Measuring Dihydrotestosterone (DHT) in Blood Serum for Research Purposes using Derivatization and LC-MS/MS

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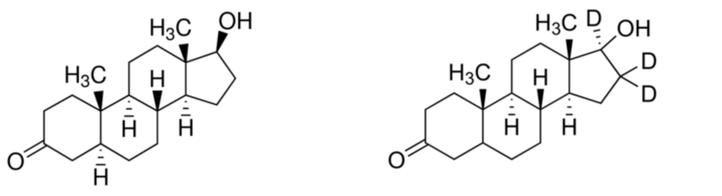
ABSTRACT

We developed an LC-MS/MS method for researchers to accurately measure dihydrotestosterone (DHT) in blood serum from 25 to 2,000 pg/mL with a throughput of at least 12 injections per hour. This was done by liquid-liquid extraction of blood serum followed by derivatization with hydroxylamine and quantitative analysis using a 4-channel high-performance liquid chromatography (LC) system coupled to a tandem mass spectrometer (MS/MS). Oxime derivatives of DHT and its D_3 internal standard were separated from sample matrix components by gradient elution through an HPLC column containing solid-core silica particles with a polar-imbedded reversed-phase bonded on its surfaces. Analytes were eluted to a heated electro-spray ionization probe of a triple-quadrupole mass spectrometer used for selected-reaction monitoring of the analytes. The desired analytical range of 25 to 2,000 pg/mL was achieved with inter- and intra-batch reproducibility of less than 7%, carryover less than 0.2% and acceptable correlation of specimen results with those from a reference laboratory. Throughputs of 12 to 24 injections/hour were achieved using one or two LC channels, respectively.

INTRODUCTION

Dihydrotestosterone (DHT), also known as 5α-dihydro-testosterone, androstanolone or stanolone, is an endogenous steroid hormone and a more potent androgen than testosterone. DHT is made by reduction of testosterone by 5α-reductase. Researchers studying the physiology of DHT and control of its biosynthesis using 5α-reductase inhibitors need to quantify this steroid within a range of 25 to 2,000 pg/mL (86.2 to 6,900 pmol/L) in blood serum. Since DHT does not ionize well by either atmospheric-pressure chemical ionization (APCI) or electrospray ionization (ESI), derivatization with hydroxylamine prior to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was necessary to reliably achieve the desired measuring range (1). Chemical structures and formulas of DHT and DHT-D3 internal standard are shown in Figure 1 along with mass-to-charge ratios (m/z) of their oxime derivatives. Fragmentation of DHT oxime is shown in Figure 2.

Figure 1. Analyte & internal standard (IS)



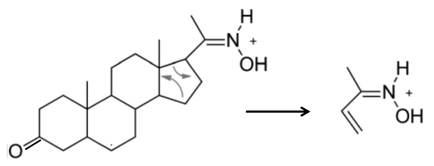
5α-Dihydrotestosterone (DHT)

Formula: $C_{19}H_{30}O_2$
Mono-isotopic mass: 290.2240
Oxime derivative *m/z* = 306.2

5α-Dihydrotestosterone-16,16,17-D_3 (DHT-D3) internal standard (IS)
Formula: $C_{19}H_{27}D_3O_2$
Mono-isotopic mass: 293.2429
Oxime derivative *m/z* = 309.3

Reactions with hydroxylamine form positive-ion oxime derivatives

Figure 2. Fragmentation of DHT oxime



Dihydrotestosterone hydroxylamine derivative

$C_4H_8NO^+$
m/z 86.060

MATERIALS AND METHODS

Sample preparation

200 µL aliquots of donor blood serum specimens, calibrators and quality control specimens (QCs), were spiked with DHT-D3 internal standard (IS) before being subjected to liquid-liquid extraction (LLE) with 2 mL methyl t-butyl ether (MTBE). After drying the MTBE extracts with heated nitrogen flow, the residue of each was reacted with hydroxylamine to form positive-ion oxime derivatives. After drying the derivative reagent, the residue of each was reconstituted with water and methanol (1:1) to a total volume of 200 µL. Then, 50 µL injections of each were made into the multi-channel LC-MS/MS system.

LC-MS/MS methods

Using one or more channels of a Thermo Scientific™ Transcend™ LX-4 HPLC system, chromatographic separation of the steroid oximes from unwanted sample components was achieved by gradient elution through a Thermo Scientific™ Accucore™ aQ column (2.6 µm, 100 x 2.1 mm), which was heated to 40ºC. Figure 3 describes chromatographic conditions.

Figure 3. Chromatographic conditions for DHT-oxime

Column 1	Accucore aQ, 2.6um, 100 x 2.1mm
Pump Type	Binary
A	Water + 0.1% Formic Acid
B	Methanol

Column heated to 40ºC

Data window start: 2 min
duration: 2 min
Method duration: 4.5 min

Start	Len	Flow	Grad	%A	%B	CD	Comments
00:00	10	0.40	Step	50.0	50.0	--->	Load sample
00:10	30	0.40	Ramp	40.0	60.0	--->	Separate analytes
00:40	60	0.40	Ramp	30.0	70.0	--->	Elute analytes
01:40	60	0.40	Step	-	100.0	--->	Wash column
02:40	110	0.40	Step	50.0	50.0	--->	Equilibrate column

The Thermo Scientific™ TSQ Endura™ triple-quadrupole mass spectrometer was used with a heated electro-spray ionization (HESI) probe to selectively monitor analytes. Selected-reaction monitoring (SRM) of two transitions (quantitation and conformation) for DHT and DHT-D3 occurred within the 2-minute data window of the 4.5-minute LC method. Ion source and MS/MS conditions are described in Figure 4. The "Electro-clean" step is described in reference 2 and helped minimize charging effects.

Figure 4. Tandem mass spectrometry conditions

Ion Source

Ion Source Type: **H-ESI**
Spray Voltage:
Spray Voltage: **Static**
Positive Ion (V): **3500.00**
Negative Ion (V): **2500.00**
Current LC Flow (µL/min): **0**
Sheath Gas (Arb): **50**
Aux Gas (Arb): **10**
Sweep Gas (Arb): **1**
Ion Transfer Tube Temp (°C): **350**
Vaporizer Temp (°C): **400**

SRM

Use Cycle Time: **True**
Cycle Time (sec): **.2**
Use Calibrated RF Lens: **False**
Q1 Resolution (FWHM): **0.7**
Q3 Resolution (FWHM): **0.7**
CID Gas (mTorr): **1.5**
Source Fragmentation (V): **10**
Chrom Filter (sec): **3**

SRM Table

Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
DHT-oxime	0	1.9	Positive	306.3	105.2	39	175
DHT-oxime	0	1.9	Positive	306.3	145.2	32	175
DHT-D3-oxime	0	1.9	Positive	309.3	96.25	34	175
DHT-D3-oxime	0	1.9	Positive	309.3	258	24	175
Electro-clean	1.9	2	Negative	200	100	30	175

Instrument control & data analysis

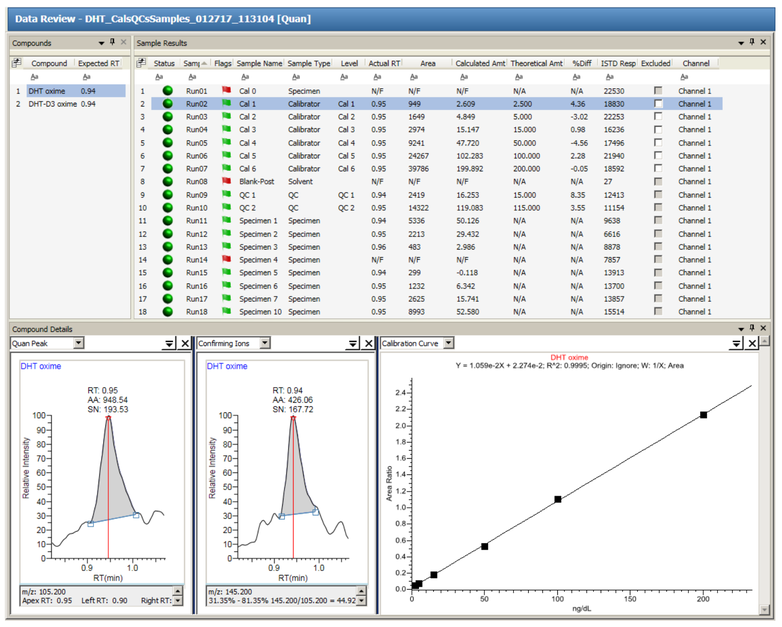
Thermo Scientific™ TraceFinder™ configured with Aria™ MX and TSQ Endura™ control software was used to control the LC-MS/MS systems, submit batches to the desired channels as well as to analyze data and report results.

RESULTS

Quantitation reliability

Typical DHT results for calibrators, QCs and specimens are show in Figure 5. The desired measuring range from 25 to 2,000 pg/mL was achieved and was consistently linear ($r^2 \geq 0.995$ with 1/X weighting). Carryover was less than 0.2%. Ion ratios (confirming/quan peak areas) averaged 54% for DHT-oxime and 46 % for DHT-D3-oxime (IS). IS peak areas among calibrators & QCs averaged 19,200 with RSDs of 12.5%. IS peak areas from donor serum specimens ranged from 6,610 to 15,500 showing an average recovery of 59%, which indicated moderate ion-suppression by matrix. However, the IS in each sample preparation adequately compensated for such matrix effects.

Figure 5. Typical DHT quantitation results



As shown in Tables 1a and 1b, intra- and inter-batch precisions among 20 replicate injections from three pools (low, medium and high DHT levels) were less than 7% and 6% coefficient of variation (CV), respectively

Table 1. Intra- and inter-batch precisions

a. Intra-batch results (pg/mL)

Run	Low	Medium	High
1	172	590	1250
2	182	550	1280
3	193	580	1390
4	190	590	1300
5	179	570	1200
6	177	570	1240
7	187	610	1210
8	174	600	1230
9	188	560	1250
10	171	570	1190
11	185	570	1300
12	160	540	1190
13	163	600	1190
14	195	520	1320
15	163	590	1260
16	173	550	1300
17	175	590	1320
18	165	580	1180
19	160	550	1310
20	184	550	1300
Mean:	**176.8**	**571.5**	**1260.5**
StdDev:	**11.0**	**23.2**	**57.2**
% CV:	**6.2**	**4.1**	**4.5**

b. Inter-batch results (pg/mL)

	Run	Low	Medium	High
Day 1	1	185	520	1140
	2	208	500	1150
	3	197	560	1150
	4	195	580	1250
	5	204	540	1220
Day 2	1	195	550	1150
	2	207	550	1150
	3	182	570	1190
	4	199	550	1170
	5	188	580	1150
Day 3	1	201	520	1140
	2	192	530	1100
	3	186	490	1190
	4	202	530	1070
	5	193	500	1130
Day 4	1	209	520	1070
	2	201	540	1070
	3	191	510	1040
	4	189	560	1040
	5	192	510	1040
Mean:		**195.8**	**535.5**	**1130.5**
StdDev:		**7.9**	**26.8**	**60.5**
% CV:		**4.0**	**5.0**	**5.3**

Accuracy assessment

Comparison of LC-MS/MS quantitation of DHT in 54 donor serum samples between our research lab and to a reference lab is summarized in Table 2. DHT values ranged from 47 to 973 pg/mL. Only 4 out of 54 results differed by more than 20% and none were more than 22.4%. The differences between the two methods averaged 2.5%, a small positive bias by our lab. The two DHT methods were equivalent within an allowable total error (TEa) of 25%.

Table 2. Comparison of donor serum DHT results (pg/mL) between reference lab (Ref Lab) and research lab (BRL).

Sample	Ref Lab	BRL	% Diff	Sample	Ref Lab	BRL	% Diff	Sample	Ref Lab	BRL	% Diff
1	151	151	0.00	19	182	182	0.00	37	937	891	-4.91
2	160	191	19.38	20	535	521	-2.62	38	174	213	22.41
3	72	76	5.56	21	1380	1492	8.12	39	552	560	1.45
4	91	105	15.38	22	286	330	15.38	40	285	284	-0.35
5	685	663	-3.21	23	380	452	18.95	41	34	35	2.94
6	281	264	-6.05	24	777	752	-3.22	42	122	142	16.39
7	92	105	14.13	25	456	489	7.24	43	139	149	7.19
8	290	295	1.72	26	266	232	-12.78	44	211	227	7.58
9	572	537	-6.12	27	1070	1080	0.93	45	319	278	-12.85
10	47	55	17.02	28	252	255	1.19	46	457	504	10.28
11	157	148	-5.73	29	691	589	-14.76	47	195	171	-12.31
12	77	80	3.90	30	1160	1240	6.90	48	117	136	16.24
13	107	130	21.50	31	1230	1160	-5.69	49	279	333	19.35
14	326	376	15.34	32	1020	804	-21.18	50	259	239	-7.72
15	462	408	-11.69	33	55	44	-20.00	51	470	480	2.13
16	76	69	-9.21	34	255	240	-5.88	52	449	493	9.80
17	66	75	13.64	35	339	311	-8.26	53	240	261	8.75
18	293	305	4.10	36	94	75	-20.21	54	267	306	14.61

Throughput

Single-channel throughput was 12 injections per hour. When multi-channeled across 2, 3 or 4 channels, the throughput increased to 24, 36 and 48 injections per hour, respectively. DHT batches were also multi-channeled with pregnenolone and estrogen batches, which utilized the same HESI source conditions.

CONCLUSIONS

Dihydrotestosterone (DHT) can be accurately measured in blood serum by this research method to achieve:

- Analytical range from 25 to 2,000 pg/mL
- Inter- & intra-batch precisions less than 7% CV and carryover less than 0.2%
- Results virtually equivalent to reference lab results
- Throughputs of 12, 24 or 48 injections per hour from a 1-, 2- or 4-channel system
- Multi-channeling with other methods utilizing the same HESI source conditions

REFERENCES

1. J. DiBussolo & M. Kozak, High-Throughput LC-MS/MS Quantification of Pregnenolone in Human Blood Serum for Clinical Research Purposes, Thermo Scientific Application Note AN-650, 2016.
2. J. DiBussolo, How to Electro-Clean APCI Sources Between Injections, Thermo Scientific Poster Note PN64408, 2016.

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