Development of Comprehensive Steroid Analysis Methods Using GCxGC-TOFMS

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Overview

A new method for the simultaneous analysis of 33 steroids in urine using GCxGC separation coupled with high resolution TOFMS was developed.

Introduction

Identification and quantitation of steroid metabolites in biological samples are essential for screening of 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, reliable detection, and identification of analytes of interest. GCxGC-HR-TOFMS provides comprehensive information about the sample, allowing study of the complete metabolome.

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.

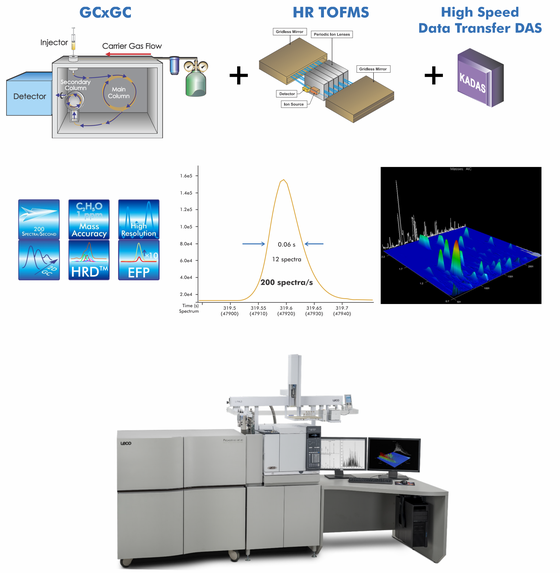

Figure 1. The concept of GCxGC coupled with High Resolution TOFMS with high speed data acquisition system.

Steroid Standards and Mixture Sample Preparation

Standards Preparation:

For this study we acquired 32 steroid standards from Steraloids and one standard from Thermo 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 °C for 1 hour
- Add 80 µl MSTFA +1% TMCS (purchased from Thermo Scientific)
- Heat and agitate at 100 °C 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.

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 °C for 1 hour
- Add 300µl MSTFA +1% TMCS (purchased from Thermo)
- Heat and agitate at 100 °C for 1 hour

Derivatization TMSI:

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

Table 1. Steroid standard list 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		

Figure 2. Example Derivatization reaction of Androsterone.

- **Methoxyamine reacts with carbonyl groups to produce methyloxime derivatives**
- **MSTFA or TMSI uses silylation to react with hydroxyl groups 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), to be used in identification of steroids in the biological matrices. In addition, the mixture of steroid standards was used for development of a chromatographic method for achieving the most efficient multidimensional separation by applying the Simply GCxGC® software tool[2]. ChromaTOF brand software (LECO, St. Joseph, MI), was used for instrument control, data acquisition, AML creation, peak finding, and compound identification.

Table 2. GCxGC and MS conditions

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

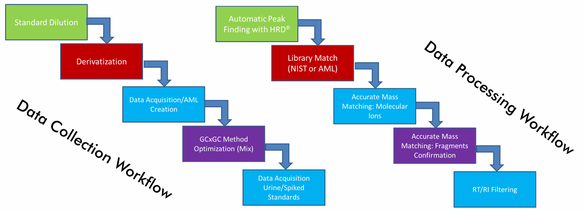

Figure 3. Data collection and data processing work flows.

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

Results and Discussion

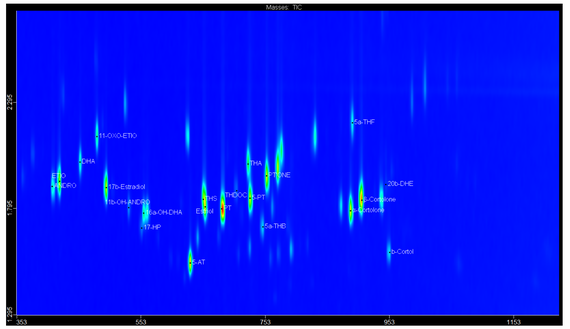

Figure 4. Contour 2D Chromatogram Plot of the derivatized mixture.

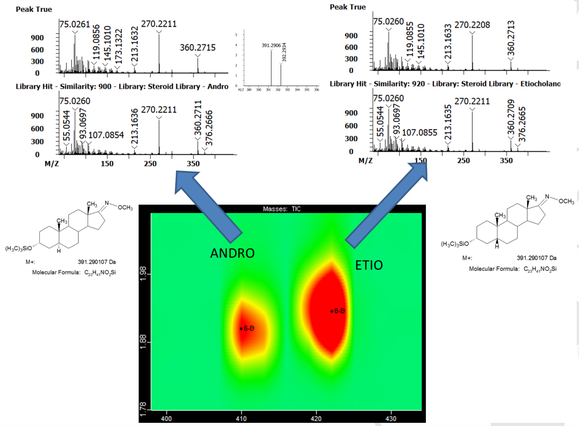

Figure 5. Contour 2D Chromatogram Plot region demonstrating an example of separation of the isomeric compounds in urine.

Conclusions

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

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