

Determination of testosterone in serum by automated sample preparation and ultra-fast LDTD-MS/MS in a cross validation study with real patient samples

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Overview

Testosterone is primarily secreted by Leydig cells in men and theca cells in women. It is the principal male sex hormone and an anabolic steroid. The measurement of serum testosterone is useful in the investigation of suspected disorders of excessive and insufficient androgen production. To achieve more specificity and accurate results, laboratories are shifting from Immunoassays to tandem mass spectrometry technology coupled to Liquid chromatography (LC) or Laser Diode Thermal Desorption (LDTD) system as a sample introduction system.

Prior to any analysis, a sample clean-up step is recommended to remove matrix components. A new automated sample preparation workflow using the Tecan® AC Extraction Plate™ is evaluated, in combination with high-throughput LDTD-MS/MS technology for sample quantification. Cross validation is performed using real patient samples.

LDTD Ionization Source

The LDTD uses a laser diode to produce and control heat on the sample support which is a 96-well plate. The energy is then transferred through the sample holder to the dry sample which vaporizes prior to being carried by a gas in an APCI region. High efficiency protonation with strong resistance to ionic suppression characterizes the ionization due to the absence of solvent and mobile phase. The LDTD provides high throughput capabilities of 6 seconds sample-to-sample analysis time, without any carry over.

AC Extraction Plate (Principle and Process)

The AC Extraction Plate is an ingenious novel consumable for sample preparation, consisting of a 96-deep well plate with wells that are coated with a proprietary material. The coating has a particularly high affinity for small apolar molecules, such as Testosterone. Sample preparation with the AC Extraction Plate is a convenient three-step process, which can be easily done manually or with an automated Liquid Handling System, such as the Tecan Freedom EVO®. In a

first step, the analytes are extracted out of an aqueous sample solution, while leaving the polar analytes and macromolecules in solution. Subsequently, the supernatant is removed, followed by a wash step to remove matrix constituents, such as phospholipids and proteins, from the wells. In a final step, the analytes are eluted from the coating by a solvent (mixture), containing acetonitrile or methanol at higher concentrations.

Sample Preparation

Extraction Plate

AC Extraction Plate (TECAN)

Extraction Procedure

- 200 µL Extraction mixture:
 - 0.92 mL NaCl (2M in water)
 - 3.18 mL Ammonium acetate (1M in water)
 - 4 mL Acetonitrile
 - 0.2 mL Testosterone-d₃ (50 ng/mL in acetonitrile)
 - 11.7 mL water
- 100 µL Sample / Cal / QC / Blank
 - Orbital shaking (1200 rpm / 10 minutes)
 - Remove loading mixture
- Add 100 µL Washing solution
 - 0.2% Formic acid in water
 - Orbital shaking (1200 rpm / 2 minutes)
 - Remove washing solution
- Add 200 µL Acetonitrile
 - Orbital shaking (1200 rpm / 5 minutes)
 - Remove washing solution
- Evaporate to dryness
- Reconstitute with 20 µL Acetonitrile
- Vortex 30 sec
- Transfer 5 µL in LazWell™ plate
- Analyze after complete solvent evaporation

Instrument settings

Mass spectrometer AB SCIEX QTrap® 5500 SelexION™ System operated in APCI positive mode.

MRM transitions used 3 µA corona discharge current and 80 msec dwell time.

Differential Mobility Spectrometry (DMS) used 4300 SV (Separation voltage) and 14 COV (Compensation voltage). Following transitions were used:

Compound	Q1	Q3	CE
Testosterone	289	97	25
Testosterone-d3	292	97	25

LDTD model S-960 operated with a gas flow rate of 3 L/min and a laser pattern ramp from 0 to 55% in 6 seconds.

Methodology

The standard curve calibration is made in charcoal stripped serum. A calibration curve ranging from 0.1 to 10 ng/mL is used. Low, medium and high QC levels are prepared. These QCs are used for intra-run and inter-run accuracy and precision measurements. The carry over is evaluated by the analysis of three blanks after the highest standard. The blank peak areas were evaluated against the mean peak area of the lower standard to determinate the interference percentage. Finally, real clinical samples were analyzed using LTD-MS/MS method and the results were compared to LC-MS/MS analysis.

Results

Linearity from a calibration curve is expressed by the correlation coefficient R presented in the following figure. All duplicate curves had a coefficient ≥ 0.997 or better.

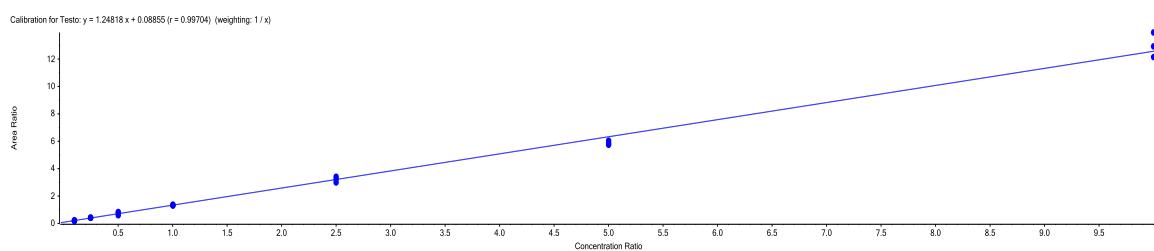


Figure 1: Calibration curve for the testosterone analysis

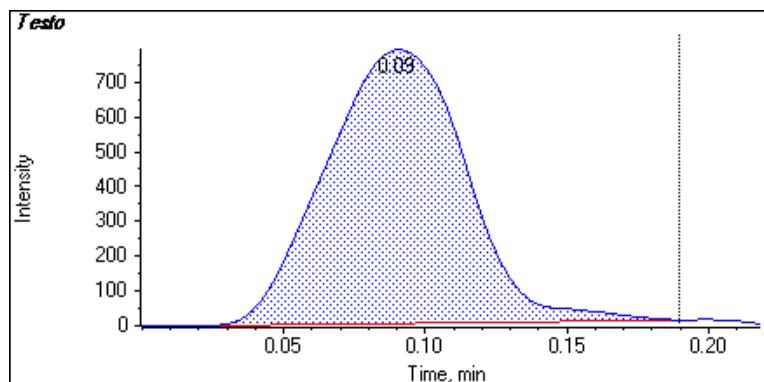


Figure 2: Typical desorption peak for testosterone analysis

All QC's are within the acceptance criteria ($\leq 15\%$) as required in the method validation for intra-run and inter-run accuracy and precision.

Statistics on cross validation with the clinical laboratory show that both methods agree, with a correlation value of R^2 : 0.9832. **Figure 3** below shows the cross validation results.

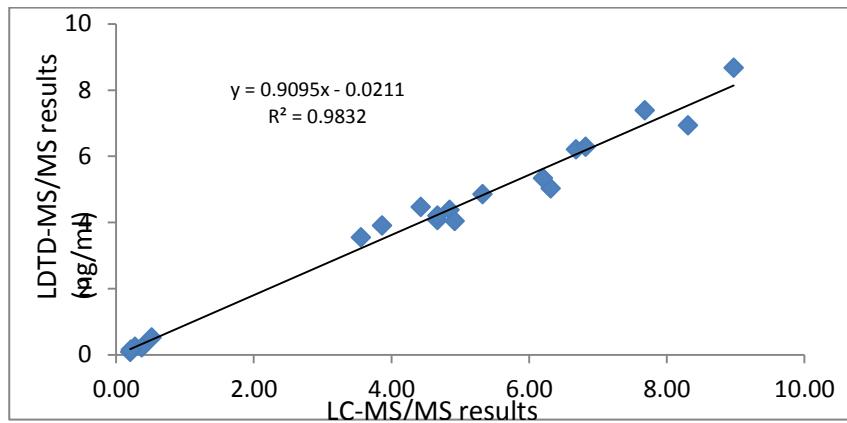


Figure 2: Cross validation results for testosterone in serum samples

Conclusion:

Method Validation of Testosterone using AC Extraction Plate combined with subsequent analysis by LDTD-MS/MS at 9 seconds per sample was achieved. Comparison by statistical analysis of this method against a reference clinical method demonstrates the ability to generate effective quantitation data at ultra-high-throughput speed.

Outlook:

Further experiments to optimize the extraction procedure and to increase the sensitivity of the assay are ongoing.