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Application News

No.C116

Liquid Chromatography Mass Spectrometry

Quantitative Analysis of Steroid Hormones Using Triple Quadrupole LC/MS/MS

Steroid hormones are heavily involved with the control of metabolism, neurotransmission, and intracellular signaling, playing critical roles in the proper functioning of the body. Further, not only do steroids play roles in sedation and seizure prevention, they are known to be effective in cancer treatment and regenerative medicine. Steroid quantitation in biological samples is therefore an important tool in clinical research. This Application News introduces the analysis of steroid hormones in human serum by LC/MS/MS following pretreatment using ISOLUTE® SLE+ (supported liquid extraction).

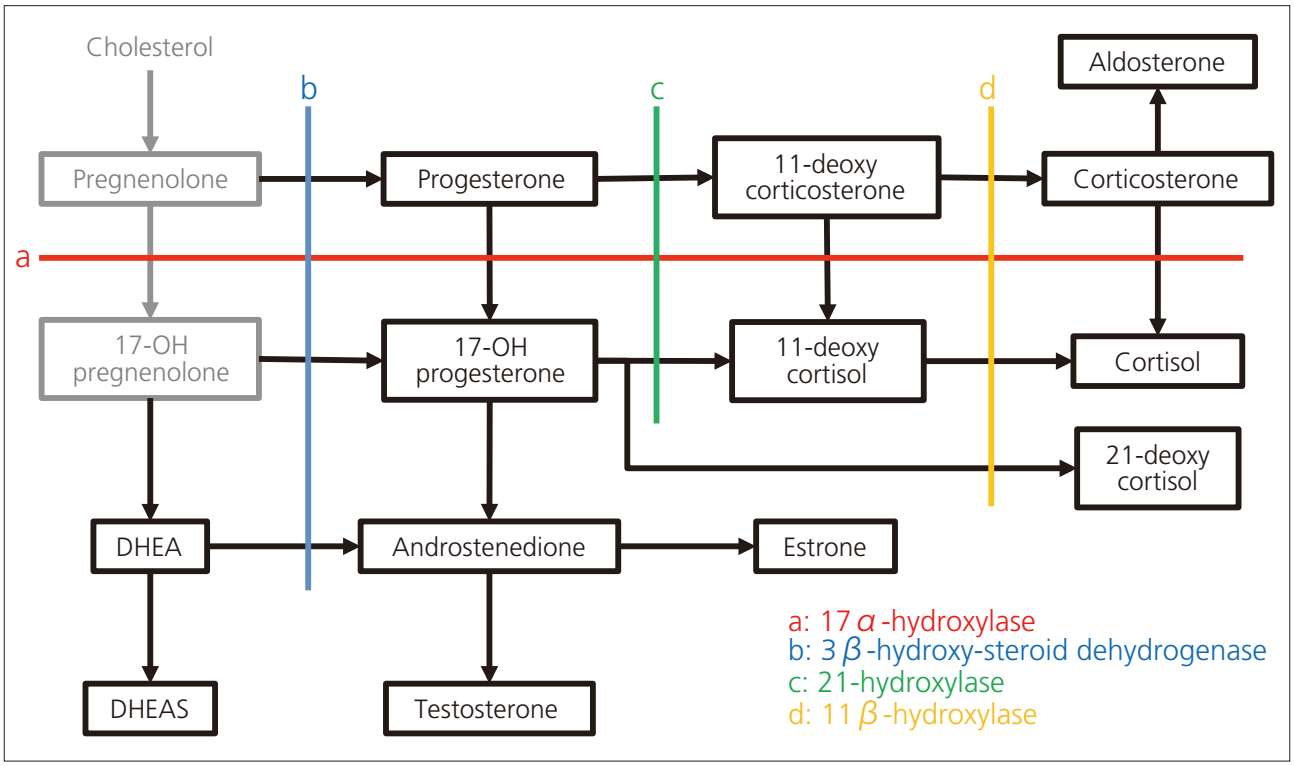


Fig. 1 Major Pathways of Steroid Biosynthesis

Table 1 Analytical Conditions

[LC] NexeraX2 System	
Column	: Shim-Pack FC-ODS (75 mm L. × 2 mm I.D., 3 µm)
Column Oven	: 40 °C
Mobile Phase A	: 5 mM Ammonium Formate - Water
Mobile Phase B	: 5 mM Ammonium Formate - Methanol
Gradient	: 35 %B (0 - 2.5 min) → 35 %B → 45 %B (2.5 - 4 min) → 45 %B → 80 %B (4 - 12 min) 95 %B (12.01 - 15 min) → 35 %B (15.01 - 18 min)
Flowrate	: 0.3 mL/min
Injection Volume	: 10 µL
[MS] LCMS-8050	
Ionization	: ESI
DL Temp.	: 110 °C
Heat Block Temp.	: 450 °C
Interface Temp.	: 370 °C
Nebulizer Gas	: 3 L/min
Drying Gas	: 7 L/min
Heating Gas	: 13 L/min

MRM Parameters

Compound Name	Group	Ret. Time (min)	Precursor *m/z*	Product *m/z* (1)	Product *m/z* (2)	Compound Name	Group	Ret. Time (min)	Precursor *m/z*	Product *m/z* (1)	Product *m/z* (2)
Aldosterone	1	5.52	361.20	315.20	343.00	Androstenedione	6	8.83	287.10	97.10	109.15
Aldosterone IS	1	5.47	367.20	349.25	331.10	Androstenedione IS	6	8.79	292.10	100.10	113.05
Cortisol	2	6.55	363.40	121.10	97.00	Testosterone	7	9.46	289.10	97.15	109.05
Cortisol IS	2	6.57	366.10	121.10	97.10	Testosterone IS	7	9.42	294.10	100.00	113.05
DHEAS	3	7.21	271.20	213.20	197.10	DHEA	8	9.03	271.20	213.20	253.15
DHEAS IS	3	7.18	277.10	219.20	203.10	Estrone	8	9.04	271.10	133.05	157.05
21-Deoxycortisol	4	7.46	347.20	311.20	121.05	11-Deoxycorticosterone	8	9.22	331.20	109.05	97.05
Coricosterone	4	7.84	347.20	121.15	97.15	17-OHP	8	9.72	331.10	97.00	109.00
Coricosterone IS	4	7.77	355.20	125.05	337.00	17-OHP IS	8	9.66	339.10	100.05	113.10
11-Deoxycortisol	5	8.04	347.20	109.10	97.05	Progesterone	9	11.34	315.20	97.05	109.10
11-Deoxycortisol IS	5	8.01	352.20	100.15	113.05	Progesterone IS	9	11.26	324.10	100.00	113.00
						DHEAS_neg	10	7.20	367.10	97.10	
						DHEAS_neg IS	10	7.17	373.10	98.00	

Type	Event#	+/−	Compound Name m/z	Time (4.653 min – 12.331 min)
MRM	1	+	Aldosterone IS 367.20>349.25, 367.20>331.10	
MRM	2	+	Aldosterone 361.20>343.00, 361.20>315.20	
MRM	3	+	Cortisol IS 366.10>121.10, 366.10>97.10	
MRM	4	+	Cortisol 363.40>121.10, 363.40>97.00	
MRM	5	+	DHEAS 271.20>213.20, 271.20>197.10	
MRM	6	+	DHEAS IS 277.10>219.20, 277.10>203.10	
MRM	7	+	21-Deoxycortisol 347.20>311.20, 347.20>121.05	
MRM	8	+	Coricosterone IS 355.20>125.05, 355.20>337.00	
MRM	9	+	Coricosterone 347.20>121.15, 347.20>97.15	
MRM	10	+	11-Deoxycortisol 347.20>109.10, 347.20>97.05	
MRM	11	+	11-Deoxycortisol IS 352.20>100.15, 352.20>113.05	
MRM	12	+	Androstenedione IS 292.10>100.10, 292.10>113.05	
MRM	13	+	Androstenedione 287.10>97.10, 287.10>109.15	
MRM	14	+	Estrone 271.10>133.05, 271.10>157.05	
MRM	15	+	DHEA 271.20>253.15, 271.20>213.20	
MRM	16	+	11-Deoxycorticosterone 331.20>109.05, 331.20>97.05	
MRM	17	+	Testosterone 289.10>97.15, 289.10>109.05	
MRM	18	+	Testosterone IS 294.10>100.00, 294.10>113.05	
MRM	19	+	17-OHP 331.10>97.00, 331.10>109.00	
MRM	20	+	17-OHP IS 339.10>100.05, 339.10>113.10	
MRM	21	+	Progesterone 315.20>97.05, 315.20>109.10	
MRM	22	+	Progesterone IS 324.10>100.00, 324.10>113.00	
MRM	23	−	DHEAS_neg IS 373.10>98.00	
MRM	24	−	DHEAS_neg 367.10>97.10	

Calibration Curves

MRM measurement was conducted for 13 types of steroid hormones. Fig. 2 shows the calibration curves obtained using a mixed standard solution, in addition the MRM chromatograms obtained using a concentration of each steroid in the vicinity of its LOQ.

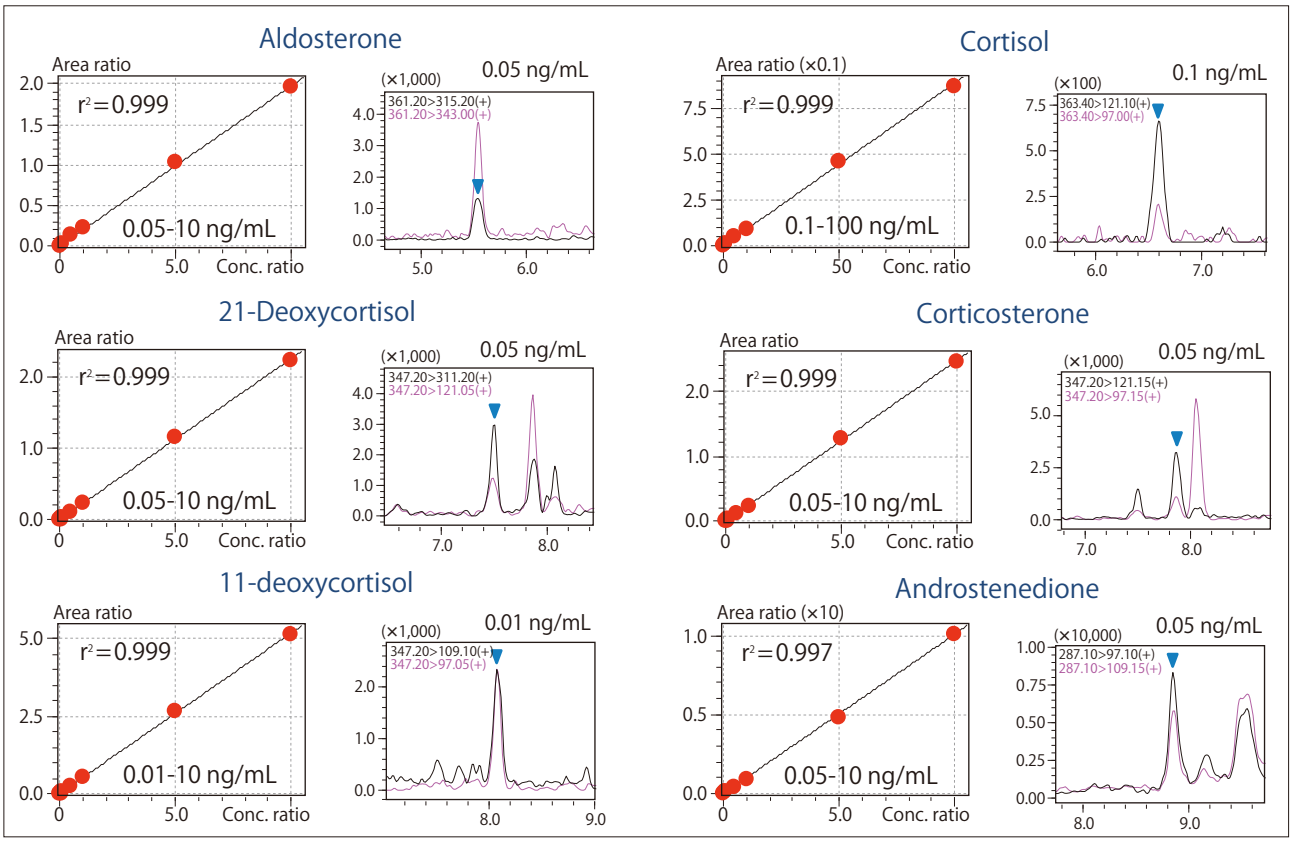
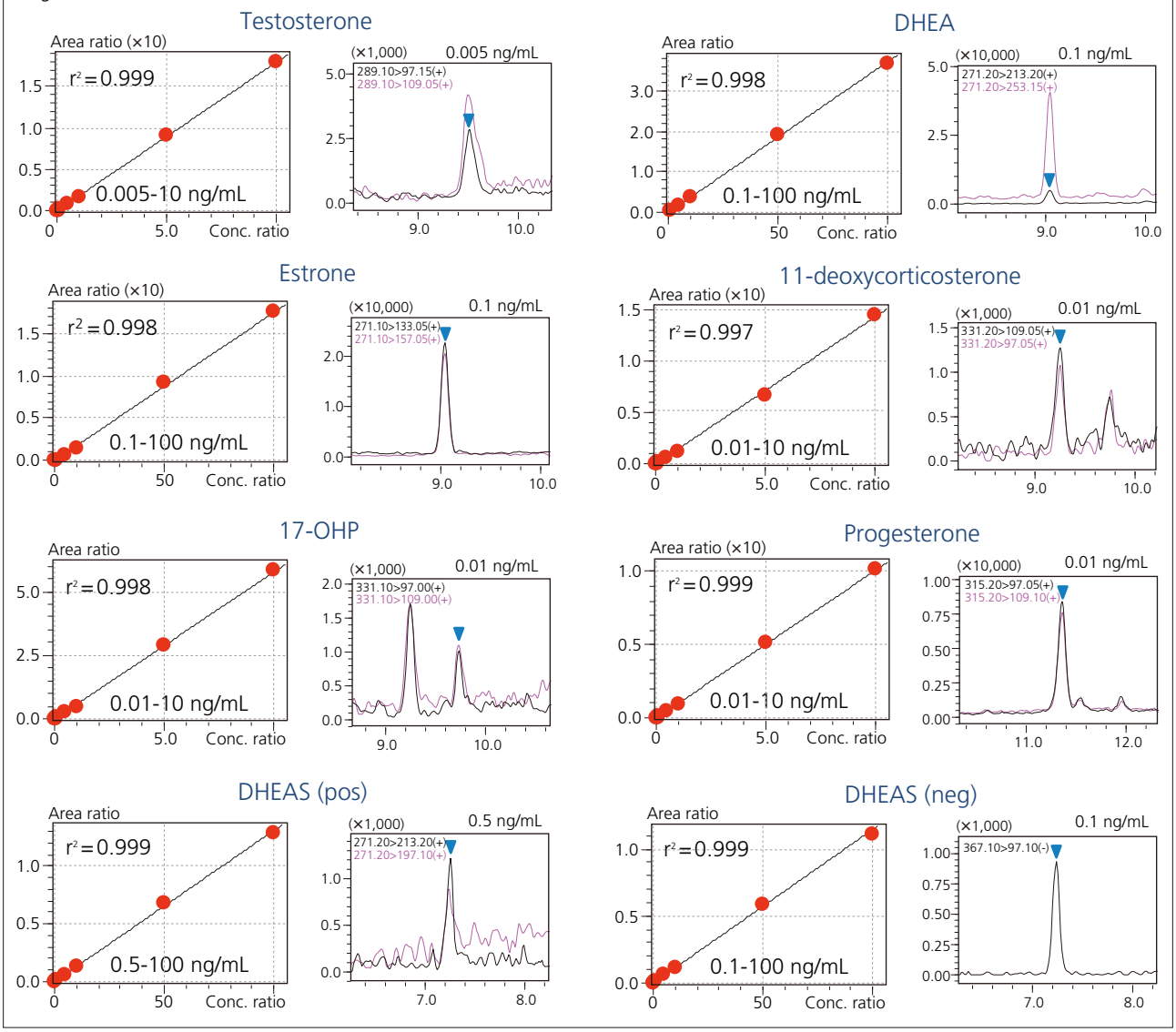


Fig. 2 Calibration Curves and MRM Chromatograms of 13 Steroid Hormones

Fig. 2 (continued)



Sample Preparation Using ISOLUTE® SLE+

Biological matrices are complex and require cleanup prior to LCMS analysis. ISOLUTE® SLE+ was used to eliminate the influence due to endogenous compounds such as proteins, phospholipids and salts. The reduction of matrix interference allows for lower levels of quantitation, and higher confidence in analytical results. The method that was used for steroid hormone extraction is outlined below.

1. Sample Preparation
   Dilute the serum sample (100 µL) with ultrapure, HPLC grade water (300 µL), and mix.
2. Sample Loading
   Dispense the prepared sample (400 µL) onto the plate and apply pressure. Wait 5 minutes for sample to completely absorb into diatomaceous earth.
3. Apply Extraction Solvent
   Add dichloromethane (900 µL × 2) to deep well plates to elute, and allow solvent to flow for 5 minutes under gravity. Then apply pressure for analyte elution.
4. Post Extraction Processing
   Transfer eluate to glass vial, and heat to dryness at 40 °C. Re-dissolve in 100 µL of solvent (mobile phase A 66 %, mobile phase B 35 %, v/v). Centrifuge and transfer supernatant onto a new plate.

Table 2 shows the concentration range added to the serum, the rates of recovery, and the matrix effect with respect to the 12 types of steroid hormones. The analysis recovery rates were determined by comparing the area values of Sample B, in which the serum was spiked with the steroids prior to pretreatment, and Sample A, in which only the serum was subjected to pretreatment, after which it was spiked with the steroids. The matrix factor was determined by comparing the area values of Sample A with that of the standard solution S.

Table 2 Recovery and Matrix Factor of 12 Steroid Hormones (n=3)

Compound	Concentration Range Added to Serum (ng/mL)	Recovery %	Matrix Factor
Aldosterone	0.05-10	94.8 %	0.13
Cortisol	0.1-100	94.7 %	0.31
21-Deoxycortisol	0.05-10	94.6 %	0.13
Coricosterone	0.05-10	107.1 %	0.30
11-Deoxycortisol	0.01-10	93.7 %	0.21
Androstenedione	0.05-10	94.2 %	0.09
Testosterone	0.005-10	80.2 %	0.00
DHEA	0.1-100	89.8 %	0.19
Estrone	0.1-100	94.3 %	0.30
11-Deoxycorticosterone	0.01-10	96.2 %	0.26
17-OHP	0.01-10	94.6 %	0.19
Progesterone	0.01-10	89.8 %	0.19

The Recoveries and matrix factor were determined according to the following equations.
Recovery = [B]/[A] ×100 Matrix Factor = 1 - [A]/[S]

Use of Ion Pair Reagents

The use of ion-pair reagents is a valid approach in analysis of ionic compounds where recovery is normally difficult. In the case of DHEAS, we were able to obtain a high recovery rate by spiking the sample with an ion pair reagent (25 mM dibutyl ammonium acetate) during pretreatment.

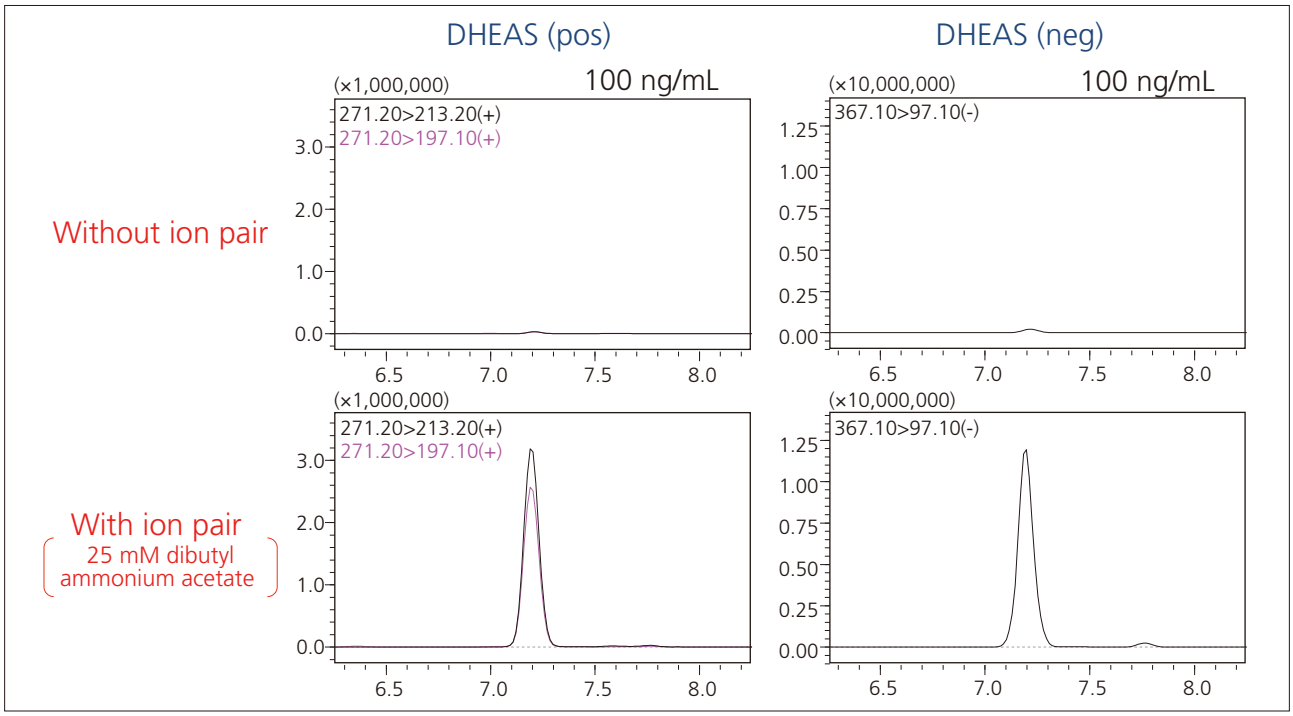


Fig. 3 Comparison of MRM Chromatograms of DHEAS With and Without Ion Pair Reagent

Table 3 Recovery and Matrix Factor of DHEAS (n=3)

Compound	Concentration Range Added to Serum (ng/mL)	Recovery %	Matrix Factor
DHEAS (pos)	0.1-100	86.2 %	0.06
DHEAS (neg)	0.1-100	89.3 %	0.11

Note)
The published data was not acquired using an instrument registered by Japanese pharmaceutical affairs law.

[Acknowledgment]
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