Impact of an Enzymatic Inhibitor on Testosterone Levels in Human Plasma vs. Serum

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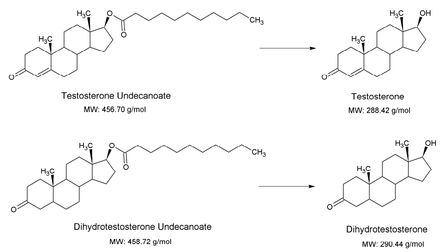
Introduction

Traditionally testosterone measurement is performed in serum. However, it has been demonstrated that when testosterone undecanoate is taken orally, testosterone must be determined in plasma containing an enzyme inhibitor to prevent the hydrolysis of testosterone undecanoate. A positive difference of up to 20% was observed between the endogenous levels of testosterone in serum compared to plasma containing anticoagulant involving enzyme inhibitor. The purpose of this work was to evaluate different anticoagulants for the measurement of testosterone. Please note that dihydrotestosterone follows the same trend but results will not be presented in this work.



Method

Testosterone and DHT are isolated from the biological matrix by an automated liquid/liquid extraction with MTBE. Chromatography is achieved using an ACE Excel 2 C18-PFP column and detection is performed on an API5000. Different collection tubes were tested: Serum, Na_2 EDTA-NaF, NaF-$K_2C_2O_4$, EDTA K_2, P800 (enzymatic inhibitor cocktail). Different proportions of NaF were also tried. Fresh whole blood from men donors was gathered to determine serum or plasma testosterone levels. Testosterone was determined in each plasma sample and compared to the testosterone level in serum from the same donor. Different donors were also tested.

Extraction Procedure

• Internal Standard:	Testosterone-d_5 Dihydrotestosterone-d_3
• Sample Volume:	300 µL
• Extraction Type:	Liquid-liquid extraction with MTBE

LC-MS/MS Analysis

Chromatographic mode:	Reversed Phase
Analytical Column:	ACE Excel 2 C18-PFP
Column Temperature:	Room Temperature
Elution mode:	Isocratic with column flush
Mobile Phase A:	Milli-Q Type Water/Acetonitrile (36/64), Ammonium formate 5mM, Formic Acid 0.1%
Flow Rate:	1.0 mL/minute
Retention Times:	2.37 minutes for T and 3.77 for DHT
Acquisition time:	4.5 minutes
Detector:	AB Sciex API 5000
Source:	TurboIonSpray, Positive mode

Results

Testosterone was validated over the dynamic range of 60-12000 pg/mL in both human NaF/$K_2C_2O_4$ plasma and serum. When different collection tubes were compared, it was observed that NaF introduces a bias of -15 to -18% on testosterone endogenous level compared to their corresponding serum tubes **(Table 1)**. When the percentage of NaF added was increased, the bias increased accordingly. EDTA K_2 and P800 showed no difference vs. serum tubes. Different donors were also evaluated and the bias was similar between each donor. EDTA-NaF collection tubes showed the lowest bias (-8.5%), due to their lower concentration of NaF (0.15%).

Table 1. Endogenous Level Difference between Serum and other Tubes

Collection Tubes	Testosterone Endogenous Level (pg/mL)	% Difference vs. Serum
Normal Serum	2872.1	
Rapid Serum Tube (RST)	2825.1	-1.64
NaF 0.43%	2477.7	-13.73
EDTA K_2	2852.9	-0.67
EDTA-NaF (0.15%)	2627.7	-8.51
NaF (0.25%)-$K_2C_2O_4$	2478.9	-13.69
NaF (1%)-$K_2C_2O_4$	2337.8	-18.60
BD™ P800	2832.9	-1.36

Table 2. Testosterone Level in Different Donors and Tubes

Matrix Lot	Serum	EDTA-NaF	% Diff. Vs. Serum	NaF-$K_2C_2O_4$	% Diff. Vs. Serum
1	3016.2	2736.3	-9.28	2542.2	-15.72
2	3092.3	2731.8	-11.66	2800.6	-9.43
3	4797.0	4241.6	-11.58	3997.1	-16.68
4	5553.9	4851.5	-12.65	4572.0	-17.68
5	5111.3	4674.5	-8.55	5076.8	-0.67
6	5761.3	4875.5	-15.37	4838.1	-16.02
7	4902.2	4322.0	-11.84	4212.0	-14.08
8	2638.0	2303.0	-12.70	2112.6	-19.92
		Mean	-11.70		-13.78

Table 3. Testosterone Concentration in Whole Blood

Tubes	T conc. (pg/mL)
EDTA	188.54
NaF/$K_2C_2O_4$	180.63
NaF-EDTA	182.68
P800	187.97

In order to determine if the bias due to NaF is related to ionic suppression or degradation in presence of NaF, freshly collected whole blood in different tubes were analyzed using the validated plasma methods. Testosterone concentrations in whole blood were compared for each tube and the results are tabulated in **Table 3**. As testosterone concentration in whole blood is similar between each tube, it suggests that NaF doesn't bring ionic suppression to the LC-MS/MS detection. It also suggests that probably the partition between erythrocytes and plasma is slightly different when using tubes with or without NaF in the anticoagulant.

The effect of NaF to the recovery or the ionization of testosterone was also studied. Quality controls were prepared at low and high testosterone concentrations in stripped plasma. One set of QCs was analyzed as is. Another set was fortified with NaF/potassium oxalate in excess. Both sets of QCs were compared. No difference was observed, showing that NaF doesn't affect the testosterone recovery or ionization **(Table 4)**.

Table 4. Comparison of Free NaF and Spiked NaF Quality Controls

Quality Controls	Testosterone Concentration (pg/mL)	
	Free NaF	NaF Spiked
Low QC (100 pg/mL)	102.7	101.0
High QC (10000 pg/mL)	10191.9	10198.2

Results

Table 5. Matrix Effect in NaF/EDTA Plasma at Low QC Level

Untreated Standard (MFQC1)				Reference Solution (RSQC1)		Calculated Matrix Factor (Analyte)	Calculated Matrix Factor (IS)	IS-Normalized Matrix Factor
Analyte Responses	Expected Concentration	Corrected Area	Internal Standard Responses	Analyte Responses	Internal Standard Responses			
117929	380	93102	1813107	106474	2043534	0.7906	0.7939	0.9958
157503	521	90693	1835622	122393	2381442	0.7702	0.8038	0.9582
133258	446	89635	1890277	121541	2369901	0.7612	0.8277	0.9196
149075	488	91644	1868682	120451	2320782	0.7782	0.8183	0.9511
745346	2673	83653	1748737	117805	2306624	0.7104	0.7658	0.9277
163849	594	82752	1703730	117877	2279679	0.7027	0.7461	0.9419
			Mean	117756.8	2283660.3			0.9490
			SD(±)					0.0270
			CV(%)					2.85

Table 6. Matrix Effect in NaF/EDTA Plasma at High QC Level

Untreated Standard (MFULOQ)				Reference Solution (RSULOQ)		Calculated Matrix Factor (Analyte)	Calculated Matrix Factor (IS)	IS-Normalized Matrix Factor
Analyte Responses	Expected Concentration	Corrected Area	Internal Standard Responses	Analyte Responses	Internal Standard Responses			
7552336	30080	7532250	1495907	9479991	1937007	0.7919	0.7695	1.0292
8101545	30221	8042300	1607232	9567454	1958527	0.8456	0.8267	1.0228
8315182	30146	8274911	1662193	9568004	1937459	0.8700	0.8550	1.0176
8284413	30188	8232821	1669784	9575087	1952321	0.8656	0.8589	1.0078
8319060	32373	7709258	1584156	9507405	1941177	0.8106	0.8149	0.9947
7851745	30294	7775545	1545014	9368296	1938006	0.8175	0.7947	1.0287
			Mean	9511039.5	1944082.8			1.0168
			SD(±)					0.0134
			CV(%)					1.32

Conclusion

The experiments tend to show that NaF causes a negative bias to testosterone concentrations when compared to serum samples. It was demonstrated that this phenomenon is not related to recovery variation or ionic suppression due to the presence of NaF. It is suggested that this bias was due to the different partition to RBC in plasma when NaF is used, compared to serum. However, as the prodrug testosterone undecanoate easily hydrolysed into testosterone when whole blood is collected without anticoagulant, it is suggested to analyze testosterone in human NaF/EDTA plasma instead of serum. NaF/potassium oxalate is also suitable for the analysis of testosterone when the prodrug testosterone undecanoate is taken orally, however the bias vs. serum is slightly higher than NaF/EDTA plasma.

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