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Free testosterone by LC/MSMS - Comparison of ultrafiltration and equilibrium dialysis

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Introduction

Circulating testosterone (T) exists in blood as free (FT) and protein-bound forms. Since FT is considered to be more reflective of the physiological actions of the hormone than total T, the measurement of FT is a better parameter to evaluate the androgen status and bioavailability.
Equilibrium dialysis (ED) is the gold standard method for separation of FT. It is a time-dependent equilibrium process, that depends on various parameters, including temperature and pH.
The objective of the project was to compare equilibrium dialysis and ultrafiltration as sample prep methods to separate FT and T. A practical LC/MSMS method needed to be validated, with an LLOQ of 1.0 pg/mL.

Analytical methods

- As T is an endogenous compound, calibration and QC samples were prepared in PBS. In each run, a male urine sample was included in duplicate as matrix control sample.
- Equilibrium dialysis was performed using the Rapid Equilibrium Dialysis device (Thermo Scientific). A volume of 0.5 mL plasma buffered with Hepes was transferred into the sample chamber and 0.75 mL of PBS dialysis buffer was transferred into the buffer chamber. Incubation was performed at 37°C for 4 hr. by placing the unit on an orbital shaker at ca. 150 rpm
- Ultrafiltration was done using Amicon Ultra 4 (Millipore) filters. Plasma was buffered with Hepes, homogenized and equilibrated for 1 hour at 37°C. Plasma ultrafiltrate was obtained by centrifugation of the mixture at 4000 rpm at 37°C for 1 hr.
- 2 mL of the ultrafiltrate samples, dialysis samples, calibration samples or QC samples was transferred into a clean test tube and internal standard was added.
- 8 mL Methyl-Tertiary-Butyl-Ether (MTBE) was added, tubes were capped and shaken for 10 minutes and then centrifuged for 5 minutes at 2000 rcf.
- The water layer was snap frozen and the organic phase was transferred into a clean tube. Subsequently, the organic solvent was evaporated to dryness.
- The residue was reconstituted with 100 µL reconstitution solvent and 50 µL was injected into the LC-MS/MS.

Fig. Rapid Equilibrium Device (Thermo Scientific)

Fig. Amicon Ultra-4 Centrifugal Filter Device (Millipore)

Analytical conditions

Analytical system	:	Applied Biosystems / MDS SCIEX API-4000 triple quadrupole mass spectrometer with Analyst software
Mode	:	Positive MRM
Interface	:	Ion spray
HPLC-System	:	Shimadzu Co-Sense system
HPLC column:		Hypersil GOLD (Thermo Scientific), 50 x 2.1 mm, 3 µm particles
Mobile phase A	:	0.1 % Acetic acid in methanol
Mobile phase B	:	Water
Flush liquid C	:	Methanol
Transitions (M/z)	:	Testosterone 289 / 109 D3-Testosterone 292 / 109

Results

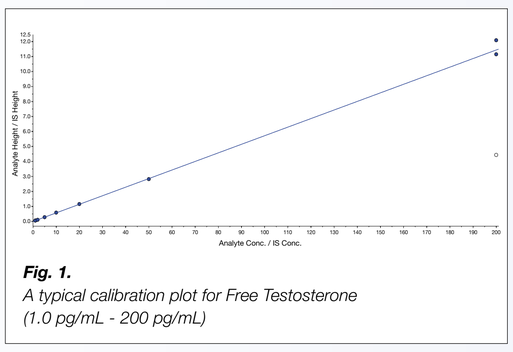


Fig. 1.
A typical calibration plot for Free Testosterone (1.0 pg/mL - 200 pg/mL)

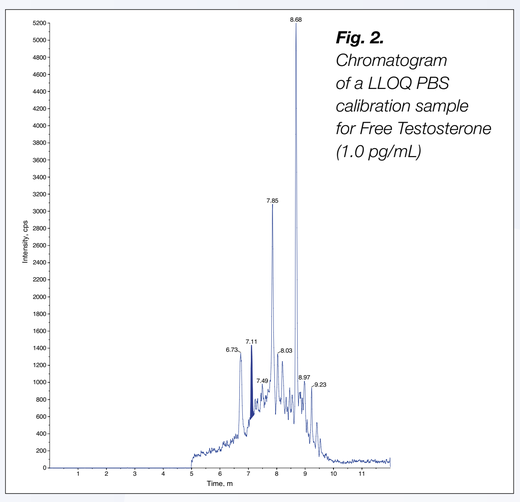


Fig. 2.
Chromatogram of a LLOQ PBS calibration sample for Free Testosterone (1.0 pg/mL)

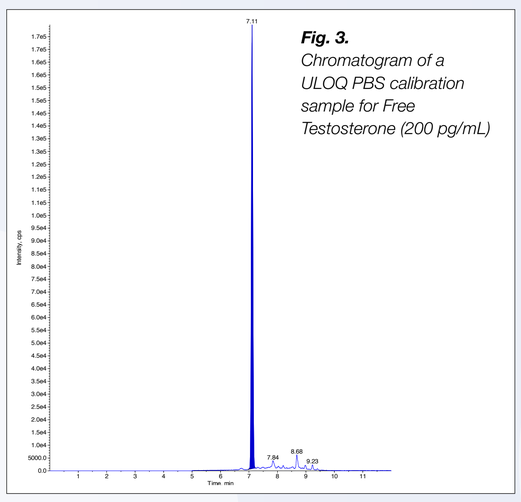


Fig. 3.
Chromatogram of a ULOQ PBS calibration sample for Free Testosterone (200 pg/mL)

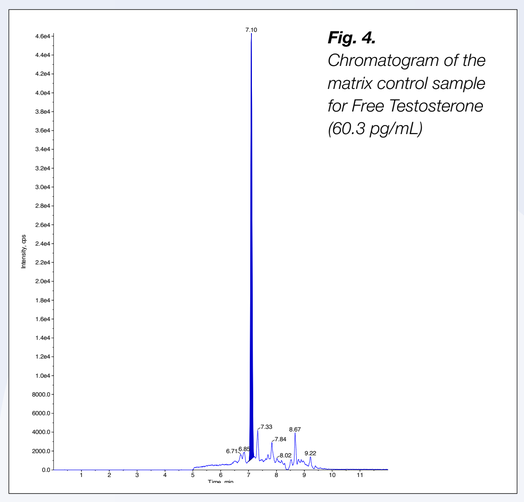


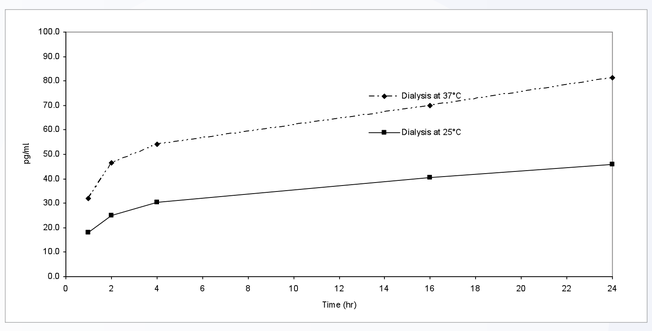
Fig. 4.
Chromatogram of the matrix control sample for Free Testosterone (60.3 pg/mL)

Name	Nominal (pg/mL)	Mean result (pg/mL)	Intra-assay precision (%)	Inter-assay precision (%)	Overall assay precision (%)	Overall accuracy (%)	n
QCL	3.00	3.20	5.0	8.7	8.8	107	18
QCM	20.0	21.7	4.0	4.2	5.5	109	24
QCH	160	176	2.8	2.6	3.6	110	24
MC	NA	60.3	5.2	12.6	13.0	NA	16

Table 1. *Accuracy and precision data for Free Testosterone in PBS QC and Matrix Control (MC) samples*

Equilibrium Dialysis (ED)

The concentration of Free Testosterone in the matrix control sample was determined using ED at 25°C and 37°C after 1, 2, 4, 16 and 24 hours of dialysis.

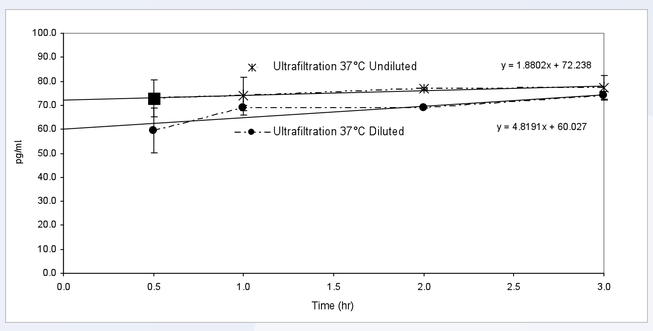


Time interval (hr)	1 hr	2 hr	4 hr	16 hr	24 hr
Mean at 25 °C (pg/mL)	17.9	25.1	30.6	40.4	45.8
CV at 25°C (%)	7.8	1.8	9.5	1.5	0.8
Change per hour at 25°C (pg/mL/hr)	-	7.2	2.6	0.8	0.7
Change per hour at 25°C (%)	-	40.3	10.3	2.8	1.7
Mean at 37°C (pg/mL)	31.9	46.4	54.1	69.8	81.3
CV at 37°C (%)	4.4	0.9	1.5	5.5	3.1
Change per hour at 37°C (pg/mL/hr)	-	14.5	3.9	1.3	1.4
Change per hour at 37°C(%)	-	45.5	8.3	2.4	2.1
Ratio 25°C/37°C (%)	**56.2**	**54.2**	**56.0**	**57.8**	**56.3**

Change per hour (pg/mL/hr) = concentration t_x (pg/mL) – concentration t_n (pg/mL) / n (hr) – x (hr)
*Change per hour (%) = Change per hour (pg/mL/hr) *100 / concentration t_x (pg/mL)*

Ultrafiltration (UF)

The concentration of Free Testosterone in the matrix control sample was determined using UF at 25°C and 37°C after 0.5, 1, 2 and 3 hours of equilibration using undiluted samples and plasma samples diluted with Hepes buffer.



Time interval (hr)	0.5 hr	1 hr	2 hr	3 hr
Mean at 25 °C undiluted (pg/mL)	-	-	34.2	-
CV at 25°C undiluted (%)	-	-	8.5	-
Mean at 37°C undiluted (pg/mL)	72.9	73.9	77.0	77.3
CV at 37°C undiluted (%)	7.6	7.8	0.4	5.0
Mean at 37°C diluted (pg/mL)	59.5	68.9	68.9	74.1
CV at 37°C diluted (%)	9.3	1.1	0.1	1.6
Ratio diluted / undiluted at 37°C (%)	**81.6**	**93.2**	**89.5**	**95.9**

Conclusion

ED and UF methods were validated for the determination of FT in human plasma with acceptable accuracy and precision, with an LLOQ of 1 pg/mL. It was shown that FT can be reliably measured following filtration of buffered plasma, equilibrated at 37°C for 1hr, through pasivated Amicon Ultra 4 (Millipore) filters at 37°C for 1hr. The UF process can be performed under controlled conditions, is simpler and faster than ED, and was therefore the method of choice for the bioanalysis of FT in a Phase I study of a new formulation of testosterone.