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Assessment of the stability of exogenous gamma hydroxybutyric acid (GHB) in stored blood and urine specimens

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Abstract. – OBJECTIVE: The aim of this work is to test the stability of exogenous GHB in whole blood and urine samples collected from living and deceased GHB free-users, spiked with known concentrations of GHB and stored at different temperatures (–20°C, 4°C and 20°C) up to 4 weeks.

MATERIALS AND METHODS: GHB was added to GHB-free ante-mortem blood and urine samples at the concentration of 5 and 10 mg/L, respectively whereas in post-mortem blood and urine specimens at 50 and 10 mg/L respectively. All samples were stored at three different temperatures: –20°C, 4°C and 20°C and extracted and analyzed at three days, 1 week, 2 weeks, 3 and 4 weeks in duplicate. No preservatives were added. GHB was quantified by GC-MS after LLE according to a previously published method.

RESULTS: Post-mortem blood specimens showed a reduction of GHB levels higher than 10% only after a period of 4 weeks of storage for samples kept at +4°C and +20°C, whereas samples stored at –20°C showed a mean reduction of 8.7%. In post-mortem urine samples, there was a mean reduction of GHB levels higher than 20% at all storage temperatures, after 4 weeks of storage. Ante-mortem blood samples showed a reduction of GHB levels lower than 10% only after 3 days of storage at –20°C and at +4°C (samples stored at +20°C showed a mean reduction of 10.4%). After 4 weeks of storage, there was a mean reduction of GHB concentrations higher than 20% at all storage temperatures. Ante-mortem urine samples showed a reduction of GHB levels higher than 10% after just 3 days of storage for samples kept at all tested temperatures. After 4 weeks of storage, there was a mean reduction of GHB concentrations higher than 25% at all storage temperatures.

CONCLUSIONS: According to our findings, it would be useful to perform GHB analysis both in blood and urine specimens within 3 days of sampling and the specimens should be stored at –20°C or 4°C in order to avoid instability issues.

Key words:
Gamma hydroxybutyric acid (GHB), Stability, Ante-mortem and post-mortem blood and urine, Forensic toxicology.

Introduction

Over the last two decades gamma hydroxybutyric acid (GHB) has been generating an increased notoriety as an euphoric and disinhibiting drug of abuse in particular in certain social backgrounds. GHB has been used in cases of drug-related sexual assault and this is why it is considered a "date rape drug", it can easily be added to drinks, in particular when the designed victim is already vulnerable due to the effect of sedative substances[1-3]. Moreover, in the 90s it was infamously popular among bodybuilders, who used it as a "steroid-accessory drug", to increase the release of the growth hormone (GH), although the scientific evidence supporting this theory is controversial[4-5].

In forensic investigations, it is necessary to determine if any GHB detected is due to endogenous production or exogenous ingestion. As a consequence of its rapid metabolism and elimination (within 4-8 hours post ingestion), concentrations both in blood and urine can drop very quickly, this is why the stability of GHB must be monitored especially in those cases where the investigation is performed after a long interval of time. It has been proved that post-mortem interval strongly affects the GHB post mortem production both in urine and blood specimens[6].

If GHB and its analogous/precursors (Figure 1) are compared to other common drugs, it is evident the small amount of informations available

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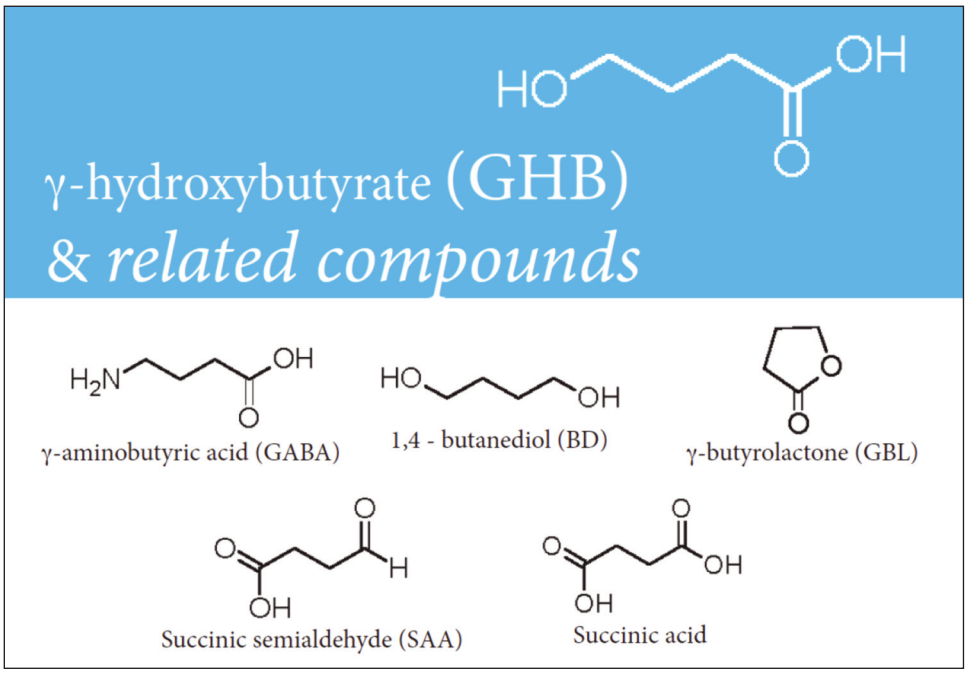


Figure 1. Chemical structure of GHB and related compounds.

regarding the trends of use and their diffusion throughout the population[3,7].

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported that although GHB consumption is usually low in Europe, there are some contexts where it together with its analogous/precursors are more often used like for example gay nightclubs[8]. More recently it has been also associated to new psychoactive substances, such as mephedrone, giving rise even to fatalities[9]. A study conducted in 2003 in the UK by the Independent Drug Monitoring Unit (IDMU) highlights that the use of GHB first reached its peak in the years between 1994 and 2002, and only few people had used it before then[10].

The aim of this work is to test the stability of exogenous GHB in whole blood and urine samples collected from living and deceased GHB free-users and stored at different temperatures up to 4 weeks. GHB was added to ante-mortem blood and urine samples at the concentration of 5 and 10 mg/L, respectively whereas in post-mortem blood and urine specimens at 50 and 10 mg/L respectively.

Material and Methods

Reagents

GHB sodium salt in methanol (1.0 mg/mL) and GHB-D6 sodium salt (0.1 mg/mL) were purchased from Cerilliant-Sigma-Aldrich (St. Louis, MO, USA); sulphuric acid (98%) and ethyl acetate were purchased from Merck (Milan, Italy); BSTFA +1% TMCS derivatising agent was purchased from Supelco (Bellefonte, PA, USA).

Extraction and Analysis

The analyses were performed following the published method suggested by Elliott[11], which has been adapted and revalidated to the conditions of the present study according to the guidelines of Peters et al[12]. A liquid-liquid extraction (LLE) following the procedure of Busardò et al[6] was used.

The GC/MS analysis was carried out using an Agilent Technologies (AT) model 6890N GC coupled with an AT mod. 5973 Inert mass selective detector and an AT 7683 Series automatic sampler at the following chromatography conditions: DB5-MS capillary column (30 m, 0.32 mm i.d.) with a temperature gradient starting at 60°C (for 2 min) and ramping to 180°C (20°C/min for 6 min) then ramping to 250°C (50°C/min for 1 min) and finally 70°C post-run. Derivatised GHB (GHB-diTMS) and GHB-D6 (GHB-D6-diTMS) were detected using the 233 and 239 ion fragments, respectively.

Sensitivity and Linearity

The lower limit of quantification (LLOQ) for GHB was defined as the lowest standard on each calibration curve (2.5 mg/L) with a peak response at least 10 times (S/N ≥ 10) that of the blank response.

Two calibration curves were used, one for blood and one for urine samples and have included 7 points, each. The following GHB calibrators: 2.5, 5, 10, 30, 40, 50 and 60 mg/L were prepared in blank (pre-screened) equine plasma for the analysis of blood samples. The following GHB calibrators: 2.5, 5, 7.5, 10, 12.5, 15 and 20 mg/L were prepared in human urine (endogenous GHB concentration determined to be < 0.5 mg/L by GC-MS) for the analysis of urine samples. Linear regression, not forced through zero, was used to produce each calibration curve.

Samples and storage conditions

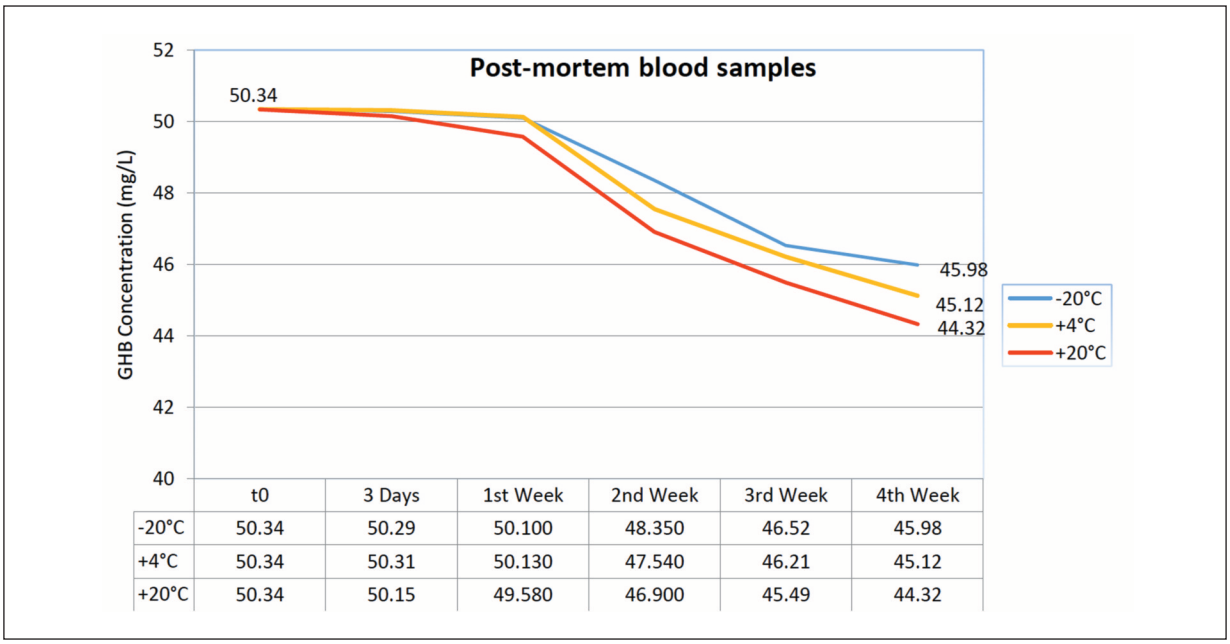
Different sources of blood and urine were obtained as follow: 10 samples of blood and 10 samples of urine came from 10 alive GHB free-subjects (mean age 39 years ± 7.19), whereas 10 post mortem blood samples and 10 post mortem urine samples were obtained from 10 cadavers (mean age 45 years ± 9.15), in which the post mortem interval was between 20 and 30 hours. The cause of death in post mortem cases was not drug related but due to natural causes in 3 cases, suicide by hanging in 2 cases, firearms injuries in 2 cases, stab injuries in 2 cases and major traumas in 1 case. For all cases the endogenous blood and urine concentrations of GHB were previously detected and they were below 0.5 mg/L. Pools of blood and urine were spiked with GHB at the following concentrations: 5 and 10 mg/L for ante-mortem blood and urine samples, respectively and 50 and 10 mg/L for post-mortem blood and urine specimens, respectively. 1 mL aliquots were transferred in 2 mL Eppendorf capped tubes and all samples were stored at three different temperatures: –20°C, 4°C and 20°C and extracted and analyzed at 3 days, 1 week, 2 weeks, 3 and 4 weeks in duplicate. No preservatives were added to the specimens.

Statistical Analysis

The statistical analysis of data was performed by using the analysis of variance in order to compare the three different storage temperatures both in ante-mortem and post-mortem blood and urine samples throughout the period of investigation up to 4 weeks, and paired *t*-test for the comparison of GHB levels between t0 and 3 days, 1, 2, 3 and 4 weeks in each group. A *p*-value lower than 0.05 ($p<0.05$) was considered statistically significant. The STATA software 11.0 version was used.

Results

The temporal trend of GHB mean values both in ante-mortem and post-mortem blood and urine samples throughout the period of investigation up to 4 weeks at three different storage temperatures is reported in Figures 2-5.



	t0	3 Days	1st Week	2nd Week	3rd Week	4th Week
-20°C	50.34	50.29	50.100	48.350	46.52	45.98
+4°C	50.34	50.31	50.130	47.540	46.21	45.12
+20°C	50.34	50.15	49.580	46.900	45.49	44.32

Figure 2. GHB mean values and temporal trend up to 4 weeks of post-mortem blood specimens spiked with 50 mg/L GHB.

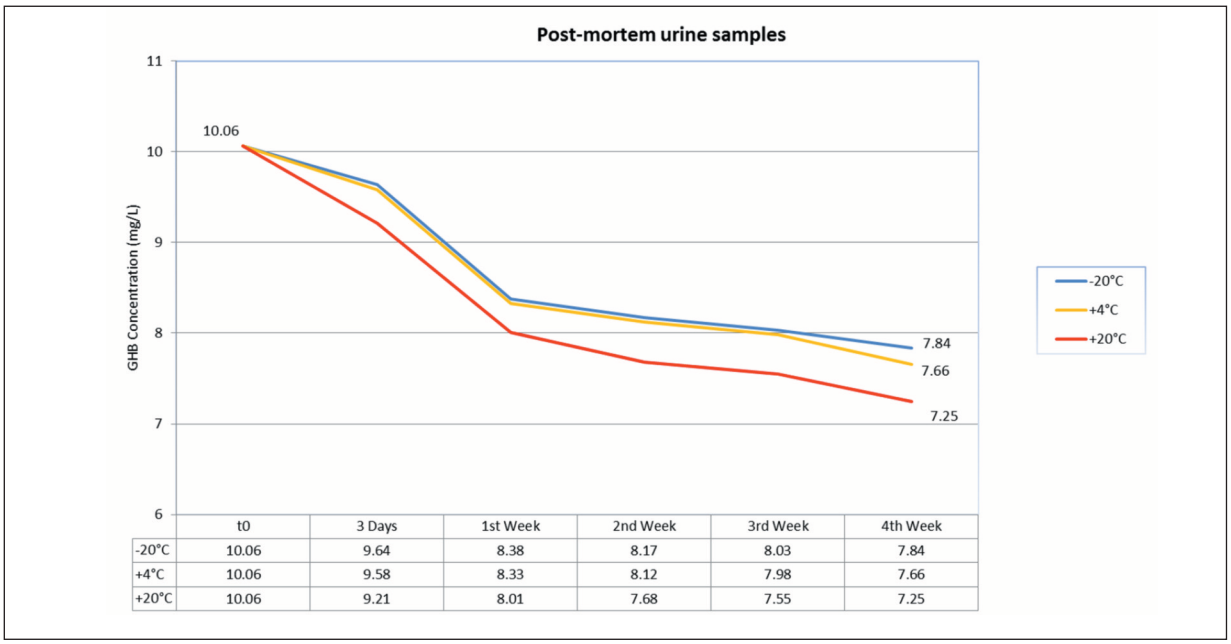


Figure 3. GHB mean values and temporal trend up to 4 weeks of post-mortem urine specimens spiked with 10 mg/L GHB.

Post-mortem blood specimens spiked with 50 mg/L of GHB showed a reduction of GHB levels higher than 10% only after a period of 4 weeks of storage for samples kept at +4°C and +20°C (mean reduction 10.4% and 12%, respectively) which was statistically significant (p<0.001), whereas samples stored at –20°C showed a mean reduction of 8.7% (p<0.01). At three days there was a mean reduction of GHB levels of 0.1% for samples stored at –20 and +4°C and a mean reduction of 0.4% for samples stored at +20°C. These decreases were not statistically

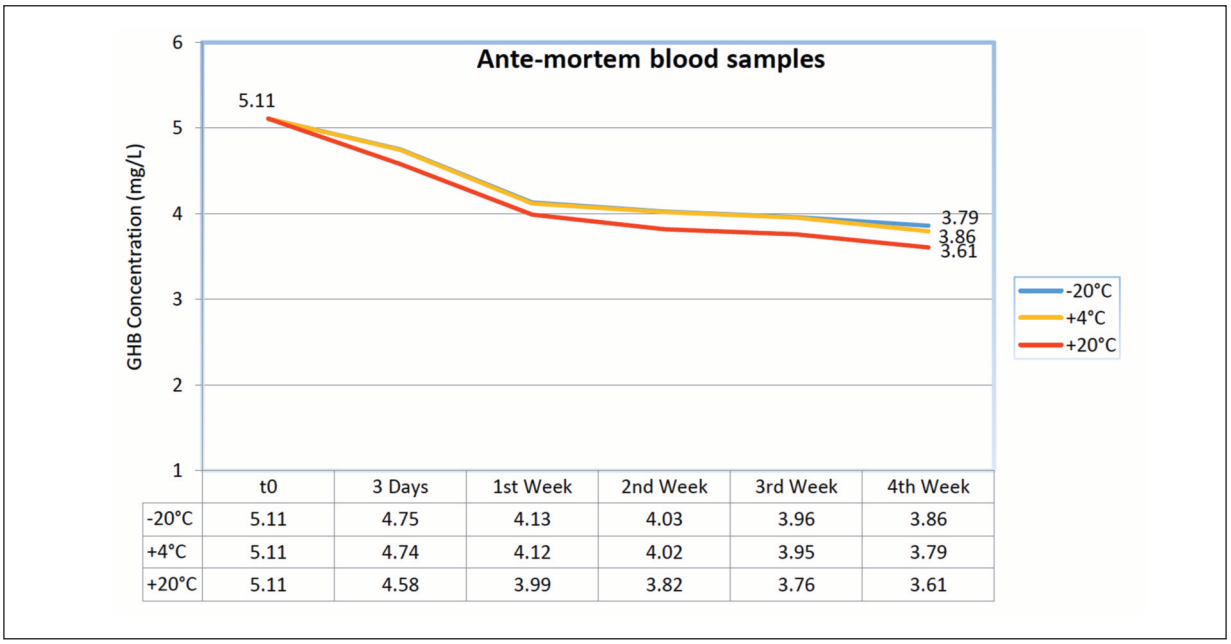
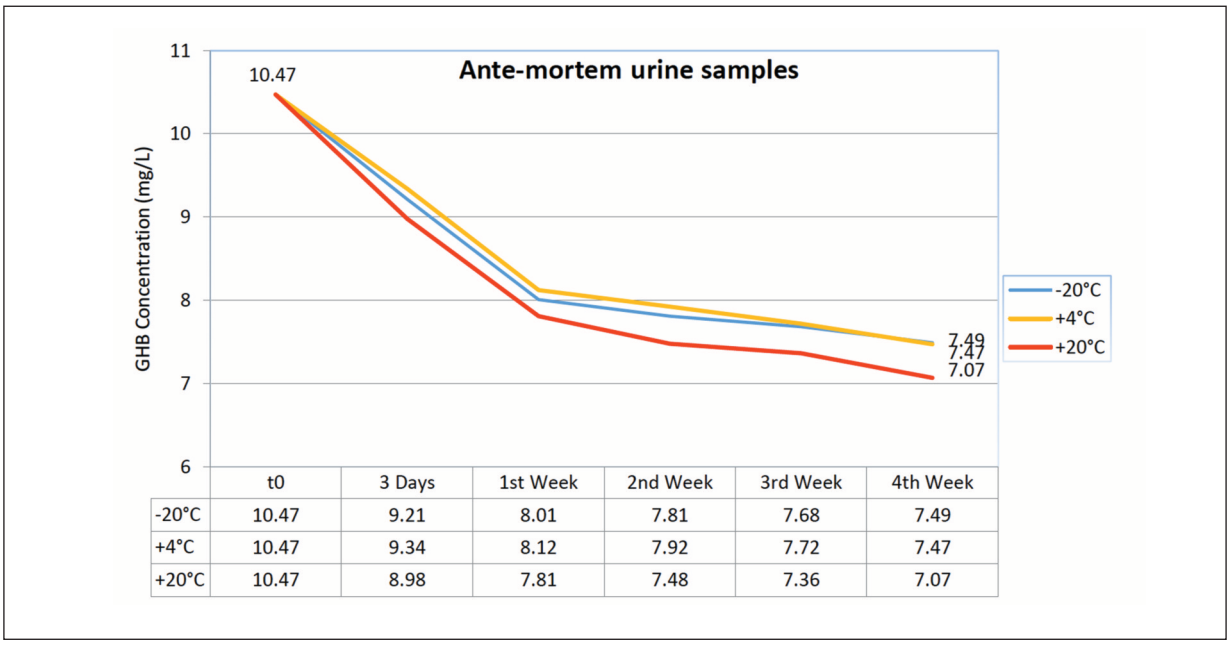


Figure 4. GHB mean values and temporal trend up to 4 weeks of ante-mortem blood specimens spiked with 5 mg/L GHB.



	t0	3 Days	1st Week	2nd Week	3rd Week	4th Week
-20°C	10.47	9.21	8.01	7.81	7.68	7.49
+4°C	10.47	9.34	8.12	7.92	7.72	7.47
+20°C	10.47	8.98	7.81	7.48	7.36	7.07

Figure 5. GHB mean values and temporal trend up to 4 weeks of ante-mortem urine specimens spiked with 10 mg/L GHB.

significant at the 5% level. The mean GHB values and the temporal trend up to 4 weeks are reported in Figure 2.

Post-mortem urine samples spiked with 10 mg/L of GHB showed a reduction of GHB levels lower than 10% only after 3 days of storage for all samples: 4.2% for samples stored at –20°C ($p<0.01$), 4.8% for samples stored at +4°C ($p<0.01$) and 8.4% for samples stored at +20°C ($p<0.001$).

After 4 weeks of storage, there was a mean reduction of GHB levels higher than 20% at all storage temperatures: 22.1% for samples stored at –20°C, 23.9% for samples stored at +4°C and 27.9% for samples stored at +20°C. These decreases were statistically significant: $p<0.001$. The mean GHB values and the temporal trend up to 4 weeks are reported in Figure 3.

Ante-mortem blood samples spiked with 5 mg/L of GHB showed a reduction of GHB levels lower than 10% only after 3 days of storage for samples stored at –20°C (7%) and at +4°C (7.2%), whereas for samples stored at +20°C there was a mean reduction of 10.4% ($p<0.001$).

After 4 weeks of storage, there was a mean reduction of GHB concentrations higher than 20% at all storage temperatures: 24.5% for samples stored at –20°C, 25.8% for samples stored at +4°C and 29.5% for samples stored at +20°C. These decreases were statistically significant: $p<0.001$. The mean GHB values and the temporal trend up to 4 weeks are reported in Figure 4.

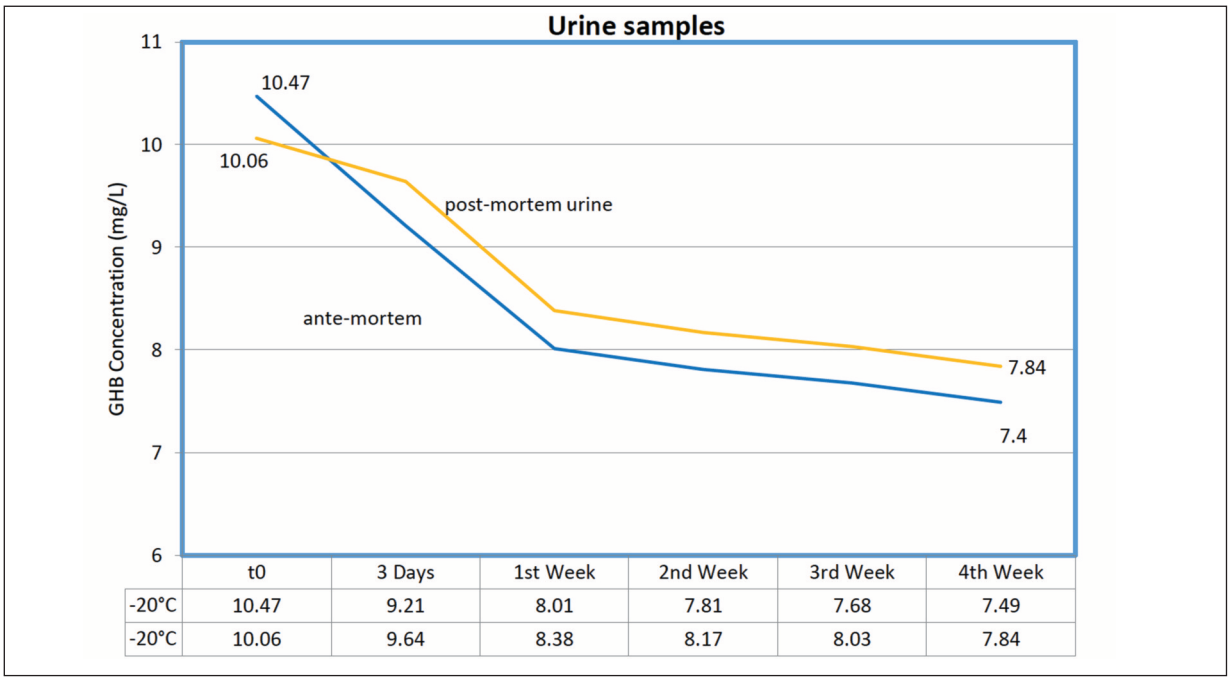
Ante-mortem urine samples spiked with 10 mg/L of GHB showed a reduction of GHB levels higher than 10% after just 3 days of storage for samples kept at all tested temperatures: at –20°C there was a reduction of 12%, at +4°C of 10.8% and at +20°C a mean reduction of 14.2%. All these reductions were statistically significant ($p<0.001$).

After 4 weeks of storage, there was a mean reduction of GHB concentrations higher than 25% at all storage temperatures: 28.5% for samples stored at –20°C, 28.7% for samples stored at +4°C and 32.5% for samples stored at +20°C. These decreases were statistically significant: $p<0.001$. The mean GHB values and the temporal trend up to 4 weeks are reported in Figure 5.

Finally, taking into consideration that both ante-mortem and post-mortem urine specimens were spiked with the same GHB concentration of 10 mg/L, the comparison of the GHB mean levels over 4 weeks did not show any significant statistical difference at all intervals of time at the 5% level as shown in Figure 6.

Discussion

For forensic purposes, toxicologists are frequently requested to repeat analyses, for this rea-



	t0	3 Days	1st Week	2nd Week	3rd Week	4th Week
-20°C	10.47	9.21	8.01	7.81	7.68	7.49
-20°C	10.06	9.64	8.38	8.17	8.03	7.84

Figure 6. Comparison of the GHB mean levels between post-mortem and ante-mortem urine samples, over 4 weeks.

son it is essential to have correct information of the stability of drugs in biological specimens for a reliable interpretation of the results obtained. Taking into consideration the storage conditions (including time, temperature, the addition or not of preservatives, etc.), drug concentrations may change significantly from when they were collected. Some drugs have shown considerable changes in stability terms during the storage period, for this reason wrong interpretations can have numerous legal consequences[13-16].

Due to the high variability of GHB endogenous concentrations as shown in the literature, even though different cut-offs have been proposed: 10 and 5 mg/L for urine and blood samples of living subjects[11,17-19] and 50 and 10 mg/L for post-mortem blood and urine samples, respectively[19,20], sometimes it is difficult to discriminate between endogenous production and exogenous administration. For this reason caution must be used in the interpretation of findings as suggested by Mari et al in the determination of a normal ante mortem GHB concentration in urine specimens[21].

Most studies performed on the formation and stability of endogenous GHB in post-mortem samples at different storage conditions, with or without preservatives, demonstrate findings which are not always consistent[17,22,23].

On the contrary, this present work has investigated the stability of exogenous GHB spiked at three different concentrations, taking into account the suggested cut-off values, in post-mortem blood and urine samples of subjects (whose post-mortem interval was between 20 and 30 hours) where the cause of death could exclude GHB exposure and in blood and urine samples of living GHB-free users. This in order to provide useful information about storage conditions and the time in which the analysis should be performed to avoid or reduce the risk of instability.

In our study, –20°C represents the best storage temperature after 4 weeks of investigation both in ante-mortem and post-mortem blood and urine specimens and there was a statistical significant difference in comparison to the other two storage temperatures ($p<0.001$).

The comparison of GHB mean values at 3 days for samples stored at –20°C and +4°C has shown no statistically significant differences at the 5% level both in ante-mortem and post-mortem blood and urine specimens. Only in one case (ante-mortem urine samples) the mean reduction of GHB levels was lower for samples stored at +4°C in comparison to –20°C (10.8% *vs.* 12%). Post-mortem blood specimens spiked with 50 mg/L of GHB have never shown a reduc-

tion of GHB levels higher than 10% throughout all period of investigation (4 weeks). Only at 4 weeks there was a reduction higher than 10% in samples stored at +4°C and +20°C (10.4% and 12%, respectively).

Currently, the literature on stability of GHB in both ante-mortem and post-mortem blood and urine samples is limited. Only few studies have been carried out on GHB stability. Chen et al[24] found that plasma GHB is stable at –20°C and room temperature for up to 9 months and 48 h, respectively. However, for the evaluation of the long-term (9 months) GHB stability in plasma at the temperature of –20°C, only three samples have been tested and two concentrations investigated (5 and 150 mg/L). Moreover, data obtained from the same study[24] suggest that GHB in processed samples is stable when stored at room temperature for 5 days and for 15 days at –20°C.

Jones et al[25] investigated the stability of GHB in 50 whole blood samples from impaired drivers, stored at 4°C for up to one year, using two different methods. The results obtained illustrated that blood GHB levels are stable when stored at 4 °C for up to 6 months.

Beránková et al[22] studied the GHB stability in blood and urine at 2 different storage temperatures, with and without preservatives. Ante-mortem and post-mortem blood and urine samples were stored with and without NaF (1% w/v) at 4 and –20°C for up to 8 months. Ante-mortem samples showed no significant GHB production. GHB concentrations up to 100 mg/L were found in post-mortem blood after 4 months of storage, at 4°C without preservatives. Storage at –20°C with preservatives found to inhibit GHB production. In post-mortem urine, only slight temporary GHB levels were found (up to 8 mg/L). Moreover, in the same report, the GHB stability was evaluated in 20 post-mortem femoral blood specimens (collected at autopsy from GHB-free individuals) within a 4-month period. After collection, samples were stored at 4°C without any preservation. Just before the analysis all the samples were treated with NaF (1% w/v), with an interval of 16-27 days from collection to fluoridation of specimens. After initial GHB analysis the specimens were stored at 4°C and re-analysis was performed after 2 and 4 months.

Original GHB concentrations of less than 10 mg/L, higher than 25 mg/L and no significant GHB levels were found in six, one and 13 blood samples, respectively. Re-analysis after 2 months of storage showed temporary GHB increase (up to 30 mg/L) with a subsequent general decrease in the following 2 months. At the end of the study the highest GHB level was found to be 6.5 mg/L[22].

Fifty-nine re-analyses were carried out in whole blood samples, of which 27 were ante-mortem samples and 32 were post-mortem samples. The samples were stored at –20°C with preservatives between 0.4 and 7.2 years. The GHB levels in 22 of the samples were below cut-off. In positive ante-mortem samples the mean concentration change from initial analysis to re-analysis was –0.8% (range –32.4-21%), while the corresponding change for the positive post-mortem samples was –7.1% (–30.4-34.4%). All negative samples were still negative when re-analyzed. Authors[13] concluded that re-analysis of these samples does not highlight any significant change in routine practice in GHB levels, when samples are stored at –20°C with fluoride preservation even up to several years.

Conclusions

GHB remains a troublesome substance and stability issues may significantly affect the interpretation of data leading to unreliable conclusions.

According to our findings, it would be useful to perform GHB analysis both in blood and urine specimens within 3 days of sampling and the specimens should be stored at –20°C or 4°C in order to avoid instability issues, which have been highlighted since the first week of investigation at all storage temperatures both in ante-mortem and post-mortem samples. Some practical recommendations are reported in Table I.

Table I. Conclusive remarks and practical recommendations.

1. Blood and urine specimens should be stored at – 20°C or 4°C.
2. Blood and urine analysis should be performed within 3 days of sampling.
3. Taking into consideration the different cut-offs caution should be used in the interpretation of GHB levels both in ante-mortem and post-mortem blood and urine samples.
4. The comparison of GHB levels both in ante-mortem and post-mortem urine specimens did not show any significant statistical difference throughout the period of investigation (4 weeks).

References

1. Schep LJ, Knudsen K, Slaughter RJ, Vale JA, Megarbane B. The clinical toxicology of gamma-hydroxybutyrate, gamma-butyrolactone and 1,4-butanediol. Clin Toxicol 2012; 50: 458-470.
2. Carter LP, Pardi D, Gorsline J, Griffiths RR. Illicit gamma-hydroxybutyrate (GHB) and pharmaceutical sodium oxybate (Xyrem): differences in characteristics and misuse. Drug Alcohol Depend 2009; 104: 1-10.
3. Nicholson KL, Balster RL. GHB: a new and novel drug of abuse. Drug Alcohol Depend 2001; 63: 1-22.
4. Frati P, Busardo FP, Cipolloni L, Dominicis ED, Fineschi V. Anabolic androgenic steroid (AAS) related deaths: autoptic, histopathological and toxicological findings. Curr Neuropharmacol 2015; 13: 146-159.
5. Busardo FP, Frati P, Di Sanzo M, Napoletano S, Pinchi E, Zaami S. The impact of nandrolone decanoate on the central nervous system. Curr Neuropharmacol 2015; 13: 122-131.
6. Busardo FP, Bertol E, Vaiano F, Baglio G, Montana A, Barbera N. Post mortem concentrations of endogenous gamma hydroxybutyric acid (GHB) and in vitro formation in stored blood and urine samples. Forensic Sci Int 2014; 243: 144-148.
7. Hibell B, Guttormsson U, Ahlström S, Balakireva O, Bjarnason T, Kokkevi A, Kraus L. The 2007 ESPAD Report. Substance Use Among Students in 35 European Countries, Stockholm, 2009. Available on line at: http://www.emcdda.europa.eu/attachements.cfm/att_77168_EN_ESPAD_summary_EN.pdf Accessed on: September 5th, 2015.
8. EMCDDA. Report on the risk assessment of GHB in the framework of the joint action on new synthetic drugs, Lisbon: European Monitoring Centre for Drugs and Drug Addiction 2002. Available on line at: http://www.emcdda.europa.eu/html.cfm/index33345EN.html Accessed on: October 20th, 2015.
9. Busardò FP, Kyriakou C, Napoletano S, Marinelli E, Zaami S. Mephedrone related fatalities: a review. Eur Rev Med Pharmacol Sci 2015; 19: 3777-3790.
10. Independent Drug Monitoring Unit. Gamma Hydroxy Butyrate Usage, Effects and UK Prices. Available on line at: http://www.idmu.co.uk/ghb.htm Accessed on: October 26th, 2015.
11. Elliott SP. Gamma hydroxybutyric acid (GHB) concentrations in humans and factors affecting endogenous production. Forensic Sci Int 2003; 133: 9-16.
12. Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int 2007; 165: 216-224.
13. Fjeld B, Burns ML, Karinen R, Larssen B, Smith-Kielland A, Vindenes V. Long-term stability of GHB in post-mortem samples and samples from living persons, stored at -20 degrees C, using fluoride preservatives. Forensic Sci Int 2012; 222: 47-51.
14. Giorgi SN, Meeker JE. A 5-year stability study of common illicit drugs in blood. J Anal Toxicol 1995; 19: 392-398.
15. Peters FT. Stability of analytes in biosamples-an important issue in clinical and forensic toxicology? Anal Bioanal Chem 2007; 388: 1505-1519.
16. Busardo FP, Kyriakou C, Tittarelli R, Mannocchi G, Pantano F, Santurro A. Assessment of the stability of mephedrone in ante-mortem and post-mortem blood specimens. Forensic Sci Int 2015; 256: 28-37.
17. LeBeau MA, Christenson RH, Levine B, Darwin WD, Huestis MA. Intra- and interindividual variations in urinary concentrations of endogenous gamma-hydroxybutyrate. J Anal Toxicol 2002; 26: 340-346.
18. Busardo FP, Jones AW. GHB pharmacology and toxicology: acute intoxication, concentrations in blood and urine in forensic cases and treatment of the withdrawal syndrome. Curr Neuropharmacol 2015; 13: 47-70
19. Busardo FP, Kyriakou C. GHB in Biological Specimens: which cut-off levels should be taken into consideration in forensic toxicological investigation? Recent Pat Biotechnol 2015; 8: 206-214.
20. Kintz P, Villain M, Cirimele V, Ludes B. GHB in postmortem toxicology. Discrimination between endogenous production from exposure using multiple specimens. Forensic Sci Int 2004; 143: 177-181.
21. Mari F, Politi L, Trignano C, Di Milia MG, Di Padua M, Bertol E. What constitutes a normal ante-mortem urine GHB concentration? J Forensic Leg Med 2009; 16: 148-151.
22. Berankova K, Mutnanska K, Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. Forensic Sci Int 2006; 161: 158-162.
23. Elliott SP. Further evidence for the presence of GHB in postmortem biological fluid: implications for the interpretation of findings. J Anal Toxicol 2004; 28: 20-26.
24. Chen M, Andrenyak DM, Moody DE, Foltz RL. Stability of plasma gamma-hydroxybutyrate determined by gas chromatography-positive ion chemical ionization-mass spectrometry. J Anal Toxicol 2003; 27: 445-448.
25. Jones AW, Gladh SA, Windberg CN, Johansen SS. Stability of gamma-hydroxybutyrate in blood samples from impaired drivers after storage at 4 degrees C and comparison of GC-FID-GBL and LC-MS-MS methods of analysis. J Anal Toxicol 2015; 39: 294-299.