Optimized Digestion and Extraction of Endogenous Gamma Hydroxybutyrate (GHB) in Human Hair

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ABSTRACT

The purpose of this study was to evaluate the efficiency and performance of digestion and extraction methods capable of identifying endogenous concentrations of gamma-hydroxybutyrate (GHB) in human head hair. Liquid-liquid extraction (LLE) and liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) were used to isolate and identify GHB from hair. A comparison of previously published hair digestion methods including enzymatic digestion (pronase E and proteinase K), chemical digestion (under alkaline conditions) and solvent agitation was conducted to examine the impact of digestion technique on quantitative results.

INTRODUCTION

GHB is an endogenous compound that is present throughout the body. The drug is also used therapeutically and recreationally. Onset of action occurs within 20 to 45 minutes of oral ingestion followed by rapid metabolism to carbon dioxide and water. Detection times in blood and urine are typically 6 and 12 hours, respectively. Following an alleged sexual assault, reporting and subsequent collection of biological evidence is often delayed beyond the detection time of the drug. In those instances, if toxicological testing is inconclusive, alternative matrices such as hair have been proposed.

Due to the mechanism of incorporation, hair provides a longer detection window than other biological matrices. However, disadvantages of hair include environmental exposure, the influence of cosmetic treatments, pigmentation and the absence of a generally accepted cut-off concentrations. Additional drawbacks include the additional time necessary for sample pretreatment. Due to its endogenous nature, interpretation of GHB in hair is complicated by the need to differentiate naturally occurring GHB from exogenous drug that may have been administered. However, analytical procedures including digestion techniques may yield different efficiencies, making it difficult to compare endogenous GHB concentrations between published studies.

Solvent-based agitation techniques have been reported as an alternative to more traditional chemical and enzymatic digestion techniques (Hari, 2013). In this study, an optimized solvent-based agitation was developed for GHB in hair, and quantitative results were compared with published chemical and enzymatic digestion techniques using sodium hydroxide, pronase E and proteinase K.

RESULTS & DISCUSSION

- Calibration models were evaluated using eight non-zero calibrators over five independent runs (Figure 1). Each calibration included three replicates of matrix-matched controls at three concentrations. Linearity was observed between 0.1 and 50 ng/mg (R^2>0.999).
- Parameters such as linearity, bias, precision, matrix effects, drug interferences, detection and quantitation limits were within acceptable ranges (Table 1).
- Pulverization and solvent agitation conditions were fully optimized. Endogenous GHB concentrations ranged from <0.2-0.79 ng/mg (Figure 2). Of the 32 samples collected, 11 were above the LOQ (0.2 ng/mg) with a majority between 0.2 and 0.4 ng/mg. These low concentrations are comparable with those of Hari *et al*. Two thirds of their samples (n=27) were <0.2 ng/mg. They also utilized a technique using agitation as their sample preparation, but used water instead of methanol as their solvent of choice.
- ANOVA was used to compare the efficiency of digestion techniques (α=0.05). The same LLE protocol was utilized following each digestion. There was no significant difference between the three pronase E protocols. However, proteinase K yielded significantly higher endogenous GHB concentrations than pronase E (p=0.0001).
- Significant differences were observed between the non-enzymatic methods (*i.e.* chemical digestion and solvent agitation) (p=0.001). Chemical digestion using sodium hydroxide was more efficient at releasing endogenous GHB from hair, compared with the fully optimized solvent agitation method. Differences between the chemical digestion, proteinase K, and the BeadRuptor agitation were highly significant (p<0.0001) (Figure 3).
- Chemical digestion yielded endogenous GHB concentrations that were more than six-fold higher than the optimized solvent-based agitation technique, and more than two-fold higher than the proteinase K enzymatic digestion. These results highlight how differences in the digestion efficiency can influence published ranges of endogenous GHB in hair.

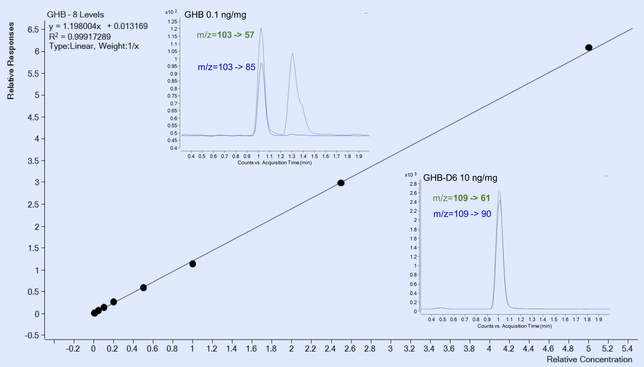

Figure 1 . Calibration model (0.1, 0.5, 1, 2, 5, 10, 25, 50 ng/mg) GHB in hair

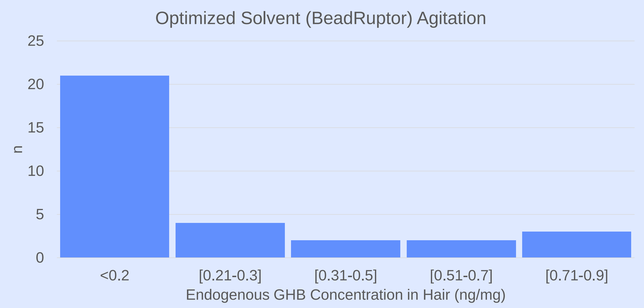

Figure 2. Endogenous GHB concentrations in drug-free volunteers using optimized solvent agitation (n=32)

Parameters	Results
LOD	0.1 ng/mg
LOQ	0.2 ng/mg
Calibration Model	0.1-50 ng/mg (Linear, weighted 1/x)
Precision	3-12% (Intra-assay CV) n=5
	7-8% (Inter-assay CV) n=15
Bias	1 to 8%
Carryover	No carryover at 50 ng/mg
Matrix & Drug Interference	No matrix interference (n=5)
	No qualitative interference (32 common drugs) No quantitative interference

Table 1. LOD, LOQ, calibration model, inter-assay precision, intra-assay precision, bias, carryover, matrix effects, and drug interferences for GHB in hair

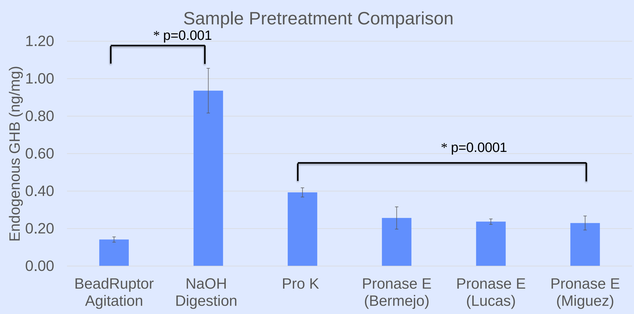

Figure 3. Efficiency of enzymatic and non-enzymatic methods of digestion (n=4)

MATERIALS AND METHODS

Instrumentation

An Agilent 1290 Infinity Liquid Chromatograph system equipped with an Agilent 6470 Triple Quadrupole Mass Spectrometer (Santa Clara, CA) was used for instrumental analysis. A Poroshell 120 EC-C18 column (100 mm x 3.0 mm x 2.7 µm) was utilized with a mobile phase consisting of 0.1% formic acid in water/acetonitrile (96:4). Separation was achieved using isocratic elution at a flow rate of 0.4 mL/min. GHB-D6 was used as the internal standard.

Analysis of Hair Samples

Hair was collected from the posterior vertex of the scalp in accordance with an IRB-approved protocol. Dichloromethane was used to decontaminate hair prior to analysis. A solvent-based agitation technique was fully optimized using an Omni BeadRuptor12. Agitation conditions, pulverization/surface area, bead type and solvents were evaluated. Maximum GHB concentrations were achieved using pulverized hair (~25 mg) agitated for 60 seconds in 2 mL tubes filled with four 2.4 mm metal beads and methanol (1 mL). Samples were centrifuged at 3500 RPM, transferred to a conical tube, and evaporated under air at 40°C. Samples were reconstituted in deionized water, acidified with 0.25 mL sulfuric acid (0.2M) and extracted with ethyl acetate (2 mL).

Sample pretreatments and digestion techniques from previously published studies were compared with the optimized solvent-based agitation. These included basic chemical digestion with sodium hydroxide (Stout, 2010), enzymatic digestion with pronase E (Bermejo, 2006; Lucas, 2000; Miguez-Framil, 2007) and proteinase K (Kronstrand, 2002).

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