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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

Zhihong O'Brien*, Noah Post, Michael Brown[1], Ajay Madan, Timothy Coon, Rosa Luo, Trudy A. Kohout

Neurocrine Biosciences Inc., 12780 El Camino Real, San Diego, CA 92130, USA

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ABSTRACT

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed to extract and quantify the androgen concentration in the rat prostate. This method introduced a novel 96-well plate format for the extraction and derivatization of testosterone (T) and dihydrotestosterone (DHT) from rat prostatic tissue that greatly simplified the sample preparation procedure. Due to the difficulty to obtain reproducible specimens with non-detectable level of androgen, a matrix-free standard solution was used for method validation. Both T and DHT calibration curves were linear over the calibration range (12.5–2500 pg) with correlation coefficient values greater than 0.9900. The intra-day and inter-day accuracy, reported as %bias, and precision, reported as %CV, of T and DHT were within ±10%. The lower limit of detection (LLOD) and lower limits of quantification (LLOQ) for both T and DHT were determined to be 5 and 12.5 pg. The validation results demonstrated the selectivity, sensitivity, accuracy, precision, linearity and ruggedness of the method, as well as the suitability of the method for simultaneous detection of T and DHT in rat prostatic tissues. The validated method was successfully applied to determine the physiological T and DHT level in rat prostatic tissues. Similarly to the serum concentration profile pattern, T and DHT intraprostatic levels peaked 2 h after lights-on and decreased after lights-off with DHT level approximately 4-fold greater than T.

1. Introduction

The clinical incidence of prostatic cancer (PCa) and benign prostatic hyperplasia (BPH) is correlated to abnormal androgenic dynamics, for instance, elevated T and DHT circulation levels in serum and male sex organs [1–3]. Recent clinical findings revealed that the main cause of death due to prostate cancer is because of recurrent prostate cancer despite castrate levels of circulating androgens following hormone treatment [4]. Testosterone and its 5-α-reductase catalyzed product, dihydrotestosterone, are the two dominant androgens in male sex organs and play important roles in differentiation of male sexuality and development of male secondary sex characteristics [5]. Study of the T and DHT dynamic changes in prostatic tissues of BPH and PCa patients is crucial for the investigation of the causes of recurring prostate cancer disease, for monitoring PCa and BPH disease progression, and for development of new treatments for PCa and BPH.

There has been significant improvement in bioanalytical assay selectivity, sensitivity and throughput of simultaneous quantification of T and DHT in serum and tissues with state-of-the-art technology, LC/MS–MS [6–11]. However, analytical methods on measurement of T and DHT from prostatic tissues are still facing challenges. Firstly, both T and DHT are endogenous steroids, thus a surrogate matrix has to be used to exclude the interferences coming from the matrix. Secondly, the current available methods to quantify T and DHT in prostatic tissue utilize a conventional sample process in tubes instead of a 96-well plate format. Therefore, a relatively higher throughput assay needs to be developed to reduce the labor intensive sample extraction process. Finally, the methodology available in the literature has insufficient description of several key steps for the adequate implementation of the technique, thus a detailed and thoroughly validated method is needed.

Circulating hormone levels of T and DHT over a 24-h cycle have been well studied in the male rat [12–15] but the corresponding prostatic tissue T and DHT levels have yet to be reported. To gain a better understanding of the role of androgenic dynamics in the cause of prostatic disorders, direct measurement of T and DHT in prostatic tissue is critical. Therefore, the objectives of this paper are to improve the currently available T/DHT prostatic tissue assay method to increase assay throughput, to validate the use of a surro-

* Corresponding author. Tel.: +1 858 270 8495; fax: +1 858 617 7830.
E-mail address: jzobrien@hotmail.com (Z. O'Brien).
[1] Current address: AnaptysBio, Inc., 10835 Road to the Cure, Suite 100, San Diego, CA 92121, USA.

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gate matrix, to clearly define the assay steps to render them easy to follow, and to apply the method to obtain the physiological T and DHT levels in the rat prostate over a 24-h cycle.

2. Experimental

2.1. Materials

Testosterone (T), dihydrotestosterone (DHT, 5α-androstan-17β-ol-3-one), testosterone-d_3 solution (T-d_3, 100 μg/ml in 1,2-dimethoxyethane), and 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FPMTS) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Testosterone and dihydrotestosterone derivative standards were synthesized in-house to help with MS condition optimization and the purities of both synthesized derivatives were tested to be 100% using LC-UV method. Water used in this study was purified in-house using a Milli-Q system Synthesis A10 (Millipore, Billerica, MA, USA). Castrated rat prostate tissues were purchased from Bioreclamation Inc. (Kicksville, NY, USA). Formic acid (≥99% pure) and dichloromethane (≥99% pure) were purchased from Acros Organics (Morris Plains, NJ). Acetonitrile (ACN) (HPLC grade), methyl *tert*-butyl ether (MTBE) (HPLC grade), and other chemicals and reagents used were obtained from Sigma–Aldrich.

2.2. Preparation of stocks, calibration standards and quality control samples

The original stock solutions of T (5 mM), DHT (5 mM) and T-d_3 as internal standard (IS) (500 ng/ml) were prepared in ACN/H_2O (60/40, v/v) and kept at −20 °C. The original stock solutions of T and DHT were diluted in ACN/H_2O (60/40, v/v) to obtain working stock solutions containing T and DHT at 10 μg/ml. The original stock solution of IS was subsequently diluted to obtain the working stock solution of 20 ng/ml. Standard solutions of T and DHT for spiking were prepared by diluting the working stock with ACN/H_2O (60/40, v/v) to achieve final concentrations of 0.1, 0.25, 1.0, 2.5, 5.0, 10, 25 and 50 ng/ml. Fifty microliter of ACN/H_2O (60/40, v/v), T-DHT spiking solution, and 50 μl of IS (20 ng/ml) were added into each of 10 tubes containing 1 ml deionized (DI) water with the final amount of added standard T and DHT as 0, 5, 12.5, 25, 50, 125, 250, 500, 1250 and 2500 pg. Since it is impossible to obtain androgen-free prostate tissues, prostatic tissue matrix was not used for the construction of the calibration curve in the routine assay. However, castrate rat prostate tissue was added into calibration curve sample preparations to demonstrate the minimal bio-matrix effect on T and DHT quantification and the validity of using surrogate calibration standards for T and DHT quantification.

Quality control (QC) samples of four concentration levels (0.25, 0.75, 2.5, and 40 ng/ml) were also prepared separately from the calibration standards to assess precision, accuracy, and recovery. QC samples were spiked with IS in the same manner as the calibration standards to achieve the following final amount values of: 12.5, 37.5, 125 and 2000 pg. The QC samples were also assayed along with the rat samples to monitor the performance of the method and to assess the integrity and validity of the rat sample assay results.

All calibration standards and QC samples were extracted, derivatized, and analyzed in the same manner as the unknown samples. All the stock solutions were stored at −20 °C and brought to room temperature prior to use.

2.3. Sample preparation

Approximately 30–70 mg frozen ventral prostate tissue was isolated on dry ice and suspended in 1 ml water spiked with 50 μl T-d_3 IS solution. Samples were homogenized with a Model Pro200 tissumizer (Pro Scientific Inc., Oxford, CA, USA) for approximately 30 s at maximum speed (30,000 rpm). Homogenized samples were extracted with 2 ml MTBE and centrifuged at 3500 rpm for 30 min at 4 °C (Beckman Coulter, Allegra™ 25R centrifuge, Fullerton, CA, USA). Samples were placed in a dry ice-acetone bath to freeze the bottom aqueous layer and allow the clean transfer of the top organic layer to a 96-well deep-well plate. The solvent was evaporated using a heated nitrogen plate evaporator (Glas-Col, Terre Haute, IN, USA) for approximately 20 min at 50 °C, and the resulting dried residue was reconstituted with 200 μl of water/methanol (80/20, v/v).

Extracts were loaded onto 96-well plate format solid phase extraction (SPE) columns (Waters Oasis® HLB μElution Plate 30 μm, 1.60 mg, Waters, Milford, MA, USA) preconditioned with 200 μl methanol and followed with 200 μl water. The loaded columns were successively washed with 200 μl of 5% methanol and 200 μl of 40% methanol, and then were eluted with 2× 100 μl of acetonitrile/isopropyl alcohol (60/40, v/v). Eluates were dried using a heated nitrogen plate evaporator for approximately 20 min at 50 °C. To increase assay sensitivity, dried T, DHT and T-d_3 were subjected to derivatization with FPMTS reagent.

Derivatization of T, DHT and T-d3 was introduced by Nakagawa and Hashimoto [11] and was modified here to accommodate the smaller scale and to achieve a high yield. Briefly, 350 μl dichloromethane, 150 μl 5% FPMTS in acetonitrile, and 6 μl triethylamine were added to the dried samples after elution from the SPE columns and incubated at room temperature for 1 h. The derivatized samples were dried as before, resuspended in 200 μl water/methanol (80/20, v/v), and purified using Waters Oasis HLB μElution columns. After successive washing with 200 μl of 5% methanol and 200 μl of 20% methanol, the samples were eluted with 2× 100 μl acetonitrile/isopropyl alcohol (60/40, v/v). Lastly, the eluates were dried and reconstituted in 200 μl water/acetonitrile (80/20, v/v), 15 μl of which was subjected to LC–MS–MS analysis.

2.4. Chromatography

The HPLC system consisted of a binary Agilent HP1100 series pump and a column heater (Agilent, Santa Clara, CA, USA) coupled with a HTC PAL autosampler (Leap Technologies, Carrboro, NC, USA). Chromatographic separation was carried out on a reverse phase Zorbax C18 (5 μm) column (3.0 mm × 150 mm) in combination with a guard column of the same type (4 mm × 4 mm i.d.) from Agilent (Agilent, Santa Clara, CA, USA). Column temperature was held at 30 °C. The mobile phase A was 100% water with 0.1% formic acid and mobile phase B was 100% acetonitrile with 0.1% formic acid. The flow rate was held constant at 0.7 ml/min. A gradient elution was performed: 0–2 min 95% A, 5% B; 6–11 min: 70% A, 30% B; 16–21 min 95% A, 5% B. The retention time for the derivatives of T, DHT and T-d_3 were 7.2, 8.9 and 7.2 min, respectively.

2.5. Mass spectrometry

Applied Biosystems API 5000 (Foster City, CA, USA) triple quad mass spectrometer equipped with an electrospray ionization interface was connected to Agilent HP1100 pump and was used to detect T, DHT and T-d_3 derivatives. The mass spectrometer was operated in the positive ion multiple reaction monitoring (MRM) mode. HPLC effluent was introduced to the ionization source from 5 to 13 min via divert valve. The spray voltage and ionization source temperature were 5000 V and 600 °C. The curtain gas (CUR), ion source gas1 (GS1), ion source gas2 (GS2) and collision gas (CAD) were set to be 40, 50, 50 and 12, respectively. Mass spectrometer conditions were optimized for strongest product ion signal intensities using T- and DHT-derivatives synthesized in-house. The optimized declustering

potential (DP), collision energy (CE) and collision cell exit potential (CXP) settings for T and DHT were 76, 37 and 26 V, and 61, 27 and 16 V, respectively. The following MRM transitions were monitored for the analytes and IS: T derivative, m/z 380.28 and 109.00; DHT derivative, m/z 382.31 and 255.20; T-d_3 derivative, m/z 383.28 and 256.20. The dwell time for each transition was 150 ms.

2.6. Assay validation

2.6.1. Quantification, linearity, lower limits of detection and lower limits of quantification

The software used for quantification was Analyst 1.4.2 (Applied Biosystems, Foster City, CA, USA). To establish the linearity, calibration curves were prepared and assayed on 4 different days. Calibration curve was constructed by plotting peak area ratios of analyte to IS versus nominal concentrations of T-DHT standards. A $1/X^2$ weighted linear regression mode was used to fit the calibration standard curve. To quantify the concentration of T and DHT in QC or unknown samples, the areas of the analyte of interests were first normalized to IS, and then compared to a standard curve. The lower limits of detection (LLOD) and lower limits of quantification (LLOQ) were determined from the analyte signal-to-noise ratio, where the LLOD is defined as the lowest concentration in which the signal-to-noise ratio is at least 3:1, and the LLOQ is defined as the lowest concentration in which the signal-to-noise ratio is at least 5:1 with intra-day and inter-day accuracy and precision $\leq\pm$20%.

2.6.2. Precision and accuracy

Intra-day accuracy and precision were evaluated from replicate analysis ($n=6$) of QC samples at four different concentrations on the same day. Inter-day accuracy and precision were evaluated from the same sets of QC samples from different days. Accuracy is the degree of closeness of the determined values to the nominal, which can be expressed as %bias and calculated using the formula: %bias = (measured − nominal)/nominal × 100%. Accuracy is considered to be acceptable when %bias is no more than ±15% of the nominal for all concentrations with the exception of standard and QC LLOQ, where $\leq\pm$20% of the nominal is considered acceptable [16]. Precision is the degree of closeness of the repeated measurements obtained from multiple sampling of the homogeneous pool, can be expressed as coefficient of variation (CV%) and calculated using the formula: CV% = (standard deviation/mean) × 100%. Precision is considered to be acceptable when CV% is less than 15% for all concentrations except standard and QC LLOQ, where $\leq\pm$20% variance estimate is considered acceptable [16].

2.6.3. Specificity

Castrate rat tissues with non-detectable levels of T and DHT were used as blank matrix for specificity evaluation. Blank samples without additions (matrix, T, DHT and IS), as well as blank samples containing only matrix were used to evaluate the specificity of the method by comparing their chromatograms with those corresponding to the T and DHT spiked samples. Interference between IS (T-d_3) and T was checked using blank samples spiked with either androgen.

2.6.4. Recovery

The absolute recovery was determined using three T and DHT QC concentrations (37.5, 125 and 2000 pg) in 6 replicates in the absence of matrix. Pure derivatized T and DHT at concentrations equivalent to underivatized T and DHT QC concentrations were used to evaluate assay recovery. The percentage recovery was calculated by comparing the peak areas of the QC samples that have undergone the derivatization and extraction process and post-extraction-derivatization water extracts spiked with pure derivatized T and DHT, assuming 100% recovery. Recovery does not need to be 100%, but the extent of analytes recoveries have to be consistent, precise and reproducible [16].

2.6.5. Matrix effect

Matrix effect was examined by comparing the slopes of the calibration curves constructed in the presence and absence of 30 mg matrix (castrate rat prostatic tissues). The accuracy of the quantification between samples with matrix and without was calculated to demonstrate the validity of using calibration solution without prostate tissue as a surrogate calibration standard. In addition, the T- and DHT-derivative signal intensity of LLOQ with and without matrix was compared to determine the effect of prostate tissue matrix on signal intensity alteration of the analytes.

2.7. Application

Adult male Sprague–Dawley rats (250–300 g) were obtained from Charles River Laboratories (San Diego, California) and single housed with a 12-h light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee. Animals were dosed with vehicle (10% PEG-400, 0.225% methylcellulose) at lights-on with care taken to minimize stress. Groups of 12 animals for each time point were sacrificed by live decapitation 2, 4, 8, 12, 18, and 24 h after dosing. Animals at the 0 time point were not dosed and sacrificed at lights-on. The prostates were immediately removed, dissected to separate the ventral lobes, and flash frozen in liquid nitrogen for physiological intraprostatic level measurement of T and DHT.

3. Results and discussions

Recently published T and DHT quantification methods in tissues, such as brains and prostates, have demonstrated the utility of high performance liquid chromatography coupled with tandem mass spectrometry (LC/MS–MS) [5,7,9,10]. However, there are still numerous challenges awaiting such as: (1) the complicated and low throughput sample extraction process described in several publications needs improvement; (2) the FPMTS derivatization and post-clean-up process requires the development of a higher throughput procedure; and (3) the difficulty of obtaining androgen-free prostatic tissue matrix for the calibration standards and the QC samples requires the development and validation of surrogate calibration standards and QC samples. Resolutions to these challenges are described as follows.

3.1. Pre-treatment of prostate tissue, derivatization and post-treatment of reaction mixture

To improve the assay throughput, method development was focused on implementing a simple one-step liquid–liquid extraction with MTBE to extract T and DHT from the homogenized tissue. A 96-well plate format based process following MTBE tissue extraction was adopted to successively perform solid phase extraction (SPE), derivatization and post-derivatization SPE clean-up with improved throughput efficiency.

To achieve required sensitivity for accurate detection of low picogram levels of T and DHT in prostate tissues, a permanently charged moiety from a derivatization reagent, like FPMTS, was introduced into T and DHT molecules via derivatization reactions (Fig. 1). Due to unclear published derivatization methods, efforts were taken to optimize the amount of derivatization reagents used, duration of the reaction and the post-reaction treatment process. To more efficiently produce the T- and DHT-derivative products, a thousand fold excess FPMTS-derivatization reagent was added into reaction mixture with an equal mole of triethylamine to FPMTS

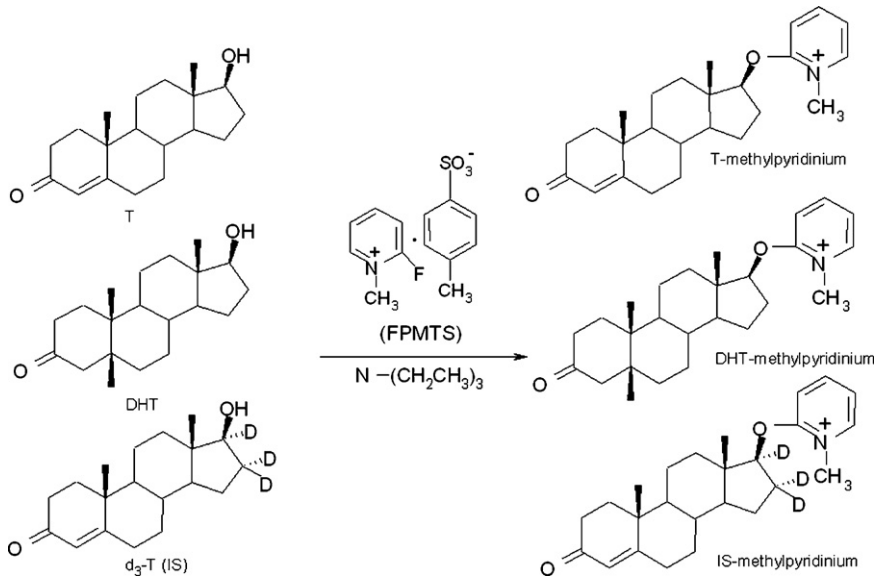

Fig. 1. The chemical structures of testosterone (T), dihydrotestosterone (DHT), testosterone-d_3 (T-d_3, IS) and their derivatives with 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FPMTS).

Table 1
Calibration curve parameters for T- and DHT-derivatives.

	T			DHT		
	Intercept	Slope	r^a	Intercept	Slope	r^a
With matrix	0.00228	0.0764	0.9942	0.000415	0.0768	0.9954
Solution without matrix	0.00622	0.0753	0.9924	0.00643	0.0725	0.9954

[a] Correlation coefficient.

to provide an alkaline medium. The derivatization reaction conditions described in the Experimental section provided satisfactory conversion from T and DHT to their respective derivatives without detectable levels of non-derivatized T and DHT in the final injection samples. Following the completion of the derivatization reaction, two types of solid phase extraction plates were evaluated for the extraction efficiency of T- and DHT-derivatives, as well as for the removal efficiency of the remaining derivatization reagents. We first tested the Water Oasis Mixed-mode Cation Exchange (MCX) 96-well SPE plates to purify the T- and DHT-derivative products following the derivatization reaction, but it failed due to extensive competition for the cation exchange binding capacity of the MCX sorbent between the positively charged derivatization reagent, FPMTS, and the positively charged T-, DHT-derivative products as well as the difficulty in differentiating the cation exchange binding strength between the products and the derivatization reagent. Secondly we tested the C18 μElution 96-well SPE plate and demonstrated adequate capability for retaining T- and DHT-derivatives on the C18 sorbent with minimum breakthroughs for the analytes and the ability to selectively exclude the derivative agent.

FPMTS-derivatization method provides permanently positively charged methylpyridyl moiety to the T and DHT molecules, which significantly enhances the ESI-MS detection sensitivities of T and DHT. However, in order to achieve maximum derivatization efficiency, an excessive amount FPMTS-derivatization reagent needs to be applied into the reaction. The biggest challenge using FPMTS-derivatization agent is therefore the removal of the excess reagent following the derivatization reaction. The removal process is achieved by using 96-well plate solid phase extraction but multiple washing followed the loading of the reaction sample solutions results in low recovery of T- and DHT-derivative products which further lowers the analytical sensitivity. A number of derivatization reagents are currently available and could be explored in the future to compare the derivatization efficiency of other reagents with FPMTS in order to select more suitable one for further enhancement of analytical detection sensitivity of T and DHT.

3.2. Calibration curves and matrix effect

Calibration curves should be prepared in the same blank matrix as the samples, however, even prostates from castrated rats may contain measurable levels of T and DHT. To determine the extent to which castrated prostatic tissue matrix affects the quantification of T and DHT, calibration curves with and without castrated rat prostatic tissues were constructed and compared in the same analytical run. The slopes of the calibration curves constructed with matrix or with ACN/H_2O (60/40, v/v) solution only and analyzed in the same run were similar, 0.0764 vs. 0.0753 for T and

Table 2
Calibration curve concentration recovery with matrix for T- and DHT-derivatives.

	Nominal amount spiked into castrated prostatic tissue matrix (pg)									
	12.5	12.5	25	25	50	125	250	500	1250	2500
Accuracy (%)										
T	94.9	105	106	105	93.9	118	88.9	93.7	111	104
DHT	92.1	104	97.5	114	102	115	85.3	92.1	116	104

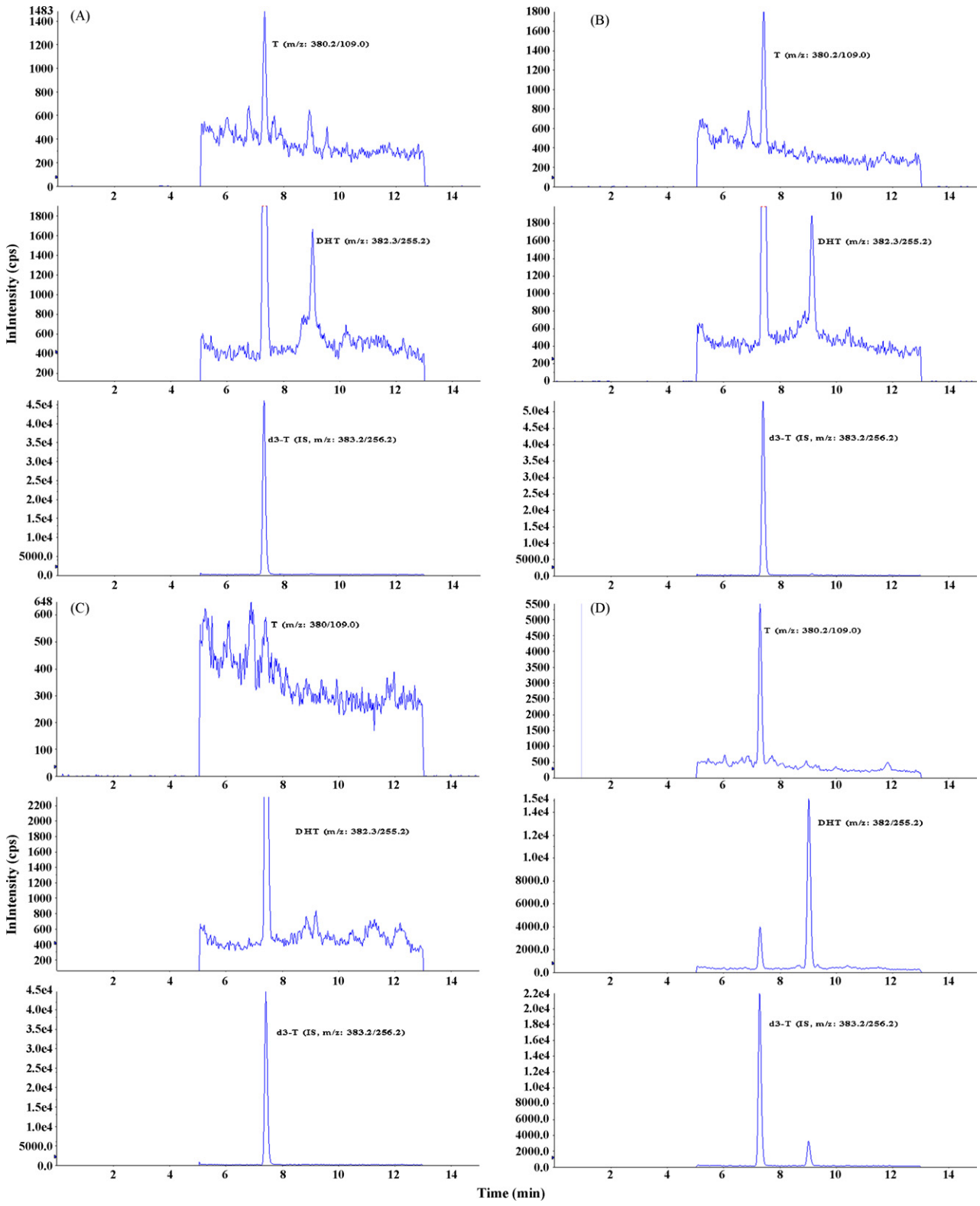

Fig. 2. Representative MRM chromatograms of the 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FPMTS) derivatives of testosterone (T: *m/z* 380.2/109.0 amu), dihydrotestosterone (DHT: *m/z* 382.3/255.2 amu), testosterone-d_3 (T-d_3, IS: *m/z* 383.2/256.2 amu) for standard solutions and extracted sample solutions: (A) LLOQ for T and DHT (0.25 ng/ml, 12.5 pg) and spiked IS solution with matrix; (B) LLOQ for T and DHT (0.25 ng/ml, 12.5 pg) and spiked IS solution without matrix, (C) internal standard (testosterone-d_3, T-d_3) extract solution and (D) rat prostatic tissue sample at 2 h after vehicle treatment and spiked with 20 ng/ml of IS.

0.0768 vs. 0.0725 for DHT, respectively (Table 1). Concentrations in calibration curve constructed with matrix were quantified using the calibration curve constructed in solution and analytical recoveries were calculated for each concentration in calibration curve with prostate matrix. The recovery values are listed in Table 2 and ranged from 88.9% to 118% for T and 85.3% to 116% for DHT. LLOQ at 12.5 pg prepared in solution had similar signal intensities of T-, DHT- and IS-derivatives as those prepared with prostatic tissue

Table 3
Intra-day and Inter-day precision and accuracy of T measurement (four runs, six replicates per run).

QCs (pg)	Intra-day			Inter-day		
	Mean ± S.D.	Precision (%CV)	Accuracy (%bias)	Mean ± S.D.	Precision (%CV)	Accuracy (%bias)
12.5 (LLOQ)	13.9 ± 0.6	4.4	10.9	12.3 ± 1.4	11.4	−1.5
37.5	33.7 ± 0.6	1.7	−10.1	34.1 ± 1.8	5.3	−9.1
125	134 ± 3.64	2.7	7.1	133 ± 7.4	5.6	6.4
2000	2010 ± 49.8	2.5	0.5	2000 ± 131	6.6	−0.2

matrix (Fig. 2(A) and (B)). These data suggest that prostate matrix had no effect on calibration curves and, therefore, calibration standards and QC samples prepared in solution were used for current method validations, as well as for the measurement of T and DHT in the collected study samples.

3.3. Specificity

Due to potential contribution of an isotopic effect on the multiple reaction monitoring (MRM) transitions between T-, DHT- and d_3-T-derivatives, both analytes and IS at their upper limit of quantitation (ULQ) were injected into the LC/MS–MS separately to investigate the interferences among the analytes and IS. Both T- and d_3-T-derivatives, with the respective precursor to product ion transitions of 380 → 109 and 383 → 256, exhibited the same retention time of approximately 7.3 min without interfering with each other. The retention time of the DHT-derivative differed from the T- and d_3-T-derivatives by approximately 1.7 min (Fig. 2). The contribution of T- and d_3-T-derivatives to the MRM mass transition of the DHT-derivative does not interfere with DHT quantification since they have distinctly separate retention times.

3.4. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

The linearity of the method was evaluated by analyzing five calibration standards (between 12.5 and 2500 pg T and DHT) in solution and one set of standards in the presence of matrix. The curve linearity was indicated by the correlation coefficients obtained from $1/X^2$ weighted linear regressions and ranged from 0.9924 to 0.9954 for both analytes (Table 1). LOD and LOQ were determined by evaluating signal-to-noise (S/N) ratios of the extracted analytes at 5 and 12.5 pg with matrix and those without matrix. Furthermore, the %Bias was also evaluated as the deviation of calculated values from the nominal values, and %CV as the precision measurement of replicates. According to well established practices [16], S/N ratios for LOD and LOQ should be at least 3 and 5, respectively. The measured S/N ratios for LOD and LOQ of both analytes of current method were greater than 5 and 10, respectively, either with or without matrix. Calculated %CV and %Bias values of less than 20% at 12.5 pg, in the presence or absence of matrix, was observed, however, greater than 20% of precision and accuracy measurement was obtained for 5 pg. Based on these data, the LODs and LOQs of T and DHT from this assay were inferred to be approximately 5 and 12.5 pg, respectively. Due to limited availability of castrated rat prostatic tissue, only two LOD and LOQ samples with matrix were evaluated. Greater accuracy could be obtained if more QC samples were to be used for LOD and LOQ evaluations. A lower LOQ could be possibly achieved by further optimizing the method, such as reducing reconstitution solution volume; modification of derivatization reaction condition; using a UPLC method for separation. Further exploration was not conducted because the method developed was well suited for current application.

3.5. Accuracy and precision

We have demonstrated that the prostatic tissue matrix has no effect on the calibration curves of both analytes, and has minor effects on the signal intensity of T-, DHT- and d_3-T-derivatives. Therefore, the accuracy and precision of this method were examined by using calibration standards and QC samples prepared in solutions. The intra-day and inter-day accuracy and precision results for T are shown in Table 3, while those for DHT are shown in Table 4. Intra-day accuracy and precision were evaluated by analysis of quality control samples at 12.5, 37.5, 125 and 2000 pg with six replicates per concentration. Intra-day bias ranged from −10.1% to 10.9% for T and −2.5% to 4.0% for DHT. Intra-day precision measured as %CV ranged from 1.7% to 4.4% for T and 5.2% to 8.7% for DHT. Inter-day bias ranged from −9.1% to 6.4% for T and −4.3% to 7.3% for DHT, while inter-day precision reported as %CV ranged from 5.3% to 11.4% for T and 5.6% to 7.3% for DHT. These results are consistent with pre-established acceptance criteria [14] since all QC samples are within ±15%. With these data, the lowest quantification limit for T and DHT prostatic tissue assay was confirmed to be 12.5 pg. It would be more evident to demonstrate the minimal matrix effect on intra-day accuracy should six replicates of matrix samples were checked against matrix-free standard curve of T and DHT. However, the matrix samples were not tested for accuracy due to limited available castrated rat prostatic tissues at the time.

3.6. Recovery

The recovery was performed with QC samples prepared in solution at three concentrations, 37.5, 125 and 2000 pg, for T and DHT. The recovery of the sample preparation was assessed by evaluating the overall process of liquid–liquid extraction, solid phase clean-up, and derivatization followed by a second solid phase clean-up. The recovery results are presented in Table 5. The assay recoveries were 71, 74 and 55% for T and 56, 59 and 45% for DHT at 37.5, 125 and 2000 pg, respectively. It is not necessary for recovery to be high but it must be robust and consistent, which was the case for our method. The source of the low recovery was not investigated, but

Table 4
Intra-day and Inter-day precision and accuracy of DHT measurement (four runs, six replicates per run).

QCs (pg)	Intra-day			Inter-day		
	Mean ± S.D.	Precision (%CV)	Accuracy (%bias)	Mean ± S.D.	Precision (%CV)	Accuracy (%bias)
12.5 (LLLOQ)	12.7 ± 0.9	6.8	1.7	12.9 ± 0.9	6.7	2.8
37.5	36.6 ± 1.9	5.2	−2.5	35.9 ± 2.0	5.6	−4.3
125	130 ± 11.3	8.7	4.0	134 ± 8.0	6.0	7.3
2000	2030 ± 173	8.5	1.3	2070 ± 151	7.3	3.3

Table 5
Recoveries of T and DHT from sample extraction.

QCs (pg)	T recovery (%)	DHT recovery (%)
37.5	71	56
125	74	59
2000	55	45

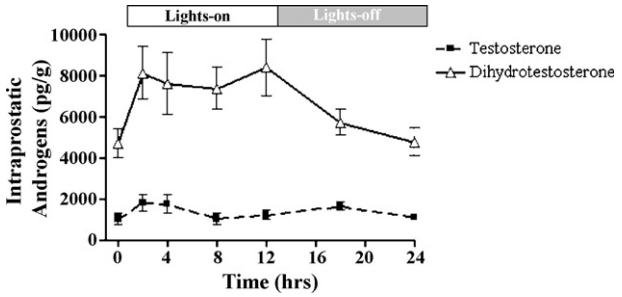

Fig. 3. Mean testosterone and dihydrotestosterone prostatic tissue concentration–time profile in 12 male Sprague–Dawley rats following single oral vehicle treatment (10% polyethylene glycol 400 and 0.225% methylcellulose).

we speculate it could be due to sample extraction or derivatization efficiencies of the method. Furthermore, only one derivatization reagent was evaluated in this study, thus it is possible that other reagents may increase the overall assay recovery and improve assay sensitivity.

3.7. Application to rat prostatic tissue sample analysis

Following validation, this methodology was used to investigate the physiological intraprostatic T and DHT concentrations in intact male rats over a 24-h light-dark cycle (Fig. 3). Circulating plasma levels of T and DHT over a 24-h cycle has been well studied [12–15], however, the corresponding T and DHT levels in the prostate has not been documented. Similarly to the serum concentration profile pattern, T and DHT intraprostatic levels peaked 2 h after lights-on and decreased after lights-off with DHT concentration level approximately 4-fold higher than that of T. It is interesting to note that while the testosterone peak is transient (duration of 2–4 h), the DHT peak is much longer lasting, and encompasses the entire lights-on period. This observation is consistent with the enhanced stability of DHT in the prostate [15].

4. Conclusions

A sample extraction method for T and DHT from rat prostatic tissue samples with high throughput was developed and validated over the range of 12.5–2500 pg of androgens. Standard solutions were proven to be appropriately used as surrogate matrix to quantify T and DHT in rat prostate tissue samples. The method was demonstrated to be sensitive, selective, accurate, precise, sufficiently linear and robust to make it suitable for analyzing preclinical rat prostatic tissue samples in a relative high throughput fashion. This validated method was successfully applied to measure rat physiological T and DHT intraprostatic levels and it may be suitable for androgen measurements in tissues or biological fluids from other species as well.

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