Minimizing Collection Tube Interfering Substances in the Analysis of Testosterone using Atmospheric Pressure Chemical Ionization (APCI)

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Overview

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is increasingly being used in the clinical laboratory for the analysis of various endogenous and exogenous compounds. Until recently, the majority of clinical assays for steroids have been based on immunoassays and radioimmunoassay which can be somewhat problematic in quantifying and differentiating steroids; these methods can suffer from cross-reactivity and a lack of low end sensitivity. The use of LC-MS/MS for the analysis of steroids has exploded in recent years, due to the ease of sample preparation combined with superior sensitivity and selectivity of the target compounds. Blood collection tube interference has previously been documented as a problem for LC-MS/MS assays. Early in the development of our method, we set out to minimize or eliminate interference introduced by blood collection tubes and other endogenous substances that might be present in the sample.

Background

Testosterone is a steroid hormone from the androgen group and is found in mammals, reptiles, birds, and other vertebrates. In mammals, testosterone is primarily secreted in the testicles of males and the ovaries of females, although small amounts are also secreted by the adrenal glands. The concentration of serum testosterone is lower in women and children and levels generally diminish in men as they age. Monitoring the concentration of testosterone serves as an aid in diagnosing and treating disease states related to hormone imbalance. Development of a clinically diagnostic method requires the ability to perform measurements of hormone levels across a broad dynamic range. As a result of our standing as a large national reference laboratory with a complex testing menu, we do not always have control or knowledge of the specimens that we receive. Subsequently, the need arose to develop a method that would be free from interferences and be able to assay multiple specimen types regardless of the collection tube submitted.

Method and Design

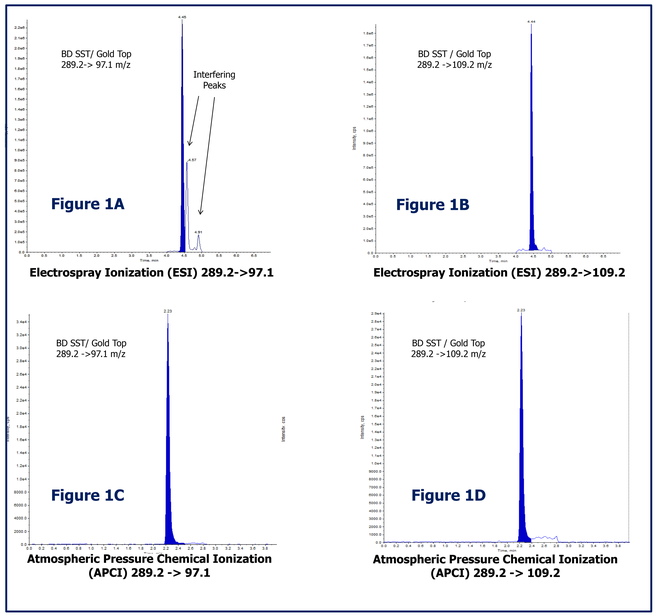
Samples were evaluated from an assortment of the most commonly encountered blood collection tubes: BD Plus Serum/CAT with Clot Activator (Red), BD SST with Gel and Clot activator (Gold), BD K_2EDTA (Lavender) and BD PST II Tubes with Gel and Lithium Heparin (Mint Green). In our study we used 43 sets of samples collected in Red, Gold, Light Green and Lavender top tubes. Samples were drawn from healthy volunteers, 21 male and 22 female; samples were extracted, prepped and injected onto a Shimadzu 20AD, Prominence HPLC system coupled to an AB SCIEX Triple Quad™ 5500 mass spectrometer. Two methods were evaluated, Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI). Both methods were run in positive ion mode.

Sample Prep: Serum (200 µl) was mixed with 20 µl of a deuterated internal standard (Cerilliant) at a concentration of 5 ng/mL, testosterone was extracted from patient serum samples utilizing a liquid-liquid extraction reagent containing 90% Hexane; 10% Methyl *tert*-butyl ether (MTBE).

ESI: Seventy microliters of sample were loaded onto a Luna 2.5 µm C18, 100 x 2.0mm (Phenomenex) column. The column was eluted with a 5.50 minute gradient from 10% - 90% of a buffer containing 80% methanol, 20% acetonitrile, 0.1% formic acid at a rate of 0.45 mL/min. Multiple reaction monitoring (MRM) chromatograms were acquired in positive ion mode using ESI under the following conditions: declustering voltage 135V, dry temperature of 600°C, curtain gas 20 psi and a dwell time of 150 msec.

APCI: Twenty microliters of sample were loaded onto a Kinetex 2.6 µm, 50 x 2.1mm (Phenomenex) column and separated at a flow rate of 0.45 mL/minute The column was eluted with a 4.5 minute gradient from 30% - 95% of a buffer containing 80% methanol, 20% acetonitrile and 0.1% formic acid at a rate of 0.45 mL/min. The mass spectrometer was set to ionize the samples using APCI and to detect in selective ion monitoring mode. MRM chromatograms were acquired in positive ion mode under the following conditions: declustering voltage 180V, dry temperature of 300°C, curtain gas 10 psi and a dwell time of 150 msec.

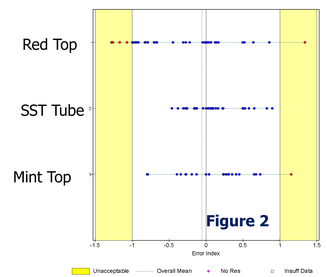
Blood Collection Tube Chromatograms

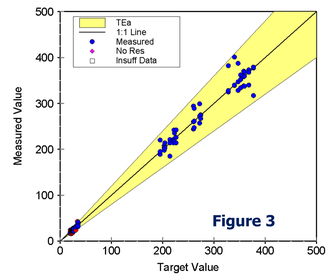


Figures 1A-D are representative chromatographs from the two unique testosterone methods. The results for the quantifying transition, 289.2 -> 97.1, when analyzed in positive ESI mode shows significant interference (see Figure 1A). This interference is not seen in the qualifying transition, 289.2 -> 109.2, under the same conditions (see Figure 1B), nor is it present in either of the transitions when evaluated in positive APCI mode, (see Figures 1C & 1D). The interference was found to be associated with the use of gel separator tubes as previously described by Run Zhang Shi, et al. Unfortunately, we were unable to obtain the necessary sensitivity using the qualifying transition 289.2 -> 109.2 to quantify testosterone, and were also unable to achieve baseline separation of the two peaks utilizing ESI. Subsequently, we chose to develop the APCI method for use in our laboratory.

Results

During the development of our assay, we observed significant interference that we attributed to gel separator tubes when ESI was used as the ionization source (see Figure 1A). In order to circumvent this we opted to use APCI to attempt to resolve the interference (see Figure 1C). In the course of the development of this assay, we needed to confirm that we had effectively removed the interfering peaks, we felt this was also an opportune time to evaluate other possible sample collection tubes. In our study we used 43 sets of samples collected in Red, Gold, Mint Green and Lavender top BD tubes; samples were collected from healthy volunteers 21 male and 22 female. All of the samples were processed and run using the APCI method over the course of 3 days. The data was entered into EP Evaluator: Multiple Instrument Correlation module. The Lavender top tubes were excluded from the study as a result of an extremely pronounced negative bias, the other two tube types were evaluated against the predicate tube type, Red top tube. We were able to show that the tube types were essentially equivalent. Further studies were performed to satisfy that we had eliminated commonly encountered endogenous interfering substances that maybe present in patient samples. We subjected our samples to high levels of Estrogen (936 ng/dL) and Progesterone (114.5 ng/dL) and analyzed the data using the EP Evaluator Interference protocol. No significant changes were observed in results due to ion suppression or interfering peaks.





Conclusion

We have developed a highly sensitive and specific APCI LC-MS/MS method suitable for the analysis of testosterone that is largely free from collection tube and endogenous interfering substances. We can accurately and precisely measure Testosterone down to levels of 3.5 ng/dL. As a result of being relatively free of interferences and cross reactivity with structurally related compounds, the LC-MS/MS method offers better sensitivity than previously employed methods used for testosterone quantification, along with higher operationial throughput and the smaller sample requirement are useful for pediatric testing.

Reference

Shi RZ, et al. Serum Testosterone quantitation by liquid chromatography-tandem mass spectrometry: Interference from blood collection tubes, Clin Biochem (2012), http://dx.doi.org/10.1016/j.clinbiochem.2012.08.008

Drake SK, et al. Potential interferences from blood collection tubes in mass spectrometric analysis of serum peptides. Clin Chem 2004:50:2398-401