A Reduced Workflow Solution for the Analysis of Gamma-Hydroxybutyrate (GHB) in Human Hair Samples Via an Automated Bead Mill as a Precursor to High Resolution-Gas Chromatography/Time-of-Flight (GC/HRT) and 2D Gas Chromatography/Time-of-Flight (GCxGC/TOF)

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Introduction

Gamma-hydroxybutyrate (GHB) is an endogenous compound and is also a drug commonly used by bodybuilders, seen in the club scene, and used in drug-facilitated sexual assaults. GHB has been shown to have an elimination rate of 18 mg/L/h, so it is typically cleared from the blood within approximately 6 h and from the urine within approximately 12 h after it is taken orally. Drugs in hair have an extended window of detection, therefore, hair is a useful alternative matrix for GHB analysis.

Techniques to interrogate hair samples have proven valuable in detecting exposure to drugs of abuse over a long sampling window. The hair matrix is challenging to work with, and a number of methods have been reported for the extraction of drugs from hair. Analysis of GHB is typically done using gas chromatography/mass spectrometry (GC/MS) following derivatization using BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) with 1% TMCS (trimethylchlorosilane). An issue with this method is that BSTFA derivatizes many small endogenous compounds including GHB and urea. When analyzing TMS derivatized samples by single quadrupole GC/MS, these small endogenous compounds tend to coelute.

This research investigates the use of High Resolution-Gas Chromatography/Time-of-Flight (GC/HRT) and 2D Gas Chromatography/Time-of-Flight (GCxGC/TOF) for analysis of human hair samples in order to determine their effectiveness in separating endogenous compounds such as GHB and urea. It also highlights the utility of these instruments as non-targeted tools which provide a more comprehensive picture of what else is in the samples. This research also compares the use of an automated bead mill and a standard cutting method for preparation of hair samples. The samples were analyzed by GC/HRT and GCxGC/TOF to determine if homogenization by the bead mill improved the efficiency of the subsequent extraction.

Methods

- Head hair samples were obtained from volunteers (A and B) and washed:
  - 3 times with DI-H_2O with sonication for 5 minutes per wash
  - 3 times with dichloromethane with sonication for 5 minutes per wash
- Dried between pieces of filter paper overnight
- Once dry, each sample was split into two parts:
  - One set was homogenized using a Biotage BeadRuptor 24 (A1,A2; B1,B2)
  - The other set was cut into 1-2 mm segments by hand using scissors (A3,A4; B3,B4)
- Two aliquots of approximately 20 mg each were weighed per sample and placed in 2 mL microcentrifuge tubes.
- A blank and five calibrators of concentrations 0.15-5.0 ng/mg (based on 20 mg of hair) were also prepared into microcentrifuge tubes.
- 1 mL of methanol and 50 µL GHB-D6 (1 µg/mL) were added to each tube
- All samples were incubated overnight at 40°C with agitation, then dried with N_2(g)
- Instrument Parameters LECO Pegasus® 4D & GC-HRT (modulation applies to GCxGC only):

Gas Chromatograph	Agilent 7890, Dual Stage Quad Jet Modulator & MPS2 Autosampler
Injection	1µL, Pulsed Splitless @ 250 °C
Carrier Gas, Flow	He @ 1.5 mL/min, Corrected Constant Flow
Column One	Rxi-5MS, 30m x 0.25mm i.d. x 0.25 µm coating (Restek)
Column Two	Rtx-200, 1.25m x 0.20mm x 0.25 µm coating (Restek)
Temp. Program	70 °C (1 min) to 320 °C @ 8 °C/min (10 min); primary oven maintained +10 °C relative to secondary oven
Modulation (Peg4D only)	4s with temp. maintained +15 °C relative to secondary oven
Transfer Line	300 °C
Mass Spectrometers	**LECO Pegasus® HT (unit mass resolution) & GC-HRT (R = 25,000)**
Ion Source Temp.	250 °C
Mass Range (m/z)	45 – 510
Acquisition Rates	200 spectra/s for GCxGC; 10 spectra/s for GC-HRT

Results – Enhanced Chromatographic Resolution

GHB and Urea were easily separated using the GCxGC/TOF instrument as illustrated for sample B1 in the surface plot expansion below (Figure 1). This resulted in high quality spectra for GHB (2TMS) and facilitated GHB quantitation (Figure 2, Table 1).

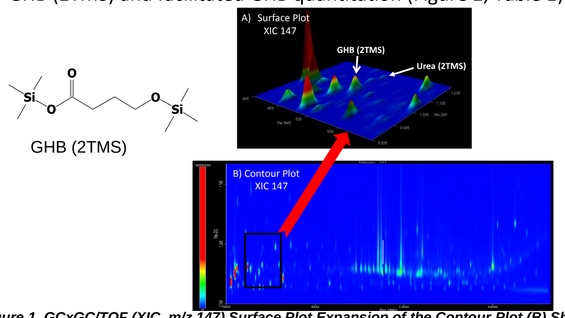
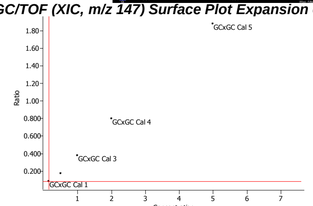


Figure 1. GCxGC/TOF (XIC, m/z 147) Surface Plot Expansion of the Contour Plot (B) Showing Separation of GHB & Urea



Standard	Area	Cert. Conc.	Calc. Conc.	Weighting
GCxGC Cal 1	13575	0.2	0.2	6.67
GCxGC Cal 2	25962	0.5	0.4	2.00
GCxGC Cal 3	63649	1.0	1.0	1.00
GCxGC Cal 4	98708	2.0	2.1	0.50
GCxGC Cal 5	282422	5.0	5.0	0.20

Figure 2. GCxGC/TOF Calibration Curve for GHB

Sample	Conc. (ng/mg)
A1 (Bead)	0.30
A2 (Bead)	0.33
A3 (Cut)	0.22
A4 (Cut)	0.24

Sample	Conc. (ng/mg)
B1 (Bead)	1.08
B2 (Bead)	0.98
B3 (Cut)	0.74
B4 (Cut)	0.80

Table 1. GHB in Samples A1-A4 and B1-B4

The hair sample extracts contained a wide variety of compounds including organic acids, diacids, bases, fatty acids (saturated & unsaturated), esters, long-chain alcohols, monoacylglycerides, and sterols. A small representative set of compounds found in sample B1 are listed in Table 2 (Ave. spectral similarity = 879/1000).

Name	Formula	R.T. (s)	Area	Similarity
Soleron	$C_6H_8O_3$	400 , 2.660	72868	893
2-Furoic Acid (TMS)	$C_8H_{12}O_3Si$	408 , 1.230	1186413	908
Levulinic Acid (TMS)	$C_8H_{16}O_3Si$	408 , 1.445	264896	948
Tarragon	$C_{10}H_{12}O$	468 , 1.070	39438	913
Malonic Acid (2TMS)	$C_9H_{20}O_4Si_2$	476 , 1.265	62241	871
3-Hydroxycaprioic Acid (2TMS)	$C_{12}H_{28}O_3Si_2$	576 , 1.030	44804	804
Phenoxyethanol (2TMS)	$C_{11}H_{18}O_2Si$	624 , 1.065	192968	925
Thymine (2TMS)	$C_{11}H_{22}N_2O_2Si_2$	664 , 1.035	17474	805
Parabanic Acid (2TMS)	$C_9H_{18}N_2O_3Si_2$	732 , 1.925	90088	842
Adipic Acid (2TMS)	$C_{12}H_{26}O_4Si_2$	756 , 1.230	412706	900
5-Oxoproline (2TMS)	$C_{11}H_{23}NO_3Si_2$	772 , 1.570	255386	901
Cinnamic Acid (TMS)	$C_{12}H_{16}O_2Si$	788 , 1.215	9561	813
Diethyltoluamide	$C_{12}H_{17}NO$	816 , 1.725	351502	928
Pimelic Acid (2TMS)	$C_{13}H_{28}O_4Si_2$	840 , 1.220	110224	899
3-Hydroxycapric Acid (2TMS)	$C_{16}H_{36}O_3Si_2$	884 , 1.010	8466	811
Propylparaben (TMS)	$C_{13}H_{20}O_3Si$	888 , 1.250	34179	878
Butylparaben (TMS)	$C_{14}H_{22}O_3Si$	968 , 1.235	207132	876
Azelaic Acid (2TMS)	$C_{15}H_{32}O_4Si_2$	992 , 1.200	89210	901
Versalide	$C_{18}H_{26}O$	1040 , 1.105	37772	822
Homosalate (TMS)	$C_{19}H_{30}O_3Si$	1164 , 1.165	2292399	922
Oleic Acid (TMS)	$C_{21}H_{42}O_2Si$	1260 , 1.020	3282718	880
9Z-Octadecen-1-ol (TMS)	$C_{21}H_{44}OSi$	1352 , 0.925	1012588	879
Monopalmitin (2TMS)	$C_{35}H_{56}O_4Si_2$	1508 , 1.025	178351	840
Batyl Alcohol (2TMS)	$C_{27}H_{60}O_3Si_2$	1556 , 0.920	742264	901
Octocrylene	$C_{24}H_{27}NO_2$	1556 , 1.410	1174756	922
Cholesterol (TMS)	$C_{30}H_{54}OSi$	1800 , 1.055	15472352	882

Table 2. Representative Compounds in Sample B1

References

1. Jones A, Eklund A, Kronstrand R. Concentration-time profiles of gamma-hydroxybutyrate in blood after recreational doses are best described by zero-order rather than first-order kinetics. J Anal Toxicol 2009;33(6):332–5.
2. Kintz P, Cirimele V, Jamey C, Ludes B. Testing for GHB in hair by GC/MS/MS after a single exposure. Application to document sexual assault. J Forensic Sci 2003;48(1):195–200.
3. Andresen H, Sprys N, Schmoldt A, Mueller A, Iwersen-Bergmann S. Gamma-hydroxybutyrate in urine and serum: additional data supporting current cut-off recommendations. Forensic Sci Int 2010;200(1-3):93–9.
4. Scott KS. The use of hair as a toxicological tool in DFC casework. Sci Justice 2009;49(4):250–3.
5. Cooper GAA, Kronstrand R, Kintz P. Society of Hair Testing guidelines for drug testing in hair. Forensic Sci Int 2012;218(1-3):20–4.

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Results –Enhanced Mass Spectral Resolution

GC/HRT analysis produced high-resolution, deconvoluted mass spectra that were searched against large databases (NIST, Wiley) and accurate mass ions that were leveraged for molecular and fragment ion formula determinations. An Analytical Ion Chromatogram (AIC) for sample A1 is displayed in Figure 3. Names, formulas, retention times, similarities and mas accuracy values for a representative set of compounds in A1 are listed below (Table 3).

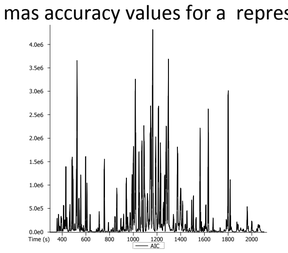


Figure 3. GC/HRT AIC for A1

Name	Formula	R.T. (s)	Area	Similarity	Ion	MA (ppm)
UREA (2TMS)	$C_7H_{20}N_2OSi_2$	486	12127269	928	$M^{+\cdot}$	-0.24
Benzoic Acid (TMS)	$C_{10}H_{14}O_2Si$	492	5448582	912	$M^{+\cdot}$	-0.32
Methylmalonic Acid (2TMS)	$C_{10}H_{22}O_4Si_2$	559	10518373	950	$M^{+\cdot}$	0.67
Serine (3TMS)	$C_{12}H_{31}NO_3Si_3$	611	4989159	938	$[M\text{-}C_4H_{11}OSi]^+$	-0.40
4-hydroxybenzaldehyde (TMS)	$C_{10}H_{14}O_2Si$	614	125596	846	$M^{+\cdot}$	0.96
5-oxo-Proline (2TMS)	$C_{11}H_{23}NO_3Si_2$	757	20156041	936	$M^{+\cdot}$	0.32
Phenylalanine (2TMS)	$C_{15}H_{27}NO_2Si_2$	849	3862385	883	$[M\text{-}C_2H_3]^+$	-0.03
Myristic Acid (TMS)	$C_{17}H_{36}O_2Si$	976	3647162	841	$M^{+\cdot}$	-6.09
Uric Acid (4TMS)	$C_{17}H_{36}N_4O_3Si_4$	1222	277571	789	$M^{+\cdot}$	0.63
9Z,12Z-Octadecadienoic Acid (TMS)	$C_{21}H_{40}O_2Si$	1266	3539559	784	$M^{+\cdot}$	-0.66
Stearic Acid (TMS)	$C_{21}H_{44}O_2Si$	1298	32507255	904	$M^{+\cdot}$	-0.83
9-Octadecenamide	$C_{18}H_{35}NO$	1375	26423908	844	$M^{+\cdot}$	-0.65
1-Monomyristin (2TMS)	$C_{23}H_{50}O_4Si_2$	1398	2094427	828	$[M\text{-}C_4H_{11}OSi]^+$	-0.03

Table 3. Representative Compounds in Sample A1

ChromaTOF HRT software was used to process data and for quantitative analysis. The calibration curve GHB (GHB-d6 internal standard) was constructed using quantitation and reference ions m/z = 233.1024 for GHB and 239.1400 for GHB-d6 (Figures 4,5). The concentrations of GHB for A and B hair samples are listed in Table 4.

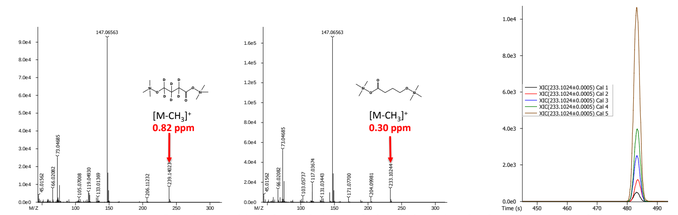
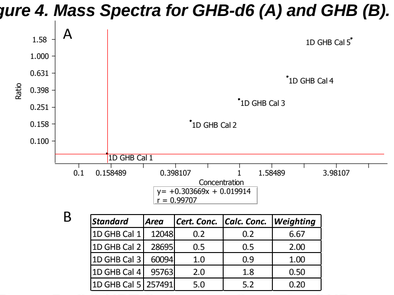


Figure 4. Mass Spectra for GHB-d6 (A) and GHB (B). (C) XICs (m/z = 233.1024) for A1-A4.



Standard	Area	Cert. Conc.	Calc. Conc.	Weighting
1D GHB Cal 1	12048	0.2	0.2	6.67
1D GHB Cal 2	28695	0.5	0.5	2.00
1D GHB Cal 3	60094	1.0	0.9	1.00
1D GHB Cal 4	95763	2.0	1.8	0.50
1D GHB Cal 5	257491	5.0	5.2	0.20

Figure 5. GC/HRT Calibration Curve for GHB

Sample	Conc. (ng/mg)	MA (ppm)
A1 (Bead)	0.41	-1.16
A2 (Bead)	0.61	-0.06
A3 (Cut)	0.28	-0.79
A4 (Cut)	0.31	0.81

Sample	Conc. (ng/mg)	MA (ppm)
B1 (Bead)	1.34	-0.18
B2 (Bead)	1.21	1.20
B3 (Cut)	1.08	0.29
B4 (Cut)	1.04	-0.62

Table 4. GHB in Samples A1-A4 and B1-B4

Discussion

Hair extracts consist of a complicated mixture of compounds transferred internally from blood or externally from sources such as conditioner, sun screen or other commercial products. Greater quantities of GHB were extracted via the "Bead" rather than the "Cut" extraction method (Table 3). In addition, greater concentrations of GHB were found in hair obtained from volunteer B.

Conclusions

- The Biotage Bead® Ruptor 24 was effective in pre-treating hair prior to extraction of drugs from the hair matrix.
- The enhanced chromatographic resolution of the GCxGC/TOF and high resolving power of the GC/HRT minimized interferences and facilitated accurate analysis of GHB in hair.