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Reliable, Sensitive, Rapid and Quantitative Enzyme-Based Assay for *Gamma*-Hydroxybutyric Acid (GHB)*

ABSTRACT: Several assays for *gamma*-hydroxybutyrate (4-hydroxybutyrate, GHB) have been developed based on the enzyme *gamma*-hydroxybutyrate dehydrogenase (GHB-DH). Enzymatic oxidation of GHB by NAD^+ is coupled to diaphorase-mediated reduction of pro-dye to yield colored product. GHB-DH from *Ralstonia eutropha* was cloned and expressed as a stable fusion protein easily purified by affinity chromatography. Quantitative initial velocity and endpoint versions of the assay in solution are described. Michaelis-Menten parameters for oxidation of GHB and ethanol were estimated. A semi-quantitative "dipstick" version of the assay on paper also is described. Both solution endpoint and "dipstick" assays are sensitive to about 0.05 mg GHB/mL using 10 μL of sample. Ethanol at concentrations possible in urine and agents used to stabilize physiological fluids for forensics analysis do not interfere significantly. The "dipstick" assay also allows detection of GHB in alcoholic beverages after evaporation of about one-fourth drop of beverage before testing. The enzymatic assay for GHB is reliable, sensitive, inexpensive and rapid.

KEYWORDS: forensic science, GHB, *gamma*-hydroxybutyrate dehydrogenase, 4-hydroxybutyrate, tetrazolium pro-dye, enzymatic detection

Gamma-hydroxybutyrate (4-hydroxybutyrate, GHB) is an endogenous metabolite in brain and peripheral organs (1,2). It has many characteristics of a neurotransmitter (3) and has been studied for potential therapeutic use in the treatment of narcolepsy, drug addiction, and symptoms of withdrawal and to induce anesthesia (4–6). However, GHB also is widely abused (7–9). At moderate doses (up to about 1 g for a 150-lb person), the psychopharmacological effect is similar to that of ethanol (10). At higher doses, GHB produces sedation and a trance-like state with loss of memory (4,9,11,12). Because it has little smell or taste, it can be ingested unknowingly. This combination of properties has made GHB a "date rape" drug that often is administered to victims in beverages (13).

GHB has a very steep dose-response relationship (14). High doses (greater than about 2.5 g for a 150-lb person) can produce serious consequences like bradycardia, decreased respiration, coma and even death, especially when consumed with other depressants of the central nervous system like ethanol (10,15–17). As it is very dangerous, GHB has been classified by the USA Drug Enforcement Administration as a Schedule I controlled substance. Because of the need to analyze for GHB in clinical, abuse, workplace, and law enforcement situations, a reliable and easy assay for GHB is desirable.

In mammalian brain, GHB is formed primarily by transamination of *gamma*-aminobutyrate to succinic semialdehyde that then is reduced by succinic semialdehyde reductase (EC 1.1.1.2) using NADPH. It is degraded primarily to succinic semialdehyde by GHB dehydrogenase (GHB-DH, EC 1.1.1.61) using NAD^+. Succinic semialdehyde is further oxidized to succinic acid that enters the Krebs cycle (3). Succinic semialdehyde reductase and GHB-DH catalyze basically the same reactions in opposite directions (18–23). Either the dehydrogenase or reductase reaction run in the direction of GHB oxidation and NAD^+ or $NADP^+$ reduction could become the basis of an assay for GHB by using reduced cofactor to form a colored product.

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Materials and Methods

Ralstonia eutropha (formerly *Alcaligenes eutrophus*) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Bactozol DNA isolation kit was obtained from Molecular Research Center Inc. (Cincinnati, OH). The synthetic oligonucleotide primers (CGGGATCCATGGCGTTTATCTACTATCTGACC) and (CGGAATTCCATGGACTGCTCAAGCATACGCCG) containing BamH1 and EcoR1 restriction sites near the 5′ ends were obtained from Invitrogen Corp. (Carlsbad, CA). BamH1 and EcoR1 endonucleases and T4 DNA ligase were obtained from New England Biolabs Inc. (Beverly, MA). GST fusion vector pGEX-2T was obtained from Amersham-Pharmacia Biotech Inc., (Piscataway, NJ). A DNA sequencing kit (Thermo Sequenase) was obtained from USB Corp. (Cleveland, OH). *E. coli* XL1-Blue cells were obtained from Stratagene (La Jolla, CA). *E. coli* pLysS cells were obtained from Novagene, (Madison, WI). Luria-Bertani (LB) medium, yeast extract and Trypticase peptone were obtained from Becton, Dickinson and Company (Cocksville, MD). Plasmid purification kit was obtained from Qiagen (Valencia, CA). Affinity resin containing covalently bound glutathione (GSH), *gamma*-hydroxybutyrate sodium salt, sodium 3,3-[(phenylamino) carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), isopropyl β-D-thiogalactoside, phenylmethylsulfonyl fluoride and aprotinin and were obtained from Sigma Chemical Corp. (St. Louis, MO). Possession and use of *gamma*-hydroxybutyrate was licensed by the United States

Department of Justice Drug Enforcement Administration and the Research Advisory Panel of California. All applicable regulations were followed. GHB masses are quoted for the acid form. Bradford reagent was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Diaphorase (1,700 international units/mg) from *Bacillus stearothermophilus* was obtained from Unitika Corp (Tokyo, Japan). Paper sheets (3MM CHR made of alpha-cotton cellulose) from which circles were cut were obtained from Whatman, Inc. (Ann Arbor, MI). Procedures for acquisition of urine specimens were approved by the Human Subjects Committee of the University of California Santa Barbara. Other materials were obtained from usual commercial sources.

R. eutropha was grown in LB medium, and total genomic DNA was isolated from a 2 mL culture using the Bactozol DNA isolation kit. Polymerase chain reaction amplification of GHB-DH coding sequence and appended restriction sites was carried out. The amplified fragment of DNA (1.1 kb) was purified by electrophoresis in agarose gel and trimmed with BamH1 and EcoR1 restriction endonucleases to generate sticky ends. The pGEX-2T fusion vector was linearized with BamH1 and EcoR1. The purified and trimmed DNA was ligated into the linearized pGEX-2T fusion vector, thus creating a circular GST/GHB-DH recombinant vector (pGEX-2T/GHB-DH). *E. coli* XL1-Blue competent cells were transformed with recombinant vector by heat shock and plated onto LB agarose plates containing ampicillin (100 μg/mL). Clones were grown in LB medium containing ampicillin, and vector DNA was isolated using the Qiagen purification kit. Sequence analysis of the recombinant vector using the Thermo Sequenase kit confirmed that the GHB-DH coding sequence was in-frame with the GST coding sequence. Cells carrying confirmed vector were grown at 37°C in LB medium containing ampicillin for amplification.

GHB-DH activity was monitored at 23°C in 1 mL of 1 mM NAD^+ in 97 mM 2-amino-2-methyl-1,3-propanediol adjusted to pH 8.5 with HCl (18). Reaction was initiated by the addition of GHB to 20 mM and monitored in a cuvette of 1 cm path length by absorbance at 334 nm due to formation of NADH. The initial velocity was estimated manually by drawing an asymptote to the curve at time zero.

GHB-DH fusion protein was expressed as follows. *E. coli* pLysS cells were transformed with recombinant vector by heat shock and plated onto LB agarose plates containing ampicillin. The transformed cells were grown in 1 L of 2 × YT medium (10 g yeast extract, 16 g Trypticase peptone, 5 g NaCl) containing ampicillin to an absorbance at 600 nm of 0.8 at 37°C. After lowering the temperature of the culture to 20°C, expression of the fusion protein was induced by addition of 0.1 mM isopropyl β-D-thiogalactoside and allowed to continue for 18–20 h. Subsequent steps were carried out at 4°C. Cells were harvested by centrifugation (1600 × g for 30 min) and resuspended in 12.5 mL of 30 mM 3-(N-morpholino)propanesulfonic acid containing 50 mM NaCl adjusted to pH 7.4 with NaOH (resuspension buffer) also containing 1 mM phenylmethanesulfonic acid and 1% aprotinin. The cell suspension was subjected to sonication on ice and clarified by centrifugation (27,000 × g for 30 min).

GHB-DH fusion protein was purified as follows. Affinity resin (4 g) containing covalently bound GSH was swelled in water. The swollen resin was packed into a 50 mL capacity chromatography column. Subsequent steps were carried out at 4°C. The resin was equilibrated by flowing 75 mL of resuspension buffer through the column. Clarified supernatant from the sonicated cells was slowly flowed into the resin, after which flow was stopped and fusion protein was allowed to adsorb to the resin for approximately 15 min. The loaded resin was washed by flowing through 75 mL of resuspension buffer containing 1% Tween 20 and additional 0.45 M NaCl and then by flowing through 75 mL of 30 mM 3-(N-morpholino)propanesulfonic acid adjusted to pH 7.4 with NaOH (wash buffer). To elute bound fusion protein, 20 mL of 30 mM 3-(N-morpholino)propanesulfonic acid containing 15 mM GSH and adjusted to pH 7.0 with NaOH was flowed into the resin, after which flow was stopped for 1 h. The resin was washed slowly with 30 mL more GSH. Fractions (10 mL) were collected throughout, and each was assayed for GHB-DH activity and polypeptide content. Three fractions containing most of the enzymatic activity were pooled. Protein in the pool was precipitated by the addition of ammonium sulfate to 75% of saturation. Precipitated protein was pelleted by centrifugation, and the clarified supernatant was decanted and discarded. The pellet was dissolved in 10 mL of resuspension buffer and placed in dialysis tubing of 10^3 M_r exclusion limit. The sample was dialyzed in 2 L of resuspension buffer that was replaced four times over a period of 24 h. The final protein concentration was determined by the method of Bradford using bovine serum albumin standard (24).

Stability of purified fusion protein was studied by mixing concentrated fusion protein (7 mg/mL resuspension buffer) with concentrated solutions of indicated potential stabilizing agents in resuspension buffer, and the solutions were stored as indicated.

The conditions for solution assay of GHB using coupled reactions were similar to those of the assay for GHB-DH. A small volume (generally 10 μL) of a sample containing GHB was added to 1.00 mL solution assay reagent containing 1 mM NAD^+, 20 μg purified fusion protein, 0.70 μg diaphorase, 80 μM of pro-dye XTT, and 97 mM 2-amino-2-methyl-1,3-propanediol adjusted to pH 8.5 with HCl. The reaction was monitored in a 1 cm cuvette at 450 nm. The spectrometer was zeroed in all cases using the same volume of water as the sample containing no GHB. Either steady-state initial velocity or end-point absorbance after at least 1 h of reaction at 23°C was determined. For initial velocities v at different GHB or ethanol concentrations S, the equation $v = V_{max}S/(K_m + S)$ was fit by non-linear regression to estimate K_m and V_{max} values given to one standard deviation. For solution endpoint assays, data for standards were fit by linear regression.

A "dipstick" assay for GHB using coupled reactions was developed as follows. Ten μL of a sample containing GHB was applied to a 6 mm circle of filter paper. In some cases the filter was warmed until ethanol and water in the sample completely evaporated. Then 10 μL of dipstick assay reagent containing 33 mM NAD^+, 17 μg of pro-dye MTT, 23 μg of fusion protein, 1.2 μg diaphorase and 670 mM 2-amino-2-methyl-1,3-propanediol (adjusted to pH 8.5 with HCl) was applied to the filter. High concentrations of buffer and NAD^+ were used to overcome the strong acidity of many alcoholic beverages and promote the forward-direction reaction. Color was allowed to develop for 2 min at 23°C, and the assay was quenched with 10 μL of 10% (v/v) acetic acid to stabilize color for photography.

Results

GHB-DH Cloning, Expression and Purification as a Fusion Protein

GHB-DH is present in the bacterium *Ralstonia eutropha*. The gene for the enzyme was identified and sequenced (GenBank™ accession L36817) by Valentin and coworkers (18). The native polypeptide has a predicted molecular weight of 40.5 kDa. It is an iron-containing, type III alcohol dehydrogenase (22).

The gene was amplified from genomic DNA by polymerase chain reaction as described in Materials and Methods. After purified polymerase product was restricted at the ends, it was ligated in-frame at the C-terminal end of the coding sequence for GST. This produced a

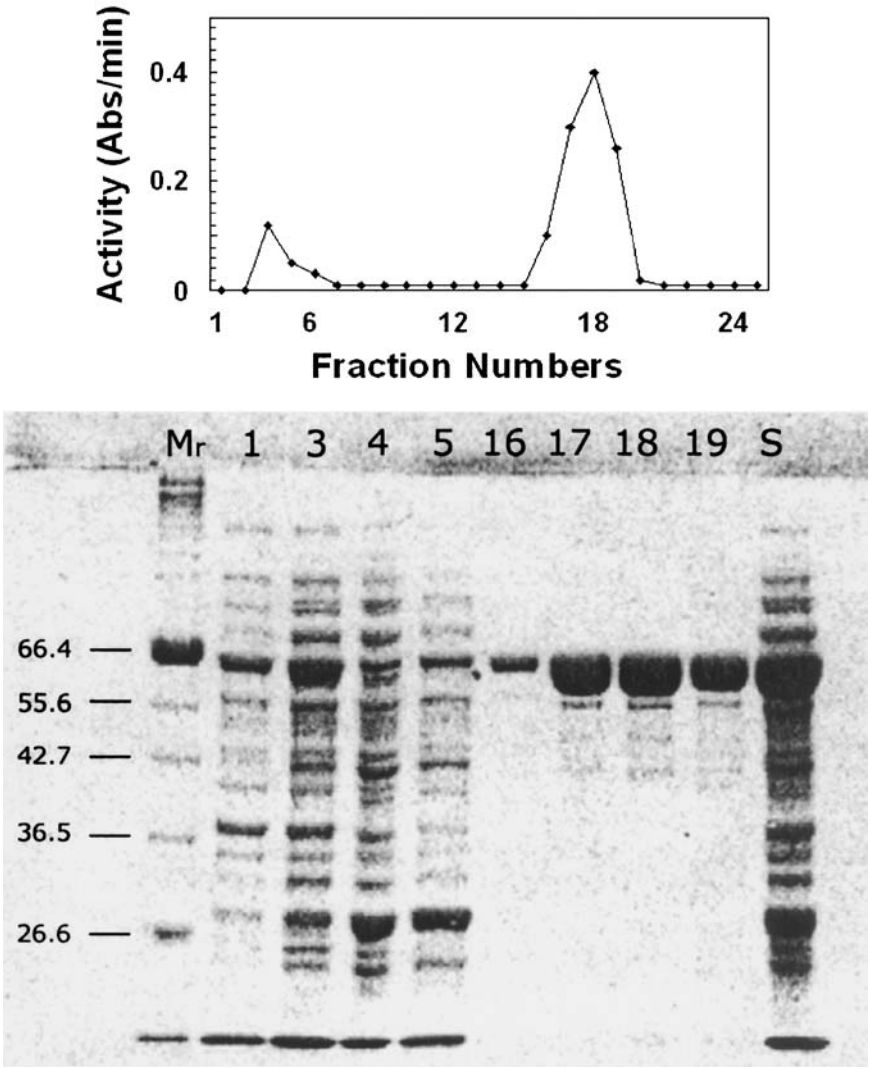

FIG. 1—*Purification of GHB-DH fusion protein. Top. Affinity chromatography on glutathione column. A 1 μL portion of each 10-mL fraction was taken for assay of GHB-DH activity. Fractions 3 and 4 are breakthrough fractions. Fractions 17, 18, and 19 were pooled. Bottom. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of chromatographic fractions. The lane labeled M_r contained protein markers whose molecular weights in kDa are indicated to the left. Numbers above lanes correspond to the fraction numbers. Lane S contains clarified supernatant before chromatography.*

recombinant gene for GST/GHB-DH fusion protein under the control of *lac* operator in the pGEX-2T expression vector, which was used to transform *E. coli*. Induction of expression at 37°C with isopropyl β-D-thiogalactoside followed by sonication of cells yielded only a little GHB-DH activity and a large amount of a new insoluble polypeptide. Induction of expression at 20°C with isopropyl β-D-thiogalactoside followed by sonication of cells produced a large amount of GHB-DH activity and a new soluble polypeptide having the same molecular weight as the insoluble polypeptide formed at 37°C.

Clarified supernatant from a 1-L culture induced at 20°C was passed through a small column containing covalently bound GSH, and unbound protein was washed through. The column was eluted with GSH in buffer, and three fractions containing large amounts of GHB-DH activity and protein of 66 kDa were obtained (Fig. 1). The expected molecular weight of fusion protein is 66 kDa. The three fractions containing large amounts of purified GHB-DH activity were pooled, and fusion protein was concentrated by ammonium sulfate precipitation and dialysis. The final yield of purified fusion protein was 213 mg at a concentration of 7 mg/mL.

Stability of Purified Fusion Protein

The dehydrogenase activity of purified fusion protein was studied after storage under different conditions (Table 1). Most of the activity was retained after 21 days at 4°C in resuspension buffer whether or not azide was present. Somewhat surprisingly, ammonium sulfate, glycerol, 2-mercaptoethanol, dithiothreitol, the reduced and disulfide forms of GSH, and EDTA all were harmful. Hence it is critical that GSH and ammonium sulfate be removed quickly after purification of the fusion protein. In other work not shown, about 60% of the activity remained after 3.5 months of storage at 4°C in resuspension buffer, and about 80% remained indefinitely after storage at −80°C in the absence or presence of glycerol. Fusion protein in resuspension buffer can be freeze dried with recovery of 86% of the activity.

GHB-DH Activity

Formation of NADH and succinic semialdehyde from NAD^+ and GHB can be monitored at 334 nm. This wavelength is not visible

TABLE 1—*Stability of fusion protein activity toward storage.**

Additions	Activity (%)†
None	82
1 mM Azide	80
50% Ammonium sulfate	40
75% Ammonium sulfate	20
5 % Glycerol	69
10% Glycerol	75
15% Glycerol	65
20% Glycerol	58
50% Glycerol	15
15 mM 2-mercaptoethanol	<2
10 mM dithiothreitol	2.6
15 mM Glutathione	<2
1 mM Glutathione disulfide	<2
10 mM EDTA	<2

* Purified fusion protein was stored for 21 days at 4°C in resuspension buffer containing the indicated reagent. It then was assayed for GHB activity.

† Relative to activity before storage in the absence of added reagent.

to the human eye and requires a spectrophotometer to monitor (not shown). The rate of reaction slows considerably during the first 2 min of catalysis because of approach to equilibrium, as the reverse-direction reaction is favored thermodynamically (25). This protocol is acceptable for qualitative monitoring of GHB-DH activity during purification and storage, but it is not a suitable assay for quantitation of GHB because of limited and therefore non-quantitative conversion of GHB and NAD^+ to products.

Assays for GHB Using Coupled Reactions

A quantitative assay for GHB requires the dehydrogenase reaction to be pulled to completion by coupling to another reaction that is favorable. Diaphorase can use NADH formed by GHB-DH and GHB to reduce a soluble, colorless pro-dye to a colored product (26). Reduction of appropriate pro-dye is favorable and makes the coupled reactions thermodynamically favorable overall. Various pro-dyes that give soluble and insoluble products are available commercially, and many colors are available.

In one implementation of this strategy, formation of soluble orange dye obtained by reduction of the tetrazolium pro-dye XTT was monitored at 450 nm in a spectrophotometer. This is a solution assay that is suitable for determination of steady-state initial velocities at different concentrations of GHB (Fig. 2). In order to make the GHB-DH reaction rate limiting, excess diaphorase activity was used. Under these conditions, the rate is nearly constant over the first several minutes of reaction. If allowed to proceed to completion, the solution assay also can be used to determine endpoint conversion at different concentrations of GHB (see below). In another implementation of the coupled reactions strategy, formation of an insoluble purple dye by reduction of the tetrazolium pro-dye MTT onto paper was monitored (see below).

Kinetic Analyses

Michaelis-Menten parameters for oxidation of GHB were determined from initial velocity data obtained with the solution assay (Fig. 3). The K_m value is 2.19 ± 0.13 mM GHB and V_{max} value is 0.049 ± 0.001 A 450/min/μg fusion protein.

While examining the stability of GHB-DH in aqueous solutions containing high concentrations of ethanol, we found that ethanol is an alternative substrate. Preliminary investigation revealed that ethanol binds to the active site of the enzyme too weakly to saturate

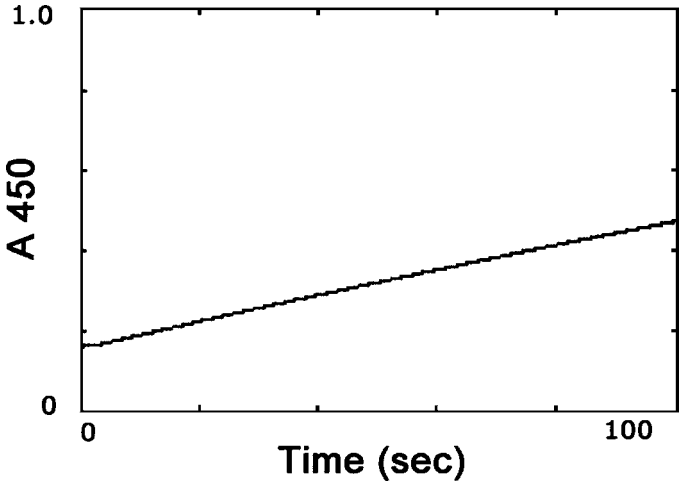

FIG. 2—*Solution assay for GHB using coupled reactions. Ten μL of 2.1 mg GHB/mL (20 mM) was added to 0.99 mL of NAD^+, fusion protein, diaphorase, and pro-dye XTT as described in Materials and Methods to initiate reduction of XTT, which was monitored by the increase in absorbance at 450 mm. More diaphorase did not increase the velocity of color formation.*

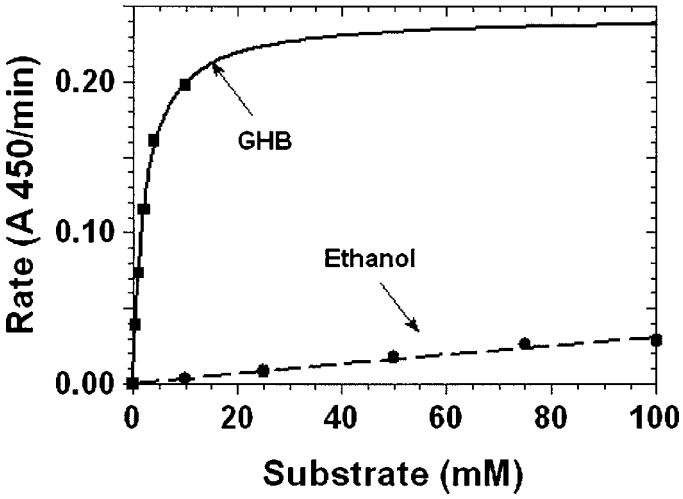

FIG. 3—*Michaelis-Menten kinetics for GHB and ethanol. Initial velocities determined as in Figure 2 for indicated concentrations of substrate in assay solution containing 5 μg fusion protein are plotted. The lines were fitted to data by regression. Data for GHB might give the impression that V_{max} is not accurately determined, but this is a visual artifact caused by plotting both sets of data on the same graph.*

it at concentrations that do not quickly denature the enzyme. This means that reliable initial-velocity data could be obtained only at substantially sub-saturating concentrations of ethanol. In order to carry out determinative regression analysis of the available data, V_{max} for ethanol was assumed to be the same as for GHB. The assumption is based on the high likelihood that the rate-determining step for oxidation of both substrates is dissociation of bound NADH (27). With this assumption, the K_m value was estimated to be 413 ± 14 mM ethanol, which is 200 times higher than the K_m value for GHB.

Endpoint Assay for GHB Using Coupled Reactions

Exogenous GHB in 10 μL of water (Fig. 4*A*), normal human urine (Fig. 4*B*), or normal human urine containing 0.47% (w/v) ethanol (Fig. 4*C*) was allowed to react to near completion, after

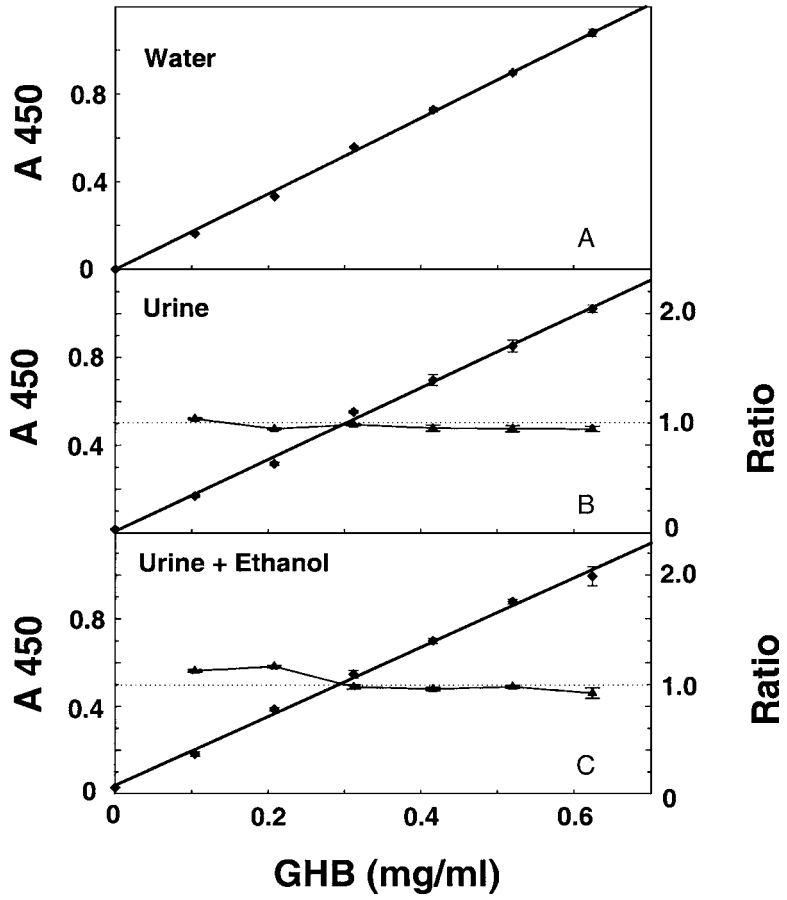

FIG. 4—*Endpoint assay for GHB using coupled reactions. Ten μL samples containing the indicated concentrations of exogenous GHB were added to 1 mL of solution assay reagent and allowed to react for 80 min to near completion, after which absorbance was read at 450 nm (♦). The ratio of absorbance obtained from GHB in urine to absorbance obtained from GHB in water also is plotted (▲). Frame A, GHB in water. Frame B, GHB in normal human urine. Frame C, GHB in normal human urine containing 0.47% (w/v) ethanol. Data show the averages of 5 determinations with 95% confidence intervals, and errors have been propagated for the ratios.*

which absorbance at 450 nm was determined. In all cases, very little color developed in the absence of exogenous GHB, and definite orange color developed with as little as 0.1 mg GHB/mL. A linear relationship between absorbance and concentration of exogenous GHB in the sample was obtained.

The absorbance obtained from exogenous GHB in urine or urine plus ethanol was divided by the absorbance obtained from the same concentration of GHB in water to assess whether urine or ethanol compromises the assay. The ratios are very slightly below 1.0 at high GHB concentrations (Fig. 4*B* and 4*C*). This occurs because urine is very slightly inhibitory to the rate of color development. The ratio is slightly above 1.0 at the lowest concentration of GHB in urine that was tested (Fig. 4*B*). This occurs because urine produces a small amount of color. Urine plus ethanol exhibited somewhat higher ratios at the lowest concentrations of exogenous GHB, presumably due to oxidation of ethanol (Fig. 4*C*). Overall, urine and urine containing a concentration of ethanol that is high for urine only slightly affect solution endpoint assay.

Limit of Detection for Solution Endpoint Assay

Samples of urine from 51 anonymous adult donors were obtained over a period of several days, stored at 4°C, and analyzed within one day of collection. GHB in urine is stable to storage over this time frame (28). The results obtained from two samples were very high outliers from the other results as determined by the modified Z-score calculation (over six medians of absolute deviation about the median), and they were removed from the analysis (29). The biochemical origin of the outliers is not known, but it is unlikely to be ethanol. Individuals with succinic semialdehyde dehydrogenase deficiency excrete elevated levels of GHB in their urine (30). Absorbance obtained from the remaining 49 samples of urine was 0.0272 ± 0.0336, corresponding to 0.017 ± 0.020 mg apparent GHB/mL (two standard deviations). Thus, the first 0.0608 absorbance corresponding to 0.037 mg apparent GHB/mL falls within the 95% confidence interval for a urine sample containing no exogenous GHB and should be subtracted from sample absorbance readings before estimating GHB in urine. This establishes a detection limit of 0.037 mg exogenous GHB/mL for this implementation of the solution endpoint assay.

"Dipstick" Assay for GHB Using Coupled Reactions

Sample volumes of 10 μL containing different concentrations of GHB were applied to paper circles, after which dipstick assay reagent containing MTT was applied. The results were assessed after 2 min of reaction. No significant color developed in the absence of GHB, and intense purple color developed in the presence of 0.1 mg or more GHB/mL of water (Fig. 5*A*), normal human urine (Fig. 5*B*), or normal human urine containing 0.63% (w/v) ethanol (Fig. 5*C*). Thus, urine and ethanol in urine do not compromise the dipstick assay.

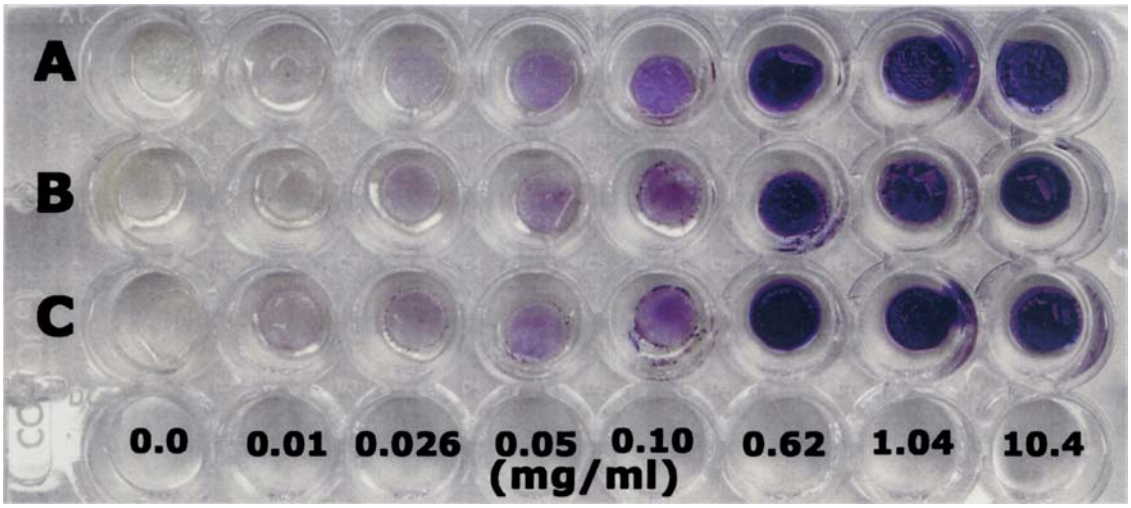

FIG. 5—*"Dipstick" assay for GHB using coupled reactions. Ten μL samples containing the indicated concentrations of GHB were spotted onto paper circles. Ten μL of dipstick assay reagent containing MTT was applied to each paper circle, and color was allowed to deposit for 2 min at 23°C as described in Materials and Methods. Row A, GHB in water. Row B, GHB in normal human urine. Row C, GHB in normal human urine containing 0.63% (w/v) ethanol.*

TABLE 2—*Dipstick precision around the cutoff.**

GHB Concentration (mg/mL)	Positive	Negative
0	0	20
0.02	5	15
0.05	15	5
0.10	20	0
0.15	20	0

* Each concentration of GHB in water was tested 20 times for 2 min of color development at 23°C.

The precision of the dipstick assay around the cutoff was assessed at concentrations of GHB above and below 0.1 mg/mL water (Table 2). Of 20 tests under each condition, no samples were positive in the absence of GHB, 25% were positive (and 75% were negative) at 0.02 mg GHB/mL, 75% were positive at 0.05 mg GHB/mL, and all samples were positive at 0.1 mg and more GHB/mL.

"Dipstick" Assays for GHB in Alcoholic Beverages

Small volumes of different alcoholic beverages containing or not containing GHB were applied to paper circles. Beverage was evaporated to dryness to remove ethanol, and dipstick assay reagent containing MTT was applied (Fig. 6). When 2.1 mg GHB/mL beverage was present, intense purple color deposited immediately. A 150-lb person who consumes 4 oz of beverage containing this concentration would ingest 0.25 g of GHB, or about one-fourth of a moderate dose. When GHB was not present, no purple color deposited. However, the color of red wine could be mistaken for a positive result, as it is similar to that of reduced MTT. Resolution of this problem is presented in the Discussion.

A titration to estimate the sensitivity of the dipstick test for GHB in some alcoholic beverages was carried out (Fig. 7). As little as 0.104 mg GHB/mL beverage, which is much less than a psychopharmacologic dose, was robustly detected.

Effects of Agents Used To Stabilize Physiological Fluids

Agents commonly used to stabilize physiological fluids for forensics analysis were tested for interference. EDTA at 10 mM, potassium oxalate at 0.2% (w/v), NaF at 1% (w/v), or NaN_3 at 0.1% (w/v), all in normal human urine, had no effect on the dipstick assay at 0.6 mg GHB/mL urine (not shown). Thus, although 10 mM EDTA is deleterious to GHB-DH activity during long-term storage, it has no apparent effect during the short time period of an assay.

Discussion

GHB-DH from the bacterium *Ralstonia eutropha* was cloned as a readily isolated fusion protein. A large quantity was obtained from 1 L of culture. The purified protein is stable during long-term storage if deleterious agents are not added. Coupling of GHB oxidation to reduction of a tetrazolium pro-dye makes the overall reaction thermodynamically favorable and results in formation of a colored product. This forms the basis of several colorimetric enzymatic assays for GHB.

GHB in a sample can be quantitatively determined by solution assay. Either an endpoint or initial-velocity approach can be used. An initial-velocity approach requires the concentration of GHB in the assay solution to be sub-saturating. The endpoint approach has the virtue that nearly all GHB in the sample is converted to soluble color. This means that endpoint assay is sensitive and the time of incubation (assuming it is long) and amounts of enzyme activities added are not critical to obtaining quantitative results. Moreover, a standard curve is not required, as one can multiply the final absorbance by the XTT conversion factor of 0.60 mg GHB/mL/absorbance to compute the concentration of GHB. However, the endpoint assay is not especially rapid, and many potential uses for a GHB test occur under conditions in which speed is required and a spectrophotometer is not available. Thus, a 2-min semi-quantitative assay on paper (the "dipstick" assay) also has been developed.

The current implementations of the solution endpoint and dipstick assays exhibit similar sensitivities, and the matrix (water, urine or beverage) has little effect on the results (but see below for comments on ethanol). The Limit of Detection is about 0.05 mg GHB/mL (a little lower for the solution endpoint assay, a little higher for the dipstick assay), the Limit of Quantitation for the solution endpoint assay is about 0.1 mg GHB/mL, and the Upper Limit of Linearity for the solution endpoint assay is about 0.6 mg GHB/mL. The

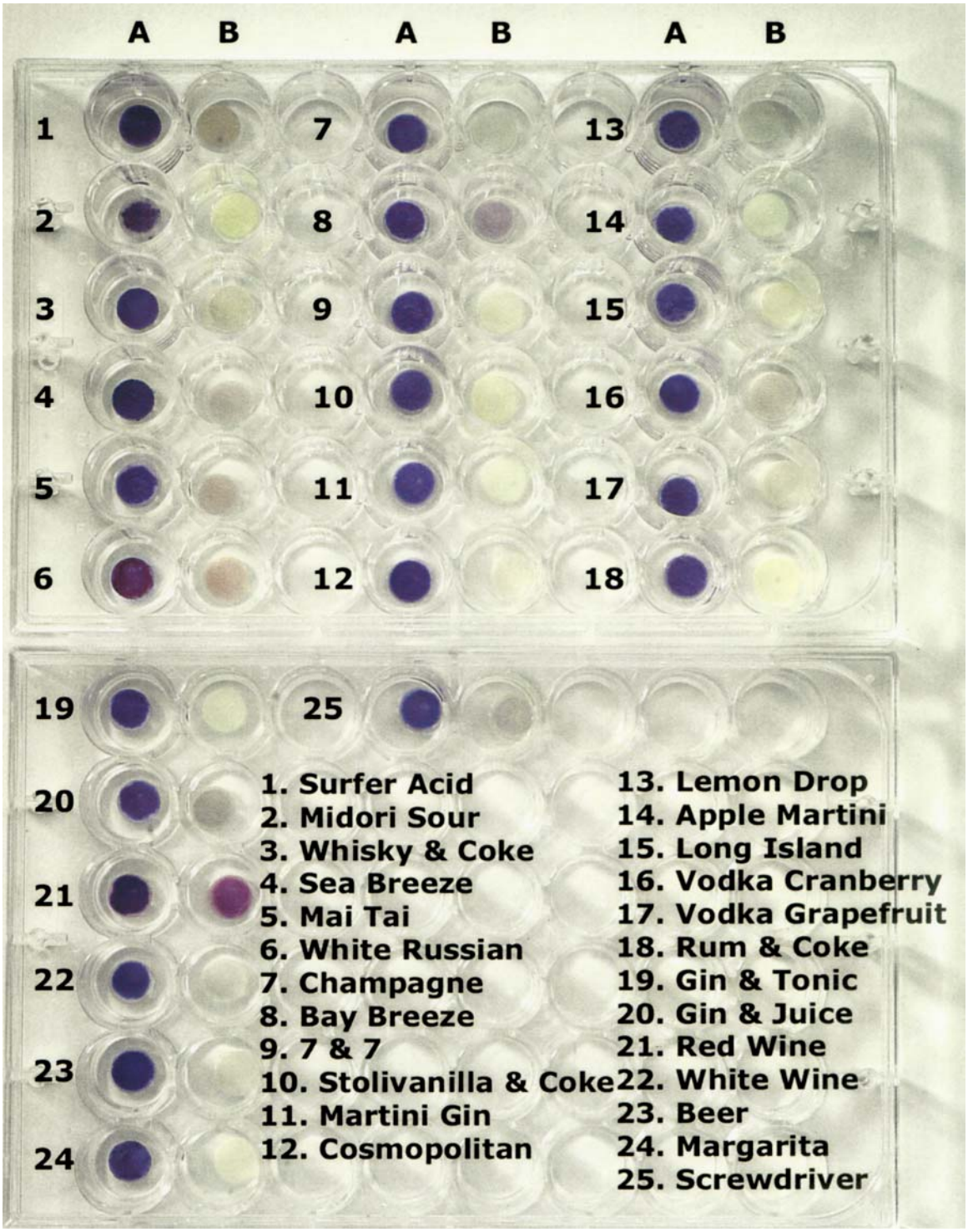

FIG. 6—*"Dipstick" assay for GHB in alcoholic beverages. Ten μL samples of various alcoholic beverages (#1–25) containing (column A) or not containing (column B) 2.1 mg GHB/mL (20 mM) were spotted onto paper circles and dried by heating for 2 minutes in a kitchen microwave oven set on high power. Ten μL of dipstick assay reagent was applied to each paper circle, and color was allowed to deposit for 2 min at 23°C as described in Materials and Methods.*

ULOL could be increased two-fold by doubling the pro-dye concentration, and the time required for the solution endpoint assay could be decreased by increasing the concentrations of enzymes.

Normal concentrations of GHB in urine are less than 0.010 mg/mL (28,31,32). In acute overdose cases, concentrations of GHB in urine average about 1 mg/mL (15,33) and are very robustly detected by the methods developed here. In fact, dilution or use of a smaller volume of urine obtained from an individual in severe overdose would be required to obtain non-saturating results.

Although ethanol is an alternative substrate for GHB-DH, it binds too weakly to yield false positives at concentrations that might occur in urine. About 1% (w/v) ethanol is threshold for false positives in both solution endpoint and dipstick tests. Dipsticks should be read at 2 min, as longer time periods can result in false positives.

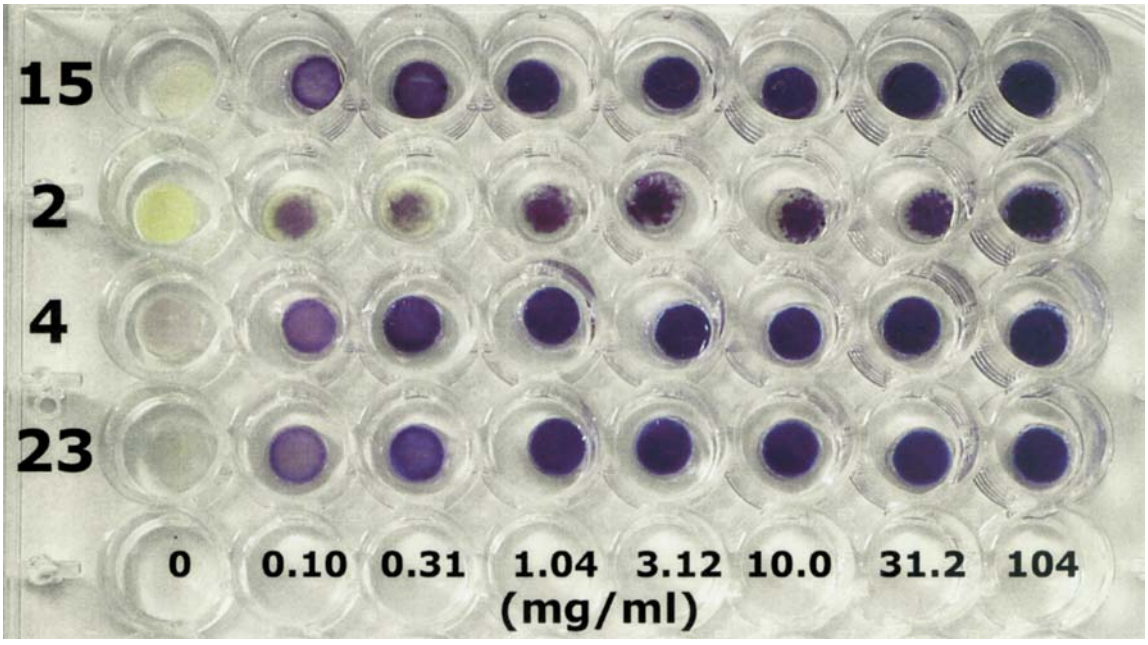

FIG. 7—*Titration of GHB in alcoholic beverages. The test was conducted as in Fig. 6, except that the indicated concentrations of GHB were present in the samples. Beverage #15 is a Long Island, #2 is Midori sour, #4 is sea breeze, and #23 is beer.*

Another potential application for a GHB test is screening of beverages. However, the high concentrations of ethanol found in typical alcoholic beverages *do* produce substantial color in 2 min. As GHB is not volatile and ethanol is, alcoholic beverages can be evaporated before testing to avoid false positives. The dipstick assay easily detects GHB at concentrations that are sub-psychopharmacologic in a 4-oz beverage.

The color of a tested beverage can affect interpretation of a dipstick result. Of 25 unadulterated alcoholic beverages tested, only red wine produced a color that could be mistaken for the presence of GHB. However, the color was present before dipstick assay reagent was applied and could be recognized not to be due to GHB by that behavior. This type of false positive also could be recognized by a novice if two pro-dyes producing different colors were painted side-by-side on the dipstick paper.

Other assays for GHB either (a) require sophisticated equipment, a skilled person and a relatively long time period to complete (34–37), or (b) use chemistry that is hazardous, multi-step and very susceptible to false positives (38–40). In contrast, the enzymatic assay for GHB is reliable, sensitive, inexpensive and rapid. Also, common stabilizers of physiological fluids do not compromise enzymatic assay. In experiments not shown here, sensitivity has been increased significantly, and GHB in samples of human serum and saliva have been assayed successfully. Further development and validation of these enzymatic screening tests for GHB might identify additional opportunities and should delineate limitations in forensics applications.

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