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Rapid colorimetric screening test for γ-hydroxybutyric acid (liquid X) in human urine

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Abstract

A rapid colorimetric test for the detection of γ-hydroxybutyric acid (GHB) is described. The ferric hydroxamate test for ester detection has been adapted to detect GHB in human urine samples from a healthy female and a healthy male subject. The assay can be performed within 5 min and with a GHB detection limit of 0.5 mg/ml when 0.3 ml of human urine is used and a GHB detection limit of 0.1 mg/ml when 1 ml of human urine is used. The colored complex indicating the presence of GHB is purple according to the assay conditions. Test results are free from the interference by alcohol, phenolic compounds and other biological chemicals under the assay conditions. In addition, the colorimetric test is free from the potential false-positive test result that could result from physiological concentrations of GHB.

Keywords: Ferric hydroxamate test; γ-Hydroxybutyric acid (GHB); γ-Hydroxybutyrolactone; Colorimetric test; Drug

1. Introduction

With the rising abuse of γ-hydroxybutyric acid (GHB), the demand for effective and rapid colorimetric screening tests for its presence in the human body is of great importance. The physiological GHB concentration in human urine is in the range of 0.5–2.0 μg/ml [1]. Typical GHB overdoes levels are greater than 1 mg/ml [2]. The reported detection limit of the existing few GHB non-enzymatic colorimetric tests is within the range of 10–0.1 mg/ml [3,4]. The objective of this study was to develop a rapid and effective colorimetric assay for GHB detection with a detection limit comparable with the existing non-enzymatic colorimetric tests. The study in this report presents a rapid and sensitive colorimetric screening test for the detection of GHB, which gives qualitative results within 5 min with a GHB detection limit of 0.1 mg/ml using the ferric hydroxamate test for esters [5]. The procedure involves the conversion of GHB into γ-butyrolactone (GBL), and its subsequent formation into a purple-colored ferric ion complex.

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2. Materials and methods

2.1. Materials

γ-Butyrolactone (GBL) and phenol was obtained from Sigma (St. Louis, MO). The sodium salt of γ-hydroxbutyric acid (GHB) was synthesized from GBL by alkaline hydrolysis with 1 M NaOH. Hydroxylamine hydrochloride ($NH_3OH{\cdot}Cl$), ferric chloride, ethanol, sulfuric acid, pH paper and sodium hydroxide were obtained from Fisher Scientific Inc. (Tustin, CA). All reagents were analytical grade and all solvents were HPLC grade. Human urine samples were obtained from a healthy female subject and a healthy male subject. Both subjects did not take any medication 24 h prior to the collection of urine samples.

2.2. Procedure for the ferric hydroxymate GHB colorimetric assay

Stock solutions of 1 mg/ml aqueous GHB, 1 mg/ml GHB in ethanol/water (1:1, v/v) and 1 mg/ml GBL were prepared daily. Urine samples that were spiked with GHB or GBL to give stock solution concentrations of 10–0.1 mg/ml were prepared daily. GBL stock solutions were used immediately after its preparation.

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About 0.3 ml of each of the following solutions was added to test tubes and assayed: (1) 1 mg/ml aqueous GHB solution, (2) 1 mg/ml GHB spiked urine sample, (3) 1 mg/ml GHB aqueous alcohol sample, (4) 1 mg/ml aqueous GBL sample, (5) 1 mg/ml GBL spiked urine sample, (6) distilled water (blank), and (7) non-spiked urine sample. To the test tubes containing the 0.3 ml samples, 0.5 ml of concentrated sulfuric acid was added. After the addition of the acid, 1 ml of a 0.5 M hydroxylamine hydrochloride ($NH_3OH{\cdot}Cl$) solution was added to each test tube. The pH of the resulting solutions was adjusted to ~10 by the addition of 2 ml of 12 M NaOH. The pH of the solutions was then adjusted to ~2 by the addition of 0.4 ml concentrated sulfuric acid. To the resulting reaction mixture, 0.2 ml of 0.7 M ferric chloride solution was then added. The instant formation of a purple-colored top layer was observed to signify the presence of GHB or GBL in samples spiked with these reagents. For samples that were not spiked with GHB or GBL, a light yellow to pale brown color was observed for the assay solutions of the newly formed top layer.

Lactone formation controls (Table 1, entries 8 and 9), which were used to evaluate the conversion of GHB to GBL for the spiked and non-spiked urine samples, were accomplished by following the same procedures except with one adjustment, 0.5 ml of distilled water was used instead of concentrated sulfuric acid at the beginning of the assay procedure. For test samples, to determine the limit of detection of the assay, serial dilutions were performed and 1 ml test samples and 0.3 ml test samples were used. The standard assay procedure was used for the phenol control experiment to test for the interference of the colorimetric assay by phenolic compounds.

Table 1
Ferric hydroxamate GHB colorimetric assay test results

Sample (medium)[a]	Final color[b]
GHB (distilled water)	Purple
GHB (urine)[c]	Purple
GHB (water/ethanol, 1:1, v/v)	Purple
GBL (distilled water)	Purple
GBL (urine)[c]	Purple
Control experiments[a]	
Distilled water	Light yellow
Urine[c]	Pale brown
GHB (urine, non-sulfuric acid treated sample)[c,d]	Pale brown
Urine[c,d]	Pale brown
Phenol (urine)[c]	Pale brown

[a] GHB, GBL and phenol concentrations are 1 mg/ml where the use of these compounds are indicated. The samples were treated with sulfuric acid.

[b] Color of top layer formed in test tube after the addition of ferric chloride solution to indicate a positive (purple) or negative (light yellow–pale brown) test result.

[c] Samples were tested from both a human male and a human female subject.

[d] No sulfuric acid was used in the assay procedure to convert GHB into GBL.

3. Results and discussion

The colorimetric test for GHB detection is a modified procedure of the ferric hydroxamate test used for the detection of organic esters [5]. By converting GHB into its lactone (GBL) using sulfuric acid it was realized that the ferric

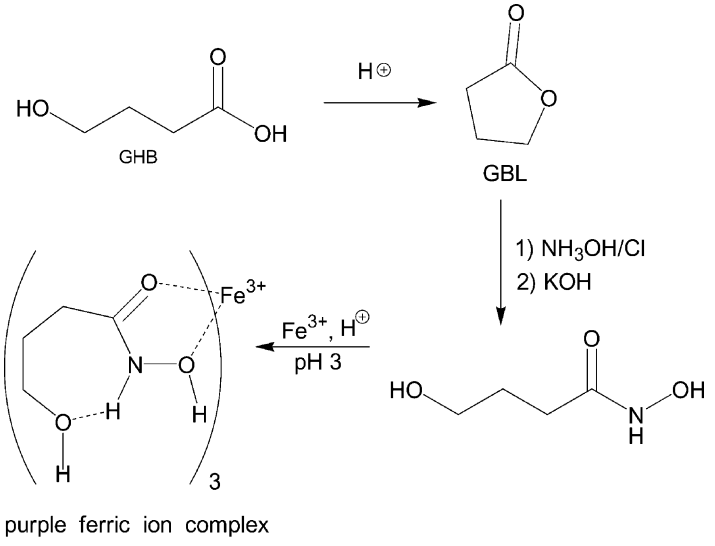

Fig. 1. Ferric hydroxamate GHB colorimetric assay reaction sequence.

hydroxamate test could be applied (Fig. 1). Previous studies have shown that the ferric hydroxamate test could be used for the detection of lactones [6,7].

According to the experimental results in Table 1, the presence of GHB in urine samples was signified by the presence of purple color from the iron complex formed in the assay solution. Comparing the results of the GHB spiked urine sample and its control (Table 1, entries 2 and 8, respectively), the GHB spiked urine sample gave a purple color, while its control showed a pale brown color. The results indicated that iron(III) did not form the purple-colored complex with GHB. The aforementioned observation proved that sulfuric acid was essential for the conversion of GHB into GBL. In addition, the results suggest that the colorimetric test is selective for the presence of GBL and is insensitive to the presence of GHB if it is not treated with acid.

Comparing the results of the GHB aqueous sample and the GHB spiked urine sample (Table 1, entries 1 and 2), both samples gave a purple color indicating that the test is not interfered by other chemicals in the urine samples. A similar conclusion can be drawn by comparing the results of the GHB spiked urine sample and the non-spiked urine sample (Table 1, entries 2 and 7, respectively), where the GHB spiked urine sample gave a purple color, while the non-spiked urine sample showed a pale brown color.

Since most GHB overdoes cases involve alcohol abuse, the effect of alcohol on the detection of GHB was evaluated by preparing a GHB aqueous alcohol (ethanol) sample (Table 1, entry 3). The result of the GHB aqueous alcohol sample was compared with the GHB aqueous sample (Table 1, entries 3 and 1). Both the samples gave a purple color, indicating that the presence of alcohol does not cause interference with the test result.

A possible interference for the proposed colorimetric test is from phenolic compounds. It is known that iron(III) forms colored complexes with phenolic compounds under neutral to basic conditions [8,9]. However, the purple-colored ferric hydroxamate complex indicating the presence of GHB was formed under acidic conditions (pH 3). Interference by phenolic compounds in the urine samples under the assay conditions at physiological concentrations was not problematic and was confirmed by spiking urine samples with phenol (Table 1, entry 10). Comparing the results of the spiked urine samples with phenol and the non-spiked urine sample (Table 1, entries 10 and 7), both the two samples gave pale brown colored top layers for negative test results. In addition, the purple-colored ferric hydroxamate complex was absent from the spiked urine samples with phenol (Table 1, entry 10). This observation confirmed that phenolic compounds in the urine samples do not interfere with the test result under the assay conditions.

By examining the results of the non-spiked urine sample with the distilled water sample (Table 1, entries 7 and 6, respectively), the non-spiked urine sample gave a pale-brown color, while the distilled water sample gave a light yellow color. The absence of the purple-colored ferric hydroxamate complex from the non-spiked urine sample (Table 1, entry 7) suggests that biological chemicals, especially the ketone bodies from the urine did not interfere with the colorimetric test results. In addition, since it is commonly known that GHB exists naturally in human urine, the absence of the purple-colored ferric hydroxamate complex from the non-spiked urine sample suggests that the GBL formed from acid treated GHB at physiological concentrations do not generate concentrations of colored complexes detectable by the naked eye for the colorimetric assay. Consequently, the colorimetric test is free from the potential false-positive test result that could result from physiological concentrations of GHB.

Experiments were performed to determine the limit of detection of GHB in urine samples. The detection limit for the presence of GHB was found to be 0.5 mg/ml using 0.3 ml of sample. However, with 1 ml of sample, a detection limit of 0.1 mg/ml could be achieved. It was found that by increasing the amount of sample used in the assay, the sensitivity of the assay could be increased. By adjusting the amount of sample used, the 0.3 ml sample assay could be used as a visual cut-off for concentrations greater than 0.5 mg/ml GHB and the 1 ml sample assay could test for GHB concentrations as low as 0.1 mg/ml.

4. Conclusion

A rapid and simple colorimetric assay for the detection of GHB in the urine samples has been developed using the ferric hydroxamate test for the detection of esters. Several quantitative analytical methods that involve the conversion of GHB to GBL have been developed for measuring GHB in biological matrices [2,10–12]. The reported assay involves the conversion of GHB to GBL and its subsequent formation into a purple-colored ferric ion complex. The assay can be performed within 5 min and with a GHB detection limit of 0.5 mg/ml when 0.3 ml of sample is used and a GHB detection limit of 0.1 mg/ml of when 1 ml of sample is used. Test results were free from the interference by alcohol, phenolic compounds and other biological chemicals under the assay conditions. In addition, the colorimetric test is free from the potential false-positive test result that could result from physiological concentrations of GHB (0.5–2.0 μg/ml in human urine) [1]. For determination of other potential false positive results, simple forensic investigations should be performed to identify the sources of interference. The reported GHB ferric hydroxamate test can be used as a rapid colorimetric screening test for human urine samples.

References

[1] G. Frison, L. Tedeschi, S. Maietti, S.D. Ferrara, Determination of γ-hydroxybutyric acid (GHB) in plasma and urine by head-space solid-phase microextraction and gas chromatography/

positive ion chemical ionization mass spectrometry, Rapid Commun. Mass. Spectrom. 14 (2000) 2401–2407.
[2] S.D. Ferrara, L. Tedeschi, G. Frison et al., Therapeutic γ-hydroxybutyric acid monitoring in plasma and urine by gas chromatography-mass spectrometry, J. Pharm. Biomed. Anal. 11 (1993) 483–487.
[3] N.R. Badcock, R. Zotti, Rapid screening test for γ-hydroxybutyric acid (GHB, Fantasy) in urine, Ther. Drug Monit. 21 (1999) 376.
[4] D.J. Koppenhaver, GHB color test, Microgram 30 (1997) 130.
[5] J.A. Feldman, S.G. Frank, T.J. Holmes Jr., Determination of di-(2-ethylhexyl) sodium sulfosuccinate and related dialkyl esters in aqueous solutions by the ferric hydroxamate procedure, J. Pharm. Sci. 60 (1971) 920–921.
[6] J.C. Legrand, J.R. Pasqualini, M.F. Jayle, Determination of steriod lactones, Bull. Soc. Chim. Biol. 44 (1962) 997–1007.
[7] R. Kringstad, Detection of unstable lactones by means of thin layer chromatography and the ferric hydroxamate test, Anal. Chem. 47 (1975) 1420–1421.
[8] E.V. Gan, H.F. Haberman, I.A. Menon, Simple and sensitive test for the determination of phenolic compounds in urine and its application to melanoma, J. Invest. Derm. 64 (1975) 139–144.
[9] V. Das Gupta, K.A. Bomer, Quantitative determination of phenol in phenolated calamine lotion USP, J. Pharm. Sci. 64 (1975) 1199–1200.
[10] J.T. Lettieri, H.L. Fung, Evaluation and development of gas chromatographic procedures for the determination of γ-hydroxybutyric acid and γ-butyrolactone, Biochem. Med. 20 (1978) 70–80.
[11] J.D. Doherty, O.C. Snead, R.H. Roth, A sensitive method for quantitative of γ-hydroxybutyric and γ-butyrolactone in brain by electron-capture gas chromatography, Anal. Biochem. 69 (1975) 268–277.
[12] T.B. Vree, E. van der Kleijn, H.J. Knop, Rapid determination of 4-hydroxybutyric acid (γ-OH) and 2-propyl pentanonate (depakine) in human plasmas by mean of gas–liquid chromatography, J. Chromatogr. 121 (1976) 150–152.