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Article

Direct monitoring of exogenous GHB in body fluids by NMR spectroscopy

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Direct monitoring of exogenous GHB in body fluids by NMR spectroscopy

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ABSTRACT

γ-Hydroxybutyric acid (GHB) is a popular drug increasingly associated to cases of drug-facilitated sexual assault (DFSA). Currently, expanding procedures of analysis and having forensic evidence of GHB intake at a long term are mandatory. Up to now, most studies have been performed using GC-MS and LC-MS as analytical platforms, which involve significant manipulation of the sample and, often, indirect measurements. In this work, procedures used in NMR-based metabolomics were applied to a GHB clinical trial on urine and serum. Detection, identification and quantification of the drug by NMR methods were surveyed, as well as the use of NMR-based metabolomics for the search of potential surrogate biomarkers of GHB consumption. Results demonstrated the suitability of NMR spectroscopy, as a robust nondestructive technique, to monitor (detect, identify and quantify) fast and directly exogenous GHB in almost intact body fluids, and its high potential in the search for metabolites associated to GHB intake.

Keywords: GHB, γ-hydroxybutyric acid, body fluids, NMR spectroscopy, metabolomics, serum, urine, drug control.

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Introduction

γ-Hydroxybutyric acid (GHB)[1] has a high impact in society as a popular substance of
40 abuse associated to cases of drug-facilitated sexual assault (DFSA) - commonly referred to as “date-rape”. GHB intake produces effects such as sleep and amnesia, which render the victim vulnerable.[2-5] Other misuses comprehends recreational purposes as a club drug and as a muscle-building supplement.[6,7] Named after “liquid ecstasy”, illicit GHB is marketed mostly as the sodium salt (sodium γ-hydroxybutyrate, NaGHB), being other related
45 compounds the lactone precursor γ-butyrolactone (GBL) and 1,4-butanediol (BD) which are both metabolized to GHB after ingestion. Misuse of the drug can cause serious medical problems including trouble breathing seizures, coma, and death.[8] Synthetic GHB, branded as Xyrem®, is also a pharmaceutical with medicinal use in the treatment of narcolepsy and alcohol withdrawal.[9,10] It is a highly regulated drug,[11] requiring patient enrollment in a
50 restricted access program.[12,13] On the other hand, GHB is a naturally-occurring metabolite of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). It is primarily found in the brain and acts as a central nervous system depressant.[1,8]

The growing number of sexual assault and overdose cases with the suspicion of GHB intake urge to adopt measures to address the problem,[14] being mandatory improving and
55 expanding current procedures for the analysis of consumed GHB, related drugs and associated metabolites in body fluids. One of the major current challenges is having forensic evidence of accidental (or deliberate) GHB consumption at a long term (for example, > 24 h after intake). The difficulty of it falls upon the rapid metabolism and excretion of exogenous GHB, meaning a short time detection window before concentration

goes back to an endogenous level (3-4 h urine and blood).[15,8] As endogenous metabolite, GHB is present in urine and blood in much lower concentration (typically 0.2 to 1 mg/L)[15,16] than shortly after drug abuse. Many studies establish a cut-off discriminant limit (10 mg/L, 0.1 mM) to distinguish external exposure from endogenous values.[10,17,18] The major pathway of endogenous GHB metabolism involves its conversion to succinic semialdehyde (SSA) and succinate by SSA dehydrogenase.[19,1] Products consistent with GHB β-oxidation, including glycolic,3-oxo-4-hydroxybutyric, and 3,4-dihydroxybutyric acids, have been described in the urinary profile of individuals with SSA dehydrogenase deficiency, suggesting this metabolic route.[20,21] Also, a recent study showed the existence of endogenous GHB glucoronide in urine.[22] To present, there are just a few works regarding the metabolism of exogenous GHB in animal models. The rapid metabolism of GHB to succinic acid in rat after intraventricular injection has been described[23,24] and the conversion of GHB to D-2-hydroxyglutaric acid in rats and baboons have been also reported.[25]

Most of the described studies regarding exogenous GHB in biofluids are carried out using procedures based on GC-MS and LC-MS as analytical platforms; also, works based on GC-FID or UV have been published.[8] While standing out for a high sensitivity, these procedures involve a considerable manipulation of the original sample previous the analysis; for instance, drug isolation by extraction and/or chromatographic processes, drug conversion to a derivative or to GBL, changes in pH, in temperature, etc.[8,26] Sample manipulation favours the loss of significant information of the original biological matrix and the introduction of errors in the measurement. As well, most of the described methods

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do not allow distinguishing between GHB, GBL or BD by the same analysis, a matter of interest in some forensic cases.[27] Finally, many of these procedures end with the destruction of the sample, preventing further analyses. In this context, NMR spectroscopy is a powerful 85 tool for body fluid profiling[28] with many inherent advantages; such as minimal sample preparation, no sample destruction, simple experimental setup and nonequilibrium perturbing. It allows the absolute quantification of compounds in a straightforward way, using an internal reference or an ERETIC (electronic reference to access in vivo concentrations)[29] signal, with no need of calibration standards curves.[30] Also, it allows to 90 follow various biochemical responses, without a preselection of metabolites, observing the response of the whole mixture. To our knowledge, only one study of ingested GHB through NMR spectroscopy has been reported - a clinical trial of the drug on saliva.[31] Apart from it, a few works of pure GHB and GBL in water and a study of spiked GHB in some body fluids have been carried out using NMR spectroscopy.[32-34]

95 With the present study, we aimed to explore the feasibility and suitability of NMR spectroscopy to monitor exogenous GHB in body fluids. To this end, a NMR-monitored clinical trial of exogenous GHB on urine and serum was carried out for the first time, and different NMR analyses were assessed. Detection, identification and quantification of the drug within biological matrices were evaluated, as well as the potential of NMR-based 100 metabolomics in the search for surrogate biomarkers indicative of drug consumption.[35,36] With this approach, we aim to facilitate and accelerate the analysis of exogenous GHB in body fluids by the use of different NMR-based methodologies, as well as to advance in the search of long term forensic evidence of GHB using a metabolomics-based approach.

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105 # Experimental Section

Reagents and Chemicals

Glycolic acid, succinic acid, 3-(trimethylsilyl)-[2,2,3,3-$^{2}H_4$]-propionic acid sodium salt (TSP), sodium dihydrogen phosphate, disodium hydrogen phosphate and sodium azide were purchased from Sigma-Aldrich S.A. (Tres Cantos, Madrid, Spain). Deuterium oxide
110 (99.96 % D) was obtained from Cortecnet (Voisins-le-Bretonneux, France). Serum tubes (Vacutainers^{TM}, 8mL) were purchased from MidMeds (Essex, U.K.).

Experimental Design

Ethical approval for the GHB administration study was obtained from the research
115 ethics committee of the King's College London Drug Control Centre (approval number CREC/06/07-30). Written informed consent was obtained from twelve volunteers (six men and six women). Males had a mean age of 25 years (21–36 years) and a mean body mass index (BMI) of 23.7 kg/m^2 and females had a mean age of 26 years (22–32 years) and BMI of 23.0 kg/m^2. Prior to the study, volunteers were assessed to be in good health. Exclusion
120 criteria included a history of liver disease, succinic semi-aldehyde dehydrogenase deficiency, and currently breast feeding. All volunteers tested negative for the current use of sedatives, recreational drugs, and pregnancy (females only). A single dose (25 mg/kg body weight) of GHB was administered in the form of Xyrem® (sodium oxybate, 500 mg/L). Urine and blood samples were taken before and at different times after dose (see
125 Brailsford *et al*. for further details).[15] Urine samples at time points -10 min, 1, 2, 4, 6, 14,

20, 24 and 30 h post-dose were analyzed. Serum samples at time points -10 min. and 1 h post-dose were analyzed.

Sample Collection, Storage and Preparation

130 For serum collection, 10 mL of whole blood was allowed to clot for 30 min in the Vacutainer[TM] in which it was collected. Each sample was centrifuged (1000 g, 10 min) and the supernatant (serum) was transferred into a polypropylene tube. A 0.5 mL aliquot of each sample was lyophilized and the dried material was storage at -80°C until analysis. For urine samples, the volunteers were asked not to void any urine at times other than those 135 specified up to 24 h post-administration. A single spot urine was collected at 30 h. Samples were centrifuged (16000 g, 10 min), aliquoted (1.8 mL) and lyophilized. The dried material was storage at -80°C until analysis. Prior to the analysis each sample was reconstituted in D_2O (0.45 mL for serum and 0.9 mL for urine) without observing any precipitate. NMR samples were prepared mixing 400 µL of the reconstituted sample with 200 µL of a sodium 140 phosphate buffered stock solution (0.2 M in D_2O, pH 7.4, containing 1 mM of TSP and 3 mM of NaN_3) directly in the 5mm NMR tube.

NMR Spectroscopy

NMR experiments were carried out on a Bruker AVANCE II 600 spectrometer 145 operating at 14.1 T (600.13 and 150.92 MHz, ^{1}H and ^{13}C frequencies respectively), fitted with a 5mm multinuclear triple resonance (TBI) probe, a z-axis pulsed field gradient, an automatic sample changer and a temperature control unit (Bruker BioSpin, Rheinstetten,

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Germany). The probe temperature was maintained at 298.0 K for all experiments. Sample handling, automation, acquisition and processing were controlled using the software

150 TOPSPIN 3.1 (Bruker BioSpin, Rheinstetten, Germany).

For urine samples 1D ^{1}H NMR experiments were performed using the pulse sequence 1D NOESY-presat.[37] For serum samples a standard CPMG (Carl-Purcell-Meiboom-Gill) spin-echo sequence[38] with presaturation of the residual water signal was used. In both cases, data were collected into 32 K data points during an acquisition time of 1.7 s and

155 using a relaxation delay (RD) of 3 s. Spectra were recorded in the time domain as interferograms (FID) across a spectral width of 9615 Hz and as the sum of 128 transients. FIDs were automatically Fourier transformed and the spectra were phased, baseline corrected and referenced.

2D experiments, ^{1}H-^{1}H COSY (Correlated Spectroscopy), ^{1}H-^{1}H TOCSY (Total

160 Correlation Spectroscopy), ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Coherence) and ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation), and 1D ^{1}H selective TOCSY experiments were acquired using standard Bruker pulse sequences and routine conditions.

1D ^{1}H qNMR experiments were performed using a standard 90° pulse-acquisition sequence with presaturation of the residual water signal. Data were recorded using the same

165 parameters described before; except for an RD of 15 s. GHB and TSP integration and quantification was performed with MestreNova 8 (Mestrelab Research S.L.) and its global spectral deconvolution (GSD) application.

NMR data acquisition for the metabolomic study on urine was conducted through 1D ^{1}H NMR spectroscopy measurements, using the 1D ^{1}H NOESY-presat experiment with the

170 acquisition and processing parameters described before. All experiments were performed

under automatized identical conditions. The temperature into the probehead was previously calibrated and maintained constant at 298.0 K. For this purpose, an equilibration delay (2 min) was left once the tube was into the magnet and prior to the shimming process in all analyses. After acquisition, resulting FIDs were automatically Fourier transformed, spectra were phased, baseline corrected and referenced. The FIDs were multiply by an exponential apodization function equivalent to 0.2 Hz of line broadening prior to the Fourier transform. The phase and baseline corrections and the calibration of all spectra were checked manually after the automatic process. The total experimental time was *ca*. 8 min per sample.

All spectra were referenced to the TSP peak resonance at (δ_H and δ_C at 0.00 ppm).

Statistical Analysis

For the statistical analysis, spectra were normalized to total intensity to avoid the influence of differences in sample concentration, and submitted to variable size bucketing. Bucket tables were then *pareto* scaled, to better take into account the variation of the small peaks, and mean centered. Orthogonal partial least squares discriminant analysis (OPLS-DA)[39] was performed in SIMCA 14 (Umetrics). Models were validated with Anova cross-validation and permutation. Random forests analysis[40] and significance analysis of metabolites (SAM)[41] were performed with Metaboanalyst.[42] Random forests were performed with 1000 trees and 7 predictors per node. SAM analysis was based on the FDR and q-value method.[43] The identification of glycolate and succinate was done by reference to reported data[44] and confirmed by standard addition ("spiking" the sample).

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Results and Discussion

195 A study based on NMR spectroscopy of body fluids urine and serum of twelve healthy sex-matched volunteers having consumed GHB was conducted for the first time. Considering the reported data regarding GHB dosage as a pharmaceutical and GHB consumption as an illicit drug,[8] a single low dose of GHB was given to each volunteer (25 mg/Kg, total amount ranging from 1.4 to 2.6 g). The administered concentration was in the 200 low dosage range of reported clinical studies (25 to 72 mg/Kg).[15] Samples were collected before (-10 min) and at several time points after drug administration (up to 30 h and 13 h for urine and blood respectively). Procedures described at NMR-based metabolomics were followed in the sample preparation (*Experimental section*). For practical reasons, samples were lyophilized and later reconstituted in half the original volume of D_2O, resulting in a 205 two-folded original concentration. However, depending on the case of study, the sample should not be necessarily lyophilized neither concentrated, speeding up the sample preparation process.[37]

Monitoring exogenous GHB: detection and identification in urine and serum

210 The first goal consisted in observing if the drug at the concentration administered was NMR-detectable using metabolomics standard conditions, in post-dose urine and serum; i.e. directly in the biological matrix and with minimum manipulation. To our knowledge, there is no reported example of this nature up to date. Samples of all twelve volunteers before (-10 min) and after (1 h) drug intake were analyzed by a standard 1D ^{1}H NMR experiment

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215 and the resulting spectra were compared. In the case of urine, a ^{1}H NOESY-presat experiment was acquired, while for serum a CPMG-presat experiment was recorded in order to minimize the contribution from macromolecules (proteins and/or lipids). Every experiment took *ca.* 8 min to perform, being the sample afterwards in perfect conditions to conduct further analyses. The comparison of pre-dose and post-dose urine spectra revealed 220 the presence of three signals at chemical shifts, δ_H, 1.80, 2.24 and 3.60 ppm detectable only in post-dose samples, which corresponded to δ_H previously described for GHB.[31-34,45] Analogous results were obtained for serum. Results showed that, using standard ^{1}H NMR metabolomics conditions, the drug was quickly detected whereas GHB at an endogenous concentration was not. As an example, **Figure 1** shows urine and serum spectra of a 225 volunteer.

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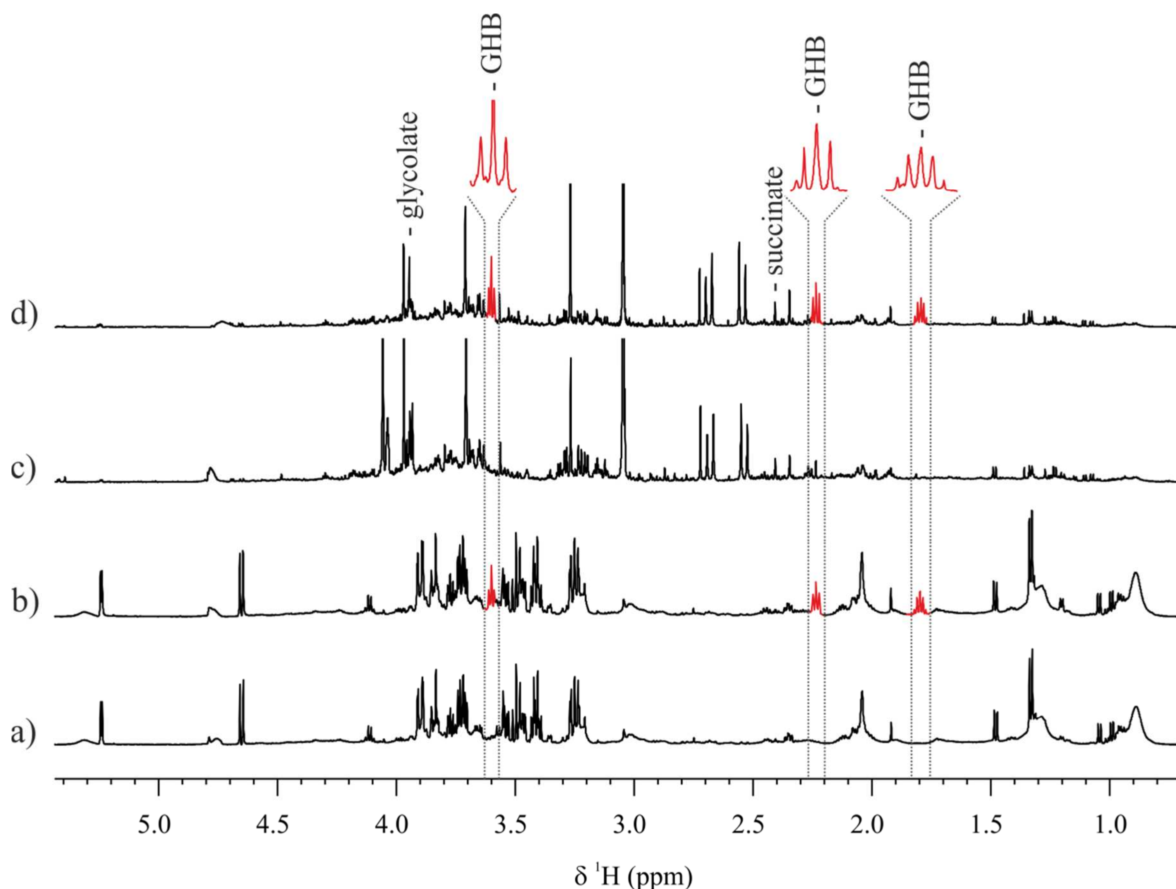

Figure 1. ^{1}H NMR spectra of body fluids from a same healthy volunteer before (-10 min) and after (1 h) GHB administration. a) Pre-dose and b) post-dose serum CPMG-presat spectra; c) pre-dose and d) post-dose urine NOESY-presat spectra. Experiments performed
230 at a magnetic field of 600.13 MHz and at 298.0 K of temperature.

After that, a complete ^{1}H and ^{13}C NMR analysis of GHB by standard 1D and 2D NMR experiments was performed in both biofluids, with the aim to identify ^{1}H and ^{13}C NMR signals of the molecule and their coupling patterns within urine and serum matrices.
235 Representative samples at 1 h post-dose were selected. Homonuclear and heteronuclear 2D NMR experiments, ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC, and

1D selective TOCSY experiments were recorded. GHB ^{1}H and ^{13}C signals were identified clearly in both matrices. As an example, **Figure 2** presents some of the results obtained for urine. The signal corresponding to protons H3 of GHB consisted on an isolated peak (a triplet of triplets) at 1.80 ppm. When H3 was selectively irradiated via 1D ^{1}H selective TOCSY, a clean filtered spectrum of GHB resulted (**Figure 2a**). This is a helpful experiment to quickly confirm the presence of the drug within complex matrices, which can be also used for quantification.[46] By the ^{1}H-^{1}H COSY experiment (**Figure 2b**) the ^{1}H-^{1}H correlation pattern of GHB was clearly observed. **Figure 2c** shows ^{1}H-^{13}C HSQC with the one-bond ^{1}H-^{13}C coupling pattern highlighted. In the ^{1}H-^{13}C HSQC correlation the unfolding of ^{1}H signals in the ^{13}C second dimension occurs, favoring the enhancement of spectral dispersion due to the broader chemical shift range of ^{13}C compared to ^{1}H. ^{1}H-^{13}C HSQC represents a useful alternative to 1D ^{1}H and 2D ^{1}H-^{1}H experiments when these are not enough for the identification of the drug in the biological sample due to severe signal overlapping.[47] Values of δ_H and δ_C of GHB detected in urine and serum (pH 7.4, 298.0 K) are gathered in **Figure 2d**. Remaining correlations of urine, analogous spectra of serum and of pure GHB are in the *Supporting Information* (Figure S1 to Figure S3).

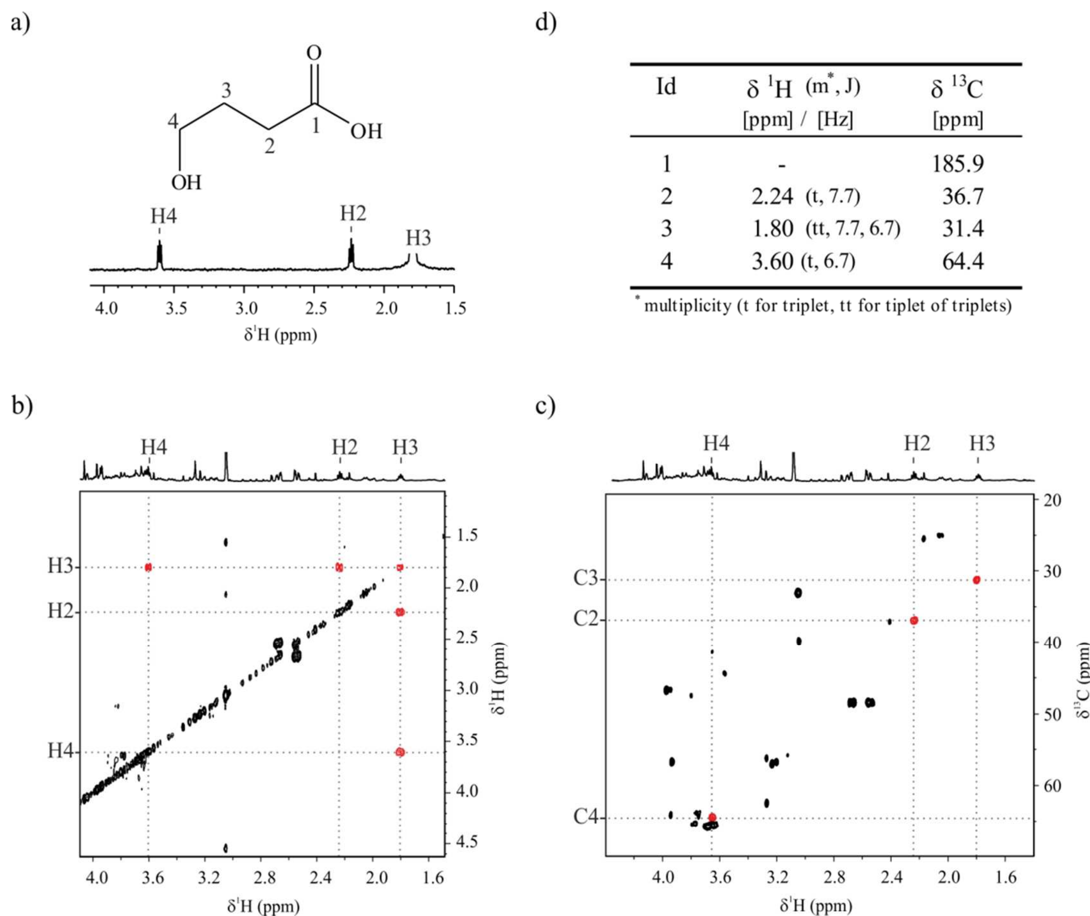

Figure 2. Selected areas of a) 1D ^{1}H selective TOCSY with saturation of H3 signal at 1.80 ppm; b) ^{1}H-^{1}H COSY and c) ^{1}H-^{13}C HSQC from a representative 1 h post-dose urine sample. d) ^{1}H and ^{13}C NMR characterization of GHB in urine and serum (pH 7.4, 298.0 K). Spectra recorded at a magnetic field of 600.13 MHz.

In all examples shown in this work GHB is present in the carboxylate form since the pH was adjusted to 7.4 (GHB pKa 4.71). However, NMR could distinguish the form of GHB in the sample (as a free acid, as a carboxylate or a mixture of both) and the

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quantification of the ratio when an equilibria between the two species exist; in that case

265 experiments must be done preserving the original ratio without adjusting the original pH.[32]

Quantifying exogenous GHB in urine by ^{1}H qNMR

The direct quantification of GHB in urine after consumption using NMR spectroscopy was assessed in a specific example. Using the samples of the experiment

270 described, the pharmacokinetics of GHB in urine was monitored through ^{1}H qNMR (quantitative NMR) as an example of the direct quantification of the drug within a body fluid. Results obtained were compared with previous ones –(performed with aliquots of the same original samples) obtained from a well stablished indirect analysis of GHB by a GC-MS based procedure.[15]

275 Samples at time points -10 min, 1, 2, 4 and 6 h post-dose were analyzed. As in the previous section, minimum sample preparation was needed, consisting mainly on the addition of a stock buffered D_2O solution in the reconstituted urine aliquot. The stock solution contained internal reference, TSP, of known concentration, to be used as internal standard for quantification. All samples were analyzed using a 1D ^{1}H qNMR experiment

280 with suppression of the residual water signal. The same acquisition and processing parameters than in a standard 1D proton experiment were applied, except for a longer relaxation delay allowing all signals to completely relax in order to provide quantitative data (*Experimental Section*). GHB H3 signal (1.80 ppm) and TSP singlet (0.00 ppm) were integrated in all spectra after deconvolution of the peaks, avoiding errors due to possible

285 low intensity overlapped signals and base line effects. The concentration of GHB in the original urine samples of each volunteer was calculated using TSP as internal standard,

applying the corresponding volume correction factor according to sample preparation and considering that H3 signal corresponds to two protons of the GHB molecule and TSP singlet to 9 protons of the TSP compound. GHB eliminated amount in each urinary void was calculated considering the total collected volume. After that, average values of concentration and eliminated amount at each time point were calculated (*Supporting Information*, Table S1). Plots of concentration and eliminated amount *vs* time are shown in **Figure 3a** and **Figure 3b** respectively. Profiles of maximum and minimum values are also plotted. The maximum concentration of GHB occurred at first post-dose collection (1 h), the mean value being 55.5 mg/L, ranging from 24.6 to 113.4. After 2 h, the mean concentration decreased significantly to 37.9 mg/L. After 4 h, GHB was just detected in one of the twelve samples (8.4 mg/L). After 6 h GHB was not detected in any volunteer's urine. As expected, curves of eliminated amount *vs* time presented an analogous profile. After 1 h, the mean GHB eliminated was 14.0 mg (ranging from 1.8 to 22.5). After 4 h, the mean eliminated amount was 8.2 mg. ^{1}H qNMR results were in accordance with those reported by Brailsford *et al.*,[15] in which aliquots of the same sample pool were analyzed. In that work, a wide pharmacokinetic study of GHB in different body fluids including urine was carried out. A procedure based on GC-MS analysis was used, where GHB was initially converted into GBL by the acidification of the sample and then a liquid-liquid extraction was carried out prior to the analysis. In that study a T_{max} of 1 h, a C_{max} of 67.6 mg/L and similar pharmacokinetic profiles (after 4 h GHB concentration were at an endogenous level) were obtained. Our results are also within the same range of other published data.[15]

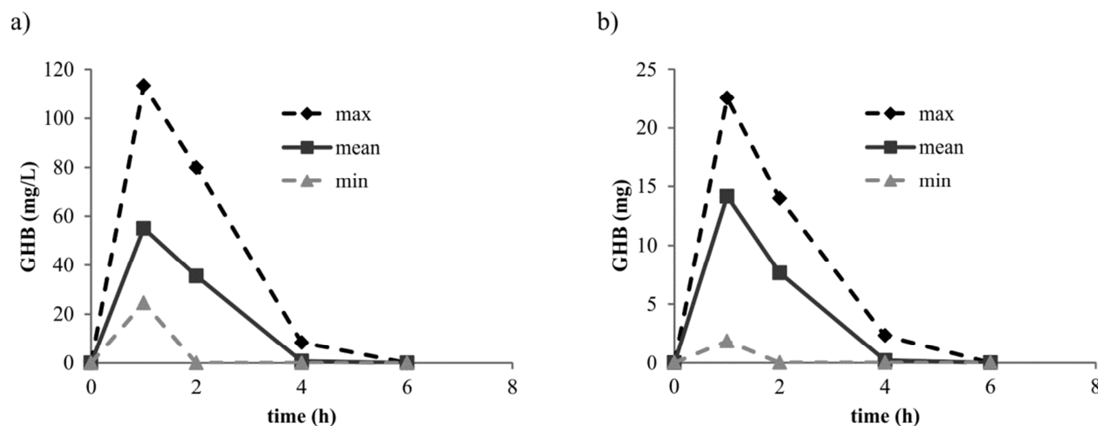

Figure 3. Pharmacokinetics of exogenous GHB in urine monitored by ^{1}H qNMR experiments. a) GHB concentration and b) GHB eliminated amount curves over time; profiles for mean (12 volunteers), maximum and minimum values.

The fact that H3 signal of GHB is nicely isolated in the proton spectrum, with minimum overlap, facilitates the quantification of the compound directly in the mixture by 1D ^{1}H qNMR. Alternatively, when situations of severe signal overlap, 2D NMR quantitative methods could yield accurate quantitative results.[48] In the present work, each 1D quantitative experiment took *ca*. 30 min, being the sample afterwards in perfect conditions to conduct further analyses. In turn, the quantification of exogenous GHB in serum samples could be carried out analogously by 1D ^{1}H qNMR experiments. In this case, the use of ERETIC[29] as reference for quantification would be recommended due to the possible presence of protein, which could bind the internal reference (TSP) and its signal would not be reliable for quantification. Using standard conditions and equipment of ^{1}H NMR-based metabolomics (128 transients, 600 MHz magnet and a conventional probe), endogenous concentration levels of GHB were not detected. Endogenous concentrations

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

may vary from 0.2 to 1 mg/L; a discriminant limit of 10 mg/L (*ca.* 0.1 mM) has been defined to distinguish external exposure from endogenous values.[15-18] With standard equipment for metabolomic studies (typically 500-600 MHz magnets with cryo- or conventional probes) concentrations up to the 0.1 mM could be detected and quantified.[48]
330 Lower detection and quantification limits can be reached using more advance equipment, i.e. higher field magnets (up to 1 GHz available), cryogenically cooled probes and/or small-volume microprobes (60 μL).[30] The sensitivity of NMR spectroscopy (and therefore the detection limit for GHB quantification) depends strongly on different aspects related to the equipment used (magnetic field, room temperature or cryogenic probe, 5 mm or 3 mmm
335 probe, etc.), the nucleus observed and the experiment acquired.[49]

Search of metabolites associated to GHB consumption by NMR-based metabolomics

After proving that exogenous GHB within urine or serum matrices can be directly
340 monitored by NMR spectroscopy, the suitability of the technique in the search of metabolites associated to GHB consumption using a metabolomics approach was assessed. The specific goal was to explore, by untargeted NMR-based metabolomics, the detection of any eventual metabolite in the biofluid with an altered concentration due to GHB ingestion; and in that case, explore its potential use as a surrogate biomarker for forensic evidence of
345 GHB consumption at a long term. The study was performed in urine as model biofluid, due to its easy accessibility and manipulation compare to serum.[15] Metabolic profiling in combination with multivariate analysis was performed in samples collected at -10 min, 1 h and 20 h after GHB administration.

Initially, pre-dose (-10 min) and 1 h post-dose samples were compared by orthogonal partial least squares discriminant analysis (OPLS-DA). A robust model was obtained where, as expected, GHB peaks (1.80, 2.34 and 3.60 ppm) were identified as the main discriminant signals in the corresponding S-plot (**Figure 4a**). Interestingly, another feature, a singlet at 3.94 ppm, stood out for its high significance in the discrimination of the two groups. The signal was initially identified as glycolate,[50] based on the δ_H and the δ_C (63.9 ppm) values and prior reported data;[44] the δ_C was obtained via an ^1H-^{13}C HSQC experiment recorded on a representative sample of the pool. The assignment was unambiguously confirmed by a subsequent standard addition ('spiking') of glycolate in the sample. The next significant discriminant feature corresponded to signal at 2.41 ppm, identified as succinate.[51] The same procedure described before was followed for the identification of succinate.[44] Another discriminant feature corresponded to peak at 4.05 ppm, identified as creatinine.[52] Results showed that, as for GHB, concentrations of glycolate and succinate increased after 1 h of drug ingestion, while for creatinine the behavior was the opposite. The relevance of glycolate and succinate features for group separation was further confirmed by SAM (significance analysis of metabolites) and random forests analysis, were they scored within the five metabolites with highest variable importance. After that, pre-dose (-10 min) and 20 h post-dose samples were compared. In the resulting OPLS-DA model, glycolate was still identified among the relevant metabolites in the S-plot, together with creatinine. Succinate was not detected as discriminant any more and, as expected, GHB neither (**Figure 4b**). As well, these results were confirmed by SAM and random forests modelling.

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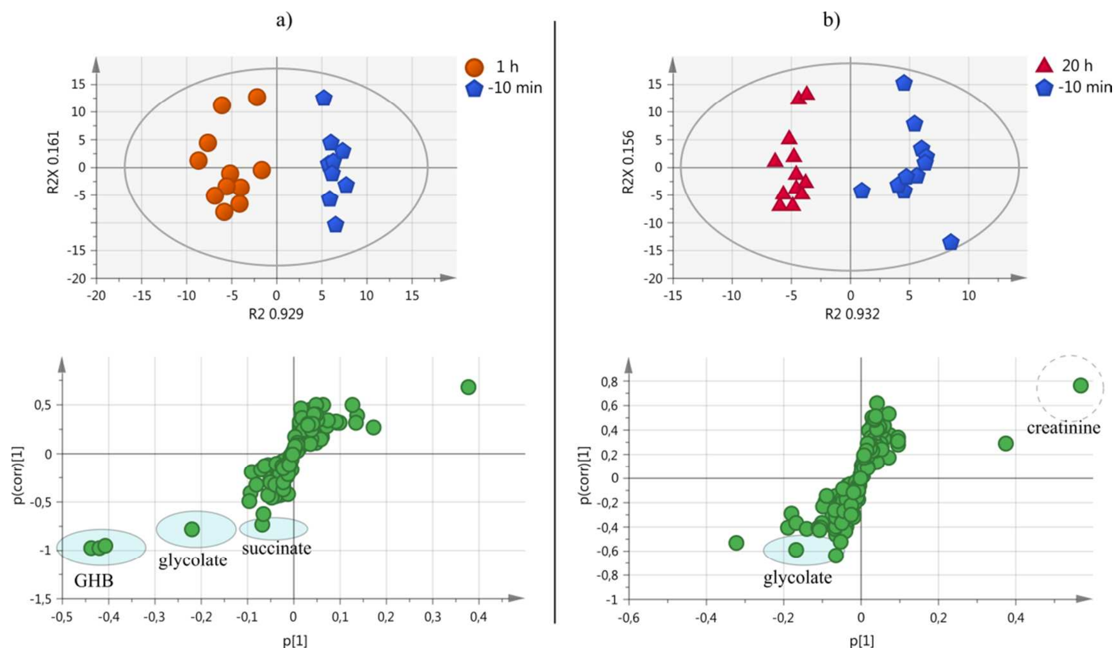

31 **Figure 4.** Urine OPLS-DA score plot and S-plot from ^{1}H metabolic profiles of samples
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33 375 before and after GHB intake. A) Comparison of -10 min pre-dose and 1 h post-dose
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36 samples; 1+1 components; R2Y(cum) = 0.93, Q2(cum) = 0.75, CV-Anova = 0.00016. b)
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38 Comparison of -10 min pre-dose and 20 h post-dose samples; 1+2 components; R2Y(cum)
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40 = 0.93, Q2(cum) = 0.65, CV-Anova = 0.004.
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45 380 Both, succinate and glycolate have been previously associated to the metabolism of
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47 endogenous GHB. Primarily, endogenous GHB is metabolized to succinic acid via
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50 oxidation of the intermediate succinic semialdehyde (SSA) by SSA dehydrogenase.[20] In the
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52 case of individuals with SSA dehydrogenase deficiency, GHB is metabolized to glycolic
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54 acid via 3,4-dihydorxybutyric acid intermediate.[21] The present study, through NMR-based
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385 untargeted metabolomics, showed that the concentrations of succinate and glycolate in urine increased significantly after GHB consumption. Results suggested that exogenous GHB (or a fraction of it) is metabolized to succinate and glycolate, which coincides with the observed in the metabolism of endogenous GHB. No presence of GHB glucoronide was observed, neither as a discriminant feature nor in a detailed analysis of 1D and 2D NMR 390 spectra of a representative sample at time point 1 h post-dose.[22] The high sensitivity of creatinine concentration in urine to factors like circadian rhythm or diet[53] made difficult to state a direct correlation between GHB ingestion and creatinine concentration just based on this experiment. **Figure 1** indicates the signals of glycolate and succinate in the ^{1}H NMR spectra of urine.

395 At that point, the evolution of the normalized relative concentrations of glycolate and succinate in urine over time (at -10 min, 1, 2, 6, 14, 20, 24, 30 h post-dose) was studied in order to assess the potential use of them as surrogate biomarkers of GHB consumption at a long term. For that, samples at the aforementioned time points were analyzed by ^{1}H qNMR experiments. The concentration of GHB was also monitored and compared to the others. 400 **Figure 5** shows the normalized relative concentration profiles of the three compounds over time. As shown, while GHB and succinate concentrations dropped rapidly to an endogenous level (at time point 6 h), glycolate concentration decreased much slower, and even after 24 h a small difference can be observed. According to these results, glycolate - with a longer detection time window that exogenous GHB – could be an interesting 405 candidate of surrogate biomarker; further targeted studies will be needed to validate its real use. To our knowledge, this is the first time that an untargeted study on the metabolism of ingested GHB has been carried out through NMR spectroscopy.

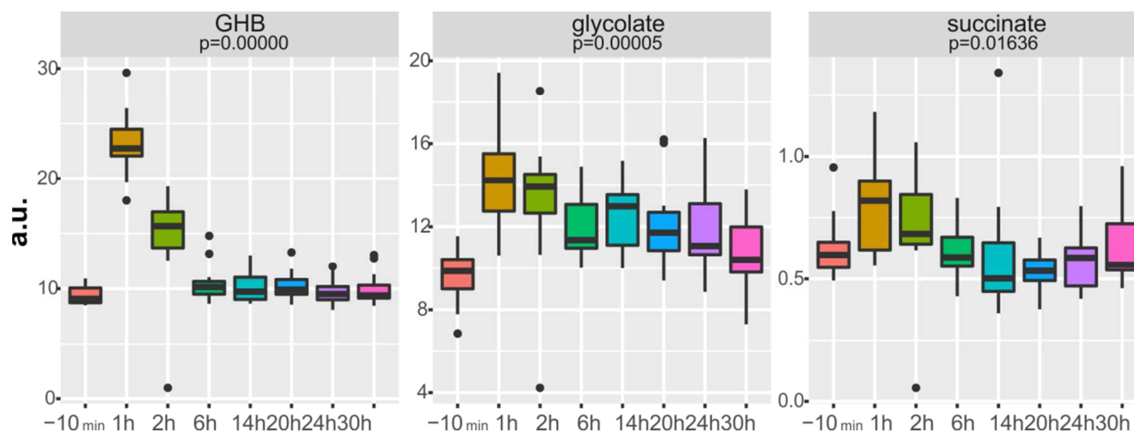

Figure 5. Boxplots of GHB, glycolate and succinate normalized relative concentrations at different time points. P-values from ANOVA are indicated.

Conclusions

The present work demonstrated the feasibility of NMR spectroscopy to monitor exogenous GHB in body fluids such as urine and serum. Unlike current procedures for the analyses of consumed GHB (mostly based on GC-MS and LC-MS), NMR spectroscopy allowed the quick monitoring of exogenous GHB within the almost intact body fluid, it yielded simultaneous interesting information of the complete matrix and it would allow to distinguish between GHB and associated drugs (GBL and BD) by the same analysis. Also, the non-destructive nature of the technique makes it compatible with further posterior analyses. The suitability of the drug detection, identification and quantification within the biological matrix and through the most appropriate NMR experiment was proved. Standard 1D ^{1}H NOESY-presat and CPMG-presat experiments allowed monitoring exogenous GHB

in urine and serum respectively, while 2D NMR correlations and 1D selective experiments allowed the unambiguous identification of exogenous GHB in both body fluids. ^{1}H qNMR applied to the direct quantitative analysis of GHB in urine in a pharmacokinetic study yielded similar results to those obtained by an indirect GC-MS procedure. The great potential of NMR-based metabolomics in the search of surrogate biomarkers to provide forensic evidence of GHB consumption at a long term was demonstrated. A significant increase of the concentration of glycolate and succinate in urine after GHB ingestion was proved, being glycolate an interesting candidate for further studies due to its longer detection time window compared to GHB and succinate. We think that these features will open up new interesting possibilities in future studies, complementing current procedures.

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Supporting Information Available

This material is available free of charge via the Internet at http://pubs.acs.org.

Figure S1, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HMBC of 1 h post-dose urine sample; Figure S2, 2D experiments, ^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC

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and 1D ^{1}H selective TOCSY experiment of 1 h post-dose serum sample; Figure S3, as Figure S2 but of pure GHB and Table S1, results of the pharmacokinetic study of GHB in urine by ^{1}*H qNMR* (PDF).

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