Research Article

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A practical ID-LC-MS/MS method for the most commonly analyzed steroid hormones in clinical laboratories

Klinik laboratuvarlarda en yaygın olarak analizi yapılan steroid hormonlar için pratik bir ID-LC-MS/MS metodu

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Abstract

Background: Analysis of steroid hormones rapidly and reliably remains a challenge in clinical laboratories as this plays an important role in evaluation of many endocrine disorders. The aim of this study was to create a steroid profiling panel by using a liquid chromatography tandem mass spectrometry (LC-MS/MS) method which was composed of the most commonly analyzed steroid hormones in clinical laboratories.
Materials and methods: Protein precipitation was performed for sample preparation. Ultra performance liquid chromatography (UPLC) system and an analytical column with C18 selectivity was chosen for chromatographic seperation. Atmospheric pressure chemical ionization (APCI) ion source was preferred for ionization, and tandem MS with triple quadrupole was used. MS scan was performed using the selected reaction monitoring mode in positive polarity. During the method validation process, test performance was evaluated for each steroid hormone, and 40 serum samples were used for method comparison with immunoassays available in our core laboratory.
Results: An isotope dilution (ID)-LC-MS/MS method was developed, in which 13 steroids can be analyzed in the same run. Test performance was quite good for the 11 steroids (cortisol, DHEA, DHEAS, total testosterone, progesterone, androstenedione, 11-deoxycortisol, cortisone, corticosterone and dihydrotestosterone) while estradiol and aldosterone performance was suboptimal considering the precision and trueness.
Conclusion: This ID-LC-MS/MS method would be useful in clinical laboratories, especially for the immunoassays having insufficient test performance and when checking for interferences in available immunoassays.

Keywords: LC-MS/MS; Immunoassay; Steroid; Method validation; Method comparison.

Öz

Amaç: Steroid hormonların hızlı ve güvenilir bir şekilde analiz edilmesi, birçok endokrin bozukluğun değerlendirilmesinde önemli bir rol oynadığı için, klinik laboratuvarlarda bir sorun olmaya devam etmektedir. Bu çalışmanın amacı, LC-MS/MS tekniğini kullanarak, klinik laboratuvarlarda en sık analiz edilen steroid hormonlardan oluşan bir steroid profil paneli oluşturmaktı.
Materyal ve Metot: Örnek hazırlığı için protein çöktürmesi yapıldı. Kromatografik ayırma için UPLC sistemi ve C18 seçiciliği olan bir analitik kolon seçildi. İyonlaştırma için APCI iyon kaynağı tercih edildi ve üçlü kuadrupole sahip tandem MS kullanıldı. MS taraması, seçilmiş reaksiyon izleme modu kullanılarak, pozitif polaritede gerçekleştirildi. Metot validasyonu işlemi sırasında, her bir steroid hormonu için test performansı değerlendirildi ve merkez laboratuvarımızdaki mevcut immün-ölçümlerle metot karşılaştırması için 40 serum örneği kullanıldı.

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Bulgular: Aynı çalışma içerisinde 13 steroidin analiz edilebildiği bir ID-LC-MS/MS metodu geliştirildi. Test performansı, 11 steroid için (kortizol, DHEA, DHEAS, total testosteron, progesteron, androstenedion, 11-deoksikortizol, kortizon, kortikosteron ve dihidrotestosteron) oldukça iyiydi; ancak östradiol ve aldosteron performansı, kesinlik ve doğruluk göz önüne alındığında suboptimaldi.
Sonuç: Bu ID-LC-MS/MS metodu; klinik laboratuvarlarda, özellikle yetersiz test performansına sahip olan immün-ölçümler için ve mevcut immün-ölçümlerdeki interferansları kontrol etmek için yararlı olacaktır.

Anahtar kelimeler: LC-MS/MS; Immün-ölçüm; Steroid; metot validasyonu; Metot karşılaştırma.

Introduction

Steroids; which are derived from cholesterol and mostly synthesized in adrenal cortex, placenta and gonads; are biological molecules functioning in regulation of many metabolic activities in organism [1]. Many endocrinological pathologies emerge because of the defects in steroid biosynthesis resulting in excess or insufficient secretion of steroids [2]. Analysis of steroid hormones in clinical laboratories plays a decisive role in diagnosis and monitoring of these diseases.

Immunoassays are the most commonly used methods for steroid analysis in clinical laboratories. In the past, radio immunoassay (RIA) methods were commonly used, but today automated instruments employing chemiluminescence immunoassay (CLIA) are widely used [3]. The major drawback of this method is the lack of specificity. The antibodies used in this methods may cross-react with other steroids or other similar molecules, and these intereferences result in serious problems [4]. Additionally, some of the commercially available kits and methods have insufficient test performance, considering the precision and trueness. Another method for steroid analysis is GC-MS which is considered as the gold standard [5]. However, this method can not be used in routine clinical laboratories because of laborious sample pretreatment steps and long analysis times. Today, liquid chromatography tandem mass spectrometry (LC-MS/MS) use is getting wider in steroid analysis [6].

The aim of this study was to create a steroid profiling panel which was composed of the most commonly analyzed steroid hormones in clinical laboratories, and had a good test performance considering the precision, trueness, analytical sensitivity and specificity.

Materials and methods

An isotope dilution (ID) LC-MS/MS method was developed, in which 13 steroids (aldosterone, corticosterone, cortisol, cortisone, 11-deoxycortisol, androstenedione, DHEA: dehydroepiandrosterone, DHEAS: dehydroepiandrosterone sulfate, DHT: dihydrotestosterone, E2: estradiol, 17α-OH progesterone, progesterone, testosterone) can be analyzed in the same run. Validated calibrators (6 point calibrators plus blank) for MS and internal standard (IS) mix (Chromsystems, Gräfelfing, Germany) were used in this study. These calibrators are serum-based standards which means they include protein to avoid matrix effect. The content of IS mix (Chromsystems, Gräfelfing, Germany) was aldosterone-d4, corticosterone-d8, cortisol-d4, cortisone-d8, 11-deoxycortisol-d5, androstenedione-d7, DHEA-d5, DHEAS-d6, DHT-d3, E2-d5, 17α-OH progesterone-d8, progesterone-d9, testosterone-d3. In this method; protein precipitation was performed for sample preparation, including the calibrators. The precipitant solution was prepared by mixing 0.3 M zinc sulfate solution ($ZnSO_4$), methanol and IS. Composition of

Table 1: LC conditions, flow gradient and tandem MS conditions.

LC conditions			
Injection volume	25 µL		
Sampler temperature	15°C		
Column flow	0.350 mL/min		
Column oven	35°C		
Analysis time	11 min		
Flow gradient	**Time (min)**	**Mobile phase A (%)**	**Mobile phase B (%)**
	0.000	50	50
	0.000	50	50
	4.000	0	100
	7.000	0	100
	7.000	50	50
	11.000	50	50
MS/MS conditions			
Ion source			APCI
Capillary temperature (°C)			400
Vaporizer temperature (°C)			300
Sheath gas pressure (Arb)			35
Aux gas pressure (Arb)			10
Discharge current (µA) (positive polarity)			4.5
Collision gas pressure (mTorr)			1.5
Vacuum (Torr)			2.1×10^{-5}
MS scan mode			SRM
Cycle time (s)			1.500
Analysis time (min)			11

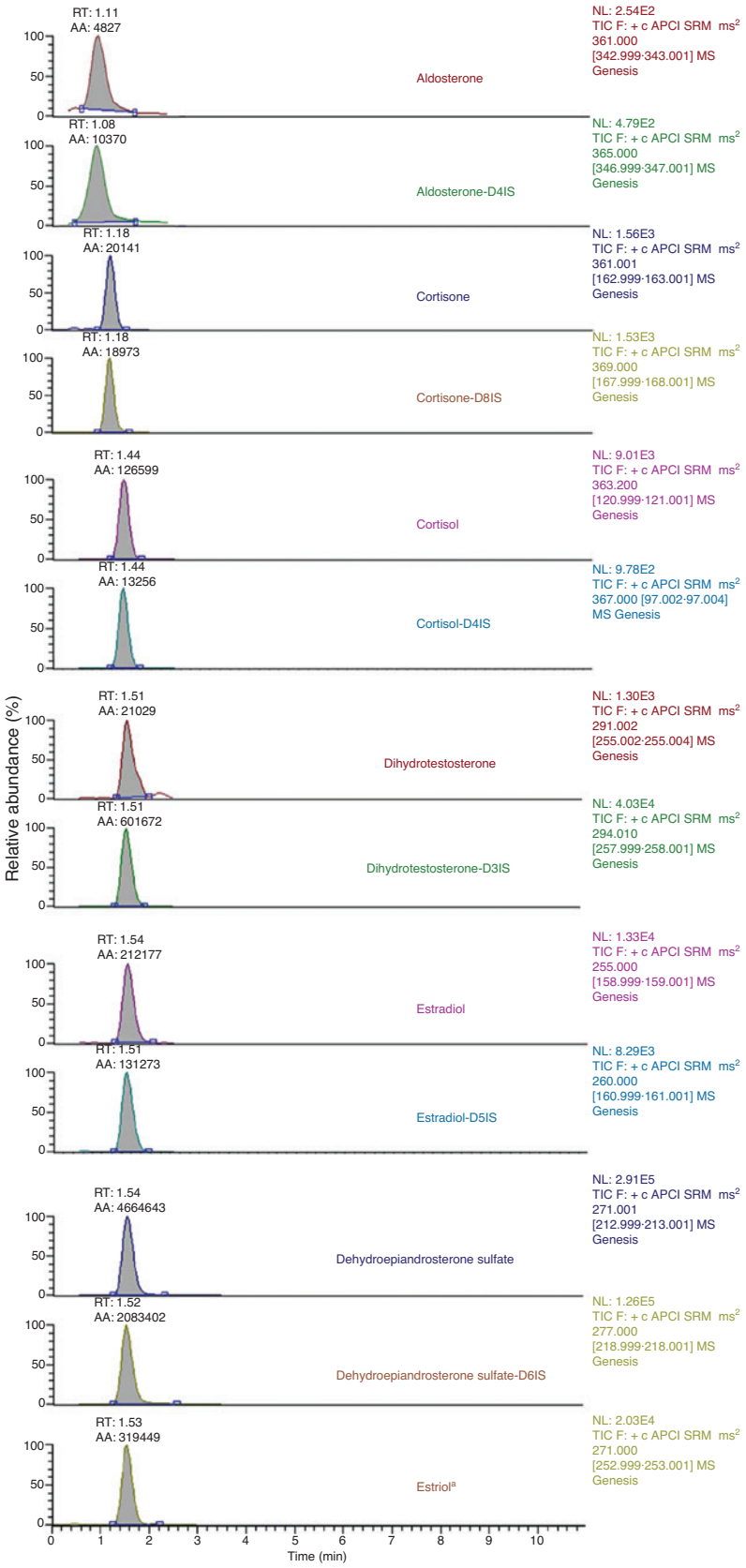

Figure 1: SRM scan of all steroids and their stable isotope standards.
(A) Chromatograms of the first 6 steroid hormones and their stable isotope dilutions eluting from the system ([a]E3 was excluded from validation process because its calibrator was not approved for MS/MS analysis.). (B) Chromatograms of the last 7 steroid hormones and their stable isotope dilutions eluting from the system.

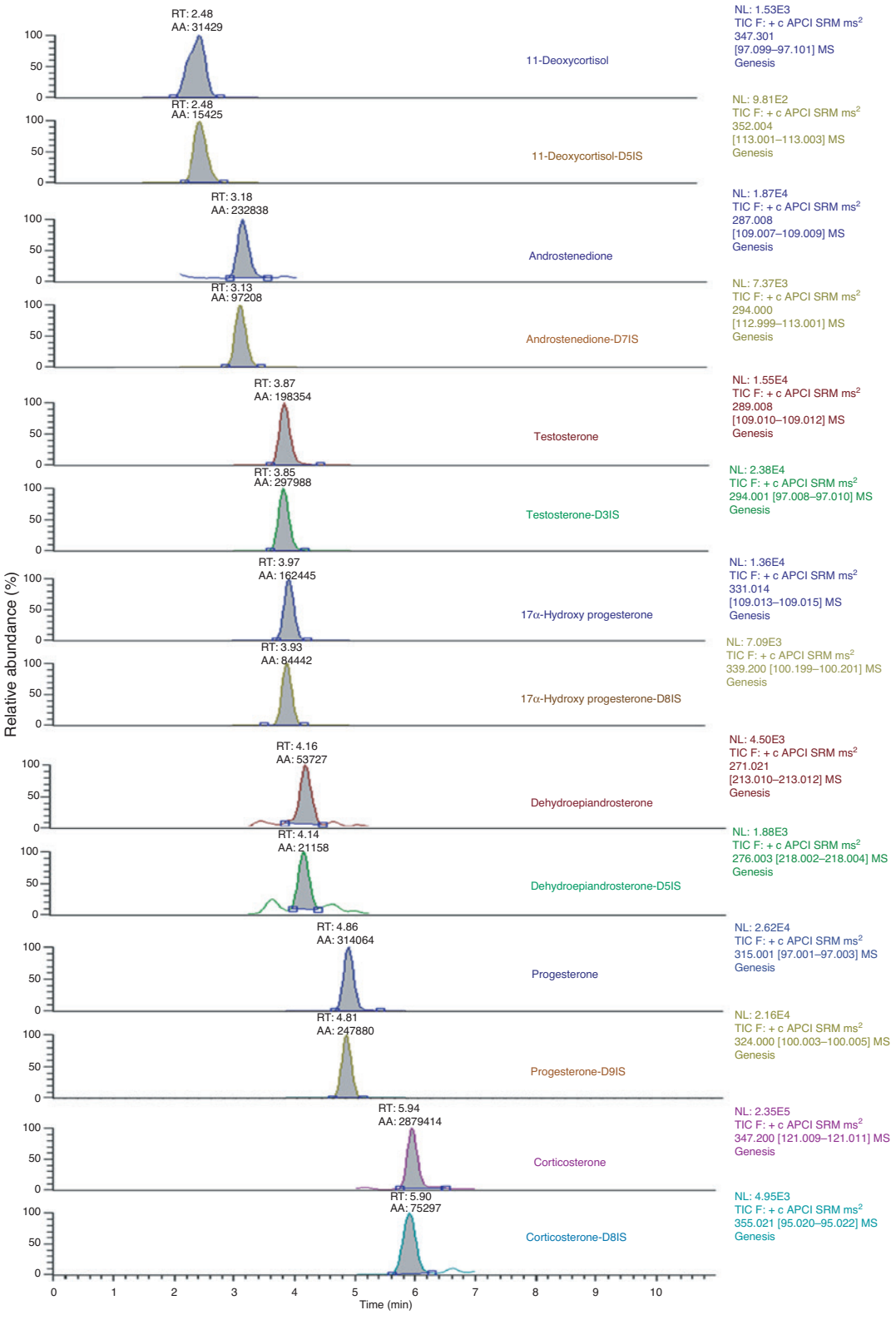

Figure 1 (continued)

the precipitant solution was IS:$ZnSO_4$:Methanol 15:20:65% (v:v:v). One hundred microliters sample and 200 µL precipitant solution were mixed and centrifuged. Supernatant part was transferred to plates and analyzed.

Ultra performance liquid chromatography system (Thermo Scientific Dionex UltiMate 3000 Rapid Separation LC System UHPLC+Focused, San Jose, CA, USA) and an analytical column with C18 selectivity (Thermo Scientific, Hypersil GOLD UHPLC Column, 50×2.1 mm, particle size 1.9 µm) was chosen for chromatographic seperation. Mobile phase A was distilled water (100%) and mobile phase B was methanol (100%). Atmospheric pressure chemical ionization (APCI) ion source was preferred for ionization, and tandem MS with triple quadrupole (QQQ) (Thermo Scientific TSQ Quantum Access MAX, San Jose, CA, USA) was used. MS scan was performed using the SRM (selected reaction monitoring) mode in positive polarity. LC-MS/MS conditions were shown in Table 1.

Method validation procedures were planned according to CLSI guidelines; and precision, trueness, limits of detection and quantitation, recovery and linearity were assessed. Besides, 40 patient samples were analyzed for steroid hormones, using the available immunoassay methods in Gulhane Training and Research Hospital, Medical Biochemistry Laboratories for method comparison study. Patients using exogenous steroid were excluded from the study. This study was approved by local ethics committee (REC number: 08/225), complied with the Declaration of Helsinki and all the subjects were given informed consent. The collected data from ID-LC-MS/MS method was compared with the data from RIA method for aldosterone (Beckman Coulter Immunotech Prague, Czech Republic) and 17α-Hydroxy progesterone (DIAsource, Louvain-la-Neuve, Belgium); CLIA method (Siemens Advia Centaur XP, Tarrytown, NY, USA) for cortisol, DHEAS, estradiol, progesterone, total testosterone; and CLEIA method (Siemens Immulite 2000 XPi, Llanberis, Gwynedd, UK) for androstenedione. MedCalc 9.2.0.1 (Ostend, Belgium) was used for statistical analysis of method comparison data.

Table 2: SRM transitions and retention time for each steroid and internal standard.

Steroid hormone	Molecular weight (g/mol)	Ionisation pattern	Precursor ion	Product ion	CE (eV)	RT (min)
Aldosterone	360	$[M+H]^+$	361	343	18	1.11
Aldosterone-d4	364	$[M+H]^+$	365	347	18	1.08
Corticosterone	346	$[M+H]^+$	347	121	35	5.94
Corticosterone-d8	354	$[M+H]^+$	355	95	40	5.90
Cortisol	362	$[M+H]^+$	363	121	30	1.44
Cortisol-d4	366	$[M+H]^+$	367	97	35	1.44
Cortisone	360	$[M+H]^+$	361	163	30	1.18
Cortisone-d8	368	$[M+H]^+$	369	168	30	1.18
11-deoxycortisol	346	$[M+H]^+$	347	97	33	2.48
11-deoxycortisol-d5	351	$[M+H]^+$	352	113	33	2.48
E3 (Estriol)[a]	288	$[M+H\text{-}H_2O]^+$	271	253	10	1.53
Androstenedione	286	$[M+H]^+$	287	109	30	3.18
Androstenedione-d7	293	$[M+H]^+$	294	113	30	3.13
DHEA	288	$[M+H\text{-}H_2O]^+$	271	213	22	4.16
DHEA-d5	293	$[M+H\text{-}H_2O]^+$	276	218	24	4.14
DHEAS	368	$[M+H\text{-}H_2SO_4]^+$	271	213	22	1.54
DHEAS-d6	374	$[M+H\text{-}H_2SO_4]^+$	277	219	24	1.52
DHT	290	$[M+H]^+$	291	255	16	1.51
DHT-d3	293	$[M+H]^+$	294	258	16	1.51
E2	272	$[M+H\text{-}H_2O]^+$	255	159	25	1.54
E2-d5	277	$[M+H\text{-}H_2O]^+$	260	161	26	1.51
17-OH progesterone	330	$[M+H]^+$	331	109	28	3.97
17-OH progesterone-d8	338	$[M+H]^+$	339	100	28	3.93
Progesterone	314	$[M+H]^+$	315	97	32	4.86
Progesterone-d9	323	$[M+H]^+$	324	100	32	4.81
Testosterone	288	$[M+H]^+$	289	109	28	3.87
Testosterone-d3	291	$[M+H]^+$	292	97	30	3.85

[a]E3 was excluded from validation process because its calibrator was not approved for MS/MS analysis.

Results

A practical LC-MS/MS method was developed for the commonly analyzed 13 steroid hormones by using stable isotope dilution for each steroid hormone (Figure 1). Ionization pattern, retention time (RT), collision energy (CE), precursor and product ions of each steroid hormone were summarized in Table 2. The first steroid eluting from the column was aldosterone (1.11 s) while the last one was corticosterone (5.94 s).

Precision of this new method was evaluated using Biorad Lyphocheck three level control materials.

Table 3: Precision performance of ID-LC-MS/MS method (units were given as μg/L).

	Level 1		Level 2		Level 3	
	Mean	**CV (%)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**
Aldosterone						
Within-day	0.164	17.49	0.717	14.81	1.225	15.88
Between-day	0.149	22.78	0.653	18.63	0.995	18.71
Cortisol						
Within-day	38.31	6.62	204.6	4.27	388.2	4.05
Between-day	35.96	9.41	197.9	6.37	364.6	5.66
11-Deoxycortisol						
Within-day	0.445	8.21	1.735	6.25	2.942	5.93
Between-day	0.452	11.18	1.612	9.82	2.785	9.38
Androstenedione						
Within-day	0.781	9.27	1.695	7.72	3.634	7.03
Between-day	0.767	13.52	1.721	12.04	3.704	11.83
DHEA						
Within-day	0.562	8.46	2.217	7.94	3.176	7.41
Between-day	0.548	11.69	2.054	9.22	2.994	8.62
DHEAS						
Within-day	547.3	4.77	1481	3.95	4227	3.58
Between-day	564.1	5.92	1522	5.46	4309	5.51
E2						
Within-day	0.053	15.82	0.224	14.67	0.407	14.02
Between-day	0.044	21.63	0.209	18.48	0.388	19.32
17α-OH progesterone						
Within-day	0.168	8.91	1.194	8.13	3.728	7.44
Between-day	0.149	11.31	1.185	11.05	3.711	9.47
Progesterone						
Within-day	0.683	5.04	6.513	4.52	20.82	4.85
Between-day	0.676	8.44	6.796	8.12	21.25	7.62
Testosterone						
Within-day	1.641	4.36	6.866	3.78	9.572	3.52
Between-day	1.633	6.82	7.027	5.77	9.668	5.37
	Calibrator 2[a]		**Calibrator 3[a]**		**Calibrator 4[a]**	
	Mean	**CV (%)**	**Mean**	**CV (%)**	**Mean**	**CV (%)**
Corticosterone						
Within-day	0.957	16.84	2.338	15.49	5.512	15.63
Between-day	0.843	21.26	2.181	20.42	5.359	18.33
Cortisone						
Within-day	2.239	9.75	4.202	9.26	9.618	9.61
Between-day	2.063	12.92	4.006	12.03	9.588	12.48
DHT						
Within-day	0.084	16.29	0.217	14.41	0.406	15.37
Between-day	0.077	20.08	0.194	19.15	0.417	18.66

[a]During precision evaluation calibrator 2, 3 and 4 were also used for repeated analysis since Biorad Lyphocheck control materials do not contain all the steroids in our panel.

Testosterone and DHEAS had the best precision performance (Table 3). Recovery studies were performed by spiking serum samples. All tests were performed duplicated. Trueness performance was the best for DHEAS and androstenedione considering the recovery data. Limit of detection (LOD) (S/N ratio is at least 3:1) and lower limit of quantitation (LLOQ) (S/N ratio is at least 10:1) were determined by analysis of the appropriate dilutions of level 1 calibrator. Accepted imprecision value was <20% for the experiments performed, during the determination and validation of LLOQ. Analytical sensitivity was worse in this method for aldosterone (20 pg/mL) and E2 (31 pg/mL) with respect to immunoassay based methods (7.64 pg/mL and 11.8 pg/mL respectively) in our core laboratory (Table 4). Linearity was assessed either.

Performance of immunoassay based methods (testosterone, DHEAS, E2, progesterone, testosterone, androstenedione, 17α-hydroxy progesterone, aldosterone) in our core laboratory were evaluated according to EURACHEM CITAC Guide [7] using the internal quality control and external quality assessment data. E2 and 17α-hydoxy progesterone performance were quite good considering the desirable specifications according to Fraser (Table 5) [8]. The results for analysis of aldosterone and estradiol with ID-LC-MS/MS method were suboptimal, considering the RIA and CLIA methods, respectively. Performance of ID-LC-MS/MS method was found to be superior when compared with CLIA method for cortisol, DHEAS, total testosterone, progesterone; chemiluminescence enzyme immunoassay (CLEIA) method for androstenedione and

Table 4: Accuracy performance, analytical sensitivity and linearity of ID-LC-MS/MS method were determined.

	Recovery study				LOD (µg/L)	LLOQ (µg/L)	Linearity study				Working range (µg/L)
	Initial (µg/L)	Added[a] (µg/L) CAL. 4	Final (µg/L)	Recovery (%)			Linearity range (µg/L)	Slope	Intercept	$S_{y/x}$	
Aldosterone	0.156	0.309	0.500	111.46	0.020	0.032	0.032–3.04	1.142	0.003	0.017	0.020–3.04
Corticosterone	4.88	5.31	9.77	93.41	0.154	0.215	0.215–47.8	0.890	0.545	1.513	0.154–47.8
Cortisol	63.06	82.8	145.9	100.14	1.82	2.12	2.12–279	1.04	–1.579	3.225	1.82–279
Cortisone	14.4	10.5	24.7	97.94	0.288	0.420	0.420–38.9	0.981	0.560	1.066	0.288–38.9
11-Deoxycortisol	2.52	1.91	4.55	106.50	0.018	0.024	0.024–13.9	1.021	0.012	0.113	0.018–13.9
Androstenedione	2.09	1.42	3.49	98.66	0.036	0.052	0.052–14	0.995	–0.028	0.081	0.036- 14
DHEA	2.91	14.7	17.9	102.19	0.283	0.518	0.518–54.3	0.957	0.286	0.689	0.283–54.3
DHEAS	1562	2033	3739	107.09	2.638	3.802	3.802–5710	1.006	1.771	3.417	2.638–5710
DHT	0.397	0.470	0.884	103.59	0.038	0.064	0.064–1.24	0.991	0.006	0.077	0.038–1.24
E2	0.248	0.500	0.737	97.77	0.031	0.047	0.047–4.85	0.919	–0.002	0.028	0.031–4.85
17α-OH progesterone	1.22	2.05	3.40	106.59	0.036	0.051	0.051–15	1.003	–0.055	0.044	0.036–15
Progesterone	14.7	5.31	20.2	104.70	0.029	0.045	0.045–25.6	1.017	–0.026	0.204	0.029–25.6
Testosterone	4.04	3.00	7.25	107.01	0.016	0.024	0.024–11.6	1.012	0.063	0.081	0.016–11.6

$S_{y/x}$, Standard deviation of y about linearity regression. [a]Level 4 Calibrator was used to spike the samples for recovery study.

Table 5: Performance of immunoassay based methods in our core laboratory.

	I (%)	B (%)	U (%)	Desirable specifications		
				I (%)	B (%)	TE (%)
Cortisol	11.23	7.47	26.97	7.6	10.26	22.8
DHEAS	14.38	9.41	34.38	3.188	7.84	13.08
Progesterone	10.15	3.89	21.75	[a]	[a]	[a]
Estradiol	10.06	4.33	21.90	11.25	8.3	26.86
Total testosterone	18.27	10.55	42.20	4.63	5.98	13.61
17-OH progesterone	8.8	11.74	29.33	9.8	13.5	29.7
Aldosterone	14.5	15.75	42.94	14.7	12.4	36.7

I, Imprecision; B, bias; U, uncertainty; TE, total allowable error. [a]No information in Westgard database.

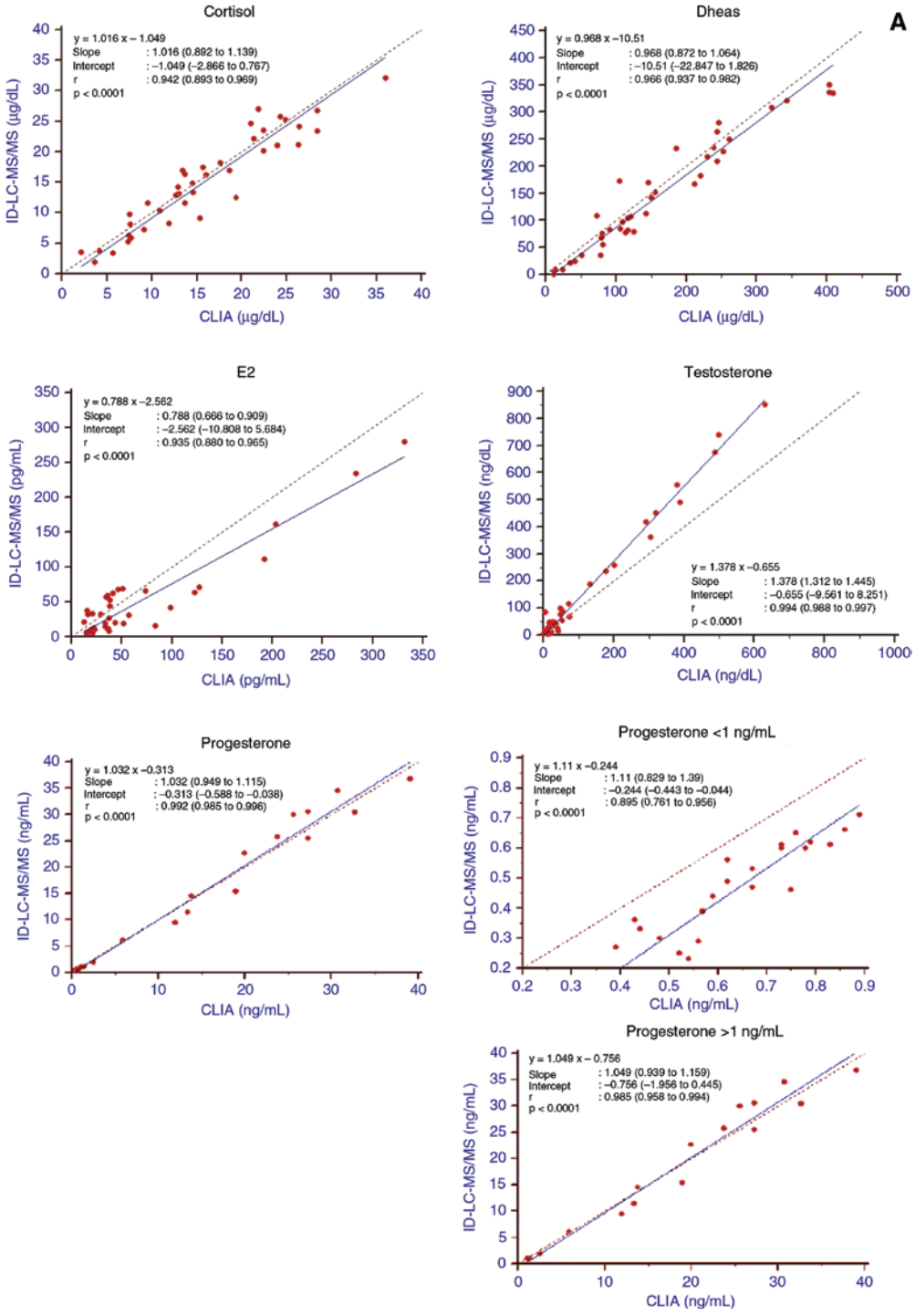

Figure 2: Correlation of ID-LC-MS/MS and immunoassay methods.
(A) Deming Regression of ID-LC-MS/MS method with CLIA method. Regression line is below "y = x line" for CLIA method which means average results of CLIA method is higher than the ID-LC-MS/MS method except testosterone. The two methods seem to be compatible with each other considering the slope, intercept and correlation coefficients. [Dashed line is y = x line. The other line is regression line. Slope intercept and correlation coefficient (r) were given with 95% confidence interval. Progesteron results were evaluated as <1 ng/mL and >1 ng/mL to increase the resolution of regression line in very high and very low levels since the progesterone levels of the samples varies in a huge range between 0.3 and 40 ng/mL]. (B) Deming Regression of ID-LC-MS/MS method with CLEIA method. Regression line is below "y = x line" for CLEIA method which means average results of CLEIA method is higher than the ID-LC-MS/MS method. However, the two methods seem to be correlated with each other. (C) Deming Regression of ID-LC-MS/MS method with RIA method. Regression line is below "y = x line" for RIA method of 17-OH Progesterone which means average results of RIA method for 17-OH Progesterone is higher than the ID-LC-MS/MS method. Contrarily, regression line is above "y = x line" for RIA method of aldosterone. However, the two methods seem to be correlated with each other.

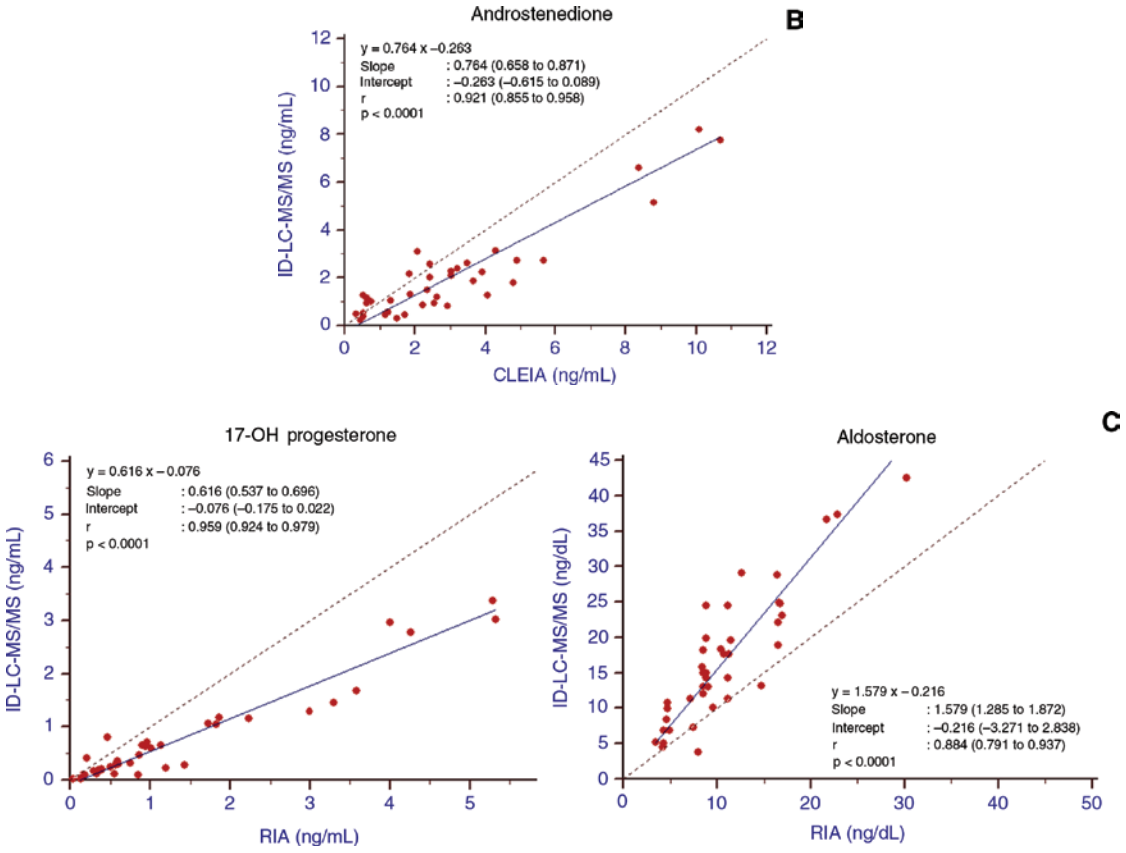

Figure 2 (continued)

RIA method for 17α-hydroxy progesterone considering the imprecision and trueness performance. Method validation data showed that the test performance of 11-deoxycortisol, cortisone, corticosterone, DHEA and dihydrotestosterone were pretty good.

Forty serum samples were analyzed with both immunoassay and ID-LC-MS/MS methods. Method comparison results revealed that aldosterone results of the two methods showed the worst correlation with a correlation coefficient of r = 0.884 (0.791–0.937, 95% confidence interval). DHEAS results of the two methods had the best correlation (r = 0.966, 0.937–0.982, 95% confidence interval) (Figure 2). According to Bland Altmann plots of the two methods, testosterone and aldosterone results of the ID-LC-MS/MS method was higher than the immunoassay methods while the others were lower in ID-LC-MS/MS method (Figure 3).

Discussion

An ID-LC-APCI-MS/MS method; having an 11 min of analysis time, with 13 steroids, two of suboptimal; has been validated. A serum sample volume of only 100 µL is required in this method. Protein precipitation was employed for pretreatment steps which made this method simple and practical. SRM mode of the tandem MS provided us high analytical sensitivity and specificity. An IS isotope was used for each steroid so the matrix effect was minimized.

There are some studies which indicate the superiority of APPI to APCI and ESI in steroid analysis. Since APPI is a soft ionization source, it creates just the ions of concern such as steroids. Thus, the suppression due to other excess ionization can be decreased resulting in a better signal-to-noise ratio [9]. Precision of APPI was reported to be better than APCI, considering the peak area/height of IS [10]. In another study, APCI was reported to be the best choice of ion source for steroid analysis except aldosterone and estradiol (APPI was suggested for these steroids) [11]. Since we could not have access to APPI ion source in our laboratory, we used APCI ion source. Many studies recommend MS scan in positive polarity except aldosterone and estradiol which are commonly scanned in negative polarity [11]. As polarity switching was not possible in our instrument, we scanned all steroid hormones in positive

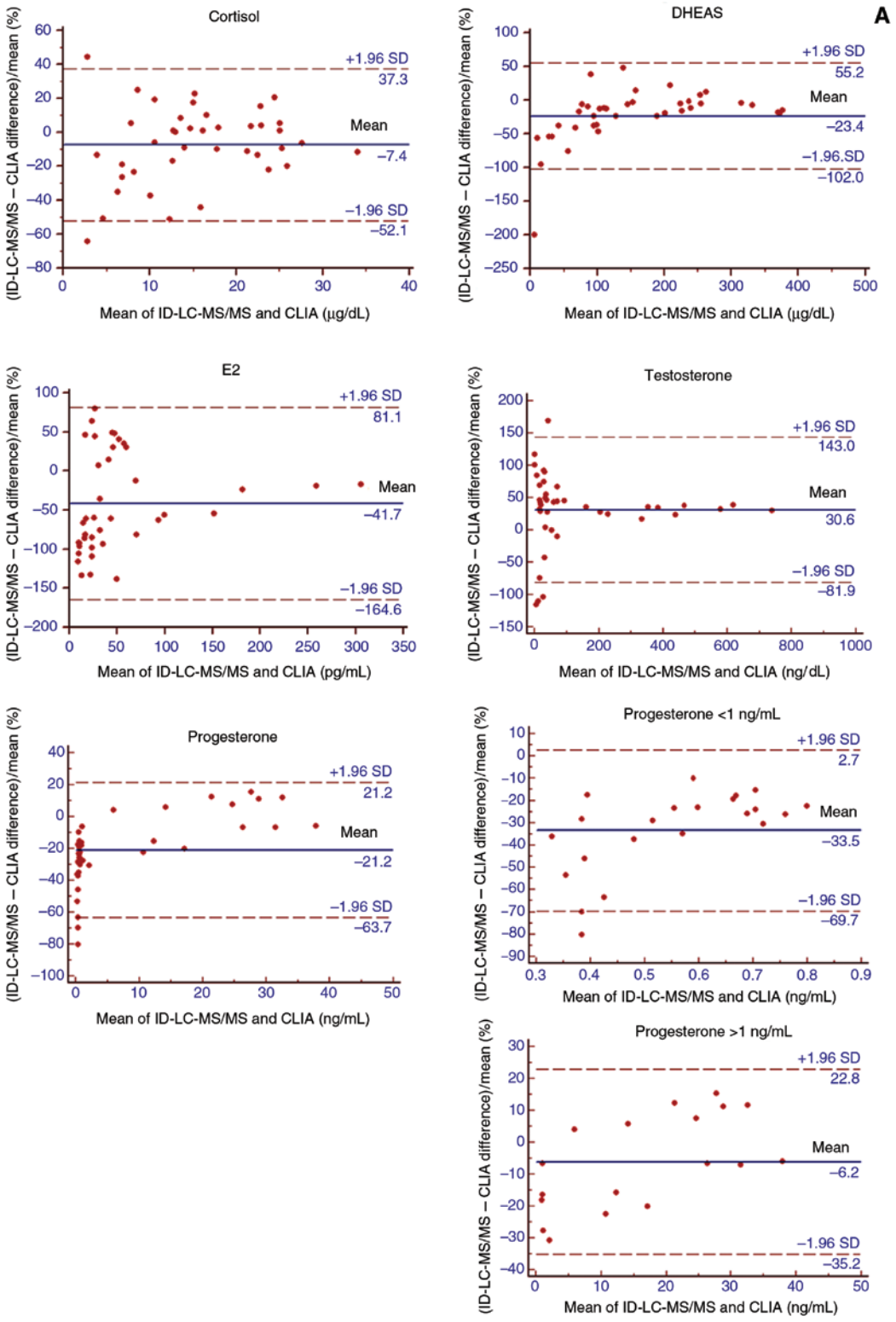

Figure 3: Percent differences against mean of the compared methods.
(A) Bland Altman plots of ID-LC-MS/MS method with CLIA method. Mean lines in the graphs are negative except testosterone which means ID-LC-MS/MS method gives lower results than CLIA method except testosterone. Bias seems to be acceptable since most of the samples are between ±1.96 standard deviation (SD). (B) Bland Altman plots of ID-LC-MS/MS method with CLEIA method. Mean line in the graph is negative which means ID-LC-MS/MS method gives lower results than CLEIA method for androstenedione. Bias seems to be acceptable since most of the samples are between ±1.96 standard deviation (SD). (C) Bland Altman plots of ID-LC-MS/MS method with RIA method. Mean line in the graph of 17-OH Progesterone is negative which means average results of ID-LC-MS/MS method for 17-OH Progesterone is lower than the RIA method for 17-OH Progesterone. Contrarily, mean line is positive for aldosterone.

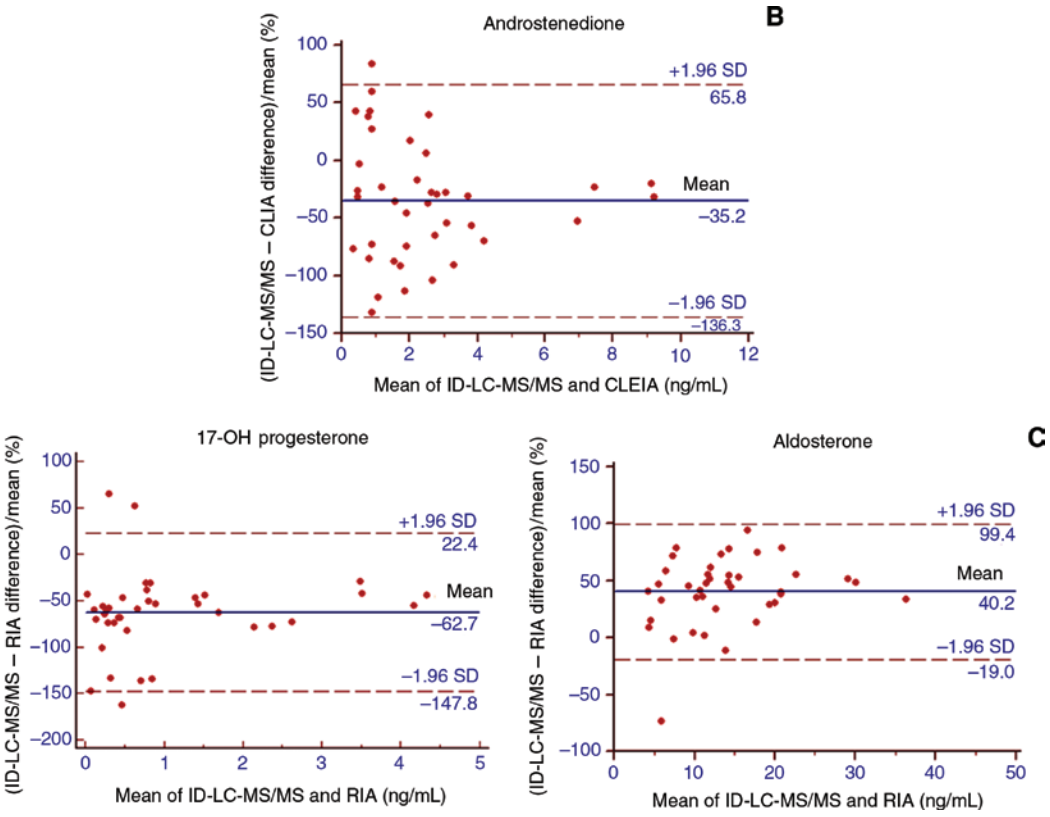

Figure 3 (continued)

polarity. These factors might be the reason for aldosterone and estradiol having suboptimal performance in our method.

This method was found to correlate well with the available validated immunoassay based methods routinely used in our core laboratory. Likely with the previous studies, ID-LC-MS/MS method gave lower results with respect to the CLIA method for cortisol [12–14], androstenedione, progesterone [14], and 17α-OH progesteron [15]. Contrarily, testosterone results were higher in ID-LC-MS/MS, which was likely with the study of Fanelli et al. [14]. Aldosterone and estradiol performance of immunoassays were better while the other steroids had superior performance in ID-LC-MS/MS method.

The most important challenge in immunoassays is lack of specificity and standardization [16, 17]. Manufacturers use different antibodies for immunoassays and the commonly used calibrators are not traceable to reference materials. This causes incompatible results. This is true for LC-MS/MS studies as well. So, we used the validated calibrators for MS in this study.

Both immunoassays and tandem MS methods have advantages and disadvantages. Automatized immunoassays allow only one steroid analysis for each run. It is simple as it does not require pretreatment steps, but much more sample volume is needed. On the other hand, LC-MS/MS methods allow many steroid analysis on the same run and require less sample volume [18]. Analytical specificity and sensitivity, and test performance of LC-MS/MS method is generally better than immunoassays [16, 17]. Additionally, LC-MS/MS methods are less prone to be affected from interferences with regard to immunoassays. However, LC-MS/MS is more complex and qulified users are needed, and some laborious and unautomatized pretreatment steps increases the duration of analysis [18]. On the other hand, reference ranges and clinical decision levels were defined according to immunoassay based methods in many guidelines. Several studies have been conducted to reevaluate these ranges according to LC-MS/MS based methods [19, 20]. Additionally, diurnal variations of many steroid hormones were assessed using LC-MS/MS methods. Reference range studies should be conducted by same time sampling, considering these diurnal variations [9, 21].

In conclusion, this ID-LC-MS/MS method would be useful in clinical laboratories, especially for the immunoassays having insufficient test performance and when checking for possible interferences in immunoassays

available. Accurate and reliable results can be obtained with high throughput with LC-MS/MS assays [22]. LC-MS/MS assays have a key contribution in diagnosis of endocrine disorders [23], and their use in steroid hormone analysis seem to be more common in the future. However, LC-MS/MS assays do not seem to replace immunoassays and they are thought to complement each other [17].

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