Determination of 18 Steroid Hormones in Human Serum Using Rapid Protein Precipitation Method Coupled With Liquid Chromatography-Tandem Mass Spectrometry

Dan Liu ; Fengmei Hu ; Chao Huang; Xianglong Zhao; Lihai Guo Shanghai AB Sciex Analytical Instrument Trading Co., Ltd., Shanghai, PR China

ABSTRACT

Steroid hormones play an important role in maintaining normal endocrine, regulating sexual function, and immune regulation. The traditional biochemical immunoassay may be inaccurate as its false-positive result. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has obvious advantages like strong specificity and high sensitivity. There are many methods for the detection of various steroid hormones in serum samples by LC-MS/MS. Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the mainly reported pretreatment methods[1-2], which are complicated, time-consuming, and expensive. Therefore, a rapid assay for the simultaneous and accurate detection of 18 serum steroid hormones was established by using rapid protein precipitation pretreatment method.

INTRODUCTION

Based on SCIEX Triple Quad™ 6500+ system, a liquid chromatograph-tandem mass spectrometry method was developed for the accurate qualitative and quantitative detection of 18 steroid hormones. Standards of 18 compounds generate standard curves at more than 7 different concentration points. Each compound showed good linear correlation within the range of standard curves. The linearity of the assay was demonstrated by all the R values greater than 0.995. All 18 compounds had low LOQ while meeting the requirements of clinical physiological concentration level. For example, LOQ concentrations were 10 pg/mL for testosterone, aldosterone and androstenedione, and 20 pg/mL for estriol, estrone and dihydrotestosterone.

In order to overcome the disadvantage of using SPE method and LLE method in the pretreatment process, the protein precipitation pretreatment method was developed as it is more convenient and easier to operate and automate.

In the process of method development, the matrix effects and recovery of analytes of different types of protein precipitators were studied. Zinc sulfate($ZnSO_4$) is a cationic precipitator, and its effect on protein precipitation is better than traditional organic reagents. Considering the precipitation efficiency and recovery, the co-treatment method of cationic precipitator ($ZnSO_4$) and organic precipitator (acetonitrile) was confirmed.

This method uses less samples and reagent, which ensures good removal efficiency of interference in complex matrix and obtains better sensitivity at the same time. The most important point is that this method is simple and fast, which only takes 5~10 mins to process a sample, saving at least 3~5 folds of time compared with traditional SPE method.

MATERIALS AND METHODS

Sample Preparation:

Only 50 µL of serum was needed, protein precipitator($ZnSO_4$ and acetonitrile) and internal standard were added, and 18 steroid hormones could be extracted from the sample at the same time through a simple vortex and centrifugation process. (Figure 1)

HPLC Conditions:

A Kinetex C18, 100x3.0 mm, 2.6 µm column at 40° C with a gradient of eluent A 0.5 mM NH_4F aqueous solution and eluent B ethanol was used at a flow rate of 450 µL/min. The injection volume was set to 15 µL. The liquid phase gradient time is 12 minutes.

MS/MS Conditions:

On the SCIEX Triple Quad™ 6500+ LC-MS/MS system with Turbo V™ source and Electrospray Ionization (ESI) probe was used. 18 steroid hormones were detected using 2 MRM transitions per compound to ensure quantitation and identification. The positive and negative ions were monitored simultaneously and *Scheduled* MRM™ mode was used.

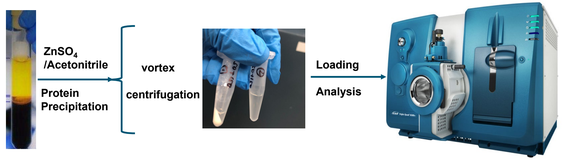

Figure 1. Pretreatment process of rapid protein precipitation method

RESULTS

Under the developed method, each compound obtains good peak shape and good response. The multi-pair isomers can also achieve baseline separation (Figure 2). The chromatogram of some compounds in human serum samples is shown in Figure 3, with good signal-to-noise ratio and good peak shape.

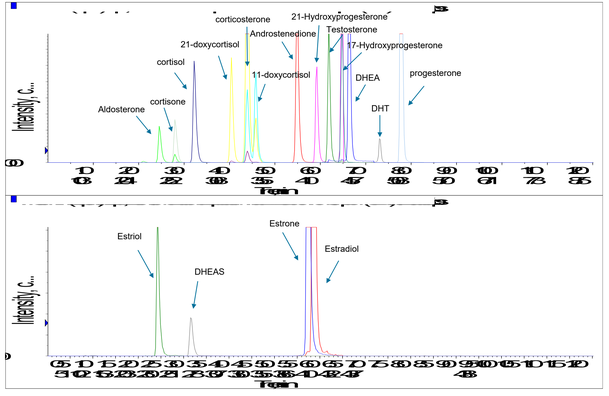

Figure 2. Detection of 18 hormones by LC-MS/MS

The results of standard curve and linear range of each substance to be measured are shown in Table1. All compounds have good linearity within their linear range, and the R value is greater than 0.995, which met the relevant requirements. The recovery of all compounds was in the range of 86.8%-113.8% when different concentrations of solution were added to serum from different human (Table 2). The results show the accuracy of this method.

Table 1. Standard curve and linear range of 18 hormones

Name	linear range	linear	R
17α-Hydroxyprogesterone	0.05 - 50	Y=0.22269x+0.00533	0.9951
Corticosterone	0.1-100	Y=0.04669x+0.00644	0.9955
Dihydrotestosterone;DHT	0.02-20	Y=0.09402x+0.00029	0.998
Pregnenolone	0.02-20	Y=0.04924x+0.00106	0.9987
Progesterone	0.05-100	Y=0.87480x+0.02038	0.9975
11-Doxycortisol	0.01-20	Y=0.64005x+0.00247	0.9974
21-Doxycortisol	0.02-20	Y=3.81310x+0.02050	0.9973
21-Hydroxyprogesterone	0.02-20	Y=1.52072x+0.01628	0.9969
Androstenedione	0.01-20	Y=1.71114x+0.00899	0.9968
Cortisone	0.05-100	Y=3.33320x+0.12860	0.9981
Testosterone	0.01-20	Y=1.51109x+0.00509	0.9967
Cortisol	0.05-100	Y=0.44718x+0.01197	0.9971
DHEA	0.25-100	Y=1.51278x-0.23593	0.9954
Aldosterone	0.01-20	Y=0.40852x-0.00197	0.9971
Estriol	0.02-20	Y=0.75346x+0.00389	0.9970
Estrone	0.02-20	Y=0.22695x+0.00025	0.9985
DHEAS	2.5-1000	Y=0.19739x+0.18901	0.9974
Estradiol	0.05-100	Y=0.87181x+0.00495	0.9952

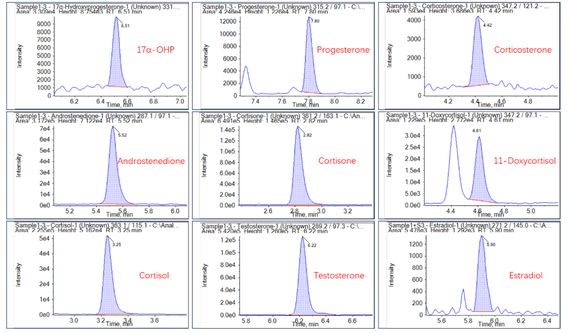

Figure 3. Chromatogram of hormones in Human Serum Sample (Part)

Table 2. Recovery of 18 hormones were added to different human serum

Analyte	R-L	R-M	R-H
17α-Hydroxyprogesterone	105.60%	102.70%	86.80%
Corticosterone	112.30%	96.90%	87.60%
Dihydrotestosterone	100.00%	104.70%	100.50%
Pregnenolone	100.00%	94.00%	105.00%
Progesterone	109.00%	95.80%	102.60%
11-Doxycortisol	106.30%	102.30%	100.40%
21-Doxycortisol	100.00%	104.50%	110.80%
21-Hydroxyprogesterone	94.70%	87.30%	86.80%
Androstenedione	104.10%	100.60%	98.30%
Cortisone	113.20%	88.50%	110.40%
Testosterone	100.60%	101.00%	97.70%
Cortisol	105.40%	105.80%	105.50%
DHEA	91.50%	106.80%	100.80%
Aldosterone	101.50%	94.10%	93.10%
Estriol	110.00%	90.00%	102.50%
Estrone	100.00%	103.40%	112.80%
DHEAS	113.80%	87.50%	107.70%
Estradiol	104.80%	90.50%	89.10%

CONCLUSIONS

A liquid chromatography-tandem mass spectrometry method for the accurate qualitative and quantitative detection of 18 kinds of hormones was established. The method has high sensitivity, good reproducibility and can meet the needs of clinical analysis. Protein precipitation pretreatment method is used for sample purification. Mass spectrometry detection parameters are optimized by SCIEX LC-MS/MS. In summary, this method has the following advantages:

- The lower limit of quantitation is pg/mL;
- Small sample size, only 50 µL sample can meet the analysis requirements;
- Pretreatment protein precipitation method is simple and fast, reducing the time of sample pretreatment;
- No need to purchase SPE consumables and equipment, saving cost;
- Positive and negative ion switching, *Scheduled* MRM™ test mode, simultaneous determination of multiple steroid hormones.

REFERENCES

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