A Novel Online Cleanup Valve Solution for Quantitative Analysis of Testosterone in Serum Utilizing LC-MS/MS

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Introduction

Determination of testosterone levels in serum is an important measurement in clinical research. Liquid chromatography triple quadrupole mass spectrometry (LC/MS/MS) is an essential tool in analytical laboratories due to its high sensitivity, specificity and excellent reproducibility. Multiple analytes can be simultaneously analyzed saving resources. This work describes a novel integrated valving and liquid metering system used for on-line sample cleanup coupled with MS/MS. An improved level of detection is possible, with minimal sample preparation, using a previously unreported configuration. This setup uses a single binary pump for analytical separation and a single online cleanup component. The online cleanup system presented here has a built-in single piston pump with solvent selection capability and user-selectable valves integrated in a single module. Using this module, a sensitive, robust and reliable analytical method was developed for the LC/MS/MS quantitation of testosterone in serum.

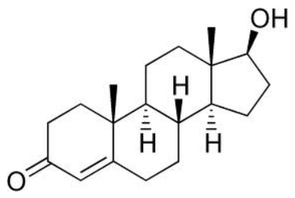

Figure 1. Structure of Testosterone

Sample Preparation

Combine 500 µL of serum with 500 µL of Acetonitrile containing 5.0 ng/mL Testosterone-d_3 internal standard, vortex for 15 second, incubate for 10 min in 5°C, centrifuge for 10 min at 5000 rpm, transfer 500 µL to injection vial containing 500µL of water, vortex and analyze by LC-MS.

HPLC Conditions

Column	Poroshell 120 EC-C18 2.1x50, 2.7 µm
SPE Cartridges	Zorbax EC-C18 2.1x12.5 mm, 5 µm
Injection Volume (µl)	40
Flow Rate (mL/min)	0.4
Mobile Phase A	Water + 5mM Ammonium Formate
Mobile Phase B	Methanol + 5mM Ammonium Formate

FlexCube Conditions

Valve Head	2-positions/10-port valve
Flow rate	1 mL/min solvent
Solvent A1	3:97 MeOH:Water
Solvent A2	1:1:1:1 ACN:IPA:MeOH:H2O

	Min.	Function	Parameter
1	0.00	Pump Vol	Pump 1mL, Flow: 0.5mL/min, Channel A: A1
2	2.00	Valve Change	Increase valve position
3	2.10	Pump Vol	Pump 1mL, Flow: 0.5mL/min, Channel A: A2
4	4.20	Pump Vol	Pump 1.5mL, Flow: 1mL/min, Channel A: A1

Mass Spec Conditions

Gas Temp (°C)	300
Gas Flow (L/min)	10
Nebulizer (psi)	45
Sheath Gas (°C)	350
Sheath Gas Flow (L/min)	11
Capillary (V)	4000

	Prec Ion	Prod Ion	Dwell	Frag (V)	CE (V)
Testosterone	289.2	109	85	135	28
Testosterone	289.2	97	85	135	24
ISTD	292.2	109	85	135	28

Results and Discussion

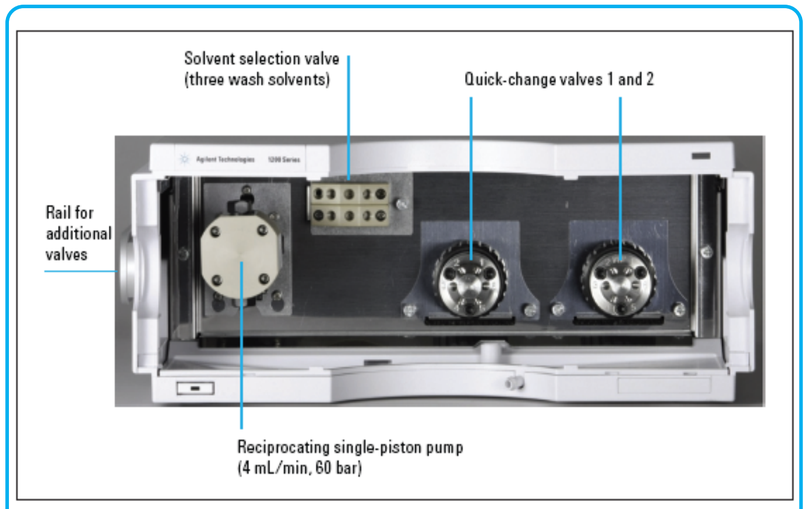

Figure 2. FlexCube Module

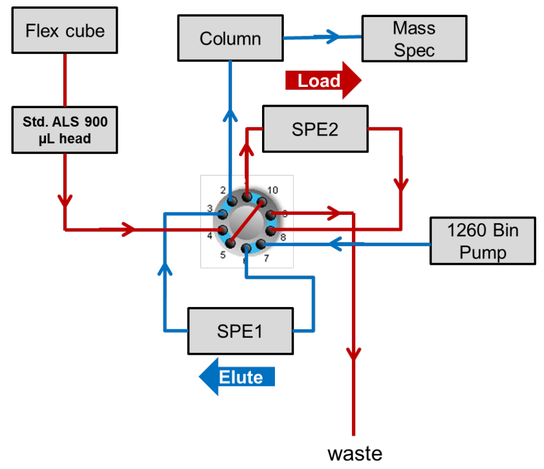

Figure 3. Agilent FlexCube LC/MS Solution with Online Sample Cleanup

A total of 500 injections of protein crashed controls and samples were analyzed for this study, divided into four sets of 125 injections per set. Each SPE cartridge was used 250 times. No significant increase in pressure were observed through the entire study. Figure 4 shows the Testosterone chromatogram with pressure traces overlay of injections 1, 100, 250 and 500. The Testosterone peak was free of matrix interferences and no ion suppression from matrix was observed.

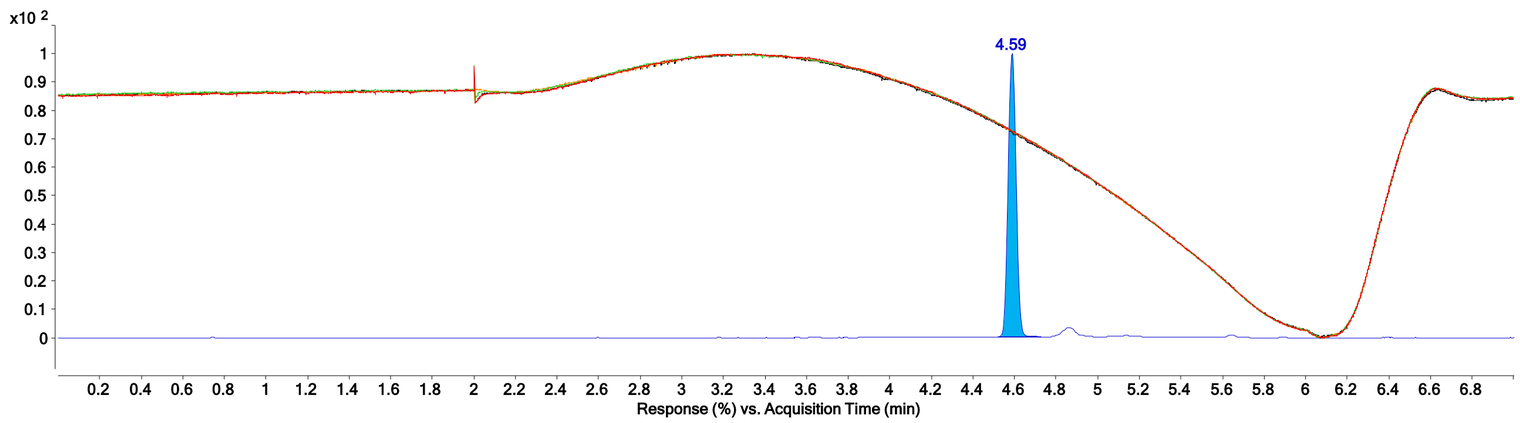

Figure 4. Testosterone chromatogram with pressure traces overlay

A calibration curve ranging from 5 pg/ mL to 100 ng/mL was found to be linear with a correlation >0.999 R^2 . Each point of the calibration curve was injected 4 times (Figure 5). Fifty injections of human defibrillated serum (Golden West Biologicals) samples were analyzed in each set. The excellent reproducibility of this method is demonstrated in figure 6, which shows an overlay of the internal standard from 50 serum injections; red traces are from SPE 1 cartridge and black traces are from SPE 2 cartridge. Retention time reproducibility shows less then 0.01% variation while area response reproducibility for SPE 1 and SPE2 were 1.5% and 1.1% receptively. The difference in area response between SP1 and SPE 2 was found to be 0.3%

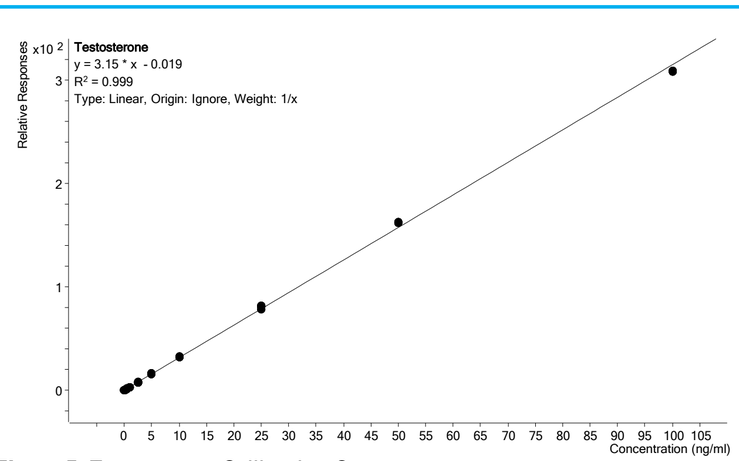

Figure 5. Testosterone Calibration Curve

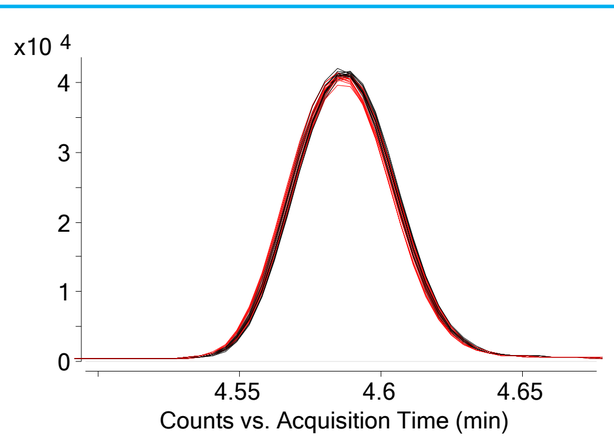

Figure 6. Overlay of 50 injections, 25 on each SPE cartridge

Conclusions

We have demonstrated a completely integrated, cost effective scheme to do online SPE coupled to LC/MS/MS for the analysis of testosterone. No special sample preparation is required. After the protein crash, the filtered sample is transferred to a vial for online SPE followed by analysis. Excellent performance of the analytical method for >250 injections of the serum samples is shown.