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TECHNICAL NOTE

TOXICOLOGY

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Enzymatic Detection of *Gamma*-Hydroxybutyrate Using Aldo-keto Reductase 7A2*,†

ABSTRACT: *Gamma*-hydroxybutyrate (GHB) is a prescribed medication as well as a drug of abuse. Its detection in various matrices for in-field forensic scientists remains a challenge. We have developed an assay that uses aldo-keto reductase 7A2 (AKR7A2) for the specific determination of GHB in various drinks. AKR7A2 was purified using Ni-affinity chromatography. The Michaelis-Menten constant for the GHB oxidation reaction was 10 mM, and the minimum detection limit was 4 mM. Ethanol was not a substrate for AKR7A2. In a coupled reaction with $NADP^+$, phenazine methosulfate (PMS), and 2,6-dichlorophenolindophenol, various beverages (orange juice, milk, soda, and numerous alcoholic drinks) containing GHB turned from blue to light yellow. In a second coupled reaction where diaphorase replaced PMS, the presence of GHB also caused the expected change of color in various beers.

KEYWORDS: forensic science, drug abuse, detection, g*amma*-hydroxybutyrate, *gamma*-hydroxybutyric acid, enzymatic, succinic semialdehyde reductase, aldo-keto reductase 7A2

Gamma-hydroxybutyrate (GHB) can be prescribed as a medication. Because of its ability to interact with $GABA_B$ receptors and probably with GHB receptors and subtypes of $GABA_A$ receptors, it is effective in the treatment of narcolepsy (Xyrem®), alcohol dependence (Alcover®), and it is also used as an intravenous anesthetic (Somsanit®) (1). Because of the same set of properties, GHB is also a drug of abuse—becoming a Schedule I Controlled Substance in the U.S.A. in 2000 (2). While GHB is not used as prevalently as other drugs of abuse, a 2009 Monitoring the Future report from the National Institute on Drug Abuse found an average usage rate of about 1% among 8th, 10th, and 12th graders in 2003–2008 (3), and the Drug Abuse Warning Network estimates 1225 annual visits in 2003–2006 for GHB-related emergency room visits (4). GHB use in drug-facilitated sexual assaults has been extensively discussed in the literature (5–8). In addition, a study based on the data from 259 patients in Sweden concluded that "intoxication by GHB has substantial morbidity and abuse of GHB has substantial mortality" (9). In 2002, the GHB-related death count was 11 in Europe (10) and 72 in the U.S.A. (2). In a publication in 2008, the forensic toxicological analysis of samples from 49 GHB-related deaths in Sweden alone was reported (11).

The development of GHB detection methods remains an important issue. Several accurate and selective methods for GHB detection in forensic laboratories have been developed and many are based on chromatography-mass spectroscopy (12–17). Additionally, NMR spectroscopy (18,19), capillary electrophoresis (20), and IR spectroscopy (21) have also been used to detect GHB. While these methods can be robust and precise, they are unsuitable for in-field work because of the required instrumentation. A relatively simple visual colorimetric method has been reported (22), and Raman spectrometers have been used for the detection of GHB (23); however, the former uses harsh chemicals and the latter requires a portable specialized instrument that might not be universally available. Commercially available "date-rape" drug test kits (coasters, cards) have been tested, but they were shown to not work in all drinks and an unadulterated drink often is needed as a comparison to make an accurate determination of the presence of GHB (24). That makes the use of these kits impractical in the majority of situations. An enzymatic assay for GHB detection has been reported; however, it produced false positives in ethanol-containing solutions and ethanol had to be evaporated prior to GHB determination, which also makes it less practical (25).

The purpose of this study was to develop an enzymatic method of detecting GHB in beverages that did not show false positives in the presence of ethanol. GHB is a substrate for at least five different enzymes. Aldehyde dehydrogenase reduces it to *gamma*-hydroxybutyraldehyde and lactonase cyclizes it into *gamma*-butyrolactone. There are three enzymes which oxidize GHB to succinic semialdehyde, namely, two aldo-keto reductases, AKR7A2 and AKR5A1, and an iron-dependent alcohol dehydrogenase

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ADHFe1 (26). We used AKR7A2, which is also called succinic semialdehyde reductase (27,28), to develop an assay that can detect GHB in various beverages without having ethanol produce false positives.

Materials and Methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. The work with GHB was permitted by the appropriate federal and state licenses.

Enzyme Purification

The pET 15b vector with a AKR7A2 gene with a six His tag, a gift from Dr. M. Picklo's laboratory (27,28), was transformed into competent BL21 (DE3) pLysS *E. Coli* (Promega, Madison, WI) using typical molecular biology protocols (29). The cells were grown overnight at 37°C in the LB media with 25 μg/mL ampicillin, spun down at 5000 × *g* for 10 min, resuspended in a prechilled buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 10 mM imidazole to a final concentration of 0.1 g wet weight bacteria/mL, and lysed with a French Press (SIM-AMINCO Spectronic Instruments, Rochester, NY) or a Branson Sonifier 450A (Branson, Danbury, CT). The lysate was centrifuged for 10 min at 20,000 × *g* at 4°C and the supernatant was collected. The Ni-nitrilotriacetic acid (Ni-NTA; Qiagen, Valencia, CA) column was equilibrated with a buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 10 mM imidazole, and the supernatant was run through the column. The column was washed with 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 100 mM imidazole. AKR7A2 was eluted from the column with 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, and 150 mM imidazole. The purified protein's buffer was immediately exchanged with a 100 mM sodium phosphate buffer (pH 6.8) using 10 kDa MWCO spin columns (Millipore, Bedford, MA). Protein concentrations were assessed via a Bradford assay using bovine serum albumin as a standard (29). AKR7A2 purification was confirmed by SDS–PAGE, Western blot (primary antibody was a gift from Dr. M. Picklo's laboratory), and MALDI-TOF Autoflex (Bruker Daltonics, Billerica, MA). Photographs were taken with BioDoc-It TM System (UVP, Upland, CA). The purified enzyme was aliquoted into approximately 20-μL fractions containing 50 μg of protein to avoid any freeze-thawing and stored at −80°C.

Kinetic Assays

A 96-well Microplate Power Wave XS Universal Spectrophotometer (Bio-Tek, Winooski, VT) or Genesis 10 UV Scanning Spectrometer (Thermo Spectronic, Rochester, NY) were used for the experimentation. All experiments were run in triplicate. For analysis of AKR7A2 activity, the reactions were followed at 340 nm with 50 μg of AKR7A2 in 0.9 M Tris–HCl (pH 8.8), 50 mM GHB, 1 mM $NADP^+$. The coupled color reactions were followed visually and also at 600 nm with 50 μg of AKR7A2 in 0.9 M Tris–HCl (pH 8.8), 50 mM GHB, 1 mM $NADP^+$, 0.05 mM 2,6-dichlorophenolindophenol (DCIP), and 0.015 mM phenazine methosulfate (PMS) (30). The reaction plates were also photographed. Michaelis–Menten constant (K_m) was calculated by fitting data using Microsoft® Excel Solver. For reactions coupled with a second enzyme, PMS was replaced by 5 μg of diaphorase from *Clostridium kluyveri*. Diaphorase reactions were monitored visually and at 600 nm in 0.9 M Tris–HCl (pH 8.8), 0.1 mM NADPH, 0.05 mM DCIP. A solution of 95% ethanol was diluted to the indicated concentrations to test the effects of ethanol. Common beverages (orange juice, soda, milk), spirits (vodka, gin, rum, tequila, and Blue Curacao), beers (classic, red amber, and porter), red wines (Zinfandel, Shiraz, Pinot Noir, Cabernet Sauvignon), and a white wine (Chardonnay) were purchased commercially and used as 30% volume of a reaction volume.

Results and Discussion

The transfection of competent BL21 (DE3) pLysS *E. coli* cells with pET 15b vector with an AKR7A2 gene was successful. The vector appeared to be "leaky"—the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) was not necessary, nor did it affect the yield of AKR7A2. The presence of AKR7A2 was not toxic to bacteria, and the overall cost for the experiments was reduced by omitting IPTG. Resuspending bacterial pellets in lysis buffer at 0.1 g/mL was found to be optimum for the maximum yield of total protein. Sonication at its optimal settings (power of 3, 40% cycle, 1 min/1 mL of lysis mixture) and lysis with French press both yielded about 10 mg of crude protein/mL of culture. The wash and elution processes were optimized for affinity Ni-NTA chromatography by the stepwise change of imidazole concentrations to yield purified AKR7A2. The buffer exchange to remove imidazole before protein storage proved to be essential as AKR7A2 lost activity after freezing in the elution buffer. The finalized protocol, which is described in Materials and Methods, yielded pure protein fractions that were analyzed by SDS–PAGE (Fig. 1). The flow-through and wash fractions were devoid of our target protein because its histidine tag bound to the Ni-NTA column, while the optimized elution fraction contained pure AKR7A2, which was removed from the column by the excess imidazole. Western analysis detected significant amounts of AKR7A2 in crude and elution fractions only, with the flow-through and wash fractions having no AKR7A2 (data not shown). According to SDS–PAGE, the estimated MW of successfully purified AKR7A2 was about 40 kDa. MALDI-TOF indicated the MW of 38,591 Da with a His tag and 37,249 Da for AKR7A2 with six His tag cleaved off by thrombin. The MW of AKR7A2, as reported in (27), was 45 kDa for SDS–PAGE and 38.8 kDa for MALDI-TOF. The cleavage of the six His tag from the recombinant AKR7A2 by thrombin did not change the specific activity of the protein (data not shown). Thus, AKR7A2-His tag was used for all enzymatic assays. Storage at −80°C did not affect the specific activity (data not shown). The method reported here differs from the protein purification procedure in (28) by the lack of use of IPTG, different imidazole concentrations, and the lack of thrombin cleavage, which saves another column purification step.

Enzymatic detection of GHB using AKR7A2 was initially run without PMS and DCIP by following the generation of NADPH at 340 nm. These reagents were then used to create an assay that

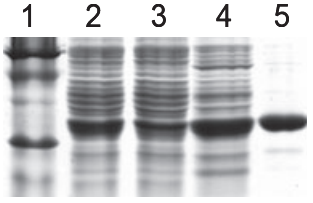

FIG. 1—*SDS–PAGE analysis of fractions of aldo-keto reductase 7A2 purification: 10 μg of MW markers (1), 30 μg of proteins from crude (2), flow-through (3), and wash (4) fractions, and 5 μg of purified protein from the elution fraction (5).*

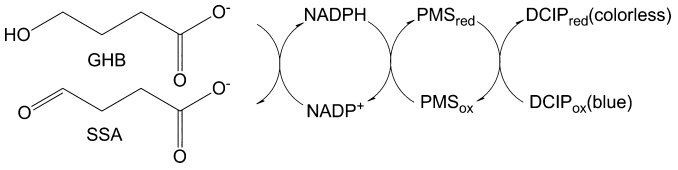

FIG. 2—*The coupled aldo-keto reductase 7A2 reaction.* Gamma-*hydroxybutyrate (GHB) is oxidized to succinate semialdehyde (SSA) to produce NADPH, which can be measured at 340 nm with a UV spectrophotometer. The addition of phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP) allows for the visualization of this reaction. PMS may also be replaced by the enzyme diaphorase.*

could be visualized or measured at 600 nm (Fig. 2). In the presence of GHB, AKR7A2, $NADP^+$, PMS, and DCIP, the dark blue solution turned light yellow in about 3 min. The increase in the amount of enzyme added yielded a linear increase in activity, as expected, indicating that reagents used were in sufficient excess and the enzyme was the limiting reagent.

The reaction's conditions and the enzyme storage conditions were optimized by investigating the effects of varying concentrations of different buffers (Tris, HEPES, MOPS, phosphate), changing pH, and checking the effects of several different reagents (NaCl, $CaCl_2$, $ZnCl_2$, PEG). The optimum conditions were found as described in Materials and Methods. Most of the variables had a small effect on the reaction, except for pH (Table 1). We observed that the AKR7A2-diaphorase-DCIP reaction for the oxidation of GHB was maximal at a pH of 8.8 and almost nonexistent at a pH of 5.5. This is in contrast to a previous report that found the activity of AKR7A2 was decreased only twofold with increasing pH from 5 to 8 for the reduction of 2-carboxybenzaldehyde (28). Considering the low pH and high ionic strength of the drinks that we are proposing to use this assay for, we used a reaction media containing 0.9 M Tris, pH 8.8. The high concentration of Tris favored the reaction, it buffered drinks well, and did not affect absorbance of most drinks studied (exceptions are noted in the text below).

The ability of the AKR7A2-diaphorase-DCIP reaction to detect varying amounts of GHB was determined (Fig. 3*A*). The Michaelis–Menten kinetic analysis revealed that the Michaelis constant was 10 mM, and the minimum detection limit (MDL) was 4 mM. K_m values for the AKR7A2 reaction with and without PMS-DCIP were almost identical to the value listed above (data not shown). The reported K_m for succinic semialdehyde reduction for AKR7A2 (direct reaction) was 0.0154 mM (28). Having the K_m of 10 mM for the reverse reaction (oxidation of GHB) is not completely unexpected. The K_m of GHB dehydrogenase for GHB was reported 2.19 mM (25), which was almost five times smaller than 10 mM, generally allowing the assay described by Bravo et al. to detect lower concentrations of GHB. However, if one considers a dose of GHB necessary to drug a person, known to be about 2 g of GHB per drink (31), the final molar concentration of GHB in our assay would be 13.2 mM in beer samples (0.36 L per large glass), 23.8 mM in soft drink and wine samples (0.2 L per glass), and 95.2 mM in strong alcoholic drinks (0.05 L per shot). Thus, K_m of 10 mM should be sufficient for the determination of GHB in those drinks using AKR7A2.

The effects of ethanol on AKR7A2 were determined by performing the AKR7A2-PMS-DCIP reaction with and without GHB at different ethanol concentrations (Fig. 3*B*). As confirmed visually and spectrophotometrically, AKR7A2 exhibited no activity in ethanol alone even at very long reaction times, and its reaction with GHB was accelerated by the presence of ethanol at concentrations at or below 12%. It is worth noting that we observed an increase

TABLE 1—*Relative specific activity of aldo-keto reductase 7A2 with diaphorase and 2,6-dichlorophenolindophenol at different pH.*

pH	5.5	6.5	7.5	8.8*
Relative specific activity (%)	0.9	2.7	27.3	100.0

*Smaller pH variations around 8.8 were also assessed, with 8.8 being optimum.

in AKR7A2 activity in the presence of ethanol up to concentration of 4%. A further increase in ethanol presumably starts denaturing the protein, and at 12% ethanol, the rate of reaction is about equal to the rate of reaction without ethanol. The reaction was stopped entirely at 40% ethanol. Thus, all subsequent assays utilizing AKR7A2 to detect GHB in beverages were adjusted so that the drink components constituted 30% of the reaction volume. So, even strong alcoholic drinks with 40% ethanol would yield the final concentration of ethanol that is 12%. It is worth emphasizing that this reaction does not use ethanol as a substrate; it is the major advantage of this assay over the assay reported in this journal earlier (25), where ethanol-containing drinks had to be heated to evaporate ethanol to avoid false positive results.

The PMS-DCIP reaction was run in strong alcoholic drinks (vodka, gin, rum, tequila, and Blue Curacao), with 1 g of GHB dose per shot (50 mL) (Fig. 4*A*). DCIP was bleached effectively and completely in all the drinks tested with the exception of Blue Curacao, which continued to have the light blue tint that characterizes the beverage. Nevertheless, the dark blue color characteristic to DCIP disappeared with the Blue Curacao, and the observed change was very obvious.

The same set of reactions, with a concentration of GHB of 2 g per 200 mL of drinks, was performed for tomato juice, orange juice, soda, and milk, as common additives to strong alcoholic drinks. While the tomato juice was too thick to record the blue color change (data not shown), this change was clearly detectable in water, orange juice, soda, and milk (Fig. 4*B*). The blue tint in orange juice and milk clearly disappeared—in this black and white photograph, the gray tint that remains is representative of the original orange/milk color and is not blue. Ethanol does not interfere with the reaction, as the controls without GHB did not change at all while the runs with GHB, both in the presence and in the absence of ethanol, were successfully bleached. This reaction, while slower, was also successful with beers (data not shown). The slow rate was expected because the concentration of GHB was only 30% above the K_m value. The color change was not observed using white wine, which produced a false positive even in the absence of AKR7A2, or any red wines, which turned dark blue in basic buffers and completely masked the blue color change (data not shown).

To overcome the problems with beer and white wine, diaphorase replaced PMS in the system, as was previously described by Bravo et al. (25). Diaphorase was 20% less active in the buffer used for AKR7A2 when compared to the diaphorase buffer recommended by the manufacturer, 0.2 M Tris–HCl, pH 8.5 (data not shown). Diaphorase was found not to be inhibited by 4% or 12% ethanol. When AKR7A2 was coupled with 5 μg of diaphorase and DCIP, the reaction exhibited some activity in solutions with ethanol, supporting the results reported in (25). Nevertheless, like with the PMS-DCIP assay, it allowed for successful visual and spectrophotometric detection of GHB in three beers tested (Fig. 4*C*). The GHB concentration was 2 g of GHB per bottle of beer. Unfortunately, this reaction did not work in wines either (data not shown).

The novelty of the method reported here allows for successful detection of GHB in alcoholic drinks using an enzymatic assay.

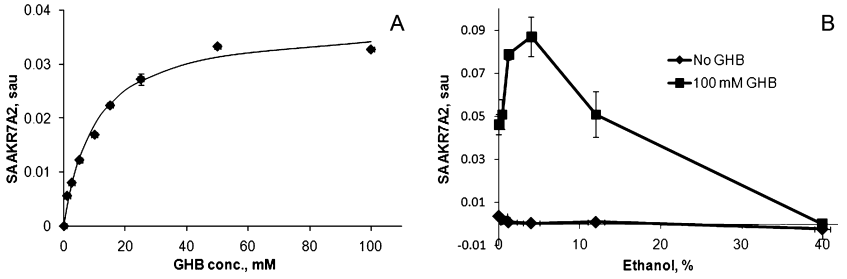

FIG. 3—*(A) Michaelis–Menten kinetics of a coupled aldo-keto reductase 7A2 (AKR7A2)-diaphorase-2,6-dichlorophenolindophenol (DCIP) reaction. Specific activity (SA) units are μmoles DCIP consumed/min per mg protein. At its maximum, the change of absorbance at 600 nm was 0.16/min. (B) AKR7A2-PMS-DCIP coupled reaction with 0%, 0.4%, 1.2%, 4.0%, 12.0% and 40.0% ethanol with and without* Gamma-*hydroxybutyrate (GHB).*

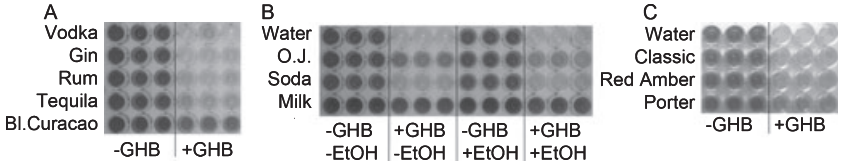

FIG. 4—*(A) Aldo-keto reductase 7A2 (AKR7A2)-phenazine methosulfate (PMS)-2,6-dichlorophenolindophenol (DCIP) reaction in the presence of strong alcoholic drinks. The final ethanol concentration in wells was 12%, and the amount of AKR7A2 was 44 μg. (B) Visible color change was observed for AKR7A2-PMS-DCIP in water, orange juice, soda, and milk containing* gamma-*hydroxybutyrate (GHB). Ethanol at 12% did not interfere. The blue tint in orange juice and milk clearly disappeared—the photograph shows gray tint that represents original orange/milk and is not blue. (C) Coupled AKR7A2-diaporase-DCIP reaction in beers with GHB (2 g per 360 mL of drink) and without GHB. The glare on this image was removed by the software. All runs are shown in triplicate.*

One limitation of this assay is its inability to detect GHB in wines. The combination of this method with other known indicator methods (22,24,25) could help alleviate the problem. Also, the MDL of 4 mM makes this assay practical only for the detection of GHB in drinks and not in bodily fluids, which may be screened by ferric hydroxymate test (22) and followed up by numerous chromatography-MS methods (12–17). When further developed, the method proposed in this study could be improved by exploring different electron accepting indicators besides DCIP. In addition, the solid-state detection of GHB (such as on a coaster) and the stability of enzymes when used in such a method could be explored.

This study takes the research of practical methods for GHB detection in various matrices for in-field forensic scientists one step closer to being more specific while staying inexpensive and free of harsh chemicals or expensive instruments.

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