Isolation, Identification, and Determination of Designer Anabolic Steroids Commonly Found in Dietary Supplements

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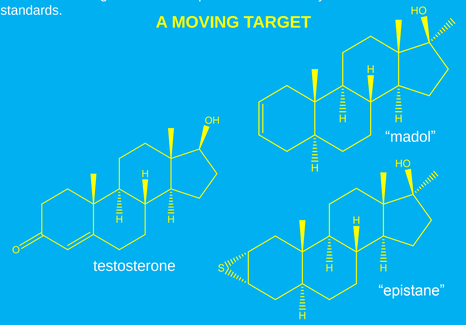
INTRODUCTION

The marketing of "pro-hormone" dietary supplements for athletes seeking to increase muscle mass, strength, endurance, and recovery time has increased dramatically in recent years. Stricter drug testing regulations have prompted some corrupt supplement manufacturers to use chemically-modified structures of existing anabolic steroids in an attempt to evade detection. These "designer" steroids are expected to convert to active hormones in the body, producing the desired effect. Although little is known about the pharmacological effects of these compounds, it is likely that they, like their banned counterparts, may cause serious long-term adverse health consequences.

The Forensic Chemistry Center (FCC) of the U.S. Food and Drug Administration has recently detected several steroid-like compounds in various dietary supplements, which could not be readily identified due to the lack of library reference spectra or commercially available standards. The general analytical approach to these emerging compounds is presented, including analysis by GC-MS, LC-MS, and/or HPLC-UV. Analytical scale high performance liquid chromatography with fraction collection is used to isolate and collect portions of designer steroids observed in samples received by the FCC. The isolated compounds are then characterized by high resolution accurate mass-mass spectrometry (HRAM-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy to elucidate their composition and structure. Quantitative analysis of these emerging substances in dietary supplements may be achieved using HPLC-UV comparison to structurally related reference standards.

A MOVING TARGET

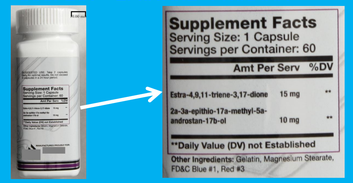


DEA CLASSIFICATION

21 U.S.C. 802(41)(A): A drug or hormonal substance is classified as an anabolic steroid by meeting the following four definitional requirements:

A. The substance is chemically related to testosterone
B. The substance is pharmacologically related to testosterone
C. The substance is not an estrogen, progestin, or a corticosteroid
D. The substance is not DHEA

SUSPECT "EPISTANE" PRODUCT



Supplement Facts
Serving Size: 1 Capsule
Servings per Container: 60

Amt Per Serv	%DV
Estra-4,9,11-triene-3,17-dione 15 mg	**
2a-3a-epithio-17a-methyl-5a-androstan-17b-ol 10 mg	**

**Daily Value (DV) not Established
Other Ingredients: Gelatin, Magnesium Stearate, FD&C Blue #1, Red #3.

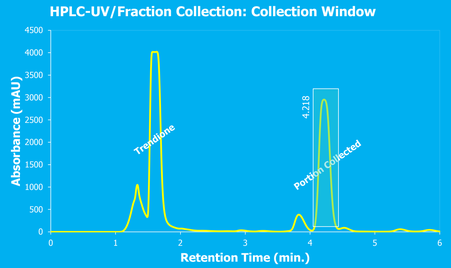
INITIAL SCREENING

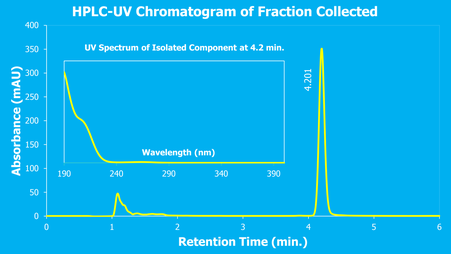
Instrumentation: Agilent GC 6890N Series with 7683 Series Autosampler, Agilent Mass Selective Detector (MSD) model 5973N, and ChemStation Software with Restek Rtx-5MS column and Agilent 1200 Infinity HPLC with Thermo Scientific Velos Pro MSD, Xcalibur Software, and a Phenomenex Luna 3µ C8(2) 100A column were used to screen the suspect sample for steroids by GC-MS and LC-MS, respectively.

Results: Initial screening of the suspect sample indicated the presence of madol, trendione, and a predominant peak with ions at *m/z* 320, 286, and 216, by GC-MS. Additionally, by LC-MS, the suspect sample was consistent with the presence of trendione, madol, and a peak with ions *m/z* 303 and 269.

HPLC-UV/FRACTION COLLECTION

Instrumentation: Agilent 1200 HPLC System with DAD detector, ChemStation Software, and Analytical Scale Fraction Collector with a Phenomenex Luna C18, 150 mm x 4.0 mm, 5 µm column were used to separate all components of the suspect sample and collect the steroid-like component of interest.



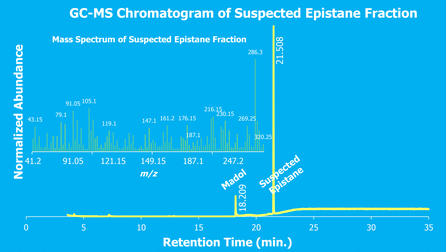


HPLC-UV Results:

- Separation of all components in the suspect sample was achieved in a 6 minute run time.
- The injection volume was increased and extraction performed to obtain a more concentrated injection. Several fractions were collected and dried to result in suspected pure epistane.
- A portion of the collected fraction was injected (using a smaller injection volume) and analyzed under the same parameters, by HPLC-UV. No additional components were detected by this method. A UV spectrum for the isolated component was obtained.

GC-MS OF ISOLATED COMPONENT

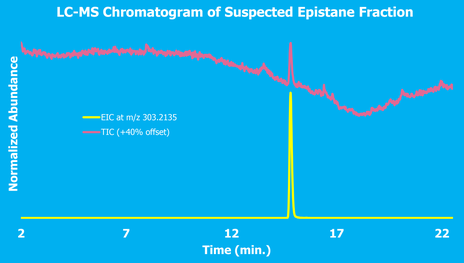
Instrumentation and method parameters for the initial GC-MS screen of the suspect product were used to analyze the isolated component.

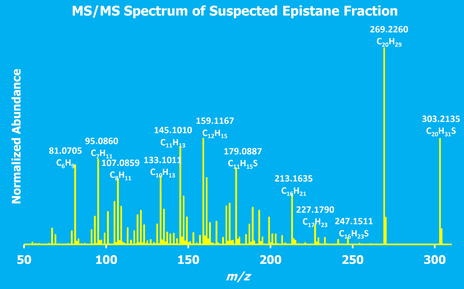


GC-MS Results: A peak with mass spectral data corresponding to madol was present in the isolated component GC-MS screen. It is likely that injection port conditions promote the conversion of the episulfide bridge to a double bond. Analysis of the fraction collected by HPLC-UV and LC-HRAM-MS did not indicate the presence of madol in the fraction.

LC-HRAM-MS

Instrumentation: Dionex UltiMate 3000 with Thermo Scientific Q-Exactive, Xcalibur Software, and a Phenomenex Luna 3µ C8(2) 100A column were used to determine the composition and purity of the fraction collected.





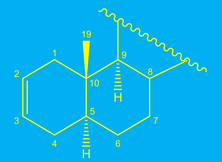
LC-HRAM-MS Results:

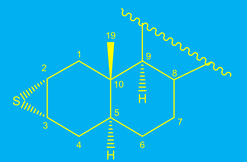
- Using LC-HRAM-MS, the fraction collected from the suspect sample exhibited a peak with *m/z* 303.2135, consistent with $[M+H-H_2O]^+$ of "epistane." No additional peaks were detected using this method.

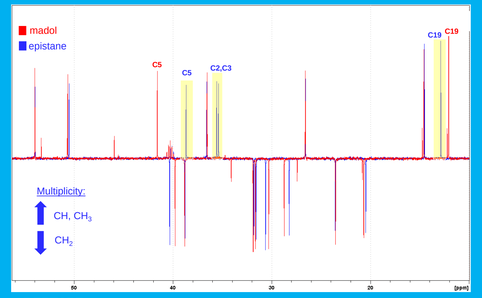
NMR OF "EPISTANE"

NMR System

- Bruker AVANCE III 500 MHz Spectrometer with 5mm BBFO probe







^{13}C NMR Assignments (δ, ppm)

Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-19
Madol	38.8	126.0	126.3	30.3	41.6	28.7	31.6	36.5	54.0	34.7	12.1
Epistane	38.7	**35.5**	**35.4**	30.6	**38.6**	28.2	31.5	36.5	54.0	35.3	**12.9**

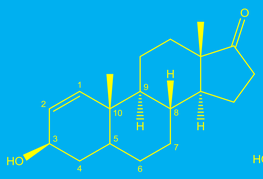
NMR Results

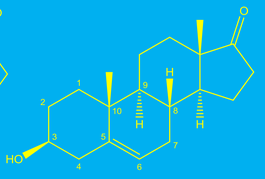
The isolated "epistane" fraction was dissolved in DMSO-d6 and analyzed by NMR using various 1D and 2D experiments. A commercial standard of the closely related "madol" compound was used as a reference for NMR spectral assignments. The ^{1}H spectrum of epistane exhibited a complex multiplet signal at δ 3.3 ppm, attributed to the methine protons at C2 and C3 of the episulfide ring. ^{13}C assignments above were made based on DEPT135, HSQC, and HMBC experiments.

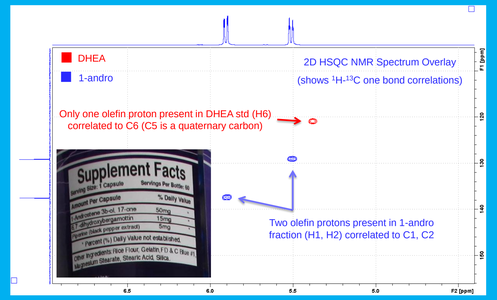
SUSPECT "1-ANDRO" PRODUCT

The previously described approach was also used to isolate and identify a second steroid-like compound known as "1-androsterone", with similar results. GC-MS provided information about the presence of the compound in a dietary supplement received by the laboratory. Separation of all components was achieved and the component of interest was collected and analyzed for structural identification and purity.

NMR OF "1-ANDRO"







QUANTITATIVE ANALYSIS

Finally, by comparing the molar absorptivities for unknowns and compounds with similar structural and/or UV spectral characteristics, it is proposed that one can determine if a certain commercially available reference standard is suitable for use in the estimation of an unknown steroid-like compound of interest. It is also proposed that differences in molar absorptivity may be used to apply a correction factor when estimating the concentration of the target compound.

CONCLUSION

Identification of steroid-like compounds for which no reference spectra or standard is available is possible using a multi-technique based approach. GC-MS is utilized as an initial screening tool, which is followed by HPLC-UV separation and subsequent isolation of the compound of interest. LC-HRAM-MS and NMR can be used to determine the composition and structure of the component isolated, as well as provide information about the purity of the fraction collected.

The proposed methodology serves as an avenue for the identification, isolation, and determination of "designer" anabolic steroids or steroid-like compounds seen frequently in dietary supplements. It provides an analytical approach to the characterization of compounds that were previously not identified.

REFERENCES

1. Masato Okano, Mitsuhiko Sato, Ayako Ikekita, Shingji Kageyama, *Analysis of non-ketoic steroids 17α-methylepithiostanol and desoxymethyltestosterone in dietary supplements,* Drugs Testing and Analysis, Volume 1, Issues 11-12, 2009, Pages 518-525