Tecan AC Extraction Plate™

Automated sample preparation for the determination of testosterone in serum by LC-MSMS

Introduction

Testosterone is a powerful androgen that is primarily secreted by Leydig cells in men and thecal cells in women. It plays a critical role in spermatogenesis and the development and maintenance of the genitalia and secondary sex characteristics in men, as well as the control of libido in both genders. The measurement of serum testosterone is useful in the investigation of suspected disorders of excessive and insufficient androgen production in adults, precocious and delayed puberty in children, and ambiguous genitalia in neonates [1-4]. For accurate, reproducible quantification of testosterone, a growing number of laboratories is shifting from immunoassay to liquid chromatography coupled with tandem mass spectrometry (LC-MSMS). The advantage of the latter is its unparalleled specificity, combined with high sensitivity and the potential for multi-analyte determination.

Prior to any analysis by LC-MSMS, a sample clean-up step is recommended to remove matrix components such as proteins, lipids, carbohydrates and salts. This application note describes a straightforward approach for the preparation of serum samples for subsequent LC-MS analysis of testosterone, focusing on a streamlined workflow suitable for automation. Compared to most conventional sample preparation methods, this new approach has fewer processing steps and is easier to automate, as well as having the advantage that the final eluate can be injected directly into an LC-MSMS system, enabling quantitative analysis without the need for reconstitution.

The extraction workflow presented here consists of simple liquid handling steps that yield highly reproducible results, and can be easily automated on a liquid handling platform. Furthermore, the AC Extraction Plate can process serum samples in a wide variety of conditions – cloudy (eg. lipophilic), white (due to high protein content), or partly red (hemophilic).

The AC Extraction Plate

The centerpiece of the new sample preparation method is the AC Extraction Plate with TICE™ (Tecan Immobilized Coating Extraction) technology. It is a 96-position, deep-well plate, with the inner surface of each well coated with a specific volume of sorptive material (phase) to approximately half the well's height. This proprietary coating acts as an extraction phase for small non-polar molecules, absorbing analytes such as testosterone from aqueous environments with high affinity, while proteins, phospholipids, carbohydrates and salts remain in solution. After rinsing the well with a wash solution to remove the matrix residues, the analyte is eluted from the coating with an elution solvent mixture containing an appropriate percentage of organic solvent. This eluate can be used directly for LC-MSMS analysis. Procedural variations are mitigated by the addition of an internal standard – in this example $^{13}C_3$-testosterone – at the beginning of the sample preparation process.

The workflow

An extraction mix containing the internal standard is prepared and transferred into a well of the AC Extraction Plate, and an aliquot of the sample (eg. serum or plasma) is added. After horizontal mixing on a shaker, the supernatant is removed, leaving the analyte(s) of interest retained in the TICE coating. A wash solution is added and the AC Extraction Plate is shaken again. The wash solution is discarded and an elution solvent added, shaking the plate again to elute the analyte from the TICE coating. The eluate is then transferred to either an LC vial or an uncoated 96-well plate, and loaded into an LC autosampler for injection. The process is depicted schematically in Figure 1.
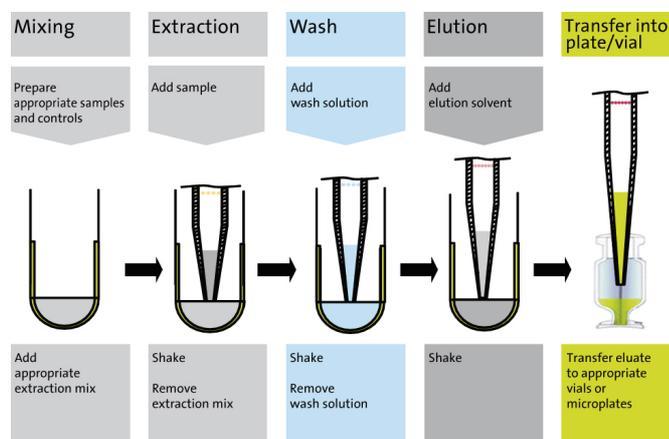

Figure 1 Schematic representation of the AC Extraction Plate workflow for the extraction of testosterone from serum or plasma.

Materials and methods

Instruments

- UPLC-MSMS system: Waters ACQUITY UPLC® system coupled with a Waters XEVO® TQ-S tandem quadrupole MS system with an electrospray ionization source.
- Tecan Freedom EVO® liquid handling system with integrated Te-Shake™ shaker (Tecan) for microplates, or a manual pipette and standalone plate shaker.

Solvents used for sample preparation

- Internal standard (IS) working solution: 1 ng/ml $^{13}C_3$-testosterone in acetonitrile:water (80:20, v/v)
- Modifier buffer: 0.33 M lithium chloride, 0.33 M ammonium formate, formic acid 0.5 % in water
- Extraction mix: modifier buffer and IS solution (2:1, v/v)
- Wash solution: 0.2 % formic acid in water
- Elution solvent: water/acetonitrile (35:65, v/v)

All solvents used were LC-MS Chromasolv® grade (Sigma Aldrich, USA). The internal standard, $^{13}C_3$-testosterone, was obtained from Cerilliant (USA).

Serum samples

Six calibrators and three quality control samples containing defined concentrations of testosterone in serum (Biocrates, Austria) were extracted. The concentration of the quality control samples covered the relevant physiological range (Table 1).

Samples (serum matrix)	Testosterone concentration (ng/ml)
Calibrator level 2	0.04
Calibrator level 3	0.2
Calibrator level 4	0.8
Calibrator level 5	3.0
Calibrator level 6	6.0
Calibrator level 7	10
QC level 1	0.339
QC level 2	1.19
QC level 3	7.26

Table 1 Concentration of testosterone in calibrators (Cal) and quality controls (QC).

Sample preparation and analysis

Calibrators, controls and samples were treated either manually, using single or eight-channel pipettes, or automated on the Freedom EVO liquid handling system, as outlined in Table 2.

Step	Procedure
1	150 µl of extraction mix was added to each well of the AC Extraction Plate
2	50 µl of serum calibrator, quality control sample or patient sample was added
3	The AC Extraction Plate was shaken horizontally for 10 min at 1,200 rpm*
4	Extraction mix was removed and 200 µl of wash solution added
5	The AC Extraction Plate was shaken horizontally for 2 min at 1,200 rpm*
6	Wash solution was removed and 200 µl of the elution solvent was added
7	The AC Extraction Plate was shaken horizontally for 5 min at 1,200 rpm*
8	The resulting eluates were transferred from the AC Extraction Plate to a non-coated microplate with a pierceable cover to minimize evaporation of solvent, then placed in a cooled autosampler (10 °C) for analysis by LC-MSMS

* Tested with the Te-Shake (optimum rpm settings may vary with the type of shaker)
Table 2 Detailed AC Extraction Plate sample preparation workflow for the extraction of testosterone for subsequent analysis by LC-MSMS.

LC-MS parameters

LC

Column and solvents:	BIOCRATES SteroIDQ® kit
Initial flow rate:	0.6 ml/min
Column:	ACQUITY UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm, Waters)
Column temperature:	45 °C
Injection volume:	10 µl

Gradient:

Time (min)	Flow rate (ml/min)	% Solvent A	% Solvent B
0	0.6	90	10
0.5	0.6	90	10
4	0.6	0	100
4.01	0.6	0	100
4.1	0.8	0	100
5.5	0.8	0	100
5.6	0.8	90	10
5.61	0.8	90	10
6	0.6	90	10
6.5	0.6	90	10

Total LC cycle time: 6.5 min

MSMS parameters

Ionization	ESI positive
Capillary voltage (kV)	4.0
Cone voltage (V)	50.0
Source offset (V)	50.0
Source temperature (°C)	150
Desolvation temperature (°C)	650
Cone gas flow (l/hr)	150
Desolvation gas flow (l/hr)	900
Collision gas flow (ml/min)	0.2
Nebuliser gas flow (bar)	7.0

Mass transitions

Testosterone (quantifier ion)	289 > 97
Testosterone (qualifier ion)	289 > 109
$^{13}C_3$-testosterone	292 > 100

Performance parameters

All experimental data was obtained using the AC Extraction Plate with the chemicals and workflow described earlier.

1. Linearity

Linear regression analysis for testosterone over six calibrator levels ranging from 0.04 to 10 ng/ml showed good linearity, with a coefficient of determination (r^2) >0.998 (Figure 2).

Compound name: Testosterone 289>97
Correlation coefficient: r = 0.999017, r^2 = 0.998035
Calibration curve: 0.908357 * x + 0.0910581
Response type: Internal Std (Ref 6), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None
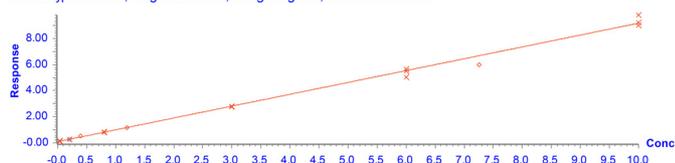
Figure 2 Calibration curve for replicate (n = 3) testosterone determinations over six calibrator levels (crosses). The calculated concentration of the three QC samples (n = 1) are represented by circles.

2. Lower limit of quantitation (LLOQ)

The LLOQ for testosterone (CV <20 % and signal-to-noise (S/N) >10) was established as 0.04 ng/ml (Figure 3). The S/N value for the analyte peak was determined from the average of triplicate extractions of 50 µl calibrator matrix samples eluted in 200 µl of elution solvent, using an injection volume of 10 µl (Figure 3).
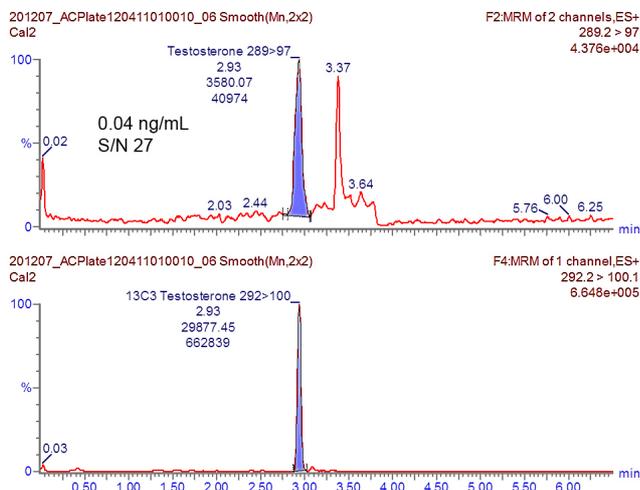

Figure 3 Chromatograms showing the testosterone analyte (top) and stable isotope-labeled internal standard (bottom) for the 40 pg/ml serum calibrator.

3. Reproducibility

Reproducibility of the extraction and analysis was determined by triplicate extractions of a set of individual female and male samples, as well as by replicate extraction (n = 5) of pools of male or female samples (Table 3).

Testosterone

	Target concentration (ng/ml)	Calculated concentration (ng/m)	Area (% CV)	IS Area (% CV)	Response (% CV)
Cal2	0.04	0.04	3.2	1.6	4.5
Cal3	0.20	0.20	2.2	1.1	2.7
Cal4	0.80	0.80	8.3	7.5	1.1
Cal5	3.0	3.0	1.0	1.0	1.2
Cal6	6.0	5.8	4.2	1.2	4.8
Cal7	10.0	10.2	4.4	7.2	3.6
W4	-	0.43	2.4	1.0	3.2
W7	-	0.20	4.6	5.6	10.4
W19	-	0.17	1.7	1.8	3.0
W18	-	0.06	1.2	4.8	4.0
W1	-	0.15	1.8	1.8	3.5
M1	-	2.26	4.0	4.1	6.7
M2	-	2.85	2.5	2.5	1.1
M3	-	0.79	2.7	2.3	4.9
M9	-	1.14	3.5	3.4	4.5
M5	-	3.54	2.3	0.8	1.7
W-Pool1	-	0.25	4.4	2.8	2.6
M-Pool1	-	6.69	2.5	5.1	5.0

Table 3 Reproducibility of calculated testosterone concentrations for calibrators, randomly selected female (W) and male (M) samples (n = 3), and pooled samples (n = 5).

4. Accuracy

Accuracy of testosterone concentrations was calculated as % bias of the target value, and ranged between -12 and +16 % for single extraction and analysis of QC samples, and from -2.6 to +2.4 % for triplicate extractions of calibrator samples.

5. Extraction efficiency and recovery

Extraction efficiency for the AC Extraction Plate method was determined by spiking samples with a standard solution at low and high testosterone concentrations, before and after extraction. Recovery was determined by comparing the internal standard in the extracted samples with the internal standard spiked into the elution solvent. Extraction efficiency and recovery for testosterone and $^{13}C_3$-testosterone were 53 and 55 % respectively.

6.   Matrix effects

A negligible ionization enhancement – 103 % – was observed for the extraction of testosterone from serum samples (values in excess of 100 % indicate ionization enhancement, and values of less than 100 % indicate ionization suppression [5]).

7.   Method comparison

The automated extraction protocol followed by UPLC-MSMS analysis was evaluated and compared to an established immunoassay method, using a set of human serum samples covering the clinically relevant concentration range of testosterone. In some cases, concentrations were below the 0.1 ng/ml lower limit of quantitation of the immunoassay. The comparison showed a correlation (r^2) of 0.993 (Figure 4).

Testosterone

$$y = 1.0434x + 0.0104$$
$$r^2 = 0.9931$$

Analyte concentration (ng/ml) **Immunoassay**

LC-MSMS
Analyte concentration (ng/ml)
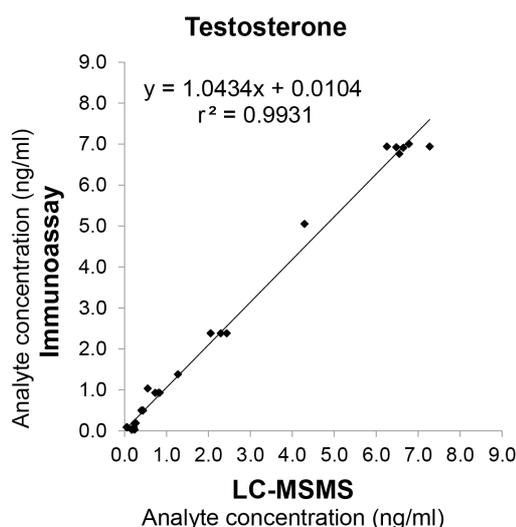
Figure 4 Comparison of the automated AC Extraction Plate method with UPLC-MSMS analysis with an established immunoassay (Elecsys Testosterone II with Cobas® e411, Roche).

Conclusion

In recent years, there has been a strong focus on automation of routine LC-MSMS analytical procedures, with particular emphasis on automated sample preparation [6]. The ideal automated solution would enable the prepared samples to flow directly into LC-MSMS without further human intervention. However, such fully automated LC-MSMS front ends have not yet found their way into routine analytical processes. By combining the AC Extraction Plate/UPLC-MSMS assay of testosterone with an automated liquid handling system such as the Freedom EVO, a completely automated workflow, from sample placement through to analysis, can be achieved.

The AC Extraction Plate offers a fast, robust and easy-to-automate analytical sample preparation method requiring minimal sample pretreatment. Time consuming, difficult to automate procedures such as protein precipitation, filtration or centrifugation are eliminated, accelerating and simplifying the entire sample preparation workflow. The extraction can be performed manually, or be easily automated on a liquid handling platform.

Automation of the AC Extraction Plate method on a Freedom EVO platform, combined with UPLC-MSMS analysis, enabled high throughput, quantitative determination of testosterone in serum with excellent assay performance. All testosterone concentrations were in the relevant physiological range. The AC Extraction Plate sample preparation procedure may also be performed manually, and is suitable for a variety of small molecule analyses.

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