A Highly Sensitive Method for the Quantitation of Testosterone and Dihydrotestosterone in Human Serum via LC-MS/MS

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PURPOSE:

The purpose of this project was to develop a highly sensitive LC-MS/MS method for the determination of testosterone (T) and dihydrotestosterone (DHT) in human serum for the measurement of T/DHT baseline levels in postmenopausal females receiving testosterone replacement therapy.

INTRODUCTION:

- DHT (a biologically active metabolite of T) is the most potent of the androgen class of compounds and is formed in the prostate gland, testes, hair follicles, and adrenal glands when 5α-reductase reduces the 4,5 T double bond.
- DHT is approximately three times more potent than T and, like T, binds to and activates androgen receptors.
- On average, the ratio of DHT:T in human serum is 1:10.
- A highly selective bioanalytical method capable of achieving very low limits of quantitation for DHT and T in human serum was needed.

CHALLENGES:

- Due to the desired DHT LLOQ (between 5 and 20 pg/mL), a derivatization was necessary.
- Several derivatization reagents were tested to obtain stable derivatives without causing interferences and/or unstable instrument response.
- DHT and T levels in postmenopausal and charcoal stripped serum were too high for either to be used as the control matrix. Therefore, various dilute matrices were screened for interferences and tested for parallel response.
- Interferences, not present in the chosen control matrix, were chromatographically separated from DHT and T in human serum samples.

METHODS AND INSTRUMENTATION:

- 2X charcoal stripped human serum diluted with ultrapure water was used as the control matrix and fortified with T and DHT at the appropriate standard concentrations prior to extraction.
- Control matrix and human serum samples (1.00 mL) were diluted with an ammonium acetate buffer and spiked with deuterated internal standards (d_3-T and d_4-DHT).
- A Zymark/Caliper Sciclone automated sample handling system was used to perform a liquid-liquid extraction with MTB ether, and the extracts were evaporated to dryness.
- Samples were derivatized by the addition of 2,3-pyridine dicarboxylic anhydride in anhydrous pyridine and incubation at 60˚C for at least one hour.
- After acidifying the samples, a second automated liquid-liquid extraction with MTB ether was performed.
- Samples were evaporated to dryness and reconstituted in an appropriate aqueous:organic solution.
- Chromatographic conditions involved a Waters, Xterra MS C18 analytical column and a mobile phase consisting of acetonitrile, water, and formic acid.
- An AB | MDS Sciex API 5000, using an ESI interface, detected negative ions in the multiple reaction monitoring mode.
- The acquisition time was 7.0 minutes.

RESULTS:

- A weighted $1/x^2$ linear regression was used for T and DHT over a concentration range of 50.0 to 10,000 pg/mL and 10.0 to 2,000 pg/mL for T/DHT, respectively.
- Inter-batch precision (% CV) and accuracy (% Bias) of T (Table 1) and DHT (Table 2) quality control samples met predefined validation acceptance criteria.
- Ten lots of serum from different donors were fortified near the LLOQ and at the high quality control concentrations. No significant matrix effect was observed. (Tables 3 and 4).
- A post-column infusion demonstrated that T, DHT, d_3-T, and d_4-DHT did not co-elute with any areas of significant suppression or enhancement (Figures 5 and 6).
- Samples having a concentration above the upper limit of the calibration standard range were diluted with blank control matrix, and results show that samples with a concentration up to 50,000 pg/mL for T and 5010 pg/mL for DHT could be quantified after the application of an appropriate dilution factor.
- The average extraction recoveries of T and d_3-T from control matrix were 76% and 77%, respectively. The average extraction recoveries of DHT and d_4-DHT from control matrix were 76% and 52%, respectively. The average extraction recoveries of d_3-T and d_4-DHT from human serum were 70% and 71%, respectively.
- Stability evaluations of T and DHT (Table 5) quality control samples met predefined validation acceptance criteria.
- Method robustness was demonstrated with multiple column lots, mass spectrometers, and extraction scientists.
- The validated batch size was 192 samples.

CONCLUSIONS:

- Parallel response between the control matrix and human serum samples was demonstrated.
- The method utilized a derivatization and double extraction to achieve low limits of quantitation and eliminate significant matrix interference.
- The bioanalytical assay for the quantitation of T and DHT in human serum met acceptance criteria for precision, accuracy, sensitivity, selectivity, and stability.
- The validation results demonstrate that a precise, accurate, sensitive, selective, rugged and reproducible assay was developed.

Table 1. T Inter-Batch Precision and Accuracy

Batch	LLOQ 50.0 pg/mL	QC A 138 pg/mL	QC B 1020 pg/mL	QC C 7520 pg/mL
Inter-Batch Mean	50.2	134	1070	7490
Inter-Batch % CV	5.2	3.8	3.1	2.6
Inter-Batch % Bias	0.4	-2.9	4.9	-0.4
n	27	27	27	27

Table 2. DHT Inter-Batch Precision and Accuracy

Batch	LLOQ 10.0 pg/mL	QC A 26.5 pg/mL	QC B 202 pg/mL	QC C 1510 pg/mL
Inter-Batch Mean	9.67	26.8	211	1480
Inter-Batch % CV	13.8	8.2	9.8	8.1
Inter-Batch % Bias	-3.3	1.1	4.5	-2.0
n	27	27	27	27

Table 3. T Selectivity and Matrix Effect Test

		Low Spike				High Spike			
Lot#	Basal Level pg/mL	Nominal Amount Spiked pg/mL	Expected Concentration pg/mL	Measured Concentration pg/mL	% Dev	Nominal Amount Spiked pg/mL	Expected Concentration pg/mL	Measured Concentration pg/mL	% Dev
1	158	50.0	208	215	+3.4	8000	8158	7700	-5.6
2	278	100	378	373	-1.3	8000	8278	7730	-6.6
3	362	100	462	446	-3.5	8000	8362	7620	-8.9
4	131	50.0	181	175	-3.3	8000	8131	7610	-6.4
5	173	50.0	223	222	-0.4	8000	8173	7380	-9.7
6	175	50.0	225	226	+0.4	8000	8175	7840	-4.1
7	87.5	50.0	138	145	+5.5	8000	8088	7830	-3.2
8	437	200	637	671	+5.3	8000	8437	8340	-1.1
9	194	50.0	244	249	+2.0	8000	8194	7760	-5.3
10	114	50.0	164	173	+5.5	8000	8114	7850	-3.3

Note: Basal levels determined by averaging the measured concentrations of three replicates of serum samples fortified with internal standard only.

Table 4. DHT Selectivity and Matrix Effect Test

		Low Spike				High Spike			
Lot#	Basal Level pg/mL	Nominal Amount Spiked pg/mL	Expected Concentration pg/mL	Measured Concentration pg/mL	% Dev	Nominal Amount Spiked pg/mL	Expected Concentration pg/mL	Measured Concentration pg/mL	% Dev
1	31.5	10.0	41.5	38.0	-8.4	1600	1632	1400	-14.2
2	82.3	20.0	102.3	103	+0.7	1600	1682	1630	-3.1
3	45.5	20.0	65.5	63.1	-3.7	1600	1646	1620	-1.5
4	44.5	20.0	64.5	61.1	-5.3	1600	1645	1530	-7.0
5	58.4	20.0	78.4	80.0	+2.0	1600	1658	1580	-4.7
6	54.5	20.0	74.5	77.0	+3.4	1600	1655	1430	-13.6
7	64.2	20.0	84.2	77.7	-7.7	1600	1664	1450	-12.9
8	33.0	20.0	53.0	51.2	-3.4	1600	1633	1600	-2.0
9	69.6	20.0	89.6	91.2	+1.8	1600	1670	1520	-9.0
10	81.4	20.0	101.4	96.5	-4.8	1600	1681	1570	-6.6

Note: Basal levels determined by averaging the measured concentrations of three replicates of serum samples fortified with internal standard only.

Table 5. Stability Evaluations

Bench-Top Stability (Hrs)	Short-Term Stability: 51 hours in polypropylene tubes at ambient temperature under white light
Processed Stability (Hrs)	Post-Preparative Stability: 167 hours in a polypropylene 96 well plate at 5˚C Processed Sample Integrity: 170 hours in a polypropylene 96 well plate at 5˚C
Freeze-Thaw Stability (Cycles)	6 freeze (-20˚C)-thaw (ambient temperature) cycles in polypropylene tubes under white light
Long-Term Storage Stability (Days)	183 days in polypropylene tubes at -20˚C

Figure 1. T Structure

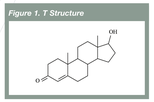


Figure 2. DHT Structure

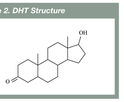


Figure 3. T Derivatization Product Structures

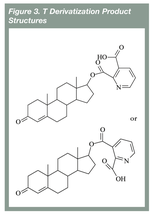


Figure 4. DHT Derivatization Product Structures

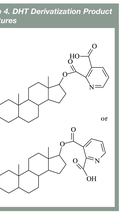


Figure 5. T Post-column Matrix Infusion

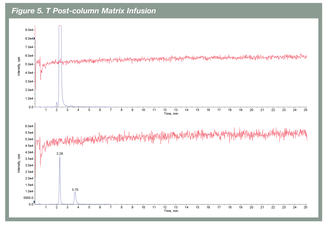


Figure 6. DHT Post-column Matrix Infusion

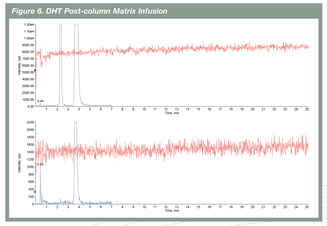


Figure 7. T (Top) and d_3-T (Bottom) Blank Control Matrix Sample

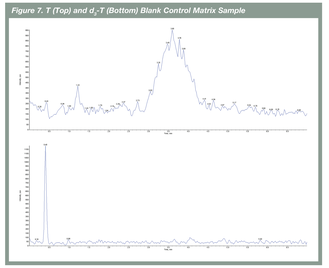


Figure 8. DHT (Top) and d_4-DHT (Bottom) Blank Control Matrix Sample

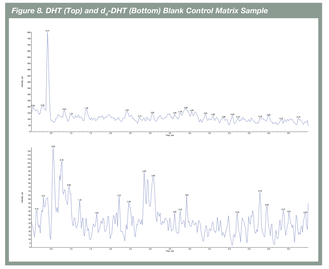


Figure 9. T (Top) and d_3-T (Bottom) Extracted LLOQ (50.0 pg/mL) Sample from Control Matrix

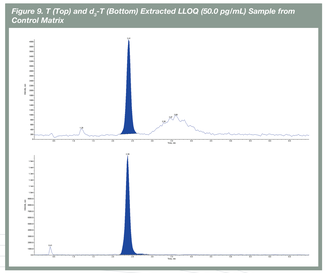


Figure 10. DHT (Top) and d_4-DHT (Bottom) Extracted LLOQ (10.0 pg/mL) Sample from Control Matrix

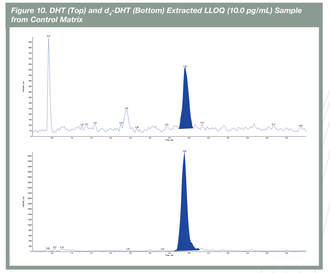


Figure 11. T (Top) and d_3-T (Bottom) Extracted Low Endogenous (64.8 pg/mL) Sample from Human Serum

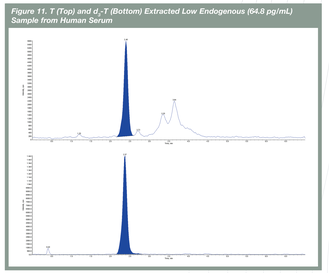
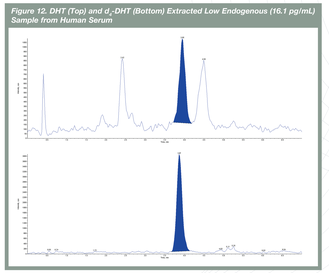


Figure 12. DHT (Top) and d_4-DHT (Bottom) Extracted Low Endogenous (16.1 pg/mL) Sample from Human Serum



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