Journal of Analytical Toxicology, 2020;44:637–650
doi: 10.1093/jat/bkaa086
Advance Access Publication Date: 5 August 2020
Special Issue

OXFORD

Special Issue

Endogenous GHB in Segmented Hair Part II: Intra-individual Variation for Exogenous Discrimination

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Abstract

The endogenous presence of gamma-hydroxybutyric acid (GHB) complicates the interpretation of results in cases where an exogenous dosing is suspected. Due to GHB's rapid metabolism and clearance following exogenous doses, hair has become a preferential matrix for confirmation of GHB exposure in drug-facilitated crimes. However, unlike blood and urine where an agreed-upon cut-off concentration for differentiation between endogenous and exogenous GHB has been made, there has been no consensus on a cut-off concentration for hair. This is due in part to the wide inter- and intra-individual variation that has been observed in endogenous GHB hair studies. A large (>50) population study of 214 donors was conducted to better understand these variations and to evaluate whether a cut-off concentration could be established for endogenous GHB in human hair. As seen in our previous study, the inter-individual variation was large, with concentrations ranging from <0.40 to 5.47 ng/mg. This range made an absolute cut-off concentration recommendation inappropriate, so an alternative approach for GHB discrimination was investigated utilizing the intra-individual variation. Male donors appeared to have greater intra-individual variation than female donors, yet it was noted that segment-to-segment variation along the length of hair had minimal change between individual donor's adjacent segments. Overall, 97.1% of the adjacent segment differences were within ±0.5 ng/mg. Therefore, instead of a recommended cut-off concentration, it appears that using adjacent segment concentration differences could be a strategy to assist in differentiating endogenous from single exogenous GHB exposure. In the absence of controlled dosing data, previously published segmented results from controlled and suspected dosing donors are examined using the adjacent segmental difference approach and the results compared to currently used ratio-based calculations.

Introduction

Although cut-off concentrations of 5 and 10 μg/mL to differentiate between endogenous and exogenous gamma-hydroxybutyric acid (GHB) have been established in blood and urine (1–11), respectively, consensus for an appropriate cut-off concentration in hair has not been reached (7, 12–26). This is in part due to the large range of endogenous GHB concentrations found in hair (0.1–12.0 ng/mg) (26), as well as the observed overlap of endogenous concentration ranges with those concentrations in controlled single-dose studies, suspected exogenous dosing cases, and chronic use/abuse reports (12–16, 19, 21–31). Because observed intra-individual variation of endogenous GHB is minimal (12, 32), an alternative solution could be to use individuals as their own reference or control to assist in identifying an outlier concentration along the length of hair that might suggest an exogenous GHB dose (7, 12–17, 19, 22–25, 27–33).

To use the intra-individual variation model, hair must be segmented for analysis to provide a time course of GHB concentrations (7, 17). Effectively applying an individual as their own reference has typically involved calculating a ratio between the suspected exogenous exposure segment and the average of all other endogenous segments, excluding the first proximal segment (7, 17, 13, 19). The United Nations Organization on Drugs and Crime (UNODC) recommends that the calculated ratio be greater than 10:1 (7). However, a controlled dosing study by Bertol et al. (19) and Kintz et al. (13) indicated that a 3:1 ratio would be more appropriate, as a documented ratio of 10:1 has not been identified to date in any other study. Some studies also observed degradation of GHB in hair as the time between exposure and hair collection increases (19, 24), which has led to hair collection ∼4 weeks after a reported exposure to improve the chances of getting a maximum ratio close to 3:1 or larger.

When calculating ratios to determine GHB exposure, it is important that the individual has a stable baseline GHB concentration. While there is large inter-individual variation, several studies have concluded that the intra-individual variation is small enough that basal levels are consistent, which allow for ratios to be evaluated (12–17, 19, 22–25, 27–32). However, not all studies include segmented results of endogenous donors (12, 13, 19, 24, 28, 31, 34, 35) or donors with exogenous exposure (12–16, 19, 22–25, 27–30). Even fewer studies have a large number (>50) of non-GHB users (12, 22, 30–32, 35) to adequately assess the intra-individual, endogenous concentration variation. One of the larger published donor studies noted that males have greater endogenous GHB variation along the length of the hair shaft (32). These results call into question whether intra-individual variation might be too large for reliable application of the ratios in hair.

A study involving eight subjects who provided every urine void over a 1-week period found the coefficient of variation (CV) was >40% for urine and concluded that a person could not serve as their own reference when differentiating endogenous from exogenous GHB exposure (1). However, Goullé (12) calculated the relative standard deviation (RSD) for 12 non-GHB users, and 11 had a %RSD <40% in 3-mm-long hair segments. While variation measurement is highly dependent on the length of hair and the number of segments, it does still show that hair is likely to have less intra-individual temporal variability than urine, allowing for an individual to potentially serve as their own reference control.

To assess intra-individual variation on a larger donor population, the analytical results of hair collected from 214 non-GHB-using donors were examined from our previous work (35). An inter-individual analysis of the hair samples from the 214 donors found a similar but smaller range of endogenous GHB concentrations, <0.40–5.47 ng/mg (35), as those previously published (0.1–12.0 ng/mg) (26). Although the inter-individual variation appears to discourage the recommendation of cut-off concentrations, a novel strategy of utilizing adjacent segment differences is presented as a potential alternative to assist with differentiating endogenous GHB from a single exogenous exposure.

Materials and Methods

The materials and methods for this study are detailed in Lloyd et al. (34) and Thomas et al. (35). Two-hundred fourteen individuals [141 females (65.9%) and 73 males (34.1%)] without any known GHB use provided hair samples cut from the vertex posterior, as close to the scalp as possible, consistent with the Society of Hair Testing (SOHT) guidelines (17). A total of 2,074 segments were obtained for analysis. The analytical method had a limit of quantitation (LOQ) of 0.40 ng/mg, and the limit of detection (LOD) was determined to be the same. Concentrations of endogenous GHB in authentic hair specimens were determined to be in the range of <0.40–5.47 ng/mg, with a median concentration of 0.72 ng/mg (35). However, there were also 19.5% of the segmental results that were <LOD/LOQ. These segments were divided into 2 groups, defined as 'observed' (301 segments) and 'non-detect' (103 segments), based on the qualitative (e.g., ion ratio, signal-to-noise ratio, etc.) and quantitative criteria that were met. Any segment with a calculated GHB concentration less than the LOQ that met all qualitative peak identification criteria was defined as 'observed.' Segments that met all qualitative identification criteria, but the internal standard area counts were below a set threshold to indicate signal suppression, were also defined as 'observed' regardless of the calculated GHB concentration. A segment where the peak failed any qualitative criterion, regardless of the calculated GHB concentration, was defined as 'non-detect.' To simplify, both groups are considered left-censored, <0.40 ng/mg, and will collectively be referred to as non-detects. The complete data set with segmented concentrations, demographics, and other anonymized donor information are available as Supplementary Material in Thomas et al. (35).

Intra-individual variability was analyzed two ways. The first method involved calculating the relative standard deviation (%RSD) of all segments for an individual with at least two quantifiable concentrations of GHB. Segments classified as non-detects were not included in the average or standard deviation calculations. A second way to assess the variability was to take the difference between two adjacent segments (i.e., segment 1 minus segment 2, segment 2 minus segment 3, etc.). If a segment did not have a calculated value (i.e., a non-detect), the associated difference(s) was excluded, as was the distal segment difference. Separately, to account for the non-detect segments and force a 'worst-case' scenario, a substitution of 0 ng/mg for all non-detect segments was also performed prior to calculation of the adjacent segment differences to avoid exclusion. Substituting 0 ng/mg for the non-detect segments was chosen based on the probability that the concentration was <0.40 ng/mg, the method's limit of detection, and would represent an extremely low concentration. To determine if the adjacent segment difference distribution was unique to this data set, segmented data results from non-GHB users in other published studies were assessed for adjacent segment differences (12, 13, 19, 24, 28). If these published studies did not provide a table with the calculated GHB concentrations for the segmental results, they were estimated from provided plots/graphs. For example, estimation of GHB concentrations was required for

Kintz et al. (13) and Chèze et al. (28). Specifically, in Chèze et al. (28), only 6 of the 13 endogenous donors (T2, T6, T7, T8, T12, & T13) in the plot could be adequately estimated. The remaining seven donors all had converging concentrations that were less than 1.0 ng/mg and were not included.

Results

From the data of the 214 donors, GHB concentrations in 97.5% of the segments were <2.00 ng/mg. When this is broken down by gender, 99.3% of female segments and 90.9% of male segments were <2.00 ng/mg. Six of the donors (5 males and 1 female) volunteered to have at least 1 additional hair sample collected later. This was to monitor intra-individual variation of endogenous GHB over longer periods of time. The longitudinal subset of data is available in the Supplementary Table SI as an Excel spreadsheet. A graphical representation of the donor trends by time can be seen in Figure 1a–f. The corresponding month for each segment represented was approximated assuming an average hair growth rate of 1 cm/month (17).

Intra-individual variability was analyzed two ways. The first method examined the relative standard deviation (%RSD) for an individual. Supplementary Figure S1 shows simple frequency plots of the %RSD for (i) all individuals, (ii) females only, and (iii) males only. In general, the intra-individual variability of measured endogenous GHB in hair was >40% in 8.9% of donors, but only 2.8% of females compared to 20.5% of males had such high %RSDs. Likewise, greater than 30% RSD was observed in 18.7% of donors. However, this approach to assess intra-individual variability does not directly reflect trends along the length of the hair.

In the second assessment, the variability in the difference between two adjacent segments was used. Figure 2a shows a frequency distribution of the 1,417 calculated differences from the population data. Using differences as a measure of intra-individual variability, 97.1% of the adjacent segment differences were within ±0.50 ng/mg, and 99.4% of the differences were within ±1.00 ng/mg. The range of adjacent segment differences observed in this data set was −1.62 to +1.70 ng/mg. In a worst-case scenario, with the addition of the substituted non-detect segments, a total of 1,858 calculated differences were plotted in a frequency distribution (Figure 2b), where 94.7% of the adjacent segment differences were within ±0.5 ng/mg and 98.5% of the differences were within ±1.00 ng/mg. All the raw segmental data with the calculated %RSD and adjacent segment differences are included in the Supplementary Tables SII and SIII, respectively, as Excel spreadsheets.

Segmented endogenous GHB results from published studies (12, 13, 19, 24, 28) along with the calculated differences can be viewed in the Supplementary Table SIV as an Excel spreadsheet. In total, from the pooled published segmented endogenous donor results, 162 differences were calculated. The distribution is shown in Figure 2c. Using differences as a measure for intra-individual variability in these published data, 85.2% of the adjacent segment differences are within ±0.5 ng/mg, and 94.4% of the differences are within ±1.0 ng/mg. The range of adjacent segment values observed in this data set is −0.5 to +5.0 ng/mg.

In the absence of a controlled dosing study within our lab, the adjacent segment difference approach to discriminate endogenous from exogenous GHB exposure was applied to the published controlled dosing studies and case reports of GHB in hair with segmented results (12–16, 19, 22–25, 27–31). The results are summarized in Table I, with any segments meeting our selection criteria as summarized in Table II (i.e., adjacent differences greater than 0.5 ng/mg and opposite in magnitude) highlighted. In total, there were 25 controlled dosing examples from 13 individuals and 12 suspected dosing examples from 11 individuals that provided segmental results (12, 13, 14, 16, 19, 22, 28, 30, 36). All controlled dosing donors met the adjacent segment difference criteria approach. Despite two inconclusive exposure cases (19, 30, 36) and two cases not meeting the single, discreet segment criteria (14, 30), the remaining seven suspected dosing donors did meet all adjacent segment difference criteria. All but one of the seven donors had segment sizes less than the 1 cm used in the external studies (12, 16, 22, 28). The ratio of the suspected dosing segment concentration to the average concentration of the basal segments, not including the first segment, for the 13 controlled dosing donor cases and 11 suspected dosing donor cases ranged from ~1.1 to 7.7 and 1.2 to 7.5, respectively. The average ratios for the controlled and suspected dosing donor cases were 4.3 and 3.1, respectively.

Discussion

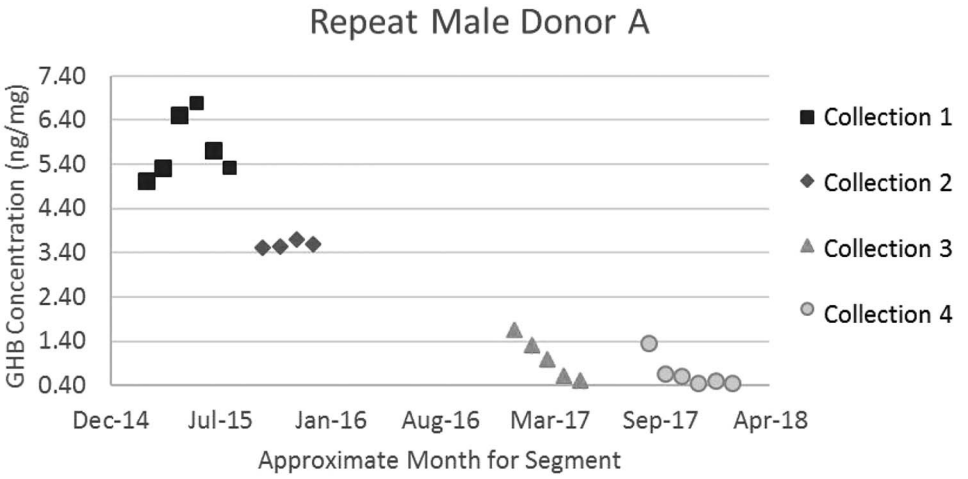
Longitudinal donor study observations

There have been few examples of the changes that could occur over time to the endogenous GHB concentrations in an individual (19, 24). The examples in Figure 1 are limited, and few conclusions can be made. However, an individual's endogenous GHB concentrations in hair are not necessarily stagnant. In the repeat examples for Female Donor A and Male Donor D, there is some overlap in the time segments. For Male Donor D, there is relatively good agreement in the overlapping time segments; however, there is some difference in Female Donor A. As the information we collect from donors is limited, it is unclear what caused the change. While there was no record of a chemical or thermal treatment for the second donation, it could have been left off or there could be other reasons that cannot be accounted for with the data collected. The remaining donors had samples collected such that there was no overlap in time and the trends were reasonably similar in Male Donors B and E, but Male Donors A and C showed dramatic changes. In any case, the goal of looking at repeat donors was to understand longitudinal changes. Some factors, such as chemical and thermal hair treatment (35, 37), have been shown to impact endogenous GHB concentrations, but there are clearly other unknown factors that could be impacting Male Donors A and C that are not fully understood. These other factors could be related to hair growth rates, the cutting/sampling of the hair, and the distribution of hair phases in the sample (38–44), but these cannot be adequately assessed with the current data and only opined as to whether they are related to the observations here. Therefore, these changes, which can be great over time for some individuals, should be considered when comparing multiple hair samples over time from the same individual.

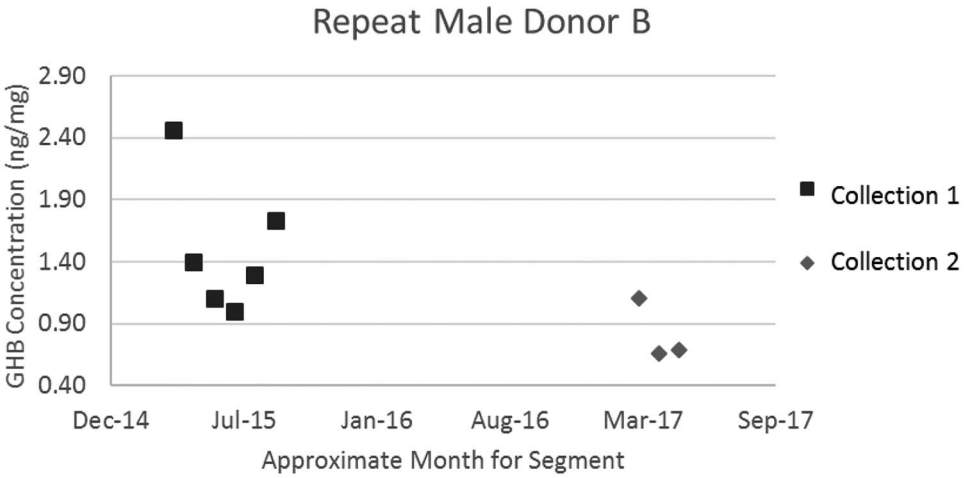
Overview of intra-individual variation observed in 214 donors

Based on the large inter-individual variation observed in our data set (<0.40–5.47 ng/mg) and previously published endogenous data sets (0.1–12.0 ng/mg) (26), an absolute cut-off concentration for discrimination of endogenous and exogenous GHB appears unsupported. Additionally, because males and females are from distinct populations (35), there would likely need to be two cut-off concentrations. For instance, 2.0 ng/mg may seem like a reasonable cut-off

a. Repeat Male Donor A (4 Collections)



b. Repeat Male Donor B (2 Collections)



c. Repeat Male Donor C (3 Collections)

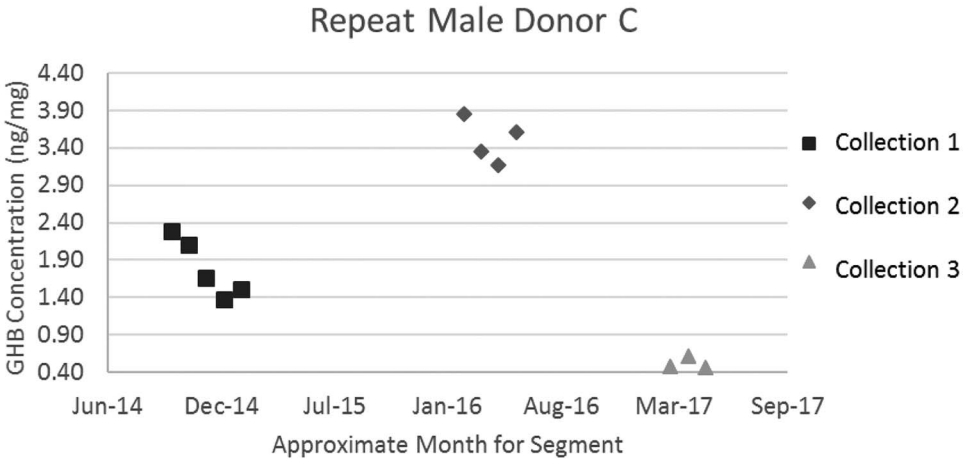
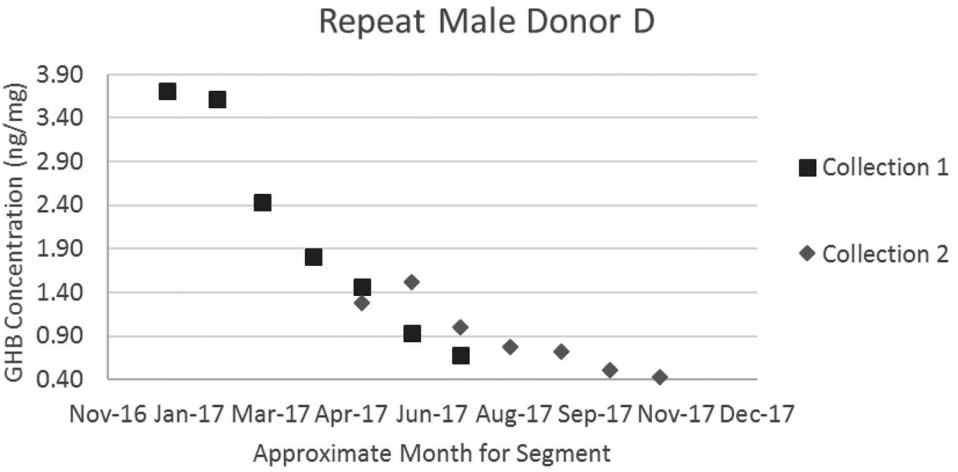
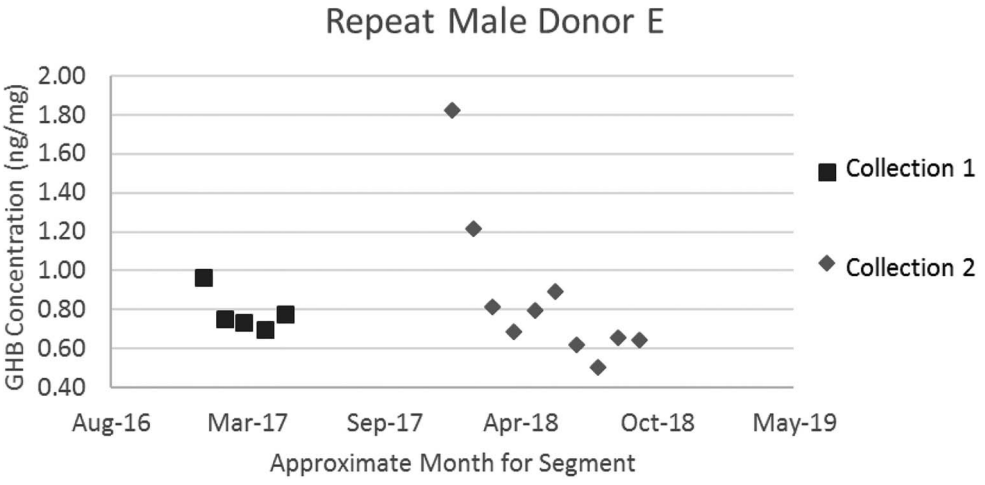


Figure 1. Repeat donor longitudinal trends in five male donors (a–e) and one female donor (f). All proximal segments are the farthest point on the right for each collection set. Concentration segment data, collection date, and other donor details can be found in Supplementary Table SI as an Excel spreadsheet.

d. Repeat Male Donor D (2 Collections)



e. Repeat Male Donor E (2 Collections)



f. Repeat Female Donor A (2 Collections)

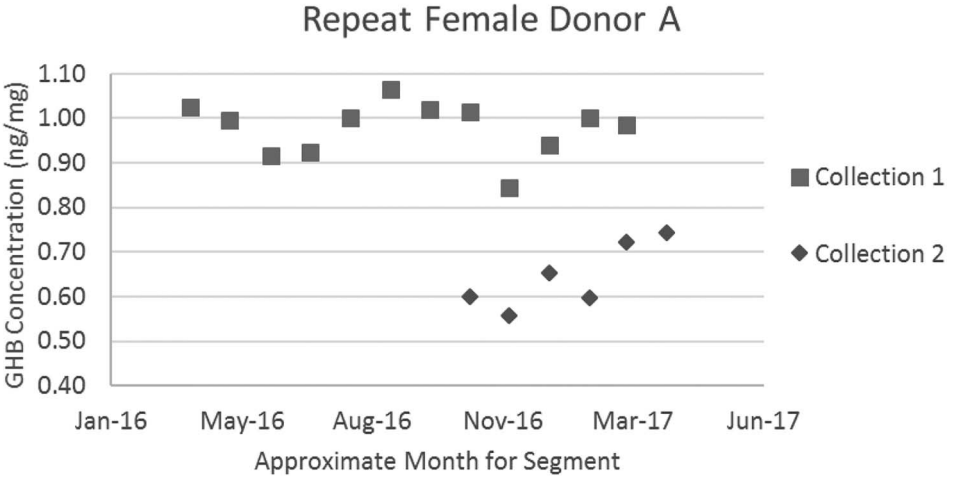


Figure 1. Continued.

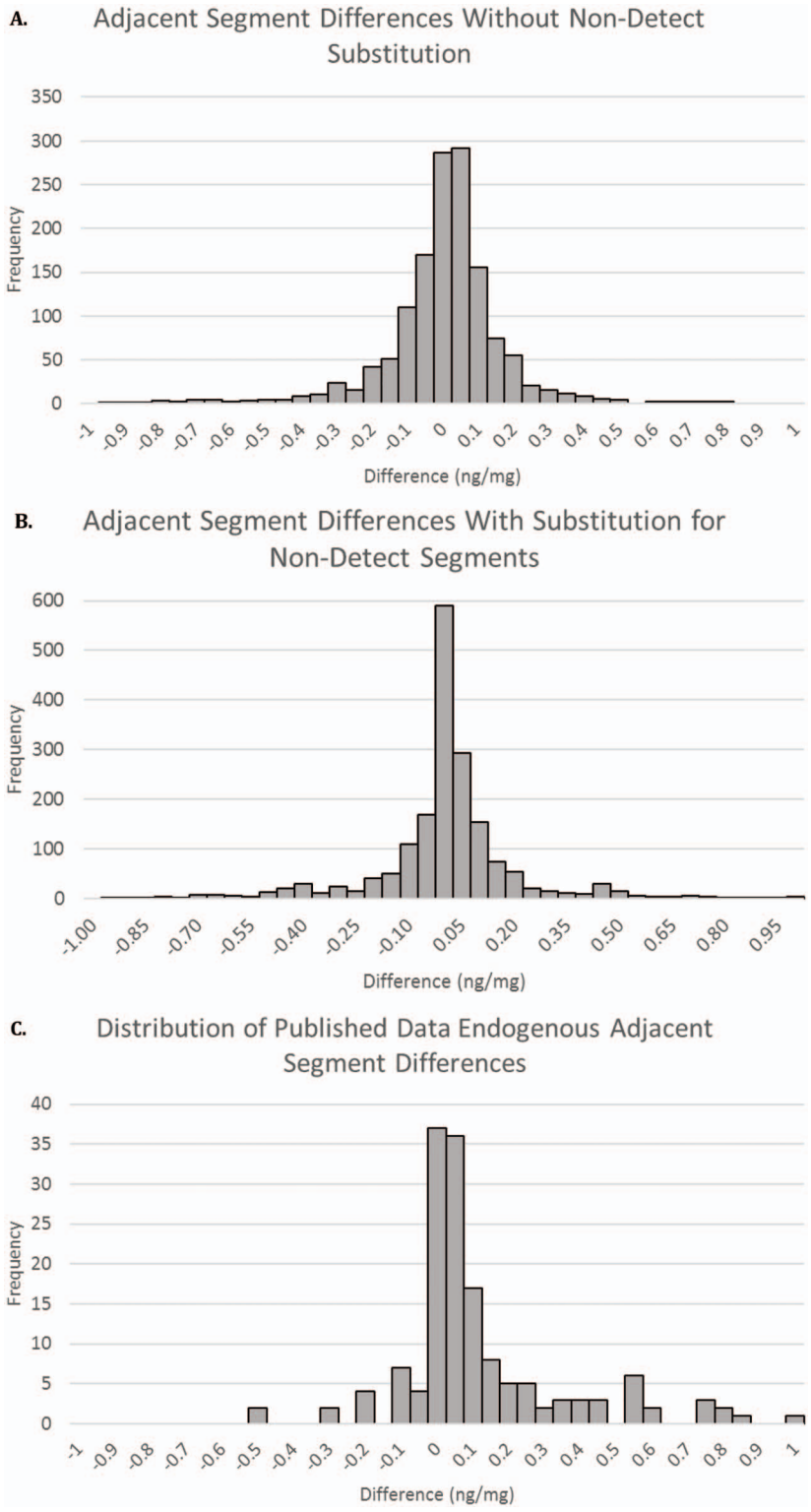


Figure 2. Adjacent segment differences frequency plots. A. Frequency plot of 1,417 adjacent segment differences without non-detect substitution. (Distribution does not include ~0.6% of the differences that were <−1.00 or >1.00 ng/mg. Differences within ±0.5 ng/mg account for 97.1% of the difference population. The mean and median differences are −0.02 and −0.01 ng/mg, respectively. The minimum and maximum differences are −1.62 and 1.70 ng/mg, respectively.) B. Frequency Plot of 1,858 adjacent segment differences with non-detect substitution. (Distribution does not include ~1.5% of the differences that were <−1.00 or >1.00 ng/mg. Differences within ±0.5 ng/mg account for 94.7% of the difference population. The mean and median differences are −0.01 and 0.00 ng/mg, respectively. The minimum and maximum differences are −4.73 and 4.22 ng/mg, respectively.) C. Frequency plot of 162 adjacent segment differences from published endogenous donor segmented results. (Distribution does not include ~5.6% of the differences that were >1.00 ng/mg. Differences within ±0.5 ng/mg account for 85.2% of the difference population. The mean and median differences are 0.22 and 0.03 ng/mg, respectively. The minimum and maximum differences are −0.50 and 5.00 ng/mg, respectively).

Table I. Controlled and Suspected Exogenous Dosing Donors with Segmented Results

Reference	Donor type	Segment size	Seg. 1	Seg. 2	Seg. 3	Seg. 4	Seg. 5	Seg. 6	Seg. 7	Seg. 8	Seg. 9	Seg. 10	Seg. 11	Seg. 12	Seg. 13	Seg. 14	Ratio[a]
(13)	Controlled Dosing 1[b]	3 mm	14.2	8	6	3	***4.7***	3.5	3.2	2.7	3.2						**1.1**
(19)[c]	Controlled Dosing 2A	5 mm	1.08	***4.8***	0.92	0.77	0.74	0.7	0.69	0.68							6.4
	Controlled Dosing 2B	5 mm	1.03	0.81	1.54	***4.4***	0.79	0.71	0.67	0.65	0.66	0.64					5.5
	Controlled Dosing 3A	5 mm	1.65	***6***	1.31	1.22	1.18	1.16	1.13	1.14							5.0
	Controlled Dosing 3B	5 mm	1.38	1.29	1.44	***5.6***	1.22	1.18	1.11	1.1	1.12	1.09					4.7
	Controlled Dosing 4A	5 mm	0.62	***1.4***	0.56	0	0	0	0	0							N/A
	Controlled Dosing 4B	5 mm	0.57	0	0	***1.28***	0.64	0	0	0	0	0					N/A
	Controlled Dosing 5A	5 mm	1.8	***6.88***	1.98	1.72	1.68	1.65	1.63	1.63							4.0
	Controlled Dosing 5B	5 mm	1.79	1.76	1.7	***6.32***	1.82	1.67	1.65	1.64	1.61	1.6					3.7
	Controlled Dosing 6A	5 mm	0.97	***5.45***	1.14	0.71	0.69	0.66	0.63	0.64							7.3
	Controlled Dosing 6B	5 mm	0.92	0.75	0.73	***4.76***	0.88	0.69	0.65	0.67	0.63	0.6					6.8
	Controlled Dosing 7A	5 mm	2.15	***5.21***	1.34	1.32	1.3	1.27	1.22	1.18							4.0
	Controlled Dosing 7B	5 mm	1.52	1.33	2.49	***4.71***	1.32	1.28	1.26	1.18	1.18	1.16					3.4
	Controlled Dosing 8A	5 mm	1.85	***7.12***	1.94	1.71	1.69	1.67	1.67	1.68							4.2
	Controlled Dosing 8B	5 mm	1.8	1.74	1.75	***6.59***	1.8	1.73	1.7	1.69	1.67	1.65					3.9
	Controlled Dosing 9A	5 mm	0.92	***3.94***	1.2	0.78	0.73	0.7	0.7	0.69							4.9
	Controlled Dosing 9B	5 mm	0.9	0.81	0.84	***3.45***	0.96	0.74	0.73	0.74	0.7	0.67					4.3
	Controlled Dosing 10A	5 mm	3.15	***6.82***	3.45	2.98	2.95	2.9	2.88	2.85							**2.3**
	Controlled Dosing 10B	5 mm	3.16	3	2.95	***5.09***	3.16	2.89	2.87	2.86	2.84	2.85					**1.8**
	Controlled Dosing 11A	5 mm	3	***5.14***	2.01	1.96	1.93	1.93	1.9	1.89							**2.7**
	Controlled Dosing 11B	5 mm	2.15	1.96	2.5	***4.11***	1.9	1.89	1.88	1.88	1.86	1.85					**2.1**
	Controlled Dosing 12A	5 mm	1.1	***7.12***	1.02	0.95	0.92	0.9	0.89	0.84							7.7
	Controlled Dosing 12B	5 mm	1.13	0.96	0.95	***6.24***	1.1	0.89	0.87	0.85	0.83	0.8					6.9
	Controlled Dosing 13A	5 mm	1.25	***4.28***	1.54	1.18	1.15	1.1	1.1	1.08							3.6
	Controlled Dosing 13B	5 mm	1.27	1.11	1.1	***4***	1.28	1.13	1.08	1.05	1.03	1					3.6
(19)[c]	Suspected Dosing 1A	5 mm	2.4	***4.96***	1.45	1.54	1.22	1.19									3.7
	Suspected Dosing 1B	5 mm	2.8	1.69	1.61	1.59	1.94	***3.95***	1.64	1.39	1.28						**2.4**
	Suspected Dosing 2	5 mm	3.11	***6.41***	2.24	1.95	1.79	1.88	1.8	***3.85***	2.3	1.89	1.48	1.57	1.61	1.59	3.4, **2.0**
	Suspected Dosing 3	5 mm	4.54	3.15	3.29	3.45	***4.19***	***3.95***	3.21								**1.2**
(12)	Suspected Dosing 4	3 mm	3.1	***5.3***	4.3	0.71[d]											7.5
(14)	Suspected Dosing 5	3 mm	1.3	0.6	0.8	***2.4***	***2.7***	0.7	0.8	0.7	0.8	0.7					3.7
(16)	Suspected Dosing 6[b]	3 mm	1	0.8	0.8	***3.5***	0.8	0.7	1	0.7							4.4
(28)	Suspected Dosing 7[b]	5 mm	7	4	***9.8***	4.5	0.8	2.5	3	3.5							3.2
	Suspected Dosing 8[b]	5 mm	20	8	6	***9.6***	6	5	2	3							**1.9**
(22)	Suspected Dosing 9[b]	10 mm	1.8	***4***	1.8	1.5	1.5	1.5									**2.5**
(30,	Suspected Dosing 10[b]	3 mm	0	0	0	0	***3.4***	***3.3***	0	0	0	0	0	0			N/A
42)	Suspected Dosing 11[b]	5 mm	1.8	1.6	***2.0***	1.7	1.7	1.5	1.4	1.3	1.4	1.3					**1.3**

All concentrations listed are in ng/mg. All bolded, italicized, and gray concentrations indicate where a controlled or suspected dosing has been identified by the study and also with the proposed adjacent difference approach. Bolded, italicized, and underlined concentrations indicate where a controlled or suspected dosing was identified in the study, but not with the proposed adjacent difference approach.

[a]The ratio was calculated by dividing the highlighted peak concentration by the average of all baseline concentration segments, not including the first segment. N/A indicates that the endogenous baseline was not sufficient to be used in calculating a ratio. Bolded ratios are for those that are less than 3:1.

[b]Concentrations were estimated from the publication's graph/chart and subject to some error.

[c]Donors for the controlled dosing study were sampled twice after GHB exposure and are listed together in the table. Additionally, the first suspected dosing donor was sampled twice, 1 and 3 months, after suspected exposure. The second suspected donor was sampled ~1 month after suspected exposure but also displayed another suspicious peak tentatively identified as another suspected exposure and was not included in the baseline average. It is not specified the length of time from post-suspected exposure when the third suspected dosing donor was collected.

[d]Listed concentration is the average of 77 distal segments. The standard deviation for these segments is 0.14 ng/mg.

Table II. Criteria for Identification of Exogenous GHB Exposure Using Local Maxima

Hair parameter	Criterion
GHB conc. Rise on proximal side of local maximum	Less or equal to −0.5 ng/mg
GHB conc. Drop on distal side of local maximum	Greater or equal to +0.5 ng/mg
Local maximum if segment size equals 1 cm	Rise and drop will occur over adjacent segments
Local maximum if segment size <1 cm	Rise and drop may be separated by one or more non-significant difference(s)

recommendation based on our female population, as only 0.7% of the female segments quantified >2.0 ng/mg. However, using the same 2.0 ng/mg cut-off for males would result in 9.1% of the segments with measurable endogenous GHB concentrations >2.0 ng/mg reported as false positives. Despite the low concentration of endogenous GHB in females, large-scale, controlled-dosing studies would be needed to validate a 2.0 ng/mg recommendation for females. The current absence of such a study supports our position that an appropriate cut-off concentration recommendation cannot be made. Instead, leveraging the intra-individual variation of endogenous GHB in hair is recommended for discrimination of exogenous GHB exposure.

To use intra-individual variation as a means of identifying exogenous exposure to GHB or GHB-related compounds, segmental analyses must be conducted (7, 17). However, there is no consensus as to how to confidently determine when a detected 'GHB spike' in a segment is significant and warrants the conclusion of exogenous exposure. Ratios of 3:1 to 10:1 of the spiked segment to the endogenous GHB segments have been recommended (7, 13, 19), but no formal threshold has been set or applied consistently in published studies (12–16, 19, 22–25, 27–31). In some cases, even a 3:1 ratio is not sensitive enough to detect exogenous exposure in a controlled dose exposure (19). Ratios are also most effective when the intra-individual variation is small. In addition, ratios are problematic because as illustrated by the data in Part I of our study (35), the concentration does not vary randomly but is largely caused by continuous longitudinal concentration trends.

Previous studies have lacked specific intra-individual variation measurements, other than to indicate the perceived variation was small and allowed for an individual to act as their own reference (12–17, 19, 22–25, 27–32). Hair does appear to have less intra-individual variation than urine (1, 12), and the majority of donors in this study, 91.1%, had %RSD values <40%. However, it was noted that a higher intra-individual variation in men was observed by Vaiano et al. (32). A few of the donors in our study with the highest %RSD values are shown in Supplementary Figure S2. In general, the lower intra-individual variation in women indicates that they may be better candidates for applying GHB ratios for exogenous exposure discrimination than men. It is currently unclear what would be causing the increased male intra-individual variation observation. An alternative approach is to use the adjacent segment differences, as this strategy could overcome the issues in individuals with greater intra-individual variation and potentially be more sensitive to exogenous GHB exposure than a ratio approach.

Adjacent segment difference false-positive rate evaluation

Based on the low %RSD and magnitude of the median value change (~0.2–0.3 ng/mg for women and ~0.5–0.6 ng/mg for men (35)), the difference in endogenous GHB concentrations between any two adjacent segments should be small and near zero. As expected, the distribution of the differences shown in Figure 2a is centered near zero. While the distribution has a symmetrical shape, it does not approximate a normal distribution. However, 97.1% of the differences are within ±0.50 ng/mg, suggesting that larger adjacent segment differences could be significant. Maintaining the assumption that our donors have not been exposed to an exogenous source of GHB, the ±0.50 ng/mg difference threshold was applied to our donor population to approximate the probability that an individual could produce a false-positive result, thereby indicating exogenous GHB exposure. Another assumption made for this testing was that a single exogenous dose would produce a segment spike with adjacent differences that are greater than 0.50 ng/mg and opposite in directional trend (i.e., −0.50 then +0.50) to indicate a discrete rise and then decrease in GHB concentration. The sign changes are an artifact of the sequential subtractions (i.e., a negative change indicates a rise in concentration and a positive change a decrease). Table II lists the criteria used for identification of exogenous GHB in adjacent segments based on a local maximum. When applying this filtering approach, only one donor met requirements close to the assumptions, donor 116, where the difference between segments 3 and 4 was −0.67 ng/mg and segments 4 and 5 was +0.40 ng/mg. While this donor does not fully meet criteria and all concentrations, including the spike being less than 2.00 ng/mg, it is being used to demonstrate an approximate false-positive rate (FPR). Using our data, the FPR estimated for this approach suggests that an un-dosed individual has an ~0.47% chance (1 in 214) of producing a significant difference or artificial spike. To estimate a confidence interval for the FPR, the Jeffrey's interval method was used. The Jeffrey's interval is known to provide more reliable coverage of the estimated confidence interval than does the more commonly used Wald interval (45). The Jeffrey's 95% confidence interval for the estimated FPR is 0.01–2.16%. The (nearly) false-positive donor had a maximum GHB concentration of 1.41 ng/mg, and the trend can be seen in Supplementary Figure S3.

To estimate a 'worst-case' FPR of randomly seeing a significant change mimicking exogenous GHB exposure, all non-detect segments were substituted with 0 ng/mg to maximize the probability of meeting the exposure decision criteria. With this substitution, three donors produced at least one pair of adjacent differences greater than 0.50 ng/mg and opposite in magnitude corresponding to a rise then fall in GHB concentration, yielding a nominal FPR of 1.40% for individuals (3 in 214). The 95% confidence interval using the Jeffery's interval method for this FPR estimate is 0.40–3.69%. Based on the worst-case scenario that most likely overestimates the true false-positive rate, the estimated FPR might be expected to be as high as 3.69% that an individual could randomly produce two adjacent changes, −0.50 ng/mg followed by +0.50 ng/mg, to indicate an increase in GHB concentration. Of course, more data on endogenous GHB measurements in hair, including higher representation from non-Caucasians, is needed to make the dataset more representative of the general US population and would be useful in better establishing

the most appropriate criteria to minimize the FPR. Further, modifying the difference threshold value to greater than 0.50 ng/mg would lead to lower FPRs in this dataset. Also, data from a large controlled GHB dosing study would be useful in establishing a practical threshold and criteria for declaring a GHB exposure.

Application of the adjacent segment difference strategy to published endogenous studies

To determine the robustness of the adjacent difference strategy, we applied this approach to previously published endogenous GHB results (12–16, 19, 22–25, 27–31). All segmented endogenous GHB results (12, 13, 19, 24, 28) with calculated adjacent differences can be seen in the Supplementary Table SIV as an Excel spreadsheet. In total, 162 differences were calculated, and a frequency distribution was determined as shown in Figure 2c. Despite containing fewer data points and varied segment sizes, the distribution looks similar to our data set distribution in Figure 2a; the main exception is more significant skewing on the right side of the distribution. This could be due to the observation in the published endogenous results that most first segments have higher GHB concentrations than the second segment, providing a positive/right skew, as well as the shorter total lengths of hair analyzed compared to our study (i.e., 3 cm vs. 12 cm). It should also be noted that the endogenous concentrations in these previously published studies were determined using different analytical methods that could also have an impact on the distribution shape and skew the data relative to our study. However, most of the published endogenous differences are still near zero, as 85.2% of the adjacent segment differences are within ±0.5 ng/mg and 94.4% of the differences are within ±1.0 ng/mg. The range of adjacent segment difference values observed in the published endogenous dataset was −0.5 to +5.0 ng/mg. The mean and median segment differences observed in these data are 0.22 and 0.03 ng/mg, respectively. When filtering for false positives, there was only one individual with a false-positive peak, donor T12 (all values <2.0 ng/mg) from Chèze et al. (28); however, this could be due to the estimation error from the graph as absolute values were not included for this donor (adjacent differences = −0.5 and +0.5 ng/mg). With this tentative detection, an estimated FPR of 3.13% (1 in 32) was calculated and is relatively similar to the estimated FPR ranges from our study. The slight increase in FPR for the published endogenous data relative to our data set may be due to the smaller number of donor samples used in this calculation.

To look for differences between the FPR in the published endogenous studies and for the FPR in this study (i.e., 1/214), Fisher's exact test was used with a null hypothesis that there was no difference between the two FPRs (46). The use of Fisher's Exact test was required because the assumptions required for testing for a difference in FPRs using the more familiar test based on the normal approximation are not met by these datasets. Based on the analysis performed, no statistically significant difference between the FPRs in the published endogenous study and this one was noted ($P = 0.244$). This confirms the similarity between our published results with previous studies and that the adjacent segment application is not specific to our data set. While better estimates of the FPR can always be made with more data, these published data (12, 13, 19, 24, 28), despite different segment sizes and analytical methods, seem to support the potential value of using adjacent segment differences as a method for the differentiation of endogenous from exogenous GHB. It is also clear that controlled dosing studies with large populations are needed to better understand the limitations of this approach and better define the recommended threshold and its corresponding false-positive rate.

Application of the adjacent segment difference strategy to published exogenous studies—controlled dosing

While some controlled dosing studies concluded that exogenous exposure might not be detected in hair (8, 29, 31), others were apparently successful in identifying controlled exogenous exposure (13, 19). It is unclear why, despite similar dosages, there is a disparity in these controlled dosing study results. However, the controlled dosing studies that provided segmented data with negative results (29, 31) also did not meet the adjacent segment difference strategy threshold for positive identification of exogenous exposure (data not shown).

Published controlled dosing studies with positive, segmented results are displayed in Table I. All segments meeting our adjacent segment difference strategy criteria are highlighted. These highlighted segments are the same ones identified in the respective published studies showing the successful application of the adjacent difference approach for exogenous GHB discrimination. However, there were some interesting observations. One donor, Controlled Dosing 1, from Kintz et al. (13) identified segment 5 as corresponding to the controlled dose exposure. While segment 5 does meet the adjacent segment difference criteria, this donor had a large net change of approximately −11 ng/mg in GHB across the 3 cm of hair tested. The large net change across the length of hair, in addition to a lower concentration just prior to segment 5, makes it difficult to conclude that the elevation is from the exogenous controlled dosing exposure, as opposed to normal variation in this donor. It should also be noted that the 3:1 ratio was not met and that criterion failed to identify the controlled dosing peak in Controlled Dosing 1. Considering the short length of hair tested for this donor (3 cm) and the substantial changes in endogenous concentration in the first few segments, it is difficult to determine the baseline GHB concentration for this donor. This could indicate that collecting and testing longer hair lengths and/or collecting samples closer to ~8+ weeks after suspected exposure would be beneficial to aid in the interpretation. The recommendation of waiting ~8 weeks was also made by LeBeau et al. (38) based on a hair collection study that factored in the amount of hair typically left behind during sample collection. Other studies have excluded anywhere from the first segment to the first centimeter of hair due to sweat contamination concerns (13, 17, 19, 21, 24, 27, 31, 32, 37), which would require lengths greater than 3 cm to be tested and a longer wait before collection to better establish pre- and post-exposure basal GHB concentrations. The Controlled Dosing 1 donor sample (13) also highlights the apparent greater segment-to-segment variability that may be observed in male donors that could complicate identification of exogenous GHB exposure.

Another controlled dosing study by Bertol et al. (19) utilized 12 volunteers who were sampled 1- and 2-month post-exposure. That study highlighted the decrease or degradation of GHB that can occur over time and the fact that even the 3:1 recommended ratio is not always sensitive enough to identify exogenous exposure in all controlled dosing experiments. However, the adjacent segment difference approach with a 0.5 ng/mg threshold was successful in identifying all controlled exogenous exposures in the same Bertol dataset, even when the 3:1 ratio threshold was not met. The smallest absolute value of the adjacent differences associated with the exogenous GHB exposure segment in this data set was 0.64 ng/mg.

Two other differences were within ±1.00 ng/mg, but the remaining 45 differences were greater than ±1.00 ng/mg.

Similar to the above controlled dosing study, Busardò et al. (24) performed multiple collections over time from a single individual with a single exogenous GHB exposure. Over the course of the year, with one sample collected and tested per month, the detected peak GHB concentration decreased by ∼50% over the time course of the repeat sampling and analysis in the study. The segmental data from this study was not available to be added to Table I, but using the published calculated ratio for this donor, it was noted that the ratio dropped below 3:1 in month 11. However, the published difference between the peak GHB concentration and the concentration in adjacent segments remained greater than the 0.5 ng/mg threshold for all 12 months, with the minimum difference listed as 1.3 ng/mg. Both studies highlight the limitations of the ratio approach, especially considering the potential for degradation of GHB in hair over time (19, 24). On the other hand, the data suggest that the adjacent segment difference approach continues to be sensitive to the identification of exogenous GHB exposure, despite the degradation concerns.

Application of the adjacent segment difference strategy to published exogenous studies—suspected dosing

The adjacent segment difference approach was applied to 12 different suspected single-dosing cases from 11 donors (Table I). It should be noted that in these 11 donors, 3 mm or 5 mm segments were analyzed, as opposed to the 1-cm segments used in our study. The region of hair that is identified and consistent with the time frame of exogenous exposure does meet the −0.5 ng/mg followed by +0.5 ng/mg difference criteria. The suspected dosing segments with acceptable differences are highlighted with bolded, italicized text (red text online version). Of the 12 examples, 8 exactly met our proposed criteria, 3 met our criteria with modification, and 1 did not.

One subject, Suspected Dosing 7 (28), had an unusually low concentration in segment 5. The inclusion of this low value does impact the average endogenous GHB concentration; without this segment, the suspected dosing peak would not meet the 3:1 ratio threshold. However, as there is no average basal GHB concentration calculation for the adjacent segment difference approach, there is no impact to the exogenous exposure conclusion. The ratio approach can be sensitive to the segments used for calculating the endogenous baseline concentration, whereas the adjacent segment difference approach seems to be mostly unaffected by those issues.

For another suspected dosing example from Bertol et al. (19) (Suspected Dosing 3), the donor segment results were not sufficient to conclude that the observed peak was caused by possible exogenous GHB exposure, even using Bertol's adjusted ratio range (2.14–5.48) (19). Using the adjacent difference approach, it was observed that the absolute difference between segments 4 and 5 and segments 6 and 7 were both greater than 0.5 ng/mg and opposite in magnitude, indicating that the possible exogenous exposure peak is actually in two adjacent segments, 5 and 6, as opposed to just a single segment. A visual graph of the differences is shown in Figure 3a, with the differences meeting the threshold criteria indicated by an asterisk. This observation of exogenous exposure occurring in two adjacent segments was also seen in two other suspected GHB exposure donors, Suspected Dosing 5 in Kintz et al. (14) (Figure 3b) and Suspected Dosing 10 in Martz et al. (30) (Figure 3c). All three of these suspected dosing donor cases (Suspected Dosing 3, 5 and 10 (14, 19, 30)) have segment differences greater than 0.5 ng/mg and opposite in magnitude; however, these are not adjacent differences as the two segments display elevated concentrations and instead separate the rise and fall differences by one difference that is small and less than 0.5 ng/mg (0.1–0.3 ng/mg). This trend would indicate that the elevated GHB concentration is not in a single segment but rather spread across two segments. These three examples, Suspected Dosing Donors 3, 5 and 10, show that segment length, as well as other factors, may have an impact on the single, discrete segment assumption, as elevated GHB concentrations were observed in two adjacent segments (14, 19, 30). The assumptions used for the adjacent segment difference approach and even for the ratio strategy are important to consider for proper application and result interpretation. It should be noted that single-dose detection in more than one adjacent segment has been reported elsewhere (39–41, 44) and the single segment spike may not be an appropriate assumption to use for all cases. Whether these observations are related to the hair growth rate and phase or other band-broadening causes is unclear (39–41). Regardless of the cause, it remains an important consideration for deciding proper segment length size and for interpreting analytical results.

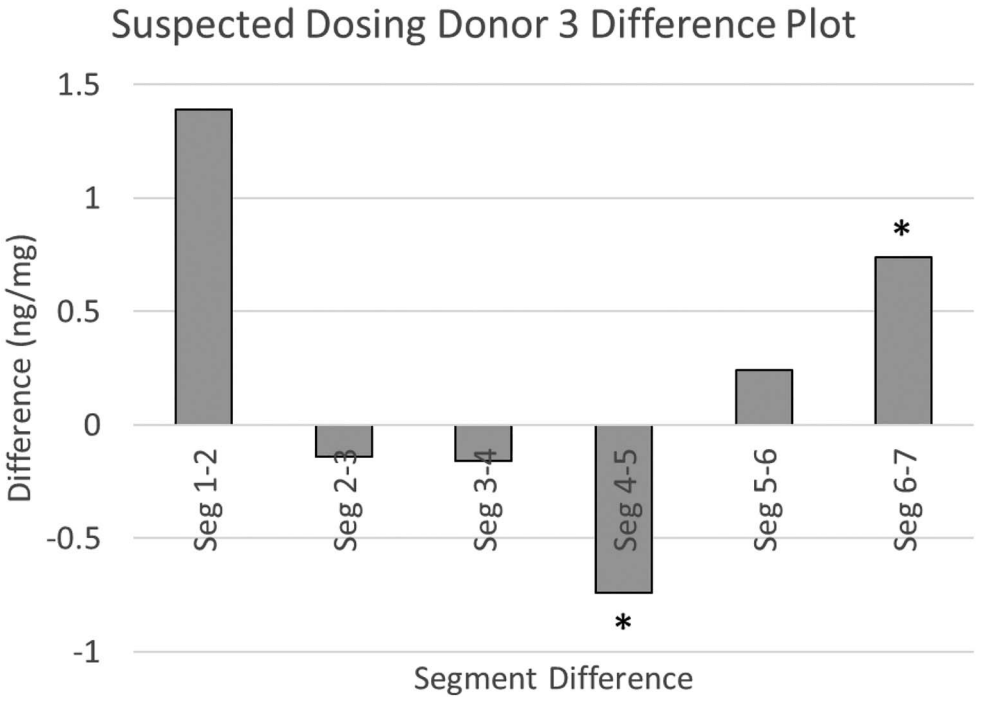
One suspected dosing donor, Suspected Dosing 11, did not meet the adjacent segment difference criteria (underlined value) for successful identification of exogenous GHB exposure (30, 36). This was previously identified with an alternative mathematical outlier approach (36). Despite the authors' conclusion of exogenous exposure, the estimated changes, −0.4 and +0.3 ng/mg, are small and well within the normal differences observed in the endogenous data distribution for both our study and the published data sets (Figure 2a–c) and lower than the other observed differences in the controlled and suspected dosing donors from Table I (absolute value range 0.64–6.1 ng/mg). Therefore, the conclusion of exogenous GHB exposure cannot be supported using adjacent segment differences. This lack of positive identification by the adjacent segment difference approach may be due to some complication that obscures interpretation, such as a low dose or hair treatment (35, 37), if the exogenous dosing did indeed occur. Alternatively, this example could indicate a lack of sensitivity by the adjacent segment difference strategy and result in a nonzero false-negative rate. It should be noted that this donor does not meet the 3:1 ratio criterion either.

Conclusions

This is the first study that measures and defines intra-individual variation in the hair of a large donor population as a means of support for using an individual as their own reference when determining a single exogenous GHB exposure. The large inter-individual GHB concentration range observed, <0.4–5.47 ng/mg, along with the absence of a large controlled dosing study makes selecting an appropriate recommended cut-off concentration to differentiate endogenous GHB from exogenous exposure ineffective. This conclusion is consistent with previous studies (7, 12–26). However, the intra-individual variation, as measured by %RSD in most of the donors, supports the use of an individual as their own reference to identify possible exogenous exposure (7, 12–17, 19, 22–25, 27–33). The adjacent segment difference criteria attempt to reduce the false-negative rate currently observed in published controlled dosing and suspected exogenous exposure cases (12–14, 16, 19, 22, 28, 30) by improving the sensitivity of the detection method. Additionally, the adjacent segment difference approach is more efficacious for individuals that have greater intra-individual variability (%RSD >40%) because it eliminates the need to determine a specific background GHB concentration.

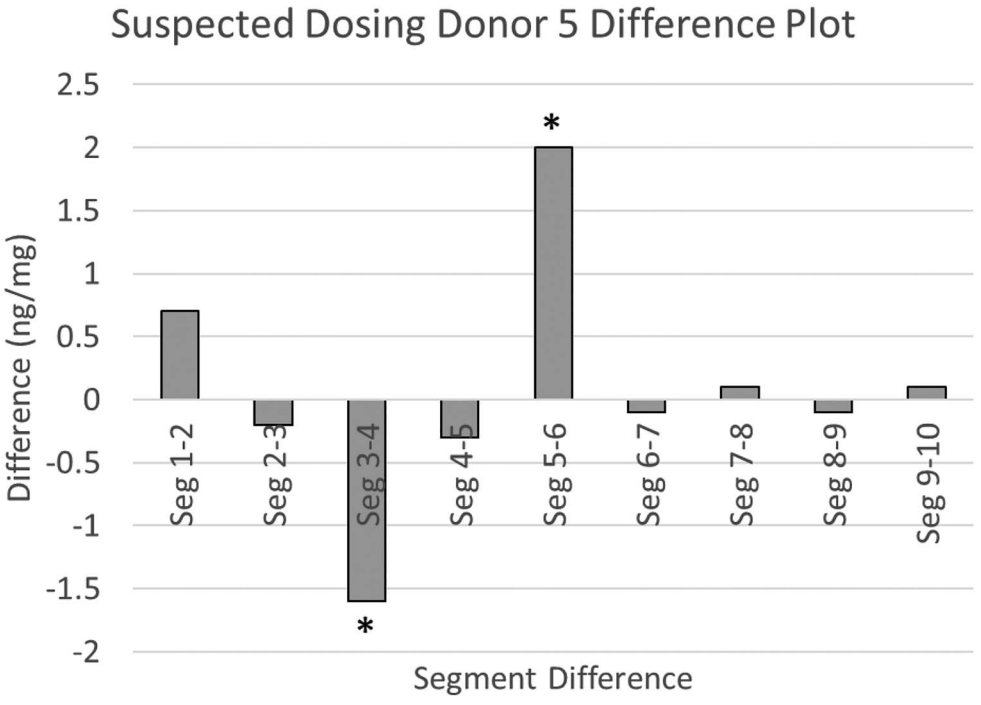
To further evaluate the suitability of the adjacent segment difference approach, several other studies need to be conducted under

A. Suspected Dosing Donor 3



*Paired segment differences that are <-0.5 then >0.5 ng/mg.

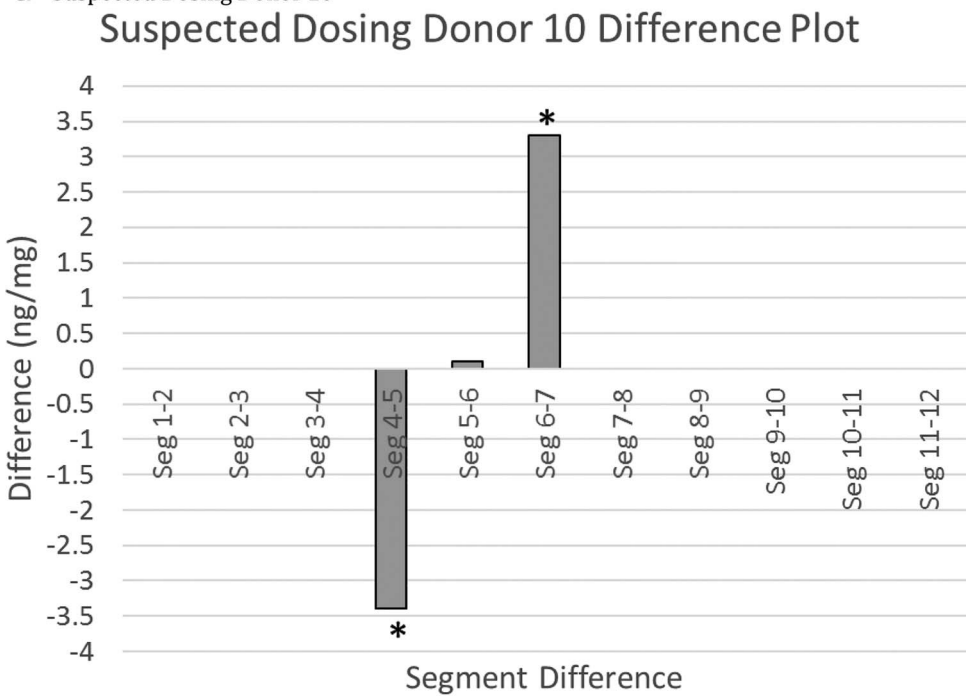
B. Suspected Dosing Donor 5



*Paired segment differences that are <-0.5 then >0.5 ng/mg.

Figure 3. Select adjacent segment difference plots A. Suspected Dosing Donor 3. B. Suspected Dosing Donor 5. C. Suspected Dosing Donor 10.

C. Suspected Dosing Donor 10



*Paired segment differences that are <-0.5 then >0.5 ng/mg.

Figure 3. Continued.

different and varying ingestion conditions. Additional endogenous data is needed to better represent non-Caucasians and to define the FPR, which is dependent on the difference threshold set. Therefore, a large donor-controlled dosing study would be advisable to determine an appropriate adjacent segment difference threshold and to define the potential false negative rate. The 0.5 ng/mg threshold applied here was for demonstration purposes and worked successfully when applied to published controlled dosing and suspected drug-facilitated crime case donors. However, the threshold can likely be better adjusted to decrease the false-positive and false-negative rates while still providing enough discrimination between endogenous and exogenous GHB exposure.

There is a clear need in the forensic toxicology community to define specific experimental criteria (e.g., segment length) for comparison of results from different laboratories so that consistent reliability of the adjacent segment difference approach can be ensured. Segment length could have a significant impact on this approach and varies widely from 3 to 20 mm in the studies we reviewed (12–16, 19, 21–32, 34, 35, 37), as does the total length of hair tested. Smaller segment lengths (i.e., 3 mm) could be problematic due to varying hair growth rates and the increased chance of a single dose appearing in multiple adjacent segments. Thus, we draw the following conclusions and suggestions: (i) the collection should take place 8 weeks or more after the suspected dosing event and a length of at least 3 to 5 cm be gathered from the scalp; (ii) GHB ratios are discouraged as a test for GHB ingestion, especially for male subjects; (iii) based on our data, a concentration above 2 ng/mg along with a positive indication from the adjacent segment difference is likely to indicate exogenous dosing; and (iv) hair preparation should be standardized/limited for parameters such as segment length and we recommend using 1 cm segments.

Overall, using the adjacent segment differences approach shows promise as an alternative strategy to identify exogenous GHB exposure that is more discriminating than the ratio approach. This approach offers unique benefits of being less susceptible to donors with larger intra-individual variation and more sensitive to exogenous exposure.

Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

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Acknowledgements

This research was supported in part by an appointment to the Visiting Scientist Program at the Federal Bureau of Investigation (FBI) Laboratory Division, administered by the Oak Ridge Institute for Science and Education, through an interagency agreement between the US Department of Energy and the FBI.

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