Development of the Comprehensive Method for Steroid Analysis by GCxGC-HR-TOFMS

Jonelle Shiel, David Alonso, and Viatcheslav Artaev | LECO Corporation, St. Joseph, MI USA

Introduction

Identification and quantitation of steroid metabolites in biological samples are essential for screening for various hormonal disorders. Many of these metabolites are closely related isomers and cannot be easily separated by LC or one-dimensional GC due to their chemical and structural similarity, which complicates or even prevents reliable analyte assignment.[1] Comprehensive two-dimensional gas chromatography (GCxGC), coupled to a high resolution high mass accuracy time-of-flight mass spectrometer (HR-TOFMS) provides dramatically enhanced chromatographic separation, an increase in sensitivity, and reliable detection and identification of analytes of interest.

Thirty-three steroids from different classes (progestogens, androgens, estrogens, glucocorticoids, mineralocorticoids) were derivatized and analyzed using GCxGC coupled to HR-TOFMS to achieve detection and reliable identification in complex matrices.

Steroid Standards Sample Preparation

For this study we acquired 32 steroid standards from Steraloids and one standard from Fisher Scientific.

Standard Preparation

- Prepare a 2 mg/ml solution in Methanol
- Pipette 10 µl into an autosampler vial
- Speed Vac to dryness for ~30 minute

Derivatization

- Add 20 µl Methoxyamine HCl in pyridine (20 mg/ml)
- Heat and agitate at 80 ºC for 1 hour
- Add 80 µl MSTFA +1% TMCS (purchased from Thermo Scientific)
- Heat and agitate at 100 ºC for 1 hour

Automation

- The GCxGC-HR-TOFMS was equipped with a GERSTEL Double Rail Autosampler. The Maestro software was programmed to automatically perform the derivatization procedure. ChromaTOF® brand software was used to collect and process data after injection.

Table 1. Lists steroid standards that were derivatized, analyzed, and used to create a User Accurate Mass Library.

Abbreviation	Trivial Name	Abbreviation	Trivial Name
SS (ISTD)	Stigmasterol	MP (ISTD)	Medroxyprogesterone
ANDRO	Androsterone	THA	Tetrahydro-11-dehydrocorticosterone
ETIO	Etiocholanolone	5α-THB	5α-Tetrahydrocorticosterone
DHA	Dehydroepiandrosterone	THF	Tetrahydrocortisol
11-OXO-ETIO	11-oxo-Etiocholanolone	5α-THF	5α-Tetrahydrocortisol
17β-Estradiol	17β-Estradiol	α-Cortolone	α-Cortolone
17-HP	17-Hydroxypregnanolone	β-Cortol	β-Cortol
11β-OH-ANDRO	11β-Hydroxyandrosterone	β-Cortolone	β-Cortolone
16α-OH-DHA	16α-Hydroxy-DHEA	Cortisone	Cortisone
PT	Pregnanetriol	Cortisol	Cortisol
5-AT	Androstentriol	20β-DHE	20β-Dihydrocortisone
THS	Tetrahydro-11-deoxycortisol	20α-DHE	20α-Dihydrocortisone
THDOC	Tetrahydrodeoxycorticosterone	20β-DHF	20β-Dihydrocortisol
Estriol	Estriol	6β-OH-F	6β-Hydroxycortisol
PT'ONE	Pregnanetriolone	18-OH-F	18-Hydroxycortisol
5-PT	Pregnentriol, 5-PT	20α-DHF	20α-Dihydrocortisol
THE	Tetrahydrocortisone		

Mixture Preparation

Mixture Preparation

- Pipette 10 µl of the 2 mg/ml of each of the standards into an autosampler vial
- Speed Vac to dryness for ~1 hour

Derivatization MSTFA

- Add 60 µl Methoxyamine HCl in pyridine (20 mg/ml)
- Heat and agitate at 80 ºC for 1 hour
- Add 300 µl MSTFA +1% TMCS (purchased from Thermo Scientific)
- Heat and agitate at 100 ºC for 1 hour

Derivatization TMSI

- Add 60 µl Methoxyamine HCl in pyridine (20 mg/ml)
- Heat and agitate at 80 ºC for 1 hour
- Speed Vac to dryness for ~1 hour
- Add 350 µl TMSI (purchased from Thermo Scientific)
- Heat and agitate at 100 ºC for 16 hours

Figure 1. Example Derivatization reaction of Androsterone. 1. Methoxyamine reacts with carbonyl group to produce methyloxime derivatives 2. MSTFA or TMSI uses silylation to react with hydroxyl group to produce trimethylsilyl (TMS) derivatives.

Methods

The analytes were run as individual components first and the resulting high resolution mass spectra were used to create a custom accurate mass library (AML). The AML assisted in the identification of steroids in the biological matrices. In addition, the standard mixture of the steroid was used for development of a chromatographic method for achieving the most efficient multidimensional separation by applying the Simply GCxGC™ software tool.[2] The *ChromaTOF* (LECO, St. Joseph, MI) brand software was used for instrument control, data acquisition, AML creation, peak finding, and compound identification.

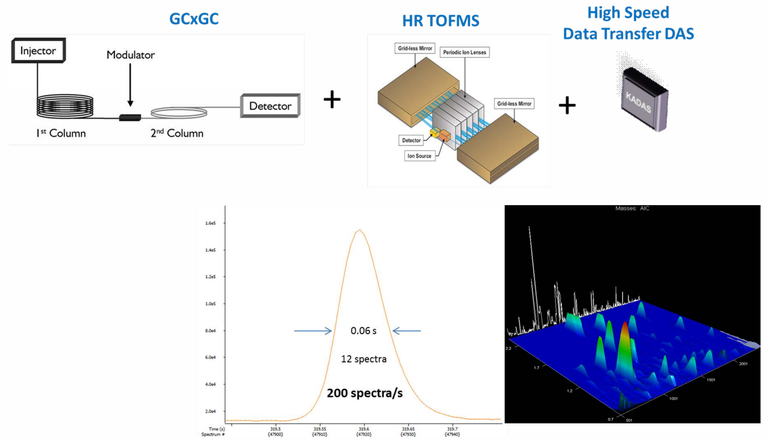

Figure 2. GCxGC coupled to HR-TOFMS concept.

Figure 3. Pegasus® HRT*4D (LECO Corp., St. Joseph, MI) – GCxGC-HR-TOFMS system used in this study.

Gas Chromatograph	Agilent 7890
Injection	1 µL, Split 100:1, 250 °C
Carrier Gas	He, 1.4 mL/min
Temperature	200 °C (0.5 min) – 300 °C at 5 °C/min
GCxGC – LECO Cryogenic Thermal Modulator	
Columns	1D: 15 m x 0.250 mm x 0.25 µm HP-1MS
	2D: 2 m x 0.250 mm x 0.25 µm BPX-50
	2D: 1.75 m in GC Oven, 0.10 m in Modulator, 0.15 m in 2D Oven
	Guard Column 1.4 m x 0.250 mm x 0 µm Uncoated
	Guard Column: 0.80 m in 2D Oven, 0.60 m in Transfer Line
Temperaure	2D Oven: +13 ºC, Modulator: +15 ºC
Modulation Period	3 seconds, Hot Pulse: 0.9 second
Mass Spectrometer – LECO *Pegasus* HRT+ 4D (R=25K@219 M/Z)	
Transferline Temperature	300 ºC
Ion Source Temperature	250 ºC
Spectra Acquisition Rate	200 spectra/second
Mass Range	40-1000

Results

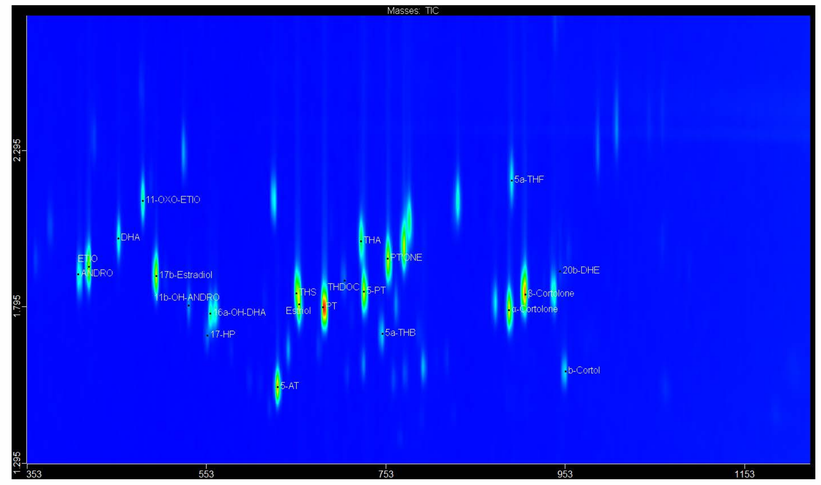

Figure 4. Contour 2D Chromatogram Plot of the derivatized mixture.

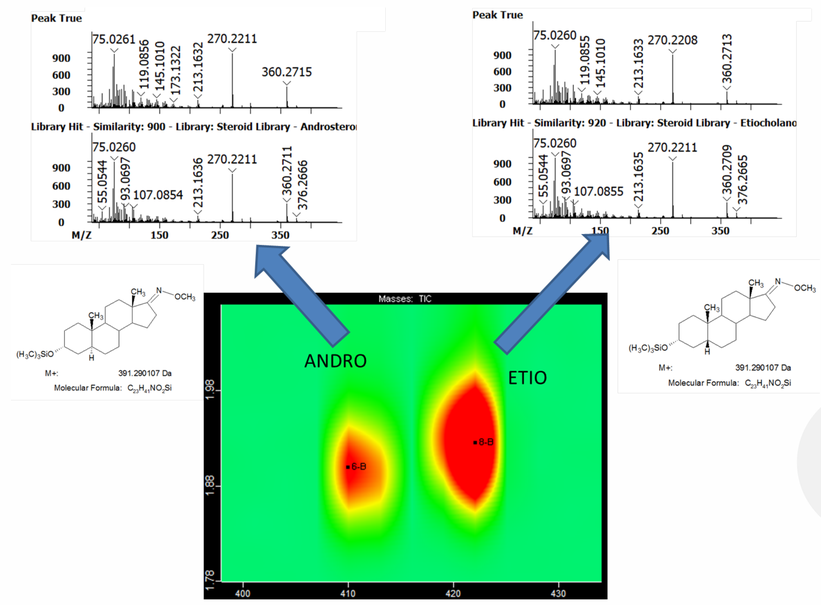

Figure 5. Contour 2D Chromatogram Plot region demonstrating an example of separation of the isomeric compounds.

Summary

- AML library was created by running individual derivatized steroid samples.
- GCxGC Method was developed allowing reliable separation of all 33 steroids in the sample.
- All 33 analytes were found in the mixture and positively assigned using accurate mass confirmation for molecular ions (when available) and major fragment ions, RI, and AML/NIST library matches.
- GCxGC-HR-TOFMS was successfully applied for non-targeted steroids identification in mixture.

Future Work

- Optimize derivatization procedures of the steroids in the matrix (urine).
- Validate the GCxGC-HR-TOFMS analysis method by using urine samples spiked with the standards mixture.

References

[1] Comprehensive Steroid Analysis By GCxGC-TOFMS, Michael Groessl et al, 288477, ThOG, Proceedings of the 65th ASMS Conference on Mass Spectrometry and Allied Topics, Indianapolis, IN, June 4-8, 2017.

[2] https://www.leco.com/simply-gcxgc

Delivering the Right Results