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Ultra-fast Multiplexed LC/MS/MS Using Newly Designed Online SPE Column for the Simultaneous Analysis of Steroid Hormones in Human Serum

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1. Introduction

The Multiplexed 4-channel LC/MS/MS system uses up to 4 multiple, alternating sample introduction streams to keep a single mass spectrometer working continuously. Online solid phase extraction (SPE) automatically reduces matrix effects in LC/MS/MS and enriches the analyte to increase reproducibility and sensitivity without complicated sample pretreatment. Combining these technologies, multiplexed 4-channel online SPE LC/MS/MS system is expected to maximize ruggedness and robustness while increasing sample throughput. (See Fig.1). This study evaluated the sample throughput and quantification of steroid hormones simultaneously in serum samples.

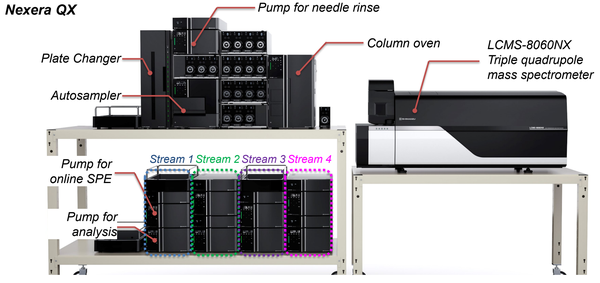

Fig1. Appearance of Nexera QX (left) and LCMS-8060NX (right). This system implements 4-channel Online-SPE-LC/MS/MS.

2. Sample Preparation and Analytical conditions

Materials

Standard solutions of 17 steroids were purchased from Merck and ultra-low hormones & steroids serum was obtained from Golden West Diagnostics, LLC.

Calibrators and QC samples

500 µL of serum calibrators and controls were mixed with 2 µL of ISTD and 1,500 µL of acetonitrile, then vortexed 1min. After centrifugation for 10 minutes at 10,000 rpm, supernatant was transferred to a new plastic tube and dried down under nitrogen. 50 µL of 50% methanol was added to the tube and transferred to an LC vial.

Multiplexed online SPE-LC-MS/MS system

The 4-channel online SPE-LC-MS/MS system was composed of Nexera QX and LCMS-8060NX. Newly designed online SPE column implements high throughput online SPE. The flow diagram in each steps and analytical conditions are shown on Table1, 2 and Figure2.

Table1. LC conditions

System:	Nexera QX
Trap Column:	Trap Column for QX [prototype] (1.0 x 50 mm)
Analytical Column	Biphenyl Column (1.7 µm, 2.1 x 50 mm)
Mobile Phase A	0.2 mM ammonium fluoride in water
Mobile Phase B	0.2 mM ammonium fluoride in methanol
Mobile Phase C	0.1% formic acid in MeOH:ACN:IPA:Water=1:1:1:1 (v/v)
Column oven temp:	20°C
Injection volume:	30 µL

Table2. MS conditions

System:	LCMS-8060NX
Ionization:	Heated ESI
Interface Temp	400°C
DLTemp:	150°C
Heat Block Temp	500°C
Nebulizing Gas	3.0 L/min
Heating Gas	15 L/min
Drying Gas	5.0 L/min

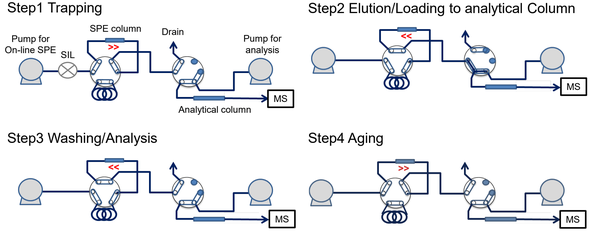

Fig2. The schematic diagram of online SPE-LC/MS/MS in each step

3. Results and Discussion

Chromatographic Separation

The MS chromatogram of 17 steroids spiked to serum at 1,000 pg/mL is shown on Fig3. Excellent chromatographic selectivity was demonstrated through baseline resolution of isobaric steroids specie (See Fig4). 17-Hydroxyprogesterone and 11-Deoxycorticosterone have same monoisotopic mass (330.2195 Da). 21-Deoxycortisol, 11-Deoxycortisol and Corticosterone have 346.2144 Da. Those isobaric groups were completely separated under this analytical condition.

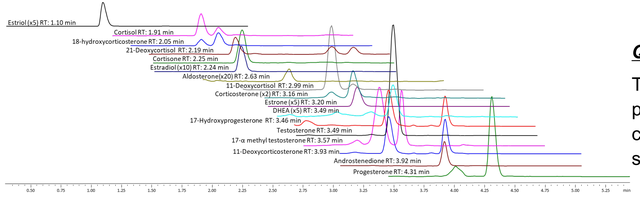

Fig3. MS chromatograms of 17 steroid hormones at 1,000 pg/mL in serum

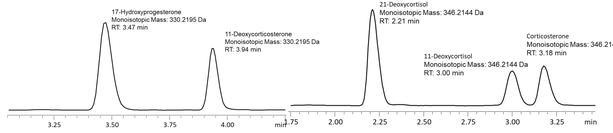

Fig4. MS chromatograms of (a) 17-Hydroxyprogesterone and 11-Deoxycorticosterone, (b) 21-Deoxycortisol, 11-Deoxycortisol and Corticosterone at 5,000 pg/mL in serum

Carryover reduction

When carryover occurs, peaks attributed to the previously analyzed sample may be observed in the subsequent chromatogram which may co-elute or interfere with desired analytes. Washing online SPE column and needle inner rinse were performed to reduce carryovers. Needle inner rinse was especially effective to eliminate residue of previous analytes (See Fig5). There was no interference peak in blank sample injected right after measurement of a sample at 10,000 pg/mL.

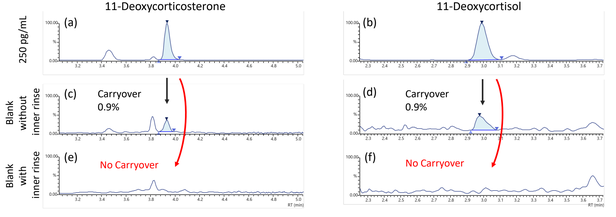

Fig5. MS chromatograms of (a) 11-Deoxycorticosterone at 250 pg/mL, (b) 11-Deoxycortisol at 250 pg/mL, (c) and (d) blank without needle inner rinse, (e) and (f) blank with needle inner rinse

Quantification results of steroid hormones in serum

The linear range of the steroids in serum was evaluated from 1 to 10,000 pg/mL using calibrators and accuracies were confirmed using 3 levels of controls. The linear regression values of R^2 were grater than 0.993 for 18 steroids and all accuracies were within 100 ± 15%. (See Table3).

Table3. Calibration range and QC results

Steroid	Linear Range (pg/mL)	LQC Spiked Conc 30 pg/mL		MQC Spiked Conc 300 pg/mL		HQC Spiked Conc 3,000 pg/mL	
		Measured Conc (pg/mL)	Accuracy %	Measured Conc (pg/mL)	Accuracy %	Measured Conc (pg/mL)	Accuracy %
11-Deoxycorticosterone	5-10,000	32.1	107	276	92	3,286	110
11-Deoxycortisol	5-5,000	30.0	100	267	89	3,330	111
17-Hydroxyprogesterone	25-10,000	27.8	93	284	95	3,037	101
Methyltestosterone	10-2,500	26.2	87	271	90	3,329	111
18-hydroxycorticosterone	2.5-10,000	28.9	96	264	88	2,900	97
21-Deoxycortisol	25-10,000	31.1	104	304	101	2,748	92
Aldosterone	10-10,000	34.2	114	286	95	2,724	91
Androstenedione	10-10,000	32.8	109	342	114	3,349	112
Corticosterone	10-10,000	28.9	96	311	104	3,155	105
Cortisol	5-10,000	29.4	98	279	93	2,760	92
Cortisone	5-10,000	27.4	91	88*	88	913**	91
DHEA	10-10,000	29.7	99	341	114	3,152	105
Progesterone	5-2,500	26.4	88	291	97	-	-
Testosterone	1-2,500	32.2	107	342	114	-	-
Estradiol	10-10,000	27.6	92	283	94	2,877	96
Estriol	5-10,000	29.0	97	297	99	3,019	101
Estrone	1-10,000	32.6	109	324	108	3,360	112

* Spiked 100 pg/mL, ** Spiked 1,000 pg/mL

Throughput improvement

The measurement time in single stream was 10.3 min. On the other hand, in case of using 4 streams it took 5.0 min which included sample clean-up on online SPE and needle inner rinse between measurements. The throughput was doubled.

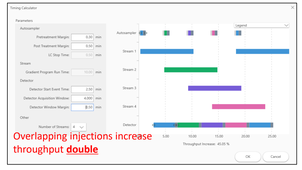

Fig6. Timing calculator on *QX Solution*

4. Conclusion

We established high-sensitivity and wide-dynamic range quantitation method of steroid hormones by a multiplexed 4-channel online SPE-LC-MS/MS system. Our method achieves excellent chromatographic separation, allowing for specific detection of isobaric species. This platform enables extraordinary sample-throughput that minimize time consuming sample pretreatment and data acquisition, which is useful for precision assay with large number of samples.

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[Authors belong to #1] Named authors are partially funded by Shimadzu Corporation.
[The author belongs to #3] The author is affiliated and funded by Shimadzu Corporation.