Enhanced Detection and Separation of Anabolic Steroids for Anti-Doping Control Screening by Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass Spectrometry (GCxGC-TOFMS)

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Key Words: GCxGC-TOFMS, Anabolic Steroids, World Anti-Doping Agency

1. Introduction

The advantages of GCxGC-TOFMS are demonstrated in this research. This application illustrates the capability of comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry to be an effective instrumental option for anti-doping control screening. Anabolic steroid screening analysis in urine is complicated, requiring sensitive instrumentation and optimized chromatographic separations. This application presents a practical method utilizing comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) for the identification and quantification of five androgenic anabolic steroids in urine. Established methods for steroid analysis in urine depend heavily on one-dimensional gas chromatographic separations and selected ion monitoring (SIM) mass spectrometry methods. This experimental method employs GCxGC to increase peak capacity and resolution in combination with time-of-flight mass spectrometry (TOFMS) detection followed by data processing with deconvolution software algorithms for positive confirmation of anabolic steroids in urine.

Stanozolol (Winstrol), 4-Hydroxystanozolol, Boldenone, 19-Norandrosterone, 17α-Methylandrostan-3α-17β-diol, and 3-Hydroxystanozolol were prepared as a steroid mixture from commercial standards. The illegal steroid Stanozolol and metabolites are known to be particularly difficult to detect and separate chromatographically. Results for the identification of 3-Hydroxystanozolol at the 2 ppb level are presented. Methyltestosterone was used as an internal standard at a concentration of 200 ng/mL. Stanozolol (Winstrol) exhibits poor chromatographic response below 10 ppb and was not included in the calibration curve development. This analysis poses a variety of challenges for the laboratory analyst. Sample preparation is difficult, labor intensive, complex, and problematic to reproduce consistently. Matrix effects from the complexity of urine often obscure detection of trace level steroids and their metabolites. The serious legal nature and difficulty in performing this analysis demands that instrumentation must be able to provide absolute confirmation and verification of the analytical results. Sample preparation utilized an established extraction and derivatization procedure for anti-doping control. Significant improvements in chromatographic resolution and peak capacity, as well as the enhanced detectability that GCxGC-TOFMS provides are shown by the results of this anti-doping control screening analysis. The application results show successful trace level identification of the five steroid standards at 2 ppb levels. This research demonstrates favorable results and practical applicability of GCxGC-TOFMS for the positive identification of anabolic steroids at the lowest allowable concentration limits in compliance with the guidelines set by the World Anti-Doping Agency (WADA). GCxGC provides increased peak capacity and enhanced chromatographic resolution not possible in one dimensional chromatography. High speed TOFMS provides fast acquisition, up to 500 Hz, that is essential for the successful acquisition and analysis of the data density needed to fully characterize low levels of steroids in complex sample matrices such as urine. The data rich files generated by GCxGC-TOFMS are processed with deconvolution algorithms which deliver qualitative identification as well as a multiple compound quantification in a single run. Results from this work show limit of detection values at or below 2 ppb for five anabolic steroids with a quantitative calibration linearity of greater than 99.9 percent.

2. Experimental Conditions

Initial sample preparation method development focused on the acid hydrolysis of conjugated steroids, extraction, and trimethyl-silyl derivatization procedures, in addition to the chromatographic (GCxGC) and mass spectrometry (TOFMS) instrumental optimization. Sample preparation was conducted with established protocols for steroid analysis in urine. (1) Steroid reference standards were purchased from Cerilliant (Round Rock, Texas) and Alltech-Applied Science Labs (Deerfield, Illinois). A five component steroid stock standard mixture was prepared in methanol. Serial dilutions were made from the steroid stock standard mixture and then spiked into 2 mL urine aliquots. Methyltestosterone was spiked into each 2 mL urine matrix sample at 200 ng/mL as an internal standard prior to addition of the steroid standard. A working five point calibration made from the stock steroid standard spiked into 2 mL aliquots of urine at 2, 10, 20, 50, and 100 ng/mL. Acid hydrolysis with beta-glucuronidase was carried out prior to solvent extraction with Methyl-tert-butyl-ether. Extracts were dried completely using nitrogen evaporation. Trimethylsilyl-derivatization was conducted with a commercially available MSTFA-ammonium-iodide-ethanethiol mixture from (Sigma-Aldrich, Saint Louis, Missouri). A 3 μL pulsed splitless injection was used to analyze each sample immediately after derivatization.

A Pegasus® 4D time-of-flight mass spectrometer (LECO, St. Joseph, Michigan) was used to generate the GCxGC-TOFMS results. The Pegasus 4D instrument was equipped with an Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, California) featuring a two stage cryogenic modulator and a secondary oven (LECO). LECO ChromaTOF® software was used for all acquisition control, data processing, and calibration curve development. A 30 m x 0.25 mm x 0.25 μm film thickness, Rxi-5ms, (Restek Corp., Bellefonte, Pennsylvania) GC capillary column was used as

the primary column for the GCxGC-TOFMS analysis. In the GCxGC configuration, a second column of 1.2 m x 0.18 mm id. x 0.20 μm film thickness, BPX-50, (SGE Analytical Science, Austin, Texas) was placed inside the secondary GC oven after the thermal modulator. Helium carrier gas flow rate was set to 1.5 mL/minute at a corrected constant flow via pressure ramps. The primary column was programmed with an initial temperature of 140°C for 0.20 minute then ramped at 20°C/minute to 170°C, next ramped at 5°C/minute to 260°C for 2.0 minutes, with a final ramp at 10°C/minute to 315°C for 12.0 minutes. The secondary column temperature program was set to an initial temperature of 145°C for 0.20 minute then ramped at 20°C/minute to 175°C, next ramped at 5°C/minute to 265°C for 2.0 minutes, with a final ramp at 10°C/minute to 320°C for 12.0 minutes. The thermal modulator was set to +20°C relative to the primary oven and a modulation time of 4 seconds was used. The GCxGC-TOFMS analysis total runtime was 39.2 minutes. The MS mass range was 45 to 750 m/z with an acquisition rate of 100 spectra per second. The ion source chamber was set to 230°C. GCxGC-TOFMS analysis was conducted immediately after sample derivatization. Subsequently, calibration curves were developed and loaded into the software for automated data processing and quantification purposes. Method development achieved an optimized GCxGC-TOFMS analysis capable of effectively separating the five anabolic steroids from the complex urine matrix at or below 2 ppb detection limits.

3. Results and Discussion

Trace level identifications of the five steroid standards mixture was achieved at the 2 ng/mL with library search match similarities of greater than 60 percent. Over 5000 peaks per sample were detected for this analysis. Figure 1 displays the three-dimensional contour plot chromatogram for the 20 ng/mL standard GCxGC-TOFMS analysis. The highlighted peaks show the labeled spiked steroids at the concentration of 20 ng/mL in urine throughout the chromatographic plane. The three-dimensional chromatogram clearly shows the ability of GCxGC to maximize separation and resolution of anabolic steroids from the complex urine matrix in both the first and second dimensions.

The two-dimensional surface plot chromatogram in Figure 2 shows a zoomed-in portion of the 20 ng/mL steroid GCxGC-TOFMS analysis where Stanozolol, 3-Hydroxystanozolol, and 4-Hydroxystanozolol elute. This figure is an excellent example of the increased peak capacity available with comprehensive two-dimensional chromatography. The Stanozolol and Hydroxystanozolols derivatize with one, two, or three trimethylsilyl groups. Each of the compounds, Stanozolol, 3-Hydroxystanozolol, and 4-Hydroxystanozolol elute in the same first dimension retention time modulation periods depicted by the arrows in Figure 2.

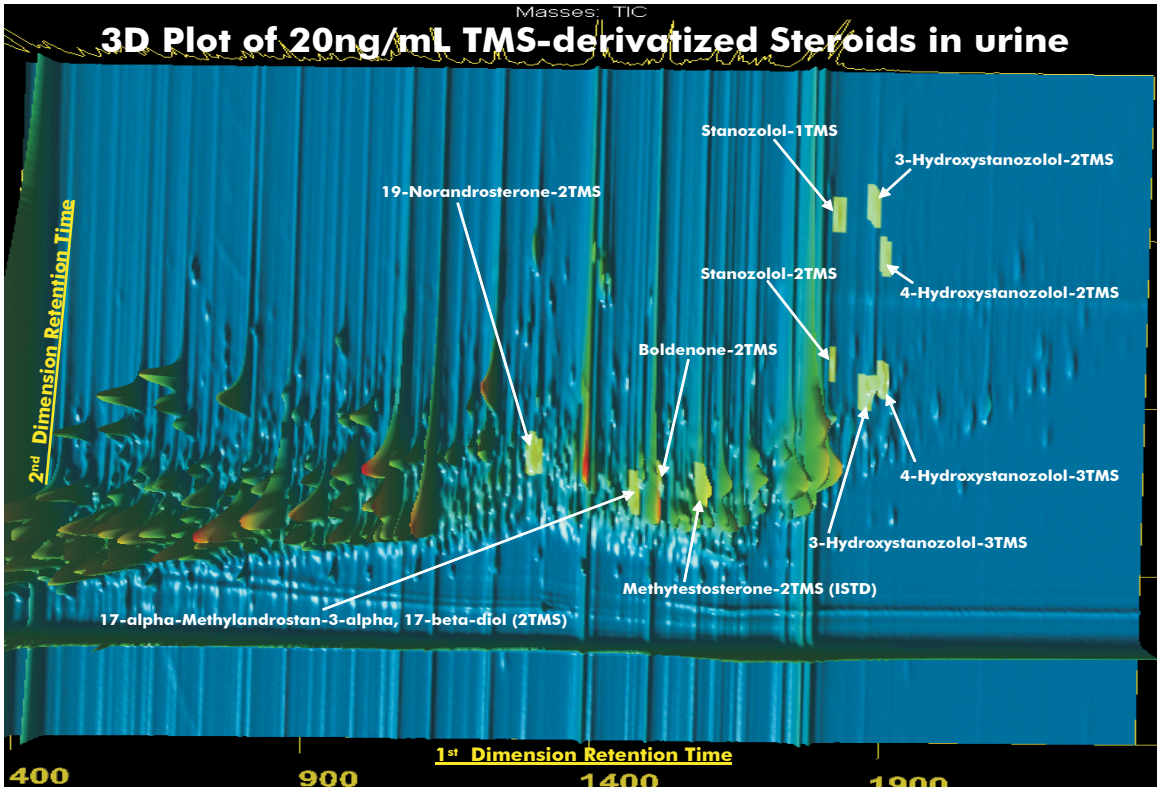

Figure 1. This three dimensional surface plot shows the GCxGC-TOFMS analysis of the 20 ng/mL sample analysis with peaks labeled for the steroids Stanozolol, 4-Hydroxystanozolol, Boldenone, 19-Norandrosterone, 17α-Methylandrostan-3α-17β-diol, 3-Hydroxystanozolol and the internal standard Methyltestosterone.

However the Stanozolol and Hydroxystanozolols which derivatized with fewer trimethylsilyl groups are completely separated in the second dimension as shown by the 1TMS and 2 TMS Stanozolol-metabolites which elute between 2.32s and 3.32s in the second dimension. Baseline resolution is achieved for all partially derivatized Stanozolol compounds that would otherwise coelute in one-dimensional chromatography further complicating the mass spectra of each. Figure 2 clearly shows an excellent example of how GCxGC fully resolves analytes thereby increasing the mass spectral identification capability of the analysis.

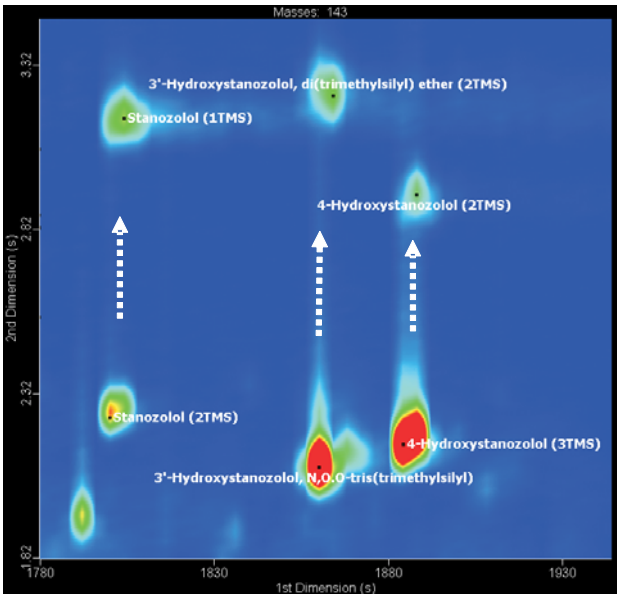

Figure 2. The GCxGC-TOFMS analysis chromatogram in Figure 2 illustrates a zoomed-in portion of the two-dimensional contour plot for the 20 ng/mL derivatized steroid standard in urine. The extracted ion chromatogram (m/z 143) displays the separation of Stanozolol, 3-Hydroxystanozolol, and 4-Hydroxystanozolol partially or fully derivatized with 1, 2, and 3 trimethylsilyl groups. This figure shows the fully resolved Stanozolol (1TMS), 3-Hydroxystanozolol (2TMS), and 4-Hydroxystanozlol (2TMS) by comprehensive two-dimensional chromatography that would otherwise be totally coeluted in one-dimensional chromatography.

Compliance with WADA Minimum Required Performance Levels (MPRL) for Detection of Prohibited Substances

Table I shows the five steroid standards and the internal standard results of the low level standards at 2 ng/mL which is the WADA cut-off limit. The column highlighted in red lists the quantitative signal to noise ratio for each steroid. S/N ratios for the five steroids ranged from 85 to 1353 at the 2 ppb concentration. The low level cut-off guidelines are achieved and exceed the limits set by WADA. Limits of detection at a 10:1 S/N were calculated from the 2 ng/mL standard quant S/N results shown in Table 1. The calculated limits of detection extend from 15 to 235 pg/mL in the part per trillion range.

Calibration Curve Development

A five point calibration curve was developed in ChromaTOF software in the concentration range of 2 to 100 ng/mL for each of the five derivatized steroid standards. Figure 3 illustrates the calibration curve developed in ChromaTOF for 17-alpha-Methylandrostan-3-alpha, 17-beta-diol (2TMS) with a first order linear correlation coefficient value of 0.99987. Calibration curve development for all GCxGC results for the 2, 10, 20, 50 and 100 ng/mL steroid standards was achieved with greater than 99.98 percent linearity. The generation of quantitative calibration curves for the five steroid standards used in this study demonstrates the practical application of utilizing GCxGC-TOFMS for anti-doping control screening.

The column headings in Table II show the steroid analyte name, absolute first and second dimension retention time, the analyte type, curve fit, the first order equation, and the calculated correlation coefficient for each standard. The calibration table developed for this research produced excellent linearity for all five steroids. Results for the calibration curve development yielded linearity of greater than 99.9 percent for the five TMS-derivatized steroid standards.

Table I. The peak table for five steroids and steroid metabolites are listed in Table I for the low level (MPRL) WADA cut-off limits at 2 ppb. The quantitative S/N ratios range from 85 to 1353 for the 2 ng/mL standards. These S/N ratios exceed the minimum required performance levels set by the WADA guidelines for all of the steroid standards.

Name	Quant S/N	Area	Concentration	1st Dimension Time (s)	2nd Dimension Time (s)	UniqueMass	Similarity
19-Norandrosterone, 3-trimethylsilyl ether, 17-trimethylsilyl enol ether	1353.20	387757	2 ng/mL	1292	1.72	315	749
17-alpha-Methylandrostan-3-alpha, 17-beta-diol (2TMS)	1064.50	271595	2 ng/mL	1464	1.50	143	857
Boldenone (2TMS)	970.86	1041076	2 ng/mL	1500	1.56	206	744
Methyltestosterone 2TMS (ISTD)	30031.00	2.4E+07	200 ng/mL	1576	1.54	301	908
3'-Hydroxystanozolol, N,O,O-tris(trimethylsilyl) deriv.	93.92	80321	2 ng/mL	1860	2.06	254	619
4-Hydroxystanozolol (3TMS)	85.12	127836	2 ng/mL	1884	2.16	143	739

Table II. The calibration table used to develop calibration curves in ChromaTOF software is shown in Table II. Calibration linearity of greater than 99.9% was achieved for all of the steroids.

Name	Absolute R.T. (sec , sec)	Type	Equation	Correlation Coefficients
19-Norandrosterone, 3-trimethylsilyl ether, 17-trimethylsilyl enol ether	1292 , 1.840	Analyte	y= +0.00590503x + 0.0106961	0.99924
17-alpha-Methylandrostan-3-alpha, 17-beta-diol (2TMS)	1464 , 1.630	Analyte	y= +0.00315231x + 0.00859919	0.99987
BOLDENONE (2TMS)	1500 , 1.680	Analyte	y= +0.0174845x + 0.0131281	0.99927
Methyltestosterone 2TMS	1580 , 1.520	Internal Standard	NA	NA
3'-Hydroxystanozolol, N,O,O-tris(trimethylsilyl) deriv.	1864 , 2.200	Analyte	y= +0.00464906x - 0.00601017	0.99911
4-Hydroxystanozolol (3TMS)	1884 , 2.170	Analyte	y= +0.00353856x - 0.00390789	0.99961

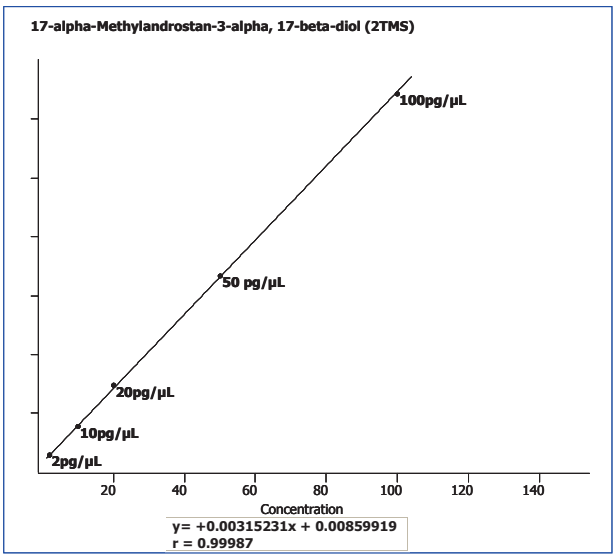

Figure 3. This five point calibration curve shows the first order linear fit for 17-alpha-Methylandrostan-3-alpha, 17-beta-diol (2TMS) in the calibration range from 2 to 100 ppb. The calculated correlation coefficient value equals 0.99987 demonstrating calibration linearity of greater than 99.98 percent.

Mass Spectral Deconvolution

Mass spectral deconvolution of trace ppb components in heavy sample matrices has greatly improved utilizing non-skewed mass spectra and fast acquisition generated by TOFMS. Figure 4 displays a deconvolution example for the trace level concentration of the derivatized anabolic steroid (19-Norandrosterone) at 2 ppb. The spiked steroid is coeluted and buried under the endogenous anabolic steroid peak, 5a'-Androst-16-EN-3a'-OL. The extracted ion chromatogram in Figure 4 shows the masses for m/z 315, 241, 57, and 117. The peak markers for the unique masses m/z 315 and 241 show the peak apex separation of approximately 20 milliseconds. The mass spectrum labeled A shows the Caliper or (total ion mass spectrum) before deconvolution. The mass spectrum labeled B displays the Peak True deconvoluted mass spectrum for 19-Norandrosterone. The mass spectra labeled C is the library search match for 19-Norandrosterone (2TMS). A library search match similarity of 74.9 percent was given for the 19-Norandrosterone (2 ppb) derivatized standard peak. This illustration clearly shows the deconvolution of a trace anabolic steroid even when it is buried in and masked under a heavy sample matrix.

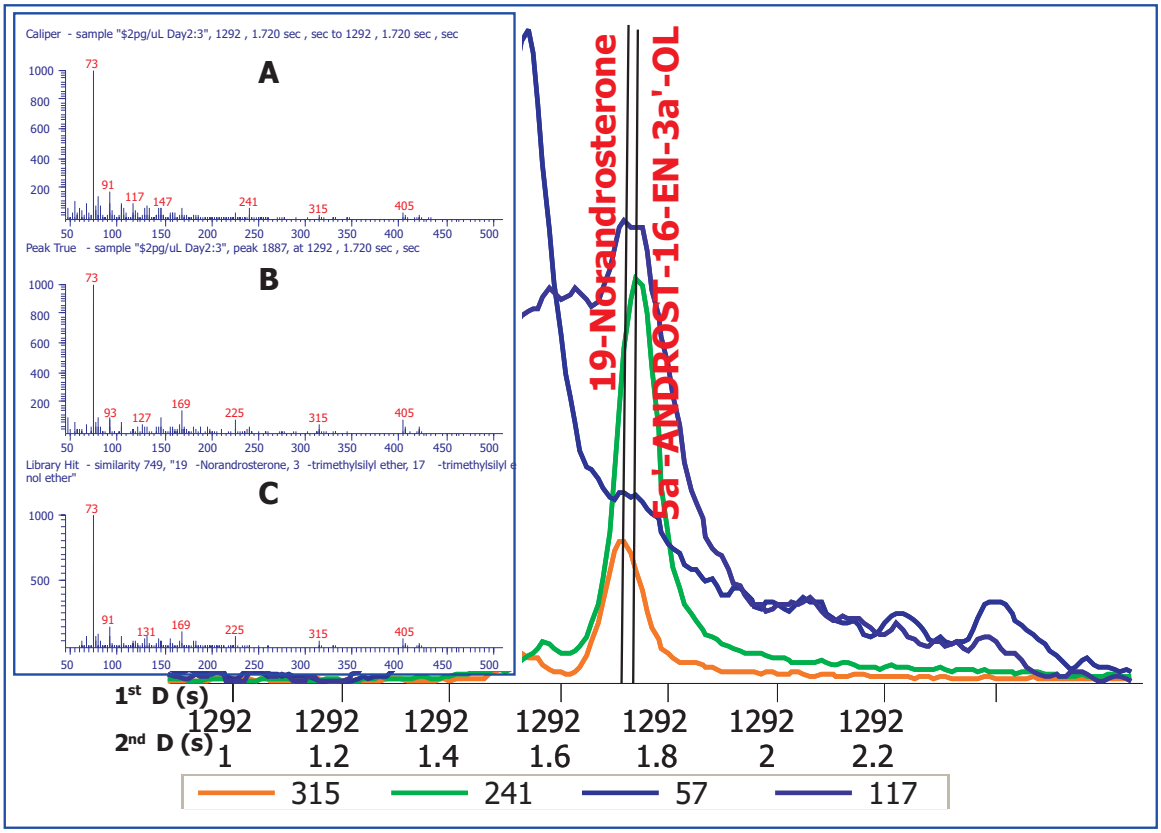

Figure 4. The extracted ion chromatogram for m/z 315, 241, 57, and 117 is displayed showing the corresponding first and second dimension retention times. The vertical labeled peak markers designate identification of the 2 ppb trace level anabolic steroid 19-Norandrosterone buried under the endogenous steroid 5-alpha-Androst-16-EN-3-alpha-OL. The mass spectra in the inset are (A) the Caliper total ion mass spectrum before deconvolution, (B) the Peak True deconvoluted mass spectrum and (C) the Library match hit with a 75% library match for 19-Norandrosterone.

4. Conclusions

This application demonstrates that steroid screening by GCxGC-TOFMS is improved significantly compared to one dimensional chromatography using single quadrupole GCMS. Excellent calibration linearity for quantitation was achieved for all analytes at greater than 99.9%. Limits of detection at part per trillion levels were calculated from acquired data for all five steroids. This research demonstrates the ability of GCxGC-TOFMS to provide enhanced analyte detectability, deconvolution of trace level concentrations of steroids, and exceptional quantitation capabilities for the analysis of illegal anabolic steroid screening in urine. The results of this experimentation show that significantly increased analytical performance was accomplished using comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOFMS) for anti-doping screening control. In conclusion this application shows favorable and practical applicability for the positive identification of anabolic steroids in urine at or below the lowest allowable concentration limits established in the guidelines set forth by the World Anti-Doping Agency (WADA).

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