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Original Article

Highly Sensitive Simultaneous Measurement of Steroid Hormone in Human Serum by Liquid Chromatography-electrospray Ionization Tandem Mass Spectrometry

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Abstract: A high sensitive simultaneous determination method of nine steroid hormones including Estrone (E1), estradiol (E2), testosterone (T), dihydrotestosterone (DHT), 17-hydroxyprogesterone (17OH-P4), androstenedione (AN), progesterone (P4), dehydroepiandrosterone (DHEA), and cortisol by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) was established in a positive mode using recently developed picolinyl derivatization. Steroid hormones were derived with picolinic acid and 2-methyl-6-nitrobenzoic anhydride, then purified by solid-phase extraction with InterSep SI cartridge. The LC-ESI-MS/MS method enhanced the specificity and sensitivity for E1, E2, T, DHT, DHEA, and cortisol. The method validation indicated that the limits of quantification for E1, E2, T, DHT, 17OH-P4, AN, P4, DHEA, and cortisol were 1, 1, 1, 2, 2, 2, 2, 20, and 100 pg/tube, respectively with acceptable accuracy and precision within ± 15%. The present method was applied to the measurement of nine steroid hormones in children's serums with high reliability and reproducibility.

Keywords: LC-ESI-MS/MS, steroid hormone, picolinyl derivatization, human serum, solid-phase extraction.

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1. Introduction

Steroid hormones, such as sex hormones and glucocorticoids play an important role in various cellular processes in the central nervous system, ranging from neurodevelopment to neurodegeneration [1]. Steroid hormones are estimated clinically to diagnose diseases associated with pathological process occurring in the adrenal gland, or other hormone-responsive organs [2]. The determination of steroid hormones can be carried out by various research methods. In the early 1940 ~ the 50s, steroids such as androgen and corticoid were first measured as Zimmerman and Porter-Silber methods on clinical chemistry [3]. Then gas chromatography-mass spectrometry (GS-MS) was developed to investigate the metabolism of steroids including androgen and cortisol metabolites in the 1960s [4, 5]. In 1969, Abraham developed the first radioimmunoassay (RIA) method to measure tracer estradiol-17β [6]. Following the first RIA, immunoassays (IAs) involved enzymatic assay were applied to determine other steroid hormones [7]. From the 1980s to the 1990s, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was introduced to analyze the level of steroids [8].

IAs were developed in parallel to MS and were generally used for analysis in clinical works due to their sensitivity, simplicity, and cost. Nevertheless, this method has several disadvantages, such as cross-reactivity with similar analytes, standardization of steroid hormone measurements between laboratories, and sensitivity issues [9]. In recent years, LC-MS/MS has been usually chosen for estimating steroid hormones in the clinical research labs because of shorter sample preparation than for GC-MS and increased specificity and sensitivity in comparison with IAs [2]. Among the hormone analysis methods, LC-MS/MS has widely been used for simultaneous quantification of steroid hormones in different biological samples through selected reaction monitoring (SRM) [10].

Bioanalysis involves the identification and quantification of a compound (drug) or their metabolite in several biological matrices (blood, plasma, serum, urine, saliva, feces, skin, hair, organ tissue). Validation of any assay is an important key to gain reliable results [11]. According to the Food and Drug Administration (FDA) guidelines, bioanalytical method validation encompasses some parameters such as calibration curve, selectivity, specificity, sensitivity, accuracy, precision, recovery, stability, and matrix effects [12]. Method validation can also be performed based on the European Medicines Agency (EMA) guideline [13]. Therefore, developing a LC-MS/MS assay for quantitative measurement of steroid hormone is essential.

Steroid hormones are compounds with significant biological activities at very low levels (nanomolar and even picomolar) through acting at intracellular receptors and nuclear receptors in the target organs such as some sex steroids (androgens and estrogens) [14]. Estrogen and androgen receptors are expressed in sex steroid-sensitive tissues during childhood, and consequently, prepubertal children are considerably influenced by the action of sex steroid hormones. Only a minor change of the concentration of sex steroids leads to a major variation in the entire activity of the involved hormone, which has a significant impact on phenotypes in children. Several reports illustrated that exposure to environmental pollutants and other endocrine disruptors have been adversely affected the development of children [15].

Sex steroids include estrogen and testosterone, which are primarily produced by the gonads. Estrogens play a vital role in development in both girls and boys. Estrogens are mainly produced by the ovaries in girls. There are three major forms of physiological estrogens in females including estrone, estradiol, estriol [16, 17]. Meanwhile, testosterone and DHT play an important role not only in the male reproductive system but in the endocrine system as well. As a result, nutritional energy-rich environments bring enormous benefits for children [18]. The concentration of steroid

hormones in children such as testosterone, estrone, and estradiol is very low. Thus, to improve sensitivity and accuracy, the derivatization method has been proposed.

The aim of this study is to develop a sensitive, reliable, and simultaneous method to quantity nine steroid hormones in human serum by using LC-ESI-MS/MS in a positive mode and picolinyl derivatization.

2. Experimental Method

2.1. Materials and Method

2.1.1. Standards

Estrone (E1), estradiol (E2), testosterone (T), dihydrotestosterone (DHT), 17-hydroxyprogesterone (17OH-P4), androstenedione (AN), progesterone (P4), dehydroepiandrosterone (DHEA), and cortisol were purchased from steroid company (USA).

2.1.2. Stable Isotope Steroids as Internal Standards

Estrone-2,3,4-$^{13}C_3$ (E1-$^{13}C_3$), estradiol-2,4,16,16-$^{2}H_4$ (E$_2$-d$_4$), testosterone-2,2,4,6,6-$^{2}H_5$ (T-d$_5$), DHT-16,16,17-$^{2}H_3$ (DHT-d3), 17-hydroxyprogesterone-2,2,4,6,6,21,21,21-$^{2}H_8$ (17-OHP-d$_8$), Progesterone-2,2,4,6,6,17,21,21,21-$^{2}H_9$ (P-d$_9$), androstenedione-2,3,4-$^{13}C_3$ (AN-$^{13}C_3$), DHEA-2,2,3,4,6,6-$^{2}H_6$ (DHEA-d$_6$), and cortisol-11,12,12-$^{2}H_4$ (cortisol-d$_4$) were purchased from CD isotopes Inc (Quebec Canada) and Otuka Company (Japan).

2.1.3. Picoline acid Anhydride Reagent

Picolinic acid (PA), 4-dimethylaminopyridine (DAP), 2-methyl-6-nitrobenzoic anhydride (MNBA) and triethylamine (TEA) were acquired from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

2.1.4. Picolinyl Derivatization Method (Picolinyl Anhydrous Reagent)

Mixed anhydride reagent was added 10 mg of DAP, 50 mg of MNBA, and 30 mg of PA in dried tetrahydrofuran (THF) 1 mL. By shaking for 10~20 min, the picolinic anhydrous suspension appeared as white crystallization in THF.

The mixed anhydride reagent (50~75 ul) and triethylamine (20 uL) respectively were added to the dried sample. The reaction mixture was allowed to stand at room temperature for 30 min. Derivatives were purified by solid-phase extraction according to similar manner as described serum analytical method.

2.1.5. Solid Cartridge Column and Its Direction

InterSep Pharma and InterSep SI cartridges were purchased from GL Sciences (Tokyo, Japan).

2.1.6. Solvents

LC-MS grade acetonitrile (ACN) and methanol (MeOH), formic acid, ethanol (EtOH), ethyl acetate (EtOAc), tetrahydrofuran (THF), acetone, and n-hexane were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Hormone Extract and Purification from Serum

Human serum (0.2 ~ 0.4 ml) was diluted with water to 1 mL and added to mixture the internal standard (IS) (0.1 ml) (cortisol-d$_4$, 1000 pg; AN-$^{13}C_3$, 17OH-P4-d$_8$, P4-d$_9$: 200 pg; T-d$_5$, DHEA-d$_6$, DHT-d$_3$, E1-$^{13}C_3$, E2-d$_4$: 100 pg in MeOH), 5% $NaHCO_3$ (1 ml). EtOAc (2.5 ml) was added and then shaken for 5 min. The mixture was centrifuged for 5 min at 3,000 × g. The aqueous phase was frozen and the organic layer was separated. The obtained organic layer was evaporated to dryness at 40 °C with centrifuged evaporator under vacuum. The extract was dissolved with MeOH (0.25 ml) and diluted with water (1 ml). The sample was transferred onto InterSep Pharma cartridge which had been successively washed with MeOH (3 ml) and water (6 ml). After washing with 30% MeOH-water (3 ml), the steroids fraction was eluted with 80% ACN-water (1.5 ml). After evaporated with centrifugal evaporator at 40°C under vacuum, the sample was reacted with reagent mixed anhydride reagent (0.1 ml) and TEA (0.02 ml) were added to the dried sample. The reaction mixture was allowed to stand for 30 min at room

temperature. The reaction mixture was diluted with hexane (1 ml), and then the sample was transferred onto InterSep SI cartridge which had been successively washed with acetone (3 ml) and hexane (3 ml). After washing the cartridge with hexane-EtOAc (3:1, v/v, 2.5 ml), the derivative was charged eluted with acetone-ACN (8:2, v/v, 2 ml), and then the eluate was evaporated to dryness at 40ºC under vacuum. The residue was dissolved in ACN-water-formic acid (60:40:0.1, v/v, 0.1 ml) and a part of the solution was subjected to LC–ESI-MS/MS.

2.3. LC-ESI-MS/MS

LC-MS/MS was performed using Shimazu 8060 triple stage quadrupole mass spectrometer equipped with a positive electrospray ionization (ESI) source (Shimadzu, Kyoto, Japan) and a Shimadzu HPLC system (LC-30AD pump, SIL-30AC auto-sampler, LC-20AB prominence liquid chromatography, CTO-20A column oven, Shimadzu, Kyoto, Japan). The analytical column was a Kinetex C18 (1.7 μm, 2.1×150 mm, Phenomenex, USA), used at 50ºC. The mobile phase consisting of 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) was used with a gradient elution of A:B = 50:50 (0-4.5 min), 50:50 to 20:80 (4.5-5.0 min), 20:80 (5.0-6.0 min), 20:80 to 0:100 (6.0-6.1 min), 0:100 (6.1-7.0 min), 0:100 to 50:50 (7.0-7.1 min) and 50:50 (7.1-8.0 min) at a flow rate of 0.45 ml/min. The following ESI conditions were used: nebulizing gas flow, 2 L/min; heating gas flow, 8 L/min; interface temperature, 400 ℃; DL temperature, 200 ℃; heat block temperature 400 ℃, drying gas flow 6 L/min.

For the quantification of steroids, the estimation ions were as follows (m/z): cortisol-PA and cortisol-d_4-PA, 469.1/267.2 and 472.1/313.2; AN and AN-$^{13}C_3$, 287.1/ 97.1 and 290.1/100.1; 17OH-P4 and 17OH-P4-d_8, 331.1/ 97.0 and 339.2/113.2; E1-PA and E1-$^{13}C_3$-PA, 376.1/157.1 and 379.1/160.1; P4 and P4-d_9, 315.2/ 97.1 and 324.2/100.1; T-PA and T-d_5-PA, 394.1/253.2 and 399.1/258.2; DHEA-PA and DHEA-d_6-PA, 394.1/175.1 and 400.2/259.2; DHT-PA and DHT-d_3-PA, 396.1/255.2 and 399.1/206.2; E2-2PA and E2-d_4-2PA, 483.1/264.1 and 487.0/266.1.

2.4. Method Validation

2.4.1. Calibration Curve

A series of steroid-free serum samples (0.4 mL) or water was spiked with cortisol (100, 500, 2000, 10000, 25000, 50000 pg); DHEA (4, 20, 80, 400, 1000, 2000 pg); AN, 17OH-P4, P4, DHT (2, 10, 40, 200, 500, 1000 pg each); E1, E2, T (1, 5, 20, 100, 250, 500 pg each) and with internal standards (cortisol-d_4, 1000 pg; AN-$^{13}C_3$, 17OH-P4-d_8, P4-d_9: 200 pg; T-d_5, DHEA-d_6, DHT-d_3, E1-$^{13}C_3$, E2-d_4: 100 pg), which was then pre-treated, derivatized and subjected to LC-MS/MS. The calibration curves were constructed by plotting the peak area ratio of steroid to its IS(Y) versus concentration of steroid (X). Subsequently, a 1/x weighting linear regression was performed for constructing the calibration curve.

2.4.2. Matrix Effect and Specificity

Human serum samples (0.2, 0.3, 0.4 and 0.4 ml spiked with cortisol, 500 pg; DHEA, 20 pg; AN, 17OH-P4, P4, DHT: 10 pg; T, E1, E2: 5 pg in 0.1 ml of MeOH) from individual volunteers were spiked with internal standards (cortisol-d_4, 1000 pg; AN-$^{13}C_3$, 17OH-P4-d_8, P4-d_9: 200 pg; T-d_5, DHEA-d_6, DHT-d_3, E1-$^{13}C_3$, E2-d_4: 100 pg /0.1 ml MeOH). The samples were added MeOH (0.1 ml), 5% $NaHCO_3$ (1 ml), EtOAc (2.5 ml) and then shaken for 5 min. These samples were subjected to extraction, purification, picolinyl derivatization, purification, and LC-ESI-MS/MS analysis as described above. The linear relationships between the serum volumes and measured values of steroids were examined for each serum. In addition, the recovery rates of added steroids were calculated.

2.4.3. Assay Precision and Accuracy

The intra-assay precision was assessed using hormone-free serum spiked with a known amount of 9 kinds of hormones (cortisol: ranged from 100 to 50000 pg; DHEA: ranged from 4 to 2000 pg; AN, 17OH-P4, P4, DHT: ranged from

2 to 1000 pg; E1, E2, T ranged from 1 to 500 pg) and internal standards (cortisol-d_4, 1000 pg; AN-$^{13}C_3$, 17OH-P4-d_8, P4-d_9: 200 pg; T-d_5, DHEA-d_6, DHT-d_3, E1-$^{13}C_3$, E2-d_4: 100 pg) on 1 day (n=5). These samples were mixed with 5% $NaHCO_3$ (1 ml), EtOAc (2.5 ml), and then extracted. The extracts were purified and derivatized. The derivatives were purified and then analyzed by LC-ESI-MS/MS according to the same manner as described above. The recovery rate was measured as described above.

The inter-assay precision was assessed by determining these samples over 3 days. The recovery rate was measured as described above. The precision was determined at the relative standard deviation.

Table 1. Liquid chromatographic, positive-ESI-mass spectral, and tandem mass spectral data of steroid or its picolinyl derivatives and its internal standards

Steroid	LC	Mass		ESI-MS; MS/MS data					SRM transition (CE, eV)	
	TR (min)	(M)	M+1	Major product ion (m/z)					Estimate steroid	Internal standard
A	1.395	468.1	469.1	309.2	291.1	267.0	249.0		469.1/267.2 (26)	472.1/313.2 (22)
B	2.124	330.1	331.1	286.1	213.1	109.1			331.1/97.0 (26)	339.2/113.2 (32)
C	4.085	314.2	315.2	123.1	109.2	97.1			315.2/97.1 (23)	324.2/100.1 (23)
D	5.768	393.1	394.1	271.2	253.2	227.2	175.1		394.1/175.1 (23)	400.2/259.2 (22)
E	4.387	393.1	394.1	271.2	253.2	211.3	197.0		394.1/253.2 (20)	399.1/258.2 (20)
F	5.870	395.1	396.1	255.2	213.2	203.2	199.1	173.0	396.1/255.2 (24)	399.1/206.2 (23)
G	2.090	286.1	287.1	97.1					287.1/ 97.1 (24)	290.1/100.1 (23)
H	3.749	375.1	376.1	157.1	106.1				376.1/157.1 (22)	379.1/160.1 (22)
I	5.965	482.1	483.1	264.1	238.1	220.1	159.1	124.1	483.1/264.1 (24)	487.0/266.1 (23)

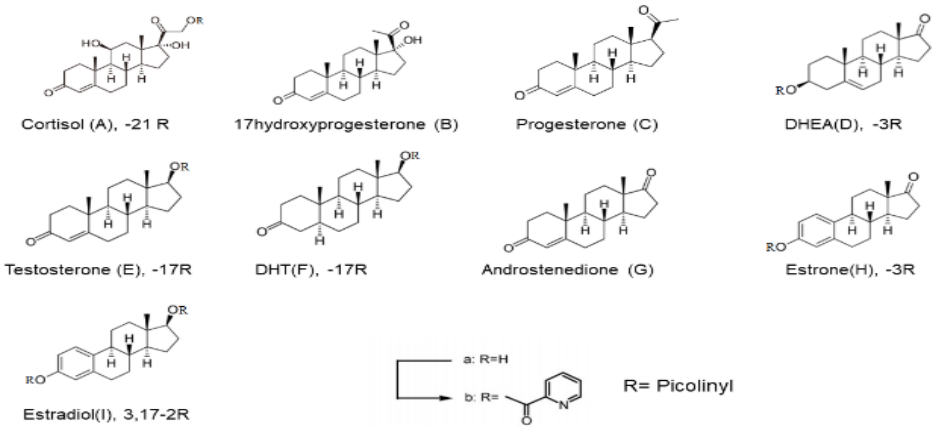

Figure 1. Simultaneous determination of steroid hormone in children serum by LC-tandem mass spectrometry.

2.4.4. Limits of Quantification (LOQs)

The steroid-free umbilical cord serum was used to determine the LOQs. The LOQ was defined at the lowest concentration on the calibration curves (<15%) and with at least 5 times the response compared to the blank response.

3. Results

3.1. HPLC-ESI Mass Spectrometry

Table 1 summarized the liquid chromatographic and positive-ESI mass spectral data and SRM transitions of the native and picolinoyl derivatives of Steroids (Figure 1; A-I) and internal standards. The picolinyl derivative compounds exhibited well-shaped chromatographic peaks shown in Figure 2.

3.2. Calibration Curves

Calibration curves were constructed for assay compounds using each of these stable isotope compounds.

Each calibration curve, as determined by linear regression analysis, exhibited good linearity with a regression coefficient of more than 0.995 as shown in Table 2.

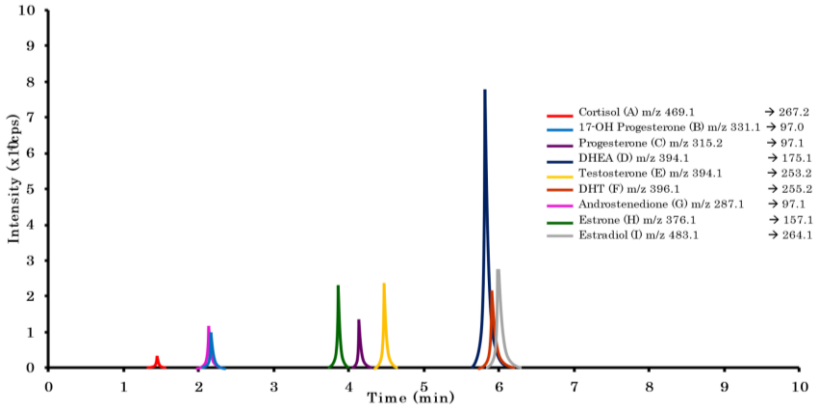

Figure 2. Typical SRM chromatograms of picolinyl derivatives of Steroids (A: 2000 pg, B, C, F, G: 40 pg, D: 80 pg, E, H, I: 20 pg, as injected amounts).

Table 2. Calibration parameters for each steroid and its derivatives

Steroid	Equation parameter						Calibration range	Correlation coefficient	Internal standard
	Slope			Intercept			pg/ injection	(r)	
A	0.000170	±	7.803E-06	0.001097	±	0.004296	100-50000	0.9998	Cortsiol-d4
B	0.006938	±	9.074E-04	0.021130	±	0.019081	2-1000	0.9995	17-hydroxyprogesterone-d8
C	0.005205	±	9.982E-04	0.000362	±	0.000560	2-1000	0.9999	Progesterone-d9
D	0.009124	±	5.966E-04	0.010829	±	0.008386	4-2000	0.9999	DHEA-d6
E	0.009617	±	6.190E-04	0.009844	±	0.019840	1-500	0.9999	Testosterone-d5
F	0.039043	±	1.365E-03	- 0.000309	±	0.017638	2-1000	0.9992	DHT-d3
G	0.003675	±	5.153E-05	0.008823	±	0.003104	2-1000	0.9998	Androstenedione-13C3
H	0.010697	±	8.940E-04	-0.002213	±	0.001329	1-500	0.9987	Estrone-13C3
I	0.009188	±	8.202E-05	0.000946	±	0.001309	1-500	0.9994	Estradiol-d4

3.3. Assay Accuracy and Precision

To determine the intra- and inter accuracy and precision, 4 different levels of the low, medium and high QC samples spiked with a known amount of 9 kinds were estimated. The results were shown in Table 3.

Both accuracy and precision for intra-assay and inter-assay were <15% at all levels except for low levels, and <20% at low levels. The intra-day accuracy and precision for added 9 kind steroids ranged from XX %~ YY % and AA % to bb%, respectively. Also, inter-day accuracy and precision were XX %~ YY % and AA % to bb%, respectively.

Table 3. Intra-day and inter-day Assay accuracy and precision

Steroid	Intra assay (n=5)						Inter assay (n=5)					
	Added	Found			Accuracy	RSD	Added	Found			Accuracy	RSD
	(pg)	(pg/mL)			(%)	(%)	(pg)	(pg/mL)			(%)	(%)
A	100	101.29	±	0.92	101.3	0.9	100	98.28	±	4.86	98.3	4.9
	500	485.22	±	29.80	97.0	6.1	500	499.30	±	22.45	99.9	4.5
	2000	1909.02	±	119.26	95.5	6.2	2000	2045.90	±	141.29	102.3	6.9
	10000	9824.67	±	668.99	98.2	6.8	10000	10114.70	±	598.33	101.1	5.9
B	2	2.22	±	0.14	110.8	6.3	2	1.98	±	0.05	99.0	2.4
	10	9.89	±	1.15	98.9	11.6	10	10.67	±	0.86	106.7	8.1
	40	44.30	±	2.11	110.8	4.8	40	40.93	±	2.72	102.3	6.6
	200	202.18	±	9.28	101.1	4.6	200	197.38	±	9.50	98.7	4.8
C	2	1.95	±	0.19	97.6	9.9	2	1.96	±	0.18	98.0	9.3
	10	9.68	±	0.73	96.8	7.5	10	9.72	±	0.51	97.2	5.2
	40	38.88	±	1.32	97.2	3.4	40	40.04	±	2.88	100.1	7.2
	200	209.82	±	0.70	104.9	0.3	200	192.85	±	16.04	96.4	8.3
D	20	19.64	±	0.91	98.2	4.6	20	20.29	±	0.92	101.5	4.6
	80	78.19	±	0.97	97.7	1.2	80	75.44	±	6.26	94.3	8.3
	400	393.72	±	5.39	98.4	1.4	400	388.59	±	26.92	97.1	6.9
	1000	997.13	±	4.92	99.7	0.5	1000	978.11	±	15.88	97.8	1.6
E	1	0.91	±	0.04	90.6	4.8	1	0.98	±	0.05	98.2	5.1
	5	4.71	±	0.05	94.1	1.1	5	4.53	±	0.31	90.7	6.9
	20	19.20	±	0.77	96.0	4.0	20	19.03	±	1.50	95.2	7.9
	100	98.40	±	2.78	98.4	2.8	100	98.50	±	3.03	98.5	3.1
F	2	1.94	±	0.08	96.9	3.9	2	1.87	±	0.16	93.5	8.8
	10	9.06	±	0.35	90.6	3.9	10	9.24	±	0.19	92.4	2.1
	40	36.22	±	1.22	90.5	3.4	40	36.90	±	2.47	92.3	6.7
	200	190.28	±	1.77	95.1	0.9	200	195.13	±	8.16	97.6	4.2
G	2	1.95	±	0.17	97.6	9.0	2	2.02	±	0.04	101.2	2.0
	10	10.04	±	0.72	100.4	7.1	10	9.87	±	0.65	98.7	6.6
	40	39.33	±	0.66	98.3	1.7	40	39.41	±	0.79	98.5	2.0
	200	198.66	±	2.28	99.3	1.1	200	198.63	±	6.12	99.3	3.1
H	1	0.94	±	0.05	93.5	5.0	1	0.97	±	0.10	97.4	10.2
	5	4.57	±	0.01	91.3	0.2	5	4.70	±	0.63	93.9	13.5
	20	18.49	±	0.59	92.4	3.2	20	20.01	±	2.18	100.1	10.9
	250	241.40	±	0.70	96.6	0.3	250	257.64	±	5.15	103.1	2.0
I	1	0.99	±	0.13	98.6	13.0	1	0.98	±	0.02	98.0	1.8
	5	4.60	±	0.55	92.0	11.9	5	4.40	±	0.12	88.0	2.7
	20	19.44	±	1.39	97.2	7.1	20	18.46	±	0.71	92.3	3.8
	250	238.10	±	8.62	95.2	3.6	250	245.13	±	11.02	98.1	4.5

Table 4. Proportionality between serum volumes and levels of estimated several steroids in children

Matrices		Cortisol			17-OH Progesterone			DHEA		
Serum	Vol	Added	Found	Accuracy	Added	Found	Accuracy	Added	Found	Accuracy
	mL	(pg)	(pg)	(%)	(pg)	(pg)	(%)	(pg)	(pg)	(%)
S-1	0.2	0	2945		0	11.9		0	22.7	
	0.3	0	4570		0	18.2		0	34.1	
	0.4	0	6002		0	23.7		0	47.2	
S-2	0.2	0	2623		0	13.1		0	92.3	
	0.3	0	3870		0	21.3		0	130.5	
	0.4	0	5107		0	29.7		0	165.9	
S-1	0.4	500	6455	90.7	10	33.8	101.5	20	66.5	96.5
S-2	0.4	500	5532	85.0	10	41.3	116.4	20	183.1	85.9
S-3	0.4	500	5750	111.1	10	49.4	104.9	20	140.5	82.8
S-4	0.4	500	6528	81.1	10	46.45	98.0	20	264.6	85.9
Mean				92.0			105.2			87.8
SD				13.3			8.0			6.0
Matrices		Estrone			Estradiol			Androstenedione		
Serum	Vol	Added	Found	Accuracy	Added	Found	Accuracy	Added	Found	Accuracy
	mL	(pg)	(pg)	(%)	(pg)	(pg)	(%)	(pg)	(pg)	(%)
S-1	0.2	0	0.4		0.2	0.26		0.2	5.31	
	0.3	0	0.71		0.3	0.70		0.3	8.29	
	0.4	0	0.85		0.4	0.68		0.4	11.73	
S-2	0.2	0	0.81		0.2	0.47		0.2	13.45	
	0.3	0	0.91		0.3	0.77		0.3	20.16	
	0.4	0	1.36		0.4	0.78		0.4	26.02	
S-1	0.4	5	5.55	94.0	5	5.62	98.8	10	20.80	90.7
S-2	0.4	5	6.02	93.3	5	5.40	92.4	10	35.08	90.7
S-3	0.4	5	5.60	90.1	5	5.50	95.7	10	30.34	88.4
S-4	0.4	5	6.26	99.3	5	5.65	98.3	10	51.60	99.4
Mean				94.2			96.3			92.3
SD				3.8			2.9			4.9
Matrices		Testosterone			Dihydrotestosterone			Progesterone		
Serum	Vol	Added	Found	Accuracy	Added	Found	Accuracy	Added	Found	Accuracy
	mL	(pg)	(pg)	(%)	(pg)	(pg)	(%)	(pg)	(pg)	(%)
S-1	0.2	0	1.77		0	1.87		0.2	2.10	
	0.3	0	2.43		0	2.90		0.3	3.37	
	0.4	0	3.37		0	3.62		0.4	5.00	
S-2	0.2	0	5.60		0	3.89		0.2	1.95	
	0.3	0	8.25		0	6.053		0.3	3.60	
	0.4	0	11.45		0	8.026		0.4	4.32	
S-1	0.4	5	8.30	98.6	10	14.14	105.2	10	12.72	77.2
S-2	0.4	5	16.57	102.3	10	20.169	121.4	10	13.99	96.7
S-3	0.4	5	14.46	81.8	10	18.087	104.8	10	16.08	101.8
S-4	0.4	5	17.82	95.0	10	23.489	108.4	10	17.39	108.8
Mean				94.4			109.9			96.1
SD				8.9			7.8			13.5

Table 5. The serum hormone levels in 9-year-old male and female children

Steroid	Units	Subjects	Serum levels		
		n	Mean	±	SD
Cortisol	ng/mL	43	25.59	±	16.35
17-hyroxyprogesterone	pg/mL	43	218.24	±	176.52
Progesterone	pg/mL	43	28.15	±	24.18
Dehydroepiandrosterone	pg/mL	43	746.78	±	472.55
Testosterone	pg/mL	43	57.04	±	51.49
Dihydrotestosterone	pg/mL	43	31.19	±	19.87
Androstenedione	pg/mL	43	200.06	±	175.56
Estrone	pg/mL	43	3.43	±	6.69
Estradiol	pg/mL	43	5.97	±	10.89

3.4. Assay Specificity

As shown in Table 4, concentrations analyzed for each volume of serum (0.2~0.4 mL) were good proportionality. Also, these samples (S1~S4) with known amount steroid standard were well agreement to these of theoretical values in concentration with excellent accuracy of aa ~ bb %.

3.5. Limit of Quantification

The limit of quantification values (LOQ) for E1, E2, T, DHT, 17OH-P4, AN, P4, DHEA, and cortisol were 1, 1, 1, 2, 2, 2, 2, 20, and 100 pg/tube, respectively.

3.6. Biomedical application

The present method was applied to the analysis of 9 kinds of steroid hormones in children's serums. The results were shown in Table 5.

4. Discussion

In this study, the simultaneous quantification of 9 kinds of steroid hormones in children was developed using LC-MS/MS method. DHEA, DHT, and estradiol having hydroxy groups are estimated highly sensitive to lead to picolinoyl derivative. The limits of quantification for E1, E2, T, DHT, 17OH-P4, AN, P4, DHEA, and cortisol were 1, 1, 1, 2, 2, 2, 2, 20, and 100 pg/tube, respectively with acceptable accuracy and precision (error < 15%) shown in Table 3.

The concentrations of androgens (T and DHT) and estrogen (E1 and E2) in human serum are very low and change with age and sex. Blood T (30-1640 pg/ml) and DHT (30-70 pg/ml) levels [19] in children were significantly lower than in adults (T: 620-6000 pg/ml [20], DHT: 100-950 pg/ml [21]). Furthermore, in contrast to females, serum levels of T and DHT in males saw a considerable increase [22]. Meanwhile, serum concentrations of E1 and E2 also were different among a range of ages, and E1 and E2 levels measured in males were significantly lower when compared with females. Especially, the serum levels of E1 and E2 in males aged 4-8 were very small, approximately 0.3 – 5.4 pg/ml [23].

The level of steroid hormones in blood has usually been estimated using radioimmunoassay or enzyme immunoassay. Nevertheless, poor accuracy and specificity due to the cross-reactivity of antisera have been observed in the immunoassay method [24]. Moreover, the limit of quantification of some LC-MS/MS methods was quite high [25]. This leads to insufficient sensitivity for measuring steroid hormones in samples from children because of low concentration.

In order to improve these limitations, using LC–ESI-MS/MS combined with the picolinyl derivatization increases the accuracy and specificity of analytical steroid hormones. The present method was applied to the measurement

of E1, E2, T, DHT, 17OH-P4, AN, P4, DHEA in human serum. As shown in Table 5, the median E2 concentration was in general significantly higher than the median E1 concentration. The serum DHT/T ratio was roughly 0.55, which was higher considerably than the normal blood level of DHT/T ratio (approximately 0.1-0.2). These results illustrated that there was a correlation between deformity in children and another biosynthesis route thought no testosterone. Therefore, the measurement of serum steroid hormones may be useful clinically for the diagnosis of some diseases.

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