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Barbital assay by reversed phase high performance liquid chromatography

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ABSTRACT

Diethylbarbituric acid (barbital), a hypnotic and sleeping aid, is assayed by reversed phase high-performance liquid chromatography utilizing a column havingoctadecylsilyl (C-18) covalently bonded onto silica. All the test samples and column solvent wasprepared in 95% ethanol and 5% water. The barbital drug efficientlysolubilized into this solventsystem and was stable for hours prior to analysis. Barbital eluted from the column consistentlyat 1.7 minutes. The minimum concentration of analyte detected in this work is0.0001680 molar which is 0.0309 milligrams/milliliter. The highest concentration utilized in this work at 0.03669 molaror 6.758 milligrams/milliliter. The standard curve utilized for test determination had correlation coefficient of Pearson's r = 0.9992 (R^2 = 0.9984). The percent recovery rates for barbital showed a mean of 97.0% and standard deviation ± 1.58% (standard error = 0.345, mode = 96.9, sample variance = 2.50, kurtosis = -1.28) and median of 97.5%. Askewness value of 0.101for percent recovery indicates the percent recovery rates are highly symmetric. The Spearmans' rsof 1.000 for actual and calculated molaritiesindicating extremely high positive correlation.

KEYWORDS

Barbital;
Dethylbarbituric acid;
HPLC;
Barbiturates.

INTRODUCTION

Barbiturates are considered to be hypnotics and sedatives[1]. Most combinedsedative-hypnoticdrugs are considered to be general depressants and depress manycellularfunctions in various organs[1]. Barbiturates candistribute throughout all tissuesand fluids if allowed to pervade in the plasma for long periods of time[1]. Barbituratesare as addicting as heroin and can be even more dangerous during withdrawal. Withrepeated use over substantial time a substantial tolerance will occur followed by anecessity torequire more drug to achieve the identical effect, but the lethal dose levelremainsessentially the same. The clinical application of barbiturates is determined bythe duration of action requiredbut with the realization that the effect ofthe drugincreases with dosage[1].

This group of agents are divided into three general categories: ultra-short active(havingeffects within a minute), short acting (with effects from 15 to 40 minutes), intermediate acting (having effects within 40 minutes to an hour), and long acting(effectslasting more than an hour)[1, 2]. The long-acting barbiturates, which includesbarbital, are clinical agents usedas anti-convul-

sants, for controlling ulcers, and highblood pressure[1]. Barbital was the first agent of this group applied for medicalpurposes[2]. All barbiturates have similar structure to barbital and general centralnervous system depressants, but will affect all excitable tissues in the body[3, 4].Barbiturates adhere to both basic mechanisms of tolerance development, which are:(1) tissue tolerance, and (2) pharmacokinetic tolerance[4, 5].

Barbital has been utilized as an antidote to cocaine, procaine hydrochloride, andother drugs applied to induce local anesthesia[6]. Treatment of acutebarbituratepoisoningis by using diuretics to stimulate urine excretion such as with sodium sulfateand glucose, but barbital requires the use of ammonium chloride[7]. Barbital anesthesiacan be controlled through diuresis[8]. Previous identificationand assay of variousbarbiturates has been accomplished at wavelengths of240 nm but requiringpHstabilization at 10 or 2[9].

Sensitive assays for barbital poisoning areneeded partly because it is utilized as acommon buffer in various biology laboratorymanipulations and incidents of poisoninghave been documented[10]. Deep ultraviolet-visible (UV) reflected optical fiber sensorwith spectrophotometric detectionhas been used for drug detection at 200 nm[11]. Agas chromatography-mass spectrometry (GC-MS) method coupled with pulse split lessinjection technique was developed for the determination of 10 sedative-hypnoticsincludingbarbital[12]. Some barbiturates, including barbital, were determined bymicellar liquid chromatography with C18 column[13]. Liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS) methodfor determination of barbital, amobarbital, phenobarbital, and secobarbital in humanplasma is been found suitable[14]. Barbital has been determined by absorption (zero-order) UV spectra and first-order derivative spectra[15]. Other assay methods includeliquid chromatography-electrospray tandem mass spectrometry[16], rapid ultravioletmonitoring[17], capillary electrophoresis[18], and electrokinetic supercharging[19].

This work presents methodology for detection and assay of barbitalaccomplished byreversed-phase high performance liquid chromatography in alcohol solvent.

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 diethylbarbituric acid (barbital)compound for use as standards and preparation of samples was obtained from Sigma-Aldrich. For the High Performance Liquid Chromatography (HPLC) analysis, an Alltech426 HPLC Pump and Linear UVS 200 detector were utilized (2051 Waukegan Road, Deerfield, Illinois 60015-1899 USA). Solvent utilized throughout the project was95% ethanol and 5% water.

Preparation of standards and test sample analysis

For preparation of standards solutions, known amounts of diethylbarbituric acid areweighed by analytical balance and dissolved in the ethanol-water solvent mixture. Themolarity of each standard is identical to preparation of standard curve: 0.0001676 molar, 0.0003336 molar, 0.006575 molar, 0.01311 molar, 0.02198 molar, 0.0255 molar, 0.02997 molar, 0.03394molar, and 0.01912 molar. This is a 114 fold range in values.All test samples containingdiethylbarbituric acid were dissolved in-and found to behighly soluble with the following solvent: 95.0% ethanol, 5.0% water. All measurementsof absorbance by HPLC and preparation of samples was accomplished utilizing thissolvent system. For column analysis by HPLC a reversed phase C-18 octadecylsilyl($C_{18}H_{37}$) bonded phase packing wasutilized for the column. The barbital analyte elutedconsistently at 1.7 minutes. Detection was accomplished by ultraviolet detector set to240 nm, rise time 0.1, range AUFS set to 1.0. The HPLC pump was set to 2900 psigand one milliliter samples were injected.

Numerical analysis

Where indicated the numerical analysis utilizing Spearman/Kendall correlation, Kruskal-Wallis test, Mann-Whitney test, 95% ellipses, and Kolmogorov-Smirnov (twosamples) wasperformed by PAST version 2.06 (copyright Hammer and Harper 1999-2011).

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Summary statistical analysis was also performed by Microsoft EXCEL (copyright2010Microsoft Corporation, Microsoft Office Professional Plus 2010). The paired t-testand Wilcoxon matched-pairs signed-ranks test was performed by GraphPadInStatversion 3.00 (Copyright 1992-1998 GraphPad Software Inc. (www.graphpad.com) forWindows 95, San Diego California USA).

RESULTS AND DISCUSSION

Determining the correct clinical dosage of barbiturate for the reduction of anxiety orsedation canbe problematic due to the difficulty of predicting the correct concentrationand that evena slight overdose can be lethal. The drug barbital acts similarly to allsedatives and hypnotics work by changing the amounts of gamma-aminobutyric acid(GABA) in the brain[1]. The sudden termination of barbital usage without medicalconsiderations can give rise to dangerous side effects. This fact calls for suitablemethods of monitoring drug contentand excretion.

Oxy-barbiturates are hypnotics of the barbiturate group of drugs in which the atomattached at the carbon-2 position is oxygen and virtually all hypnotic barbiturates areoxy-barbiturates[1]. All oxy-barbiturates have been shown to be soluble in alcohol[1].Consequently ethanol is chosen as a solvent for solubilizing this hypnotic compoundand due tolow absorbance at most wavelengths. Barbital has an ionization exponentvalueat 25° Cof pKa = 7.8[1]. In rodents the delay of activity for this hypnotic afterintravenous administration is measured to be 22 minutes[1]. As much as 65% to 90%ofa total dose of barbitalis excreted unchanged in humans[1]. For the barbituratesgroupin general, an increase inplasma alkalinity such as through hyperventilation orincrease in absorbed $NaHCO_3$ willincrease drug urine excretion[1]. These facts andthecontinued clinical use of barbituratesprompts methods for their detection.

The relative molecular structure of diethylbarbituric acid (barbital, barbitone, 5, 5-diethylpyrimidine-2, 4, 6(1*H*, 3*H*, 5*H*)-trione) is presented in Figure 1 with SMILESnotationO=C1NC(=O)NC(=O)C1(CC)CC, molecular mass = 184.193 g/mole, andpolarizability. The pharmacokinetic half-life of thiscompound is 27.1

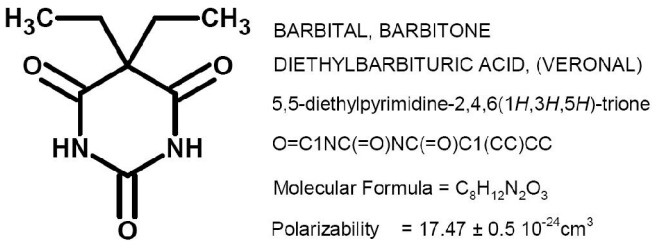

Figure 1 : Molecular structure of barbital (diethylbarbituric acid) with SMILES and formula designation

hours to 33.5hours[1]. The solid compound dissolved readily in 95% ethanol/5% water and wasstable throughoutthe study. Samples of all types were kept at room temperature. Allsamples were prepared in clean glass and air tight tubes prior to injection. Prior tosample injection, a solvent-only injection was done to stabilize detectionbackgroundand setting of detector.

A standard curve applied here is presented in Figure 2 showing extremely highcorrelation (Pearson's r = 0.9992) of molarity to area of elution (μV). The coefficientof determination R^2 = 0.9984 indicating an extremely good fit of the regression lineto the data and extremely high prediction efficiency by this model. In addition thisvalue of R^2 reflects a 99.84% explanation of the data by this model. The actual linearequationy = 3037353.9x + 851.04 (r = 0.9992) for the standard curve. The minimumconcentration of barbital detected in this method is0.0001680 molar which is 0.0309milligrams/milliliter (30.9 micrograms/milliliter or 30.9 parts

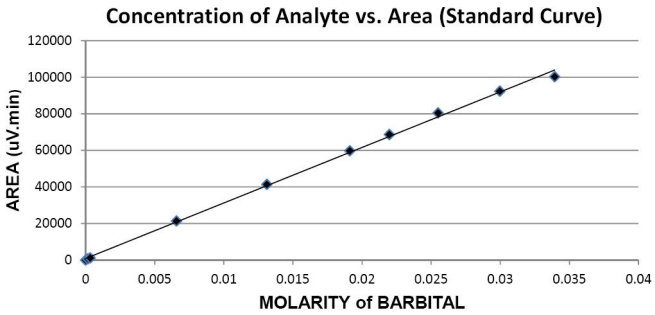

Figure 2 : A standard curve of barbital determination having r = 0.9992, R^2 = 0.9984, generalequation of line y = 3037353.9x + 851.04.

per million) with the highestconcentration at 0.03669 molar (6.758 milligrams/milliliter).

An assay by ultraviolet-visible spectrometer is not useful because of negligibleabsorbance by barbital in ethanol solvent. This is shown clearly in Figure 3, wherebarbital dissolved in ethanol has absorbance values less than 0.02 from thewavelength of 320 nm to

700 nm. These absorbance values show no consistencyin trend and are consistently very low numerically.

A typical elution profile is introduced in Figure 4, showing an individual peak elutingconsistentlyat 1.7 minutes. For this plot, minutes are independent variable and (μV) asthe dependent variable. The solvent mixture for column elution was 95% ethanol and5% water. The HPLC pump was set to 2900 psig and one millili-

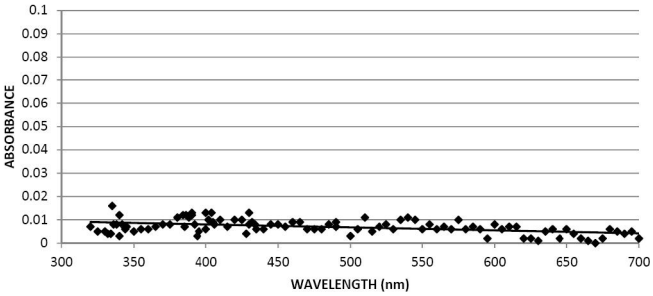

Figure 3 : Plot of barbital absorbance as a function of wavelength.

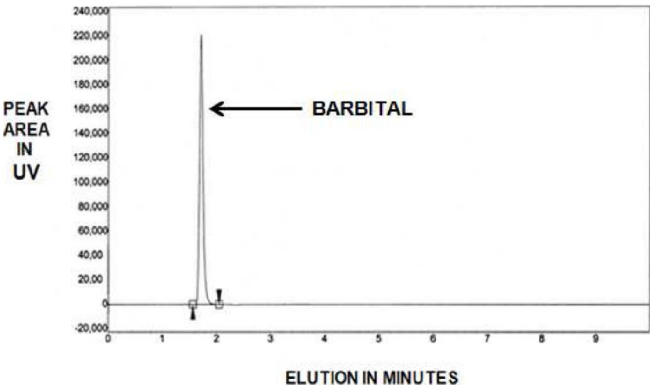

Figure 4 : A typical chromatogram of barbital sample. A single peak elutes consistently at 1.7 minutes..

ter samples wereinjected. Detection wavelength set to 240 nm consistently.

A number of known samples of about 0.001 molar were injected into the instrumentwith variation of wavelength of detection. The findings presented in Figure 5 indicatethat as wavelength of detection decreases from 280 nm to 210 nm the absorbanceincreases. Clearly setting the detector wavelength at 280 nm would give very poordetection of barbital with very low level of sensitivity. As the detector wavelengthapproaches 210 nm the absorbance values for barbital increase but interferencefrom the solvent and detection noise could be problematic. The detection wavelengthof 240 nm selected for this methodology is an excellent choice that allows sensitivityand accuracy for analyte identification and quantitation.

Test samplesof any origin are encompassed within the 95% confidence ellipses(ie. 95% confidence interval). The standard curve values of barbital concentration fortest samples fell within the 95% confidence interval (ie. 95% of all entry values)[20].Shown in Figure

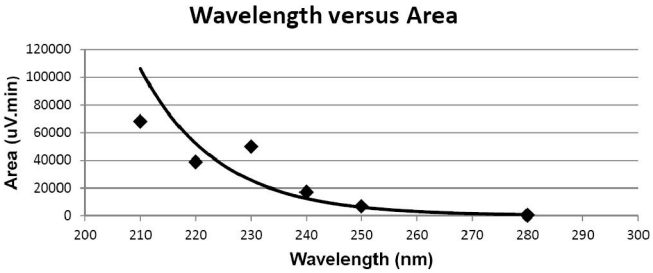

Figure 5 : Variation of barbital absorbance at constant molarity concentration and change in detection wavelength.

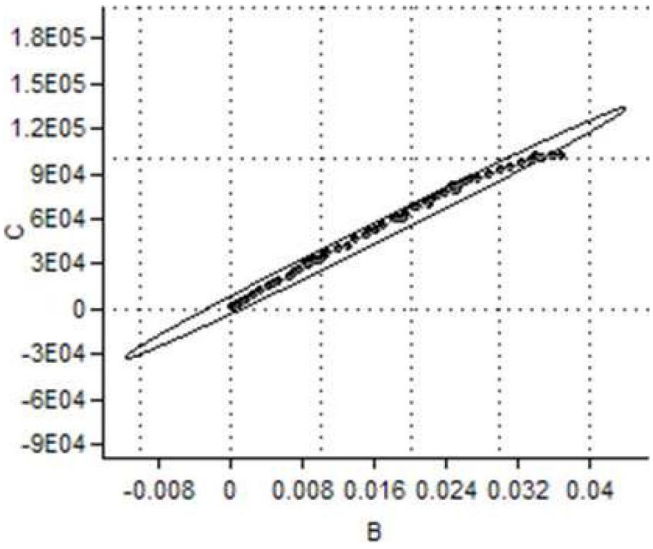

Figure 6 : Data contained within 95% confidence ellipses. Axis are computed as C (y-axis) and B (x-axis) for this determination

6, this outcome indicates very high inclusion and consistency of theanalysis[20].

Determination of molarity viz a vie by standard curve under conditions describedwas accurate and consistent. Comparison of actual molarity by calculated molarityutilizing a standard curve are viewed in TABLE 1 Determination of Molarity. ThePearson's r correlation amongst actual and calculated molarity is extremely highat 0.9982. Summary of statistics for actual and calculated molarity values areviewed in TABLE 2. The Spearman/Kendall correlation for statistical outcome of TABLE 2 has Spearman's rs =1.000 (Pearson's r =

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TABLE 1 : Actual and calculated nolarity

Calculated Molarity	Actual Molarity	Percent Recovery
0.03189	0.0335	95.2
0.03164	0.03236	97.8
0.03041	0.03119	97.5
0.02882	0.02871	99.6
0.02801	0.02741	97.8
0.02783	0.0266	95.4
0.02736	0.02606	95
0.02545	0.02522	99
0.02544	0.02494	98
0.02423	0.02319	95.5
0.02043	0.01945	95
0.01925	0.01879	97.5
0.01087	0.01037	95.2
0.01014	0.009959	98.1
0.009842	0.00955	96.9
0.007341	0.007012	95.3
0.00533	0.005239	98.2
0.004342	0.004328	99.7
0.003339	0.003239	96.9
0.002502	0.0024581	97.9
0.0009464	0.000995	95.1

TABLE 2 : Statistics of sample recovery

Summary Statistic	Calculated Molar Concentration	Actual Molar Concentration
Sample Variance	0.000124	0.000125
Kurtosis	-1.68738	-1.62045
Skewness	-0.23452	-0.14611
Mean	0.017877	0.017646
Standard Error	0.002429	0.002441
Median	0.02043	0.01945
Standard Deviation	0.011129	0.011188
Range	0.030944	0.032505
Minimum	0.000946	0.000995
Maximum	0.03189	0.03350

0.9988), indicating perfect positive correlation, with Mann-WhitneyU = 50 which is less than the critical value of U = 73 ($\alpha = 0.05$)indicating the twosets of statistical values come from identical populations[21]. Inaddition theKruskal-Wallis test outcome of p = 0.7624 indicates no statistical significantdifference between the two sets of results viewed in TABLE 2. These results clearlydemonstrating that actual molarity and calculated molarity are not significantlydifferentat all and can be considered but the same.

The determined percent recovery rates (see TABLE 1, third column) for barbital showeda mean of 97.0% and standard deviation ± 1.58% (standard error = 0.345, mode = 96.9, sample variance = 2.50, kurtosis = -1.28, skewness = 0.101) and median of 97.5%. Askewness value of 0.101 indicates the percent recovery rates are highly symmetric. Ofthese values the minimum is 95.0% to maximum of 99.7%, having a range of 4.7%[17].A negative value for kurtosis (-1.28) indicate low peakedness or platykurticdistribution[22, 23].

In so far as the relationship between calculated molarity from standard curvelinear equation (see TABLE 1, first column) to the actual molarities (see TABLE 1, second column) of test samples the Paired t test showed highly effective and accuratepairing withnear zero difference between populations (one-tailed P value is 0.0678, two-tailedP value is 0.1356). The Spearmans' rsof 1.00 for actual and calculatedmolarities of test samples indicated extremely high positive correlation in addition to theKruskal-Wallis test P = 0.8307(P > 0.05) indicating there is no statistical differencebetween these two sets of values. In addition a Kolmogorov-Smirnov results of P =0.9999 (P >.05) indicate thatcalculated and actual molarities are of the samedistribution (P > 0.05 so data isessentially normally distributed)[21, 23].

CONCLUSIONS

In summation, the sedativediethylbarbituric acid (barbital) is identified andquantifiedby reversed phase high-performance liquid chromatography utilizingoctadecylsilyl covalently bonded onto solid silica. Utilizing solvent 95% ethanol and 5%water the barbital solubilized readily and was stable for several hours prior to analysis.Ananalytepeak was consistently observed at 1.7 minutes. The minimum concentrationofanalyte detected in this work is0.0001680 molar (0.0309 milligrams/milliliter or30.9 parts per million) with the highest concentration at 0.03669 molar(6.758 milligrams/milliliter). A standard curve showeda correlation coefficient ofPearson's r = 0.9992 with coefficient of determination of $R^2 = 0.9984$, with the linearmodel explaining 99.84% of variance found in data. Percentrecovery

was highlyefficient. This approach clearly is effective and accurate for thedetection of barbital.

ACKNOWLEDGEMENTS

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