Introduction

The measurement of circulating testosterone concentration is important in evaluating hypogonadism, gynecomastia, sexual dysfunction, infertility in men, androgen excess in women, and puberty and sexual differentiation in children. The analysis of testosterone by LC/MS/MS has emerged as the method of choice due to its high specificity, sensitivity, and accuracy [1]. However, the measurement of testosterone in women and pre-pubertal children using LC/MS/MS analysis is still challenging due to the substantially lower circulating levels of testosterone and limited sample volume from pediatric patients. A derivatization has to be employed for enhancing the detection of testosterone in LC/MS/MS analysis [2]. We report here a non-derivatized and rapid LC/MS/MS method for the analysis of testosterone in human serum to a desired low level of 0.5 ng/mL in small volumes of serum. In addition, we also include the other steroids in this reported study.

Experimental Conditions

HPLC System
Shimadzu UPLC system with LC-20 AD, Binary Pump and SIL-20 AC, Autosampler

MS Detector
AB Sciex QTRAP® 5500 System with ESI or APCI

Ionization Sources

Ionization Sources

IS Parameters

Table 1. Analyte Characteristics and Conc. (for Figure 1)

| Component         | M. Formula | MW/KDa | 1/4 Conc. | Conc.     | Slope     | Y = aX + b  \
|-------------------|------------|---------|-----------|-----------|-----------|-------------------|
| Estradiol         | C_{18}H_{22}O_{4}  | 271.21531 | 100 | 1.32 | 2.07x + 0.0184  
| Cortisol          | C_{21}H_{28}O_{5}  | 336.2122 | 100 | 1.0 | 2.07x + 0.0184  
| Androstenedione   | C_{19}H_{22}O_{4}  | 271.21531 | 100 | 1.0 | 2.07x + 0.0184  
| Testosterone      | C_{19}H_{28}O_{4}  | 291.2190 | 100 | 1.0 | 2.07x + 0.0184  
| Dihydrotestosterone| C_{19}H_{26}O_{4}  | 271.2150 | 100 | 1.0 | 2.07x + 0.0184  
| Progesterone (P4) | C_{21}H_{26}O_{5}  | 315.0971 | 0.1 | 2.07x + 0.0184  
| Estrone           | C_{18}H_{22}O_{5}  | 307.2190 | 100 | 1.0 | 2.07x + 0.0184  
| Testosterone 1    | C_{20}H_{26}O_{4}  | 315.0971 | 0.1 | 2.07x + 0.0184  
| Dihydrotestosterone| C_{21}H_{26}O_{4}  | 291.2292 | 5.0 | 2.07x + 0.0184  

Results

Figure 1. Recovery of Steroids from Human Serum by SPE

Figure 2. LC/MS/MS Responses on Hormones in Different Mobile Phases at ESI *

Figure 3. Comparison of LC/MS/MS Responses of Steroids with ESI (-ve) and APC1 (-ve) probes

Figure 4. Testosterone in Human Serum at LLOQ Level

Figure 5. Testosterone Standard Calibration Curve

Discussion of Results

HPLC Elution - The Selection of Mobile Phases

In the analysis of neutral compounds by LC/MS, it is common practice to employ volatile weak acids as additives to mobile phase to enhance the ionization. Contrary to this our study, results showed a phenomenon of "wrong-way-round ionization" [3]. As Figure 2 shows the ultra-polarized 0.1% formic acid in LC/MS grade water as aqueous mobile phase significantly suppressed the ESI+ signals of testosterone and other neutral hormones compared to the mobile phase of freshly collected Milli-Q DI water without acidic additives. At the same time the mobile phase of LC/MS grade water is a proven higher intensity for testosterone and other neutral steroids than DI Water/MeOH as mobile phase. Using methanol as organic modifier improved the sensitivity as shown in Figure 2 because of its acidic functionality in gas phase of ionization compared to the commonly used acetone/ether modifier. The higher back pressure resulting from the increased viscosity of methanol/water mixtures was overcome by utilizing a core-shell Kinex 2.6 µm column which provided UHPLC efficiency at much lower pressure (roughly 40-60% lower) than the columns made with 2-µm fully porous particles. Due to this lower operational pressure, a relatively higher flow rate (0.6-0.8 ml/min) to 50 to 2 x 3 mm ID column could be achieved to a fast analysis time of 3.5 min/speaker.

Table 2. Quantification of Testosterone in Human Serum

Performance Validation Results

Conc. Mean (ng/mL) Intra Assay (%) Inter Assay (%) [LOQ] 85.6 ± 110.9 (n = 9) 83.1 ± 113.3 (n = 9) 0.18 0.17 Relative Error 3% 2% 2% Precision (RSD %) 12.8 ± 7.11 (n = 9) 11.45 ± 7.11 (n = 9) 0.0 ± 2.0 (n = 6) 27.8 ± 2.10 (n = 6) 1.0 ± 0.8 (n = 6) 4.0 ± 0.5 (n = 6) 1.0 ± 0.3 (n = 6) 500 ± 0.5 500 ± 0.5 500 ± 0.5

*Conc.: 0.18 ng/mL

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Ionization Sources - ESI* or APC1

Figure 3 demonstrates that the ESI* probe provided significantly higher intensities for testosterone, DHT and cortisol than APC1 probe.

Method validation for the analysis of Testosterone

The results demonstrate linearity with value of R ≥ 0.9995 as shown in Figure 6.

The method accuracy is found to be 83.113% at Lower Limit of Quantification (LLOQ), 88-100% on 971 female serum, and 97-114% on NIST 971 male serum by intra- or inter-assays. The method precision is <15% RSD at LLOQ level and <5% RSD on the NIST 971 female or male serum samples as shown in Table 2. The LLOQ is 0.05 µg/mL for testosterone using this method shown as Table 3.

Conclusions

A non-derivatized, sensitive, and rapid LC/MS/MS method for the analysis of testosterone in human blood has been developed.

Methanol as organic modifier without additives as mobile phase enhances the intensities of neutral steroids in LC/MS/MS.

For cortisol and Methyl-Q DI water/MeOH mobile phase without acidic additive is a wrong-way-round-right-way for increasing the sensitivity of detection to neutral steroids by LC/MS/MS.

Utilizing a core-shell 2.6 µm column provides UHPLC efficiency at lower than sub-2 µm porous particle column at relative high flow rate for achieving high speed LC/MS/MS analysis for testosterone.

References