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Ultra-rapid procedure to test for γ-hydroxybutyric acid in blood and urine by gas chromatography–mass spectrometry

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Abstract

Gamma-hydroxybutyric acid (GHB) is a substance naturally present within mammal species. Properties of a neurotransmitter or neuromodulator are generally suggested for this substance. GHB is therapeutically used as an anaesthetic, but can be used for criminal offences (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. Twenty μl of blood or urine were pipetted into a glass tube, followed by 20 μl GHB-d_6 and 45 μl acetonitrile. After vortexing and efficient centrifugation, the supernatant was collected and evaporated to dryness. The residue was derivatized with BSTFA+1% TMCS for 20 min at 70 °C. After injection on a 30-m HP5 MS capillary column, GHB (*m*/*z* 233, 204 and 147) and GHB-d_6 (*m*/*z* 239) were identified by mass spectrometry. The procedure was linear from 1 to 200 mg/l for both blood and urine. Precisions were in the range 4 to 11%. The method appears simple, specific and rapid as an accurate result can be obtained within 1 h.

Keywords: γ-Hydroxybutyric acid

1. Introduction

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

GHB is a substance naturally present within mammalian species. Properties of neurotransmitters or neuromodulators are generally suggested for this substance [1,2].

Doses of 10 mg/kg cause amnesia; 20–30 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 include 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 be beneficial 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

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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).

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 cases [8].

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 10–12 h [11]. 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 sodium fluoride) 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 [12,13].

In a controlled human study where 60 mg/kg dose was administered, saliva did not enhance the window of detection [9]. Sweat [14] or hair [15] can also be collected to document GHB exposure. However, these specimens remain uncommon.

Immunoassays for GHB detection are not available.

Even if colorimetric [16] or micellar electrokinetic chromatography [17] procedures have been published, most of the papers use gas chromatography coupled to mass spectrometry. Essentially, two approaches have been developed: GHB can be detected after cyclization to gamma-butyrolactone in strong acid medium [18,19] or directly after liquid–liquid extraction [20–22]. At this time, it is not possible to indicate which performs best.

After adaptation of a paper of Verstraete et al. [23], we have developed the following procedure, applicable in both blood and urine.

2. Materials and methods

2.1. Specimen

Blood and urine specimens were obtained from emergency units of the hospitals close to Strasbourg, or from the autopsy room of the Institute. Blood was preserved in EDTA tubes, while urine samples remained unpreserved. Both blood and urine were stored at +4 °C.

2.2. Chemicals and reagents

Acetonitrile was HPLC grade (Merck, Darmstadt, Germany). BSTFA + 1% TMCS (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane) was purchased from Fluka (Saint-Quentin Fallavier, France). GHB and GHB-d_6 were purchased from Promochem (Molsheim, France).

2.3. GHB extraction

The procedure is identical, irrespective of whether the test is for blood or urine.

Twenty μl of blood or urine were treated with 45 μl of acetonitrile, in the presence of 20 μl of GHB-d_6 (25 mg/l) used as internal standard (I.S.). After agitation and centrifugation (10 000 *g* for 15 min), the supernatant was collected and evaporated to dryness under nitrogen flow. The residue was derivatized by adding 35 μl BSTFA + 1% TMCS, then incubated for 25 min at 70 °C.

2.4. GC–MS procedure

A 1-μl aliquot of the derivatized extract was injected into the column of a Hewlett-Packard (Palo Alto, CA, USA) 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 × 0.25 mm I.D. × 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 70 °C, maintained for 1 min, to 100 °C at 10 °C/min, then

to 295 °C at 30 °C/min and maintained at 295 °C for the final 1 min.

The detector was a Hewlett-Packard 5973 operated in the electron ionization mode. The electron multiplier was operated at 1900 V.

Data were recorded in full scan and ions monitored were: m/z 233, 204 and 147 and m/z 239 for GHB and GHB-d_6, respectively (the underlined ions are used for quantitation).

2.5. Method validation

A standard calibration curve ($n=3$) was obtained by preparing standards containing 1, 10, 50, 100, 150 and 200 mg/l of spiked GHB. It was not possible to find any GHB-free blood and urine, but these calibrations were achieved using fresh material and tested to be lower than 2 mg/l of the drug.

Within-batch and between-batch precisions for GHB were determined using fresh blood and urine, spiked at 10 and 100 mg/l with GHB.

The detection limit (LOD) was evaluated by diluting blood and urine with physiological serum until a response equivalent to three times the background noise was observed.

3. Results and discussion

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.

Figs. 1 and 2 are typical chromatograms (+electron impact) obtained from authentic blood and urine specimens. The blood and urine concentrations were 24 and 32 mg/l, respectively.

Selected ions and retention times of GHB and the deuterated internal standard are reported in Table 1. The ion of GHB (m/z 233) corresponds to the demethylated molecular ion; the confirmatory ion (m/z 147) was common to both GHB and the deuterated internal standard, but did not cause any interference.

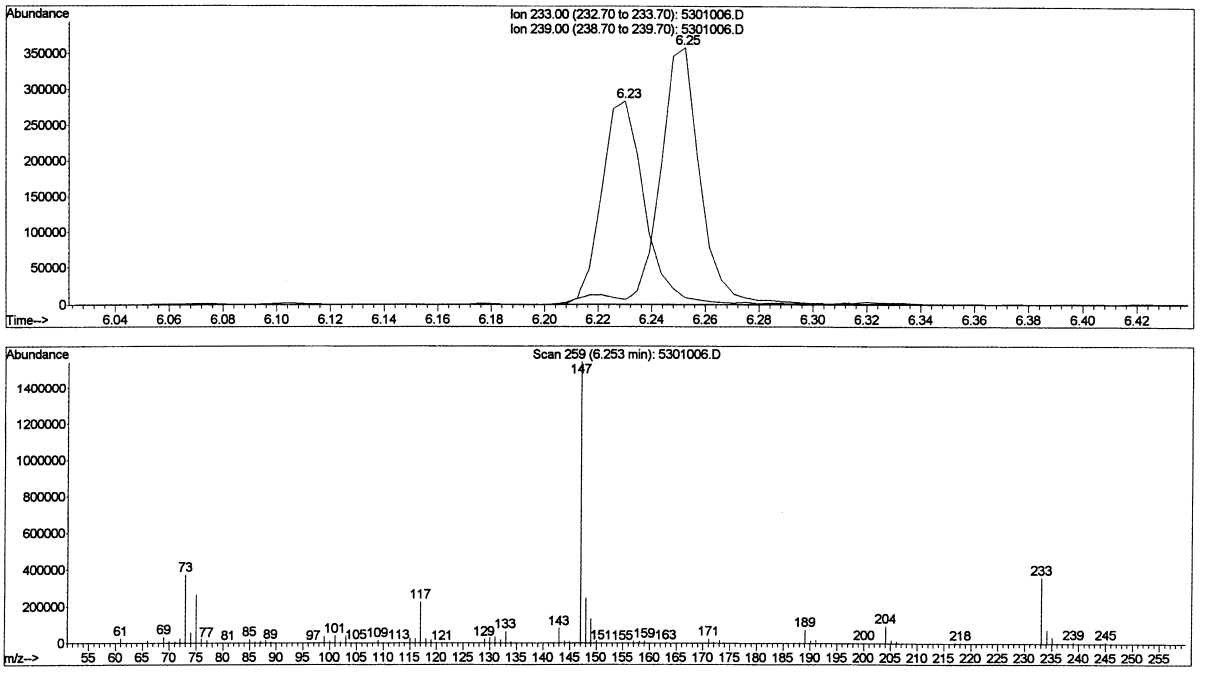

Fig. 1. Chromatogram obtained after extraction by the established procedure of a blood specimen. GHB was quantified at the concentration of 24 mg/l. Top, SIM chromatogram; bottom, electron impact of GHB.

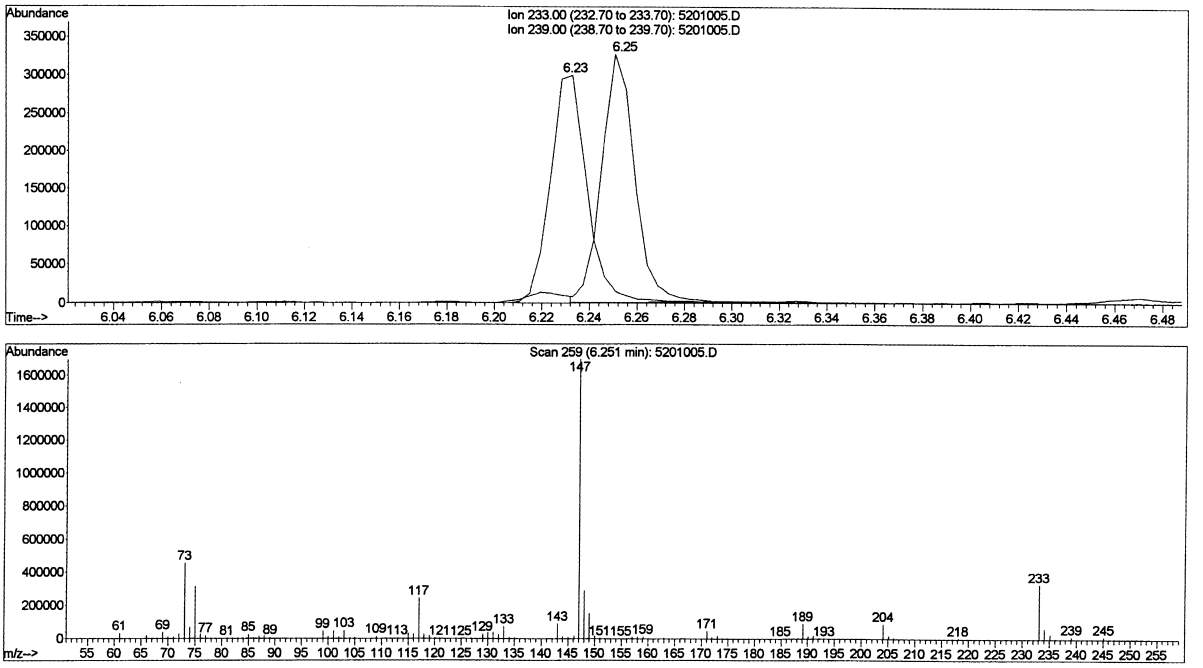

Fig. 2. Chromatogram obtained after extraction by the established procedure of a urine specimen. GHB was quantified at the concentration of 32 mg/l. Top, SIM chromatogram; bottom, electron impact of GHB.

The calibration curve corresponds to the linear regression between the peak-area ratio of GHB to I.S. and the final concentration of the drug in spiked blood and urine.

In blood, responses for GHB were linear in the range 1 to 200 mg/l. From three independent calibrations, the correlation coefficients ranged from 0.991 to 0.998.

In urine, responses for GHB were linear in the range 1 to 200 mg/l. From three independent calibrations, the correlation coefficients ranged from 0.990 to 0.997.

In blood, the within-batch precisions were 4.6 and 8.2%, as determined by analyzing eight replicates of specimens spiked with GHB concentrations at 10 and 100 mg/l, respectively. The between-batch precisions were 5.7 and 9.1%, as determined by analyzing eight replicates of specimens spiked with GHB concentrations at 10 and 100 mg/l, respectively.

In urine, the within-batch precisions were 6.5 and 10.5%, as determined by analyzing eight replicates of specimens spiked with GHB concentrations at 10 and 100 mg/l, respectively. The between-batch precisions were 7.7 and 11.3%, as determined by analyzing eight replicates of specimens spiked with GHB concentrations at 10 and 100 mg/l, respectively.

The limit of quantitation was the first point of the calibration curve, that is 1 mg/l, close to the endogenous levels.

Limits of detection were 0.1 and 0.2 mg/l for blood and urine, respectively.

Derivatives were stable at least for 24 h. Formation of the trimethylsilyl (TMS) derivatives appears to be appropriate to obtain optimal peak shapes and

Table 1
Selected ion (m/z) and retention times for GHB and the internal standard

Analyte	Retention time	Ions (m/z)
GHB	6.25 min	233, 204 and 147
GHB-d_6	6.23 min	239

Ions underlined are used for quantitation.

intense molecular ions. Potential conversion of GHB to GBL during the preparation process did not occur, as verified during the initial steps of development.

To avoid false positive results and therefore cautious interpretation of data, it is mandatory to collect blood with EDTA.

Since GHB is present in both blood and urine 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 [19]. At this Institute, the following positive cut-offs are used:

- blood from living people and collected with EDTA: 10 mg/l;
- post-mortem blood: 50 mg/l;
- urine (from living people and post-mortem): 10 mg/l.

4. Conclusion

This sensitive, specific and reproducible method developed is suitable for the detection and quantification of GHB in human blood and urine, as it was always possible to detect endogenous levels of the drug, whatever the specimen submitted for analysis. It appears easy and particularly rapid, as a valid result can be offered within 1 h.

This technology may find useful applications, but the definition of legally defensible cut-off values would require more data.

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