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High-Throughput Analysis of Testosterone and DHEA in Human Serum by LDTD-MS/MS: Towards Increased Diagnostic Accuracy

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OVERVIEW

Purpose

- Use of selective adduct formation in doped APCI to analyze Testosterone and DHEA (isobaric compound) in serum in shotgun approach.

Method

- Liquid-Liquid Extraction (LLE).
- Samples dried and analyzed by LDTD-MS/MS with dopant in carrier gas.

Quantification

- Linearity: r^2> 0.99 over the calibration range.
- Inter-run accuracy, values between 97.7% and 105.9% were obtained and the precision results were lower than 11.4%CV.
- Samples analyzed with a runtime of 8 seconds using LDTD-MS/MS technique.

INTRODUCTION

The determination of serum testosterone and dehydroepiandrosterone (DHEA) concentrations facilitates the diagnosis and treatment of various diseases. In addition to testosterone, the primary androgen hormone, many other androgenic hormones play an important role in the diagnosis and treatment of various diseases. Since the introduction of steroid immunoassays, antibody affinity-based methods have been widely used to measure bioactive steroids in biological fluids due to their high sensitivity. However, these techniques are limited by the specificity of antibodies for epitopes, which may lead to cross-reactions with structurally similar steroids, thereby reducing the specificity of the method. To obtain a rapid analytical method to separate testosterone and DHEA, isobaric compound, a specific APCI ionisation and extraction method and an LDTD-MS/MS analytical method was developed.

LUXON Ionization Source:

The Luxon Ion Source (**Figure 1**) is the second-generation sample introduction and ionization source based on the LDTD technology for mass spectrometry. The Luxon Ion Source uses a Fiber-Coupled Laser Diode (**Figure 2**) to obtain unmatchable thermal uniformity giving more precision, accuracy and speed.

The process begins with dry samples which are rapidly evaporated using indirect heat. The thermally desorbed neutral molecules are carried into a corona discharge region. High-efficiency protonation and strong resistance to ionic suppression characterize this type of ionization and is the result of the absence of solvent and mobile phase. This thermal desorption process yields high-intensity molecular ion signal in less than 1 second sample-to-sample and allows working with very small volumes.

Figure 1 *Luxon Ion Source*

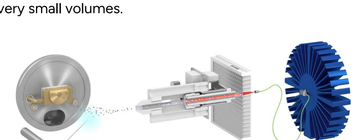

Figure 2 *Schematic of the Luxon Ion Source*

METHOD

Liquid-liquid extraction

The infusion of Ammoniac in the LDTD carrier gas is optimized to get the maximum signal of both adduct and protonated molecules. The calibration curve and quality controls are spiked into an artificial matrix (20 mg/mL BSA in PBS). 12.5 µL of internal standard solution and 250 µL of samples are mixed (1000 rpm, 5 s). 1000 µL of a mixture of Hexane/MTBE (90:10) are added to the sample. Tubes are mixed (1000 rpm, 30 s) and centrifuged (14000 rpm, 4 min). 500 µL of the upper layer are transferred into a borosilicate tube (10X75mm) and samples are evaporated until dryness. Samples are then reconstituted using 100 µL of Acetonitrile:Water mix (1:1). 4 µL are spotted onto a LazWell plate and then analyzed by LDTD-MS/MS.

Instrumentation

- Ion Source: Luxon Ion Source SH-960, Phytronix
- Mass Spectrometer: LCMS-8060, Shimadzu

Luxon Parameters

Laser power pattern:

- Increase laser power to 65% in 3 sec.
- Hold 2 second.
- Carrier gas flow: 4.5 L/min (Air) with infusion of NH_4OH (1% in water) at 2 µL/min.

MS Parameters

- APCI (+)
- Corona Needle Voltage : 5 kV
- CID: 250 kPa
- Time: 10 msec

Table 1 *MRM transitions for LDTD-MS/MS*

	Transition	CE (eV)
Testosterone	289.3 → 109.1	-27
IS Testosterone-d_3	292.3 → 109.1	-27
DHEA	306.3 → 253.2	-17
IS DHEA-d_6	312.3 → 259.3	-17

RESULTS

Validation

Calibration curves ranging from 100 to 10000 pg/mL for testosterone and 200 to 10000 pg/mL for DHEA are prepared in BSA (20 mg/mL dilute in PBS buffer). A set of QCs are prepared in the same matrix. Replicate extractions are deposited onto a LazWell™ plate and dried before analysis. The peak area against the internal standard (IS) ratio is used to normalize the signal.

Linearity

The calibration curves are plotted using the peak area ratio and the nominal concentration of standards. For the linearity test, the following acceptance criterion is used:

- Linear regression (r) must be ≥ 0.995
- **Table 2** shows the determination coefficient of six different runs. **Figure 3** shows typical calibration curve for Testosterone.

Table 2 *Determination coefficient for curves*

	Determination coefficient (r^2)	
Run	Testosterone	DHEA
1	0.99876	0.99818
2	0.99804	0.99698
3	0.99699	0.99390
4	0.99786	0.99464
5	0.99705	0.99747
6	0.99738	0.99356

RESULTS

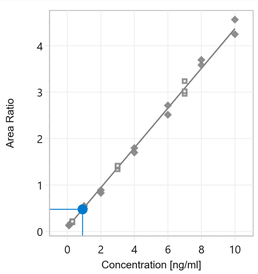

Figure 3 *Typical calibration curve for Testosterone*

Precision and Accuracy

Eight calibration points and three QCs (QC-L, QC-M and QC-H) were prepared in BSA (2% in PBS buffer) as a negative matrix and used to validate the method. The peak area against the internal standard (IS) ratio was used to normalize the signal. Replicate extractions are deposited on a LazWell™-plate and dried before analysis. The following acceptance criteria were used:

- Each standard and QC must not exceed 20% CV at lower concentration and 15% CV for the other concentration.
- Each standard and QC must be ±20% of the nominal value (%Nom) at lower concentration and ±15% of the nominal value (%Nom) for the other concentrations.

Table 3 *Inter-run Precision and Accuracy*

Testosterone	QC-L	QC-M	QC-H
Conc (pg/mL)	300	3000	7000
N	18	18	18
Mean (pg/mL)	304.5	3178.3	7392.5
%CV	11.4	4.1	3.8
%Nom	101.5	105.9	105.6
DHEA	**QC-L**	**QC-M**	**QC-H**
Conc (pg/mL)	600	3000	7000
N	18	18	18
Mean (pg/mL)	598.1	3146.5	7277.5
%CV	9.3	6.6	7.7
%Nom	99.7	104.9	104.0

For the inter-run precision/accuracy experiment, each QC (low, medium and high) sample set was analyzed in triplicate on six different runs. **Table 3** shows the inter-run precision and accuracy results for testosterone and DHEA. The %CV was below 15% CV and the %Nom within 15% of nominal value.

Stability

Wet stability of sample extracts:

Following the extraction, the sample extracts are kept at 4°C in closed containers protected from light. After 1 day, sample extracts are spotted on a LazWell™ plate, dried and analyzed. Precision and accuracy of QC samples are reported in **Table 4**. All the results are within the acceptable criteria range for 1 day at 4°C.

Dry stability of samples spotted on LazWell:

Extracted samples are spotted onto a LazWell™ plate, dried and kept at room temperature for 1 hour before analysis. The precision and accuracy results of QC samples are reported in **Table 4.** All the results are within the acceptable criteria range for 1 hour at room temperature.

Table 4 *Wet and dry stability*

Testosterone	Wet stability (1 day / 4°C)			Dry stability (1 hour / RT)		
	QC-L	QC-M	QC-H	QC-L	QC-M	QC-H
Conc (pg/mL)	300	3000	7000	300	3000	7000
N	3	3	3	3	3	3
Mean (pg/mL)	304	3120.0	7230.2	295.9	3123.3	7137.1
%CV	4.4	4.4	6.4	10.8	3.5	3.1
%Nom	101.3	104.0	103.3	98.6	104.1	102.0
DHEA	**Wet stability (1 day / 4°C)**			**Dry stability (1 hour / RT)**		
	QC-L	QC-M	QC-H	QC-L	QC-M	QC-H
Conc (pg/mL)	600	3000	7000	600	3000	7000
N	3	3	3	3	3	3
Mean (pg/mL)	623.9	3179.3	6871.0	615.8	3096.2	7052.7
%CV	6.9	5.0	4.4	10.9	0.9	3.5
%Nom	104.0	106.0	98.2	102.6	103.2	100.8

Cross-validation study

Real patients' plasma samples (N=6: three males and three females) have been tested with this method to correlate with results obtained by traditional LC-MS/MS. The percentage difference between the values is evaluated. A difference below 20% is obtained. Results are reported in **Table 5**.

Table 5 *Cross validation results*

	Testosterone			DHEA		
	LC (pg/mL)	LDTD (pg/mL)	%Diff (%)	LC (pg/mL)	LDTD (pg/mL)	%Diff (%)
M1	6752	5623	-18.2	3127	3199	2.3
M2	11170	10373	-7.4	1745	2128	19.8
M3	11059	9188	-18.5	3656	3732	2.1
M4	421	435	3.3	3724	3102	-18.2
M5	156	2083	0.2	3491	3499	0.2
M6	9113	218	9.1	2148	1854	-14.7

CONCLUSION

- **Specific adduct formation to get selective MRM transition to separate isobaric molecules.**
- **Efficient Liquid-Liquid Extraction (LLE) is used to extract Testosterone and DHEA in serum samples.**
- **High-throughput analysis using LDTD-MS/MS.**
- **Accuracy, precision, and stability within the acceptance criteria.**
- **Sample-to-sample analysis of 8 seconds.**

COI Disclosure: I have a financial relationship with Phytronix Technologies as a salaried employee.