TRANSFORMING PROMISING IDEAS INTO COMMERCIAL REALITY

Determination of Testosterone in Plasma instead of Serum: When is it needed?

Ann Lévesque, Ph. D.

November 20th, 2014

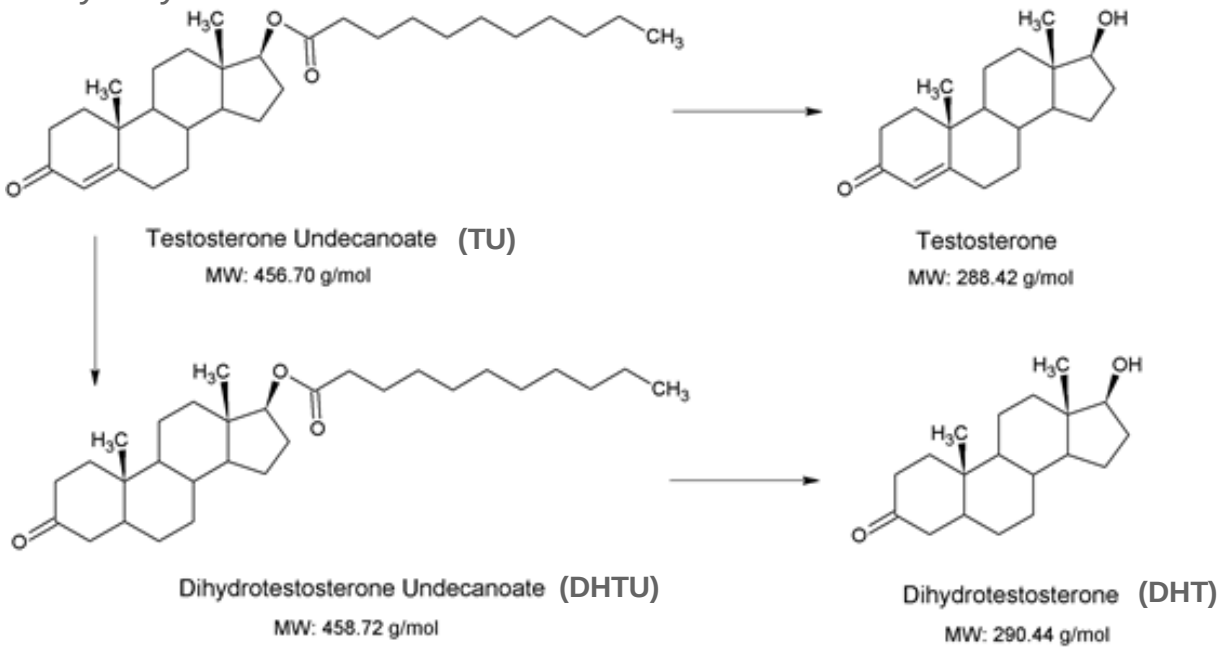
inVentiv Health clinical

Introduction

- Endogenous androgens are responsible for normal growth and development of the male organs and for maintenance of secondary sex characteristics.
- Testosterone undecanoate (TU) is the most commonly used treatment options for hypogonadism.
- TU capsules are currently available on the market as well as the injection form.
- TU is orally bioavailable contrary to testosterone oral dosing.

A Little Bit of Chemistry

- Testosterone undecanoate, also named testosterone undecylate, is an ester of testosterone.
- TU is metabolized into dihydrotestosterone undecanoate (DHTU).
- Esters may be easily hydrolyzed, specifically in whole blood, into the steroid and the fatty acid side chain moieties due to non-specific esterases.
- Uncontrolled hydrolysis may cause overestimation of testosterone concentrations.
- TU can be hydrolyzed to testosterone and DHTU to DHT.



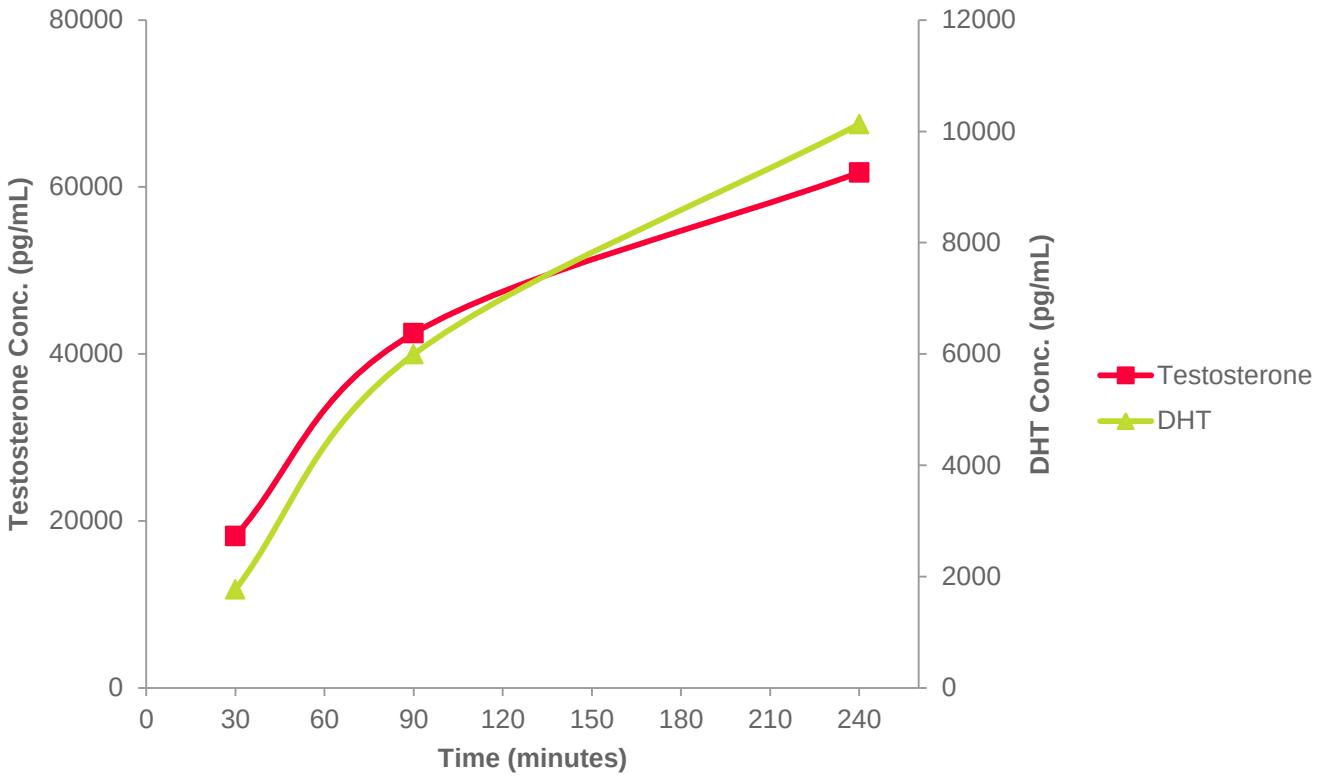
Context

- Testosterone and DHT are usually analyzed in serum.
- As a support to clinical studies, the stability of the analytes of interest has to be proven.
- The extent of the degradation of the pro-drug (TU) has to be evaluated.
- Multiple experiments were performed to evaluate the impact of TU and DHTU degradation on testosterone and DHT concentrations after blood collection.
- We will determine if serum is the appropriate matrix in studies with TU oral administration.

Experiment #1: Degradation of TU and DHTU in Whole Blood

- TU was added at a final concentration of 1 600 ng/mL and DHTU at 800 ng/mL to freshly collected whole blood without any additive (serum).
- Aliquots were set aside for 30, 90 and 240 minutes at room temperature.
- Aliquots were centrifuged and serum was harvested.
- Testosterone and DHT were determined by LCMSMS using an automated liquid-liquid extraction.
- The initial endogenous level of testosterone and DHT was also quantified.

Experiment #1: Degradation of TU and DHTU in Whole Blood



Endogenous level: Testosterone: 145 pg/mL; DHT: 89 pg/mL

- Extensive increase of Testosterone and DHT concentrations over time is observed.

Experiment #2: Impact of TU Concentrations on Testosterone

- Different TU concentrations from 15 to 700 ng/mL were added to freshly collected whole blood without any additives.
- Aliquots were set aside for 30 and 60 minutes at room temperature.
- Aliquots were centrifuged and serum was harvested.
- Resulting serum was analyzed for testosterone using the LCMSMS assay.
- Concentrations were compared to the endogenous level.

Experiment #2: Impact of TU Concentrations on Testosterone

TU Concentration Added (ng/mL)	Duration of incubation (min)	Concentration Testosterone Measured (pg/mL)	%Difference vs. TU=0 (%)
0	0	237.5	N/Ap
15	30	367.6	54.8
	60	458.1	92.9
100	30	1526.6	542.8
	60	2531.8	966.0
300	30	3060.2	1188.5
	60	5321.9	2140.8
700	30	7324.9	2984.2
	60	12628.1	5217.1

- Testosterone concentration increases over time.
- Even low TU concentrations have a significant impact.

Experiment #3: TU Degradation: Comparison of Additives

- Whole blood was collected in different collection tubes containing different additives or none.
- TU at a concentration of 600 ng/mL was added to the whole blood.
- Aliquots were incubated for 30 minutes at room temperature after clotting for the serum and 50 minutes at 4°C for the plasma.
- Aliquots were centrifuged and testosterone was analyzed by LCMSMS assays in the serum and the plasma.

Experiment #3: TU Degradation: Comparison of Additives

Anticoagulant	Matrix Analyzed	Incubation Period (min)	Testosterone Concentration (pg/mL)		
			Comparison Sample	Stability Sample	% Change
Without anticoagulant not fortified with TU (endogenous level)	Serum	N/Ap	3706	N/Ap	**N/Ap**
Without anticoagulant	Serum	30 at RT	12702	19678	**54.9**
Rapid Serum Tube (RST) without anticoagulant	Serum	30 at RT	8144	12283	**50.8**
NaF 0.43%	Serum with additives	30 at RT	9924	15921	**60.4**
EDTA K_2	Plasma	50 at 4°C	4419	5290	**19.7**
NaF (0.15%)-Na_2EDTA	Plasma	50 at 4°C	3928	4525	**15.2**
NaF (0.25%)-$K_2C_2O_4$	Plasma	50 at 4°C	4092	4611	**12.7**
BD™ P800 containing Protease and Esterase Inhibitors	Plasma	50 at 4°C	4796	7203	**50.2**

- The % change is higher at room temperature.
- NaF-Na_2EDTA and NaF/$K_2C_2O_4$ tubes kept at 4ºC gave the lowest conversion.

Experiment #4: Comparison of NaF-Na_2EDTA and NaF-$K_2C_2O_4$

- Whole blood was collected from 8 donors in NaF-Na_2EDTA or NaF-$K_2C_2O_4$ collection tubes.
- TU at a concentration of 600 ng/mL was added to the whole blood.
- Aliquots were set aside for 10 and 60 minutes at 4°C.
- Aliquots were centrifuged and the plasma testosterone was analyzed by the LCMSMS assay.
- We compared the levels of testosterone.

Experiment #4: Comparison of NaF-Na_2EDTA and NaF-$K_2C_2O_4$

Donors	Testosterone Concentration (pg/mL)					
	NaF-Na_2EDTA			NaF-$K_2C_2O_4$		
	10 min 4°C	60 min 4°C	% Change	10 min 4°C	60 min 4°C	% Change
1	3052	3504	**14.8**	3830	3823	**-0.2**
2	3321	3560	**7.2**	3792	3755	**-1.0**
3	5141	5026	**-2.2**	5378	5298	**-1.5**
4	5571	5534	**-0.7**	5965	5403	**-9.4**
5	5608	5578	**-0.5**	5566	6045	**8.6**
6	5266	5591	**6.2**	6082	6099	**0.3**
7	5621	5107	**-9.2**	6645	5261	**-20.8**
8	2837	3134	**10.5**	3746	3437	**-8.2**

- No degradation of TU is observed.
- No significant difference is observed between donors.

Method Validation

- Complete validation was done for the plasma containing NaF-Na_2EDTA in accordance with the most recent FDA and EMA validation guidelines.

	Testosterone	DHT
Matrix	NaF-Na_2EDTA Plasma	
Range (pg/mL)	100-30000	50-5000
Internal Standard	Testosterone-d_5	Dihydrotestosterone-d_3
Extraction	Liquid-Liquid	
Mobile Phase	Methanol/Water/Acetic Acid	
Column	ACE Excel-2 C18-PFP	
Masses	290.4→97.0 amu	291.3→255.4 amu
LCMSMS	API 5000	

Method Validation

- We tested all the usual parameters of a validation such as the accuracy and precision, the matrix effect and the different stability assessments.
- Stability tests were performed with QCs fortified with TU, DHTU, DHT and testosterone glucuronide.

	Testosterone		DHT	
Stabilities	% Bias		% Bias	
	Low QC (192 pg/mL)	High QC (4300 pg/mL)	Low QC (400 pg/mL)	High QC (23000 pg/mL)
Freeze/Thaw at -20°C (4 Cycles)	-14.6	-10.2	-5.8	-3.3
Freeze/Thaw at -80°C (4 Cycles)	-14.9	-10.7	-5.8	-3.4
Short-Term at RT for 23 hrs	-6.1	2.5	-2.5	0.2
Short-Term at 4°C for 21 hrs	-9.1	-0.5	-4.2	0.6
Long-Term at -20°C for 14 days	-8.7	-4.4	-1.9	-1.8
Long-Term at -80°C for 14 days	-8.1	-5.4	2.1	-3.1
Whole Blood on ice for 110 min	-6.1	0.3	-11.1	8.3
Post-Preparative at RT for 69 hrs	5.1	8.6	14.0	10.1

- Acceptance criteria: 67% total QCs must be within ± 15% of bias
- 50% QCs per level must be within ± 15% of bias
- Mean % Bias of QC samples ± 15%

Clinical Study

- Oral TU was given twice daily.
- Serum and NaF-Na_2EDTA plasma were collected and testosterone, DHT, TU and DHTU were quantified in plasma and serum.
- Comparison of serum and plasma concentrations was done. Pharmacokinetic parameters (Ln-transformed AUC_{0-24} and C_{max}) were assessed.
- For testosterone, results obtained from serum and plasma cannot be considered equivalent since the AUC_{0-24} and C_{max} values are out of the acceptance criteria for the ratio (serum/plasma) and 90% confidence interval (CI) since the ratio was 132.25% for the AUC_{0-24} and 135.48% for the C_{max}.
- For DHT, although the ratio (serum/plasma) and the 90% CI for the AUC_{0-24} are within the 80-125% acceptance criteria, serum and plasma cannot be considered equivalent due to significant difference between C_{max} values (132.42%).
- For TU and DHTU, serum and plasma are considered equivalent in terms of pharmacokinetic analysis.

Conclusions

- The conversion of TU to testosterone is extensive and continues over time in whole blood when no additives are present. We demonstrated that DHTU is also hydrolysed into DHT.
- The temperature of sample handling influences the hydrolysis, with conversion being more extensive at room temperature.
- The addition of esterase inhibitors is important to prevent the hydrolysis of the undecanoate moiety.
- The method was successfully validated in human NaF-Na_2EDTA plasma.
- A clinical study demonstrates that the conversion of TU and DHTU when serum samples were collected was also observed in real human study samples.
- The instability of TU and DHTU observed in serum samples makes a bioanalytical method for testosterone based on plasma sample analysis more appropriate due to the inaccuracy of the results obtained in serum.

Acknowledgements

From SOV Therapeutics:

- Om Dhingra, Ph.D., Founder, Chairman of the Board, President and CEO
- James Bernstein, Ph.D., Chief Scientific Officer

From inVentiv Health Clinical:

- Sylvain Lachance, B.Sc., Bioanalytical Scientifc Expert
- Stéphanie Gagnon, B.Sc., Pharmacokineticist
- Caroline Savard, Research Scientist
- Nathalie Pelletier, Bioanalytical Projects Coordinator
- Nadine Boudreau, M.Sc., Bioanalytical Associate Director
- George Scott, Ph.D., Bioanalytical Vice-President

