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Analytical Products

Application Note

Sample Preparation of Serum Prior to Determination of Free Testosterone

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Introduction

The industry's gold standard for sample preparation of free hormones from serum has been associated with equilibrium dialysis.[1] It is the free portion of hormones, including testosterone, that is responsible for the biological activity.[2] Free testosterone accounts for approximately 1-2% of total testosterone.[3] In males this generally falls in the range of 20 – 230 pg/mL and for females in the range of 0.6 – 10 pg/mL (0.8-1.4% of total).[4] Solid phase microextraction (SPME) is a relatively new method to employ for measurement of free concentration and one that has recently been incorporated into a 96-pin device for use with conventional well plates. Bioanalytical SPME, or BioSPME, has been shown to be a fast sample preparatory technique.[5]

Methods

The BioSPME sample preparation method for the 200 µL samples utilized a Supel™ BioSPME C18 96-pin device with a Hamilton® Starlet system. The method includes multiple steps but was developed to have a total process time of 1 hour (**Figure 1**). A volume of 500 µL was used for the conditioning, acetonitrile, and wash solution, water. The desorption and derivatization steps were performed off-line. The acetonitrile desorption solution, 50 µL, contained 25 pg/mL D3-testosterone prior to derivatization. Derivatization was performed with an addition of 200 mM hydroxylamine hydrochloride, 100 µL, at 60 °C for 20 min with agitation at 600 rpm.[6] The free concentration of testosterone in serum samples was determined using simultaneously extracted calibrators (10 – 200 pg/mL) prepared in phosphate buffered saline and analyzed by an Agilent 1290 LC utilizing an Ascentis® Express C18 (5 cm x 2.1 mm, 2.7 µm) column connected to an AB Sciex 6500+ TripleQuad™. Quantifier and qualifier transitions were utilized for the natural and isotopically labelled testosterone and derivatized testosterone (**Table 1**).

Injection volumes of 60 µL of the final prepared samples of the total 150 µL were used to accommodate reinjections if required.

Free testosterone in male serum samples were previously determined by externally validated equilibrium dialysis-based methods at respective laboratories (Lab A, Lab L, and Lab Q). Samples were purchased from Lab A and were previously tested. The serum samples sent to Labs L and Q were collected in collaboration with the Clinical & Translational Science Institute at Penn State University. Aliquots of these samples were submitted for testing with the remainder of samples kept at -80 °C. A second extraction was performed after one month in the -80 ºC freezer. A total of 30 unique serums samples were tested using BioSPME in randomized analysis. In general, samples were tested in duplicate or triplicate depending on availability of sample quantity. Images of the actual samples are shown in **Figure 3**.

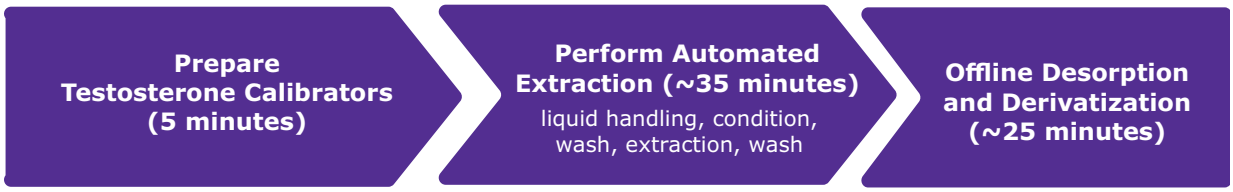


Figure 1. Overview of sample preparation prior to analysis by LC-MS/MS. A detailed outline of the automation is shown in **Figure 2**.

Table 1. Transitions monitored

Analyte	Q1	Q3	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Testosterone – 1	289.2	97.2	40	85	10	51	10
Testosterone – 2	289.2	109.2	40	85	10	33	10
D3-Testosterone – 1	292.2	97.2	40	85	10	51	10
D3-Testosterone – 2	292.2	109.2	40	85	10	33	10
Te – NHOH – 1	304.2	112.0	40	85	10	33	10
Te – NHOH – 2	304.2	124.0	40	85	10	30	10
D3–Te-NHOH – 1	307.2	112.0	40	85	10	33	10
D3–Te-NHOH – 2	307.2	124.0	40	85	10	30	10

*Te-NHOH represents the derivatized testosterone with hydroxylamine

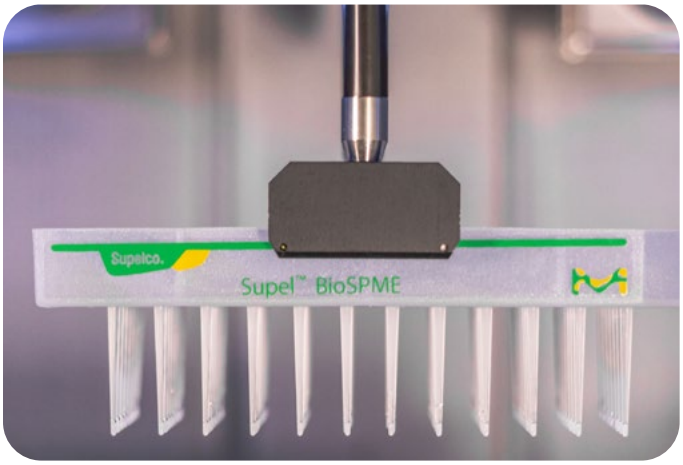
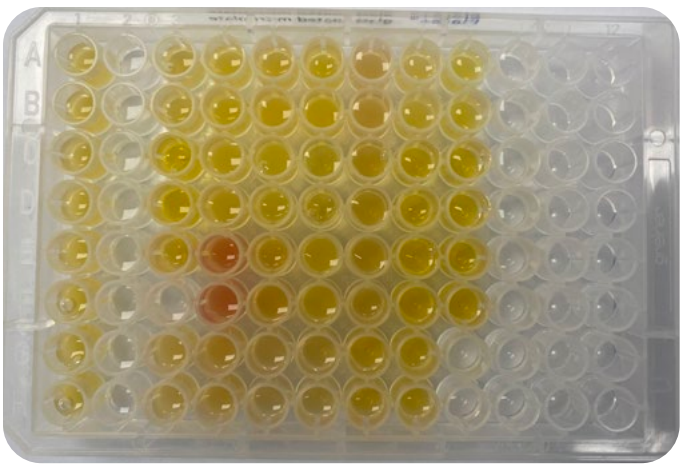


Figure 2. (Right) Overview of the steps the Hamilton® Starlet Robot performs in the automated version. (Above) Grippers from Hamilton® Starlet moving the Supel™ BioSPME Device.

Liquid Handling	Optional	• Condition Plate is filled with 500 µL of acetonitrile in corresponding consecutive wells • Wash Plate is filled with 500 µL of water in corresponding consecutive wells
Conditioning	20 min, static	• Robot grips the BioSPME Pin Tool from the parked position and transfers to the condition plate and submerges the pins in acetonitrile for 20 mins under static conditions.
Wash	10 sec, static	• Robot transfers the BioSPME from the conditioning plate to the wash plate and submerges the pins for 10 secs under static conditions.
Extraction	10 min, 37 °C at 1200 rpm	• Robot transfers the pin device to the extraction plate allowing extraction of the free testosterone to occur. The heated shaker is at 37 ºC and is mixing 1200 rpm for 10 minutes. • Glass-lined well-plate to prevent non-specific binding.
Wash	1 min, static	• Robot transfers the BioSPME device from the extraction back to the initial wash solution for 1 min under static condition.
Park		• Upon the second wash, the robot will transfer the BioSPME device back to the Home position. • Desorption and Derivatization will occur off-line



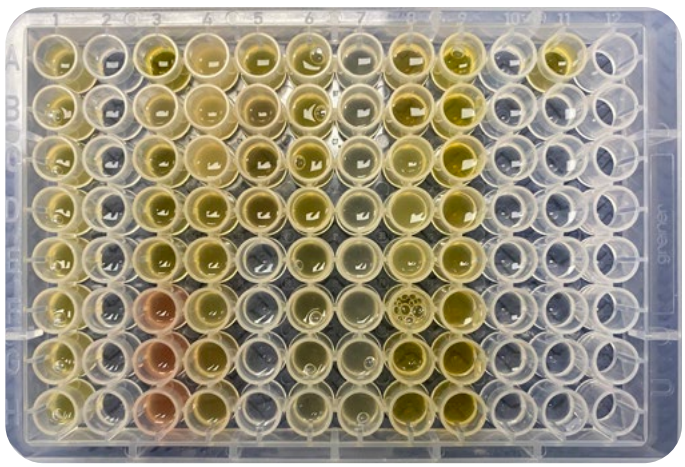


Figure 3. Actual samples for extraction 1, left, and extraction 2, right. Each loaded well contained 200 µL of either serum sample (yellow/red) or a calibrator prepared in phosphate buffered saline. Serum samples were not excluded if they appeared lipemic (cloudiness from lipids/fats), icteric (yellowing from bilirubin), or hemolytic (presence of ruptured red cells)

Results:

The instrumental limit of detection, LOD, and lower limit of quantification, LLOQ, of the derivatized testosterone, Te-NHOH, was determined by serial dilution and n=12 injections (**Table 2**). The LOD was 0.2 pg/mL and LLOQ of the quantifier transition, Te-NHOH-1, was 1 pg/mL, RSD of 8.0%. The LLOQ of the qualifier transition, Te-NHOH-2, was 1 pg/mL, RSD of 9.5%. The peak integration ratio for the quantifier/qualifier was 1.10, RSD 15.1%. The sensitivity was achieved by replacing the 20 µL standard injection loop on Agilent 1290 LC instrument with a 100 µL loop to allow for larger injection volumes. Representative chromatograms of the diluted samples down to 0.2 pg/mL are available in **Figure 4**.

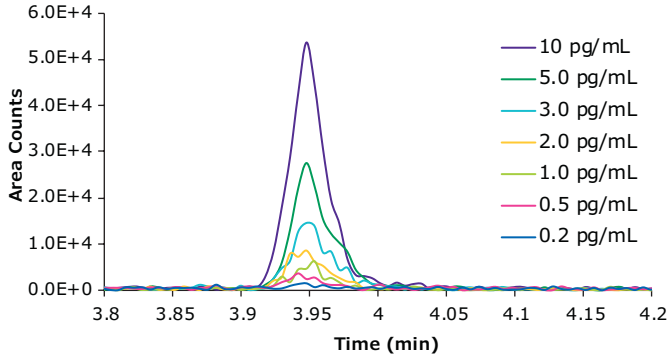


Figure 4. Chromatogram of Te-NHOH-1 at various concentrations ranging from 10 pg/mL down to 0.2 pg/mL in (1:2 acetonitrile:water).

Table 2. Accuracy and precision for injection of various concentrations of derivatized testosterone in 1:2 acetonitrile:water and the peak ratios for transitions monitored (n=12).

	Te-NHOH-1 Quantifier Transition			Te-NHOH-2 Qualifier Transition			Ratio Peak Area Counts Te-NHOH-1/Te-NHOH-2	
pg/mL in solution	pg/mL determined	RSD	S/N	pg/mL determined	RSD	S/N	Avg	RSD
0.2	0.13	68.0	3.8	< 0.00	n/a	13.1	1.31	66.1
0.5	0.43	20.6	4.6	0.22	47.4	32.1	1.00	20.7
1.0	1.06	8.0	30.5	0.80	9.5	62.4	1.09	15.1
2.0	2.05	5.3	55.3	1.76	4.6	112.5	1.17	9.0
3.0	3.17	5.1	77.2	2.87	6.7	160.9	1.17	8.5
5.0	5.15	3.2	161.7	4.99	2.9	294.5	1.16	4.1

The extracted calibration curve for free testosterone quantification, range of 10 – 200 pg/mL, had a R = 0.9964 and 0.9936 using a 1/(x*x) regression **Figure 5**. Representative total ion chromatograms of three different samples are presented in **Figure 6**. Correlation plots between the free testosterone determined by validated equilibrium dialysis (Lab A, range 20.3 – 194.4 pg/mL) and the

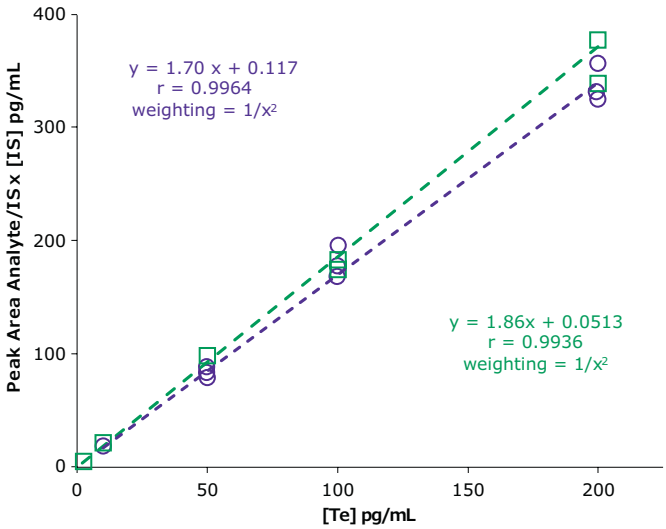


Figure 5. Extracted calibration curves from phosphate buffered saline for determination of free testosterone. Purple circles – extraction 1 and Green squares – extraction 2.

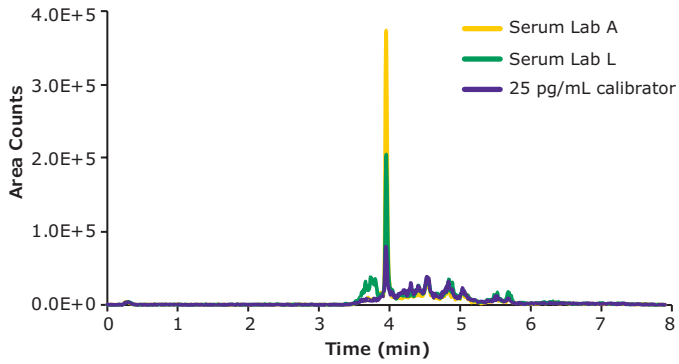


Figure 6. Total ion chromatograms for three different samples: 25 pg/mL extracted calibrator (purple), Lab L serum sample (green), and Lab A serum (yellow).

BioSPME prior to LC-MS/MS yields a linear correlation of y = 0.917x – 6.23, r^2 = 0.954 (extraction 1) and y = 0.956x – 1.57, r^2 = 0.976 (extraction 2). Extraction 2 was performed over a month later after undergoing a freeze/thaw cycle and storage at -80 °C in the interim. (**Figure 8**). Including the additional samples from Labs L and Q, linear correlations of y = 0.923x – 6.37, r^2 = 0.921 (extraction 1) and y = 0.941x + 0.15, r^2 = 0.960 (**Figure 9**). In either correlation graph, the r^2 is above 0.92 and indicates almost ideal correlation between the two methods. When considering the existence of a y-intercept, it may result from a couple of different reasons. These include different sample preparation, instrumentation, and analysts. Another variable that is unaccounted is the state of the serum samples as some of the samples were pretested (and underwent freeze thaw cycles) while others were shipped across the country.

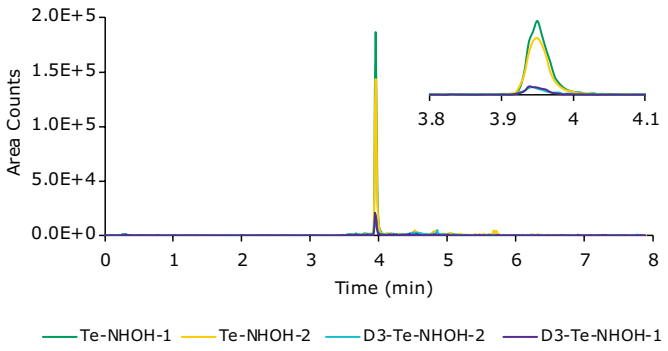


Figure 7. Representative chromatogram (Lab A #1) showing the transitions monitored. Zoomed-in window to highlight the peak.

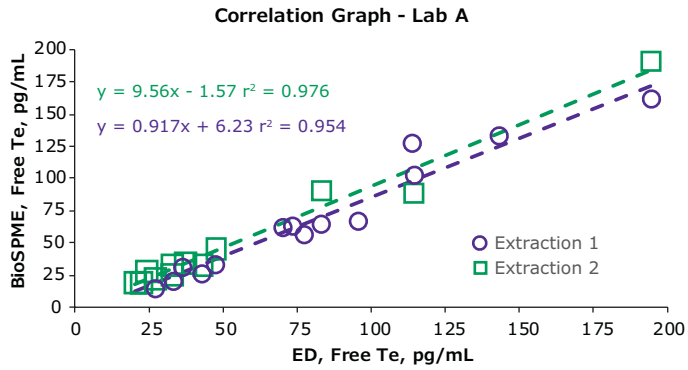


Figure 8. Correlation of free testosterone for Lab A samples between two different methods; BioSPME, determined at Merck, and equilibrium dialysis, pre-determined externally using equilibrium dialysis. Purple circles for extraction 1, and Green squares for extraction 2.

The derivatized internal standard peak area counts, D3-Te-NHOH-1, on a per well basis were monitored and used as a quality check. An average internal peak area count across all tested wells was 5.84 x 10^4 ± 0.61 x 10^4 (RSD 10.6%) and 6.40 x 10^4 ± 0.58 x 10^4 (RSD 9.1%) for extraction 1 and extraction 2 respectively. The percent difference from the average for extraction 1 and extraction 2 is shown in **Figure 10**. This uncertainty is contributed from the pipetting for desorption and derivatization plus from LC-MS/MS measurement. In all samples, the amount of underivatized testosterone was below the limit of detection.

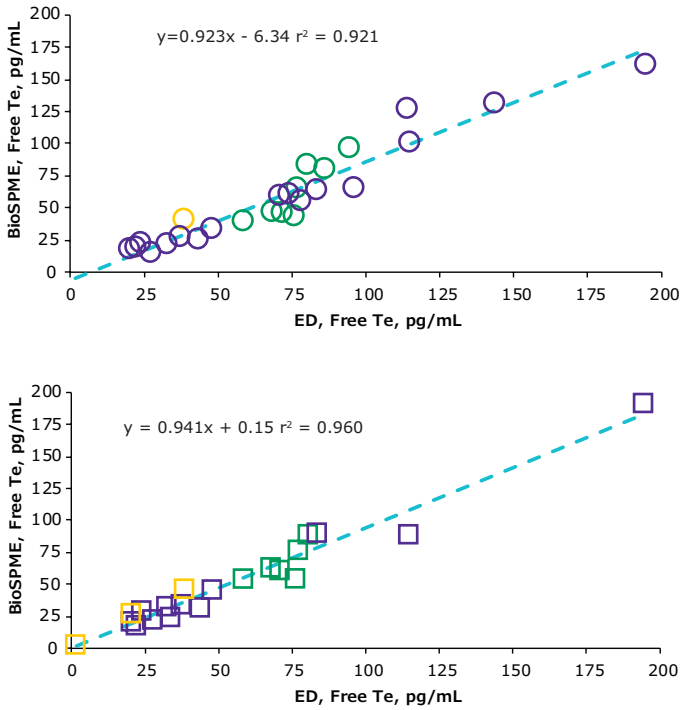


Figure 9. Correlation of free testosterone for all samples; Lab A (purple), Lab L (yellow), and Lab Q (green) between two different methods; BioSPME, determined by Merck, and equilibrium dialysis, determined externally using equilibrium dialysis. Circles for extraction 1; squares for extraction 2.

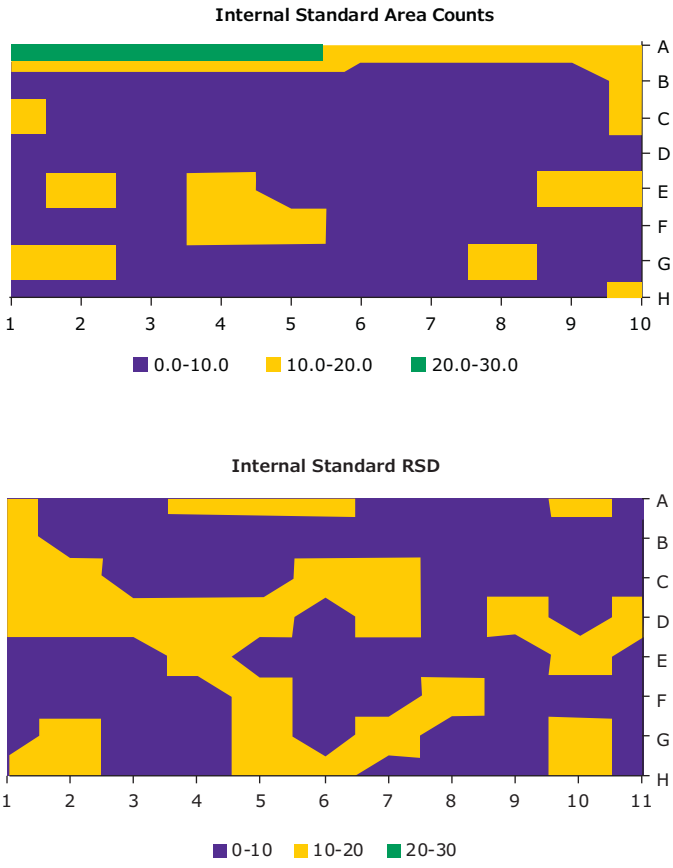


Figure 10. The percent difference from the average internal standard area counts, D3-Te-NHOH-1, Avg = 5.82 x 10^4 for extraction 1 (top) and Avg = 6.40 x 10^4 for extraction 2 (bottom), on a per well basis across the plate. Columns 2 and 10 were testosterone calibrators, remaining columns were samples.

Conclusion:

A BioSPME extraction method prior to analysis by LC-MS/MS was developed, and the evaluated results showed strong correlation (r^2 = 0.92 – 0.96) for serum samples analyzed by externally validated equilibrium dialysis LC-MS/MS for free testosterone. The BioSPME method was automated by using a Hamilton® Starlet Robotic system and can be adapted to other robotic liquid handlers that have gripper functionality. The time to process one 96-well plate was approximately an hour. The developed LC-MS/MS detection method used derivatization of the final extract by hydroxylamine hydrochloride to increase the sensitivity for detection of free testosterone.

References

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Water, LC-MS LiChrosolv®	**1.00029**
Testosterone solution 1.0 mg/mL in acetonitrile, ampule of 1 mL, certified reference material, Cerilliant®	**T-037**
Testosterone-d3 (16, 16, 17-d3) solution 100 µg/mL in acetonitrile, ampule of 1 mL, certified reference material, Cerilliant®	**T-046**
Nunc™ 96 DeepWell™ plate size 96 wells, U-bottom natural polypropylene wells, maximum volume 1.3 mL, non-sterile	**P8241**
Corning® Thermowell PCR 96 well plates 96 well plate, Thermowell PCR plate, polypropylene, conical bottom, clear, 25/cs	**CLS6551**
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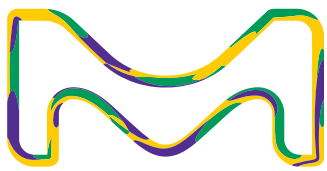

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