Screening designer steroids in urine using ultra-high resolution time-of-flight mass spectrometry and liquid chromatography time-of-flight mass spectrometry with comprehensive fragmentation

Kevin Siek[1], Erica A. Guice[2], Jeffrey S. Patrick[1], Joe Binkley[1] 1 - LECO Corporation, Separation Science Division, St. Joseph, MI 49085; 2 – Western Slope Laboratory, LLC, Troy, MI 48083

Background

Introduction

Abused anabolic steroids (AAS) are used to enhance the performance of athletes in competition. The variety of compounds being used is dynamic and directed towards defeating analytical methods. Urine is the most common matrix for analysis and provides a significant challenge to analyst and instrument. Triple quadrupoles (QqQ) are historically used for their selectivity and sensitivity with a compromise in the absence of comprehensive data. High performance time-of-flight mass spectrometry provides an alternative to QqQ by leveraging different selectivity mechanisms and offering comprehensive data. The use of high performance time of flight mass spectrometry interfaced to UHPLC is applied to the detection and quantitation of steroids (including boldenone, clenbuterol, DHEA, nandrolone, formestane and others) in urine samples after enrichment using solid-phase extraction. The high resolving power and mass accuracy achieved is available in fragment ions which are leveraged to enhance selectivity in a fashion similar to the MRM on a QqQ.

Experimental

Materials - Methandrostenolone, stanozolol, 3-hydroxystanozolol, and 3-hydroxystanozolol-d3 (deuterated internal standard) were obtained from Cerilliant. Clostebol, 6β-hydroxyboldenone, and oxandrolone were obtained from Alltech. Urine samples were obtained from an anti-doping surveillance program.

Sample Preparation - Samples were prepared using conventional procedures of hydrolysis followed by solid phase extraction. Details have been given by Guice [1].

UHPLC and Flow-Injection -An Agilent 1290 series pump and autosampler were used for flow injection sampling. The injection volume was 3 µL, and the flow rate was 300 µL/min. Consecutive duplicate injections of each sample were made. An injection was made approximately every 30 seconds, allowing sufficient time for flushing of the injection system between injections.

UHPLC separation was achieved using a 2.2 µm Restek Ultra II C18 50 mm x 2.1 mm ID column. The flow rate was 360 µL/min. The mobile phase composition was 10% B at time zero, followed by a linear gradient to 40% B at 0.25 minutes, and a linear gradient to 100% B at 6.25 minutes. 100% B was held isocratically for 1.5 minutes prior to re-equilibration at 10% B for 1.25 minutes. The injection volume was 5 µL, and the column temperature was 40°C.

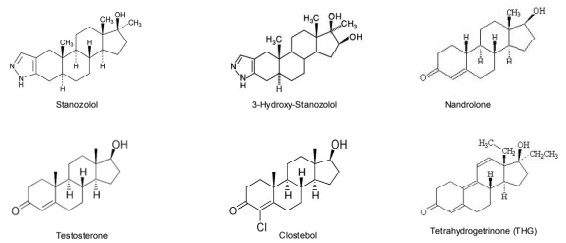
For both modes of analysis mobile phase B was methanol and mobile phase A was an aqueous solution of 26 mM formic acid and 1 mM ammonia, prepared by adding 1 mL formic acid and 0.1 mL 25% aqueous ammonia to 1 liter of water.

Mass Spectrometry Detection was achieved using a LECO Citius™ LC-HRT high resolution time-of-flight mass spectrometer with an electrospray ionization source operated in positive ion mode.

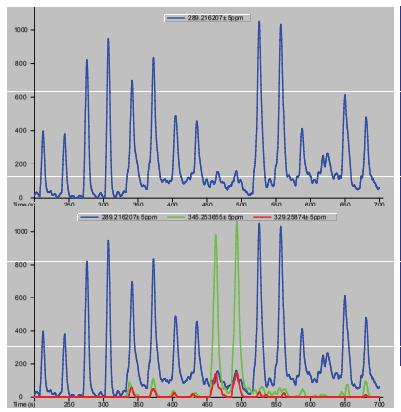
Flow Injection Analysis –
Ultra High Resolution Mode (R = 100,000(fwhm))
Mass Range 160 – 600 m/z
Acquisition Speed – 3 spectra/second
Calibration with polyethylene glycol

HPLC-
High Resolution Mode (R = 50,000(fwhm))
Mass Range 50 – 525 m/z
MSc² Offset as noted in results
Acquisition Speed – 3 spectra/second (alternating between low and high energy)
Calibration with polyethylene glycol

Representative Targets for Analysis

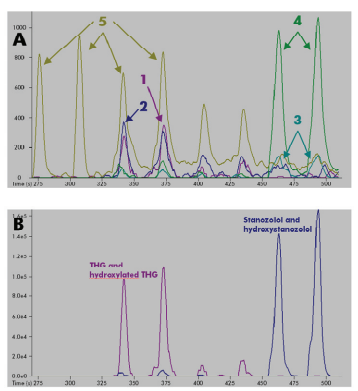


Direct Analysis Using High Resolution Time of Flight



High-throughput monitoring of testosterone and its isomers by m/z 289.2162 (5 ppm window)

Global analysis by high resolution MS captures the reason why one sample may be low: elevated levels of AAS's as shown by accurate m/z XIC



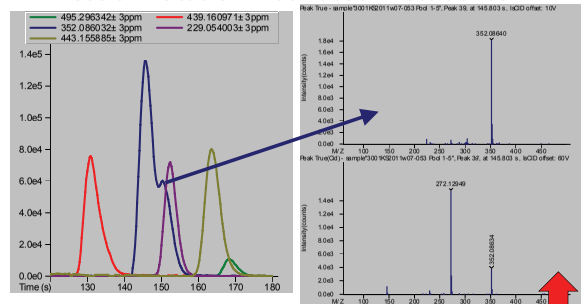
A segment of the ultra high resolution screening analysis

A: Extracted ion traces: THG (1), Hydroxylated THG (2), Stanozolol (3) Hydroxylated Stanozolol (4), and Testosterone (5)

B: eXIC - Multiple signal correlation traces: greater than zero only if signals for an unmetabolized steroid and its hydroxylated metabolite correlate within a few injection peak. Good agreement of signal intensity is observed between replicate injections. Enhancement derives from segregated accurate mass signals

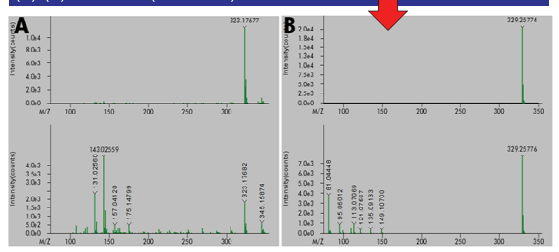
UHPLC/MS Analysis Using High Performance TOFMS

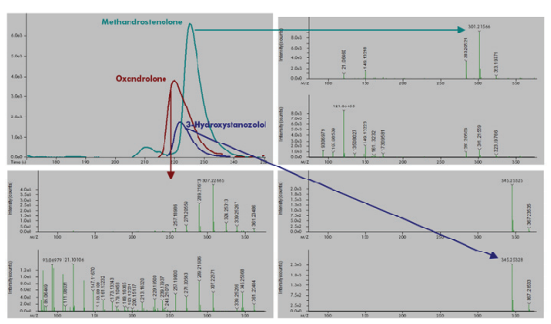
Conjugates identified by neutral loss annotation – sulfated metabolite



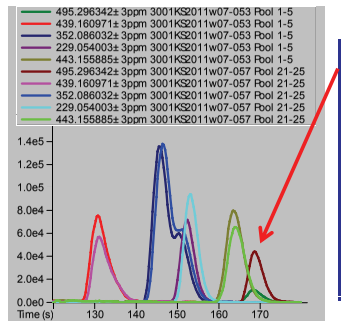
Deconvolved spectra of co-eluting drugs showing MS and MSc² spectra fr a sulfated metabolite (Difference = 79.95685)

Deconvolved spectra (MS and MSc²) of
(A) Clostebol (50 eV CID) and
(B) (B) Stanozolol (65 eV CID)





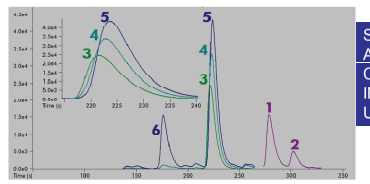
Deconvolved spectra of co-eluting steroids, each at collision offsets of 10 V (top) and 60 V (bottom)



SELECTIVE ANALYSIS FOR DIFFERENTIAL QUANTITATION OF METABOLIC CHANGES

Analysis of endogenous metabolites using accurate m/z signal extraction (3 ppm window). Samples are two pools of different conditions

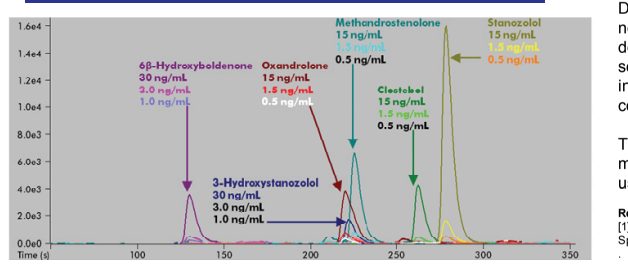
Preganediol glucuronide (m/z = 495.296342) shows multifold change between conditions



SELECTIVE ANALYSIS OF A GROUP OF RELATED COMPOUNDS (STANOZOLOL) IN URINE SAMPLE USING **eXIC** CAPABILITIES

Stanozolol-related analytes detected in a sample
1: Stanozolol; [M+H]+ = 329.259, principal fragment = 81.045
2: Stanozolol-related; [M+H]+ = 329.259, principal fragment = 81.045
3: 3-Hydroxystanozolol-d3 (IS); [M+H]+ = 348.272, principal fragment = 97.040
4: 3-Hydroxystanozolol; [M+H]+ = 345.254, principal fragment = 97.040
5: Hydroxystanozolol-related; [M+H]+ = 345.254, principal fragment = 81.045
6: Hydroxystanozolol-related; [M+H]+ = 345.254, principal fragment = 81.045

Linearity of response of six steroids **eXIC** using MSc² and correlated precursor and fragment ions. Concentrations evaluated from 0.15 through 150 ng/ml (5 µL injection).



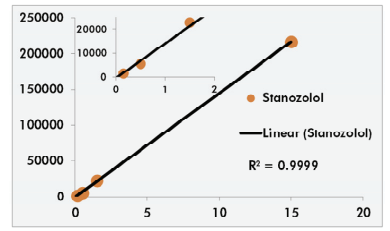
Linearity and Quantitation

Linearity of Response for Steroid Analytes

Results obtained using eXIC and MSc² analysis with selected accurate m/z pairs.

Concentration / ng mL⁻¹	6β-Hydroxyboldenone	3-Hydroxystanozolol	Concentration / ng mL⁻¹	Formebolone
300	402390	197330	150	437306
30	48481	73498	15	49787
3.0	4792	1488	1.5	9674
1.0	2292	327	0.5	3783
0.30	1469	n/a	0.15	197
Slope	2668	1214	Slope	2895
r^2	0.999649	0.999995	r^2	0.999992

Concentration / ng mL⁻¹	Methandrostenolone	Clostebol	Stanozolol
150	772486	319661	1897209
15	84447	36905	214595
1.5	10629	3345	22791
0.5	3264	900	5519
0.15	939	87	1323
Slope	5131	2460	12615
r^2	0.999994	0.999994	0.999996



Linear Regression of the eXIC signal from Stanozolol. This shows linearity as well as sensitivity in response.

Conclusions

Flow-injection electrospray ionization high resolution mass spectrometry can process sample at rates competitive with immunoassays. This requires high resolving power (ca. 100,000) and system stability/mass accuracy (mass drift over short times) in the low ppm range.

As resolving power of commercial instruments continues to increase, this approach will become more practical for general screening.

Mass spectrometry with comprehensive collision induced dissociation (MSc²) takes full advantage of a high resolution mass analyzer. By providing the capability for retrospective data analysis and makes complete precursor and product ion spectra available for confirmation of analyte identity.

Deconvolution facilitates accurately distinguishing precursor and product ions of near-dead co-elutions and produces spectra of high integrity. Signals from deconvolved spectra can be adaptively combined to retrospectively extract highly selective quantitative information (eXIC) regarding any known or unknown analyte in the sample. Sensitivity remains independent of the number of signal combinations extracted.

The data described above demonstrate the overall utility of high performance TOF mass spectrometry in the qualitative and quantitative analysis of drugs of abuse using different steroids as challenging examples.

Reference
[1] Guice, E.A.: Anabolic Androgenic Steroid Testing by Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometry in Urine. Conference Proceedings Mass Spectrometry Applications to the Clinical Lab (2011)