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Simultaneous determination of multiple androgens in mice organs with liquid chromatography tandem mass spectrometry

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ABSTRACT

Prostate cancer (PCa) is the most commonly diagnosed cancer in men worldwide. It is essentially dependent on potent androgens, such as testosterone (T) and dihydrotestosterone (DHT). The precursors of T and DHT, which includes androstenedione (A4) and dihydroepiandrosterone (DHEA), and also the metabolites of DHT, 5α-androstane-3α,17β-diol (3α-Diol) and 5α-androstane-3β,17β-diol (3β-Diol) are able to affect the development of PCa. Therefore, it is important to simultaneously determine all these key androgens. This study aims to develop and validate an LC–MS/MS quantification method to simultaneously detect and quantify the six related androgens, including T, DHT, A4, DHEA, 3α-Diol, and 3β-Diol in limited sample volume. The sample preparation involved liquid extraction with methyl *tert*-butyl ether (MTBE), following by chemical derivatisation with hydroxylamine. The limits of quantitation for T, DHT, A4, and DHEA were 0.05 nM and 3α-Diol and 3β-Diol were 0.5 nM with S/N ratio of at least 5:1 by using 100 μL samples.

1. Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer in men worldwide [1]. With the groundbreaking discovery by Hodges and Huggins, they found that PCa is actually a hormone-dependent cancer [2–6]. Its survival and progression is essentially reliant on androgens such as testosterone (T) and dihydrotestosterone (DHT). Since then, androgen deprivation therapy (ADT) has always been the main form of treatment for advanced PCa. It is very effective during the initial two to three years of the treatment. However, recurrence of the disease is almost certain [4–9]. At this stage, it is currently termed as the "castration resistant prostate cancer" (CRPC) [2,10].

CRPC patients have been associated with poor prognosis and the median survival rate is roughly about 30 months only [11]. One of the more commonly accepted reasons for the occurrence of CRPC is the intracrine synthesis of T and DHT [12]. It allows for PCa to progress, despite the seemingly minute levels of T in serum after ablation. In fact, evidence showed that AKR1C3 expression is often increased in localized, advanced PCa or CRPC [12]. However, it is often found in minimal levels in normal prostate epithelia [13]. It is the critical enzyme responsible to convert the adrenal androgen, A4, into T, as well as 5α-dione into DHT [12]. Inhibition of AKR1C3 should not interfere with the metabolism of glucocorticoid since, it acts further downstream of 17-hydroxylase/17,20-lyase. As such, it is expected that there should have lesser associated side effects and becomes a reasonable target for treatment of CRPC [12].

An important point to note is that AKR1C3 shares close resemblance to three other enzymes, namely AKR1C1, AKR1C2, and AKR1C4 [13,14]. Drug that does not selectively inhibits AKR1C3 will be undesirable as it will affect the other isoforms, which will likely cause side effects. AKR1C4 is mainly present in liver [14] and not in prostate. On the contrary, AKR1C1 and AKR1C2, are present in the prostate as for AKR1C3 [13]. They are very important too as AKR1C1 and AKR1C2 metabolise and deactivate DHT into its two metabolites, 5α-androstane-3β,17β-diol (3β-Diol) and 5α-androstane-3α,17β-diol (3α-Diol), respectively [14].

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3α-Diol is usually considered as an inactive androgen to AR [15,16], whereas 3β-Diol is a selective ligand for estrogen receptor β (ERβ) [15,17,18] instead of androgen receptor (AR). In fact, 3β-Diol being a selective ERβ ligand can activate ERβ, which is often associated with beneficial outcomes such as anti-proliferation of cancerous cells. Thus, selective inhibition of AKR1C3 is imperative for treating CRPC.

Since, T and DHT, their precursors and the DHT's metabolites all contribute in one way or another, to the development of PCa, it is important to simultaneously determine the multiple androgens. In this study, our focus is mainly to develop a liquid chromatography tandem mass spectrometry (LC–MS/MS) method that can simultaneously detect and quantify the six key androgens, namely DHEA, A4, T, DHT, 3α-Diol and 3β-Diol. These are known to be involved in few of the reported pathways to synthesise T and DHT to maintain their intracrine levels that trigger the relapse of PCa after ADT. The method was fully validated and tested on some mice organs samples to verify its actual applicability.

2. Materials

Dihydroepiandrosterone, dihydrotestosterone, internal standard, deuterated-estradiol (d_5-E2), and hydroxylamine hydrochloride of purity 97% were all obtained from Sigma–Aldrich (St. Louis, MO, USA). Androstenedione and testosterone were obtained from Toronto Research Chemicals (New York, ON, Canada). 5α-androstane-3β,17β-diol and 5α-androstane-3α,17β-diol were obtained from Steraloids (Newport, RI, USA). Dimethoxycurcumin was obtained from Invitrogen (Shanghai, China). Internal standard, deuterated-testosterone (d_3-T) of purity 98% was obtained from CDN isotopes.

Solvents used for LC–MS/MS measurement were all of HPLC grade, unless otherwise stated. Formic acid, methyl *tert* butyl ether (MTBE) from Sigma–Aldrich (St. Louis, MO, USA), acetonitrile (ACN), and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Ultra-pure water was obtained using Milli-Q water purification system (Millipore, Bedford, MA, USA).

Phosphate buffered saline (PBS) was obtained from Vivantis Inc., US. Bovine albumin serum (BSA) was obtained from Sigma–Aldrich (St. Louis, MO, USA).

3. Experimental

3.1. Preparation and extraction of calibration standards and quality controls

Stock solutions and standards of A4, DHEA, DHT, T, 3α-Diol, and 3β-Diol were prepared and kept at −20 °C, wrapped with foil. Calibration curves for each of the compounds in 1 g/L of PBS/BSA matrix were established. For the calibration standards, they were prepared by spiking 20 μL of each of the standard mixture of DHEA, A4, T, DHT, 3α-Diol, and 3β-Diol in methanol into 80 μL of blank PBS/BSA solution to make up a total of 100 μL solution. And the final calibration standards prepared for the six analytes, DHEA, A4, T, DHT, 3α-Diol, and 3β-Diol were ranged from 0.01 to 20 nM in the PBS/BSA. 10 μL of internal standard mixture of 5 nM d_3-T and 500 nM of d_5-E2 was spiked into each of the 100 μL sample. Four quality controls (QC) with concentrations 0.05 nM, 0.5 nM, 2 nM, and 10 nM, respectively, were also prepared in the same matrix. These samples were extracted using liquid–liquid extraction of 1 mL of MTBE and vortexed vigorously for 2 min. This was followed by centrifugation at 13,000 rpm for 3 min at room temperature. The supernatants were transferred into clean 2-mL eppendorf tubes and extraction was repeated another time. The combined extracts were dried under gentle stream of nitrogen gas at 40 °C. The dried residue was reconstituted with 60 μL of 0.1 M hydroxylamine hydrochloride in 3:7 methanol:water and reacted at 70 °C for 15 min. It was allowed to cool and centrifuge at 14,000 rpm for a min. 20 μL of the final solution was injected for the LC–MS/MS analysis.

3.2. Preparation of samples for stability studies

Three sets of triplicates of the four QCs in 1 g/L of PBS/BSA were prepared for the various stability tests. One set of triplicates of the four QCs were prepared and stored immediately at −80 °C for a month until ready for long term stability test analysis. Another set of triplicates were stored at −80 °C and thawed at room temperature of 24 °C after 24 h span. These samples were subjected to three rounds of freeze–thaw cycles for freeze–thaw stability determination. Last set of triplicates of the four QCs were left at room temperature of 24 °C for 4, 6, 8, and 24 h, respectively, before analysis to study the short term stability of all the androgens spiked in PBS/BSA under room temperature conditions. For all the different sets of stability samples, their responses were compared against responses from triplicates of a control set prepared with the same concentrations of the androgens that were freshly prepared on the day of extraction for LC–MS/MS analysis. These samples, together with the reference set were spiked with 10 μL of internal standard mixture on the day of analysis before extraction.

3.3. Sample preparation for mouse organ samples

The C57BL/6 or B6 mice were intraperitoneally (i.p.) injected with 75 mg/kg of dimethoxycurcumin. Triplicates of the organs were collected from three mice at 20 min, which was the timepoint where the drug reached its C_{max} based on previously conducted pharmacokinetic study [19]. The organs were immediately stored at −80 °C after collection. Before extraction, the organs were thawed at room temperature and rinsed with saline buffer before drying and weighing. Each organ was then transferred into a clean 2-mL eppendorf tube and homogenised. 10 μL of internal standard mixture with 5 nM of d_3-T and 500 nM of d_5-E2 was spiked into the homogenised sample. Similar extraction procedure was adhered to as mentioned in Section 3.1. However, an additional step was taken where these tissues samples were ultrasonicated in a water-bath sonicator for 10 min after each round of addition of extraction solvent. They were centrifuged at 6000 rpm for 10 min before transferring the supernatants into clean eppendorf tubes. Immediately after obtaining the chromatograms, samples were stored back at −80 °C and LC–MS/MS quantification analysis was performed. Some samples that exceeded the upper limit of the calibration ranges of the respective analytes were diluted and re-analysed until the concentrations of the analytes fell within the calibration ranges established. Suitable volume of each of the affected sample was transferred to a clean tube and top up to 60 μL with the 0.1 M hydroxylamine hydrochloride in 3:7 methanol:water derivatising agent. These diluted samples were vortexed vigorously for 1 min and centrifuged at 14,000 rpm for another minute. 20 μL of the sample was injected and re-analysed under LC–MS/MS. Dilution factor was accounted for the concentration of the internal standard used in the sample after the dilution.

4. LC–MS/MS analysis

UPLC was carried out on an Agilent 1290 Infinity liquid chromatography system with a binary pump. An Agilent Eclipse Plus C18 RRHD (2.1 mm × 50 mm, 1.8 μm particle size) column was used for separation. The two eluents were ultrapure water with 0.1% formic acid (solvent A) and acetonitrile (solvent B) and it was delivered at a total flow rate of 0.5 mL/min with column temperature at 40 °C. 20 μL of each sample was injected and injector needle was

Table 1
Summary of the MRM transitions for the six androgens.

Analyte	MRM transitions (m/z)	Optimised collision energy (V)	Optimised cell accelerator voltage (V)	Retention time (min)
A4-oxime	m/z 317 → 112(quantifier)	25	3	1.91
	m/z 317 → 124(qualifier)	29	3	
DHT-oxime	m/z 306 → 105(quantifier)	38	3	2.14
	m/z 306 → 107 (qualifier)	28	3	
T-oxime	m/z 304 → 124(quantifier)	25	3	2.03
	m/z 304 → 112.1(qualifier)	29	3	
DHEA-oxime	m/z 304 → 253(quantifier)	15	3	1.79
	m/z 304 → 213(qualifier)	15	3	
d_3-T-oxime (Internal standard for A4, T, DHT and DHEA)	m/z 307 → 124 (quantifier)	25	3	2.03
	m/z 307 → 112(qualifier)	29	3	
3α-Diol	m/z 257 → 161(quantifier)	15	1	2.37
	m/z 257 → 147(qualifier)	15	1	
3β-Diol	m/z 257 → 161(quantifier)	15	11	2.14
	m/z 257 → 147(qualifier)	15		
d_5-E2 (Internal standard for 3α- and 3β-Diol)	m/z 260 → 161(quantifier)	10	3	2.01
	m/z 260 → 135 (qualifier)	10	3	

then washed with acetonitrile. The column was first equilibrated at 20% B and increased to 40% B in 0.5 min and maintained for another minute. Thereafter, B was increased to 60% within 0.1 min and maintained for another 0.9 min. At the 3rd min, B was further increased to 90% and maintained for another min before decreasing to 20% within a minute. This was maintained for 1 min before ending the sample run. The total runtime was 6 min with constant flow rate of 0.5 mL/min throughout. During the run, the first 0.8 min and the last 2.5 min were diverted to waste so as to prevent any interferences in the ion source of the MS detector. Samples were kept in the autosampler at 6 °C throughout the runs.

The separated analytes was detected and analyzed by the Agilent 6490 Triple Quadrupole (ifunnel) mass spectrometer with agilent jet stream (AJS) ESI source under positive ion mode. Multiple reaction monitoring (MRM) was used for the quantification of each analyte. MS and source parameters were optimized and the following were used for the analysis. MS parameter, delta EMV: +250 V; source parameters used were gas temperature: 200 °C; gas flow: 14 L/min; Neubuliser: 20 psi; Sheath gas temperature: 250 °C; Sheath gas flow: 11 L/min; capillary (+): 3000 V; nozzle voltage: 1500 V(+); ifunnel high pressure RF: 200 V and low pressure RF: 110 V. Table 1 shows the summarized MRM transitions and their respective optimized collision energies, cell accelerator voltages, and also the retention times for the detected analytes. Nitrogen gas was used as the curtain and collision gas. All data were recorded and processed by using the Agilent Mass Hunter B06.00 (Agilent, Foster City, CA).

Based on the chemical structures for the two analytes, 3α-Diol and 3β-Diol, they do not undergo derivatisation under the same sample preparation conditions like the other four androgens. And their internal standard was d_5-E2, which also do not undergo derivatisation under the reaction conditions used during sample preparation. The concentrations of the androgens were quantified using an eleven-point calibration curve of peak area ratio against the concentration ratio of the analyte to internal standard.

4.1. Method validation

Interday ($n = 9$) and intraday ($n = 6$) assays were conducted for the calibration standards in PBS/BSA to determine the various validation parameters such as accuracy, coefficient of variation (CV%), lower limit of quantification (LLOQ), limit of quantification (LOQ) and regression values. LOQ and LLOQ were determined based on signal-to-noise (S/N) ratio of 10:1 and 5:1, respectively. Blank PBS/BSA solution containing only internal standard was analysed, so as to study if there is any matrix effect from the background.

5. Results and discussion

5.1. LC–MS/MS method development for simultaneous detection of six key androgens

This method was developed to simultaneously detect and quantify the six key androgens reported to be involved in the biosynthesis of T and DHT. For DHEA and A4, they are recognised to be involved as the precursors to these potent androgens, T and DHT in the biosynthetic path. And 3α- and 3β-Diols are the metabolites from the conversion of DHT. Therefore, it is important to detect these six related androgens.

Apart from that, it is also important to not only detect them but also to be able to quantify them concurrently. T can be formed from A4, its only precursor through the help of AKR1C3 enzyme [12]. As such, administration of a range of drug doses should be able to cause changes to the concentration levels of T, which in turn can reflect the dose-response curve of the drug's inhibition profile on AKR1C3 enzyme. Thereafter, IC_{50} of the drug for inhibition of AKR1C3 could be elicited. And with simultaneous measurements of especially 3α- and 3β-Diols, which were formed through catalysis of AKR1C2 and AKR1C1 respectively, dose response curves for inhibition profiles of AKR1C1 and 2 can also be obtained at the same time. And the IC_{50} values of AKR1C1 and AKR1C2 can also be elicited too. From which, this enables the study of selectivity of the drug for inhibition of AKR1C3 to be performed with convenience, greater accuracy and confidence.

Nevertheless, achieving high sensitivity to all the analytes is an issue, especially for the two diols within the same sample run. This is because only DHEA, A4, T, DHT, and internal standard, d_3-T are able to undergo chemical derivatisation with hydroxylamine to enhance their sensitivities. As for the remaining two diols, their chemical structures do not permit them to undergo the same chemical derivatisation with hydroxylamine. This implied that in order to achieve analysis for all the six androgens in the same sample within the same run, enhancement of signals to the two diols will not be achievable. To worsen matters, upon addition of hydroxylamine and reacting at 70 °C for 15 min, it degraded the responses of the diols by about 10% (data not shown). But when the reaction was performed at 60 °C for the same length of time, all the analytes' signals decreased. Therefore, derivatisation was allowed to proceed at 70 °C for 15 min. Based on the reported levels of 3α-Diol (0.728 nM) and 3β-Diol (0.841 nM) found in the sera of males measured using GC–MS under selected ion monitoring mode (SIM) [20], we wanted to develop a simultaneous quantification method where not only DHEA, A4, T, and DHT can be detected with high sensitivities, but

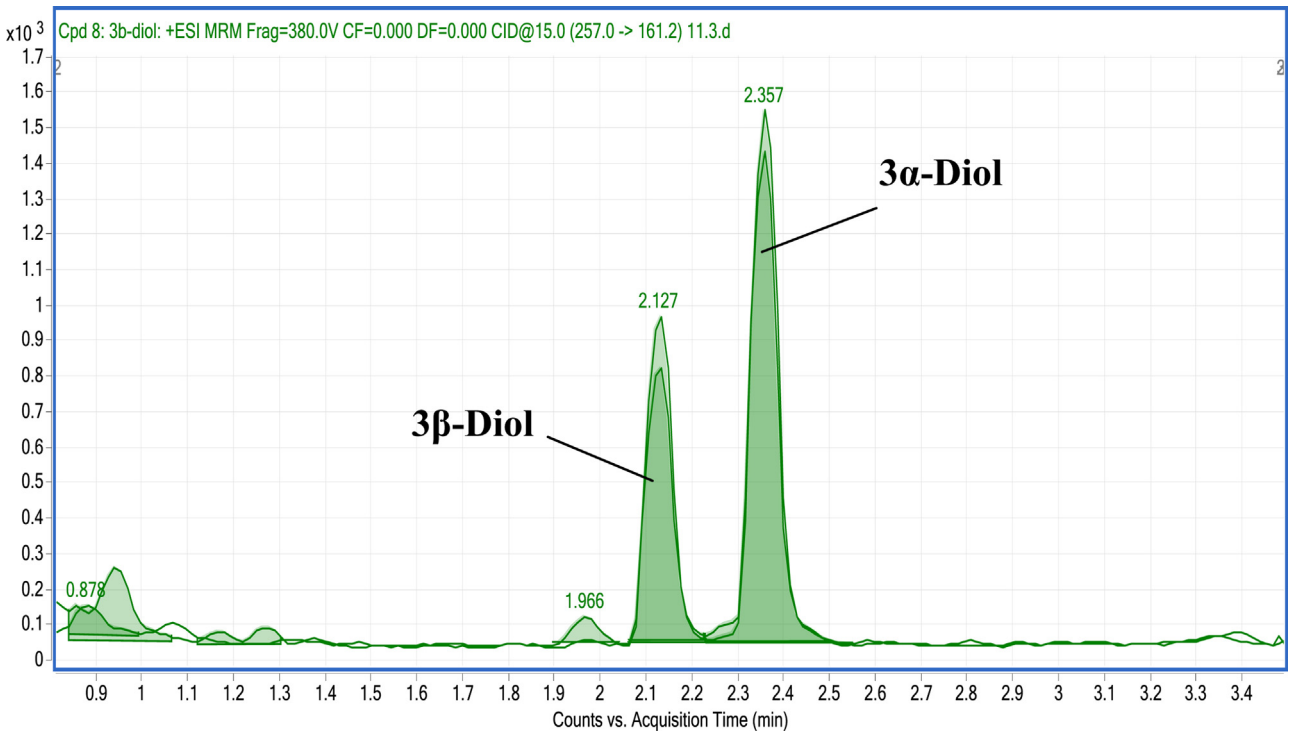

Fig. 1. Chromatogram of complete separation of the two isomers, 3α-Diol and 3β-Diol.

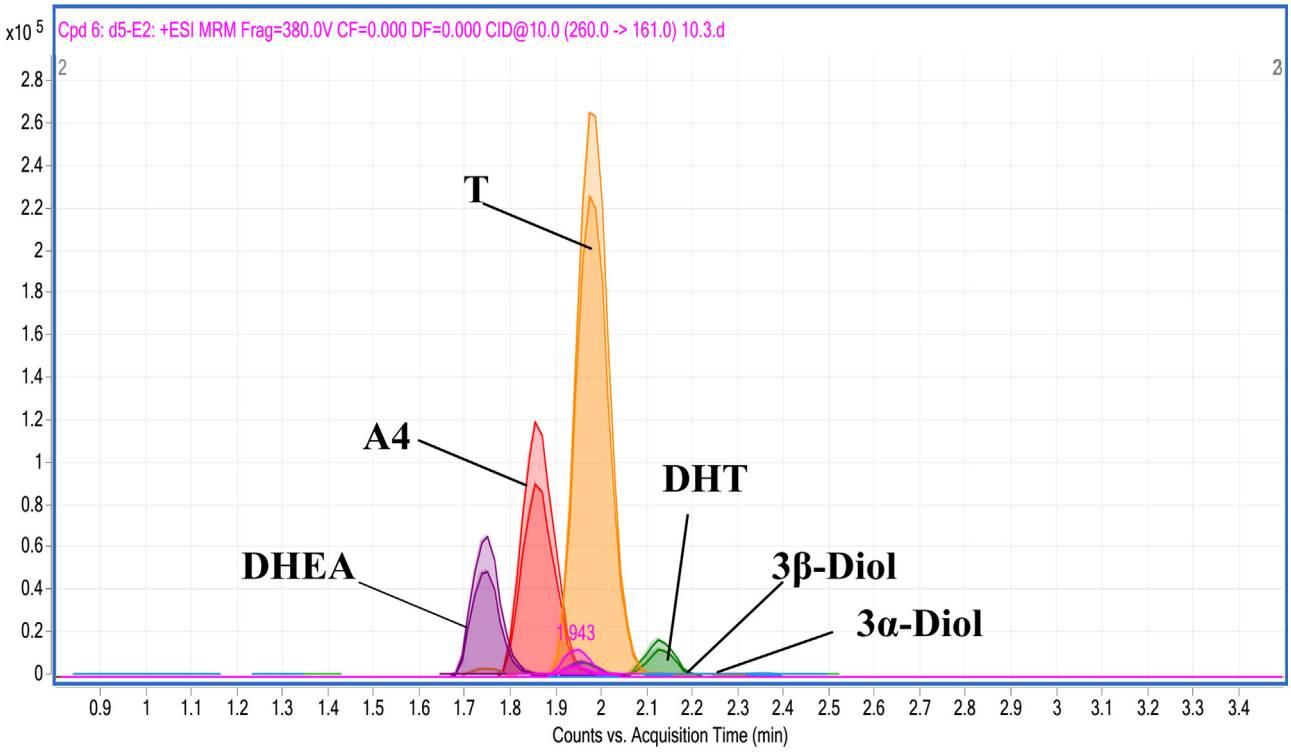

Fig. 2. Typical chromatogram of separation of the six androgens.

also be able to make accurate measurements on the two diols with at least inclusion of this threshold concentration level at 0.5 nM.

Separation of all these analytes with high structural similarities under the liquid chromatographic (LC) conditions was challenging, especially with these two diols. The two diols bear near-identical resemblance in structures and the MS/MS detection would be a problem as the two diols share the exact MRM transitions for their fragmentations. The only solution is still to achieve an LC separation especially for these two diols before MS detection. In this study, LC separation was eventually achieved for these two isomeric diols with a C18 column, together with the other four analytes within a short elution time of 6 min. They were able to be separated (Fig. 1) just like a reported case of the isomers of testosterone [21]. To the best of our knowledge, this should be the first report of using LC–MS/MS for simultaneous detection and quantification of the six androgens, including these two diol isomers (Fig. 2).

Table 2
Summary of typical calibration curves equations for each analyte.

Analyte	Calibration curve equations	Regression coefficients, r^2
DHEA	$y = 3.023400x + 0.004514$	0.99
A4	$y = 5.951754x + 0.012070$	0.99
T	$y = 13.823719x + 0.001739$	0.99
DHT	$y = 0.809512x + 0.007938$	0.99
3α-Diol	$y = 0.253581x + 0.006101$	0.98
3β-Diol	$y = 0.140521x + 0.002804$	0.99

5.2. Validation of the LC–MS/MS method

Linear regressions of 1/y weighting were achieved for the analytes, DHEA, A4, T, and DHT in 1 g/L of PBS/BSA. Linear regressions without any weighting were also obtained for 3α- and 3β-Diol in the same matrix. Typical calibration curves equations with their respective regression coefficients, r^2 for the six androgens were summarised in Table 2.

The validated ranges for DHEA, A4, T, and DHT were from 0.05 to 20 nM, with LOQ for DHEA, A4, and T to be experimentally determined to be 0.05 nM with S/N ratio of at least 10:1. For DHT, it is less responsive but still able to achieve LLOQ of S/N ratio of at least 5:1 at concentration 0.05 nM. As for both 3α- and 3β-Diol, the validated ranges were from 0.5 to 20 nM in the same matrix with LLOQ to be 0.5 nM with S/N ratio of at least 5:1. Based on the Guidance for Industry Bioanalytical Method Validation [22], all the validated data for accuracies and coefficient of variation (CV%) were within acceptable ranges for the six analytes (Tables 3a–8b).

With the use of QCs prepared in 1 g/L of PBS/BSA, the recoveries and matrix effect were determined. From the chromatograms of the blank matrix, there were small but relatively insignificant peaks with the same retention times as DHEA, A4, and T in the matrix. Whereas for DHT, 3α-Diol and 3β-Diol, no peak with the same retention times as the three analytes was observed in the matrix. So, the developed method of detection for these six androgens was still quite specific. All the data were analyzed and calculated with the Agilent MassHunter Quantitative Analysis B.05.02 software. All the data exhibited the robustness and reproducibility of this current method was for the measurement of the six androgens in PBS/BSA.

Table 3a
Validation of DHEA for interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.046	0.045	0.045	0.045	0.000	1.0	90.6
0.1	0.094	0.097	0.098	0.096	0.002	1.9	96.4
0.5	0.506	0.491	0.476	0.491	0.015	3.0	98.2
1	0.992	0.957	1.061	1.003	0.053	5.3	100.3
2	2.085	2.051	2.040	2.059	0.023	1.1	102.9
5	5.386	5.429	5.127	5.314	0.163	3.1	106.3
10	10.595	10.774	9.988	10.452	0.412	3.9	104.5
15	14.918	14.524	14.355	14.599	0.289	2.0	97.3
20	19.172	19.637	20.781	19.863	0.828	4.2	99.3

Validation of DHEA for interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.049	0.055	0.054	0.053	0.004	6.7	105.1
0.5	0.495	0.518	0.483	0.499	0.017	3.5	99.7
2	1.940	2.065	2.068	2.024	0.073	3.6	101.2
10	9.345	10.136	9.287	9.589	0.475	5.0	95.9

Table 3b
Validation of DHEA for intraday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.059	0.058	0.058	0.049	0.049	0.058	0.055	0.005	8.8	110.5
0.1	0.109	0.095	0.090	0.087	0.087	0.097	0.094	0.009	9.1	93.9
0.5	0.471	0.511	0.526	0.496	0.533	0.497	0.506	0.022	4.4	101.1
1	1.055	1.021	1.074	1.059	0.962	0.975	1.024	0.047	4.6	102.4
2	1.948	1.801	1.897	1.809	1.877	2.148	1.913	0.128	6.7	95.6
5	5.239	5.194	4.682	4.533	5.117	5.585	5.058	0.387	7.7	101.2
10	9.937	10.770	9.384	10.614	10.400	11.988	10.516	0.880	8.4	105.1
15	14.883	16.187	15.238	13.632	14.673	16.755	15.228	1.116	7.3	101.5
20	19.293	18.899	20.696	18.043	21.496	18.806	19.539	1.297	6.6	97.7

Validation of DHEA for intraday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.052	0.043	0.043	0.043	0.045	0.047	0.046	0.004	7.8	91.1
0.5	0.46	0.439	0.459	0.471	0.405	0.427	0.444	0.025	5.5	88.7
2	2.04	2.111	1.765	2.052	1.847	1.815	1.938	0.146	7.5	96.9
10	9.705	9.897	10.296	11.321	9.288	10.588	10.183	0.719	7.1	101.8

Table 4a
Validation of A4 for interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.046	0.049	0.049	0.048	0.002	4.1	96.0
0.1	0.097	0.095	0.098	0.096	0.002	1.6	96.4
0.5	0.491	0.476	0.454	0.474	0.019	4.0	94.7
1	0.973	0.935	0.997	0.968	0.031	3.2	96.8
2	1.879	1.915	1.939	1.911	0.030	1.6	95.5
5	4.841	5.139	4.799	4.927	0.186	3.8	98.5
10	10.325	10.593	9.689	10.202	0.464	4.6	102.0
15	14.573	14.769	14.461	14.601	0.156	1.1	97.3
20	20.566	19.997	21.440	20.667	0.727	3.5	103.3

Validation of A4 for interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.047	0.053	0.048	0.050	0.003	7.0	99.1
0.5	0.417	0.466	0.446	0.443	0.025	5.6	88.6
2	1.772	1.851	1.966	1.863	0.097	5.2	93.1
10	9.463	10.002	9.425	9.630	0.322	3.3	96.3

Table 4b
Validation of A4 for intraday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.059	0.059	0.059	0.043	0.059	0.059	0.056	0.006	11.4	112.7
0.1	0.096	0.086	0.101	0.091	0.092	0.095	0.093	0.005	5.3	93.3
0.5	0.452	0.436	0.476	0.448	0.488	0.423	0.454	0.025	5.4	90.8
1	0.881	1.055	0.903	0.886	0.861	0.862	0.908	0.074	8.1	90.8
2	1.906	1.794	1.875	1.606	1.919	2.106	1.868	0.164	8.8	93.4
5	5.455	4.977	4.990	4.818	5.000	5.149	5.065	0.218	4.3	101.3
10	10.019	10.591	9.702	10.563	10.536	11.563	10.496	0.634	6.0	104.9
15	15.947	17.019	14.785	15.667	14.978	16.087	15.747	0.812	5.2	104.9
20	19.133	18.502	19.735	17.845	21.211	18.969	19.232	1.158	6.0	96.2

Validation of A4 for intraday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.052	0.046	0.042	0.047	0.055	0.053	0.049	0.005	10.3	98.4
0.5	0.469	0.485	0.457	0.462	0.420	0.465	0.460	0.021	4.7	91.9
2	1.757	1.755	1.804	1.983	1.676	1.747	1.787	0.104	5.8	89.3
10	8.886	9.413	9.254	11.360	9.313	9.497	9.621	0.878	9.1	96.2

Table 5a
Validation of T for interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.048	0.047	0.049	0.048	0.001	2.1	96.1
0.1	0.094	0.094	0.096	0.095	0.001	1.2	94.7
0.5	0.505	0.503	0.483	0.497	0.012	2.5	99.4
1	0.983	0.990	1.036	1.003	0.028	2.8	100.3
2	2.008	2.049	2.028	2.028	0.020	1.0	101.4
5	5.082	5.312	4.916	5.103	0.199	3.9	102.1
10	10.391	10.281	9.763	10.145	0.335	3.3	101.5
15	15.007	14.643	14.609	14.753	0.220	1.5	98.4
20	19.575	19.925	20.972	20.158	0.727	3.6	100.8

Validation of T for interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.048	0.048	0.051	0.049	0.002	4.0	97.7
0.5	0.466	0.518	0.472	0.486	0.028	5.8	97.1
2	1.924	2.016	2.031	1.991	0.058	2.9	99.5
10	9.782	10.140	9.518	9.813	0.312	3.2	98.1

Table 5b
Validation of T for intraday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy(%)
0.05	0.057	0.057	0.058	0.058	0.058	0.059	0.058	0.001	1.5	115.4
0.1	0.117	0.093	0.113	0.088	0.089	0.089	0.098	0.013	13.5	97.9
0.5	0.473	0.508	0.499	0.466	0.476	0.449	0.478	0.022	4.6	95.7
1	0.979	0.986	0.970	0.981	0.910	0.939	0.961	0.030	3.1	96.1
2	2.069	1.886	1.943	1.837	1.955	2.150	1.973	0.117	5.9	98.6
5	5.482	4.979	4.937	4.998	5.364	5.642	5.234	0.301	5.8	104.7
10	10.006	10.229	9.860	10.575	10.122	11.731	10.420	0.686	6.6	104.2
15	14.997	15.797	15.197	15.219	14.578	15.773	15.260	0.467	3.1	101.7
20	19.016	18.803	20.591	17.253	21.796	18.989	19.408	1.577	8.1	97.1

Validation of T for intraday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.059	0.054	0.047	0.046	0.059	0.046	0.052	0.006	11.9	103.7
0.5	0.500	0.506	0.460	0.519	0.440	0.444	0.478	0.034	7.1	95.6
2	1.883	1.957	1.922	2.132	1.840	1.921	1.943	0.101	5.2	97.1
10	10.032	9.703	10.158	11.442	9.365	10.369	10.178	0.713	7.0	101.8

Table 6a
Validation of DHT for interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.054	0.054	0.056	0.055	0.001	2.0	109.4
0.1	0.100	0.101	0.105	0.102	0.002	2.4	101.8
0.5	0.482	0.451	0.461	0.465	0.016	3.5	92.9
1	0.926	0.906	0.981	0.938	0.039	4.2	93.8
2	1.942	1.895	1.833	1.890	0.055	2.9	94.5
5	4.787	4.918	4.518	4.741	0.204	4.3	94.8
10	9.830	10.519	9.437	9.928	0.548	5.5	99.3
15	15.226	14.440	15.302	14.989	0.477	3.2	99.9
20	20.400	20.603	21.349	20.784	0.499	2.4	103.9

Validation of DHT for interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.05	0.048	0.045	0.053	0.049	0.004	7.5	97.3
0.5	0.446	0.514	0.469	0.476	0.035	7.3	95.2
2	1.813	1.856	1.957	1.875	0.074	3.9	93.8
10	9.830	10.654	9.712	10.065	0.513	5.1	100.7

Table 6b
Validation of DHT for intraday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean Conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.062	0.062	0.047	0.066	0.064	0.055	0.059	0.007	12.1	118.5
0.1	0.103	0.092	0.103	0.108	0.095	0.093	0.099	0.007	6.8	98.9
0.5	0.440	0.433	0.431	0.480	0.476	0.464	0.454	0.022	4.9	90.8
1	0.988	0.979	0.837	0.869	0.843	0.811	0.888	0.076	8.6	88.8
2	1.926	1.708	1.721	1.869	1.844	1.934	1.834	0.099	5.4	91.7
5	4.868	4.577	4.546	4.818	4.674	4.915	4.733	0.156	3.3	94.6
10	9.477	9.783	9.638	10.686	9.120	11.193	9.983	0.790	7.9	99.8
15	14.298	15.829	14.451	13.301	12.993	14.693	14.261	1.021	7.2	95.0
20	19.703	22.292	23.764	20.266	22.581	22.103	21.785	1.520	7.0	108.9

Validation of DHT for intraday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.05	0.058	0.059	0.060	0.049	0.060	0.058	0.057	0.004	7.6	114.3
0.5	0.432	0.594	0.474	0.545	0.567	0.507	0.520	0.061	11.7	103.9
2	1.934	2.135	2.216	2.046	1.765	2.129	2.037	0.164	8.1	101.9
10	11.588	11.351	11.680	11.839	11.115	11.407	11.497	0.258	2.2	114.9

Table 7a
Validation of 3α-Diol for interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.5	0.495	0.507	0.522	0.508	0.013	2.6	101.6
1	0.962	0.92	1.029	0.97	0.055	5.6	97
2	2.02	1.863	1.997	1.96	0.084	4.3	98
5	4.804	5.106	4.832	4.914	0.167	3.4	98.3
10	9.71	9.593	9.014	9.439	0.373	3.9	94.4
15	15.197	15.197	15.244	15.213	0.027	0.2	101.4
20	20.046	20.047	20.35	20.147	0.175	0.9	100.7

Validation of 3α-Diol for interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.5	0.51	0.476	0.497	0.494	0.017	3.5	98.9
2	1.881	2.191	2.147	2.073	0.168	8.1	103.6
10	10.433	9.427	10.107	9.989	0.513	5.1	99.9

Table 7b
Validation of 3α-Diol for intraday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.5	0.57	0.562	0.595	0.542	0.562	0.537	0.561	0.021	3.7	112.2
1	0.989	1.186	1.132	1.015	1.029	1.07	1.07	0.075	7	107
2	2.106	1.945	1.872	1.828	2.198	1.947	1.983	0.142	7.1	99.1
5	4.651	4.558	4.644	4.392	4.563	5.179	4.665	0.269	5.8	93.3
10	11.455	9.013	10.836	8.955	8.919	11.286	10.077	1.238	12.3	100.7
15	13.073	12.743	14.083	12.518	14.437	14.65	13.584	0.918	6.8	90.5
20	19.072	21.791	22.362	20.22	21.846	21.351	21.107	1.231	5.8	105.6

Validation of 3α-Diol for intraday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy(%)
0.5	0.54	0.512	0.452	0.515	0.556	0.578	0.525	0.044	8.3	105.1
2	2.382	2.226	2.139	1.909	1.8	2.362	2.136	0.238	11.2	106.8
10	11.638	11.033	11.006	11.763	10.866	10.318	11.104	0.531	4.8	111

Table 8a
Validation of 3β-Diol for interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.5	0.579	0.464	0.415	0.486	0.084	17.3	97.2
1	1.101	0.938	0.913	0.984	0.102	10.4	98.4
2	2.225	2.005	2.055	2.095	0.115	5.5	104.8
5	5.006	4.827	4.766	4.866	0.125	2.6	97.3
10	10.154	10.609	8.367	9.710	1.185	12.2	97.1
15	14.592	15.120	15.026	14.912	0.282	1.9	99.4
20	20.199	19.654	20.849	20.234	0.598	3.0	101.2

Validation of 3β-Diol for interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated interday concentration (nM)			Mean conc. (nM)	Interday validation summary		
	Day 1	Day 2	Day 3		Stdev	CV (%)	Accuracy (%)
0.5	0.520	0.428	0.434	0.461	0.052	11.2	92.1
2	2.199	1.880	1.955	2.011	0.167	8.3	100.6
10	9.589	11.287	9.818	10.231	0.921	9.0	102.3

5.3. Stability of DHEA, A4, T, DHT, 3α-Diol and 3β-Diol in 1 g/L of PBS/BSA

Different types of stability test were conducted on the six androgens in the matrix to understand the rate of degradation of these androgens during storage and sample preparation. Three types of stability tests, namely freeze–thaw, long, and short term stability tests were conducted. Four QCs samples were prepared and were termed as lowest, low, mid, and high QC. Nevertheless, the lowest QC here did not meet the acceptable validation requirements for 3α- and 3β-Diol. So, only the remaining three QC standards of low, mid and high concentration levels were used for stability analysis for 3α- ad 3β-Diols. The mean recoveries for the six androgens in the QC standards were obtained when compared against reference

Table 8b
Validation of 3β-Diol for intraday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.5	0.595	0.487	0.599	0.575	0.527	0.601	0.564	0.047	8.3	112.8
1	1.026	1.104	1.186	1.071	1.020	1.118	1.087	0.063	5.8	108.7
2	2.178	2.094	2.346	2.264	2.280	1.887	2.175	0.166	7.6	108.7
5	4.808	5.248	4.850	4.954	4.777	5.796	5.072	0.394	7.8	101.4
10	10.564	9.473	9.395	8.749	9.373	9.462	9.503	0.587	6.2	95.0
15	12.362	13.593	14.793	13.764	15.347	14.010	13.978	1.034	7.4	93.2
20	17.210	21.189	22.700	19.337	23.331	22.099	20.978	2.311	11.0	104.9

Validation of 3β-Diol for intraday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (nM)	Estimated intraday concentration (nM)						Mean conc. (nM)	Intraday validation summary		
	1st run	2nd run	3rd run	4th run	5th run	6th run		Stdev	CV (%)	Accuracy (%)
0.5	0.464	0.448	0.473	0.589	0.493	0.495	0.494	0.050	10.1	98.7
2	2.372	2.081	2.373	2.178	2.274	2.020	2.216	0.149	6.7	110.8
10	10.938	10.404	11.276	11.336	10.581	11.484	11.003	0.437	4.0	110.0

Table 9
Average range of recoveries for the samples at lowest[a], low, mid and high concentrations for short & long term and freeze–thaw stability test.

Analyte	Range of recoveries (%)					
	At 4 h	At 6 h	At 8 h	At 24 h	Long term	Freeze–thaw
DHEA	100.9–111.3	88.2–115.9	86.1–111.4	89.7–108.9	88.2–98.1	88.7–100.1
A4	102.1–130.0	90.6–105.0	94.4–106.1	99.1–106.3	100.2–106.6	88.4–101.1
T	103.0–117.2	93.3–102.0	93.4–100.3	94.6–118.5	86.7–96.4	86.1–95.0
DHT	89.8–134.2	99.2–125.7	95.8–131.3	80.2–116.5	89.1–126.8	89.7–230.8
3α-Diol	0–114.0	0–106.2	40.3–111.0	8.9–108.2	0–102.1	0–99.3
3β-Diol	0–77.4	0–71.5	0–90.4	0–94.1	0–81.6	0–139.9

[a] The lowest QC referred here was not used for analysis for 3α-Diol and 3β-Diol as its concentration fell out of quantifiable ranges of these two analytes.

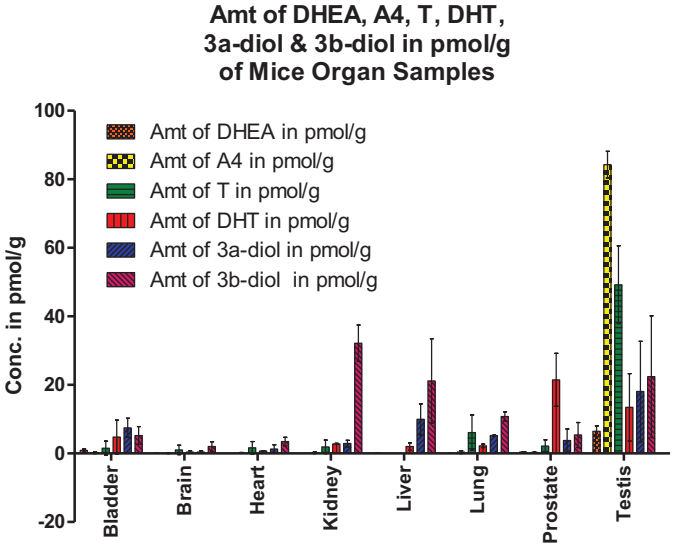

Fig. 3. Diagram showing the respective levels of androgens in the mice organs using LC–MS/MS detection.

set of QC standards of the freshly-spiked samples with the same concentrations as shown in Table 9.

From the results, DHEA, A4, T, and DHT were relatively stable in PBS/BSA matrix under the different test conditions as observed from the average recoveries from the three stability tests. DHEA did not suffer from much degradation when samples were kept under room temperature for 4 h but can possibly degrade by about 15% when kept at room temperature beyond 4 h. DHEA was also relatively stable under storage at −80 °C for one month and can withstand three freeze–thaw cycles, keeping the degradation to within 12%.

As for A4, T, and DHT, these androgens did not undergo much degradation under all short & long term and freeze–thaw stability conditions.

For 3α- and 3β-Diols, they were not that stable, especially for their low concentrations samples. The two diols in the low QC samples became negligible after all the conditions described in the three types of stability tests. But their mid and high QC samples were relatively more stable even when kept for long term under −80 °C or subjected to three rounds of freeze–thaw actions. On comparison, 3β-Diol enjoyed greater stability than 3α-Diol.

5.4. Detection of the levels of androgens-of-interest in mouse organs after administration of dimethoxycurcumin

In order to further verify the applicability of this LC–MS/MS method, a simple detection and quantification was performed on some mice organ samples. Fig. 3 shows the amounts of the six androgens-of-interest detected from the various organ samples collected from triplicates of mice. From here, it can be seen that the potent androgens such as T and DHT were relatively high in the male reproductive organs such as prostates and testes samples and is a reasonable result. In fact, among the rest of the organs tested, the testes samples were observed to contain the highest amounts of all the six androgens detected, including the adrenal androgen, A4 and DHT metabolites, 3α- and 3β-Diols. Additionally, 3β-Diol was observed to be present in relatively high levels in several other organs such as in kidneys, livers and lungs. This is a reasonable result as testes, kidneys, livers. and lungs are actually reported to have high expressions of ERβ [23]. And since 3β-Diol is known to be a selective endogenous ligand to ERβ [15], it is not surprising that it is present in higher concentrations in these organs. This result demonstrates that this LC–MS/MS method can be used to simul-

taneously determine the six androgens in limited volumes of the homogenized mouse organ samples.

6. Conclusions

A convenient, fast and robust LC–MS/MS method has been successfully developed and validated to simultaneously quantify six related androgens that are involved in the biosynthesis of T and DHT which leads to the occurrence of CRPC. To the best of our knowledge, this is the first reported LC–MS/MS method which allows the simultaneous quantification of these six androgens, including the two isomeric diols, 3α- and 3β-Diols. The validated method has been tested on some mice organs and was proven to be able to detect and accurately quantify these androgens.

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References

[1] E.S. Antonarakis, M.A. Carducci, M.A. Eisenberger, Novel targeted therapeutics for metastatic castration-resistant prostate cancer, Cancer Lett. 291 (1) (2010) 1–13.
[2] A.A. Shafi, A.E. Yen, N.L. Weigel, Androgen receptors in hormone-dependent and castration-resistant prostate cancer, Pharmacol. Ther. 140 (3) (2013) 223–238.
[3] B.J. Feldman, D. Feldman, Development of androgen independent prostate cancer, Nat. Rev. 1 (2001) 34–45.
[4] E.A. Mostaghel, P.S. Nelson, Intracrine androgen metabolism in prostate cancer progression: mechanisms of castration resistance and therapeutic implications, Best Pract. Res. Clin. Endocrinol. Metab. 22 (2) (2008) 243–258.
[5] S. Koochekpour, Androgen receptor signaling and mutations in prostate cancer, Asian J. Androl. 12 (5) (2010) 639–657.
[6] E.A. Mostaghel, B. Montgomery, P.S. Nelson, Castration-resistant prostate cancer: targeting androgen metabolic pathways in recurrent disease, Urol. Oncol. 27 (3) (2009) 251–257.
[7] T. Karantanos, P.G. Corn, T.C. Thompson, Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches, Oncogene 32 (49) (2013) 5501–5511.
[8] O. Acar, T. Esen, N.A. Lack, New therapeutics to treat castrate-resistant prostate cancer, Sci. World J. 2013 (2013) 379641.
[9] K.E. Knudsen, T.M. Penning, Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer, Trends Endocrinol. Metab. 21 (5) (2010) 315–324.
[10] R.B. Montgomery, E.A. Mostaghel, R. Vessella, D.L. Hess, T.F. Kalhorn, C.S. Higano, L.D. True, P.S. Nelson, Maintenance of intratumoral androgens in metastatic prostate cancer: a mechanism for castration-resistant tumor growth, Cancer Res. 68 (11) (2008) 4447–4454.
[11] A.R. Hamid, M.J. Pfeiffer, G.W. Verhaegh, E. Schaafsma, A. Brandt, F.C. Sweep, J.P. Sedelaar, J.A. Schalken, Aldo-keto reductase family 1 member C3 (AKR1C3) is a biomarker and therapeutic target for castration-resistant prostate cancer, Mol. Med. 18 (2012) 1449–1455.
[12] A.O. Adeniji, M. Chen, T.M. Penning, AKR1C3 as a target in castrate resistant prostate cancer, J. Steroid Biochem. Mol. Biol. 137 (2013) 136–149.
[13] M.G. Dozmorov, J.T. Azzarello, J.D. Wren, K.M. Fung, Q. Yang, J.S. Davis, R.E. Hurst, D.J. Culkin, T.M. Penning, H.K. Lin, Elevated AKR1C3 expression promotes prostate cancer cell survival and prostate cell-mediated endothelial cell tube formation: implications for prostate cancer progression, BMC Cancer 10 (2010) 672.
[14] M.C. Byrns, Y. Jin, T.M. Penning, Inhibitors of type 5 17beta-hydroxysteroid dehydrogenase (AKR1C3): overview and structural insights, J. Steroid Biochem. Mol. Biol. 125 (1–2) (2011) 95–104.
[15] D. Dondi, M. Piccolella, A. Biserni, S.D. Torre, B. Ramachandran, A. Locatelli, P. Rusmini, D. Sau, D. Caruso, A. Maggi, P. Ciana, A. Poletti, Estrogen receptor b and the progression of prostate cancer: role of 5a-androstane-3b,17b-diol, Endocr. Relat. Cancer 17 (2010) 731–742.
[16] A.O. Adeniji, B.M. Twenter, M.C. Byrns, Y. Jin, M. Chen, J.D. Winkler, T.M. Penning, Development of potent and selective inhibitors of aldo-keto reductase 1C3 (type 5 17beta-hydroxysteroid dehydrogenase) based on *N*-phenyl-aminobenzoates and their structure-activity relationships, J. Med. Chem. 55 (5) (2012) 2311–2323.
[17] V. Guerini, D. Sau, E. Scaccianoce, P. Rusmini, P. Ciana, A. Maggi, P.G.V. Martini, B.S. Katzenellenbogen, L. Martini, M. Motta, A. Poletti, The androgen derivative 5α-androstane-3β,17β-diol inhibits prostate cancer cell migration through activation of the estrogen receptor β subtype, Cancer Res. 65 (12) (2005) 5445–5453.
[18] P. Picciarelli-Lima, A.G. Oliveira, A.M. Reis, E. Kalapothakis, G.A. Mahecha, R.A. Hess, C.A. Oliveira, Effects of 3-beta-diol, an androgen metabolite with intrinsic estrogen-like effects, in modulating the aquaporin-9 expression in the rat efferent ductules, Reprod. Biol. Endocrinol. 4 (51) (2006) 1–10.
[19] S.F. Soh, C.K. Huang, S.O. Lee, D. Xu, S. Yeh, J. Li, E.L. Yong, Y. Gong, C. Chang, Determination of androgen receptor degradation enhancer ASC-J9® in mouse sera and organs with liquid chromatography tandem mass spectrometry, J. Pharm. Biomed. Anal. 88 (2014) 117–122.
[20] F. Jacolot, D. Picart, F. Berthou, H.H. Floch, Determination of 5α-androstane-3α, 17β-diol and 5α-androstane-3β, 17β-diol in human plasma by selected ion monitoring, Biomed. Environ. Mass Spectrom. 13 (8) (1986) 389–394.
[21] V. Bellemare, F. Faucher, R. Breton, V. Luu-The, Characterization of 17α-hydroxysteroid dehydrogenase activity (17α-HSD) and its involvement in the biosynthesis of epitestosterone, BMC Biochem. 6 (12) (2005) 1–11.
[22] Guidance for Industry Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) Center for Veterinary Medicine (CVM), BP, 2001. p. 1–22.
[23] K. Maruyama, H. Endoh, H. Sasaki-Iwaoka, H. Kanou, E. Shimaya, S. Hashimoto, S. Kato, H. Kawashima, A novel isoform of rat estrogen receptor beta with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regulator of estrogen action, Biochem. Biophys. Res. Commun. 246 (1998) 142–147.