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Urinary excretion and effects on visual placing response in mice of gamma-valero-lactone, an alternative to gamma-hydroxy-butyrate for drug-facilitated sexual assault

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ABSTRACT

Γ-Valero-lactone (GVL) is a freely marketed organic solvent and food additive. GVL is also an underrated legal substitute for γ-hydroxybutyric acid (GHB), and it is sold under the trade name "Tranquili-G". GVL is usually not included in forensic drug testing, even though it has been reported in drug-facilitated sexual assaults (DFSA).

To date, few studies verified the effectiveness of GVL as a hypnotic/narcotic substance, and no data are available on its urinary excretion profile. This work aims to fill this knowledge gap and lead to the introduction of GVL in forensic drug testing, especially when DFSA is suspected.

To monitor the timeframe of GVL activity *in vivo*, we have assessed the effects on sensorimotor responses in visual placing tests carried out on CD-1 mice at a dose of 400 mg/kg of GVL, administered by gastric gavage. In parallel, samples of the *in vitro* and *in vivo* metabolism studies were analyzed using a rapid and cost-effective analytical procedure based on gas chromatography coupled to mass spectrometry. Our data confirmed that GVL impairs visual placing response in mice in the first 4 h after the intake, and they also show that GVL is a less potent substitute of GHB. The urinary excretion profile of GVL is consistent with the results of the behavioral study, with a maximum of excretion in the first 5 h after the intake. GVL is only detectable in the first 0–8 h after the intake. Our data confirmed the same rapid urinary excretion rate of GHB in urine and the related forensic implications, with the risk of possible false-negative results, especially when DFSA is suspected.

1. Introduction

Gamma-valero-lactone (GVL) is the 4-methyl analog of gamma-butyrolactone (GBL), a synthetic compound that acts as a pro-drug in humans, being rapidly metabolized to gamma hydroxy butyrate (GHB). The latter is an endogenous compound biosynthesized from γ aminobutyric acid (GABA) in the GABAergic neurons (Abanades et al., 2006; Busardo and Jones, 2014). Apart from its physiological roles and activities, GHB has also been abused both as a recreational and/or a date rape-drug, depending on the doses of intake (Degenhardt et al., 2002; Bosch et al., 2015). Specifically, GHB abuse in cases of drug-facilitated sexual assaults (DFSA) is known, and several cases have been reported (Kintz, 2007; Stillwell, 2002; Dinis-Oliveira and Magalhães, 2013; Hall and Moore, 2008). In addition to the above effects, GHB is also suspected to stimulate growth hormone release, and therefore assumed also by bodybuilders (O'Cornell et al., 2000). Such a peculiar combination of biopharmacological effects led to the illicit use of GHB, both as a recreational and/or a date rape- and/or a performance enhancing drug, depending on the doses of intake. Furthermore, GHB detection is complex: its presence in urine can be detected up to about six hours after the intake (Haller et al., 2006; Brailsford et al., 2012; Abanades et al., 2007), which entails significant forensic implications

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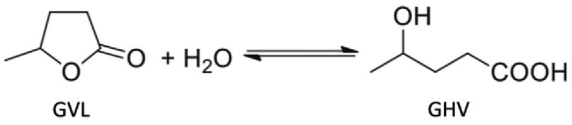

Fig. 1. GVL and the free acid form, GHV, pH-dependent aqueous equilibrium (Carter, 2004; Andresen-Streichert et al., 2013).

for its recreational use, particularly in DFSA, where rape is often reported with a significant delay.

GHB has become a controlled substance in different countries, including Italy, where it is listed in Table IV of Italian law, specifically in the Presidential Decree No 309 of 9 October 1990 (DPR 309/90: "Consolidation of the laws governing drugs and psychotropic substances, the prevention, treatment and rehabilitation of drug addicts" (Tabella, 2002)). Therefore, consumers try to find possible legal analogues, such as γ-butyrolactone (GBL), 1,4 butanediol (BD) (Lee et al., 2014; Brunt et al., 2014; Andresen et al., 2011), and GVL: freely marketed as a green solvent, frequently used by the food industry as a food additive (Horváth et al., 2008), and available on the web market with the name "Tranquili G" (Marinetti et al., 2012), GVL can also be seen as a potential substitute of GHB as a "recreational drug".

The chemical structure of GVL is characterized by a lactone ring containing a methyl group in the fifth position of a dihydrofuran-2(3H)-one ring. The structural similarity between GBL and GVL leads also to similarities in their physico-chemical and biopharmacological properties. The lactone form of GVL exhibits a pH-dependent equilibrium with the open ring and active form, γ-hydroxy-valeric-acid (GHV) (Zink et al., 2005; Wong et al., 2017) (see Fig. 1), known as 4-methyl-GHB (Marinetti et al., 2005). The same pH-dependent equilibrium is reported for the lactone (GBL) and the free acid form of GHB (Ciolino et al., 2001). In the human body, the lactone forms of GVL and GBL are converted to their active open form, GHV and GHB, by human lactonase. The rate of this conversion is faster for GVL than for GBL (Carter et al., 2005).

The effects of GVL have been investigated in previous works (Marinetti et al., 2012; Carter et al., 2005; Carter, 2004). Based on published studies, GVL produces, in intoxicated subjects, GHB-like effects, such as sedation, catalepsy, and muscular weakness (Andresen-Streichert et al., 2013). These effects, together with a set of very peculiar features, such as being colorless, tasteless, rapidly soluble and rapidly absorbed and eliminated from the body of victims, led to its illegal use as a sexual "date-rape drug" (LeBeau et al., 2015). Our research group has recently compared the sensory alteration, sedative effect, and motor impairment induced by GVL with those induced by GHB in mice (Arfè et al., 2019).

To date, only three cases of GVL intake in humans have been reported, one of which suspected as a DFSA" (Andresen-Streichert et al., 2013). The few cases reported are probably imputable to the fact that routine drug testing in hospitals generally does not cover GVL . Indeed, the uptake of GABA analogs poses significant challenges for forensic laboratories involved in their detection, due to pH equilibrium, high volatility, and polarity of the compound of interest, which requires several sample pre-treatment considerations.

In addition to its possible abuse for DFSA, GVL may also be of interest in sport doping. Apart from its putative effects on growth hormone (hGH) release, GVL can also mimic the action of common narcotics, so that it could be seen as a potential performance enhancing drug: both as an alternative to either hGH and insulin growth factor 1 (IGF-1), as well as a narcotic, not yet included in the list of prohibited substances and methods of the World Anti-Doping Agency (WADA.

This work aims to assess the pharmacokinetic profile of GVL, considering specifically its metabolic pathways and its urinary excretion profile, in the aim of setting up a rapid and cost-effective method useful in forensic toxicology. More specifically, we estimated the *in vivo* effects of GVL by a visual placing test, that is a practical and sensitive behavioral test, able to disclose the potential disperceptive effect of sedative/hypnotic drugs that alter sensorimotor reaction, like cannabinoids, opioids (Bilel et al., 2020), and GHB (Arfè et al., 2021). The above effects should proceed consistently to impairment of consciousness and retrograde amnesia, that are the main effects produced by GVL as a sexual abuse drug. In parallel, we carried out both *in vitro* metabolism studies employing human liver microsomes (to define the biotransformation pathways of GVL), and *in vivo* controlled administration studies in mice (400 mg/kg), to monitor the urinary excretion of GVL and its metabolites.

2. Materials and method

2.1. Chemical and reagents

GVL, γ-caprolactone (used as the internal standard for excretion study: ISTD), and GHB (sodium salt) were purchased from LGC Standards S.r.l (Milan, Italy). For behavioral and metabolism studies GVL and GHB were dissolved in saline (0.9% NaCl) solution that was also used as vehicle and were administered by gastric gavage by means of a special probe (Plastic Feeding Tubes, 18 ga x 30 mm; Instech Laboratories, Inc. Plymouth Meeting, PA, United States).

The reagents and solvents were all analytical grade and purchased from Sigma-Aldrich (Milan, Italy), *i.e.*, formic acid, sodium phosphate, sodium hydrogen phosphate, potassium carbonate, potassium hydrogen carbonate, acetonitrile, methanol, chloroform, ethyl acetate, and tert-butyl methyl ether. The human liver microsomes (HLM, from 20 Caucasian male and female donors of different ages), were purchased by Corning Incorporated (Woburn, Massachusetts, United States), and all the reagents used for the *in vitro* metabolism experiments (*i.e.*, sodium phosphate buffer, NADPH regenerating system (sol A and sol B) containing, $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) were supplied by Corning Incorporated (Milano, Italy). The enzyme mixture β-glucuronidase (from *Helix Pomatia*) used for the enzymatic hydrolysis of the glucorono-conjugates was purchased from Roche (Monza, Italy).

2.2. Animals and dose selection

15 male ICR (CD-1®) mice, 25–30 g (Centralized Preclinical Research Laboratory, University of Ferrara, Italy) were group-housed (5 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm), exposed to a 12:12 light-dark cycle (light period from 6:30 AM to 6:30 PM) at a temperature of 20–22 °C and humidity of 45–55% and were provided *ad libitum* access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. As already described in our previous publications (Ossato et al., 2015; De-Giorgio et al., 2020), doses were chosen based on behavioral and neurological effects reported in human volunteers, using interspecies dose scaling and basing on previous preliminary investigations (Haller et al., 2006; Arfè et al., 2021). Mice were divided in three groups, each one of 5 mice: the first group received 400 mg/kg of GVL; the second group the same dose of GHB, and the third group (*i.e.* the control group) the administration vehicle only (0.9% saline).

2.3. Behavioral tests

The disabling effect on sensorial responses of GVL and of GHB, at a dose of 400 mg/kg (oral gavage), was verified by the visual placing test, which evaluated the ability of the mouse to integrate the visual, tactile (from vibrissae stimulation) and vestibular information necessary to prepare the correct landing of the mouse on the ground (Lambert et al., 2016). The Visual Placing response test is performed using a tail suspension modified apparatus able to bring down the mouse towards the floor at a constant speed of 10 cm/sec (Ossato et al., 2015).

Briefly, CD-1 mice were suspended 20 cm above the floor by an adhesive tape that was placed approximately 1 cm from the tip of the tail. The downward movement of the mouse is videotaped by a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile s.r.l, Gemonio, Varese, Italy) placed at the base of the tail suspension apparatus. Movies were analyzed off-line by a trained operator who does not know the drug treatments performed. The frame-by-frame analysis allows to evaluate the beginning of the reaction of the mouse while it is getting closer to the floor. The first movement of the mouse when it perceives the floor is the extension of the front legs. When the mouse starts the reaction, an electronic ruler evaluates the perpendicular distance in millimeters between the eyes of the mice and the floor. The untreated control mice perceive the floor and prepare to contact at a distance of about 27 ± 3.2 mm. Visual Placing response was measured at 0, 30, 60, 120, 180, 240, 300-, 360-, 420- and 480-min post GHB and GVL administration. Behavioral tests are conducted into a thermostatic (temperature 20–22 °C, humidity about 45–55%) and light-controlled (about 150 lux) room in which there is a background noise of about 40 ± 4 dB. Before the experimental sessions, each mouse is placed in the box, and it is handled and trained every other day (once a day) for a week (three days of training in total) in order to get used to the environment and to the experimenter. All experiments were performed between 8:30 AM to 2:00 PM. Experiments were conducted in blind by trained observers working together in pairs (Ossato et al., 2015).

2.3.1. Data and statistical analysis

In visual placing sensorimotor response data are expressed as the percentage of baseline. All the numerical data are given as mean ± standard error of the mean (SEM) of 4 independent experimental replications. The statistical analysis of the effects of the individual substances over time was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. The statistical analysis was performed with the program Prism software (GraphPad Prism, USA). The results are reported as estimate F with tabulated F, at specific *p*-value.

2.4. In vivo *experiments*

The urinary excretion profile was studied employing the murine model for both the phase I and phase II (glucorono-conjugation) metabolism, through protocols already described in previous publications (Camuto et al., 2020; Chieffi et al., 2020; Tirri et al., 2021). Two groups of 5 mice, in metabolic cages, were selected, a single dose of 400 mg/kg of GVL was administered by oral gavage to the first group. The second group of 5 mice was selected as control group for urine blank samples. Urine samples were collected from 9:00 AM for each mouse, every hour in the first 8 h and every 3 h in the range 24–36 h, for a total of 12 urine samples for each mouse. Urine blank samples from the mice control group were also collected in the same time intervals.

2.5. In vitro *experiments*

For the *in vitro* metabolism studies, GVL was first incubated with human liver microsomes (HLM) in order to estimate only the phase-I biotransformation reactions involved. *In vitro* metabolism studies were performed by HLM from a pool of 20 mixed Caucasian male and female donors of different ages in order to minimize the effect of intra-individual variation. The urinary excretion profile was subsequently studied employing the murine model for both the phase I and phase II (glucorono-conjugation) metabolism, with the aim of estimating the excretion window of this substance and defining its diagnostic marker(s) of intake.

All incubation conditions for GVL, *i.e.*, proteins and substrate concentrations, buffer and solvent types, incubation time (evaluated in the range 0–48 h), were optimized, starting from protocols already published and used by our group (Camuto et al., 2020; Chieffi et al., 2020; Tirri et al., 2021; Mazzarino et al., 2013). The final incubation medium also contained GVL 8 mM, 200 μL of phosphate buffer 0.8 M, magnesium chloride 3.3 mM, $NADP^+$1.3 mM, glucose-6-phosphate 3.3 mM and glucose-6-phosphate dehydrogenase 0.4 U/mL, in a total volume of 250 μL. Samples were pre-warmed at 37 °C for 5 min, and the phase I reactions were started with the addition of HLM. After incubation at 37 °C for the selected experimental time, 250 μL of ice-cold acetonitrile were added, to terminate the phase I reactions. The samples were then transferred into an ice bath for the further precipitation of the proteins in the assay medium. The precipitate was subsequently separated from the supernatant by centrifugation at 21,000 g (15,000 rpm) at room temperature for 10 min. Each set of assays also included a negative control sample containing all reaction mixture components except the enzymatic proteins to monitor the potential non-enzymatic reactions, leading to the possible formation of degradation products. Each incubation experiment was performed in triplicate. The mean concentration values recorded between 15 min and 48 h of incubation of GLV with HLM were compared to those of the sample at 0 min. The comparison was performed by means of a one-sample-test a statistical test used to determine whether an unknown population mean is different from a specific value based on the calculated t value compared with the tabulated P value. Statistic tests were performed with Prism 8 – GraphPad software as well as all the graphics.

2.6. GC–MS analysis

2.6.1. Sample pre-treatment

GVL was analyzed as the lactone form after liquid-liquid extraction at pH 7.4 (phosphate buffer 0.8 M) followed by analysis by GC–MS. Sample extraction was optimized by testing different solvents (*e.g.*, chloroform, ethyl acetate, *tert*-butyl methyl ether) and solvent volumes (1, 2, 5, 7 mL). The sample pre-treatment protocol was developed using 50 µL of blank urine from control mice, spiked at different concentrations of GVL (100–5000 ng/mL) to a final volume of 2 mL. The sample was added with 50 µL ISTD (γ-caprolactone, final concentration 100 ng/mL) followed by 100 µL of phosphate buffer 0.8 M (pH 7.4) and 5 mL of *tert*-butyl methyl ether. After 20 min of mild shaking at 30 °C, samples were centrifugated at 3000 rpm for 2 min and transferred into an ice bath for 5 min. The organic layer was then evaporated to dryness under a nitrogen flow at 30 °C and solved with 50 µL of *tert*-butyl methyl ether for the analysis by GC–MS.

For the controlled administration studies, a volume between 10 and 200 µL of urine, depending on the expected concentration of GVL as estimated following a first explorative analysis, was added with water to a total volume of 2 mL and then treated with the protocol described above. The extraction procedure was repeated three times to guarantee the complete extraction of all the potential phase I metabolites of GVL before the enzymatic hydrolysis. The three organic layers were collected and evaporated to dryness under a nitrogen flow at 30 °C. The final residue was dissolved in 50 µL of *tert*-butyl methyl ether and then analyzed by GC–MS. The aqueous layer was stored up for the phase II metabolism studies and subsequently added with 100 µL of phosphate buffer 0,8 M (pH 7,4), 50 µL of the standard solution of γ-caprolactone (final concentration 100 ng/mL) and 50 µL of β-glucuronidase for the hydrolysis of glucorono-conjugates metabolites, after 1.5 h of incubation at 55 °C. This led to the conversion of phase II metabolites into phase I form allowing their analysis in GC–MS. Samples were then extracted following the same procedure described for the phase I metabolism.

2.6.2. Instrumental conditions

Samples were analyzed using an Agilent 6890/5973A gas chromatography-mass spectrometry (GC–MS) system (Agilent Technologies, Milan, Italy), operating in electron ionization (70 eV) mode, using a fused-silica capillary column (cross-linked (5%-Phenyl)-methylpolysiloxane, length 17 m, i.d. 0.20 mm, film thickness 0.33 µm) (HP-5, Agilent Technologies, Milan, Italy).

Table 1
Molecular mass, characteristic ions, and retention time of GVL and internal standard. Diagnostic ions for the determination of the LOD are reported in boldface.

Compound	Mass	Diagnostic ions (*m/z*)	Retention time (min)
GVL	100	**56, 85**, 86, 99, **100**	4.04
ISTD	114	56, 70, 85, 86, 114	5.52

The gas chromatographic conditions were as follow: the carrier gas was helium at a constant pressure rate of 15 psi; injection volume was 2.0 μL with inlet operating in pulsed splitless mode (purge flow split 10 mL/min at 0.5 min injection pulse pressure 25 psi until 0,30 min); temperature program was, 50 °C (0.5 min-hold), 10 °C/min to 100 °C, 40 °C/min to 310 °C, hold for 3 min; the transfer line and injection temperature operated at 280 °C. The acquisition was performed in selected ion monitoring (SIM) mode.

2.7. Method validation

The method was qualitatively validated for GVL defining selectivity, limit of detection (LOD), recovery, carryover and repeatability of relative retention time (RRT) and of relative abundances of characteristic ion fragments (RA). The developed method was validated according to the ISO 17025 and WADA-guidelines for screening methods. The normality of the distribution was previously checked with the Shapiro-Wilk test so that outliers could be checked with the Dixon test in accordance with ISO 17025.

To this end, the parameters required for the validation of a qualitative screening procedure for a non-threshold substance were assessed. Selectivity was evaluated with the analysis of mice urine blank samples. In view of the potential application of the method also on human urine, selectivity was also evaluated by analyzing the blank human urine samples from twenty volunteers. For the validation of the other parameters, blank human urine samples were thus employed.

The presence of possible interfering compounds was verified by the analysis of blank urine samples, to exclude the presence of compounds sharing the same diagnostic fragment ions of GVL and eluted at the same retention time. The limit of detection (LOD) was evaluated by progressive dilution of spiked urines from a concentration of 500 ng/mL down to a concentration still giving a signal to noise (S/N) value ≥ 3 for all the three diagnostic ion fragments selected and reported in Table 1. Carryover was assessed by the analysis of urine samples spiked at a concentration of 20 times the LOD. Recovery was evaluated on eight different urine samples spiked with GVL at a concentration at the LOD and 3 times the LOD. Repeatability was evaluated on 20 blank urine samples spiked with GVL at the LOD, calculating RRT and RA. The repeatability of RRT was estimated as the ratio of the retention time of GVL and ISTD expressed as CV%. The RA was calculated by dividing the ion trace area of the lowest diagnostic ion (*m/z* 100) by the area obtained from the ion trace of the most abundant diagnostic ion (*m/z* 56). The repeatability of RA was also expressed as CV%.

3. Results and discussion

3.1. Evaluation of the visual placing response

The visual placing response did not change in vehicle-treated mice over the 8 h of observation (Fig. 2). Oral administration of GHB and GVL (400 mg/kg) reduced the visual sensorimotor response in the visual placing test in mice (Fig. 2; significant effect of treatment ($F_{(2231)} = 166.7$, $p < 0.0001$), time ($F_{(10,231)} = 35.87$, $p < 0.0001$) and time x treatment interaction ($F_{(20,231)} = 14.97$, $p < 0.0001$). The effect caused by GHB administration was effective at 10 min (inhibition ~24% respect to saline treatment) and maximally after 60 min (inhibition ~82.5% respect to saline treatment) from drug administration. The maximum inhibitory effect was maintained for up to 3 h and then tends to progressively decrease, to completely disappear 4 h after GHB administration. GVL is less effective than GHB in reducing the visual sensorimotor response in the visual placing test in mice (Fig. 2). In fact, the inhibitory effect caused by GVL is significant at 60 min, and its maximum effect (inhibition ~30% with respect to saline treatment) is significantly lower than those induced by GHB (Fig. 2). The inhibitory effects caused by GVL also disappeared 4 h after oral administration of the compound.

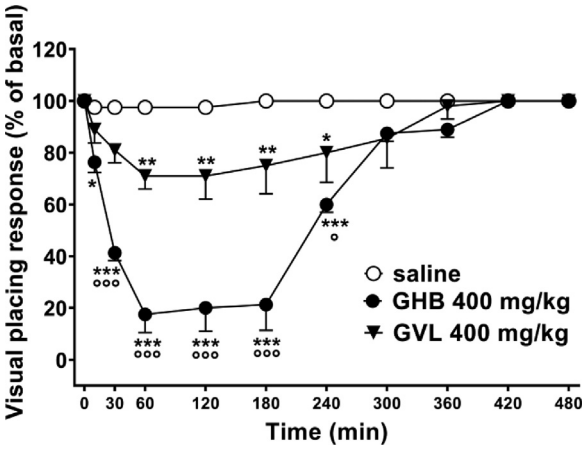

Fig. 2. Effect of the oral administration of GHB and GVL (400 mg/kg) on the visual placing test in mice. Data are expressed (see "Materials and methods") as the percentage of baseline and represent the mean ± SEM of 5 animals for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* saline. $^{\circ}p < 0.05$, $^{\circ\circ\circ}p < 0.001$ *versus* GVL.

3.2. Metabolism study

The samples of both *in vitro* and *in vivo* metabolism studies were analyzed by GC–MS technique operating in SIM mode. The characteristic ions were selected based on the fragmentation pattern observed after full scan analysis of a standard solution of GVL (Fig. 3) and full scan analysis of blank urine samples and spiked urine samples. An extracted chromatogram of a positive sample is reported in Fig. 4. The specific diagnostic ions were selected from those that did not show significant interference in the matrix. The specific retention time and diagnostic ions transition for GVL and ISTD y-caprolactone were reported in Table 1.

3.2.1. Validation results

The following validation parameters were estimated as described in the experimental section: selectivity, limit of detection, recovery, carry over, RTT and RA. The results obtained satisfy the ISO17025 and WADA criteria for a qualitative method (World Anti-Doping Agency (WADA), 2019, 2021). The selectivity was evaluated with blank urine samples analyzed with the protocol described in the experimental part. No interfering compounds were identified at the same retention time and with the same diagnostic fragments of the GVL. The developed method described showed no carryover at the concentrations tested with spiked urine samples. Data obtained from the ten recovery samples analyzed shown a normal distribution of data checked with Shapiro-Wilk test, and no outliers were found with Dixon test (data not reported). GVL shows a recovery near 60% at a concentration at 3-times the LOD and near 50% at LOD. The RRT show good repeatability (CV% lower than 0,5%), which is compliant with the WADA criteria for a non-isotopic internal standard (World Anti-Doping Agency (WADA), 2019). The repeatability of RA shows CV% lower than 15%. The results of LOD recovery, RRT, and RA with their CV% are reported in Table 2.

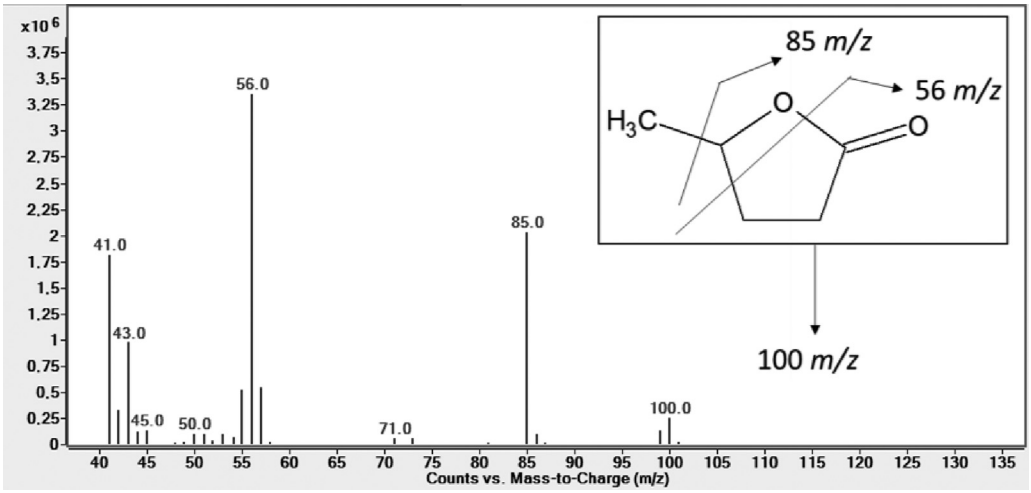

Fig. 3. Extracted mass spectrum of GVL obtained after the injection of a standard solution of GVL in GC–MS with the representation of characteristic fragmentation selected to identify GVL (*i.e., m/z* 56,85,100).

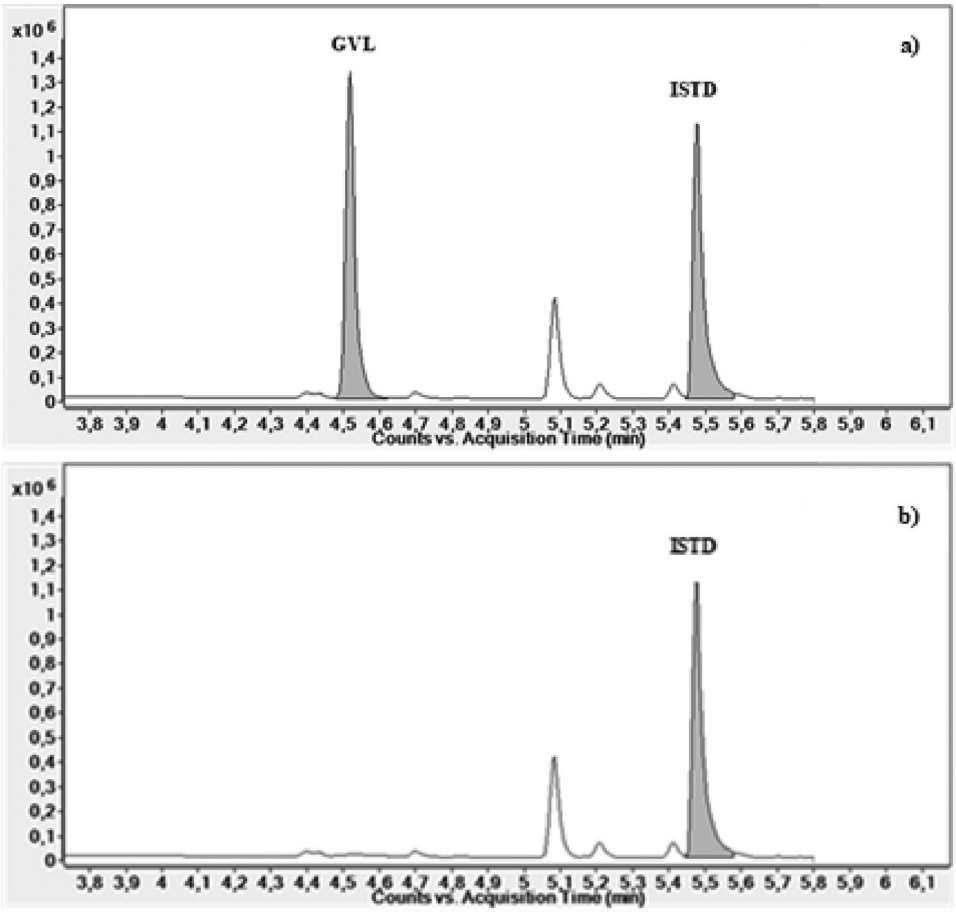

Fig. 4. Extracted chromatogram of a positive sample analyzed in SIM mode (a) compared to a blank sample (b).

3.2.2. In vivo *experiments*

For the i*n vivo* excretion studies, an appropriate volume of sample (10 to 200 µL) was selected depending on the time of collection, given the higher concentration of GVL in the first 0–2 h, which progressively decreases over the entire range. More in details, in the first 2 h after the administration of GVL in mice, an amount of 10 μL of the sample was found to be the optimal volume giving an adequate GC–MS signal in terms of peak intensity and shape. Increasing volumes were used over the range of 2–8 h (20 μL up to 200 μL) for the same purpose. The same volume was assayed for each mouse. The results are reported in Fig. 5 for both phase I and phase II GVL excretion and are expressed as the ratio of GVL to ISTD signals over the range studied and are corrected for the volume of samples used for analysis. Rapid excretion of GVL confirms GHB-like behavior. Indeed, these data are comparable with GHB excretion that shows a detectability around 3–6 h after the intake with a subsequent decrease to concentrations below the endogenous level (*i.e.*, GHB < 10 mg/L (Haller et al., 2006; Brailsford et al., 2012; Abanades et al., 2007)).

Table 2
Estimated parameters for a qualitative method in compliance with the WADA criteria for a non-isotopic internal standard. Limit of detection (LOD), percent recovery (Rec%) estimated at two different concentrations (LOD and 3xLOD), relative retention time (RRT), relative abundances of characteristic fragments (RA) with calculated percent coefficient of variation (CV%).

Rec.% (LOD)	Rec.% (3xLOD)	CV% (LOD-3xLOD)	LOD (ng/mL)	S/N
56	64	10.2–9.8	**6.5**	3.5
RRT	**CV%**	**RA**	**CV%**	
0.73	0.14	**4.6**	3.9	

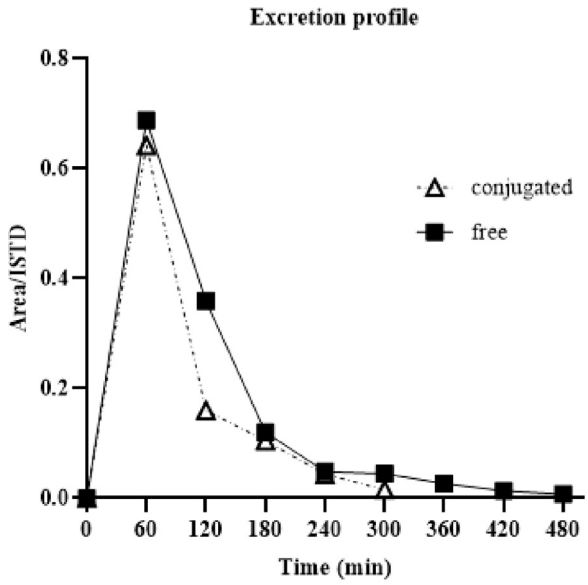

Fig. 5. Excretion profile of GVL both of phase I (free) and phase II (conjugated) calculated as the ratio of GVL and internal standard peak areas.

No phase I metabolites of GVL were detected along the entire collection time interval, as expected given the similarity between GVL and GHB. These data are in agreement with the results of the *in vitro* experiments (see next section), where the incubation of GVL for 48 h showed no statistically significant decrease in the levels of GVL (see Table 2). These data demonstrate that, in our experimental conditions, there is no evidence of CYP450-mediated oxidative metabolism of GVL.

Concerning the phase II excretion, GVL conjugation was observed in the first hour after the administration, when the levels of glucuronate-GVL were roughly the same as the unconjugated GVL; while after 2 h from the administration, the excretion of unconjugated GVL prevails. From the 3rd to the 5th hour, free and conjugated GVL show again similar excretion intensities (see Fig. 5). Conjugated GVL became undetectable after the 5th hour (Figs. 5 and 6).

The overall excretion of GVL, analyzed as the lactone form after conversion of the phase II conjugate products by enzymatic hydrolysis, is reported in Fig. 6 and shows a highest excretion within the first 3–4 h of GVL administration, with a consistent decrease in the following hours. These data are consistent with the affection of the visual placing test that is observable in the first 4 h after the intake of GVL. Furthermore, the maximum effect on visual placing response coincides with the maximum excretion at 1 h after administration. The significantly lower excretion after 4 h by the administration suggests that the amount of circulating GVL is probably too low to affect the visual placing response. The phase II metabolism is comparable with GHB, that is converted to the glucorono-conjugated form both for endogenous and exogenous GHB (Petersen et al., 2013; Wang et al., 2016; Mehling et al., 2017).

The rapid excretion of GVL poses analytical problems related to its identification in biological samples. Furthermore, as is known for

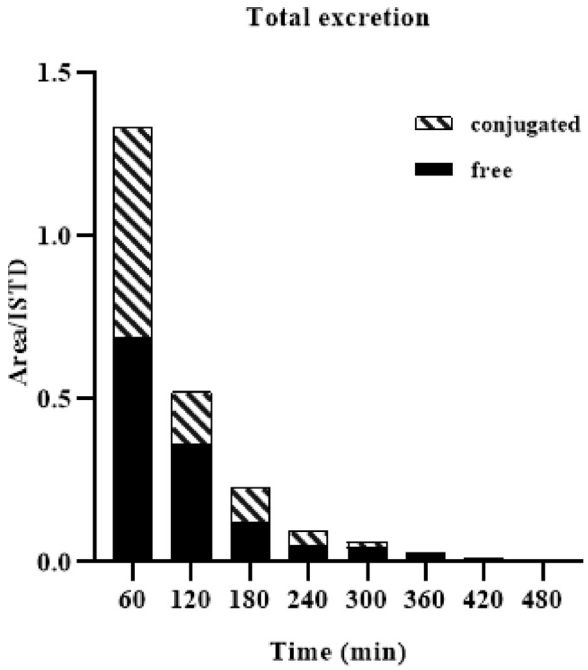

Fig. 6. Total excretion profile, as the sum of free and conjugated GVL, calculated by the ratio of GVL and ISTD.

Table 3
Relative area, time interval, average, standard variation (Sd), and CV% for incubation of GVL with HLM. The results of the one-sample *t*-test were reported as *P*-value (P), t calculated (t), and degrees of free (df). The calculated *t*-value for 9 degrees of freedom was compared with the tabulated *P*-value for 10 samples at a 95% confidence level ($\alpha = 0.05$). Since $t > P$, there are no significant differences between the two averages 0′ *vs* 15′–48 h interval.

Time	R. Area		
0′	0.0514		
15′	0.0580	**Average**	**0.0537**
30′	0.0690	**Sd**	**0.0008**
60′	0.0494	**CV%**	**1.5147**
90′	0.0621	**one-sample *t*-test**	
180′	0.0590	***P* = 0.411**	
240′	0.0411	***t* = 0.8619**	
360′	0.0407	**Df = 9 $\alpha = 0.05$**	
12h	0.0617	**0′ *vs* incubation not significantly different**	
24h	0.0515		
48h	0.0473		

GHB, phase II metabolism does not extend the excretion window (Mehling et al., 2017).

3.2.3. In vitro *experiments*

The most repeatable results were obtained using methanol as substrate solvent (the total amount of methanol in the final assay was 1%), a substrate concentration of 8 μM, a protein concentration of 0.5 mg/mL, in phosphate buffer 0.1 M at pH 7.4.

After 48 h of incubation with HLM, no phase I metabolites were detected. Subsequently, the decrease of GVL over different incubation times was evaluated in order to understand whether GVL was not metabolized. With this aim different incubation times were selected starting after 0 min to 48 h, a one-sample *t*-test was performed to estimate variation by incubated samples and 0 min control sample. P value ($P = 0.411$) shows no significant difference between these two groups. These results indicate that GVL was not metabolized by CYP450 (see Table 3). However, other metabolic pathways for the *in vivo* metabolism are possible.

4. Conclusion

To the authors' best knowledge, this is the first work that evaluates the effects of GVL on visual placing response, at the same time following its urinary excretion profile, after the administration of a dose of 400 mg/kg to ICR (CD-1®) male mice. Our results show that GVL, similarly to GHB, impairs visual placing response, suggesting its detrimental effect on sensorimotor responses. This impairment could be related to the sedative/hypnotic effects of these classes of compounds as reported in intoxicated subjects. Therefore, GVL could be a potential substitute for GHB, and its detection in samples collected in the framework of cases of DFSA, driving under the influence of drugs (DUID), or anti-doping tests (mostly for its potential effect in stimulating growth hormone release), could be of high utility in forensic toxicology.

The analysis of GVL in biofluids seems to present the same analytical challenges encountered for GHB, due to a short half-life and a consequent very rapid excretion in urine, without the formation of long-term metabolites. The newly developed analytical method was qualitatively validated in terms of selectivity, limit of detection, carry over, matrix effect, recovery, and repeatability. The sample pre-treatment is rapid and effective, and the choice to analyze GVL in the lactone form allows to simplify the pretreatment procedure, without the need for solid-phase excretion nor derivatization. The pre-treatment protocol ensures the total conversion of glucuronide-GVL into GVL that, based on our results, can be selected as a suitable diagnostic marker of intake. The limitation of our study is due to the fact that, for ethical reasons, the experimental data refer to studies *in vitro* and on animal models, actual pharmacokinetic profile in human requiring additional studies to be described in all details.

Compliance with ethical standards

Experimental protocols performed in the present study were in accordance with the new European Communities Council Directive of September 2010 (2010/63/EU), a revision of the Directive 86/609/EEC, and were approved by the Italian Ministry of Health (license n. 335/2016-PR) and by the Ethics Committee of the University of Ferrara. Moreover, adequate measures were taken to minimize the number of animals used and their pain and discomfort.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Cristian Camuto: Conceptualization, Methodology, Validation, Data curation, Formal analysis, Writing original draft. **Raffaella Arfè:** Conceptualization, Data curation, Visualization. **Micaela Tirri:** Conceptualization, Data curation, Visualization. **Xavier de la Torre:** Supervision, Data curation, Formal analysis. **Monica Mazzarino:** Supervision, Formal analysis, Writing review & editing. **Matteo Marti:** Supervision, Funding acquisition. **Fabio De-Giorgio:** Funding acquisition, Writing review & editing. **Francesco Botrè:** Project administration, Supervision, Methodology, Writing review & editing

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.etdah.2021.100028.

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