Measurement of exogenous gamma-hydroxybutyric acid (GHB) in urine using isotope ratio mass spectrometry (IRMS)

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Aim: The objective of the study was to check if gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) is a suitable method to differentiate between endogenous and exogenous GHB in urine. **Methods:** In the presented study, a controlled and monitored administration of 2.25 g GHB (Xyrem®) to one participant was carried out. Spot urine samples were collected once before administration and at different time intervals after the intake (2 h; 4 h; 6 h; 8 h; 12 h; 24 h; 48 h and 72 h). Concentrations of GHB were measured by LC/MS/MS and compared to carbon isotope ratios (CIR) analyzed by GC/C/IRMS. **Results:** The administered GHB was found to have a CIR of $\delta^{13}C_{VPDB}$ = -28.6 ± 0.1 ‰. For endogenous GHB prior to the administration a CIR of -23.7 ‰ was detected. After 6 hours the concentration of GHB was 4.2 µg/ml and therefore already below the accepted cut-off-value of 10 µg/ml. In comparison, the CIR was found to be -25.7 ‰, so an influence of the exogenous content could still be assumed after 6 hours. **Conclusion:** Due to its fast elimination, GHB intake in this study could not be demonstrated unambiguously for longer than 4 hours using LC/MS/MS as well as GC/C/IRMS.

1. Introduction

GC/C/IRMS is a very well established method in doping analysis for distinction of endogenous steroids from additional intake of synthetic products [1, 2, 3]. Aim of the study was to check if this method is able to differentiate between endogenous and exogenous GHB in urine samples. The carbon isotope ratio (CIR) is expressed as $\delta^{13}C$ and reported in parts per thousand (per mil ‰). All measurements are related to the international standard Vienna Pee Dee Belemnite (VPDB) based on the equation:

$$\delta^{13}C[‰] = \frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{VPDB}} - 1 \quad (1)$$

Gamma-hydroxybutyric acid (GHB) is a naturally occurring substance found in the central nervous system, which is structurally related to the neurotransmitter GABA (gamma-amino butryric acid) since it is a minor metabolite of GABA [4].

Due to its fast elimination a GHB intake can only be verified in a very short detection window [5]. On the one hand, exogenous and endogenous GHB can be differentiated on the basis of the found concentration by LC/MS/MS applying the cut-off level of 10 µg/ml in urine [6, 7, 8]. On the other hand, carbon isotope ratios (CIR) should enable as well the discrimination between endogenous compound and synthetic products of GHB by GC/C/IRMS measurements [9, 10]. Therefore, the objective was to compare both methods by analysis of the same post administration urine samples.

2. Material and Methods

Materials: Standard solution of GHB and corresponding deuterium-labelled internal standard of GHB-d6 were obtained from LGC Promochem. All chemicals and solvents were of analytical grade.

Clinical trial: In the presented study, a controlled and monitored administration of 2.25 g GHB (Xyrem®) to one participant was carried out. Spot urine samples were collected once before administration and at different time intervals after the intake (2 h; 4 h; 6 h; 8 h; 12 h; 24 h; 48 h and 72 h). The study was approved by the local ethics committee. Concentrations of GHB were measured by LC/MS/MS and compared to CIR analyzed by GC/C/IRMS. For comparison of both methods only urine samples up to 12 hours after the intake were considered.

GC/C/IRMS: Since GC/C/IRMS analysis determines CIR after combustion of the analyte to CO_2, an extensive purification before separation by GC is necessary in order to avoid coelution of other compounds and the target analyte. Up to 3 ml of each urine sample were concentrated to about 0.2 ml using a speed vac (60°C, <20 mbar). High performance liquid chromatography (HPLC) cleanup of the concentrate and collection of the fraction containing GHB were performed on an Agilent 1100 HPLC system coupled to a FOXY R1 fraction collector. The conversion of GHB into GBL by acidic hydrolysis was followed by a liquid-liquid extraction using dimethyl sulfoxide (DMSO) [9]. GC/C/IRMS measurements were carried out on a Delta V Plus IRMS (Thermo) connected to a Trace 1310 GC via a GC IsoLink (oven temperature at 950°C) and a ConFlow IV. Injections were performed by a TriPLus RSH autosampler with 2.5 µL at 280°C in splitless mode. Separation of analytes took place on a J&W Scientific DB-17MS column. Peak purity and identity was checked by the hyphenated ISQ single quadrupole mass spectrometer. As reference standard with each injection 5α-androstan-3β-ol was used.

LC/MS/MS: After the addition of deuterated internal standard to 100 µl urine, a simple and fast protein precipitation by addition of 200 µl acetonitril was performed. Due to the high polarity of GHB a Nucleodur HILIC column was used. Isocratic elution of 80 % pure acetonitril (solvent B) and 20 % water with 5 mM ammoniumacetate (solvent A) was utilized for separation within 15 minutes at a flow rate of 0.2 ml/min. The LC/MS/MS system consisted of a Shimadzu HPLC system coupled to an API 4000 triple-quadrupole mass spectrometer from AB Sciex. The measurement was performed in the negative ionisation mode. The following MRM transitions were employed for the compound of interest (*m/z*, 103 → 57 and 103 → 85) and internal standard (*m/z*, 109 → 61 and 109 → 90).

3. Results and Discussion

A requirement for the discrimination of endogenous and exogenous GHB by CIR measurements is a significant difference between endogenous GHB and the synthetic products. First of all the administered GHB (Xyrem®) was measured and found to have a CIR of $\delta^{13}C_{VPDB}$ = -28.6 ± 0.1 ‰. For the endogenous GHB of the participant prior to the administration a CIR of - 23.7 ‰ was detected.

All results of the LC/MS/MS and GC/C/IRMS measurements are listed in Table 1 and shown in Figure 1. A maximum GHB concentration of 254 µg/ml was found in the first urine sample collected after intake using LC/MS/MS measurement. At this concentration a CIR of -28.7 ‰ could demonstrate the presence of exogenous GHB as well using GC/C/IRMS.

Tab. 1. Results of the LC/MS/MS and GC/C/IRMS measurements.

Time [h]	GHB conc. [µg/ml]	CIR of GHB [‰]
0	1.6	- 23.66
2	254	-28.66
4	66.9	-28.42
6	4.2	-25.72
8	1.5	-24.11
12	1.5	-24.29

After 6 hours the concentration of GHB was 4.2 µg/ml and therefore already below the accepted cut-off-value of 10 µg/ml. In comparison, the CIR was found to be -25.7 ‰, so an influence of the exogenous content could still be assumed after 6 hours. However, GHB administration could not be verified for more than 4 hours.

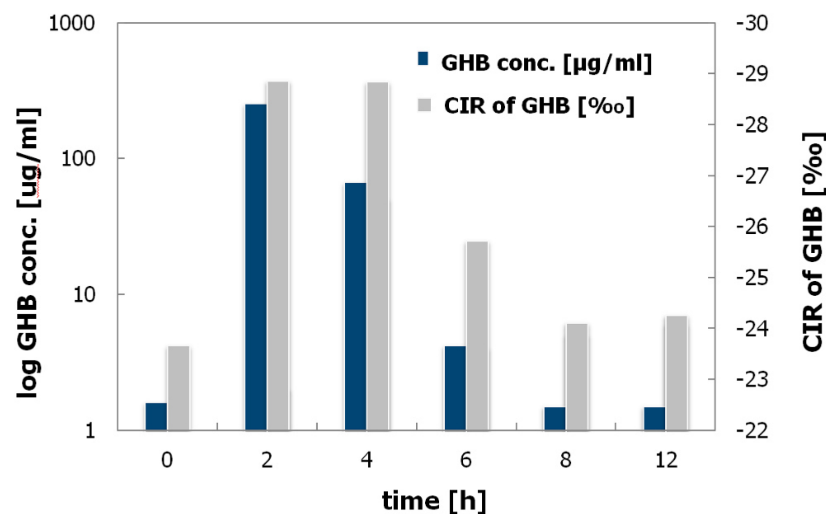

Fig. 1. Results of the LC/MS/MS and GC/C/IRMS measurements.

4. Conclusion

GC/C/IRMS was proven to be a suitable method for the distinction of exogenous and endogenous GHB. Nevertheless, due to its fast elimination, GHB intake in this study could not be demonstrated unambiguously for longer than 4 hours using LC/MS/MS as well as GC/C/IRMS. Since synthetic products of GHB exhibit a wide range of CIR values [10], the potential of the presented method should be investigated more extensively in further studies.

5. References

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