-hydrazino-4-(trifluoromethyl)-pyrimidine (HTP), has been developed. We describe the advantages of this procedure, over others currently in use; we present proof of validation for the accuracy and precision of the method; and we describe an application of the new method, namely, an assessment of the impact of a reduction in expression of the NADPH-cytochrome P450 reductase (*Cpr*) gene on circulating and tissue levels of T and DHT. The latter study employed a transgenic mouse model (known as the *Cpr*-low mouse); in this mouse, the *Cpr* gene is hypomorphic, leading to decreased expression of CPR protein, and consequent decreases in the activities of microsomal cytochrome P450 (P450) enzymes, in all organs examined [31].

Materials and methods

Materials

T, DHT, dehydroepiandrosterone (DHEA), androsterone, and HTP were purchased from Sigma-Aldrich (St. Louis, MO). [2,2,4,5,6-²H₅]-Testosterone (D₅-T) was purchased from Cambridge Isotope Laboratories (Andover, MA). 17 α -methyltestosterone (MT) and 17 α -methylidihydrotestosterone (MDHT) were purchased from Steraloids (Wilton, NH). Charcoal-stripped fetal bovine serum (CS-FBS) was purchased from Hyclone (Logan, UT). HPLC-grade hexane was purchased from J. T. Baker (Phillipsburg, NJ). Ethyl acetate was purchased from Anachemia (Sparks, NV). LC-MS grade acetonitrile, methanol, and water were purchased from Fisher Scientific (Pittsburgh, PA). Acetic acid was purchased from Mallinckrodt (Hazelwood, MO).

Derivatization

Aliquots of T and DHT standards (in methanol) and tissue or serum extracts were evaporated to dryness in glass tubes, under N₂. The residues were redissolved in 50 μ l of acetonitrile, and 50 μ l HTP reagent (0.2 mg/ml in dry acetonitrile containing 0.05% trifluoroacetic acid). The reaction mixtures were transferred to 2-ml glass vials (National Scientific, Rockwood, TN), sealed, and incubated at 60 °C for 1 h. The reactions were stopped by cooling on ice for 5 min. The reaction mixtures were then evaporated to dryness under N₂. The derivatives were dissolved in 50 μ l of dry acetonitrile, and transferred to 250- μ l inserts in the auto-sampler for LC-ESI-MS/MS analysis. The reaction of HTP with T is depicted in Figure 1.

LC-ESI-MS/MS

All samples were analyzed on an Applied Biosystems/MDS Sciex API 4000 Q-Trap mass spectrometer equipped with a turbo ion spray source, and interfaced with an Agilent 1200 Series liquid chromatograph. The system was operated, and data were analyzed, with the Analyst software (Applied Biosystems/MDS Sciex), version 1.4.2. The mass spectrometer was operated in the positive ionization mode. Instrumental parameters were optimized during direct infusion of standards with solvent consisting of 10% A (0.1% acetic acid in water/acetonitrile at 9:1 (v/v)) and 90% B (acetonitrile), at a flow rate of 0.2 ml/min. The [M+H]⁺ ions of T, DHT, the T-HTP derivative, and the DHT-HTP derivative were identified by LC-ESI-MS, with Q1 operated in full scanning mode in the range of m/z 200 to 1000. A product ion spectrum was obtained for each compound. Multiple reaction monitoring (MRM) was used for quantitative analysis. Nitrogen was used as the curtain gas (setting at 35), gas 1 (setting at 35), gas 2 (setting at 60), and the collision gas (setting High). The ionspray voltage was set at 5500, and the gas temperature was set at 500 °C. The declustering and entrance potentials were 80 and 10 V, respectively. The collision energy (CE) and collision cell exit potential (CXP) for each MRM transition are given in Table 1.

A Luna Phenyl-Hexyl column (2.0 $\times$ 150 mm, 3- μ m particle size; Phenomenex, Torrance, CA) was used for HPLC. The mobile phase consisted of 0.1% acetic acid in 90% H₂O/10% acetonitrile (A) and 100% acetonitrile (B). A linear gradient was performed, with initial hold at 30% B for 1 min, increasing to 90% B over 8 min, a hold at 90% B for 4 min, and a return to 30% B over 1 min. The column was equilibrated at the initial condition for 8 min, prior to the next injection. The injection volume was routinely set at 10 μ l, the flow rate was 0.2 ml/min throughout, and the column was at ambient temperature. For LC-MS/MS analyses, data were collected between 7 and 14 min; column effluent before 7 min and after 14 min was diverted to waste.

High-resolution Orbitrap MS

High-resolution MS was performed with a Thermoelectron LTQ XL Orbitrap MS operating in the positive-ion electrospray MS/MS mode. High-resolution production spectra were recorded over the m/z range of 120 to 600, with a resolution of 60,000 at m/z 400, and a mass accuracy within 2 ppm. The $[M+H]^+$ ion of bis(2-ethylhexyl)phthalate at m/z 391.2843 served as the lock mass. The normalized collision energy was 24 eV, the ion injection time was 500 μ s, the capillary temperature was 300 °C, and the sheath gas flow rate was at setting 13, without auxiliary flow. The electrospray source was at 4.0 kV, the capillary voltage was 46; the tube lens voltage was 99, and the source current was 100 μ A. Samples were introduced into the MS in a mobile phase consisting of 0.2% formic acid/acetonitrile (30:70), via an Agilent 1200 nanoflow HPLC system, at a flow rate of 50 μ l/min.

Preparation of T and DHT standard solutions and calibration curves

Methanolic stock solutions of T and DHT (at 1 mg/ml) were used to prepare working standards of T and DHT (at 0.1, 1, 10, 100, and 1000 ng/ml), by serial dilution into methanol. All standards were stored in amber vials at –80 °C. CS-FBS was used as the matrix for preparation of the calibration curves. The calibrators were prepared by the addition of appropriate amounts of working standards of T and DHT to 0.1 ml of CS-FBS, to give the final concentrations, for T (at 0.1, 0.25, 0.5, 1.5, 5, 15, 50, and 150 ng/ml) and for DHT (at 0, 0.5, 1, 2, 5, 10, 20, and 40 ng/ml).

Animals

Male and female wild-type (WT) C57BL/6 and Cpr-low mice [31], at 2 to 3 months of age, were obtained from breeding stocks maintained at the Wadsworth Center. Animal-use protocols were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. The animals were sacrificed by euthanasia with CO₂. Blood samples, collected through cardiac puncture, were kept on ice for 1 h, prior to centrifugation at 13,000 g for 10 min at 4 °C. Tissue and serum samples were stored at –80 °C until use.

Sample preparation

Brain, seminal vesicle, and testis samples were thawed, weighed, and homogenized in physiologic saline at a concentration of 100 mg/ml. Ovaries and prostates were thawed, and then homogenized in 1 ml saline per ovary or prostate. A Polytron (Kinematica, model GT 10–35) was used for tissue homogenization. A 20- μ l aliquot of each homogenate was saved for protein determination, by use of the bicinchoninic acid method (Pierce; Rockford, IL).

For determination of T and DHT in tissue samples, 1 ml of the homogenate was used; for serum samples and calibrators, 0.1 ml of mouse serum or CS-FBS was added to 0.9 ml of saline. All samples were fortified with 200 pg of MT and MDHT, and then extracted with 8 ml of 60% hexane/40% ethyl acetate; the tubes were gently shaken in a horizontal shaker for 1 h at room temperature. After centrifugation at 1000 g for 10 min, the organic phase was transferred to a new 15-ml glass tube, and then evaporated to dryness under N₂. The residues were redissolved in 0.4 ml of methanol; the samples were further diluted with 1.6 ml of water, and then purified by solid-phase extraction (SPE) on Isolute C₁₈ cartridges (200 mg/3 ml) (Biotage; Charlottesville, VA). The C₁₈ cartridges were first activated with 5 ml of methanol, and then equilibrated with 3 ml of H₂O. After loading of the diluted samples (2 ml, containing 20% methanol), the cartridges were washed with 2 ml of 10% methanol. The analytes were eluted in 2 ml of methanol and evaporated to dryness under N₂. The residues after SPE were resuspended in 50 μ l of dry acetonitrile, combined with 50 μ l of HTP reagent for derivatization (as described above), and then analyzed by LC-MS/MS.

Lower limits of quantitation (LLOQ), recovery, and matrix effect

LLOQs, for the analyses of T and DHT as HTP derivatives, were evaluated according to the U. S. Food and Drug Administration LLOQ guidance ($S/N \geq 5$, precision of 20%, and accuracy of 80–120%). For determination of the recoveries of T and DHT, known amounts of T and DHT were added to CS-FBS either *before* the hexane/ethyl acetate extraction, or *after* the SPE, and the ratios (*before:after*) of the amounts detected for each analyte in the two types of samples were calculated. Internal standards were added to CS-FBS before extraction. Potential ion suppression effects of the matrix were evaluated by comparing peak areas of known amount of T or DHT spiked in CS-FBS extract (after SPE, as described above), to the peak areas of the same amounts of T or DHT, respectively, spiked in solvent.

Method validation

The method for determination of T and DHT in tissue and serum samples was validated in terms of inter-batch accuracy and precision. For each batch, 0.1 ml of CS-FBS was diluted with 0.9 ml of saline, fortified with a fixed amount of internal standards (200 pg each of MT and MDHT), and combined with appropriate amounts of T and DHT working standards, to achieve T and DHT concentrations of 0.15 and 0.75 (low level), 2 and 4 (medium level), and 8 and 8 ng/ml (high level), respectively. The samples were extracted, derivatized, and analyzed by LC-ESI-MS/MS (using MRM). Three validation batches were prepared for evaluation of the accuracy and precision of the method; each batch was analyzed on a different day. Each batch included a double blank (no analyte or internal standard), a blank (no analyte), a set of calibration standards, with duplicates at each T and DHT concentration, and four replicates of samples spiked with low, medium, and high levels of T and DHT.

Results and discussion

Derivatization of T and DHT for LC-MS analyses

The formation of T-HTP via derivatization of T with HTP is shown in Figure 1; the formation of DHT-HTP from DHT and HTP was analogous. As compared to positive atmospheric pressure chemical ionization (APCI), positive ESI provided better ionization efficiency for both T and DHT derivatives (data not shown). The mass spectrum of T-HTP is dominated by the protonated molecule, $[M+H]^+$, at m/z 449 (data not shown). The product ion spectrum of T-HTP under collision-induced dissociation (CID) showed formation of two dominant product ions, at m/z 257 and at m/z 269 (Fig. 2A); the fragmentation pathways appeared to involve cleavages of the steroid A and B rings, consistent with the pathway reported for the fragmentation of un-derivatized T [32].

The fragmentation pathway proposed in Figure 2A is further supported by the fact that ions at m/z 257 and m/z 269 are also observed in the product ion spectrum of the $[M+H]^+$ molecule of MT-HTP, at m/z 463 (Supplemental Fig. S1A). Fragmentation by neutral loss of fragments containing the steroid D ring, with or without a C-17 methyl group, could be expected to give rise to ions of the same m/z values in the spectra of T-HTP and MT-HTP, if the ions observed are representative of portions of the steroid A and B rings, as we have proposed. High-resolution production spectra of the $[M+H]^+$ ions of T-HTP and MT-HTP were consistent with the proposed fragmentations through the steroid A and B rings, producing ions consistent with the empirical formulas $C_{12}H_{12}N_4F_3$ and $C_{11}H_{12}N_4F_3$ (Table 2).

The ions at m/z 257 and m/z 269, arising from the $[M+H]^+$ of T-HTP, showed a high degree of stability: attempts at further collisional activation were ineffective. When the m/z 449→269 and m/z 449→257 MS/MS transitions were monitored, in the quantitative analysis of serum samples, the sensitivities obtained for the two transitions were similar. However, when tissue samples were analyzed, lesser extents of matrix-derived interference in the MS/MS ion

chromatograms were observed for the m/z 449→257 transition than for the m/z 449→269 transition. Therefore, the m/z 449→257 transition was used for quantitation, and the m/z 449→269 transition was used for confirmation, of T levels in tissue and serum samples.

The mass spectrum of DHT-HTP is also dominated by the protonated molecule, $[M+H]^+$, at m/z 451. As shown in Figure 2B, m/z 288, 260, and 164 are the most prominent fragment ions in the production spectrum of DHT-HTP. We propose that the ion at m/z 164 represents the protonated trifluoromethylpyrimidine moiety, whereas the ions at m/z 288 and 260 arise from the steroid ring system. The ion at m/z 288 could be formed through heterolytic cleavage of the hydrazone N-N bond, with the charge carried by the steroid fragment. The m/z 260 fragment could arise from the neutral loss of C_2H_4 from the m/z 288 ion, an interpretation supported by the observation of m/z 302 and 274 ions in the product ion spectrum produced by collisional activation of the $[M+H]^+$ for MDHT-HTP, at m/z 465 (Supplemental Fig. S1B). The shifts of 14 Da over the corresponding ions in the spectra of the non-methylated steroids indicate that the methyl group at C-17 is present in these fragments. This evidence of the intact methyl-substituted steroid D ring strongly supports the contention that the ion at m/z 302 arises from cleavage of the hydrazone N-N bond, a pathway analogous to the fragmentation proposed for the product ion spectrum of DHT-HTP. The high-resolution production spectra of the $[M+H]^+$ ions of DHT-HTP and MDHT-HTP are consistent with the occurrence of fragmentation at the N-N bond, neutral loss of C_2H_4 , and cleavage of the protonated trifluoromethylpyrimidine moiety, giving rise to ions consistent with empirical formulas $C_{19}H_{30}ON$, $C_{17}H_{26}ON$ and $C_5H_5N_3F_3$ (Table 2).

For quantitative analysis of DHT, more matrix interferences were observed when monitoring the 451→288 transition than when monitoring the 451→260 transition. Therefore, the m/z 451→260 transition was used for quantitation, and the m/z 451→288 transition was used for confirmation, both for method validation and for determination of DHT levels in tissue and serum samples.

Optimization of the derivatization reaction

Trifluoroacetic acid (at final concentration of 0.025%) is required for the derivatization reaction. The derivatization efficiencies were similar, when either ethanol or acetonitrile was used as the reaction solvent. The reaction time course was investigated over the range of 5 to 120 min. The reaction was completed by 1 h. Therefore, the optimized conditions for HTP derivatization consisted of an incubation of the reaction mixture in dry acetonitrile, containing 0.025% trifluoroacetic acid, at 60 °C for 1 h. As compared to the LOD for un-derivatized T, the LOD ($(S/N)>5$) for derivatized T was 10 fold lower (0.2 vs. 2 pg on column). The LOD for derivatized DHT (1 pg on column) was 40 times lower than that for un-derivatized DHT (40 pg on column).

Stability of the HTP derivatives

In order to prevent possible hydrolysis of the derivative, we removed the trifluoroacetic acid by evaporating the reaction mixture to total dryness under N_2 , and then resuspended the residues in dry acetonitrile; the amounts of un-derivatized DHT and T detected in this final preparation were <5% of the respective total amounts of originally added T and DHT (data not shown). The HTP derivatives stored in dry acetonitrile were stable at 4 °C; the amounts remaining after 24 h were 100% for T-HTP and 88% for DHT-HTP.

LC-MS analysis

In preliminary studies, several new derivatization reagents (not shown) were tested in order to identify ones that show both satisfactory ionization efficiency, and adequate chromatographic behaviors, for determination of T and DHT. All of the derivatives tested formed *cis* and

trans isomers; the pair of isomers of the HTP derivatives of T or DHT were detected as single peaks in LC-MS analysis (Fig. 3, A and B), whereas the pairs of isomers for all other types of derivatives of T or DHT were detected as separate peaks (not shown). Therefore, HTP was chosen for further studies.

It was difficult to achieve baseline separation of the HTP derivatives of T and DHT, under the HPLC conditions tested. We estimate that, with 2 ng of derivatized T on column, the signal detected as DHT-HTP was equivalent to ~ 8 pg (the peak area ratio for m/z 451→260 from the same amount of separately injected T-HTP (Fig. S2, B) and DHT-HTP (Fig. S2, A) was ~0.4%). Conversely, with 2 ng of derivatized DHT on column, the signal detected as T-HTP was equivalent to ~ 2 pg (the peak area ratios for m/z 449→257 from the same amount of T (Fig. S2, A) and DHT derivatives (Fig. S2, C) was ~0.1%). Since physiological levels of T are usually higher than the levels of DHT, the co-elution of DHT with T is unlikely to interfere with detection of T, but such co-elution could result in an overestimation of DHT levels in those tissues where T is present at much higher levels than is DHT.

DHEA, an abundant endogenous steroid with the same molecular weight as that of T, is a major source of potential interference in LC-MS/MS measurement of T [27,30,33]. Therefore, we determined the potential interference by DHEA in our assay for T. DHEA-HTP and T-HTP were not baseline-separated under the HPLC conditions tested. With 2 ng of derivatized DHEA on column, the signal detected as T-HTP was equivalent to ~ 1.2 pg (the peak area ratios for m/z 449→257 from the same amount of DHEA and T derivatives were ~0.06%). Therefore, DHEA is unlikely to interfere with determination of T in tissues or serum. Notably, androsterone-HTP and DHT-HTP were baseline-separated under the LC conditions used, and thus there are no concerns about potential interference by androsterone for the determination of DHT.

Analysis of T and DHT in tissue and serum samples—MT and MDHT were used as the respective internal standards for quantitation of T and DHT. D₅-T was also routinely included for confirmation of the retention time of T; however, it was not used for quantitation of T, because the deuterium labels of D₅-T were unstable under the conditions for derivatization. The relative retention times of the MT and MDHT, compared to those of T and DHT, are shown in Figure 3(C and D). No endogenous MT or MDHT was detected in any of the biological samples.

For quantitative analysis of T and DHT in biological samples, calibration curves (over the range of 0.10 to 150 ng/ml for T, and 0.5 to 40 ng/ml for DHT) were prepared, with use of 0.1 ml of CS-FBS as the matrix. The calibration curves were plotted with use of concentration ratios of analytes to internal standards (T/MT or DHT/MDHT) as the x-axis, and peak area ratios of the analytes to internal standards as the y-axis. The calibration curves for both T and DHT showed excellent linearity over the concentration range used ($R^2 > 0.998$; Supplemental Fig. S3). The reconstructed ion chromatograms do not show any significant background peaks in the un-spiked CS-FBS samples (Fig. 4, A and C). The recoveries of T and DHT were nearly identical to those for their internal standards under a variety of extraction conditions. The matrix effects (ion suppression) of the CS-FBS on the measurements of T and DHT were determined to be 23% and 25%, respectively (n=4).

The results of inter-batch validation assays for the quality-control (QC) samples (spiked at 0.15 and 0.75, 2 and 4, and 8 and 8 ng/ml, for T and DHT, respectively) are shown in Supplemental Table S1. The accuracy of the method was evaluated by determination of the mean relative errors. The precision of the method was evaluated by determination of the relative standard deviation (SD). The intra-batch accuracy values of the spiked samples, at all three levels tested, were in the range of -7.8%~0.92%, while the relative SD values were ≤7.4%.

The LLOQ for T was found to be 0.10 ng/ml (as determined by analysis of CS-FBS samples spiked with T at various concentrations), with a relative SD of 15.6%, a mean relative error of -6.5%, and an S/N ratio for the analyte exceeding 5 (n=6 determinations). The LLOQ for DHT was 0.50 ng/ml, with a relative SD of 3.9%, a mean relative error of -4.6%, and an S/N ratio for the analyte exceeding 5 (n=6 determinations). The MS/MS ion chromatograms of T and DHT at LLOQ are shown in Figure 4(B and D). Notably, the LLOQ achieved in our study for DHT (0.5 ng/l), through the use of the HTP derivatization technique and an API4000 Q-TRAP, is higher than that reported by Shiraishi and coworkers (~0.02 ng/l), who utilized the more sensitive API5000 LC-MS instrument and microbore chromatography [33]. Undoubtedly, the sensitivity of our assay can be further increased by improvements in LC conditions and by utility of a MS with greater sensitivity; furthermore, the ability of our technique to provide a confirmatory MS/MS transition is valuable for the identification of potential interferences in complex sample matrices such as tissue extracts.

As a first application of the new assay method, we determined the levels of T and DHT in the serum, brain, and ovary of female Cpr-low and WT mice, as well as in the serum, testis, seminal vesicle, and prostate of male Cpr-low and WT mice (Tables 3 and 4; and Supplemental Fig. S4 and S5). DHT was not detected in any of the samples from females; whereas T was detected in both males and females. T levels were significantly higher (by 25 fold) in the ovary, as well as in the serum and brain (both by ~2.5 fold) of the Cpr-low females, than in the corresponding tissues of the WT females. As expected, serum T levels were much higher in males than in females; the mean serum T levels in females (100 pg/ml) were at the LLOQ of the T assay. In general, serum levels of T, and tissue levels of T and DHT, appeared to be higher in the Cpr-low males than in the WT males. However, only serum levels of T and tissues levels of DHT in the seminal vesicle were significantly higher in the Cpr-low males than in the WT males.

In mammals, T is mainly synthesized by the testis in males [2] and by the ovary in females [1]; T is transported from the testis or the ovary to other organs via the circulatory system. It is still not clear how the systemic and tissue levels of T are regulated; complex networks, including events that modulate the rates of steroid biosynthesis and degradation [34;35;36] as well as the abundance of androgen-binding proteins [37], are assumed to be involved.

In the Cpr-low mice, the expression of CPR was down-regulated in all organs tested [31]. Therefore, all CPR-dependent activities, including those of the P450 enzymes, were suppressed. However, since microsomal P450 enzymes are involved in both the biosynthesis (e.g., CYP17) and the degradation (e.g., CYP19, CYP3A, CYP2A) of T, the impact of an organism-wide suppression of P450 activities on tissue as well as on systemic T levels will likely reflect the collective effects on biosynthetic as well as metabolic P450 enzymes in various tissues. Thus, our finding, that the suppression of CPR-dependent enzyme activities in the ovary resulted in a dramatically higher T levels in this organ (Table 3), suggests that the T-degradation pathways (particularly CYP19) were more strongly affected than were the T-biosynthesis pathways, in the ovary of the Cpr-low mice. A less likely alternative is that the highly elevated T levels in the ovary result from an increased T storage and/or reduced T secretion, given the higher systemic levels of T in these animals, as compared to WT mice.

In contrast to the 25-fold higher T levels seen in the ovary, the serum level of T was only 2.5-fold higher in the Cpr-low females than in WT females (Table 3). This relatively small effect of Cpr suppression on serum T levels could be due to the relatively high residual P450-mediated T-degradation enzyme activities in the liver of the Cpr-low mouse [31]; in that regard, systemic degradation of T is thought to be mainly carried out by hepatic enzymes, including P450s [38,39]. The levels of T in the brain were also ~2.5-fold higher in the Cpr-low females than in WT females (Table 3). This result is consistent with a previous report that T could freely cross

the blood-brain-barrier [40]; thus, the higher systemic T would lead to proportionally higher T levels in the brain.

The present finding of the very high T levels in the ovary of the Cpr-low mice, along with our previous finding of high serum T levels in these mice, could explain the infertility phenotype previously observed in Cpr-low females [31]. Notably, elevated levels of T in the ovaries of women could also cause polycystic ovary syndrome and infertility [4].

Similar to the finding for female Cpr-low mice, the serum levels of T were significantly higher in the Cpr-low males than in the WT males (Table 4). However, the tissue levels of T in the testis (the main organ of synthesis), seminal vesicle, or prostate were not significantly different between the Cpr-low and WT males; this observation suggests that the tissue levels of T in the male mice are regulated both by local mechanisms and by systemic clearance.

DHT levels in the testis and prostate were not significantly different between the Cpr-low and WT males; however, DHT levels in the seminal vesicles of the Cpr-low males were significantly higher (1.6 fold) than those in vesicles of the WT males (Table 4). In contrast to the infertility observed for the Cpr-low females, no obvious reproductive dysfunctions were observed for the Cpr-low males. Therefore, the physiological effect of the elevated tissue levels of DHT in the seminal vesicle of the Cpr-low males necessitates further investigation.

Conclusions

A novel method was developed and validated for simultaneous quantitative analysis of T and DHT, as HTP derivatives, in various mouse tissues. Compared to the previously described derivatization methods for determination of T and DHT, our method is more attractive for quantitation of T and DHT in tissues, in two ways. Firstly, the product ion spectra of the HTP derivatives of both T and DHT are dominated by structurally informative analyte-specific fragment ions that provide both primary and confirmatory MRM transitions, for quantitative analysis. Secondly, the HTP derivatives of T and DHT are eluted as single peaks, thereby improving the sensitivity and selectivity of the method. An initial application of this method, to determinations of the physiological levels of T and DHT in the serum, brain, ovary, testis, seminal vesicle, and prostate of Cpr-low and WT mice, led to the intriguing finding of dramatically higher T levels in the ovary of the female Cpr-low mice, as compared to the ovarian levels in the WT females. Thus, our method, which allows determination of T and DHT levels in various tissue samples from individual mice, should have broad application for the characterization of transgenic/knockout mouse models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Proposed scheme for the formation of T-HTP via reaction of HTP with T.

Figure 2.

Product ion spectra of T-HTP (A) and DHT-HTP (B) derivatives. Proposed fragmentation patterns of the $[M+H]^+$ ion of the T-HTP (A) and DHT-HTP (B) derivatives under CID are also shown.

Figure 3.

MS/MS ion chromatograms for HTP derivatives of T (A, 449/257), DHT (B, 451/260), and their internal standards MT (C, 463/257) and MDHT (D, 465/274), obtained from medium QC samples. The minor peaks in panels B and D represent background signals (not produced by the standard).

Figure 4. MS/MS ion chromatograms for T-HTP and DHT-HTP in blank CS-FBS (A and C) and LLOQ samples (B and D).

Table 1

Collision energy (CE) and collision cell exit potential (CXP) for MRM transitions employed for the detection of the HTP derivatives of T, DHT, MT, and MDHT

Compound	MS/MS transition	CE (eV)	CXP (volt)
DHT-HTP	451→288	38	18
DHT-HTP	451→260	49	16
T-HTP	449→257	50	21
T-HTP	449→269	50	14
MDHT-HTP	465→302	38	18
MDHT-HTP	465→274	49	16
MT-HTP	463→257	50	21
MT-HTP	463→269	50	14

Table 2

High-resolution MS/MS analysis of the HTP derivatives of T, MT, DHT, and MDHT Product ions of the T-HTP $[M+H]^+$ ion at m/z 449.25

Formula	Measured mass	Theoretical mass	Delta (ppm)
$C_{12}H_{12}N_4F_3$	269.1010	269.1009	0.54
$C_{11}H_{12}N_4F_3$	257.1010	257.1009	0.44
$C_5H_5N_3F_3$	164.0429	164.0430	-0.56
Product ions of the MT-HTP $[M+H]^+$ ion at m/z 463.27			
Formula	Measured mass	Theoretical mass	Delta (ppm)
$C_{12}H_{12}N_4F_3$	269.1008	269.1009	-0.06
$C_{11}H_{12}N_4F_3$	257.1008	257.1009	-0.10
$C_5H_5N_3F_3$	164.0428	164.0430	-1.02
Product ions of the DHT-HTP $[M+H]^+$ ion at m/z 451.27			
Formula	Measured mass	Theoretical mass	Delta (ppm)
$C_{19}H_{30}ON$	288.2318	288.2322	-1.33
$C_{17}H_{26}ON$	260.2007	260.2009	-0.77
$C_5H_5N_3F_3$	164.0427	164.0430	-1.75
Product ions of the MDHT-HTP $[M+H]^+$ ion at m/z 465.28			
Formula	Measured mass	Theoretical mass	Delta (ppm)
$C_{20}H_{32}ON$	302.2476	302.2478	-0.71
$C_{18}H_{28}ON$	274.2166	274.2165	0.35
$C_5H_5N_3F_3$	164.0427	164.0430	-0.85

Table 3

Levels of T in brain, ovary, and serum of the WT and Cpr-low female mice

Strain	T levels		
	Serum (pg/ml)	Brain (pg/mg protein)	Ovary (pg/mg protein)
WT (n=4)	100 ± 18 ^a	0.77 ± 0.16	36 ± 28
Cpr-low (n=5)	250 ± 67 ^b	2.0 ± 0.94 ^c	900 ± 525 ^c

^aFor samples with T levels below the LLOQ, a 2nd injection was made after the remaining samples were concentrated.

^bP < 0.01, compared to the WT mice (Student's *t*-test)

^cP < 0.05, compared to the WT mice (Student's *t*-test)

Table 4

Levels of T and DHT in serum, testis, seminal vesicle, and prostate of the WT and Cpr-low male mice

Strain	Steroid levels					
	Serum (pg/ml)		Testis (pg/mg protein)		Seminal vesicle (pg/mg protein)	
	T	DHT	T	DHT	T	DHT
WT (n=6)	3700 ± 3130	978 ± 810	120 ± 85	4.0 ± 4.0	52.5 ± 61.7	81.7 ± 62.0
Cpr-low (n=6)	13700 ± 8500 ^a	1660 ± 980	193 ± 81	6.0 ± 2.0	113 ± 57	130 ± 47

^ap < 0.05, compared to the WT mice (Student's t-test)