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Detection and quantitative assay of 2-thiobarbituric acid from inert solid and solubilized samples by spectrometer

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ABSTRACT

The thiobarbiturates differ from other barbiturates in having a sulfur atom to replace the carbonyl oxygen at the C-2 position. The drug 2-thiobarbituric acid was soluble in 95% ethanol and 5% water for sensitive detection and measurement. All samples were examined at 340 nanometer wavelength. An ultraviolet-visible instrument with one centimeter glass cuvettes was suitable for this assay. The molar extinction coefficient at 340 nm was 349.9 Liter/(cm)(mole). An absorbance spectrum for 2-thiobarbituric acid in the identical solvent used for assay is accomplished. The standard curve ranged from 0.0000457 molar to 0.003830 molar, an 84x fold concentration span. The lowest concentration assayed in this study was 0.006588 grams per liter (6.588E-06 grams per milliliter) with the highest concentration analyzed at 0.5521 grams per liter (5.5521E-04 grams per milliliter). This methodology was found to be consistent during analysis and highly sensitive.

KEYWORDS

2-thiobarbituric acid;
Barbiturates;
Spectrometry;
Thiobarbiturates.

INTRODUCTION

The heterocyclic organic compound 2-thiobarbituric acid (2-thioxodihydro- pyrimidine-4,6(1H,5H)-dione) is considered to be a sedative and hypnotic drug referred to as a member of the group of general depressants called thiobarbiturates[1]. Structural features of thiobarbiturates include a sulfur atom in place of the carbonyl oxygen at the C-2 position in contrast to barbiturates retaining the oxygen atom at the C-2 position or oxybarbiturates. In positioning a sulfur atom to replace the carbonyl oxygen at the C-2 position does produce a greater lipid solubility and more rapid onset activity than the corresponding oxybarbiturates[1,2].

Thiobarbiturates in general tend to be more toxic than the oxybarbiturates and with some important changes in their pharmacodynamics[1]. Thiobarbiturates are utilized in medicinal applications generally as anesthetics which are administered intravenously or rectally[1,2]. Derivatives of 2-thiobarbituric acid have found various applications in pharmacological and analytical applications with other types of derivatives being investigated for their anti-cancer and anti- viral activities[3].

Various kinds of lipophilic thiobarbiturates are being studied for clinically useful applications as medica-

ments in the treatment of convulsions and anesthesiology[4]. Previous studies have shown that various derivatives of thiobarbituric acid have been shown to inhibit the enzymatic activity of the hepatitis C virus (HCV) NS5B polymerase[5]. Tyrosinase has been demonstrated to play various roles in organisms and studies are focusing on the regulation of tyrosinase activity. Previous studies have shown that the inhibitory effect of thiobarbituric acid on tyrosinase is a potentially usefull reversible noncompetitive activity[6].

In any case 2-thiobarbituric acid is utilized as a reagent for assaying malondialdehyde which is among products found from lipid peroxidation[7-9]. Malondialdehyde is one of several low-molecular-weight products formed from the decomposition of certain primary and secondary lipid peroxidation products[8,9]. These type of assays are important in handling of oxidative stress in critical care environments[9].

As a consequence of the various uses of 2-thiobarbituric acid there is a growing need for methodologies of analytical assay for thiobarbiturates in general due to their clinical, pharmacological, industrial, and bio-analytical application. Ultraviolet differential spectrophotometry has been applied for measuring various thiobarbiturates previously[10]. Additionally, assay by thin layer chromatography has been successfully accomplished[11]. The methodology presented in this study is an accurate and highly sensitive approach for measurement of the drug 2-thiobarbituric acid.

EXPERIMENTAL

Reagents and instrumentation

All reagents applied as solvents were analytical grade and obtained from Sigma-Aldrich (P.O. box 14508, St. Louis MO 63178 USA). The 2-thiobarbituric acid (2-thioxodihy- dropyrimidine-4,6(1H,5H)-dione) as a light yellow powder, with only slight water solubility used for standards or preparation of samples was obtained from Eastman Organic Chemicals (P.O. Box 431, Kingsport, Tennesee, USA or AstraTech, 1 Deer Park Dr. Suite C, Monmouth Junction, New Jersey, 08852 USA).

The absorbance readings were collected from Milton Roy Spectronic 21D UV- Visible Spectrophotometer with stable precision 10nm bandwidth/spectral slit-width high resolution continuous wavelength range (A.L.T., 12 Colton Road East Lyme, CT 06333). One centimeter width glass cuvettes were used throughout. The analyte 2-thiobarbituric acid dissolved readily and remained stable in 95% ethanol and 5% water. Solvent utilized throughout the project was 95% ethanol and 5% water.

Sample analysis parameters for spectrophotometric analysis

All samples of 2-thiobarbituric acid were dissolved in and found to be highly soluble with the following solvent: 95.0% ethanol, 5.0% water. All measurements of absorbance or percent transmittance was accomplished utilizing Milton Roy Spectronic 21D UV-Vis Spectrophometer by this solvent system. Samples of 2- thiobarbituric acid were carefully dissolved in solvent of 95 % ethanol and 5% water then examined by spectrometer in 1 cm glass cuvettes. This proved to be efficient and permitted very highly sensitive determination of the analyte. All analytical samples and solvent mixtures were stored in glass containers and sealed air tight until use. To obtain the absorbance spectrum an amount of 0.02728 grams of 2-thiobarbituric acid was dissolved in 100 mL of 95% ethanol and 5% water for scanning from 320 nm to 600 nm. Distilled water was utilized throughout.

For extraction of 2-thiobarbituric acid from inert solid then the following steps were followed: 1) Obtain a 2 mL size portion of the solid material and place into a fresh glass sealable sample tube; 2) Place 4 mL to 8 mL of solvent 95% ethanol and 5% water and vortex briskly for one minute; 3) Allow the suspension to settle; 4) Vortex briskly for one minute; 5) Repeat steps (3) and (4); 6) Allow the suspension to settle or filter or centrifuge to pellet debris; 7) The supernatant can be measured directly at 340 nm (or diluted appropriately to obtain measurement of absorbance). Note that contaminants soluble in the alcohol solvent will interfere with measurements.

The molecular properties of 2-thiobarbituric acid such as formula weight, octanol/water partition coefficient Log P, polar surface area, and Rule of 5 where stated were determined by utilizing chemical informatics system of Molinspiration (Molinspiration, Nova ulica,

SK-900 26 Slovensky Grob, Slovak Republic).

Numerical analysis

Where indicated the numerical analysis utilizing Spearman/Kendall correlation, 95% ellipses, and normality test was performed by PAST version 2.06 (copyright Hammer and Harper 1999-2011). Summary statistical analysis was also performed by Microsoft EXCEL (copyright 2010 Microsoft Corporation, Microsoft Office Professional Plus 2010). The one sample t-test and unpaired t-test with Welch correction, Wilcoxon test, and Mann-Whitney test was performed by GraphPad InStat version 3.00 (Copyright 1992-1998 GraphPad Software Inc. www.graphpad.com) for Windows 95, San Diego California USA) and Graph Pad online statistical analysis (www.graphpad.com/quickcalcs/index.cfm).

RESULTS AND DISCUSSION

The detection and measurement of 2-thiobarbituric acid (as well as other thiobarbiturates) is necessitated due to its application in clinical environments as an anesthetic[1], industrial uses for the bio-analytical assay of lipid peroxidation events[6,8,9], and anti-viral studies with potential general use[3]. A technique presented here can be accomplished utilizing simple or more advanced ultraviolet-visible (UV-Vis) spectrometers and is an effective and facile process. Some general advantages of UV-Vis based spectrometry are: 1) simpler operation; 2) lower cost; and 3) non-destructive determination of analyte (e.g. the 2-thiobarbituric acid can be retrieved from the solvent mixture). Also UV-Vis absorption spectroscopy has been used in the clinical laboratory and industrial facilities for many years and universal in its application in analytical chemistry.

The drug compound 2-thiobarbituric acid ($C_4H_4N_2O_2S$) shown in Figure1 is a member of the thiobarbiturates group of sedative/hypnotic barbiturates and considered distinct from oxybarbiturates. It has a polar surface area of 58.196 Angstroms^2, octanol/water partition coefficient of Log -0.937, and zero violations of the Rule of 5[12], which identifies this thiobarbiturate as an effective oral drug.

One major aspect of selecting a suitable solvent for analysis by UV-Vis spectrometry is the effective dissolution of the analyte which was accomplished here by use of the polar solvent of 95% ethanol and 5% water. This means was easy to perform and convenient. All samples prepared in 95% ethanol and 5% water were kept in air tight glass sample tubes until use. The complete absorbance spectrum collected for 2-thiobarbituric acid in 95% ethanol and 5% water is shown in Figure 2. A known amount of 2-thiobarbituric acid was dissolved in 95% ethanol and 5% and measuring absorbance values at the respective wavelengths settings. After complete analysis the final value for molar absorptivity (å) is determined to be 349.9 Liter/(cm)(mole). At wavelength 400 nm the absorbance approaches zero and remains up to 700 nm. At 320 nm wavelength a significant background absorbance occurred from the alcohol solvent utilized in this study however at 340 nm there is no background due to an ethanol/water solvent system. Consequently all determinations for the drug 2-thiobarbituric acid in this solvent system is accomplished at 340 nm.

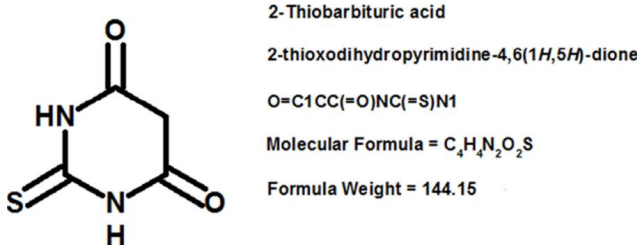

Figure 1 : Structure and properties of 2-thiobarbituric acid. The molecular structure, common name, SMILES notation, and formula presented here for the sedative/hypnotic compound 2-thiobarbituric acid.

A standard curve represents the relationship between two quantities. They are used to determine the value of an unknown quantity from one that is more easily measured. The standard curve applied for assay is presented in Figure 3 over a broad range of molarity values. The standard curve utilized in this study ranged from 0.00004570 molar to 0.003830 molar (a 84x wide range in molar concentration values). By mass per volume this is a range from 0.00659 grams per liter (or 6.59E-06 grams per milliliter) to 0.5521 grams per liter (or 5.521E-04 grams per milliliter).

A 95% confidence ellipses is a two-dimensional analog of a confidence interval which can be visualized on a 2-way plot[13]. A 95% confidence ellipse, from within 95% of the data points are expected to lie, en-

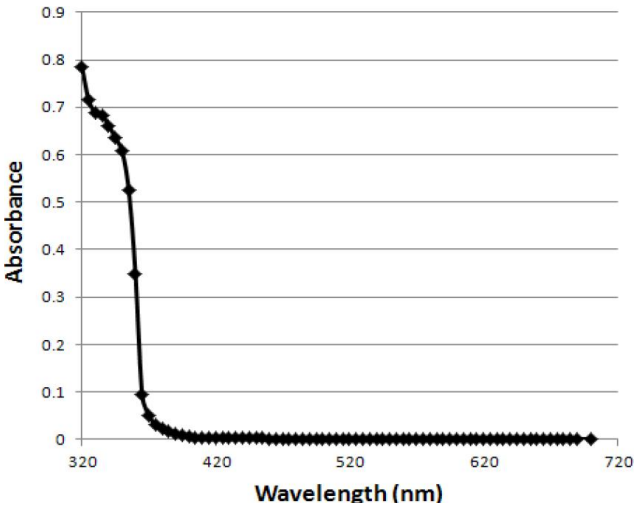

Figure 2 : Absorbance spectrum of 2-thiobarbituric acid from 320 nm to 700 nm obtained in glass cuvettes and 95% ethanol with 5% water.

compassed all entries of the standard curve as well as all analytical samples taken for completion of this study inclusive of solubilized and extract from inert solid. This outcome affirms the high level of consistency and reproducibility that was established in this determination for 2-thiobarbituric acid. Molarity values and absorbance values for these standards pass the normality test and therefore are well-modeled by a normal distribution[14]. The equation of the line derived from the standard curve is: y = 349.8696x + 1.7E-05. The Pearson's r correlation is r = 1.000, with the coefficient of determination R^2 = 1.000 indicating that this regression line fits the data well. The coefficient of determination, R^2, is useful because it gives the proportion of the variance (fluctuation) of one variable that is predictable from the other variable. It is a measure that allows estimating the level of certainty in making predictions from a certain model and is the ratio of the explained variation to the total variation[14].

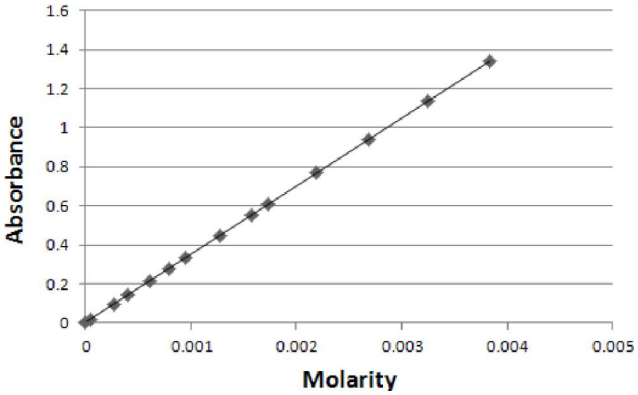

Figure 3 : Standard curve of assay by spectrophotometer. The standard curve utilized in this study ranged from 0.00004570 molar to 0.003830 molar (a 84x wide range in molar concentration values).

Comparison of actual molarity and calculated molarity based upon the equation of the linear standards plot of absorbance against molarity, is presented in TABLE 1. The percent recovery is accurate at essentially 100 % at both high and low levels of concentration of 2-thiobarbituric acid. The two groups of molar data of actual and calculated values did not pass the normality test and do not describe a normal distribution. The unpaired t test performed on actual and calculated molarities indicate no reason to conclude that the overall means of these two groups differ (two tailed P value is 0.9997 then with critical value P = 0.05)[14]. Similarly the Wilcoxon test for actual and calculated molarities (see TABLE 1) provides a large value of P indicating no reason to conclude the overall medians of these two sets of values differ (P = 0.1573 then with critical value P = 0.05). The Mann-Whitney test showed that actual and calculated molar values have the same distribution of scores with no significant difference between these two groups (absolute value of z at 0.01639 with $z_{.05}$ = 1.645; two tailed P value is 0.9869 with critical value P = 0.05; calculated U = 390.5 with critical U = 272)[14].

Extraction of analyte from inert solid support

The solvent used for spectrometer assay was found to be suitable for extracting the thiobarbiturate drug from various inert solid supports which in turn provides a means to assay the 2-thiobarbituric acid from inert solid materials. From large and small granules of sand and silica-glass the dried thiobarbiturate compound was dissolved into 95% ethanol and 5% water prior to UV-Vis spectrometric assay as described. The liquid portion of the extraction steps is kept in air tight containers but can be filtered to remove solid aggregates present or centrifuged to pellet the same.

A control specimen consisted of dissolved thiobarbiturate that was dried to powder with-in the same glass containers utilized throughout the study. These samplings can be measured by an identical standard curve prepared as described previously. Repeat samplings were examined as described with results that are statistically repeatable and descriptive.

TABLE 1 : Percent recovery of analyte

Actual Molarity	Calculated Molarity	Percent Recovery
0.002644	0.002644	100
0.002424	0.002424	100
0.002681	0.002681	100
0.002909	0.002910	100
0.003087	0.003087	100
0.003244	0.003244	100
0.003830	0.003830	100
0.003587	0.003587	100
0.0001486	0.0001486	100
0.0002744	0.0002743	100
0.0003172	0.0003172	100
0.0004001	0.0004001	100
0.001720	0.001721	100
0.0001200	0.0001200	100
0.0004944	0.0004944	100
0.0007059	0.0007059	100
0.0001972	0.0001972	100
0.0002086	0.0002086	100
0.0002286	0.0002286	100
0.001849	0.001849	100
0.003040	0.003041	100
0.002832	0.002832	100
0.0003658	0.0003658	100
0.001300	0.001300	100
0.00156	0.001561	100
0.002578	0.002578	100
0.0003715	0.0003715	100
0.002892	0.002892	100

Statistical examination of measurements showed the process to be consistent and descriptive of the quantity of drug present (see TABLE 2). A test for outliers performed by Grubb's test (also referred to as extreme studentized deviate or ESD) for comparison of 12 control runs with all test samples showed no outliers among the numerical values of absorbance and molarity concentrations. For all control and test sample measurements the numerical values of molarity and absorbance pass testing for normality and therefore follow a normal distribution.

In addition all molar and absorbance numerical values of controls (1-12) and testing samples (13-29) fall within 95% ellipses, indicating these numbers are within 95% confidence intervals. Unpaired t test with Welch correction for comparing control values (1-12) to tests (13-29) show that the mean of the controls do not differ from the overall mean of tests (two-tailed P value is 0.4808 and greater than critical value P = 0.05)[14]. In addition the t-test comparison of means indicates that the mean molarity value for controls is not significantly different from mean molarity of all test samples (13-29) where two-tailed P value is 0.4563 and critical value P = 0.05. Extracting by alcoholic solvent from inert solid material thus appears an effective tool for measuring the presence of 2-thiobarbituric acid that is deposited. This methodology using alcohol-water solvent provides an accurate and sensitive means to measure the amount of 2-thiobarbituric acid in specimens of pharmaceutical origin.

TABLE 2 : Extraction from solid materials

Number	Sample Tested	Molarity	Absorbance
1	control	0.0003858	0.135
2	control	0.0004115	0.144
3	control	0.0003744	0.131
4	control	0.0004115	0.144
5	control	0.0003773	0.132
6	control	0.0003858	0.135
7	control	0.0003601	0.126
8	control	0.0003887	0.136
9	control	0.000383	0.134
10	control	0.0004258	0.149
11	control	0.0004058	0.142
12	control	0.0004201	0.147
13	large sand particles	0.0004601	0.161
14	large sand particles	0.0004258	0.149
15	large sand particles	0.0004173	0.146
16	small sand particles	0.0004173	0.146
17	small sand particles	0.0004487	0.157
18	small sand particles	0.0004373	0.153
19	small sand particles	0.0004544	0.159
20	small sand particles	0.000403	0.141
21	small sand particles	0.0004344	0.152
22	small silica particles	0.0003401	0.119
23	small silica particles	0.0003544	0.124
24	small silica particles	0.0003058	0.107
25	small silica particles	0.0003715	0.13
26	small/large silica beads	0.0003315	0.116
27	small/large silica beads	0.0003058	0.107
28	small/large silica beads	0.0002887	0.101
29	small/large silica beads	0.0003144	0.11

CONCLUSION

The sedative/hypnotic 2-thiobarbituric acid is determined in an alcohol-water solvent system by UV-Vis spectrometer using glass cuvettes at 340 nm wavelength. A standard curve is efficacious for use with this approach with very high linearity and correlation (Pearson's r = 1.000, R^2 = 1.000) with absorbance to molarity of set standards. The lowest concentration assayed in this study was 0.006588 grams per liter (6.588E-06 grams per milliliter) with the highest concentration utilized at 0.5521 grams per liter (5.5521E-04 grams per milliliter). The standard curve utilized spanned an 84x breadth of molarity concentrations. Comprehensive statistical analysis indicated that accuracy by percent recovery of analyte is extremely high. Extraction removal from inert solid support by the identical alcohol-water solvent effectively measured the analyte. The methodology for the assay of 2-thiobarbituric acid is applicable to clinical, pharmaceutical, environmental, and industrial usage.

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ABBREVIATIONS

UV-Vis, ultraviolet visible; nm, nanometers; ESD, extreme studentized deviate; cm, centimeter.

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