

HIGH THROUGHPUT ANALYSIS OF PLASMA TESTOSTERONE USING LC-MS/MS

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Overview

A rapid LC-MS/MS method for the analysis of plasma testosterone is presented. The sensitivity of the method allows accurate measurement of testosterone in women. The assay has good precision (intra-assay CV<5%, inter-assay CV<8%) and the validity is demonstrated by the analysis of patient samples (n=14). Calibration is linear to 50 nmol/L.

Introduction

Testosterone is the main androgen secreted by the Leydig cells of the testes and its production increases during puberty. Women produce about 5 to 10% as much testosterone as do men. Hirsutism is defined as the excessive growth of terminal hair in women and children in a distribution similar to that occurring in postpubertal men.¹

Radioimmunoassay (RIA) is currently the most widely used technique for measuring the level of total circulating testosterone (both protein-bound and non-protein-bound forms).² Individual RIA methods differ mainly in the mode of sample preparation, in the quality of antisera used, and in the nature of the steroid-protein conjugate used to generate the antiserum. Gas chromatography combined with mass spectrometry (GC-MS) remains the reference method for testosterone measurement and is often used to assess the bias of routine immunoassay methods. A high performance liquid chromatography (HPLC) method that does not require derivatisation has also been described for testosterone.³

Almost all testosterone antisera show some degree of cross-reactivity with dihydrotestosterone (DHT) but show negligible cross-reactivity with other androgens. Assays that use antisera generated against the C-19 position give maximum analytical specificity with respect to endogenous steroids. However, cross-reactions with 19-norsteroids that are used in contraceptive preparations can cause problems with some analytical methods. In most clinical situations, estimation of testosterone without prior separation of DHT is permitted because plasma concentrations of DHT are only 10 to 20% of those for testosterone. Moreover, testosterone and DHT are the two most important androgens in the systemic circulation; even when a method measures the levels of both of them, clinically useful information about the total androgen load is obtained.

Materials and Methods

Patient samples for this study were generously supplied by Leeds General Infirmary, Leeds. Blood samples were collected into Vacutainer tubes and centrifuged. d₂-Testosterone (internal standard) was purchased from CDN Isotopes (Quebec, Canada).

Standards were prepared using spiked addition of testosterone into plasma (Sigma, Poole, UK). For comparative purposes, total testosterone concentrations were also measured using a radioimmunoassay.

Mass Spectrometry

A Micromass Quattro micro triple quadrupole mass spectrometer fitted with a **Z** SPRAY ion source was used for all analyses. In positive electrospray ionisation mode in the presence of ammonium acetate and formic acid, testosterone forms a strong protonated molecule (m/z 289, **Figure 1a**) that can be fragmented by CID to form a stable product ion (m/z 97, **Figure 1b**).



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Sample Preparation for LC-MS/MS

- Methanolic internal standard d2-testosterone 10 nmol/L (100 μL) was added to plasma or standard (50 μL)
- Vortex mix for 1 min. and centrifuge for 10 min.
- Inject 10 µL of the supernatant

Chromatography

Chromatography was performed using a Merck SpeedROD RP-18 column (5µm, 4.6mm x 50mm) eluted with 75% aqueous methanol containing 2mM ammonium acetate and 0.05% formic acid at a flow rate of 0.6 mL/min. Testosterone and d_2 testosterone were monitored in MRM mode using the transitions m/z 289>97 and m/z 291>99, respectively (**Figure 2**). The analysis time was less than 4 minutes, injection-to-injection.



Figure 2. LC-MS/MS analysis of plasma testosterone spiked standards (A) 0 nmol/L, (B) 50 nmol/L. Upper traces are for d₂-testosterone internal standard and lower traces are for testosterone.

Results

Plasma was spiked with testosterone up to 50nmol/L to generate standards that span the same range as current RIA methods. Standards were analysed in duplicate and processed using QuanLynx v4.0 to generate a linear calibration curve (**Figure 3**). The y-intercept of the regression line represents the endogenous concentration of the plasma testosterone. The corrected standard curve is shown in **Figure 4**.



Figure 3. Calibration curve for LC-MS/MS analysis of testosterone. Values are from duplicate measurements

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Figure 4. Corrected calibration curve for LC-MS/MS analysis of testosterone

The intra- and inter-assay precision was assessed using two patient samples and shown to have good reproducibility (**Table 1**).

Plasma Testosterone Concentration (nmol/L)				
	Intra-assay (n=10)		Inter-assay (n=5)	
	$Mean \pm SD$	%CV	$Mean \pm SD$	%CV
Low	6.9 ± 0.32	4.6	6.5 ± 0.84	8.3
High	34.9 ± 0.64	1.8	35.4 ± 1.4	3.9

Table 1. Analytical precision of the LC-MS/MS assay for plasma testosterone. The coefficient of variation (CV) was calculated as SD/mean for the analysis of n replicate extractions of low and high, plasma QC samples.

To assess the overall performance of the assay as a routine method in a clinical laboratory, plasma samples were analysed by the LC-MS/MS method and compared with the existing RIA assay. A positive correlation was observed between the LC-MS/MS method and the RIA (**Figure 5**).



Figure 5. Linear regression analysis of the plasma testosterone concentrations determined by LC-MS/MS and RIA in 14 patient samples

Conclusion

- We have developed a rapid LC-MS/MS procedure for the analysis of plasma testosterone and present preliminary validation data.
- The procedure is simple and requires a small sample volume. This makes it a suitable method that can be incorporated into a routine clinical laboratory for high-throughput analysis.
- The method is precise and comparable to current RIA methods.
- Method developments in tandem mass spectrometry are providing a flexible and costeffective solutions to many problems encountered in the modern laboratory.

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