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**A-038 An Improved Multiplexed HTLC-HESI-MS/MS Method for the Measurement of Total Testosterone in Serum**

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**BACKGROUND:** Testosterone is an anabolic steroid produced primarily in the testicles of males and mainly in the ovaries of females. Imbalance of testosterone is the primary cause of hypogonadism in males, hirsutism and virilization in females, osteoporosis, and diabetes mellitus. Accurate and fast-turnaround measurement of testosterone is critical for diagnosis, prevention, and treatment of testosterone-related diseases in adults and children. In a previous study, testosterone was derivatized with methoxyamine and hydroxylamine in a multiplexed high turbulence liquid chromatography with heated-electrospray ionization-tandem mass spectrometry (HTLC-HESI-MS/MS) method. Using these reagents, differential mass-tagging of testosterone allowed different specimens to be combined in a single injection to increase assay throughput. However, interference was observed for hydroxylamine-derivatized testosterone in serum collected in serum separator tubes (SST). In the current study, our objectives were to (1) replace hydroxylamine with a new derivatizing agent not subject to interference and (2) develop an improved HTLC-HESI-MS/MS method for multiplexed total testosterone measurement.

**METHODS:** Healthy male and female donor specimens were collected in SST and red-top tubes. Serum was mixed with stable isotope-labeled testosterone as internal standard (ISTD) prior to protein precipitation. Testosterone in the supernatants was derivatized, enriched by solid phase extraction, dried, and reconstituted. Specimens were injected on the HTLC system for on-line extraction, transferred to analytical columns, and eluted using binary gradient. Testosterone derivatives and ISTDs were detected and analyzed on a Triple Quadrupole Mass Spectrometer (Thermo) equipped with HESI. Ionization and multiple reaction monitoring (MRM) scan parameters were optimized for maximized ion transmission and sensitive, specific, and stable quantitation. MRM transitions were used as quantifiers and qualifiers for both testosterone derivatives. Also assessed were ion suppression using T-column infusion, specimen stability by storage in SST tubes at 4 °C, isobaric interference from dehydroepiandrosterone (DHEA), and carryover.

**RESULTS:** Intra- and inter-assay coefficients of variation (CV%) for total testosterone levels were <5% at 12, 80, 250, and 1200 ng/dL for both testosterone derivatives. Limits of quantitation (LOQs) for both derivatives were <1 ng/dL. Calibration linearity was verified across the measurement range of 2.5–2000 ng/dL. Both agents were equivalent in derivatizing and recovering testosterone from serum based on a comparison of their quantitative results ( $R = 0.991$ , bias =  $-3.8$  ng/dL). No interference was observed in serum collected in SST. Serum specimens collected in SST and red-top tubes from same donors gave equivalent results ( $R = 0.991$ , bias =  $0.2$  ng/dL). Ion suppression was calculated as less than 20% for both derivatives. CV% of the ion-ratios of both derivatives were less than 15% and were stable across the linear range. Testosterone levels were stable in SST for 72 h (at 4 °C) with the serum not separated from the gel. No isobaric interference and no carry over were observed.

**CONCLUSION:** We have developed and validated an improved multiplexed HTLC-HESI-MS/MS testosterone assay using new derivatizing agents. The current method allowed accurate, rapid, specific, and stable analysis of serum total testosterone. SST is an acceptable specimen type for the current method. Precision, accuracy, linearity, ion suppression, and sample stability in SST were validated.