Evaluation of Prelude SPLC and TSQ Endura Mass Spectrometer in Research Analysis of Testosterone in Human Serum

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Application Note 609

Key Words
Testosterone, TurboFlow, sample preparation liquid chromatography, SPLC, mass spectrometry, human serum

Goal
To evaluate a Thermo Scientific™ Prelude SPLC™ coupled to Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer by using two quantitative methods, one using online Thermo Scientific™ TurboFlow™ sample cleanup and one using offline sample cleanup, for the analysis of testosterone in human serum for clinical research.

Introduction
The analysis of testosterone in human serum for clinical research is currently performed using many different sample preparation procedures. In this note, we evaluated a Prelude SPLC system coupled to a TSQ Endura MS (Figure 1) in the analysis of testosterone using two sample preparation methods: online sample cleanup and offline sample cleanup. Each method uses specific chromatography, but both share the same mass spectrometer settings and parameters.

Experimental
Sample Preparation
Two different sample preparation methods were used.

Online sample preparation using TurboFlow on the Prelude SPLC system
The first method was an online cleanup sample preparation using a TurboFlow column with separation by an analytical column. Aliquots (200 µL) of serum sample were placed into microcentrifuge vials. The samples were then diluted with 200 µL of water/methanol (1:1, v/v) containing internal standard. After this, they were vortexed, centrifuged, and the supernatant was transferred to a clean HPLC vial. A 100 µL aliquot of each sample was then injected onto a TurboFlow column.

Figure 1. Prelude SPLC system coupled to a TSQ Endura MS

Offline sample preparation using liquid-liquid extraction
The second sample preparation method was offline liquid-liquid extraction (LLE) with methyl-t-butyl ether followed by two-fold sample concentration. A 50 µL aliquot of processed sample was injected onto the analytical column.

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Chromatography

Analytical columns and mobile phases for both online cleanup and offline cleanup methods are listed in Table 1.

The sample preparation liquid chromatography method implementing online extraction is presented in Table 2a. The liquid chromatography method used in analysis of offline extracted samples is presented in Table 2b.

Table 1. Online TurboFlow (SPLC) and offline LLE (HPLC) conditions

	Online TurboFlow	Offline LLE
TurboFlow Column	Thermo Scientific™ Cyclone-P™ column, 50 x 0.5 mm	NA
Analytical Column	Thermo Scientific™ Accucore™ aQ column, 2.6 µm, 50 x 3.0 mm	Accucore aQ column, 2.6 µm, 50 x 3.0 mm
Loading Mobile Phase A	10 mM ammonium formate, 0.05% formic acid in water	NA
Loading Mobile Phase B	10 mM ammonium formate, 0.05% formic acid in methanol	NA
Loading Mobile Phase C	Isopropanol/acetonitrile/acetone (45:45:10)	NA
Eluting Mobile Phase A	10 mM ammonium formate, 0.05% formic acid in water	10 mM ammonium formate, 0.05% formic acid in water
Eluting Mobile Phase B	10 mM ammonium formate, 0.05% formic acid in methanol	10 mM ammonium formate, 0.05% formic acid in methanol

Fisher Chemical brand solvents were used in all mobile phases.

Table 2. LC methods: (a) online cleanup method and (b) offline cleanup method

(a)

			Loading							Analytical			
Step	Start	Sec	Flow	Grad	%A	%B	%C	Tee	Loop	Flow	Grad	%A	%B
1	0.00	40	2.00	Step	100.0	-	-	-	Out	0.60	Step	90.0	10.0
2	0.67	60	0.20	Step	10.0	90.0	-	T	In	0.60	Step	90.0	10.0
3	1.67	5	2.00	Step	10.0	90.0	-	-	In	0.60	Step	33.0	67.0
4	1.75	60	2.00	Step	10.0	90.0	-	-	In	0.60	Step	33.0	67.0
5	2.75	30	2.00	Step	-	-	100.0	-	Out	0.60	Step	-	100.0
6	3.25	45	1.50	Step	100.0	-	-	-	Out	0.60	Step	90.0	10.0

(b)

Step	Start	Sec	Flow	Grad	%A	%B
1	0.00	20	0.40	Step	95.0	5.0
2	0.33	5	0.40	Step	60.0	40.0
3	0.42	210	0.40	Ramp	20.0	80.0
4	3.92	85	0.40	Step	0.0	100.0
5	5.33	70	0.50	Step	95.0	5.0

Mass Spectrometry

The mass spectrometry method utilized a heated electrospray ionization (HESI) source. Data were collected in selected-reaction monitoring (SRM) mode (Table 3).

Table 3. SRM transitions

Analyte	Precursor Ion *m/z* (Q1)	Product Ions *m/z* (Q3)	CE	RF lens
Testosterone	289.2	97.2, 109.2	26	102
Testosterone-$^{13}C_3$	293.4	100.2	21	109

Method Validation

Calibration standards in the range of 20.0 to 10,000 pg/mL for the online sample preparation method and 10.0 to 10,000 pg/mL for the offline sample preparation method were prepared in charcoal stripped serum (CSS). Quality control (QC) samples were prepared in CSS at three levels: 60, 450, and 8,000 pg/mL for both methods. Accuracy and precision were determined by analyzing five replicates of all three quality control levels over three days and quantitating them using calibration curves injected at the beginning and end of each batch run. Matrix effects were determined by comparing QCs prepared in matrix to QCs prepared in neat solution and also by monitoring internal standard signal throughout the batches containing donor samples. Additionally, a dilution study was performed by diluting donor matrix with charcoal stripped serum. Lastly, carryover was assessed by dividing the total analyte signal at the lower limit of quantitation (LLOQ) by the total analyte signal found in the matrix blank after injecting the upper limit of quantitation (ULOQ). This cannot exceed 20% of the LLOQ signal.

Results and Discussion

Both methods met requirements for analysis of testosterone in plasma or serum. The limit of quantitation, calibration range, and carry-over limit for both methods are shown in Table 4. Intra- and inter-assay precision data is presented in Table 5.

Table 4. Method performance

	Online Cleanup	Offline Cleanup
LOQ	20 pg/mL	10 pg/mL
Calibration Range	20–10,000 pg/mL	10–10,000 pg/mL
Carry-over Upper Range	10,000 pg/mL	10,000 pg/mL

Table 5. Method precision

	%RSD					
	Online Cleanup			Offline Cleanup		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
Intra-assay	<11.4	<5.3	<2.2	<14.2	<8.3	<5.8
Inter-assay	8.3	4.3	3.1	11.4	6.7	4.5

Representative calibration curves are shown in Figures 2 and 3. Figure 4 presents chromatograms for the lowest calibration standards. Limited matrix effects were observed in both methods.

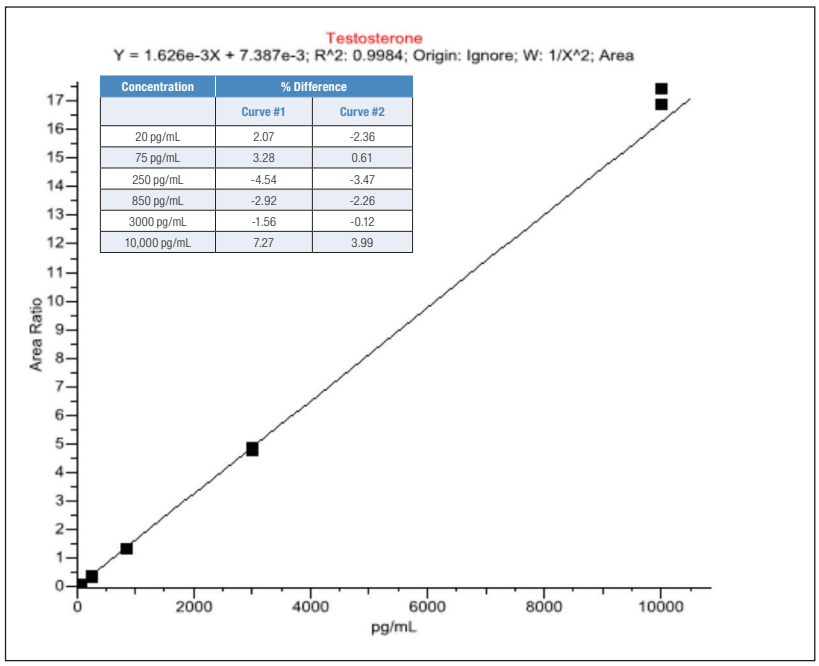

Figure 2. Representative calibration curve collected with online cleanup method (calibration standards in duplicates)

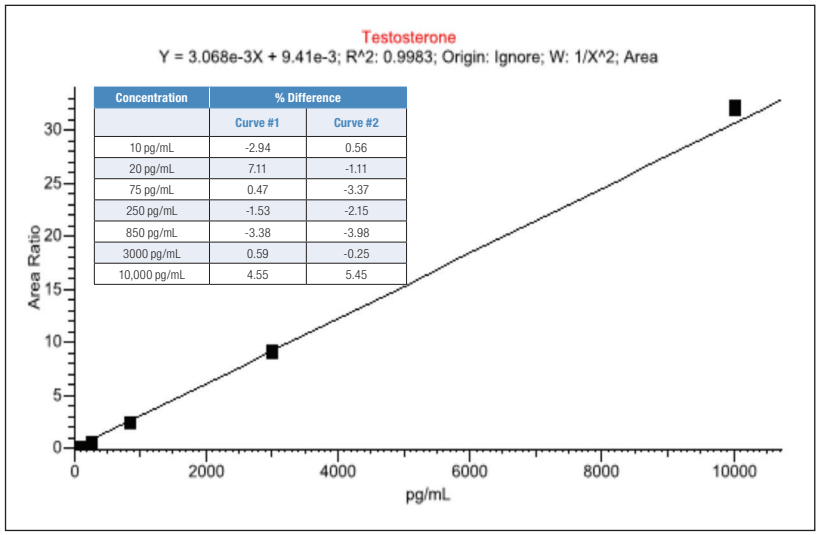

Figure 3. Representative calibration curve collected with offline cleanup method (calibration standards in duplicates)

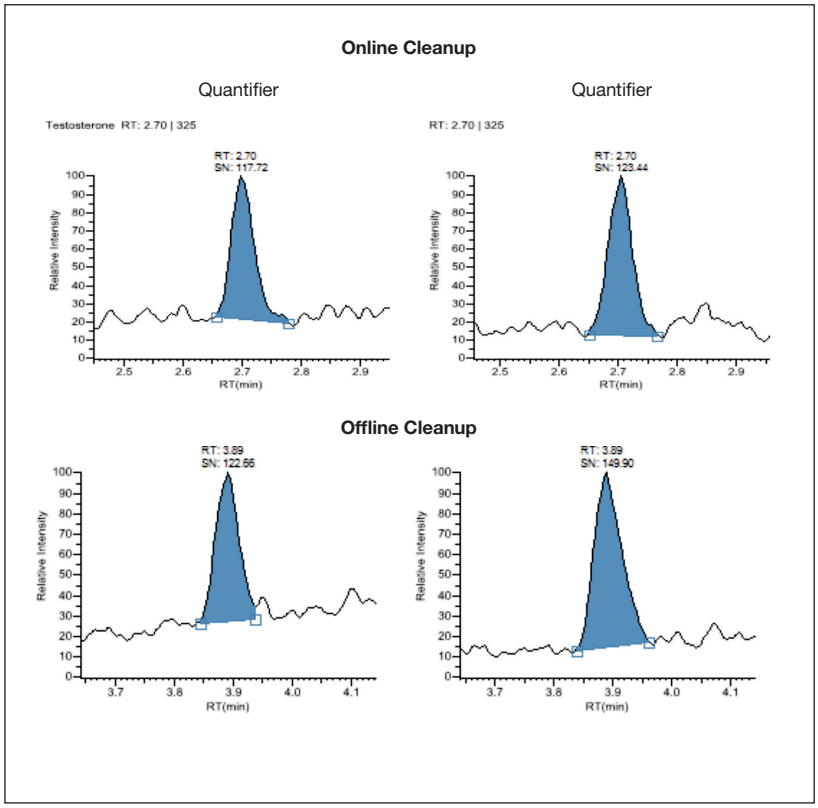

Figure 4. Chromatograms of the lowest calibration standards showing quantifying and qualifying ions: 20 pg/mL for online cleanup method and 10 pg/mL for offline cleanup method

Table 6 presents the results of the dilution study performed using the TurboFlow online cleanup method. Figure 5 shows internal standard signal across calibration standards and donor samples.

Table 6. Dilution study results for online cleanup method

Dilution Factor	Expected pg/mL	Experimental pg/mL	% Recovery
No dilution		4172	
x2	2086	2089	100%
x4	1043	1061	102%
x8	521	512	98.2%
x16	261	252	96.6%

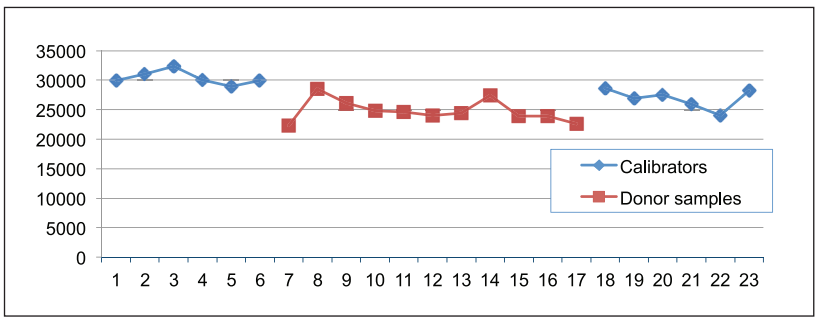

Figure 5. Internal standard signal across calibration standards and plasma donor samples. Data collected with online cleanup method

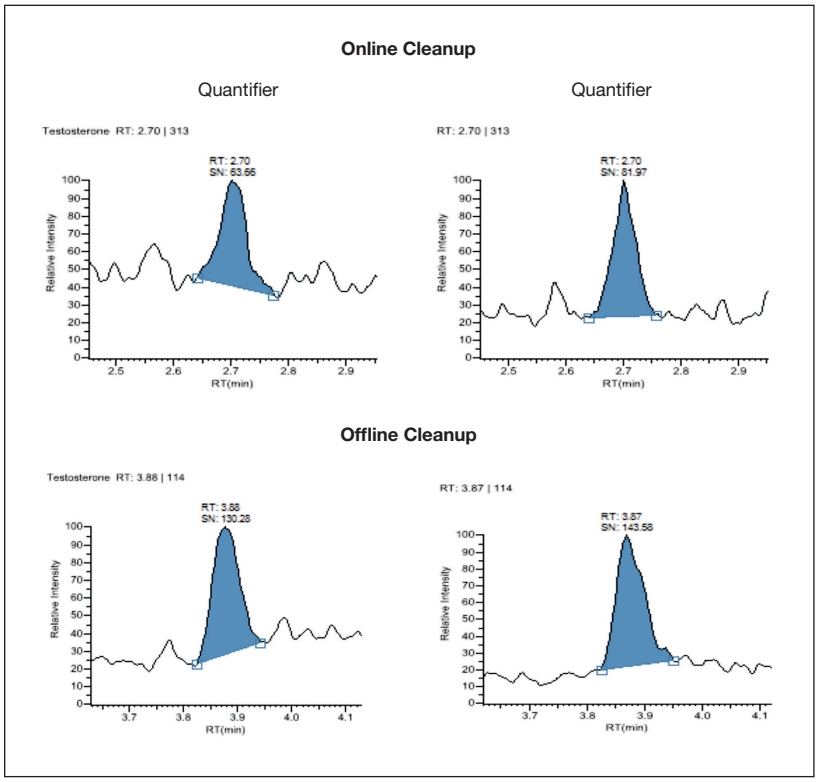

Figure 6. Chromatograms of donor sample at concentration of 20 pg/mL showing quantifying and qualifying ions and analyzed with online and offline cleanup methods

To double throughput, both methods can be multiplexed on the dual-channel Prelude SPLC (Figure 7).

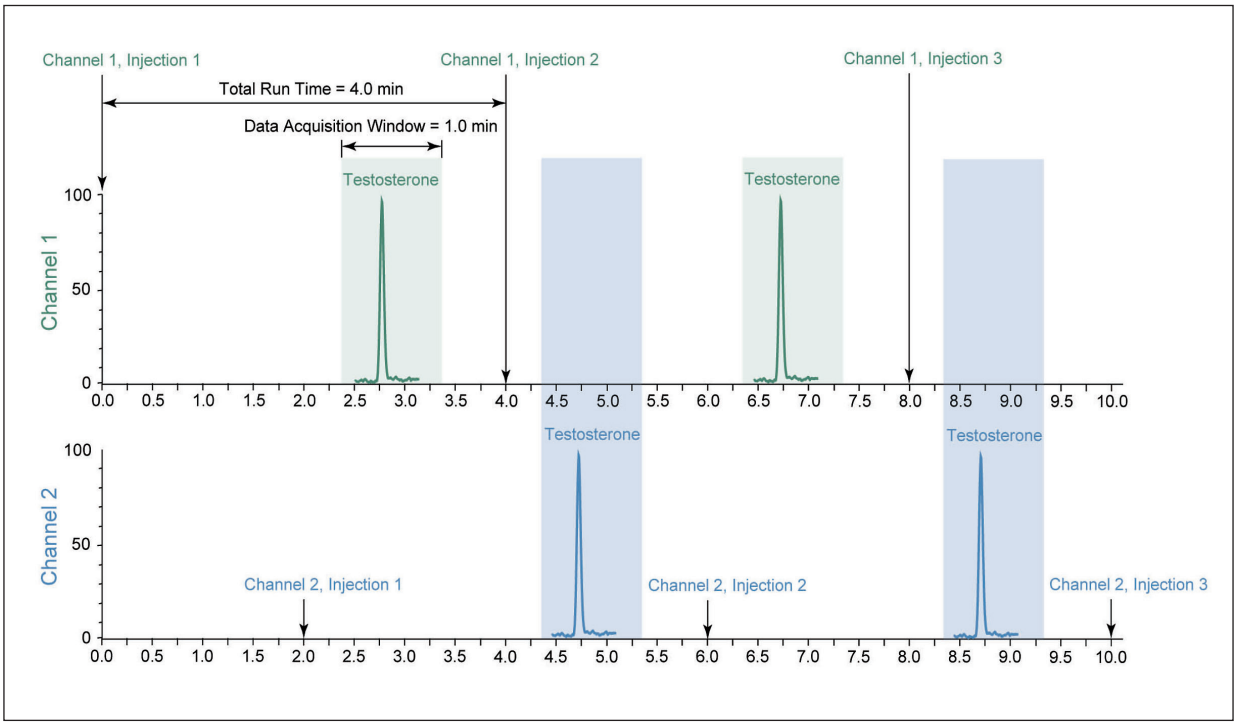

Figure 7. Multiplexing online sample cleanup method with the dual-channel Prelude SPLC

Conclusions

The performance of a Prelude SPLC system coupled to a TSQ Endura mass spectrometer was successfully evaluated in analysis of testosterone in human plasma for research using either online sample or offline sample cleanup. The LC methods on the Prelude SPLC system provide two short run times of 4 minutes for the online cleanup method and 6.5 minutes for the offline cleanup method. Limited matrix effects were observed in both methods. The high sensitivity of the TSQ Endura mass spectrometer allows low limits of quantitation in both methods.

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