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TECHNICAL NOTE

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Chemical Composition and Structure of the Microcrystals Formed Between Silver(I) and γ-Hydroxybutyric Acid and γ-Hydroxyvaleric Acid

ABSTRACT: This study examined microcrystals formed by silver with γ-hydroxybutyric acid (GHB) and γ-hydroxyvaleric acid (GHV), the five-carbon analog of GHB, in the presence of silver, copper, and lanthanide nitrates. Distinct microcrystals formed with silver (+1) and lanthanum (+3) ions but not with the copper (+2) ions. The crystals formed with GHB were distinctly different than those formed with GHV and in all cases, the drug microcrystals were easily distinguishable from reagent crystals. X-ray diffraction analysis provided definitive structure for the microcrystals. The morphological differences between the silver-GHB and silver-GHV crystals were characterized using simple measurements such as size and angles provided by image recognition software. The utility of the test for casework was demonstrated using spiked beverage samples.

KEYWORDS: forensic science, drug analysis, GHB, γ-hydroxyvaleric acid, GHV, microcrystal tests, X-ray diffraction, crystallography, crystal morphology, image analysis

Microcrystal tests have been used for the analysis of drug and other analytes since the 1800s. Crystals that form in the presence of an analyte and reagent are identified based on their morphology and behavior when observed using polarized light microscopy. However, the specific chemical identity of the crystals is rarely known. While it is recognized that crystal tests are of value (1–4), their place in the forensic analytical scheme is no longer central. This can be attributed to the lack of analysts trained in microcrystal recognition and the subjective nature of such identifications. Presumptive and screening tests combined with instrumental methods further reduce the need for crystal tests. However, there are types of evidence not amenable to traditional color-based presumptive tests. In such cases, a viable crystal test would be useful, particularly if the crystal is distinctive and can be unambiguously associated through known structure and quantitative measurements to a specific drug or analyte.

Examples of such a situation are predator drugs delivered to victims in drinks. Of current concern is γ-hydroxybutyric acid (GHB) and related compounds (5,6). The matrix in which drugs such as GHB are delivered is complex and often colored, making it difficult or impossible to employ tests based on color changes. Even if a color test is feasible and yields a positive result, color differences are not commonly associated, even anecdotally, with specific compounds. GHB provides a case in point. Many of the color test reagents used to screen samples for this drug are acid/base indicators (7) that target the weak acid character of GHB. A positive result, even when it can be obtained, is only marginally informative in comparison with information provided by a positive microcrystal test. However, this is only true when crystal morphology can be unambiguously assigned to the specific drug or analyte in question.

In this study, microcrystal tests were evaluated for GHB and γ-hydroxyvaleric acid (GHV), a 4-methyl substituted analog of GHB which produces comparable psychotropic effects (6). GHV was included as it appears to be an emerging drug threat similar to GHB that is likely to present similar analytical challenges (6). The closed-ring lactone forms of GHB and GHV (γ-butyrolactone (GBL) and γ-valerolactone (GVL), respectively) were also tested as some fraction of the drug will be in the closed form depending on the pH of the matrix (8–10).

The crystal test reagents selected were ones previously reported as useful for GHB and included a silver/copper reagent (8). This reference described the use of a solution containing 1% each of Ag^+ and Cu^{2+} tested with GHB and related four-carbon compounds as well as with other drugs. Distinctive crystals were produced for GHB within 5 min at the periphery of the drop with a reported detection limit of *c.* 2.0 mg/mL (0.2% w/w). No crystals formed with GBL. A report was located referring to a lanthanum reagent (11), although the reference was described on a webpage that provided little specific information. The webpage has since been removed, but lanthanum is a logical choice for crystal testing given its size and charge. Therefore, it was included for evaluation.

A key goal of the present study was the unambiguous assignment of chemical composition and structure of microcrystals. One effective method to characterize a crystal is X-ray diffraction (XRD). This crystallographic method works well with large atomic weight atoms such as gold, platinum, silver, and copper that are used in many forensic microcrystal tests. Although XRD has been

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used in forensic applications (12–18), a literature search failed to identify any studies in which this technique has been applied to microcrystals of interest or current use in forensic chemistry.

XRD can be conducted on powders or using a single ordered crystal as was the case here. Exposing the crystal to X-ray radiation generates a series of internal reflections and interactions. The resulting patterns of constructive and destructive interaction can be related to unit cell structure and this was the technique incorporated here. Additional characterization was accomplished using image analysis and measurement techniques. The large crystals formed in the presence of GHB and GHV were proven to have incorporated the drug into the crystal cell structure in an orderly and reproducible fashion.

Methods and Materials

GHB, GBL, and GVL were purchased from Sigma Aldrich (St. Louis, MO). GHV was synthesized in-house from the GVL and the identity and purity confirmed using NMR analysis. To perform the crystal test, one drop of sample solution containing the analyte of interest was placed on a glass microscope slide, followed by a drop of crystallizing reagent. For initial work, aqueous solutions of the two analytes were also examined. The resulting solutions of analyte and reagent on the slide were dried at room temperature and examined using a Leica DMLP polarizing microscope (Leica Microsystems USA, Bannockburn, IL) at a total magnification of ×100. Photographs were taken using a QICAM QIMAGING Fast 1394 camera using Image Pro Plus® software. Individual crystals were isolated using micromanipulation techniques and diffraction data was obtained with a Siemens P4 diffractometer (Bruker AXS USA, Madison, WI) equipped with a CCD detector and then analyzed using SHELXTL crystallographic software.

The crystallizing reagents used are summarized in Table 1. All were prepared from solid standards (8) at a concentration of 1.0% (w/v) for the ion from nitrates and hydrated nitrate salts. For initial testing, solutions of 1000 p.p.m. GHB, GHV, GBL, and GVL were prepared in methanol and distilled water. Methanol was originally used to speed drying of the crystals but was abandoned due to excess spreading on the microscope slides. Case samples were simulated using a light beer, Coca-Cola® (Coca-Cola, Atlanta, CA), Sprite® (Coca-Cola), a white wine, and mixed drinks consisting of vodka/cranberry juice and tequila/Sprite®. Samples were spiked at 1.0% of GHB and GHV (separately) and tested at this concentration and at dilutions as described below. The stock and diluted samples included the concentrations typically seen in drug facilitated sexual assault cases (8).

Extractions of the simulated case samples were performed using a previously published extraction technique (9). Approximately 5 mL of the sample was placed in a large test tube and acidified to a pH of 2–3 using dilute HCl and measured using 0–14 pH paper. The acidified solution was extracted with chloroform and the organic layer discarded. The aqueous layer was saturated with NaCl and extracted with ethyl acetate. The ethyl acetate portion was dried with gentle heating.

TABLE 1—*Test reagents.*

Single Ion (0.1% w/v)	Combined Ions (1% each w/v)
Ag^+	Ag^+ and Cu^{2+}
Cu^{2+}	Ag^+ and La^{3+}
La^{3+}	La^{3+} and Cu^{2+}
	$Cu^{2+}/Ag^+/La^{3+}$

Results and Discussion

Distinctive rectangular and parallelogram-shaped crystals were observed for GHB and GHV samples respectively with reagents containing silver and lanthanum. No crystals formed using copper ion alone, nor were crystals formed when the solution of all three ions was used. The microcrystals observed in this study match the descriptions and dimensions of those reported in the earlier work (8). No crystals formed for either of the closed-form lactones (GVL and GBL) with any of the microcrystal reagents. Representative photomicrographs are as shown in Fig. 1.

Generally, the crystals formed within 10 min and developed from the periphery of the drop. The crystals persisted when dry, indicating that the time to observation is not a critical variable. The crystals formed in the presence of lanthanum were too fragile to transfer to the XRD and were not studied further. This fragility may be attributable to a less stable crystal structure resulting from the insertion of a 3+ ion in the lattice, but this hypothesis could not be explored or confirmed. All subsequent work undertaken and described utilized the 1% solution of silver nitrate.

The width of the GHV crystals was typically larger (40–60 μm) than those associated with GHB (20–40 μm), particularly early in the crystal formation process. Some of the GHV crystals developed ragged terminal edges in the latter stages of development. Crystals were also observed with reagents alone, but these dendritic (copper/silver metal), rhombus, and hexagonal shaped crystals were distinctly different from those generated in the presence of the drugs. In all trials, three to five separate slides were prepared as replicates and photomicrographs of several crystals at different reagent concentrations were studied to generate measurements as summarized in Table 2.

Contrary to the earlier report (8), the presence of copper ion was not required for crystal formation; microcrystals formed readily with reagents containing only silver nitrate and only lanthanum nitrate. This may be related to different methods of reagent preparation. The present work used hydrated nitrates as starting materials whereas the earlier work relied on a copper nitrate derived from a copper chloride solution. This solution was treated with silver nitrate to precipitate out the chloride as AgCl, but silver ions likely remained in the solution. Regardless, the description of the GHB microcrystals from this earlier work closely matches what was obtained here.

Structural analysis using XRD revealed that in the case of the GHB crystal, the asymmetric unit within the unit cell consist of two Ag^+ and two chelating GHB anions producing a dimeric $[Ag(O_2CCH_2CH_2CH_2OH)]_2$ unit (Fig. 1). This dimeric molecular unit is connected to adjacent $[Ag(O_2CCH_2CH_2CH_2OH)]_2$ units via a pair of Ag–O bonds. This arrangement affords an extended, nearly planar molecular strand directed parallel to the facial plane of the crystal lattice. The immediate coordination geometry about each Ag^+ is pseudo-square planar. The four atoms bonded to each Ag^+ ion consist of a carboxylate O atom from each chelating butyrate ligand, the other silver atom, and a bridging carboxylate O atom from an adjacent symmetry-related dimer.

The molecular strands held together by intermolecular hydrogen bonds between the terminal hydroxyl groups located at the perimeter of each strand. The resultant three dimensional structures consist of layers of these hydrogen bonded strands, and is further ordered by weaker Ag–O interactions between parallel layers. The structure is highly organized and shows no indication of disorder with the lattice.

In the case of GHV, the crystallographic asymmetric unit consists of three Ag^+ and three GHV anions producing a trimeric [Ag

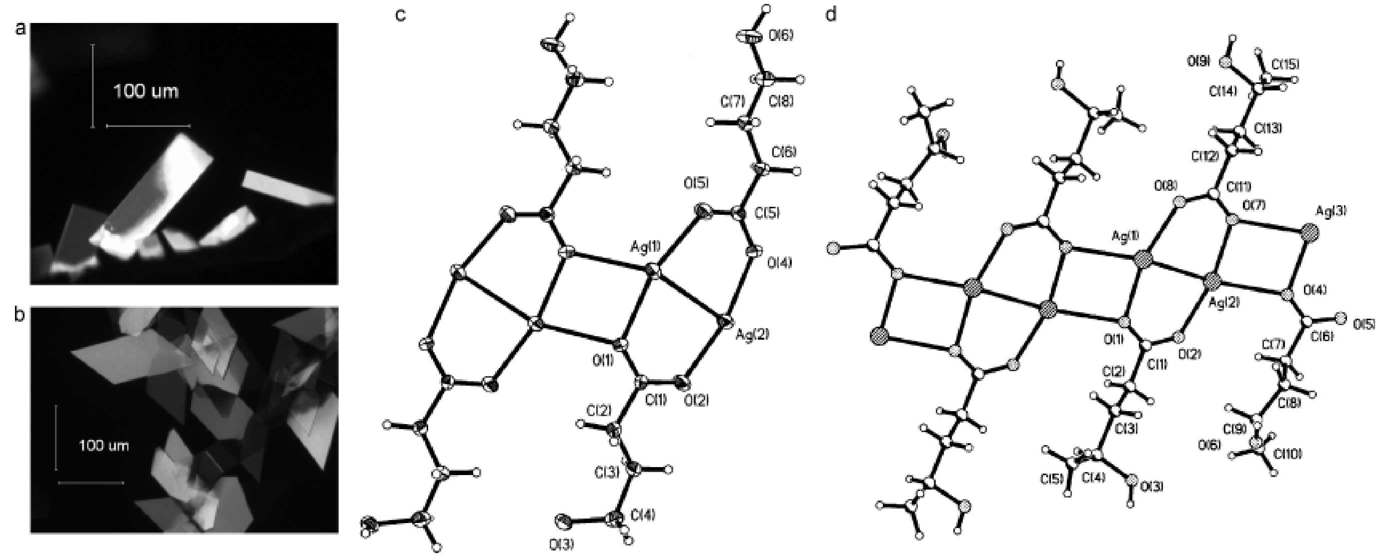

FIG. 1—*(a) Photomicrograph of crystal formed with γ-hydroxybutyric acid and silver ion and structure determined by X-ray diffraction (right, c). (b) Photomicrograph of crystal formed with γ-hydroxyvaleric acid and silver ion and structure determined by X-ray diffraction (right, d).*

$(O_2CCH_2CH_2CMeHOH)]_3$ unit. The solid state structure of this system is comparable with that observed for the Ag–GHB analog. However, the hydrogen-bonding network between the terminal hydroxyl groups of adjacent strands is disrupted by the crystallographic disorder of the chiral CHMeOH group for two of the three GHV chelating ligands. The spatial arrangement of atoms about each Ag is also pseudo-square planar and weak Ag–O interactions similarly exist between the parallel layers within the lattice. The presence of the methyl group of the GHV anion lowers the overall lattice symmetry from monoclinic for $[Ag(O_2CCH_2CH_2CH_2OH)]_{2n}$ to triclinic for $[Ag(O_2CCH_2CH_2CMeHOH)]_{3n}$, thus explaining the difference in morphology (rectangle vs. parallelogram) for GHB and GHV, respectively.

The behavior of the crystals under crossed polars was consistent with their thin plate structure. They appeared gray and showed maximum brightness at 45°, as can be seen with some of the crystals shown in Fig. 1. Michel–Levy colors indicated that the Ag–GHB structure was thicker compared with the Ag–GHV. Thin-film interference colors were also observed. Knowledge of the crystal structure explains why the lactone (ring) forms of the two drugs did not form the characteristic crystals. An open chain form is required for the drug moiety to be incorporated into the crystal. The pH of the crystal test reagent was nearly neutral and not sufficiently basic to drive the lactone ring to open. In no case was copper or lanthanum detected in the crystal structure. Given that the crystals formed in the presence of silver ions alone were indistinguishable from those formed when other ions were present, it is unlikely that either of the other ions (lanthanum or copper) were ever incorporated in the crystal structures.

Analysis of spiked water samples using silver solutions showed the distinct crystals observed previously for GHB and GHV over a concentration range of 1–0.01% (w/v of the drug). Distinctive crystals were also observed in the sodas (Coke® and Sprite®) and the Sprite®/tequila drink, but not in the wine, beer, or the mixed drink sample of vodka and cranberry juice. It was thought that the thicker viscosity of these samples when dried played a role in this inhibition. To remove interfering components, the beer, wine, and mixed drink samples were extracted as previously described by acidification, chloroform wash, and ethyl acetate extraction.

Success of the extraction of the spiked samples was verified using a validated HPLC analysis (19,20) which showed two peaks consistent with GHB and a smaller GBL peak. However, when the residue was rehydrated and a drop tested with silver nitrate solution, no characteristic crystals were seen despite repeated attempts. It is possible that the extraction procedure concentrated the GHB/GHV as well as substances that interfered with crystal formation. Sugars are one possibility that would not be detected using the HPLC method applied here.

Further attempts to produce crystals using a toluene wash of the samples (8) and dilution were also unsuccessful. An important clue to the observed problems may lie in the initial tests, which showed that the tequila and Sprite® drink produced crystals while the mixed drink consisting of cranberry juice and vodka did not. It is conceivable that juice and related fermentation products such as beer, wine, and juice contain extractable compounds at sufficient concentrations to inhibit or interfere with crystal formation. Further investigation is underway.

TABLE 2—*Crystal descriptors.*

Sample	Shape	Average Internal Angle ($n = 7$)	Standard Deviation	%RSD	Confidence Interval (95%)
GHB (1000 ppm) $AgNO_3/CuNO_3$	Rectangular	90.4°	0.59	0.66	± 0.50
GHV (1000 ppm) $AgNO_3/CuNO_3$	Parallelogram	Acute: 49.3°	1.74	3.56	± 1.6
		Obtuse: 126.0°	1.03	0.81	± 0.95
GHB (5000 ppm) $AgNO_3/LaNO_3$	Rectangular	89.9°	0.39	0.44	± 0.29
GHV (5000 ppm) $AgNO_3/LaNO_3$*	Parallelogram	N/A	N/A	N/A	N/A

*Crystals formed were irregular with many appearing broken. Angle measurements were not possible.
GHB, γ-hydroxybutyric acid; GHV, γ-hydroxyvaleric acid.

Conclusions

Microcrystal tests can fulfill a niche as presumptive tests when color-based tests are not feasible. Here, it was possible to identify GHB and GHV in some complex matrices and to clearly differentiate the two based on crystal morphology. When the atomic arrangement of a crystal can be positively determined and associated with a specific drug, the microcrystal test is no longer a presumptive test but a conclusive one. The key is the ability to recognize the crystal and apply quantitative measures such as size and angles to verify analyst interpretation. The method can be applied to many types of beverages, but for wine, beer, and some mixed drinks, some type of additional sample extraction and preparation will be needed before the method can be generally applied to casework.

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