GHB

Gamma-Hydroxybutyric Acid

Enzymatic Assay

This product is for research use only
It is not intended for use in diagnostic procedures

KK-GHB-U

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INTENDED USE

The BÜHLMANN gamma-hydroxybutyric acid (GHB) kit has been designed for the direct and quantitative determination of GHB in human urine and serum. The assay can be adapted to clinical chemistry analyzers according to specific instrument protocols.
This product is for research use only. It is not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

$$GHB + NAD^+ \xrightarrow{\text{Rec. Enzyme}} Product + NADH + H^+$$

The GHB is converted to its metabolite, succinic semialdehyde (SSA), by the action of GHB-Dehydogenase (Rec. Enzyme) and oxidized nicotinamide adenine dinucleotide (NAD^+). The increase in absorbance at 340 nm resulting from the reduction of NAD^+ into NADH is proportional to the amount of GHB in the sample.

REAGENTS SUPPLIED AND PREPARATION

Reagents[1)]	Quantity	Code	Reconstitution
Incubation Buffer	1 vial 12 ml	B-GHB-IB	Ready to use
Cofactor lyophilized NAD^+	1 vial	B-GHB-CF	Add 5.6 ml of deionized water[4)]
Enzyme lyophilized recombinant Enzyme	2 vials	B-GHB-E	Add 4.2 ml of deionized water[4)]; **do not vortex**
Calibrators[2)] lyophilized GHB	2 vials	B-GHB-CASET	Add 2 ml of deionized water[4)]
Controls Low / High[3)] lyophilized GHB in human urine	2 vials	B-GHB-CONSET	Add 2 ml of deionized water[4)]

Table 1

1) All reagents contain sodium azide (<0.1%) as preservative.
2) After reconstitution the GHB concentration of the Calibrators is 10 and 100 mg/L, respectively.
3) The Controls contain lot-specific amounts of GHB. Refer to the QC data sheet for actual concentrations.
4) For reconstitution see chapter Procedural Notes.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
The unopened kit components are stable at 2 - 8 °C, **except Cofactor that must be stored at -20 °C upon receipt.** Do not use reagents after expiration of the kit.	
Opened / Reconstituted Reagents	
Incubation Buffer	At 2-8 °C until the expiration date
Cofactor	Store for up to 2 months at 2-8 °C
Enzyme	
Calibrators	
Controls	

Table 2

WARNINGS AND PRECAUTIONS

- B-GHB-CASET and B-GHB-CONSET contain GHB at <100 mg/L. At this concentration GHB is considered as non-toxic.
- All reagents contain sodium azide at <0.1%. Sodium azide may react with copper or lead and form explosive compounds in metal drain lines. Upon disposal, flush with a large volume of water to prevent azide build up.
- Do not ingest any reagents.
- Use only deionized H_2O for reconstitution. See chapter Procedural Notes.
- Calibrators and Controls contain components of human urine. Thus the risk of transmitting pathogens of known or unknown origin cannot be completely excluded.
- Urine samples should be handled as potentially infectious.
- All products and samples containing human material should be handled in accordance with good laboratory practice using appropriate precautions.

MATERIALS REQUIRED BUT NOT PROVIDED

- Accurate pipetting devices (1, 5 or 10 ml pipettes)
- Test tubes/rack
- Clinical chemistry analyzer with an optical filter at 340 nm
- Deionized water
- 0.9 % saline solution

SPECIMEN COLLECTION AND STORAGE

- Depending on the dead volume of the analyzer, ~110 µl sample volume must be transferred into the instrument specific sample tubes. Less than 10 µl of urine or serum sample is needed to run the test. For details refer to the instrument manual.
- Urine: Collect urine (pH 5-8) without additives, keep the samples refrigerated, and have them filtered or centrifuged. Collect the supernatant.
- Serum: Refer to page **Fehler! Textmarke nicht definiert.** to learn about the interference of hemolytic, lipemic or icteric samples. Collect blood into plain tubes (no anti-coagulant), avoid haemolysis, leave to clot for one hour, centrifuge for 10 minutes at approximately 1500 x g at room temperature (18-28 °C), and collect the supernatant.
- Sample storage: Urine and serum samples can be stored at 2-8 °C for at least two weeks. For longer storage keep the samples frozen at -20 °C. GHB stability in samples has intensively been investigated (8).

PROCEDURAL NOTES

Reconstitution of Reagents:

- Calibrator and Control vials are under vacuum. Thus, it is mandatory to remove the caps slowly and carefully in order to prevent loss of material.
- Cofactor (B-GHB-CF), Calibrators (B-GHB-CASET) and Controls (B-GHB-CONSET): Vortex the vials for 30 seconds after reconstitution or use a suspension mixer for 15 minutes.
- Enzyme (B-GHB-E): **DO NOT VORTEX!** Use a suspension mixer for 15 minutes at room temperature (RT) until the lyophilized Enzyme has completely dissolved. Alternatively, gently swivel the vial after reconstitution, leave it 15 minutes at RT, and gently move it upside-down afterwards.
- On board, the Enzyme can be kept up to 2 months at a temperature up to 15 °C.

ASSAY PROCEDURE

GHB enzymatic assay is designed for automated analysis on clinical chemistry analyzers. It has to be programmed as a three reagent test. For details refer to the analyzer specific protocols.
The following protocol has been established for KoneLab 30 (Thermo) and is used here as a general example for the test procedure. For applications on other instruments, we will provide assistance.

1. 100 µl of Incubation Buffer (R1)
   + 8 µl of urine or serum sample (S)
   + 7 µl of deionized water
2. + 50 µl of Cofactor (R2)
   → Incubate for 1.5 - 2 min at 37 °C
3. + 85 µl of Enzyme (R3)
4. $T_{0\ min.}$: Measurement at 340 nm (Blank)
   → Incubation for 5-6 min at 37 °C
5. $T_{5\text{-}6\ min.}$: Measurement at 340 nm

RESULTS

Calibration

Use endpoint mode at 340 nm. The absorbance obtained after reading 1 (M1) and 2 (M2) is calculated using the absorbance differences (M2-M1). The standard curve will be calculated from the two calibrators using a linear regression mode. Refer to Table 3 and Figure 1 for typical results and standard curve. Depending on the instrument it might be necessary to read additionally at a secondary wavelength (e.g. 700 nm).

Calibration is stable for at least 2 weeks. Calibration of the assay should be carried out every 14 days, when a fresh vial of Enzyme is reconstituted or when the controls are out of the confidence range.

Samples and Controls

The absorbance at 340 nm is recorded for the Calibrators and the GHB concentration for each Control and sample is calculated as indicated above.

CALCULATION

Automated procedure: For calculation of the results use the endpoint mode with two calibrators (10 and 100 mg/L). Refer to the instrument manual for further details.

Dynamic range: 5 - 230 mg/L

STANDARDIZATION

The GHB Calibrators have been calibrated against commercially available GHB quantified with HPLC.

QUALITY CONTROL

- The values of the Low and High Control provided with the kit must be within the lot specific range indicated on the corresponding QC data sheet. Otherwise, the standard curve has to be re-calibrated.
- It is Good laboratory practice (GLP) to record the following data for each assay: kit lot number, reconstitution dates of kit components, results for Calibrators and Controls, and internal urine or serum pool.
- The precision and expected values of standard curve and controls should be within established limits. The confidence limits for the controls are lot-specific and printed on the QC data sheet delivered with the kit.
- Reliable results will only be obtained by using precise laboratory techniques (current GLP guidelines) and by accurate attention of this instruction for use.

LIMITATIONS

Samples exceeding 230 mg/L should be diluted 1:10 with 0.9% saline solution (1 volume sample + 9 volumes saline solution). The results have to be multiplied by 10.

If the OD of the urine samples exceeds 1.0 OD at 340 nm, results may be falsely positive.

Positive results should be confirmed by alternative methods.

Substances and/or factors other than those investigated in the specificity study may interfere with the test and cause false results.

PERFORMANCE CHARACTERISTICS

Assay performance characteristics have been determined on KoneLab 30.

Limit of Blank (LoB): <1 mg/L. The LoB has been established by repeated measurements of blank values (0.9% NaCl, n= 60) in accordance with CLSI protocol EP17-A.

Limit of Detection (LoD): 1.5 mg/L. The LoD has been established by repeated measurements of two samples containing 4.8 and 6.2 mg/L GHB (n= 40 and 10, respectively) in accordance with CLSI protocol EP17-A (Table 4).

Limit of Quantification (LoQ): Urine: 5.0 mg/L; serum: <5.0 mg/L. The LoQ of urine and serum was determined by three measurements of three urine and serum samples at concentrations between 3 and 16 mg/L. The sensitivity at 10 % CV was determined to be 5.0 and <5.0 mg/L, respectively.

Precision: Repeatability: ≤5 % CV; Between run: <10 % CV; Between day: <5 % CV; Total precision: <10 % CV. The precision of urine and serum samples has been determined in accordance with CLSI protocol EP5-A2 by repeated measurements in two runs per day over a period 10 – 20 work days (Table 5).

Dilution linearity: Urine 100 - 105 %; serum 99 - 100 %. Three urine and two serum samples with elevated GHB concentration have been diluted with 0.9% NaCl solution (Table 6). The mean value is 103 % in urine and 100 % in serum. It is linear between 5 and 230 mg/l.

Recovery: Urine: 95 - 107 %; serum: 106 - 113 %. Three different urine and serum samples have been spiked with increasing amounts of GHB. The mean value is 100 % in urine and 110 % in serum (Table 7*).*

Specificity: The substances listed in Table 8 have been analyzed between 10 and 1000 mg/L to determine the enzyme specificity.

INTERFERING SUBSTANCES

Therapeutic drugs and drugs of abuse: The **therapeutic drugs** and **drugs of abuse** listed in Table 9 and Table 10 were evaluated according to the approved guideline for interference testing in clinical chemistry, EP7-A2 (7), on the KoneLab 30. No interference has been observed up to the listed concentrations.

The interference of **Ethanol** is shown in Figure 2. Up to 3‰ the measured GHB concentration is below 10 mg/L. 1 g/L Ethanol raises the GHB value by 3 mg/L.

Serum Indices: No interference is detected with the following substances up to the listed concentrations: **Triglycerides** (**Intralipid®** 275 mg/dL; equivalent to 7.7 mmol/L triglycerides), **conjugated bilirubin** (360 µmol/L; 30 mg/dL), **unconjugated bilirubin** (513 µmol/L; 30 mg/dL) or **haemoglobin** (3.1 mmol/L; 500 mg/dL) on KoneLab 30.

METHOD COMPARISON

34 urine samples have been compared to a published IC-GHB method (6) (Figure 3):

KK-GHB = y = 1.07x IC-GHB - 15.66; R^2 = 0.997

Figure 1: **Example of Standard Curve**

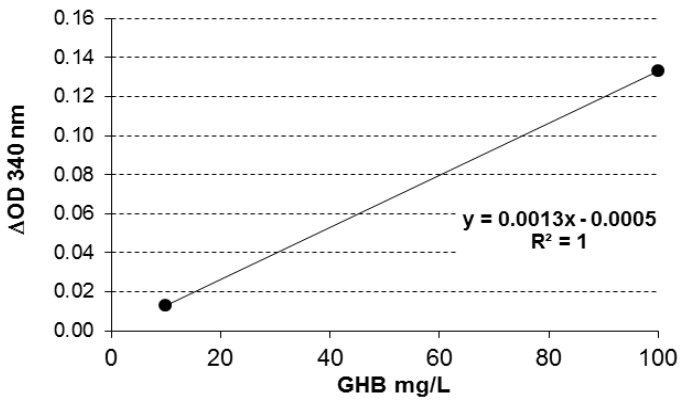

Table 3: **Example of Results**

Calibrators (2 replicates)	**Mean** (ΔOD)	**SD** (ΔOD)	**CV** [%]
10	0.013	0.0001	0.7
100	0.124	0.0025	2.0

Controls (2 replicates)	**Values** (ΔOD)	**Replicates** [mg/L]	**Mean** [mg/L]	**CV** [%]
low	0.017	13.0	13.0	0.3
	0.017	13.1		
high	0.096	76.7	76.7	0.0
	0.095	76.6		

Table 4: **LoB , LoD and LoQ**

LoB [mg/L]	**LoD** [mg/L]	**LoQ** [mg/L]
<1	1.5	Urine: 5
		Serum: <5

Table 5: **Assay Precision**

Sample [mg/L]	**Total Precision** [%]	**Repeatability (Within Run)** [%]	**Between Run** [%]	**Between Day** [%]
Urine				
11.2	8.6%	4.9%	7.0%	0.0%
55.3	4.5%	3.0%	3.0%	1.5%
100.3	5.2%	1.0%	2.9%	4.2%
Serum				
12.0	9.7%	5.0%	8.3%	0.0%
55.2	4.1%	2.1%	3.6%	0.0%
106.6	3.7%	1.4%	3.5%	0.0%

Table 6: **Dilution Linearity**

Example 1 of 3	**Dilution**	**Observed** [mg/L]	**Expected** [mg/L]	**O/E** [%]
Urine 1	1:1	238.6	287.3	83
	1:1.5	170.6	191.5	89
	1:2.25	119.9	127.7	94
	1:3.4	85.1	85.1	100
	1:5.1	58.8	56.7	104
	1:7.6	41.1	37.8	109
	1:11.4	27.8	25.2	110
	1:17.1	19.6	16.8	117
	1:25.6	13.0	11.2	116
	1:38.4	8.3	7.5	112
Mean urine 1				**103**
Mean urine 2				**105**
Mean urine 3				**100**
Mean urine				**103**
Serum 1	1:1	239.9	295.3	81
	1:1.5	184.0	210.9	87
	1:2.25	132.7	140.6	94
	1:3.4	93.7	93.7	100
	1:5.1	66.1	62.5	106
	1:7.6	45.1	41.7	108
	1:11.4	30.5	27.8	110
	1:17.1	20.0	18.5	108
	1:25.6	12.4	12.3	101
	1:38.4	7.9	8.2	96
	1:57.7	5.1	5.5	94
Mean serum 1				**99**
Mean serum 2				**100**
Mean serum				**100**

Table 7: **Spiking Recovery**

Example 1 of 3	**Native** [mg/L]	**Spiked with** [mg/L]	**Observed** [mg/L]	**Expected** [mg/L]	**O/E** [%]
Urine1	<1.5	10	10.1	10.0	97
		25	15.2	15.0	91
		50	49.1	50.0	98
		100	90.5	100.0	95
Mean urine 1					95
Mean urine 2					98
Mean urine 3					107
Mean urine					**100**
Serum1	<1.5	10	11.8	10.0	107
		25	18.3	15.0	118
		50	54.7	50.0	110
		100	102.6	100.0	104
Mean serum 1					110
Mean serum 2					113
Mean serum 3					106
Mean serum					**110**

Table 8: **Enzyme Specificity**

Component/substrate	Substrate specificity
γ-hydroxyvaleric acid, (GHV)	<0.1 %
γ-butyrolactone, (GBL)	4.0 %
1,4-butanediol, (1,4-BD)	<0.1 %
α-hydroxybutyric acid, (AHB)	<0.1 %
β-hydroxybutyric acid, (BHB)	<0.1 %
γ-hydroxyvaleric acid, (GHV)	<0.1 %
succinic acid	<0.1 %
γ-valerolactone, (GVL)	<0.1 %

Interferences

Table 9 **Interference of therapeutic drugs**

Therapeutic drugs	No interference up to	
Amikacin	53.2	µmol/L
Caffeine	84.5	µmol/L
Carbamazepine	75.4	µmol/L
Cyclosporine	480	nmol/L
Digoxin	4.43	nmol/L
Ethosuximide	970	µmol/L
Gentamicin	18.6	µmol/L
Lithium	1.77	mmol/L
Lithium (Vitros)	2.25	mmol/L
Methotrexate	7.78	µmol/L
Paracetamol	1.44	mmol/L
Phenobarbitone	223	µmol/L
Phenytoin	74.9	µmol/L
Primidone	58.8	µmol/L
Salicylic acid	2.86	mmol/L
Theophylline	177	µmol/L
Tobramycin	19.5	µmol/L
Valproic acid	998	µmol/L
Vancomycin	20.3	µmol/L

Table 10 **Interference of Drugs of abuse**

Substance	No interference up to	
Amphetamines (d-Methamphetamines)	1250	ng/mL
Barbiturates (Secobarbital)	375	ng/mL
Benzodiazepines (Oxazepam)	390	ng/mL
Cannabinoids (11-Nor-Δ-9-THC-9-COOH)	65	ng/mL
Cocaine (Benzoylecgonine)	500	ng/mL
LSD	1	ng/mL
Methadone	375	ng/mL
Opiates (Free Morphine)	2500	ng/mL
Phencyclidine	31	ng/mL
Propoxyphene (Norpropoxyphene)	375	ng/mL
Nortryptyline	375	ng/mL

Figure 2 **Interference of Ethanol**

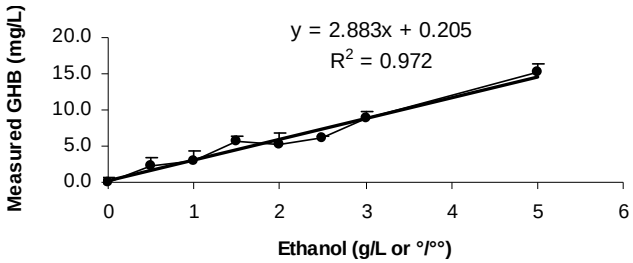

Figure 3 **Correlation between KK-GHB and IC-GHB**

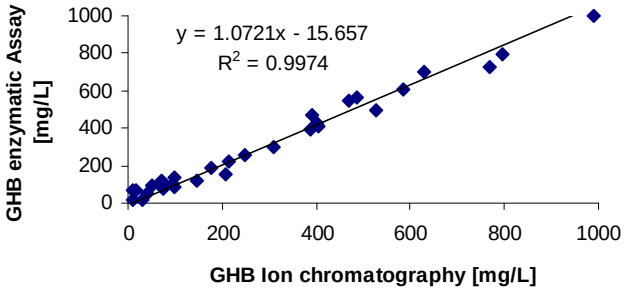

Table description: "Calculation" (page 3), "Performance Characteristics" (page **Fehler! Textmarke nicht definiert.**), and "Interpretation of Results" (page **Fehler! Textmarke nicht definiert.**).

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Symbol	Explanation
	Use By
	Catalogue number
	Batch code
	*In Vitro* Diagnostic Medical Device
	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature limitation
	Upper limit of temperature

Symbol	Symbol
	Incubation Buffer
	Cofactor
	Enzyme
	Calibrator A
	Calibrator B
	Control Low
	Control High

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Patented
US 8,609,359 B2
EP 2 253 711 B1