Supporting Information


Delivering Enhanced Testosterone Replacement Therapy through Nanochannels

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1 Plasma Testosterone Analysis by LC-MS

Testosterone solution (2 mg ml\(^{-1}\)) for the preparation of standard curves was prepared in methanol and stored at -30 °C until needed. Deuterated testosterone (D3) dissolved in methanol (2 mg/ml) and diluted to a final concentration of 25 fmol D3 per 60 µl into 100% acetonitrile was used as the internal standard.

Fresh dilutions of the stock testosterone solution were prepared by serial dilutions in 60% methanol/40% water containing 0.1% ammonium acetate for standard curve generation. A minimum of 10 dilution calibration points (in duplicate) were measured each time. Linearity was typically greater than 5 log scales (with \(r^2 > 0.99\)), with a typical limit of detection [LOD] of 60 attomole testosterone injected on column (equivalent to ~1.7 pg ml\(^{-1}\) original plasma concentration detectable at 10 µl LC-injection volume), with a typical lower limit of quantification [LLOQ] of 100 attomole testosterone on column (equivalent to ~2.8 pg ml\(^{-1}\) original plasma concentration) defined with a signal-to-noise ratio >10.

Sample preparation: 20 µl of EDTA-treated plasma was mixed with 60 µl of the internal D3 standard, mixed by vortexing for 1 min, incubated for 15 min at room temperature followed by centrifugation at 14,000 x g for 10 min to separate precipitated proteins. Supernatant was separated and dried completely under vacuum using Speedvac. Dried residue was re-suspended in 40 µl of 60% methanol/39.9% water/0.1% ammonium acetate and 10 µl aliquots were injected (in duplicate) on column for testosterone analysis. A calibration curve was prepared using the same procedure each time during the sample analysis.

Liquid Chromatography (LC) Conditions: LC separation of samples was carried out on a Waters Acquity UPLC system (Waters Corp, Millford, MA) using an Acquity UPLC BEH C-18 column
(2.1mm X 50mm; 1.7 μm particle) and a matching guard column. Column was maintained at 35 °C for analysis. Solvent A was 99.9% water + 0.1% ammonium acetate; Solvent B was 99.9% methanol + 0.1% ammonium acetate. Column was initially equilibrated with 60% solvent B at 0.4 ml/min flow rate for sample injection and was maintained at 60% B for 1 min. LC gradient was changed from 60% to 75% solvent B over 30 s, then from 75% solvent B to 99% solvent B over 0.1 min and held at 99% solvent B for an additional 0.4 min. LC gradient was then switched to 60% solvent B at a flow of 0.6 ml min⁻¹ for 2 min to re-equilibrate column prior to the next injection. Total gradient time was 4 min with approximately a 5 min run-time for one sample injection to other. Testosterone and the internal standard (D3 testosterone) eluted at a retention time of 1.7 min under these conditions.

**Mass Spectrometry (MS) Conditions:** Mass spectrometry was carried out on a Waters Xevo TQ tandem quadrupole mass spectrometer operated in the SRM/MRM mode under positive ion electrospray conditions, with a source temperature of 150 °C, desolvation temperature of 500 °C, and desolvation nitrogen gas flow of 1000 L/h. MS and MS/MS conditions were optimized at capillary voltage of 0.5 kV, cone voltage 36 V, collision energy 22 V, and dwell times of 0.146 s for each transition. Molecular transitions routinely monitored for quantification of testosterone was 289 to 97 and 292 to 97 for D3 internal standard. Instrument control and data acquisition was accomplished using MassLynx v4.1 software (Waters Corp., Milford, MA) and sample quantification by TargetLynx (v4.1) software (Waters Corp. Milford, MA). Data was quantified relative to the external standard curve for the same molecular transition of testosterone and normalized to percent recovery for every sample relative to the D3 internal standard. Average amount of testosterone was reported from the duplicate assays. Typical CVs between replicates were < 5%.
2 Phase Solubility Studies

Phase solubility studies of testosterone were carried out using different concentrations of HPCD and a linear trend was observed for up to 1mM HPCD concentration (Fig. S-1). TES solubility at 25 and 37 °C was determined by extrapolation of the linear portion of the phase solubility trend to ‘0’ HPCD concentration and stability constants of testosterone inclusion complex at 25 and 37 °C was determined using the following relationship:

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\text{Stability Constant} = \frac{\text{slope}}{\text{y-intercept}} \times (1 - \text{slope})
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![Figure S-1. Phase solubility studies of testosterone](image)
Figure S-2. Degradable testosterone pellet exhibited biphasic release pattern resulting in very high plasma levels initially which decreased constantly with time.