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Determination of barbituric acid, utilizing a rapid and simple colorimetric assay

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Abstract

Barbituric acid is widely used in the manufacturing of plastics, textiles, polymers, and pharmaceuticals. Three assay methods are presented, which can utilize either aqueous or solid samples. The detection of as little as 10 μg of barbituric acid can be accomplished, either as an aqueous or solid sample, when using micro reaction tubes. The red–violet endpoint is easily discerned and results upon the formation of a violuric acid derivative. A Spot Test protocol is described which allows for a positive/negative indication of barbituric acid presence. The Spot Test is sensitive to as little as 18.75 μg/ml of barbituric acid. The construction of a Standard Curve for assaying multiple samples and over longer time periods is demonstrated, and is shown to be linear from 18.75 μg/ml to 2.25 mg/ml of barbituric acid. Spectrophotometer readings are made from an absorption peak appearing at 530 nm. The molar absorptivity of the violuric acid derivative is calculated to be 31.4 per mol/l per cm. The micro reaction tube assay will quantitate as little as 10 μg of barbituric acid through interpolation with controls containing a known amount of analyte. Inorganic salts such as NaCl, NaN_3, LiBr, and $CaCO_3$ do not interfere with endpoint determination. Many organic compounds (also pharmaceuticals) do not inhibit the reaction.

Keywords: Barbituric acid; Violuric acid; Barbiturates

1. Introduction

The compound barbituric acid (2,4,6-trihydroxypyrimidine; malo-nylurea) is widely used in the manufacturing of plastics [1], pharmaceutical preparations [2], indicators [2], textiles [2,3], and polymers [4]. This compound was introduced by von Baeyer in 1863 from a fusion of urea and malonic acid [5]. It is known that barbituric acid itself has no affect on the central nervous system [5], however it is a precursor to medicinal barbiturates which can be lethal in excessive amounts [5–7]. Other work has shown that in mice, barbituric acid will cause liver and kidney weight increase [8], and induce hyperglycemia and glucose intolerance [9]. The solid residue of this compound is irritative to the skin, eyes, and respiratory tract when contact has occurred (M.S.D.S.). Barbituric acid is also a precursor to derivatives

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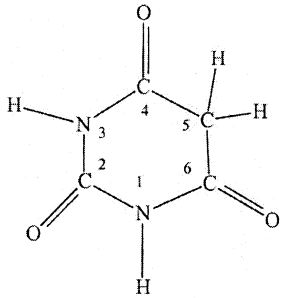
that have been shown to have antimicrobial activity [10,11], and for tumor inhibitory agents [12]. The molecular structure shown:

(1)



BARBITURIC ACID

Derivatives of barbituric acid are obtained by placing alkyl or aryl groups in exchange of the hydrogens at position 5 (see 1). These derivatives have sedative and hypnotic effects [13]. Sedative barbiturates are general depressants, which depress nerve, skeletal, and cardiac muscle. Barbiturate poisoning directly affects the cardiovascular system and can induce coma, with other effects varied depending on the particular barbiturate. Although tolerances for sedative barbiturates can evolve, this does not alter the lethal overdose concentration. Barbiturate compounds diffuse into all tissue and organs in vivo, including the brain and crossing of the placenta barrier [13].

The major route of elimination in vivo occurs in the liver, and by oxidation at the C-5 position (see 1). Cleavage of the barbituric ring occurs only slightly. Although cleavage of the barbituric acid ring occurs only slightly, toxic effects appear at dose exposures of amounts greater than the clinically applied levels of the sedative forms. Examples of toxic activity include the overwhelming of the detoxification mechanisms of the liver and disturbance of intra- and extra-cellular osmotic balance [13].

Members of sedative barbiturates can be classed as long-acting, short-acting, and ultra-short-acting. Phenobarbital, a long-acting member, is administered up to 120 mg/day. Amobarbital, a short-acting member, is administered up to 150 mg/day. Sodium thiopental, ultra-short-acting, is administered intravenously up to 0.2 g/treatment. Lethal dose levels vary widely, and depends on the type, dose, route of administration, and patient.

The colorimetric assays presented for barbituric acid determination are specific to its structural ring form as a prerequisite to the formation of the colored violuric acid derivative. Sensitivity of detection are to parts per million. In addition, these protocols allow the analysis of samples as aqueous mixtures, dust particles, or other solid samples.

2. Materials and methods

2.1. Reagents

All chemicals were obtained from Sigma–Aldrich, P.O. Box 14508, St. Louis, MO 63178, USA. A Perkin–Elmer Lambda 6 and Spectronic 21D were used for spectrophotometric analysis with 1 cm quartz cells.

2.2. Assay method utilizing spectrophotometer

Obtain a 1.0–3.0 ml aqueous sample for assay, or alternatively dissolved a solid sample into an equivalent volume, then add 0.50 ml of aqueous saturated $NaNO_2$. Add 0.10 ml of 2.0 M acetic acid and mix. Add sufficient distilled water to bring to a final volume of 4.00 ml, and mix. Read absorbance at 530 nm wavelength with UV–Vis spectrometer. The formation of known Standards follows the same steps using a stock aqueous barbituric acid mixture at 3.000 mg/ml to acquire the desired concentration levels. Use water, $NaNO_2$, and acetic acid only as a mixture for the 'blank'.

2.3. Assay method utilizing micro reaction tubes

To quantitate 10–30 μg amounts of barbituric acid the aqueous sample or solubilized solid specimen must not be greater than 10 μl in volume, which is then placed into an appropriate micro reaction tube. Add 10 μl of saturated $NaNO_2$, then 2.0 μl of 2.0 M acetic acid, mix, and observe for a red–violet endpoint. Standards of desired concentration values are made following the same

steps and then used for comparison and interpolating concentrations of test samples. A stock barbituric acid mixture at 3.000 mg/ml provides amounts at 3 μg/μl and is convenient for this purpose. Known controls at 12, 15, 18, 21, 24, and 27 μg of barbituric acid, are representative and convenient amounts to use with this protocol.

2.4. Spot test assay for barbituric acid

For rapid positive/negative indication of barbituric acid place 90 μl of saturated $NaNO_2$ with 90 μl of aqueous sample, mix, then add 20 μl of 2 M acetic acid. A red–violet color production is indicative of barbituric acid. A positive control having a known amount of barbituric acid and a negative control with no barbituric acid should be run simultaneously. A stock solution of barbituric acid at 3.000 mg/ml provides a convenient means to generate desired concentrations.

2.5. Evaluation of non-interfering substances

Following the spot test protocol, 100 μg of barbituric acid and 100 μg of each of the organic or inorganic compounds listed in Table 1 were shown to be non-interfering with the discernment and interpretation of the definitive colorimetric endpoint upon violuric acid production.

Table 1
Compounds evaluated to have no interference with the colorimetric reaction for determination of barbituric acid are shown here

Organic compounds	Inorganic salts
Aspirin	NaN_3
Quinine	NaCl
Nicotinic acid	$CaCO_3$
Nicotinamide	LiBr
Inositol	$Na_2B_4O_7{\cdot}10H_2O$
Caffeine	K_2HPO_4
Cholesterol	
L-proline	
p-Nitrobenzoic acid	
Maleic acid	
Myristic acid	
D-Tartaric acid	
Benzenesulfonamide	

A broad range of organic functional groups and inorganic salts are represented here.

2.6. Evaluation of aqueous, dust, and solid samplings

Aqueous mixtures of barbituric acid were prepared in a range of 1.0–2.0 mg/ml for determination by standard curve. To demonstrate application to dust aliquots, an amount of 4.0 mg of barbituric acid was finely ground in mortar and pestle, then randomly scattered over a 24×24 in.^{2} surface area. A cotton-tipped applicator stick then systematically swapped to the area back and forth. The cotton tip was washed into a plastic tube with minimal water, allowed to dry, then subjected to the Spot Test (see above). For alternate solid samples, equal amounts of inorganic salts were mixed with barbituric acid, and then subjected to the spot test as described.

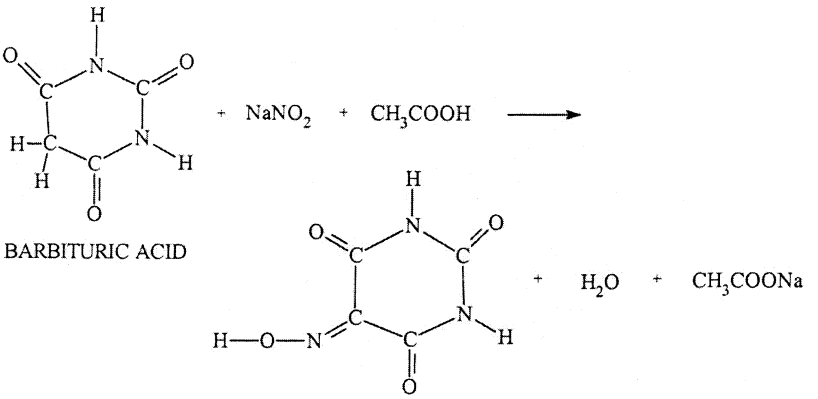
3. Results and discussion

The red–violet barbituric acid derivative, violuric acid, is formed upon reaction of aqueous nitrite and barbituric acid in an acidic solution. The colored endpoint is strong and easily recognized. The reaction does not cause any significant temperature alterations. Formation of the violuric acid product proceeds quickly at room temperature, less than 1 min, and is stable for up to 24 h at 25 °C. The rapid formation of the colorimetric response which is clearly visible to the naked eye is an advantage over other analytical methodologies. Glass or plastic containers are suitable for these reactions. The reagents used are stable at 25 °C for >10 weeks, in which they retain their reactivity with barbituric acid over that time span.

An aqueous mixture or solid residue containing barbituric acid can be used in all analytical methods described. Analysis was carried out in glass and plastic containers of suitable size. The colorimetric assays are versatile and easily interpreted. When barbituric acid is placed with acetic acid and sodium nitrite, the resulting products include a nitrosation in the five position of barbituric acid and formation of the tautomeric violuric acid [2].

The violuric acid formed is a purple salt, and is stable at 25 °C for up to 24 h. The chemical reaction proceeds rapidly and can be represented as follows:

CHEMICAL REACTION



BARBITURIC ACID

VIOLURIC ACID

Solutions of known barbituric acid concentrations are utilized for standard curves, which is demonstrated in Fig. 1. Linear regression analysis of the smooth line, which is formed reveals a correlation coefficient of 1.00, indicating a high level of linearity. The graph shows the relationship of the absorbance at 530 nm, which is plotted, versus concentration of barbituric acid expressed as milligrams per milliliter in the aqueous solutions. Calculation of the molar absorptivity of the purple violuric acid derivative product with these sample solutions produced a consistent value (using Beer's Law) of 31.37 per mol/l per cm, having population standard deviation of 0.71 per mol/l per cm. Using a prepared standard curve allows the rapid and accurate quantitation of barbituric acid with numerous samples.

The graph of Fig. 1 represents the absorbances of mixtures having concentrations expressed as mg/ml of barbituric acid. The linearity of the standard curve is demonstrated over a 100-fold range of concentration. Their respective molar concentrations and absorbances are (molar/absorbance): (0.0176 M/0.553), (0.0117 M/0.371), (0.00878 M/0.283), (0.00586 M/0.176), (0.00293 M/0.0920), (5.82E-04 M/0.0210), (2.93E-04 M/0.0160), (1.46E-04 M/0.0070). Sedative barbiturate compounds (i.e. phenobarbital, veronal, etc.) have alkyl and aromatic groups substituted in the five position of the parent structure, and will not react to create interfering signals due to the blocking of =NOH formation by these same attached C-5 groups.

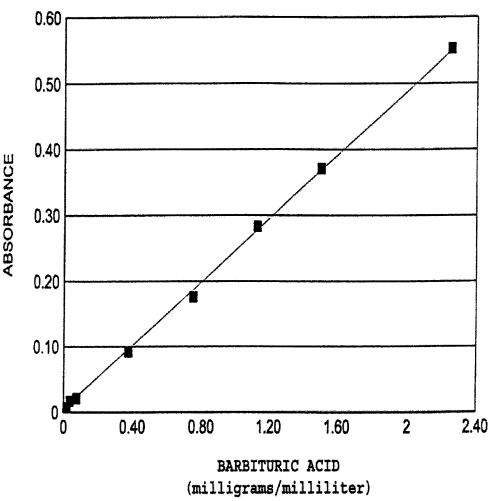


Fig. 1. The standard curve for the assay of barbituric acid is developed for a range of 0.01875–2.25 mg/ml of analyte. Absorbances are of the colored reaction product violuric acid, which are read at 530 nm wavelength in 1 cm cells. The results are highly linear, having a correlation coefficient of 1.000 and slope of 16.33 per mg/ml) when analyzed by linear regression.

For the rapid spot test described in Section 2 the limit of detection is less that 18.75 μg of barbituric acid per milliliter of solution. This analysis method is sensitive and produces a easily recognized red–violet endpoint. A positive control of a known amount of barbituric acid is recommended, and a negative control having no barbituric acid. Small glass reaction tubes are beneficial, and assist in endpoint determination. All other aspects of reaction descriptions that are given above also apply to the spot test procedure. To demonstrate the applicability of these protocols for mixtures having other organic compounds, the spot test examination was applied to mixtures containing 100 μg of barbituric acid plus 100 μg of one of the following compounds: aspirin, quinine sulfate, nicotinic acid, nicotinamide, inositol, L-proline, caffeine, and cholesterol. Organic functional groups represented in the selected compounds include carboxylic acids, ether groups, alcoholic hydroxyl groups, tertiary amines, secondary amines, amide groups, cyclic amide structures, alkyl chains, and structures bearing aromatic rings. Various inorganic salts were tested in a similar manner, salts such as NaCl, LiBr, NaN_3, carbonate and phosphate salts. See Table 1 for listing of classes of compounds evaluated as non-interfering. The red–violet endpoint observed upon violuric acid formation remained strong, easily recognized, and consistent despite an equal background presence of the amino acid, vitamin, drug, inorganic salt or metabolite compound listed previously.

This is demonstrated in Fig. 2, in which the colorimetric endpoint of violuric acid is clearly discerned in test samples which also contains 100 μg of one of the background compounds described previously. The level of color produced in each sample is consistent and based upon violuric acid produced

Application of the same test procedure in the presence of sulfanilamide and L-tryptophan (an essential amino acid) produced a yellow oily product which nullifies the red–violet endpoint and interferes with the interpretation of the barbituric acid spot test. This is demonstrated in Fig. 3, where a positive and negative samples are seen (samples A and B, respectively), with L-tryptophan (sample C) and sulfanilamide (sample D).

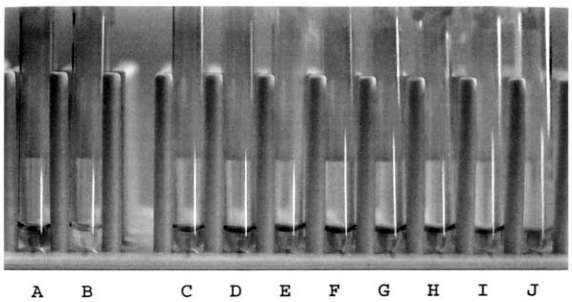


Fig. 2. Complex organic compounds do not interfere with formation of the colorimetric endpoint as demonstrated here with the spot test samples. The red–violet endpoint is still clear and easily recognized despite the presence of 100 μg of a background organic compound. The following samples contain 100 μg of barbituric where indicated and 100 μg of a second compound as described: (A) barbituric acid (positive control); (B) no barbituric acid (negative control); (C) aspirin/barbituric acid; (D) quinine sulfate/barbituric acid; (E) nicotinic acid/barbituric acid; (F) nicotinamide/barbituric acid; (G) inositol/barbituric acid; (H) L-proline/barbituric acid; (I) caffeine/barbituric acid; (J) cholesterol/barbituric acid.

The micro reaction tube procedure utilizes volumes of 22 μl, and is sufficiently small to allow the use of capillary tube containers, which functioned well and provided a convenient means to make interpolative comparisons with known controls. Also suitable are micro-centrifuge tubes of

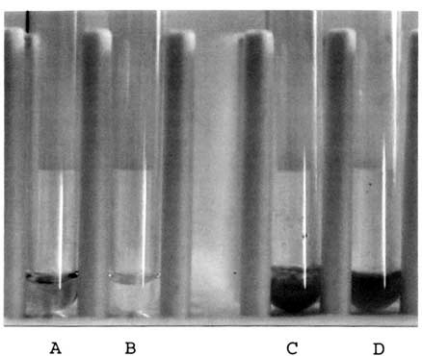


Fig. 3. Some amine compounds may produce a competing colored complex which nullifies the red–violet endpoint of the violuric acid, for the spot test methodology as described in Section 2. Shown here are two examples, where 100 μg of barbituric acid is present as indicated with 100 μg of an organic amine compound. (A) barbituric acid (positive control); (B) no barbituric acid (negative control); (C) L-tryptophan/barbituric acid; (D) sulfanilamide/barbituric acid.

either glass or plastic make. The red–violet endpoint of violuric acid formation is recognizable with the use of samples at concentrations of 12, 15, 18, 21, 24, 27 μg amounts, which permits quantitation by comparative interpolation. As little as 10 μg can be visually detected. A stock barbituric acid mixture at 3.000 mg/ml will dispense 3 μg/ml for assay controls.

The assay techniques presented here are competitive to other methods, and have significant advantages. Protocols presented here are similar in sensitivity to approaches using reactions with *p*-benzoquinone [14], and *p*-dimethylaminobenzaldehyde [15], which also require longer incubation periods for color production (the procedure here requiring < 60 s). Potentiometric determination of barbituric acid requires expensive equipment, complicated data interpretation, and works with a toxic organic mercury compound [16]. Similar detection sensitivity was accomplished by another spectrophotometric methodology [17], however it utilizes the controlled substance chloral hydrate, has longer incubation periods, and does not have the ability for micro reaction assay or spot testing. Very expensive equipment utilizing capillary electrophoresis with electrochemical detection, have been applied as a means for barbituric acid and 2-thiobarbituric acid identification [18].

Background testing with various compounds produced no interfering signals. However the same tests performed with L-tryptophan (amino acid) and sulfanilamide demonstrated an important feature included in the use of nitrite ion. Sulfanilamide has a primary aryl amine and L-tryptophan contains both a primary and secondary amine site [19], all of which may react with nitrous acid, which is formed when NO_2^- is treated with an acid. Aliphatic primary amines and aromatic primary amines react with NO_2^- in acidic solution to produce various alkenes and alcohols [20]. The general reaction can be shown as follows:

$$\text{R-NH}_2 + \text{NO}_2^- + 2\text{H}^+ \rightarrow \text{R}^+ + 2\text{H}_2\text{O} + \text{N}_2 \rightarrow \text{alcohols, alkenes}$$

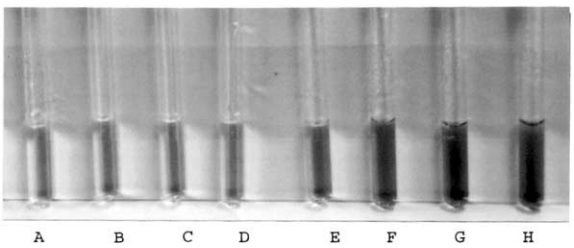


Fig. 4. Demonstration of the spot test protocol applied to samples having different amounts of barbituric acid. (A) no barbituric acid (negative control); (B) aspirin only (no color); (C) quinine sulfate only (no color); (D) inositol only (no color); (E) barbituric acid at 40 μg; (F) barbituric acid at 90 μg; (G) barbituric acid at 140 μg; (H) barbituric acid at 180 μg.

Secondary amines may react with acidic NO_2^- to form *N*-nitrosoamines, which are considered strong carcinogens [20]. The general reaction may be represented as shown below:

$$(\text{R})_2\text{NH (secondary amine)} + \text{NO}_2^- \text{ (nitrite ion)} + \text{H}^+ \rightarrow (\text{R})_2\text{N–N} = \text{O } (N\text{-nitrosomaine}) + \text{H}_2\text{O}$$

The results of the above reaction will produce a strong yellow product nullifying the violuric acid endpoint (Fig. 3).

Fig. 4 demonstrates the colorimetric endpoint formation with various amounts of barbituric acid obtained when utilizing the spot test, and examples of negative results (no color formation) with other organic compounds such as aspirin, quinine sulfate, and inositol. Discernable color formation and consequent endpoint determination are the result with 40, 90, 140, and 180 μg amounts of barbituric acid. A discernable colorimetric gradation occurs with the increase of barbituric concentration.

For dust samples evaluated with the spot test procedure (Section 2) the presence of barbituric acid was determined in all samples gathered in this manner or similar. For solid residues mixed with an inorganic salt, again the assay successfully indicated the presence of barbituric acid in all the proper samples. Results for this colorimetric determination are represented in Fig. 5, where no color is observed in the negative control (Sample A), color produced in Sample B having 40 μg of barbituric acid (positive control), color observed

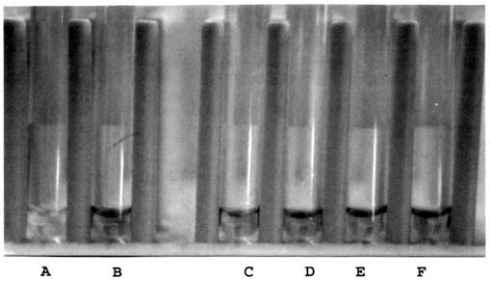


Fig. 5. Results for spot test determination of barbituric acid in dust collection samples and solid samples having inorganic salt contaminants is demonstrated here. Sample (A) no barbituric acid (negative control); (B) barbituric acid at 40 μg; (C) positive result for barbituric acid dust collection by cotton-tipped applicator (Section 2); (D) positive result for barbituric acid powder contaminated with LiBr; (E) positive result for barbituric acid powder contaminated with NaCl; (F) positive result for barbituric acid powder contaminated with NaN_3.

in barbituric acid dust collection (Sample C), color is observed in Samples D, E, and, F for solid samples having inorganic salts as contaminants (LiBr, NaCl, and NaN_3, respectively). All aqueous samples determined by means of the standard curve were accurately evaluated to within 3% percent of the correct value. This provides a recovery of 97% or greater.

Comparable sensitivity to tens of μg/ml was achieved by use of cyclic voltammetry and controlled-potential coulometry [21]. A sensitivity of 0.1 μg/ml is accomplished with the use of a ruthenium/cerium complex chemiluminescence [22].

Three protocols for a highly sensitive determination of barbituric acid are presented, which are simple to perform and easy to interpret. Small volumes of mixtures may be utilized for the Micro Reaction Tube protocol and larger volumes for the Spot Test. The total reaction volumes for each protocol are optimized to allow for maximum sensitivity and convenience of application. Numerous samples may be analyzed accurately and with high sensitivity by construction of a standard curve which is also demonstrated here. The techniques are also very specific and can be used when various other organic compounds may be present in the desired sample matrix. The protocols are rapid and may be applied to solid and aqueous samples.

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