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Validation of a sequential extraction and liquid chromatography–tandem mass spectrometric method for determination of dihydrotestosterone, androstanediol and androstanediol–glucuronide in prostate tissues

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ABSTRACT

Androgens are key mediators of prostate development and function, a role that extends to the development of prostate diseases such as benign prostatic hyperplasia (BPH) and prostate cancer. In prostate, DHT is the major androgen and reduction and glucuronidation are the major metabolic pathways for DHT elimination. A streamlined method for quantitation of dihydrotestosterone (DHT), 5α-androstan-3α,17β-diol (3α-diol), and 3α-diol glucuronide (diol-gluc) was established and validated for use with archived prostate tissue specimens to facilitate examination of the roles of the underlying metabolism. This involved a sequential 70/30 hexane/ethyl acetate (hex/EtOAc) extraction of steroids, followed by an ethyl acetate extraction for diol-gluc. Derivatization of the hex/EtOAc fraction with2-fluoro-1-methylpyridinium *p*-toluene-4-sulfonate (FMP) was used to enhance sensitivity for hydroxyl steroids and liquid chromatography–tandem mass spectrometry (LC–MS/MS) was utilized for analysis of both fractions. The method was validated with calibration standards followed by recovery assessment from spiked samples of BPH and normal prostate. Lower limits of quantitation (LLOQ) were 50 pg/g, 20 pg/g and 100 pg/g for DHT, 3α-diol and diol-gluc, respectively for extracts from 50 mg equivalents of tissue. Prepared samples were stable for up to three weeks at 4 °C and 37 °C. The method provides excellent sensitivity and selectivity for determination of tissue levels of DHT, 3α-diol, and diol-gluc. Furthermore, this protocol can easily be extended to other hydroxyl steroids, is relatively straightforward to perform and is an effective tool for assessing steroid levels in archived clinical prostate samples.

1. Introduction

Prostate cancer (PCa) is the most diagnosed cancer in men in western countries, and its incidence is increasing steadily worldwide [1–4]. It has been estimated in 2011 that there would be approximately 25,500 cases diagnosed in Canada and 240,890 cases diagnosed in USA [5,6]. Early detection methods, such as digital rectal exams and assessment of serum prostate-specific antigen (PSA), together with adjuvant medical therapies have led to a decline in PCa-associated mortality over the past 10 years [7–11]. In spite of these early detection methods and therapies, PCa remains the third most common cause of death due to cancer and it was estimated that 4100 Canadian and 33,720 American men would die from the disease in 2011 [5,6].

Androgens, acting through the androgen receptor (AR), are required for prostate development and normal prostate function; however, they are also linked to the development of diseases, especially benign prostatic hyperplasia (BPH) and PCa [12–14]. Approximately 80–90% of PCa is dependent on androgen levels at initial diagnosis [13,15]. In the systemic circulation, testosterone is the major androgen; whereas in the prostate, testosterone is predominantly converted by 5α-reductase to the more active metabolite 5α-dihydrotestosterone (DHT) (Fig. 1) [16–19]. DHT

Abbreviations: DHT, dihydrotestosterone; 3α-diol, 5α-androstan-3α,17β-diol; diol-gluc, 3α-diol glucuronide; BPH, benign prostatic hyperplasia; FMP, 2-fluoro-1-methylpyridinium *p*-toluene-4-sulfonate; LC–MS/MS, liquid chromatography tandem mass spectrometry.

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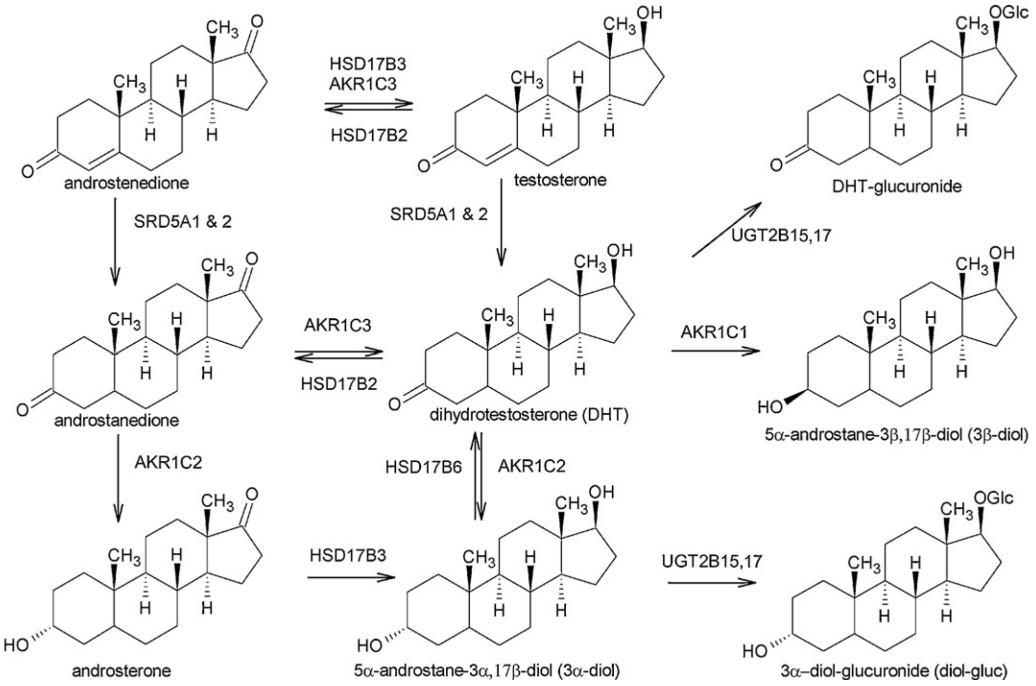

Fig. 1. Metabolic scheme for the synthesis and deactivation of androgen steroids that can occur within the prostate. Enzyme abbreviations: AKR, aldo-ketoreductase; HSD3B, 3β-hydroxysteroid dehydrogenase; HSD17B, 17β-hydroxysteroid dehydrogenase; SRD5A, steroid 5 α-reductase; UGT2B, UDP-glucuronosyltransferase.

is the most potent natural ligand for AR and its concentrations are found to be several times higher than that of testosterone in prostate tissue [20–23]. As a result, DHT has an integral role in AR signaling, prostate function and pathogenesis.

Reductive metabolism and glucuronidation are the two major metabolic pathways for DHT elimination in the prostate. Reductive metabolism involves the conversion of DHT to the androstanediols, 5α-androstane-3α,17β-diol (3α-diol) or 5α-androstane-3β,17β-diol (3β-diol), by the aldo-keto reductases,AKR1C1 and AKR1C2, with the α-diol thought to be the major metabolite [24,25] and potentially also an important androgen in the induction of PCa [26–28]. To a lesser extent, DHT can be converted to 5α-androstanedione via 17β-hydroxysteroid reductase [29]. Deactivation and elimination of DHT in the prostate by glucuronidationproceeds predominantly with the formation of glucuronide conjugateswith the help of UDP-glucuronysyltransferase (UGT) enzymes [30–33]. These enzymes can act upon DHT itself or on the reductive metabolites such as the androstanediols.

A sensitive and straightforward method for determination of both DHT and its primary metabolites in prostate tissue is necessary to improve our understanding of the clinical progression of this disease. In recent years, liquid chromatography/electrospray ionization–tandem mass spectrometry (LC–MS/MS) has become a preferred method for quantification of steroid hormones over other more conventional methods such as immunoassay, thin-layer chromatography, or gas chromatography–mass spectrometry [34]. Compared to these methods, LC–MS/MS exhibits superior assay characteristics for accuracy, sensitivity, specificity, sample preparation requirements and capacity for multiplexing analysis of multiple steroids [35–37]. While conjugated steroids such as glucuronides are inherently good substrates for detection by ESI, the physicochemical properties of many neutral steroids like DHT and the diols result in low proton affinities, inefficient ionization and low sensitivity. Therefore, chemical derivatization procedures, such as 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FMP), have been developed to enhance the detection sensitivity of these steroids [38–44].

Here we present a sensitive and accurate method for simultaneous quantification of DHT, the dominant driver of the AR in prostate tissue, the major metabolites 3α- and 3β-diol, and their corresponding glucuronides validated for use with extracts from archived, snap frozen, embedded tissue samples of prostatectomy-derived clinical samples. The method involves three steps: (1) a sequential extraction protocol [hexane/ethyl acetate (hex/EtOAc) extraction for steroids, followed by EtOAc extraction of the glucuronides], (2) FMP derivatization of the steroid fraction and, (3) LC–MS/MS analysis of the resulting two sample streams. Given the endogenous levels of these analytes in most samples, calibration and validation was carried out on standards alone with qualification for tissue samples performed using benign prostatic hyperplasia (BPH), normal prostate (NP) and spiked BPH and NP tissue homogenates.

2. Materials and methods

2.1. Standards, chemicals and reagents

5α-Androstan-3α,17β-diol (3α-diol), 5α-androstan-3β,17β-diol (3β-diol), 5α-androstan-17β-ol-3-one (DHT) and 5-androstan-3α,17β-diol-17-glucosiduronate (diol-gluc) are from Steraloids (Newport, RI, USA). 5α-Androstan-3β,17β-diol-16,16,17-d3 (d3-diol), 5α-androstan-17β-ol-3-one-16,16,17-d3 (d3-DHT) and 4-androsten-17β-ol-3-one-16,16,17-d3 (d3T) are from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Additional steroids assessed were 1,3,5(10)-estratrien-3α,17β-diol (estradiol-α), 1,3,5(10)-estratrien-3β,17β-diol (estradiol-β), 5-pregnen-3β-ol-20-one (pregnenolone), 3β-hydroxyandrost-5-en-17-one (DHEA), 5-androsten-3β,17β-diol (androstenediol), 4-androsten-17β-ol-3-one (testosterone), 4-pregnen-17-ol-3,20-dione (hydroxyprogesterone), 5α-androstan-3α-ol-17-one (androsterone) and pregnan-3,17-diol-20-one, all from Steraloids. 2-Fluoro-1-methylpyridinium *p*-toluenesulfonate (FMP) and triethylamine (TEA) are from Sigma (Oakville, Ontario, Canada). Hexane (hex), methanol (MeOH), dichloromethane ($CHCl_2$) and acetonitrile (ACN) were Optima® grade from Fisher (Ottawa, Ontario, Canada). Ethyl acetate (EtOAc) was Chromasolv® grade from Sigma and formic acid (FA) was LC–MS grade from Fisher. Water was 18 MΩ purified in house (Millipore RO unit; Billerica, MA, USA). Solid phase extraction (SPE) cartridges were 1 ml, 50 mg, C18 from Waters Corp. (Milford, MA, USA).

Stock solutions of 1 mg/ml in MeOH were prepared for all steroid standards and internal standards. Additional stocks solutions of 10 μg/ml were made up from each of the 1 mg/ml stocks by dilution with MeOH. In addition, working solutions of 1 and 10 ng/ml of (i) a 3α-diol + DHT mixture, and (ii) diol gluc were prepared in 70% MeOH. Internal standard working solutions of (i) 5 ng/ml d3-diol + 20 ng/ml d3-DHT (IS), and (ii) 10 ng/ml d3T (IS-2) were also prepared in 70% MeOH. Finally, a working solution of a 5 ng/ml 3α-diol + 50 ng/ml DHT mixture in 70% MeOH was prepared for use with in the spike recovery determinations from BPH and NP tissue homogenates.

2.2. Calibration

Into 2 ml glass tubes, aliquots from 1 and 10 ng/ml stocks of 3α-diol + DHT were used to create a series of calibration samples: 0, 0.01, 0.02, 0.05, 0.2, 0.8, 2.0, 5.0 and 20.0 ng/ml. 20 μl of IS was added to each tube and these calibration samples were dried in the Centrivap centrifugal vacuum evaporator at room temperature overnight. The dried residues were derivatized with FMP as described in Section 2.4 with the exception that they were redissolved in at a final volume of 200 μl 50% MeOH. Calibration samples were prepared in triplicate except for the 0.8 ng/ml level, for which there were six replicates. Ultimately, the samples were derivatized with FMP (Section 2.4), dried overnight in the Centrivap at room temperature, and then transferred to LC vial inserts for LC–MS/MS analysis.

For the glucuronide calibration samples aliquots from 1 ng/ml and 10 ng/ml stocks of diol-gluc were added, along with 20 μl of IS-2 and a sufficient amount of 50% MeOH to produce a final volume of 200 μl. These samples were vortex mixed and transferred to a LC vial insert for LC–MS/MS analysis (Section 2.5). With the exception of the 20 ng/ml concentration, the same series of calibration levels as for the 3α-diol + DHT was generated.

The prepared calibration samples for DHT, 3α-diol and diol-gluc were analyzed as noted in Section 2.5. Data processing was carried out using QuanLynx™ (Waters). The area under curve (AUC) of individual peaks was obtained and the analyte/internal standard AUC ratio was used for calibration using a linear fit with a 1/× weighting including the origin.

2.3. Tissue extraction

Informed consent was obtained for all specimens according to ethics guidelines at Vancouver General Hospital and The University of British Columbia. BPH tissue was obtained from patients undergoing transurethral resection of the prostate (TURP). Archived prostatectomy samples were from the Vancouver Prostate Centre tissue bank. Two methods of tissue homogenization were used. TURP samples were minced, cross-scalped and diluted with two volume equivalents water prior to homogenization on ice to a smooth consistency with a PowerGen 125 homogenizer (Fisher). TURP homogenates were pooled, vortex mixed, aliquoted into 2 ml samples and frozen at −80°C until use. Tissue-Tek® optimum cutting temperature (OCT) embedded prostate tissue from banked samples was quickly thawed, OCT was washed away with 18 MΩ water and the specimen was blotted, weighed into a 2 ml plastic screw top tube, and immediately placed on ice. Approximately two volume equivalents of water and several 2.3 mm zirconia/silica beads (BioSpec Products; Bartlesville, OK, USA) were added and homogenization was carried out with a Precellys® 24 bead mill using four 20-s cycles at 6800 rpm. All weights and volumes were recorded to ensure correct normalization of final analytical data. All bead mill homogenized samples were also stored at −80 °C prior to analysis.

For recovery experiments to qualify the method for use with calibration standards, 100 or 200 μl aliquots of BPH or NP homogenate were added to 4 ml glass tubes followed by 5 μl IS. Spiked (with DHT and 3α-diol) and non-spiked versions of the homogenates were prepared as outlined in Section 2.6.2. Samples were vortex mixed after which 20 μl of 1 M HCl and 2.5 ml of 70%/30% hex/EtOAc was added. Samples were again vortex mixed and then rotated at room temperature for 30 min. Tubes were centrifuged to ensure good phase separation and the supernatant was transferred to a fresh glass tube. The extraction was repeated and the extracts pooled prior to drying in the Centrivap at room temperature. The remaining homogenate following extraction was frozen at −80 °C prior to further EtOAc extraction.

To further reduce background signals, the hex/EtOAc fraction was subsequently subjected to SPE usinga1 ml C18 SPE cartridges pre-equilibrated with 1 ml ACN followed by 1 ml of water. Dried samples were re-dissolved in 100 μl 70% MeOH, layered onto the equilibrated cartridge and 100 μl of water rapidly added to the barrel of the SPE cartridge to dilute the loaded extracts solvent concentration to a final 35% MeOH. This was drawn into the sorbent by vacuum, then washed with 1 ml water, followed by 1 ml of 10% MeOH and eluted with 1 ml of ACN. The final eluate was dried overnight using the Centrivap at room temperature prior to undergoing FMP derivatization (Section 2.4).

Any hex/EtOAc supernatant remaining on the residual homogenate stored at −80 °C was discarded and the homogenate then thawed. 5 μl of IS-2 was added. Spiked (with diol-gluc) and non-spiked versions of the homogenates were prepared as outlined in Section 2.6.2. The samples were vortex mixed and 500 μl of EtOAc added. The samples were again vortex mixed, and then rotated at room temperature for 30 min. Tubes were centrifuged to ensure good phase separation and the supernatant was transferred to a fresh tube. The extraction was repeated and the extracts pooled prior to drying in the Centrivap. The residue was redissolved in 50 μl of 50% MeOH and centrifuged at 15,000 × g for 5 min. The clarified supernatant containing the glucuronide fraction was transferred to an LC vial insert for LC–MS/MS analysis.

2.4. Derivatization reaction

Aliquots of steroid and internal standards along with the extractions from BPH and NP tissues were dried in glass tubes using a Centrivap centrifugal vacuum evaporator at room temperature overnight. FMP derivatization solution was prepared fresh immediately prior to each use by adding $CHCl_2$ to FMP powder in a glass tube to produce a 20 mM final concentration. TEA was then dissolved into the FMP/$CHCl_2$ solution at 4 μl/ml. 500 μl of this derivatization solution was used for each of the dried standards or tissue extractions. Vials were vortex mixed, and then rotated at room temperature for 1 h. 50 μl of MeOH was then added and the vials rotated an additional 15 min to consume any remaining unreacted FMP. The resulting product was then dried in the Centrivap at room temperature, redissolved in 50 μl of 50% MeOH, transferred to a 1.5 ml eppendorf tube and centrifuged at 15,000 × g for 5 min. The clarified supernatant was transferred to a LC vial insert for LC–MS/MS analysis (Section 2.5).

2.5. LC–MS/MS

Analysis was carried out with a Waters Acquity UPLC coupled to a Quattro Premier XE using a 2.1 mm × 100 mm BEH 1.7 μM C18 column for both the FMP derivatized and glucuronide samples. MassLynx^{TM} 4.1 (Waters) was used for instrument control.

For the FMP samples, the following water/ACN gradient (with 0.1% FA)was used: 0 min, 10%; 0.5 min, 10%; 1 min, 20%; 7 min, 30%; 13 min, 35% (%ACN). This was followed by a column flush of 95% ACN and re-equilibration for a total run length of 18 min. Column temperature was 35 °C and injection volumes were 15 μl. The MS was set at unit resolution, capillary was 1.5 kV, source and desolvation temperatures were 120 °C and 300 °C respectively, desolvation and cone gas flows were 1000 L/h and 50 L/h and the collision cell pressure was held at 4.6e−3 mbar. Cone voltages and collision energies were optimized for each of the ions selected for multiple reaction monitoring (MRM).

For the glucuronide samples, the following 2 mM ammonium acetate/MeOH + 0.1% FA gradient was used: 0 min, 20%; 0.2 min, 20%; 1 min, 40%; 6 min, 80% (% MeOH). This was followed by a column flush of 95% MeOH and re-equilibration for a total run length of 11 min with column temperature and injection volume the same as for FMP samples. The MS was run slightly below unit resolution to improve sensitivity, capillary was set to 3.0 kV, source and desolvation temperatures were 120 °C and 300 °C respectively, desolvation and cone gas flows were 1000 L/h and 50 L/h and the collision cell pressure was held at 6.7e−3 mbar. As for FMP derivatives, cone voltages and collision energies were optimized for each of the ions selected for monitoring by MRM.

2.6. Assay validation and qualification for tissue analysis

Due to the presence of endogenous steroids in the BPH and NP tissue homogenates intended for use as matrix in the validation protocols, the assay characterization is divided into two components. The accuracy, precision and detection limits achieved with standards alone were determined. This was followed by an assessment of analyte recovery from spiked homogenates to qualify the use of these neat calibration preparations for application to tissue extracts. A single spiking level was chosen that was sufficiently above endogenous levels such that reasonable statistics could be obtained from the difference calculations. Matrix effects were examined by post-spiking and qualitatively assessing chromatographic profiles from homogenate extracts as well as individual clinical prostate specimens. An additional follow-up experiment comparing a series of spiking levels in homogenate with corresponding calibration samples was also carried out and is presented as Supplementary material. Short term 4 °C versus 37 °C stability was also assessed. In addition, a panel of additional steroids was used to demonstrate assay specificity as well as general applicability for other hydroxyl steroids.

2.6.1. Precision and accuracy

Calibration samples as prepared in Section 2.2 were used to determine assay precision and accuracy. For intra-assay performance, samples were prepared in triplicate except for the QC equivalent of 0.8 ng/ml ($n = 6$). The mean and standard deviation (stdev) were determined. Precision was defined by %RSD (stdev/mean × 100%) and accuracy was defined as %bias[(measured − nominal)/nominal × 100%]. Inter-assay performance was assessed in triplicate with identical calibration sets prepared an additional two times over the course of three months. Averages of the means from the three independent datasets were used in the above calculations except for DHT where one dataset was omitted due to a contaminated FMP batch.

2.6.2. Spike recovery from tissue

Spike recoveries from BPH and NP homogenates were used to determine assay performance for tissue matrices. For BPH, six replicates each of spiked versus non-spiked homogenate were compared to equivalent standard ($n = 3–4$) in order to assess extraction efficiency and recovery. With the sequential extraction protocol outlined in Section 2.3, it was necessary to have three groups of extracts. Non-spiked homogenate (A), 3α-diol + DHT and diol-gluc spiked prior to the initial hex/EtOAc extraction (B), and diol-gluc spiked prior to the second EtOAc extraction (C). Homogenates were spiked such that the final concentration of 3α-diol, DHT and diol-gluc were 0.5 ng/ml, 5 ng/ml and 1 ng/ml), respectively. The BPH spike recovery assessment was run twice, once with 200 μl and again with 100 μl of homogenate. A similar recovery assessment was carried out for NP except only duplicate samples using 200 μl of homogenate were prepared for each of two separate recovery tests.

2.6.3. Specificity and matrix effects

Blank samples, blank extracts and initial clinical test specimens, some of which had very low analyte levels, were assessed to identify any potential background interference or shoulders on peaks that might negatively impact quantitative assay characteristics. The extent of ion suppression was determined by post-spiking parallel homogenate extracts and 50% MeOH blanks, and then comparing the AUC of each peak in the presence or absence of matrix. A combined steroid sample containing estradiol-α, estradiol-β, pregnenolone, DHEA, androstenediol, testosterone, hydroxyprogesterone, androsterone and pregnan-3,17-diol-20-one was also analyzed to confirm the general applicability of the method and to identify any potential interferences.

2.6.4. Sample stability

Four sets of samples were prepared for both the 3α-diol + DHT and diol-gluc. Two sets were made up as 0.8 ng/ml calibration standard equivalents and two sets were made using BPH homogenates additionally spiked with the same 0.8 ng/ml equivalent (each of the sets were in triplicate). One of each set was maintained at 4 °C while the other was maintained at 37 °C. The samples were run on days 0, 1, 2, 4, 7 and 19, recapped, and returned to their incubation conditions after each run. Stability was assessed by normalizing to the day 0 response.

3. Results

The LC–MS/MS method for 3α-diol and DHT that we have developed for evaluation of our archived prostatectomy specimens confirms the utility of FMP derivatization that others have reported previously and provides a more streamlined workflow as well as the capacity to include diol-gluc as part of the analytical capability. The general usefulness to most hydroxyl containing steroids is also demonstrated; however, method validation focuses only on 3α-diol, DHT and diol-gluc as described below.

3.1. Analytical method

3.1.1. LC–MS/MS

The diol-FMP derivatives were observed primarily as permanent doubly charged parent ions at m/z 238.2 for 3α-diol and 3β-diol or as a permanent singly charged parent ions at m/z 366 with the loss of the methylpyridinium and dehydration due to in-source fragmentation (Fig. 2a). When fully optimized, only the doubly charged parent was evident as shown in for d3-diol (m/z 239.7). Product ion spectra are dominated by the hydrated methylpyridinium fragment (m/z 110) common to all FMP derivatives (Fig. 2b). 3α-Diol and 3β-diol derivatives were monitored using m/z 238.2 > 110 and

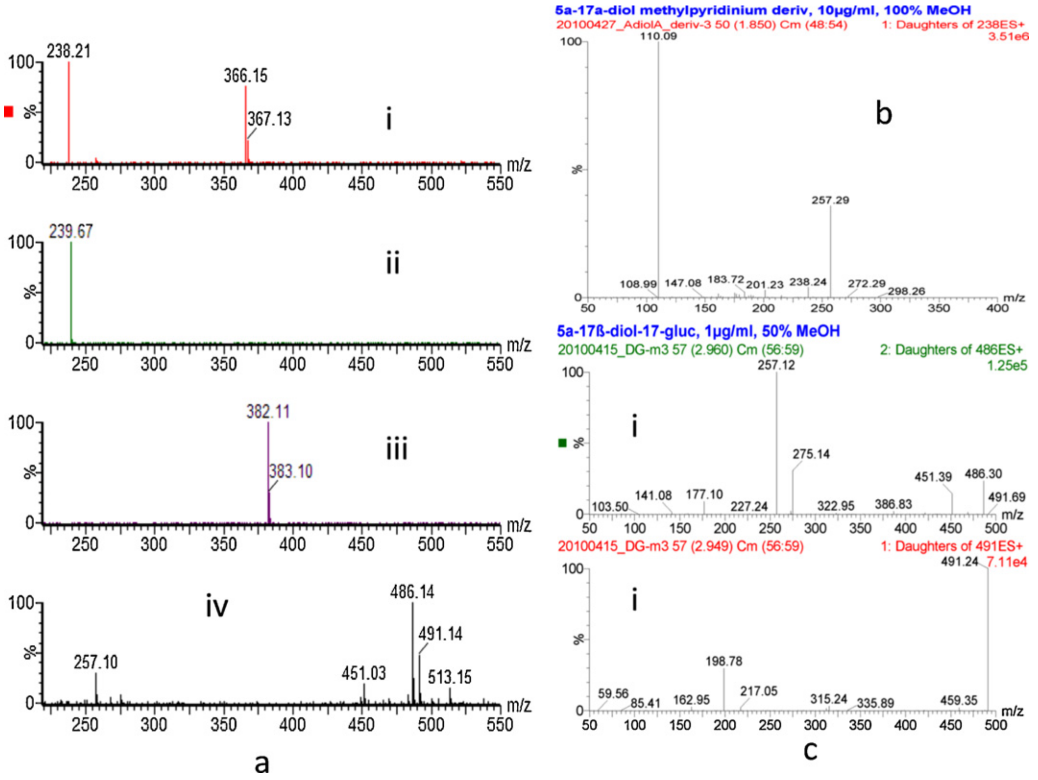

Fig. 2. (a) ES+ scans of: FMP derivative of 3α-diol at moderate (i) and low (ii) cone voltage showing doubly protonated parent (*m*/*z* 238) and in source generated fragment (*m*/*z* 366); FMP derivative of DHT with *m*/*z* 382 (iii); and of diol-gluc with *m*/*z* 486 as the ammonium and *m*/*z* 491 the dehydrated formic acid adducted species (iv). (b) Product ion spectrum of *m*/*z* 238 from the FMP derivative of 3α-diol. (c) Product ion spectrum of *m*/*z* 486 (i) and 491 (ii) of diol-gluc.

239.7 > 110 was used for the d3-3β-diol IS with retention times of 6.50 and 6.55 min, respectively, for the 3α and 3β and 6.55 min for the d3-3β-diol. Baseline separation of the 3α-diol and 3β-diol could not be achieved using a BEH C18 column (Fig. 3c); however, the presence or absence of particular isomers is still possible with this column chemistry and a rough estimate of relative abundance can be made based on the shouldering of the broadened peak evident in tissue extracts. With the use of a chiral column (Daicel 150 × 4.6 Chiralpak IA-3, 3 µm) the 3α- and 3β-diol can be separated (Fig. 3e) however sensitivity is compromised 5–10-fold and if sample size were not limiting, this would be an option. DHT and d3-DHT-FMP derivatives were observed primarily as permanent singly charged parent ions at *m*/*z* 382.2 and 385.2, both with a retention time of 10.9 min and were monitored using *m*/*z* 382.2 > 110 and 385.2 > 110 transitions, respectively. Isotopic purity of both deuterated internal standards was sufficiently high such that spill over into diol or DHT transitions was negligible.

Diol-gluc was observed to have significant yield of both diol-NH_4^+ and the dehydrated diol-FA^+ and the fragmentation pattern of both ions is shown in Fig. 2c. Both ions were selected for monitoring using *m*/*z* 486.1 > 257.1 and 491.1 > 199 transitions and *m*/*z* 292 > 97 was used for the d3T internal standard. Retention times for the diol-gluc and d3T were 5.75 min and 5.85 min, respectively.

FMP derivatives of estradiol, pregnenolone, DHEA, androstenediol, testosterone, hydroxyprogesterone, androsterone and pregnan-3,17-diol-20-one were also prepared and analyzed using *m*/*z* 228 > 110, *m*/*z* 408 > 110, *m*/*z* 380 > 110, *m*/*z* 237 > 255, *m*/*z* 380 > 110, *m*/*z* 422 > 110, *m*/*z* 380 > 110 and *m*/*z* 426 > 110, respectively. Yield for hydroxyprogesterone was very poor. As well, the permanently doubly charged parent expected for pregnan-3,17-diol-20-one was virtually nonexistent, with only the singly charged monoderivative forming. This is presumably a reflection of steric hindrance inhibiting the transformation of the 17-ol of these molecules. A similar LC–MS/MS method using a simple linear gradient was used for separation of this derivatized steroid series for which representative chromatograms are shown in Fig. 4. Whether the epi isomers of testosterone or androsterone can be separated from their counterparts with this method is not known but is quite possible given the already partial separation of the early eluting 3α- and 3β-diol; use of a chiral column would almost certainly result in good baseline separation.

3.1.2. *Specificity*

Substantial increases in background are evident in chromatograms of derivatized standards compared with neat standards. Previous reports [42] suggest that an SPE step is necessary following derivatization to reduce interference due to high levels of derivatizing agent remaining in samples. We have found this step can reliably be eliminated by quenching the reaction with MeOH and using well optimized UPLC separations, leaving peaks of interest clear of interfering peaks. Sub femtomole on-column amounts can be resolved for 3α-diol and DHT (Fig. 5a and b) with baseline separation from artifact peaks well maintained for large numbers of injections. The presence of additional matrix effects is expected for tissue extracts compared with the simple standards. The extent of this impact can be estimated from tissue extracts in which very low levels of 3α-diol and DHT are observed. A comparison of standards (Fig. 5a and b) with extracts from archived prostatectomy specimens (Fig. 7a and b) indicates that only a small increase in background is evident for 3α-diol, however a substantial increase occurs in the DHT channel. Artifact peaks arising from matrix do not substantially affect 3α-diol but a low level peak is observed directly following DHT. Detection limits for 3α-diol from extracts are thus similar to those of standards, whereas for DHT a reduction in sensitivity is expected.

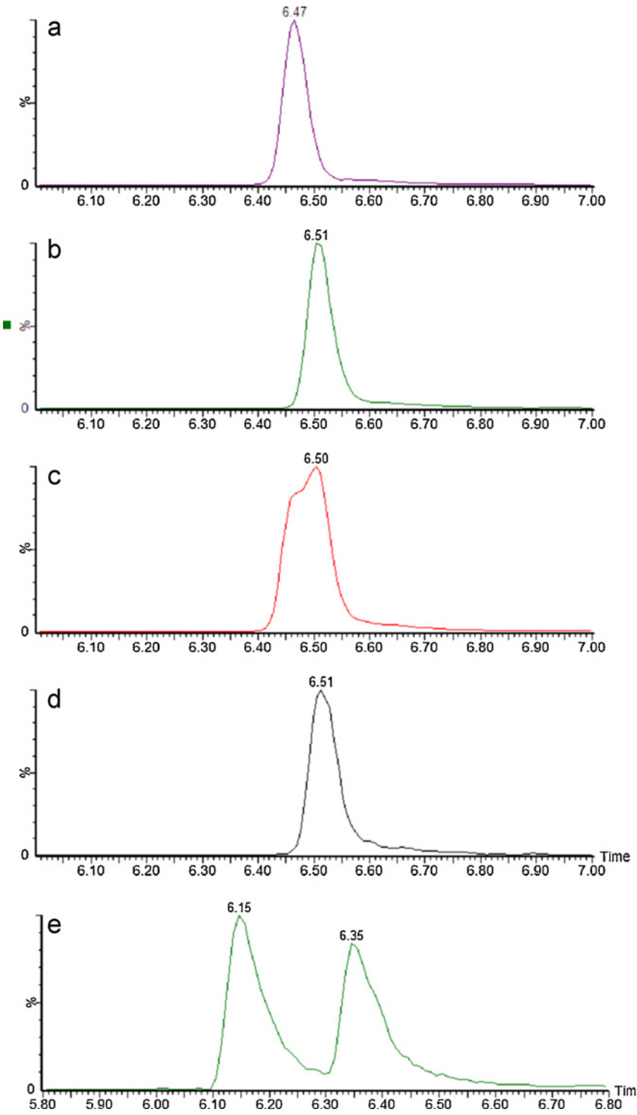

Fig. 3. Chromatograms of 3α-diol (a), 3β-diol (b), pooled 3α- and 3β-diol (c) and deuterated 3β-diol (d) using a 2.1 mm × 100 mm BEH C18, 1.7 μm column. Partial resolution of the 3α- and 3β-isomers is possible under optimized reverse phase conditions. Complete separation of 3α- and 3β-diol (6.15 and 6.35 min, respectively) is possible using a Daicel150 × 4.6 ChiralpakIA-3, 3 μm column (e) however sensitivity is 5–10-fold less.

Table 2
Precision and accuracy for 5α-androstan-3α,17β-diolglucuronide (diol-gluc); $n = 3$ for intra-assay statistics except for 0.8 ng/ml standard ($n = 6$) and $n = 3$ for inter-assay statistics; RSD is relative standard deviation; %bias is deviation of measured from expected.

Std (ng/ml)	Diol-gluc intra-assay			Diol-gluc inter-assay		
	Mean	%RSD	%Bias	Mean	%RSD	%Bias
0.01	0.007	20.8	−26.7	0.007	4.3	−28.4
0.02	0.025	20.3	26.7	0.015	59.8	−24.1
0.05	0.060	12.6	19.3	0.059	5.6	18.1
0.2	0.197	13.8	−1.3	0.212	12.2	6.0
0.8	0.771	12.0	−3.6	0.763	7.2	−4.6
2	2.023	1.6	1.1	2.025	1.1	1.2
5	5.022	4.0	0.4	5.020	1.2	0.4

The background issues associated with derivatization are not applicable to the diol-gluc standards and very clean baselines are obtained (Fig. 5c). As for the derivatives, a comparison of standards (Fig. 5c) with archived prostate specimen extracts (Fig. 7c) is used to identify potential matrix issues. Similar to 3α-diol, only a small increase in background is evident for diol-gluc and sensitivity is thus also similar to that of standards. In some tissue extracts, an additional peak was also observed immediately following the diol-gluc but this is not a consistent matrix associated peak. Given that 3α-diol and 3β-diol separate to some degree, it is possible that this peak may be 3β-diol-gluc, however a standard to confirm this possibility was not available to us at the time.

Ion suppression as measured by the ratio of AUCs obtained from spiked BPH homogenates compared to spiked 50% MeOH indicated that there was essentially no difference between responses from extracts compared with standards. Also, no interference was observed from the steroid cocktail described in Sections 2.6.3 and 3.1.1.

3.1.3. *Calibration and sensitivity*

Calibration curves were generated from a series of seven or eight calibration samples prepared as noted in Section 2.5 ranging from 0.01 ng/ml to 20.0 ng/ml. All were prepared in triplicate except for a 0.8 ng/ml ($n = 6$). Linear regression analysis with the curve including the origin and a 1/× weighting resulted in $R^2 > 0.995$ for fits of all data. Relative signal intensity for 3α-diol was ∼2.5 greater than DHT and diol-gluc about 1/10th of DHT based on AUC. The FDA guidance for Industry Bio analytical Method Validation criteria define lower limits of quantification (LLOQ) a speak to peak signal to noise ratio (PtP S/N) > 5, percent relative standard deviation (%RSD) <20% and accuracy and precision ±20% of nominal. LOQ criteria are %RSD <15% and accuracy and precision ±15% of nominal. Based on these criteria, the lower limits of quantification (LLOQ) are approximately 10 pg/ml, 20 pg/ml and 50 pg/ml respectively for 3α-diol, DHT and diol-gluc (0.5 fmol, 1 fmol and 1.5 fmol on column; PtP S/N 15, 9 and 9) (Fig. 5, Tables 1 and 2), with LOQs at approximately 2× higher levels. LOQ in the context of tissue homogenates is discussed in Section 3.1.5.

3.1.4. *Accuracy and precision*

Deviation from nominal calibration values using the average from three replicates or $n = 6$ for 0.8 ng/ml is designated as intra-day

Table 1
Precision and accuracy for 5α-androstan-3α,17β-diol (3α-diol) and dihydrotestosterone (DHT); $n = 3$ for intra-assay statistics except for 0.8 ng/ml standard ($n = 6$); $n = 3$ for 3α-diol and 2 for DHT inter-assay statistics; RSD is relative standard deviation; bias is deviation of measured from expected.

Std (ng/ml)	3α-Diol intra-assay			3α-Diol inter-assay			DHT intra-assay			DHT inter-assay		
	Mean	%RSD	%Bias	Mean	%RSD	%Bias	Mean	%RSD	%Bias	Mean	%RSD	%Bias
0.008	0.009	22.5	9.2	0.007	15.3	−6.9	0.009	17.6	17.5	0.009	12.6	7.9
0.016	0.013	11.0	−18.0	0.015	23.4	−3.8	0.015	39.3	−8.8	0.016	9.1	−2.6
0.04	0.037	7.1	−8.0	0.040	17.4	−0.7	0.035	9.9	−12.1	0.035	2.4	−13.6
0.2	0.219	6.5	9.6	0.221	5.7	10.7	0.211	4.6	5.7	0.212	0.1	5.8
0.8	0.813	5.0	1.6	0.826	1.5	3.2	0.791	5.0	−1.2	0.786	0.8	−1.7
2	2.015	4.3	0.8	2.104	4.1	5.2	2.115	4.8	5.8	2.071	3.0	3.6
5	4.945	3.3	−1.1	5.044	1.9	0.9	5.086	4.5	1.7	5.150	1.8	3.0
20	18.95	5.4	−5.2	19.37	2.0	−3.1	19.81	3.3	−0.9	19.80	0.1	−1.0

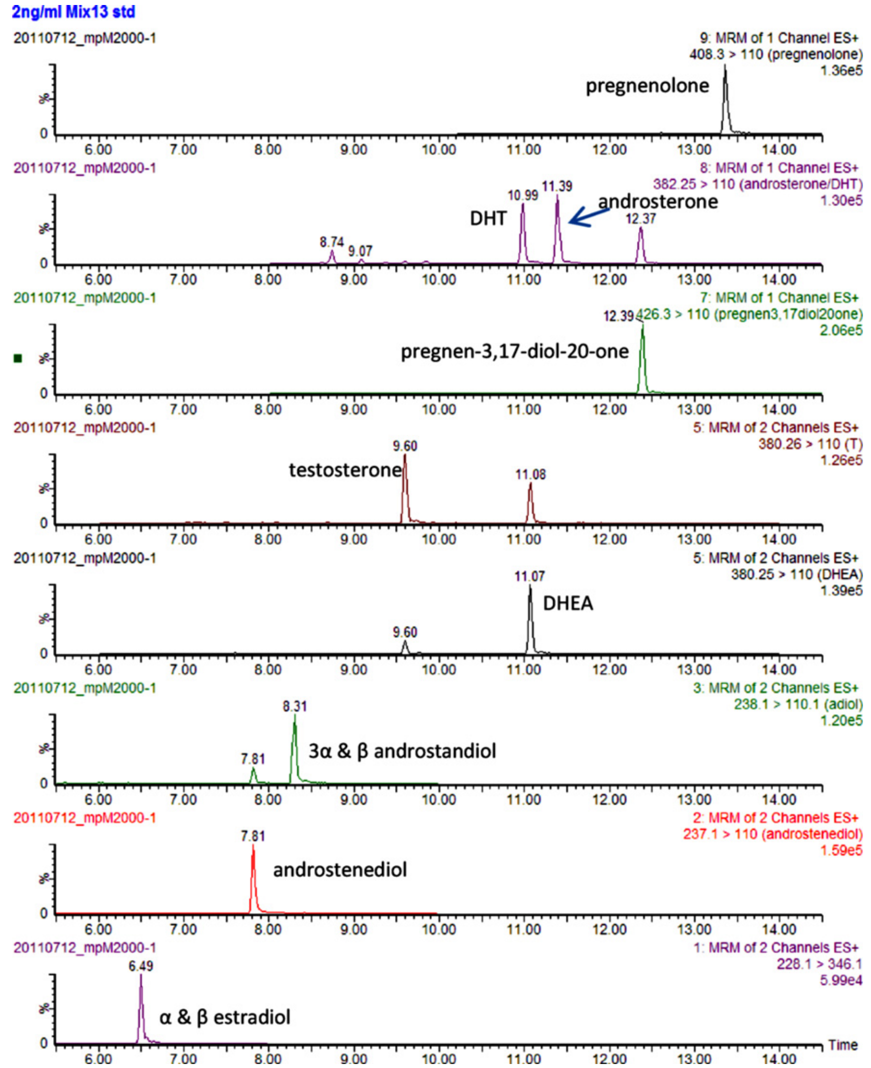

Fig. 4. Chromatograms of FMP derivatives of pregnenolone, DHT and androsterone, pregnan-3,17-diol-20-one, testosterone, DHEA, androstanediol, androstenediol, estradiol demonstrating potential use of the developed assay for additional hydroxysteroids.

%bias in Tables 1 and 2 and inter-day %bias uses the mean of these averages from three replicate calibrations carried out over the course of two months. As noted above in Section 3.1.3, acceptable accuracy is typically considered to be ±15% and ±20% bias near LLOQ. Based on these criteria, 3α-diol, DHT and diol-gluc measurements all conform to these intra- and inter-assay requirements across the calibration range to within the lower limits of quantification stated in Section 3.1.3 (10, 20 and 50 pg/ml respectively). Using the same 15% and 20% criteria for precision which is calculated as the %RSD of the averages noted above, 3α-diol, DHT and diol-gluc precision characteristics are the same as for accuracy except for a single high DHT intra-assay %RSD due to an outlier (Tables 1 and 2).

3.1.5. *Recovery from tissue*

Ideally, calibration standards are made up in the matrix from which one is intending to determine analyte concentrations. However, demonstrating equivalency between neat and matrix derived calibration samples does make the simple calibrants applicable to the tissue extracted samples. Given the presence of endogenous 3α-diol, DHT and diol-gluc in BPH and NP, it is necessary to demonstrate that neat calibration standards will qualify for use with tissue homogenates. The use of isotopic ally labeled identical compounds as internal standards generally provides such universal calibration utility. Recovery estimates provide a measure both of the extraction efficiency as well as qualifying neat calibration standards for use with tissue extracts. A single spiking level similar to that of the endogenous levels in BPH and NP was chosen in order to achieve reasonable statistics which is adequate considering the relatively narrow range of concentrations expected in specimens and inherently tight correlations expected using isotopic dilution. Spiking levels resulted in 0.5, 5.0 and 1.0 ng/ml increases in 3α-diol, DHT and diol-gluc respectively in final prepared samples. Table 3 summarizes the spike recovery from four separate analyses. BPH-1 and BPH-2 were carried out with 200 μl and 100 μl of homogenate, respectively, for each of the non-spiked and spiked groups ($n = 6$ for both groups). An additional sample group was required for diol-gluc, i.e. non-spiked, initially spiked, and spiked after the first hex/EtOAc extraction, in order to determine both overall extraction efficiency as well as that for the second EtOAc extraction alone. NP-1 and NP-2 were carried out similar to BPH-1 but were only completed in duplicate due to sample constraints. Recoveries for 3α-diol and DHT range from 80% to 105% and precision is within 20% for all measurements, with most ±15% or better. 3α-Diol and DHT chromatograms from spiked and non-spiked BPH are in Fig. 6a and b. Recovery of diol-gluc ranged from 75% to 105% for the second extraction and 70–95% for the overall sequential processing. Precision better than ±15% was obtained for all except one of the NP groups for which it was very high (72%), due in part to the

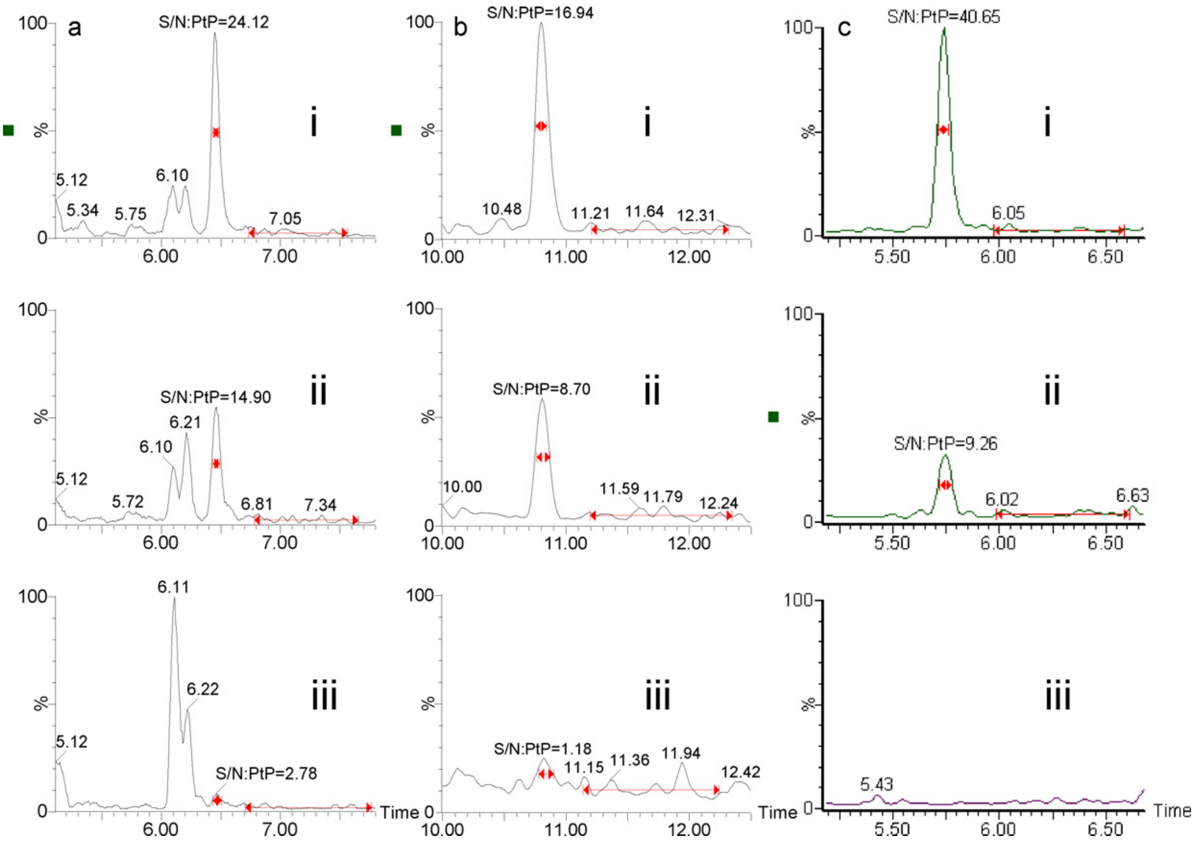

Fig. 5. (a) Assay sensitivity for FMP derivatized α-diol standards is demonstrated using 1 fmol (i), 0.5 fmol (ii) and 0 fmol (iii) on column responses. (b) Similarly, sensitivity for derivatized DHT standards is shown with 2.5 fmol (i), 1 fmol (ii) and 0 fmol (iii) on column responses and also for (c) diol-gluc standards with 6.4 fmol (i), 1.6 fmol (ii) and 0 fmol (iii) on column.

fact that these were only duplicates. Typical chromatograms for diol-gluc recovery are in Fig. 6c. The difference between overall and the second extraction alone is approximately 15%, representing the loss encountered during the first extraction which can be applied as a correction factor during the final calculation of tissue levels. Slightly greater variability in the diol-gluc data compared with the 3α-diol and DHT is likely due to the sequential nature of the extraction as well as the fact that no deuterated diol-gluc was available for use as IS. A follow-up experiment was also carried out in triplicate with a range of spike levels with a BPH homogenate preparation compared to neat standards to demonstrate that intra-assay characteristics are similar regardless of concentration or matrix. 100 μl homogenates were spiked with seven different concentrations ranging 0–16 ng/ml DHT, 0–1.2 ng/ml 3α-diol and 0–1.6 ng/ml diol-gluc. Overall extraction efficiencies, bias from expected values, calibration slopes and comparisons of applied neat calibrations versus standard additions assessment for determination of endogenous levels are in Supplementary Table 1 for 3α-diol and DHT and Supplementary Table 2 for diol-gluc. Neat standards and spiked homogenate exhibit excellent correlation except at the lowest and highest concentration spikes as expected for the various calculated parameters. Endogenous levels determined using neat calibration standards versus standard addition are within 10% of one another for 3α-diol and DHT. Based on an 80% average extraction efficiency determined for diol-gluc, values correlate within 11%.

As noted in Section 3.1.3, the LLOQ for tissue extracts will be higher than for standards. An estimate of this difference can be made by comparing baseline noise. On this basis, where the lower limits of quantification (LLOQ) for the calibration standards are 20 pg/ml, 10 pg/ml and 50 pg/ml for DHT, 3α-diol and diol-gluc, respectively, we would expect approximately 50 pg/ml, 20 pg/ml and 100 pg/ml, respectively, in tissue extracts. From initial clinical sample runs it is also evident that there is a peak that cannot be baseline separated from DHT in some specimens (Fig. 7b). This interfering peak is variable in terms of its occurrence and amplitude, however it is typically smaller than DHT when it appears. Integration by using a drop line from the peak valleys is generally well fit by the software and an accurate measure of the DHT AUC is still possible.

3.1.6. Sample stability

The identical samples were analyzed several times over the course of almost three weeks. While a slight trend of increasing AUC was apparent due to evaporation, the internal standard normalized levels did not change over this course of time. All 3α-diol and DHT data was within ±10% of the day 1 level. Most diol-gluc data was also within ±10% of the day 1 level with 3 points falling within ±15%. There was no trend in any of the datasets indicating the derivatized steroids as well as the glucuronide are quite stable whether stored at 4 °C or 37 °C or whether they are made up as calibration samples or as tissue extracts.

4. Discussion

Androgens, acting via the androgen receptor (AR), are key drivers of prostate development, growth, differentiation, and function. Certain androgens, primarily testosterone and DHT, are strongly associated with increased cellular proliferation of the prostate in patients with PCa, with testosterone found predominantly in the circulation and DHT in the prostatic tissue. The elimination and blockade of these androgens remains the cornerstone of clinical PCa management, and the ability to measure

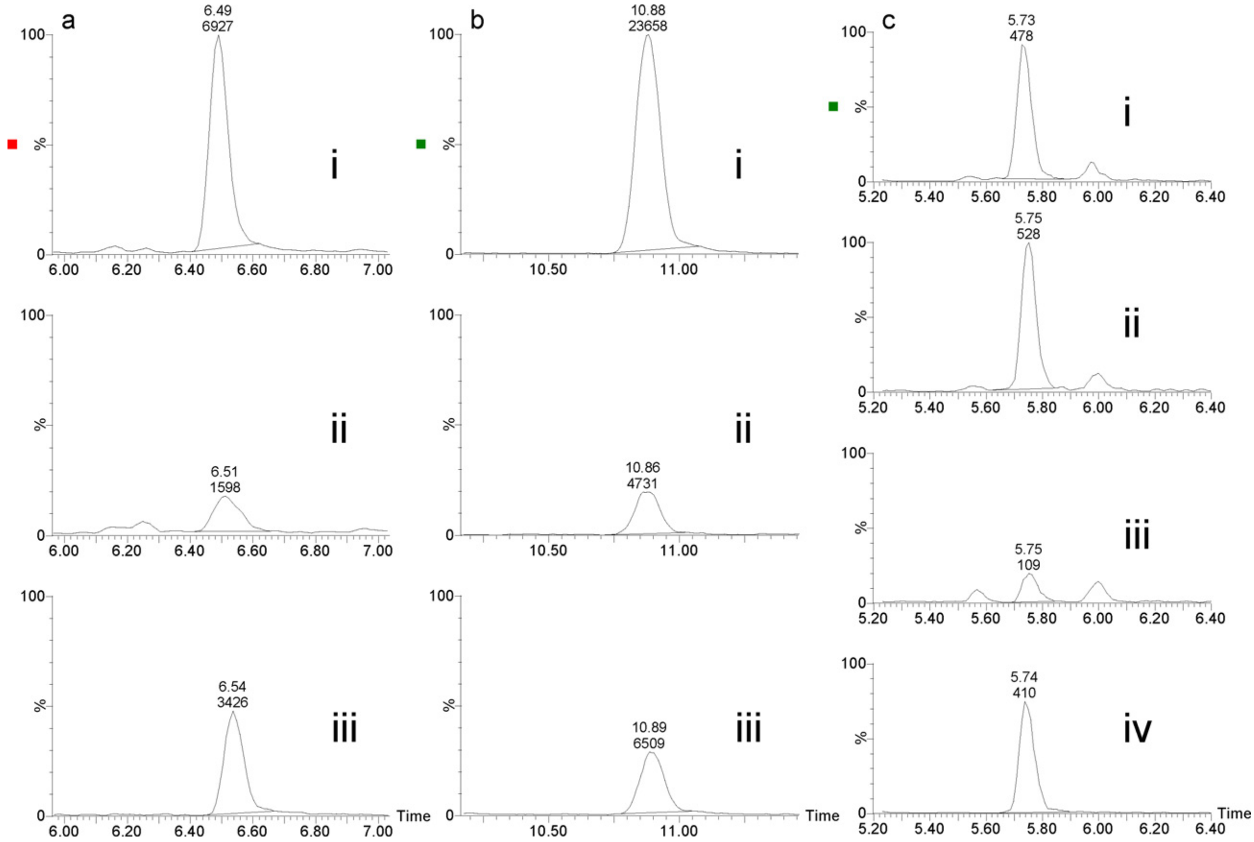

Fig. 6. Chromatograms from FMP derivatized extracts of BPH homogenate: (a) spiked with 0.5 ng/ml (i), 0 ng/ml (ii) 3α-diol and 0.5 ng/ml d3-diol internal standard (iii); (b) spiked with 5 ng/ml (i) and 0 ng/ml DHT (ii) and 2 ng/ml d3-DHT internal standard (iii). (c) Diol-gluc chromatograms from BPH homogenate extracts with initial 1 ng/ml spike (i), similar spike but prior to 2nd extraction (ii), no spike (iii) and of a 0.8 ng/ml diol-gluc standard (iv). This represents tissue levels of 0.23 ng/g diol, 2.35 ng/g DHT and 0.25 ng/g diol-gluc (0.78, 8.1 and 0.53 nM respectively) for this BPH specimen.

endogenous androgen levels is critical to understanding the underlying mechanisms involved in PCa initiation, progression and eventually the acquisition of the more aggressive “castrate resistant” PCa phenotype at which point these front line therapies become ineffective. Several reports indicate that DHT levels sufficient to activate AR are present in prostate in spite of androgen withdrawal therapy [14,45,46]. The dynamics of both the formation and elimination of DHT in prostate is necessary for a better understanding of its role in disease progression. We have implemented and validated a novel and sensitive approach that enables us to measure the levels of DHT and its two major metabolites androstan-3,17-diol and androstan-3,17-diol glucuronide from a single prostate tissue sample.

The use of LC–MS/MS is becoming the method of choice for the quantification of the very low levels of neutral steroids present in biological samples. In combination with different chemical derivatization strategies targeting either keto or hydroxyl moieties to overcome inherently poor ionization, highly sensitive, selective, linear assays are possible. Dansy chloride for instance is very useful and reliable for steroids containing phenolic moieties; dansyl hydrazine was expected to be effective for our purposes but we obtained poor yields. While protocols variously introducing ionized, amino or other groups [38–44] have been successful in enhancing response with these poorly ionizing steroids, they are in many cases quite involved and require sensitive reagents and stringent conditions. In the course of our method development for DHT, 3α-diol and diol-gluc in archived human prostate tissue, we found FMP derivativatization of DHT and 3α-diol to be a reasonably robust and easy to use chemistry with no sample cleanup required prior to LC–MS analysis. A straightforward sequential extraction allowed determination of diol-gluc from the same sample and residuals from these extractions could then also be used successfully for DNA extraction and SNP analysis. The resulting LC samples, whether derivatized steroid or glucuronide, standards or tissue extracts, were all stable at both 4 °C and 37 °C for at least three weeks.

The generation of only one either singly or doubly charged product with no residual substrate remaining was observed when derivatizing 10 μg/ml DHT, 3α-diol and other hydroxyl steroid standards, indicating complete conversion with large excess capacity for low to sub ng/ml samples. An exception is the pregnan-17-ol group which appears to be sterically hindered. 4-Pregnen-17-ol-3,20-dione undergoes essentially no conversion and only a single methylpyridinium adds to pregnan-3,17-diol-20-one. Given that there is an abundance of hydroxyl moieties, it would be preferable to select a unique fragment for analyte quantitation however the yield of *m*/*z* 110 common to all derivatized species was by far the greatest and provided the best sensitivity. It is thus not surprising that several artifact peaks are evident in the chromatographic separation of the diol and DHT and initial attempts using fast gradients were not effective. The combination of increased peak capacity with the use of UPLC separation and optimization of gradient conditions resulted in adequate separation being achieved to the extent that no post derivatization clean-up was necessary. Attempts with tissue extracts without using SPE before derivatization showed some promise initially, however the resulting data was not as reliable indicating that inclusion of an SPE step is preferred for routine analysis. Well resolved peaks were obtained for all standards. An interfering peak was evident in some prostate tissue extracts such that baseline resolution of DHT was no longer achieved; however accurate determination of the DHT AUC was

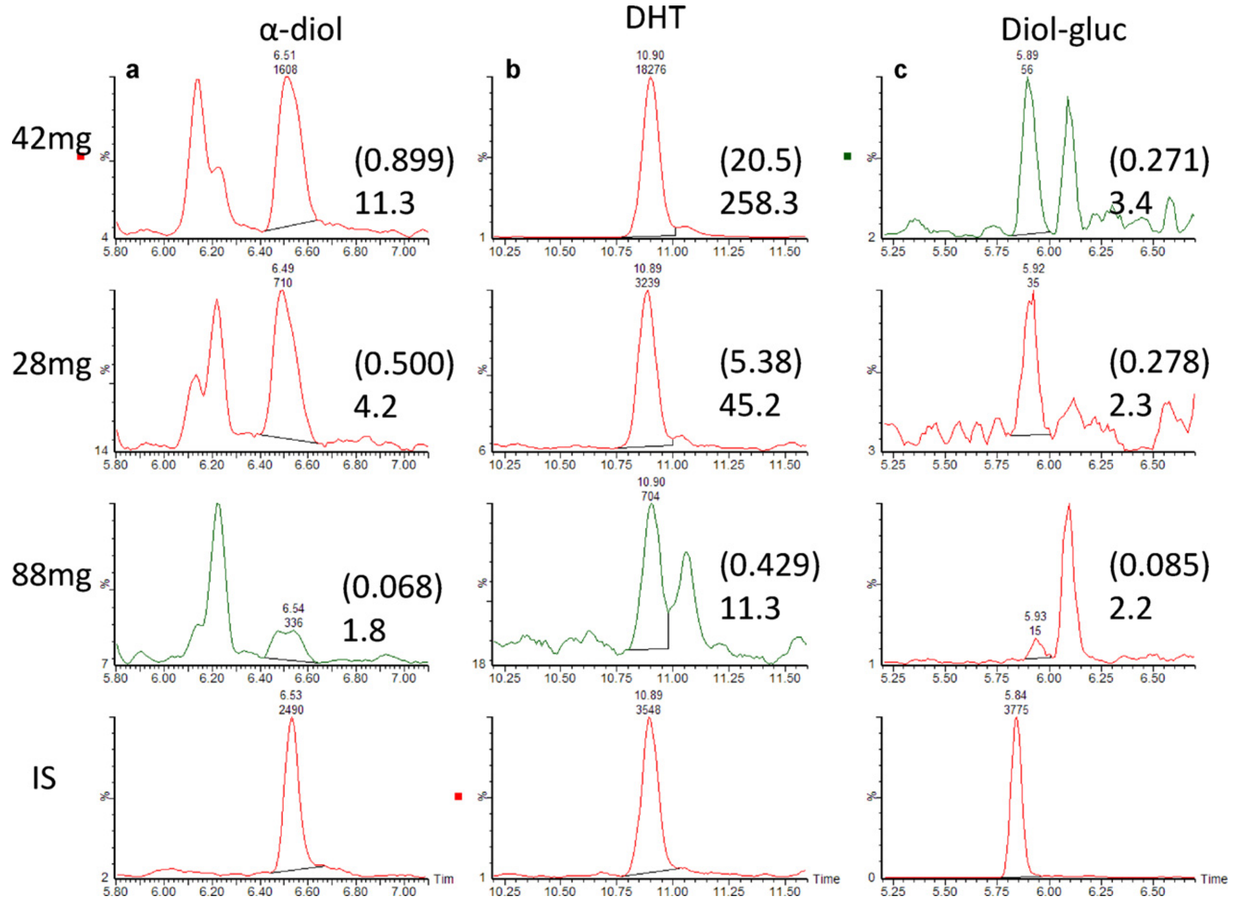

Fig. 7. Chromatograms from extracts of archived prostatectomy specimens for FMP derivatized α-diol and DHT (a) and (b), and diol-gluc (c). Specimen weight is at left of panels, tissue levels (nM) are in brackets, with the on column analyte level for the injection shown below in fmol.

still possible. With the optimized BEH C18 separation, 3α-diol and 3β-diol were resolved to some degree, with peak shouldering suggesting that the 3α-diol is the predominant isomer in most prostate tissues subsequently examined, however only an estimate of the ratio is possible and quantitative analysis includes both isomers. With the use of a chiral column, essentially baseline separation was possible, unfortunately at the expense of sensitivity. The current method validation would be expected to hold for the chiral column but with LLOQ impacted five to tenfold. Validation studies with the standards generated calibration curve linearities greater than $R^2 = 0.995$ with intra and inter day accuracies and precisions that were all within 20% down to the LLOQ of 10 pg/ml and 20 pg/ml, respectively, for 3α-diol and DHT. The recovery study to qualify the use of simple standards for use as calibrants for the tissue extracts indicated analytical recoveries of 80–100% were routinely being achieved for both 3α-diol and DHT. This would be expected given the use of chemically identical deuterated internal standards and the low ion suppression observed. The quantification limits in tissue are not as low as for standards with estimates of 20 pg/ml and 50 pg/ml respectively for 3α-diol and DHT based on relative background in the chromatograms. This translates to tissue LLOQs of 20 pg/g and 50 pg/g for 50 mg tissue equivalents, levels which are similar or better than other current methods.

Retention of the majority of the diol-gluc in the residual from the first extraction facilitates its measurement as well. This component of the assay would also have benefited from the use of a deuterated internal standard but this was unavailable. In its place, d3T was used which proved to be effective in spite of its very different physicochemical properties, with a retention time very similar to that of the diol-gluc and little to no ion suppression for both analyte and IS possibly due to the sequential manner of extraction. Due to differences in extraction characteristics, the d3T must be added prior to the second extraction with EtOAc. The amount of diol-gluc retained in the first residual was sufficiently consistent however, such that reliable overall extraction efficiencies could still be obtained. Similar to 3α-diol and DHT, diol-gluc standards generated calibration curve linearities greater than $R^2 = 0.995$ with intra and inter day accuracies and precisions that were all within 20% down to the LLOQ which in this case was 50 pg/ml. The comparable recovery study required an additional arm, as recoveries from just the second extraction as well as total recovery needed to be evaluated. Overall analytical recoveries ranged from 65% to 95% and recovery from just the second extraction ranged from 75% to 105%. The mean difference between extractions was approximately 15% for the individual datasets, representing the loss of diol-gluc during the first extraction and which can be used as a normalization factor to be applied to the individual extracts. Similar results were observed in a follow-up experiment demonstrating parallel behavior of neat standards and extracted samples with a range of BPH spiking concentrations (Supplementary Table 2). While ion suppression is negligible, greater variability is evident in the second compared to first extract of the series, which would be substantially reduced with the use of the aforementioned deuterated a diol-gluc internal standard. A tissue LLOQ estimate, again based on relative chromatographic background, is approximately 100 pg/g for 50 mg tissue equivalents. An additional peak is evident in some tissue extracts which appears directly after the diol-gluc but is variable in its intensity. Considering that

Table 3
Percent recoveries (%Rec) of 5α-androstan-3α,17β-diol (3α-diol), dihydrotestosterone (DHT) and 3α-diolglucuronide (diol-gluc) from benign prostatic hyperplasia (BPH) and normal prostate (NP) tissue homogenate; concentrations are not normalized; $n = 6$ for BPH and $n = 2$ for NP in each dataset using 200 μl of pooled homogenate except BPH2 (100 μl); two spike recoveries are calculated for diol-gluc, with spike either only prior to extraction (init) or only prior to sequential 2nd extraction (2nd); RSD is relative standard deviation.

Data set	(ng/ml)	3α-Diol				DHT				Diol-gluc					
		Spike	Homog	Homog + spike	%Rec	Spike	Homog	Homog + spike	%Rec	Spike	Homog	Homog + spike (2nd)	Homog + spike (init)	%Rec (2nd)	Total %Rec (init)
BPH 1	Conc	0.331	0.218	0.534	86.7	4.870	3.921	8.123	80.3	1.032	0.400	1.174	1.107	75.0	68.6
	%RSD	5.5	16.6	6.4		2.6	6.4	14.0		6.7	6.5	9.0	11.0		
BPH 2	Conc	0.451	0.091	0.542	99.5	4.597	1.735	6.181	96.3	0.834	0.163	0.881	0.715	86.1	66.2
	%RSD	4.7	18.6	1.9		7.3	18.4	1.7		4.0	19.1	5.1	5.4		
NP 1	Conc	0.428	0.075	0.437	84.6	4.725	1.603	6.236	98.0	1.160	0.163	1.361	1.145	103.3	84.6
	%RSD	3.1	8.8	6.9		1.2	8.1	0.1		6.6	11.0	15.1	7.3		
NP 2	Conc	0.485	0.064	0.458	81.2	4.982	1.429	5.705	85.8	1.234	0.161	1.371	1.257	104.3	94.4
	%RSD	4.4	7.2	12.0		5.5	14.9	2.4		2.6	72.0	6.1	13.8		

some separation of 3α-diol and 3β-diol is evident, it may possibly indicate the presence of 3β-diol-gluc. Without the availability of 3β-diol-gluc standard we were not able to test this hypothesis.

Extension of the validated method to other steroids is possible in its present format. Inclusion of the appropriate deuterated internal standards should provide the same reliability that we observe for 3α-diol and DHT. Good quantitative estimates are possible even with the existing internal standard pair used in this assay. Similar LLOQs in the range of 20–50 pg/g for 50 mg tissue equivalents would be expected, however extending the validation to these targets was beyond the scope of this method development.

Metabolic enzymes have critical roles in regulating endogenous androgen levels and factors that alter their function may have a significant impact on a person's risk profile in disease progression. One potential factor, which to date has received little attention, is naturally-occurring genetic variation in the metabolic genes. Few studies have looked at the impact of allelic variants in metabolic enzymes and risk for PCa development. Some groups have looked at the association of PCa and polymorphisms in UGT enzymes, namely UGT2B15 and UGT2B17 [30–32,47–49]. Besides the UGTs, polymorphisms in the HSD3B2 gene along with the SRD5A2 and SRD5A1 have also been associated with PCa risk and aggressiveness [50,51]. Little is known about the impact of allelic variants on PCa development and progression for the remaining metabolic enzymes (Fig. 1 HSD17B and AKR1C), an area of current interest for members of our group. If an association between allelic variants and increased risk for PCa can be established, we may be able to apply this understanding toward identifying this cohort or to predict potential for aggressive phenotypes.

The sequential extraction method that we have developed, coupled with an efficient and streamlined derivatization protocol and LC–MS/MS analysis of the resulting fractions, provides us with an efficient means to examine key components associated with our research questions, namely the endogenous levels of both one the main drivers of prostate development and function and participant in the malignant pathology as well as its metabolites. This method for determination of DHT, androstane-3α,17β-diol, and 3α-diol glucuronide, validated in prostate tissue, is a highly sensitive and selective technique with potential for use with other hydroxyl containing steroids as well as other tissues.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.031.

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