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Testing for GHB in Hair by GC/MS/MS after a Single Exposure. Application to Document Sexual Assault

ABSTRACT: Gamma-hydroxybutyric acid, or GHB, is a substance naturally present within mammal species. Properties of neurotransmitter or neuromodulator are generally given to this substance. GHB is therapeutically used as an anesthetic, but can be used for criminal offenses (date-rape drug).

It appears that the window of detection of GHB is very short in both blood and urine, and therefore its presence is very difficult to prove after a rape case.

In order to document single exposure, we investigated the use of hair.

Hair was collected one month after the allegated event in order to sample the corresponding period after regular growing.

After rapid (2 min) decontamination with dichloromethane, the hair shaft was cut into 3-mm segments. They were overnight incubated in 0.01 *N* NaOH in the presence of GHB-d_6, followed by neutralization and extraction in ethyl acetate under acidic conditions. GHB (precursor ion m/z 233, product ions m/z 147 and 148) was tested by GC/MS/MS (Finnigan TSQ 700) after derivatization with BSTFA + 1% TMCS.

Physiological concentrations ($n = 24$) were in the range 0.5 to 12.0 ng/mg, with no influence due to hair color. No variation of concentrations was observed along the hair shaft in controlled subjects, except for the proximal segment, due to an incorporation through sweat. This demonstrates that endogenous levels for each single subject are constant during hair growth.

A controlled human administration of 25 mg/kg to a volunteer demonstrated that a single exposure to GHB is detectable in hair after segmentation.

In a case of rape under influence, a clear increase of the corresponding segment (about 2.4 ng/mg) in time was observed, in comparison with the other segments (0.6 to 0.8 ng/mg).

This study demonstrates that a single exposure to GHB in a case of sexual assault can be documented by hair analysis when collected about one month after the crime.

KEYWORDS: forensic science, GHB, rape, hair, sexual assault, GC/MS/MS

Although considered as a new drug of abuse, gamma-hydroxybutyrate, or GHB, has been used clinically since the 1960s as an intravenous anesthetic. It was also investigated for treatment of insomnia, of alcohol and opiate withdrawal syndrome, and in cerebrovascular disorders.

GHB is a substance naturally present within mammalian species. Properties of neurotransmitter or neuromodulator are generally given to this substance (1,2).

Doses of 10 mg/kg cause amnesia; 20 to 30 mg/kg cause sleep; and doses of 50 mg/kg or higher produce anesthesia. Illicit use of GHB typically involves doses of 35 mg/kg (3). People exposed to GHB involve: bodybuilders who believe that the drug stimulates the release of growth hormone, even if this activity is still under debate (4); ravers or club attendees for its intoxicating effects, such as euphoria, reduced inhibitions, sedation, and muscle relaxation that can benefit after ecstasy abuse (5); drivers as a result of recreational abuse (6); and victims of drug-facilitated sexual assault (7).

The purported enhancement of sexuality, coupled with a possible abrupt coma-inducing effect, ease of administration in spiked drinks, and potential amnesia have resulted in the use of GHB as an assault-related drug. GHB is also attractive to rapists as it is readily available (Internet, on the street, in dance clubs or fitness centers).

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A study examining the presence of various drugs in urine obtained from 3303 individuals who claimed to have been sexually assaulted and believed that drugs were involved found ethanol to be the most common date-rape associated drug, being present in 41.1% of the cases. GHB was present in 3.0% of the total cases. However, the authors stated an important caveat when interpreting the results. They discussed the fact that late sampling of the specimens and therefore complete metabolism and elimination of the drug can lead to possible underestimation of the total number of the cases.

This appears as the key issue when dealing with GHB. Following oral administration, even at doses up to 60 mg/kg, the drug is cleared from the blood within 6 h (9,10). GHB is excreted in urine in small amounts in the free form within 10 to 12 h (11). In a controlled human study where a 60 mg/kg dose was administered, saliva did not enhance the window of detection (9). Sweat collected either by a sweat patch or a cosmetic pad can be used to document exposure when sampling is done 12 h after the crime (12). However, the interpretation of the findings must be done cautiously due to the presence of physiological concentrations. The presence of GHB in unpreserved blood (for example with EDTA or NaF) from living people, in post-mortem blood, or even in urine can be a confusing artifact in determining the role of GHB in a forensic situation (13,14).

In order to address these questions, we investigated the potential use of hair to document a single exposure to GHB.

Material and Methods

Specimen

Hair strands were obtained from a 19-year-old girl who claimed to have been sexually assaulted after drinking a soft drink spiked with a drug. She had no memory of the crime and went to the police five days after the rape. After contact with the police, this laboratory recommended to wait for about one month in order to have the corresponding growing hair between the root and the tip. Full-length hair samples (8 cm long) were taken at the surface of the skin from the vertex and stored in plastic tubes at room temperature.

Controlled hair specimens ($n = 24$) to establish the physiological GHB concentrations were obtained from laboratory personnel. In complement, segmental analyses were achieved using two hair strands collected from this population.

In a controlled human study, we orally administered to a 41-year-old man (weighing 67 kg) a 25 mg/kg dose dissolved in 100 mL of water. Hair was collected one month after this single exposure.

Hair collection and oral administration of GHB were not in conflict with the policies of the local ethical committee.

Chemicals and Reagents

Dichloromethane and ethyl acetate were HPLC grade (Merck, Darmstadt, Germany). All other chemicals were of analytical grade and provided by Merck. BSTFA + 1% TMCS was purchased from Fluka (Saint-Quentin Fallavier, France). GHB and GHB-d_6 were purchased from Promochem (Molsheim, France). Synthetic melanin was purchased from Sigma (Saint-Quentin Fallavier, France).

GHB Extraction

The hair was decontaminated twice using 5 mL of methylene chloride for 2 min at room temperature and then cut into 3-mm segments over a length of 3 cm (ten segments). A longer decontamination time will reduce the concentration of GHB in the hair.

About 5 to 10 mg of decontaminated hair were incubated in 0.5 mL 0.01 *N* NaOH, 16 h at 56°C, in the presence of 10 ng of GHB-d_6 used as an internal standard. After cooling, the homogenate was neutralized with 0.5 mL 0.01 *N* HCl, and 3 mL of ethyl acetate were added together with 0.1 mL of 0.01 *M* H_2SO_4.

After agitation and centrifugation, the organic phase was evaporated to dryness under nitrogen flow. The residue was derivatized by adding 20 μL BSTFA + 1% TMCS and 20 μL ethyl acetate, then incubated for 20 min at 60°C.

GC-MS/MS Procedure

A 1-μL aliquot of the derivatized extract was injected into the column of a Hewlett Packard (Palo Alto, CA) gas chromatograph (6890 Series). The flow of carrier gas (helium, purity grade N55) through the column (HP5-MS capillary column, 5% phenyl-95% methylsiloxane, 30 m by 0.25 mm ID by × 0.25 mm film thickness) was 1.0 mL/min.

The injector temperature was 270°C, and splitless injection was employed with a split valve off-time of 1.0 min. The column oven temperature was programmed to rise from an initial temperature of 100°C, maintained for 1 min, to 295°C at 30°C/min, and maintained at 295°C for the final 5 min.

The detector was a Finnigan TSQ 700 operated in the electron ionization mode and in selected reaction monitoring. The precursor ions, m/z 233 and 239 for GHB and the IS, respectively, were selected in the first quadrupole. The common product ions, m/z 147 and 148, were selected in the third quadrupole after collision with argon at a cell pressure at 0.62 mTorr. The collision offset voltage was −8 V. The electron multiplier was operated at 1900 V.

Method Validation

A standard calibration curve ($n = 3$) was obtained by adding 1 (0.2 ng/mg), 2.5 (0.5 ng/mg), 5 (1.0 ng/mg), 25 (5.0 ng/mg), 50 (10.0 ng/mg), and 100 (20 ng/mg) ng of GHB to 5 mg of synthetic melanin in suspension in 0.5 mL 0.01 *N* NaOH. It is acknowledged that the use of melanin may not fully substitute for the use of real hair as a matrix. However, it was not possible to find hair that was free of endogenous GHB.

Within-batch precisions for GHB were determined using hair that was obtained from three laboratory volunteers (to get three different concentrations) and previously pulverized in a ball mill and homogenized.

Relative extraction recovery was determined by comparing the representative peak area of GHB extracted from 5 mg of melanin spiked at the final concentration of 1 ng/mg with the peak area of a methanolic standard at the same concentration.

The detection limit (LOD) was evaluated with decreasing concentrations of GHB spiked in melanin until a response equivalent to three times the background noise was observed.

Results

Validation Results

Cleaner chromatograms were obtained when 0.01 *N* NaOH was used instead of the classic 1.0 *N* NaOH. Although the analytes were stable under alkaline conditions, the digestion of the hair was incomplete using 0.01 *N* NaOH.

Under the chromatographic conditions used, there was no interference with the analytes by any extractable endogenous materials present in hair. There were no blank effects.

In order to obtain optimum selectivity, which is of paramount importance in forensic science, the selected reaction monitoring (SRM) technique was applied. It is desirable to produce an intense ion signal that is characteristic for the target compound. Selectivity and sensitivity are extraordinarily increased by almost completely suppressing the noise level. A chromatogram obtained from an authentic hair specimen at 0.73 ng/mg is shown in Fig 1. Selected ions and retention times of GHB and the deuterated internal standard are reported in Table 1. The precursor ion of GHB (m/z 233) corresponds to the demethylated molecular ion; the two product ions (m/z 147 and 148) were common to both GHB and the deuterated internal standard, but did not cause any interference.

The calibration curve corresponds to the linear regression between the peak-area ratio of GHB to IS and the final concentration of the drug in spiked melanin.

Responses for GHB were linear in the range 0.2 to 20 ng/mg. From three independent calibrations, the correlation coefficients ranged from 0.989 to 0.998.

The within-batch precisions were 11.8, 10.4, and 8.9%, as determined by analyzing eight replicates of 5 mg of hair obtained from the three subjects with GHB concentrations at 0.66, 1.30, and 2.45 ng/mg, respectively.

The extraction recovery ($n = 3$) was determined to be 81.8%. The limit of detection of GHB was 0.1 ng/mg, using a 5-mg sample. This limit of detection can be improved by using a larger

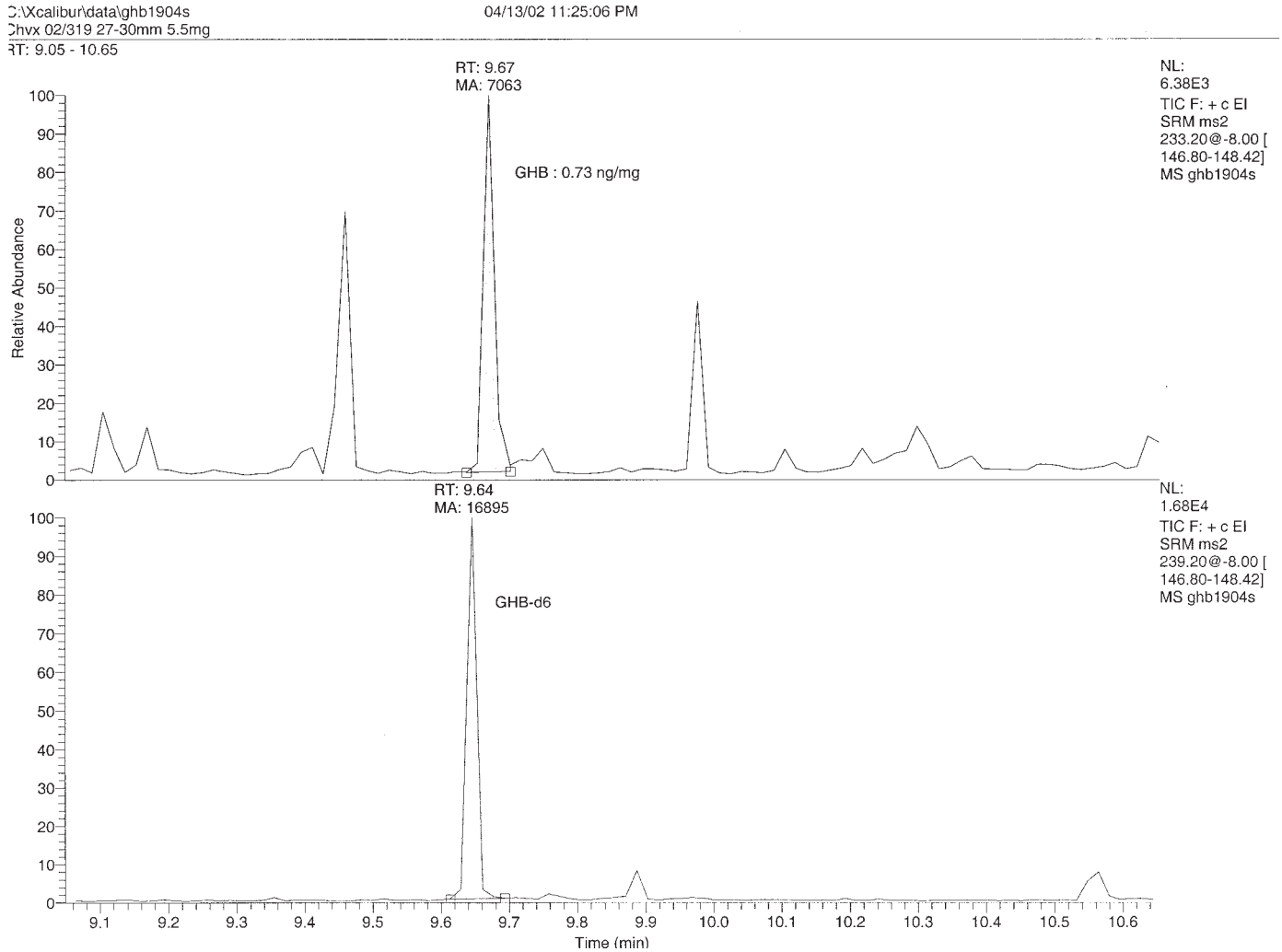

FIG. 1—*SRM chromatogram obtained after extraction by the established procedure of a 5-mg hair specimen of a laboratory volunteer who never took GHB. GHB was quantified at the concentration of 0.73 ng/mg.* Top: *GHB with its product ion at m/z 147 from the ion at m/z 233, and* bottom: *GHB-d_6 with its product ion at m/z 147 from the ion at m/z 239.*

TABLE 1—*Selected ion (m/z) and retention times for GHB and the internal standard.*

Analyte	Retention Time	Ions, m/z
GHB	9.67 min	233 to 147 and 233 to 148
GHB-d_6	9.64 min	239 to 147 and 239 to 148

amount of hair. The limit of quantitation was the first point of the calibration curve, that is, 0.2 ng/mg below the endogenous levels.

Derivatives were stable at least for 24 h. Formation of the TMS derivatives appears to be appropriate to obtain optimal peak shapes and intense molecular ions. Potential conversion of GHB to GBL during the preparation process did not occur, as verified during the initial steps of development.

Applications

Twenty-four specimens obtained from male ($n = 8$) and female ($n = 16$) volunteers were analyzed. In all cases, only the first 3 cm from the root were tested, that is, the classic procedure in this laboratory when hair is used to verify drug history. The quantitative analyses demonstrated the presence of GHB in all 24 hair samples at concentrations of about 0.5 to 12.0 ng/mg, confirming endogenous GHB content in hair. There was no difference in endogenous content between hair of various colors or belonging to subjects of different sex. Mean measured concentrations were 2.21 ± 0.57 and 2.47 ± 0.69 ng/mg for males and females, respectively. The same results were obtained between hair samples of different colors (black, $n = 10 : 2.37 \pm 0.68$ ng/mg; brown, $n = 6 : 2.21 \pm 0.71$ ng/mg; blond, $n = 8 : 2.44 \pm 0.39$ ng/mg).

Segmentation into 3-mm pieces from two subjects (blond and black) from the previous population of volunteers is represented Fig. 2. Maximal concentration is observed at the root part, probably due to incorporation through sweat and the absence of washout by normal hygiene practice. It seems that there is a need to wait for a growing phase of 2 to 3 weeks to obtain a long-term basal concentration. From sweat patch experiments, it is known that endogenous concentrations of GHB in sweat are high (12).

Increase of response from GHB after a controlled administration of 25 mg/kg to a volunteer is evident from Fig. 3, demonstrating

that a single exposure can be detected in hair. Although not published, records from the police suggest that 30 to 50 mg/kg is the dose that is used by rapists.

This method was used to document administration of GHB in a rape case. Hair of the victim was cut about one month after the offense. Segmentation revealed an increase of GHB concentrations at the corresponding time (Fig. 4), confirming exposure. This result was not challenged by the rapist, who was arrested several days after the assault.

The dosed subject has a peak concentration in Segment 1.2 to 1.5, the victim in Segment 0.9 to 1.2, although in both cases hair collection occurred about one month after exposure. This can be explained by a difference in the rate of hair growth and the position of the scissors above the scalp.

Discussion

Drugs used to facilitate sexual assaults can be difficult to detect (active products at low dosages, chemical instability), possess amnesic properties, and can be rapidly cleared from the body (short half-life).

In 1999, several authors (15) have developed recommendations and guidelines for toxicological investigations used to document impairment due to drugs in cases of sexual assault. Hair was suggested as a valuable specimen in situations where, as a result of a delay in reporting the crime, natural processes have eliminated the drug from typical biological specimens, such as blood and urine. From the literature, it is obvious that GHB exposure cannot be established more than 8 to 12 h after administration. Hair will therefore allow improved detection of GHB. While there are a lot of papers focused on the identification of drugs in hair following chronic drug use, those dealing with a single dose are very scarce. Data are available for codeine (16), cocaine (17), selegiline (18), and flunitrazepam (19). All the authors were able to target the drug in hair after a dose corresponding to a therapeutic dosage.

As GHB is an endogenous constituent, it is thought that small quantities may be transferred from the circulation to hair follicle and sweat and consequently incorporated in the hair structure. This

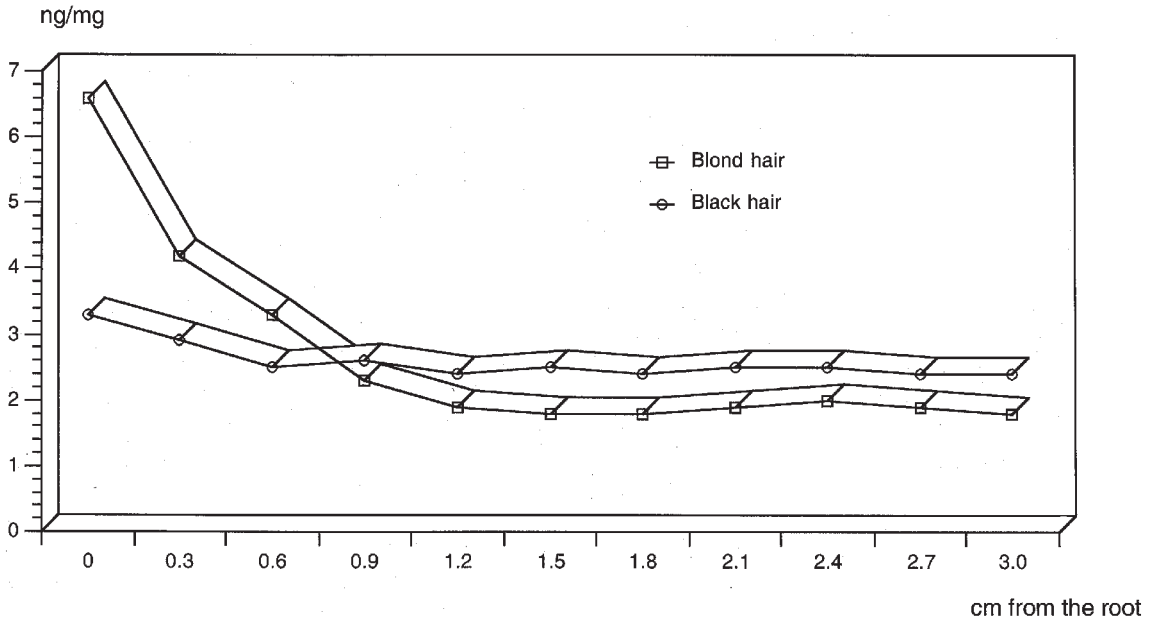

FIG. 2—*Hair segmentation from two volunteers never exposed to GHB, documenting endogenous GHB levels.*

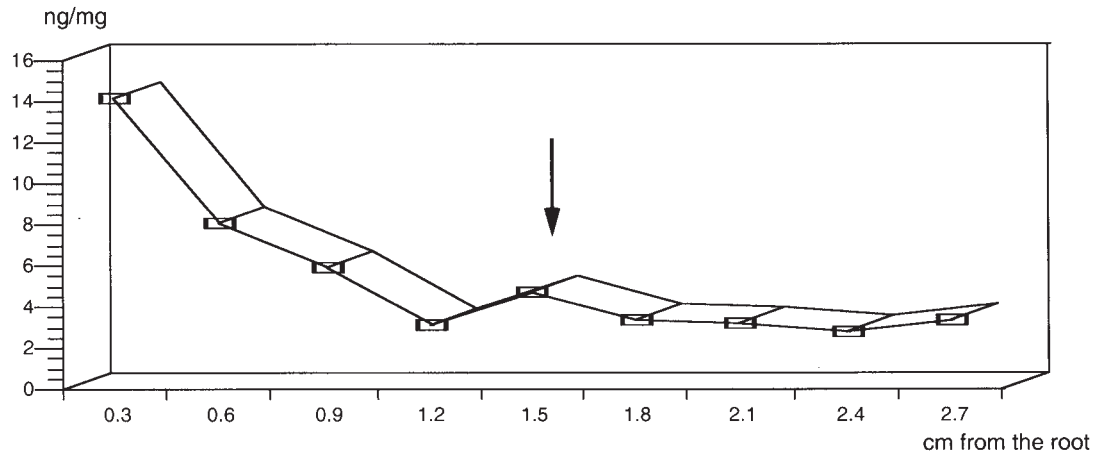

FIG. 3—*Hair segmentation from a controlled subject who orally received a 25 mg/kg dose of GHB one month before collection.*

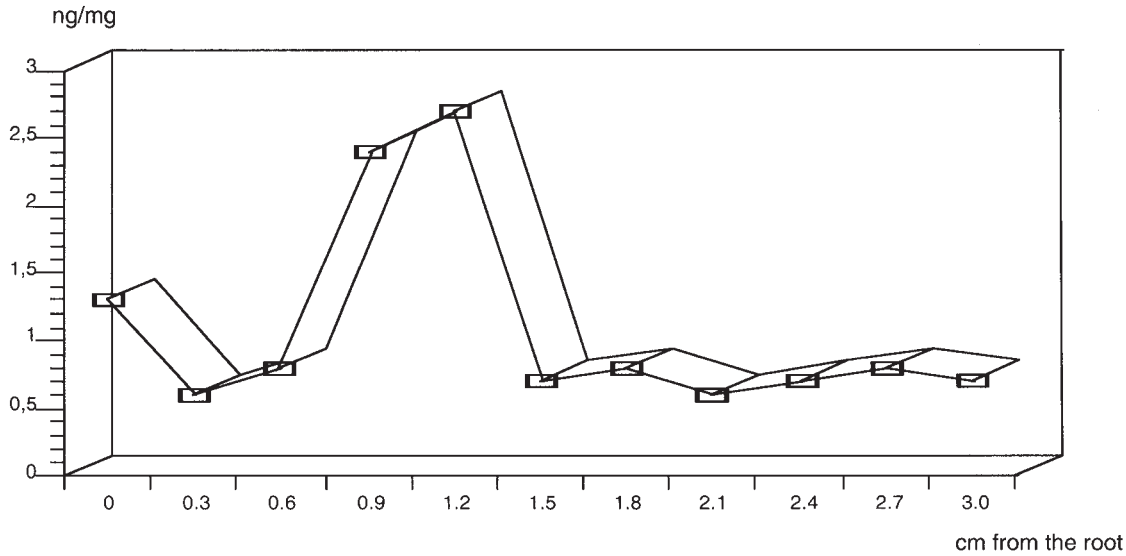

FIG. 4—*Hair segmentation from a girl raped under the influence of GHB. Hair was collected about one month after the offense.*

was first demonstrated in 1995 by Ferrara et al. (20), with an average concentration of endogenous GHB from 30 Caucasian male and female subjects of 0.53 ± 0.20 ng/mg, with no difference between sex and hair color. Our study confirms the lack of influence from sex and hair color, but our measured concentrations are higher. In a recent report, Kalasinsky et al. (21), in a GHB fatal overdose case, did not observe detectable amounts of the drug by segmental hair analysis (with a limit of detection at 1 ng/mg). However, hair root bulb results were highly positive: 47.4 and 2221 ng/mg washed versus unwashed, respectively.

Since GHB is present in the hair of the general population under physiological concentrations, toxicologists must be able to discriminate between endogenous levels and a concentration resulting from exposure. The implementation of a cut-off concentration must be done cautiously, due to the wide distribution of endogenous concentrations, from 0.5 to 12.0 ng/mg. The solution is to use each subject as his own control. From the demonstration that physiological concentrations are stable along the hair shaft, except at the root part, one can suppose that exposure will lead to a peak concentration that can be detected. Use of tandem mass spectrometry is mandatory because of the low amount of hair that needs to be tested as a consequence of the short 3-mm segments that need to be analyzed. To avoid difficulties in interpretation due to potential contamination by sweat, it is necessary to wait for 3 to 4 weeks before hair collection. This will permit the migration of the GHB spot along the length of the hair shaft.

Finally, another potential application of hair analysis would be the discrimination between GHB and gamma-butyrolactone (GBL) and 1,4-butane-diol (1,4-BD). These compounds are rapidly metabolized into GHB, but are not excreted into the urine unchanged in appreciable amounts. For a forensic point of view, it can be of interest to determine which drug was used, but obviously this cannot be achieved by testing urine because the metabolites are common. Hair should be able to both confirm exposure and identify the exact nature of the parent compound (e.g., GHB, GBL, or 1,4-BD in cases of positive urine for GHB) because it has been accepted by the scientific community that the parent compound is generally the major analyte that is incorporated in hair, even if these compounds are rapidly metabolized and have a short t1/2, as is, for example, the case with heroin (22). Thus, hair analysis would discriminate GHB exposure from other preparations containing GBL or 1,4-BD. This was recently used to discriminate nandrolone from other 19-norsteroïds in doping control (23).

Conclusions

This sensitive, specific, and reproducible method developed is suitable for the detection and quantification of GHB in human hair, as it was always possible to detect endogenous levels of the drug, whatever the specimen submitted for analysis.

Hair analysis may be a useful adjunct to conventional drug testing in sexual assault. It should not be considered as an alternative to urinalysis, but as a complement. This technology may find useful applications, but the definition of legally defensible cut-off values would require much more data.

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