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Detection and Assay of Vitamin B6 (Pyridoxine Hydrochloride) Utilizing Isocratic High Performance Liquid Chromatography

Ronald Bartzatt[1*], Purnima Gajmer[1], Mai Han Cassandra Nguyen[1] and Alexandra My-Hanh Tran[1]

[1]University of Nebraska, 6001 Dodge Street, Durham Science Center, Omaha, Nebraska 68182, USA.

Authors' contributions

This work was carried out in collaboration between all authors. Author RB designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PG, MHCN and AMHT managed the analyses of the study and operated the instruments. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To demonstrate an analysis for vitamin B_6 from commercial aqueous nutritional drinks and solid tablets, utilizing isocratic conditions with high performance liquid chromatography (HPLC) and UV detection at 290 nm.
Study Design: Vitamin B_6 in the form of pyridoxine hydrochloride is assayed by HPLC from various samples.
Place and Duration of Study: Department of Chemistry, Durham Science Center, University of Nebraska, Omaha Nebraska from May to August 2016.
Methodology: Utilizing a reversed-phase C-18 column with eluent solvent (19% ethanol, 77%

**Corresponding author: E-mail: rbartzatt@unomaha.edu;*

water, 4% acetic acid), the samples were prepared in sample solvent (19% ethanol, 81% distilled water). Detection of vitamin B6 was accomplished at 290 nm. Analysis of samples was done following solubilizing in aqueous conditions having ethanol at 10% to 20% (v/v). Column pressure at 1900 psig, rise time 0.1 with flow rate 1.0 mL/minute. Elution peak for vitamin B6 occurred consistently at 1.6 minutes. Nutritional samples, aqueous samples, and solid pills were prepared in aqueous solvent with various levels of ethanol.

Results: Levels of vitamin B6 detected were as low as 4.4029×10^{-5} molar to 7.8081×10^{-4} molar. Sensitivity for vitamin B6 was highest at 290 nm. Reverse phase isocratic conditions is shown to be effective for determination of vitamin B6 in aqueous based samples. Standard curves applied are highly linear in range from zero to 7.8081×10^{-4} molar ($y = 112{,}521{,}145.5x + 2{,}818.6$), having coefficient of determination ($R^2 = 0.9948$) with very strong positive correlation coefficient ($r = 0.9974$). Percent recovery of vitamin ranged from 95% to 105%. Amounts of vitamin present in drinks from same manufacturer were consistent.

Conclusion: Utilizing reversed phase column, isocratic solvent conditions with ethanol in water, and a UV detector set at 290 nm is effective for determination of vitamin B6. Ethanol-water solvent system is effective. Vitamin B6 was found in various amounts in nutritional drinks tested.

Keywords: Pyridoxine hydrochloride; vitamin B6; vitamin; HPLC; isocratic.

ABBREVIATIONS

HPLC: High Performance Liquid Chromatography
UV : Ultraviolet
LOQ : Limit of Quantification
LOD : Limit of Detection

1. INTRODUCTION

Pyridoxine is one of three forms of vitamin B6 that occurs in natural sources [1]. The other two forms are known as pyridoxal and pyridoxamine, with all three forms differing upon the substituent on the carbon atom of position 4 of the pyridine molecule [1]. All three forms of vitamin B6 are converted in the body to pyridoxal phosphate [1]. The form pyridoxal phosphate is vital in metabolism and serves as a coenzyme for various metabolic transformation of amino acids (decarboxylation, transamination, and racemization) and metabolism of tryptophan [1]. Pyridoxine hydrochloride occurs as white crystals that are soluble in aqueous solution.

It is generally accepted that pyridoxine is essential in human nutrition. If a patient is determined to be deficient in other members of the B vitamin family, then the presumption is taken that vitamin B6 is deficient as well. Therefore, pyridoxine therapy can be followed in addition to therapy with other members. However, lesions of the skin and central nervous system is associated particularly with pyridoxine deficiency [1].

Adequate vitamin B6 intake is known to be inversely correlated with cardiovascular disease, stroke, diabetes, and cancer [2]. Deficiency of pyridoxal 5'-phosphate portrays symptoms to include depression, nervousness, impaired immune response, and irritability [2]. Severe vitamin B6 deficiency can be a causative factor of sideroblastic microcytic anemia due to depressed hemoglobin synthesis, seizures that are refractory to conventional medications, convulsions, and peripheral neuropathy [2]. Previous studies have shown that vitamin B6 with folate can be protective against nasopharyngeal carcinoma [3]. Large doses of vitamin B6 (100 mg per day) will suppress pro-inflammatory cytokines (IL-6, TNF-α) in patients having rheumatoid arthritis [4]. Studies have shown that 16% of children having sickle cell disease have low vitamin B6 levels [5]. Nutrient deficiencies are shown to be associated with sickle cell disease [5]. Adequate levels of vitamin B6 and B2 are important to sustain in non-small cell lung cancer patients as it is found to be reduced in red blood cells [6].

Vitamins have been classified into water-soluble and fat-soluble groups, both of which can be effectively determined utilizing reversed-phase high performance liquid chromatography (HPLC) [7]. A suitable method to analyze vitamins B2, B3, B6, B12, E, K, D, A, C, and β-carotene in okra by use of reversed-phase HPLC has been presented [8]. Reverse-phase approach of HPLC is effective for assay of B1, B3, B6, folic acid and B12 from tablet forms of vitamin supplements [9].

Methodology to identify and quantify nicotinamide, pyridoxine, riboflavin, and thiamin from various foods have also been ascertained [10]. Water-soluble vitamins such as B1, B2, B3,

and B6 can be identified to quantify from germinated chickpeas [11]. Liquid chromatography-tandem mass spectrometry has been shown to be equivalent to HPLC for the assay for vitamin B6 [12]. In another methodology, acid phosphate is used to dephosphorylate vitamin forms of pyridoxal, pyroxamine, and nicotinamide in digested sample extract prior to liquid chromatography-tandem mass spectrometry [13]. Additional methodologies for the determination of vitamin B6 include techniques of spectrofluorometric, derivative spectrofluorometric, and capillary zone electrophoresis [14].

This study presents methodology to assay for the pyridoxine hydrochloride form of vitamin B6 by use of reverse-phase isocratic high performance liquid chromatography (HPLC). This is accomplished for various drinks and tablets.

2. METHODOLOGY

2.1 Reagents and Instrumentation

All solvents were analytical grade and obtained from Sigma-Aldrich (St. Louis MO 63178 USA). The pyridoxine hydrochloride (vitamin B6) compound for use as standards and preparation of samples was obtained from Fisher Bio Reagents (Fair Lawn, New Jersey 07410). For the high performance liquid chromatography analysis, an Alltech 426 HPLC Pump and Linear UVS 200 detector were utilized with reversed-phase isocratic conditions for analysis of all mixtures (Deerfield, Illinois 60015-1899). The HPLC Alltech instrumentation is controlled by computer interface.

2.2 Instrument Settings, Preparation of Standards and Test Sample

For analysis by HPLC, a reversed-phase C-18 octadecylsilyl ($C_{18}H_{37}$) bonded phase column packing was utilized. The pyridoxine hydrochloride analyte eluted consistently at 1.6 minutes. Detection was accomplished by ultraviolet detector set to 290 nm, rise time 0.1, range AUFS set to 1.0. The HPLC pump was set to 1900 psig and one milliliter per minute flow rate. Vitamin B6 would elute at 1.6 minutes consistently. Actual volume injected into the column is about 20 microliters. The dead time of eluting non-retained species is 1.5 minutes and calculated based on relationship, dead time= t_o = volume/flow rate = 1.5 mL/1.0 mL/min.

Column solvent utilized throughout the project was made for a total volume of 1000 mL by adding 200 mL of 95% ethanol, 760 ml of distilled water, and 40 mL of glacial acetic acid (stock at 17.4 molar). Therefore, the working concentrations were 19% ethanol, 0.969 molar acetic acid, and 77% water (v/v). Sample solvent was used for solubilizing vitamin B6 in various test samples: 19% ethanol, 81% distilled water. Stock standard of vitamin B6 was made by dissolving 1.0865 grams of pyridoxine hydrochloride into 100 mL of sample solvent, making a mixture of 0.052835 molar.

Filtering of commercial tablet samples of non-solubilized particles, where necessary, was accomplished by Whatman 6900-2502 GD/X 25 Sterile Syringe Filter, 25 mm, 0.2 Micron, PVDF Filtration Medium, with suitable plastic syringe.

Aqueous samples varied in consistency, ranging from clear and colorless to color by various dyes. These were analyzed following filtering out of any particles if necessary and adding ethanol to make a final level of 19% ethanol to enhance solubilizing of vitamin B6. Nutritional drinks were previously clarified from the commercial origin, but made 19% ethanol (v/v) before injection into HPLC.

Solid vitamin sources were initially ground to fine powder by mortar and pestle. For vitamin tablets, the finely ground (mortar and pestle) pellet was dissolved in solvent of 10% ethanol (v/v) and distilled water. The amount of ethanol present in any mixture that was injected into the HPLC was not less than 10% (v/v). The eluent solvent for the entire study remained as 19% ethanol (v/v), 77% distilled water (v/v), and remaining volume made up by acetic acid up to 0.696 molar (stock at 17.4 molar). Then a measured amount of the fine powder is placed into suitable sizes of volumetric flasks (1.000 Liter). The material is solubilized in a mixture of 10% ethanol (v/v) and 90% distilled water. The prepared samples were analyzed with 24 hours, filtering was accomplished by Whatman 6900-2502 GD/X 25 Sterile Syringe Filter.

2.3 Statistical Analysis and Properties Determination

Where indicated the numerical analysis utilizing Kruskal-Wallis test, Mann-Whitney test, univariate, F and T tests, one-way ANOVA, and correlation between sets of data 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) and PAST v. 2.06. The Grubb's test for outliers (extreme studentized deviate) was performed by Graph Pad In Stat version 3.00 (Copyright 1992-1998 Graph Pad Software Inc. (www.graphpad.com) for Windows 95, San Diego California USA). Determination of 95% confidence intervals was accomplished by Method Validator version 1.1 (copyright Philippe Marquis).

Molecular properties of pyridoxine hydrochloride were determined utilizing Molinspiration cheminformatics http://www.molinspiration.com/ (Molinspiration Cheminformatics, Nova ulica, SK-900 26 Slovensky Grob, Slovak Republic).

3. RESULTS AND DISCUSSION

The molecular structure of pyridoxine hydrochloride is shown in Fig. 1. It consists of a pyridine ring (a heterocyclic organic compound) with one methyl group substituent and two hydroxymethyl substituents. The Log P value of -0.55 indicates the compound is hydrophilic (i.e. higher values of Log P indicate more lipophilic attribute).

Pyridoxine hydrochloride readily dissolved into the test samples solvent having 19% (v/v) of ethanol in distilled water (for standards) or aqueous health nutritional samples that were made to be 19% ethanol (v/v) by addition of 95% ethanol. The entire study was carried out with isocratic conditions, as explained above. It is usually preferred to work in isocratic conditions, whereby the mobile phase composition remains constant. An advantage of isocratic system include the column being equilibrated all the time and does not suffer from fast chemical changes [15]. Other considerations that make isocratic elution conditions preferable include, [15]: 1) when samples for testing contain less than 10 weakly retained components; and 2) the gradient baseline would impede trace analysis.

For determining the suitable solvent for sample preparation and HPLC column elution, the effects of structure substituents of vitamin B6 were considered. Initially the pyridine ring of the vitamin is more lipophilic (Log P = 0.70) and remains lipophilic upon addition of methyl group ($-CH_3$) (Log P = 0.75). However, addition of a hydroxyl group (-OH) and two hydroxymethyl ($-CH_2OH$) substituents increases hydrophilic tendency to Log = -0.55 (see Fig. 1). Hydroxyl groups show hydrogen bond donor, while the nitrogen atom in the ring are hydrogen bond acceptor, therefore an aqueous base solvent is preferred for solubility. For application of reversed phase HPLC, water is considered a weak solvent [16]. Being polar, water repels the hydrophobic analyte into the stationary phase with more effectiveness than any other solvent, with the result of increasing retention times (making water a weak solvent). An organic modifier (ethanol in this study) is added (only one modifier type at a time) so that the analyte is no longer as strongly repelled into the stationary phase. Therefore the analyte will spend less time in the stationary phase and therefore elute earlier [16]. As progressively more organic modifier is added to the mobile phase, the analyte retention time will continue to decrease [16]. Low molecular weight alcohols, including ethanol, are a common organic modifier [16], and ethanol was found to be effective for this HPLC analysis.

Another facet that is important to determine is the wavelength setting of the UV-Vis detector to signal the elution of vitamin B6 from the instrument. Ideally, the wavelength of detection is set to the wavelength by which the maximum absorbance of the desired analyte occurs in the solvent mixture chosen for HPLC determination. To determine the optimal wavelength of detection of vitamin B6 upon elution from HPLC a set of aqueous (81% v/v) and ethanol (19% v/v) samples having identical concentration of vitamin B6 ((7.4416×10^{-4})) were injected using identical column and instrument settings. Having only the wavelength of detection varied, the wavelength showing maximum absorbance was selected. The wavelengths evaluated were: 250 nm, 260 nm, 270 nm, 280 nm, 290 nm, and 300 nm. Maximum absorbance for vitamin B6 occurred at 290 nm.

The outcome of this determination is plotted and shown in Fig. 2. The maximum absorbance is clearly shown to be at 290 nm. This is the wavelength setting for the detector in this determination of vitamin B6. Absorbance at wavelengths below 290 nm quickly drop lower and approach zero intensity at 250 nm and 260 nm. Formation of standard curves and subsequent analysis of test samples was conducted with detection wavelength at 290 nm. From this plot of absorbance and intensity (see Fig. 2) the wavelength with the highest absorbance is again the wavelength of the

absorption peak, λmax. At this wavelength the spectrophotometric method is most sensitive for the analyte vitamin B6.

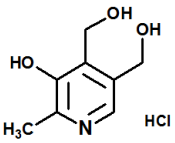

Fig. 1. The molecular structure of pyridoxine hydrochloride (4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol hydrochloride), which has hydroxyl groups (-OH). The formula weight is 205.64 grams per mole, Log P = -0.55, and chemical formula of $C_8H_{11}NO_3$-HCl

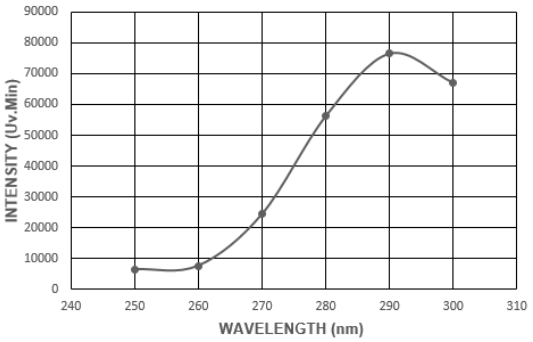

Fig. 2. Determination of wavelength for detection with HPLC analysis. Mixtures in 19% ethanol and 81% distilled water were prepared with identical concentrations of vitamin B6 at 0.00074416 molar concentration. The intensity of vitamin B6 detection examined from 300 nm to 250 nm registered a maximum absorbance intensity at 290 nm. Formation of standard curves and subsequent analysis of test samples was conducted at 290 nm

A standard curve acquired for the assay of vitamin B6 is shown in Fig. 3. The relationship is highly linear having a correlation coefficient of 0.9974, indicating a very strong positive relationship. The range of detection runs from 4.4029×10^{-5} molar to 7.8081×10^{-4} molar. The equation of the line is determined to be: y = 112,521,145.5x + 2,818.6. The limit of detection (LOD) is taken to be here at 4.4029×10^{-5} molar. This is the concentration having a signal to noise ratio of 2.688 (calculated by Signal/Noise = 6720.5 uv.Min/2500 uv.Min).

The limit of quantification (LOQ) is 1.9606×10^{-4} molar (calculated from LOQ= 10(standard deviation of y-residuals/slope of line). The coefficient of determination becomes R^2 = 0.9948, or 99.48% of the variance in the dependent variable (Intensity) is predictable from the independent variable (Concentration). Vitamin B6 consistently eluted at 1.6 minutes.

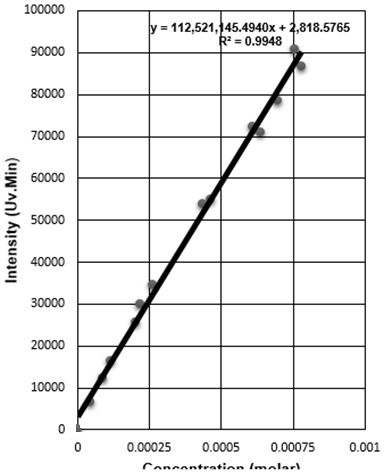

Fig. 3. A standard curve for analysis of vitamin B6 by HPLC. The coefficient of determination is R^2 = 0.9948, indicating 99.48% of the variance in the dependent variable is predictable from the independent variable. The correlation coefficient is 0.9974 (very strong positive relationship). Vitamin B6 (pyridoxine hydrochloride) elutes at 1.6 minutes

The 95% confidence interval for the slope is from 1.0587×10^8 to 1.1917×10^8. The 95% confidence interval for the intercept is from -407.24 to 6044.39.

The standard curve is highly linear with values found to be contained within a 95% ellipses (see Fig. 4). The confidence region is determined so that should a set of measurements to be repeated many times and a confidence region calculated, then on average (95%) the confidence region will include the true values of the set of variables [17,18,19]. This outcome indicating consistent determination as shown in Fig. 4.

Recovery of the analyte need not to be 100%, but the extent of recovery of the analyte should be consistent, precise and reproducible. This was accomplished in this study. The record of recovery rate for vitamin B6 is presented in Table 1. The values of calculated molar values is determined from the molarity of the mother stock solution of a known amount of vitamin B6 dissolved in aqueous solution that is 19% ethanol

(v/v). These are compared to the measured molar value that is obtained from injection into the HPLC instrumentation. The Grubbs' test, also known as extreme studentized deviate test, is a statistical test used to detect outliers in a univariate data set [17,18]. For all 42 values of calculated molar values (see Table 1) the Grubb's test indicated no outliers (P = .05, two sided). The mean value of calculated molar values is 5.2184×10^{-4} ± 0.00017 molar. Likewise, the Grubb's test indicated there are no outliers for corresponding measured molar values (P = .05, two-sided). The mean value for measured molar values is 5.2605×10^{-4} ± 0.00017 molar. The actual percent recoveries of analyte by HPLC also showed no outliers by Grubb's test (P = .05, two-sided) (see Table 1). The mean value for the percent recoveries of analyte is 101% with a standard deviation of 2.4%.

Comparison of calculated molar values and measured molar values statistically gave a linear correlation r value of 0.9967, indicating a very strong positive relationship [17,19]. Analysis of calculated molar values and measured molar values by Kurskal-Wallis test indicates the two sets of values have equal medians (P = .85, with actual medians of 0.00056194 and 0.00056302 for calculated and measured molar values, respectively). The F and T tests for these two groups of data indicated the mean of any numerical difference is zero (P = .91). The Mann-Whitney test for equal medians, also indicated the populations have equal medians (P = .86). The one-way ANOVA analysis of the two groups indicates clearly that all group means are the same (P = .93).

Interpreting results by skewness quantifies how symmetrical the numerical distribution of calculated molar values, measured molar values, and percent recovery [17,19]. Skewness for these values are -0.8623, -0.9281, and -0.5213, respectively. Negatively skewed distribution is a skew to the left or to lower values. However, kurtosis (quantifies shape matching to Guassian distribution) for calculated molar values, measured molar values, and percent recovery is 0.2388, 0.2638, and -0.2661, respectively. Positive kurtosis indicates a more peaked distribution rather than a Gaussian distribution for calculated molar values and measured molar values. Percent recovery values have a negative kurtosis and therefore flatter than Gaussian distribution.

The Passing and Bablok regression analysis is for non-parametric regression analysis that is particularly suitable for method comparison studies and allows comparing two measurement methods [19,20]. It is a statistical procedure that allows estimation of an analytical method's agreement and possible systematic bias between them [20]. It is robust, non-parametric, and not sensitive to distribution of errors and data outliers. The method presumes a linear relationship exists within the data measured [19,20].

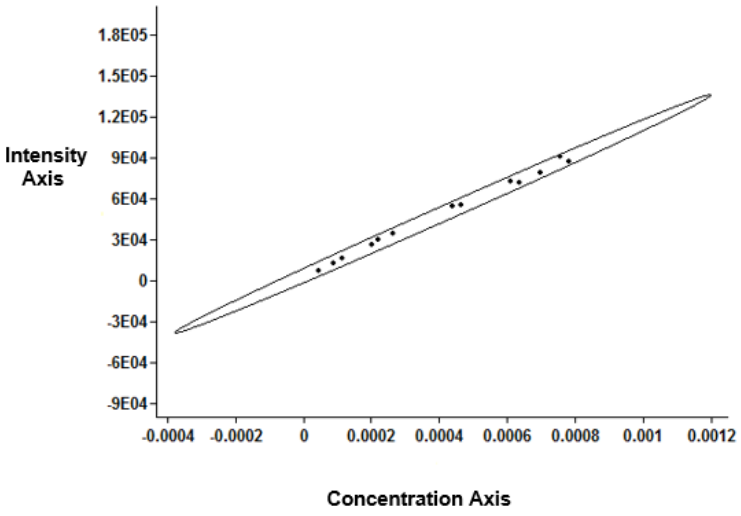

Fig. 4. 95% ellipses analysis of standard curve entries. Basically it is the smallest ellipse that will cover 95% of the points. This confidence ellipse defines the region that contains 95% of all samples that can be drawn from the underlying distribution. Note that all the points of the standard curve are contained in the 95% ellipses

Table 1. Percent recovery of vitamin B6

Number	Calculated molar value	Measured molar value	Percent recovery
1	4.3665×10^{-4}	4.4077×10^{-4}	101
2	3.9331×10^{-4}	4.0555×10^{-4}	103
3	8.4522×10^{-4}	8.2323×10^{-4}	97.4
4	6.9520×10^{-4}	7.0901×10^{-4}	102
5	6.5228×10^{-4}	6.6255×10^{-4}	102
6	6.0930×10^{-4}	6.1316×10^{-4}	101
7	5.2312×10^{-4}	5.3132×10^{-4}	102
8	1.7553×10^{-4}	1.8238×10^{-4}	104
9	5.2312×10^{-4}	5.2487×10^{-4}	100
10	4.3664×10^{-4}	4.4462×10^{-4}	102
11	7.3804×10^{-4}	7.3955×10^{-4}	100
12	6.5228×10^{-4}	6.3268×10^{-4}	97.0
13	5.6624×10^{-4}	5.4920×10^{-4}	97.0
14	4.7992×10^{-4}	5.0030×10^{-4}	104
15	2.682×10^{-4}	2.6358×10^{-4}	100
16	1.7553×10^{-4}	1.8192×10^{-4}	104
17	1.3176×10^{-4}	1.3691×10^{-4}	104
18	5.8348×10^{-4}	5.7684×10^{-4}	99.0
19	4.1066×10^{-4}	4.2084×10^{-4}	102
20	4.6262×10^{-4}	4.6437×10^{-4}	100
21	2.0176×10^{-4}	2.0342×10^{-4}	101
22	1.1423×10^{-4}	1.1972×10^{-4}	104
23	6.2650×10^{-4}	6.3095×10^{-4}	101
24	5.4038×10^{-4}	5.4598×10^{-4}	101
25	6.8662×10^{-4}	7.0854×10^{-4}	103
26	6.6946×10^{-4}	6.4940×10^{-4}	97.1
27	6.3510×10^{-4}	6.4686×10^{-4}	101
28	6.0069×10^{-4}	6.1966×10^{-4}	103
29	5.5763×10^{-4}	5.8769×10^{-4}	105
30	5.1449×10^{-4}	5.2706×10^{-4}	102
31	7.1234×10^{-4}	6.7677×10^{-4}	95.0
32	4.4531×10^{-4}	4.6357×10^{-4}	104
33	6.9520×10^{-4}	7.1834×10^{-4}	103
34	6.0069×10^{-4}	5.9776×10^{-4}	99.5
35	6.7804×10^{-4}	6.7261×10^{-4}	99.1
36	6.0069×10^{-4}	6.3224×10^{-4}	105
37	6.6087×10^{-4}	6.3780×10^{-4}	97.0
38	6.3510×10^{-4}	6.5071×10^{-4}	102
39	6.1790×10^{-4}	6.2917×10^{-4}	102
40	4.2799×10^{-4}	4.2418×10^{-4}	99.1
41	5.1449×10^{-4}	5.2338×10^{-4}	102
42	4.2799×10^{-4}	4.2450×10^{-4}	99.2

Analysis of calculated molar values in comparison to measured molar values shows an excellent relationship by Passing and Bablok regression (see Fig. 5). The 95% confidence interval for the y-axis intercept includes zero and the 95% confidence interval for the slope includes 1. Therefore there are no constant differences between the calculated and measured values, and these values can be used interchangeably. Therefore, the calculated molar values are representative of the measure molar values.

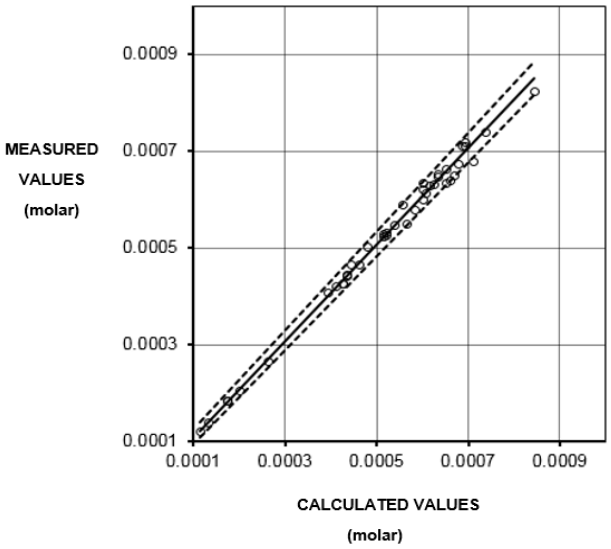

Fig. 5. Passing-Bablok regression analysis of calculated values versus measured values (see Table 1). The 95% confidence interval for the y-axis intercept includes zero and the 95% confidence interval for the slope includes 1. Therefore there are no constant differences between the calculated and measured values, and these values can be used interchangeably. The slope is 0.9966 with 95% confidence interval of 0.9670 to 1.0191

The pyridoxine hydrochloride eluted consistently at 1.6 minutes. This elution time at 1.6 minutes for vitamin B6 was consistent for all commercial vitamin containing drinks and commercial vitamin tablets. Examples of chromatograms obtained after elution from HPLC are shown in Fig. 6. The chromatograms are presented from top to bottom, as follows: an example of standard example showing vitamin B6 elution at 1.6 minutes which was consistent, vitamin water sample in orange flavor, vitamin water sample in lemonade flavor, and vitamin water sample in acai blueberry flavor. These vitamin water samples originated from the same manufacturer and were pre-filtered to clearness prior to public distribution. The commercial samples were inspected for maintained clearness prior to preparation for injection into instrumentation.

Presented in Fig. 6, is an example of a chromatogram for a standard sample (top). Also presented are chromatograms of various commercial drinks analyzed in this study. Coming from the identical manufacturer, clearly visible are numerous peaks appearing generally from 1 minute elution time to 3.5 minute elution time. Acai blueberry product has an additional peak at 5.6 minutes. The example of a standard injection clearly shows a peak at 1.6 minutes. With other peaks present in commercial samples, nevertheless, the peak of vitamin B6 elution remains adhering to 1.6 minutes. Any commercial product that contained food coloring, eluted the food coloring without causing effects on column flow rate or elution time of vitamin B6.

All commercial samples (pre-cleared by the manufacturer) were prepared the same day of opening to avoid any potential microbial contamination. Commercial samples were made to be ethanol 19% (v/v) by addition of 95% ethanol to a suitable volume. Upon addition of ethanol no precipitation of contents within commercial products occurred. At no time during the study did blockage of column or perturbation of instrument settings occur. Commercial products that were pre-cleared by the manufacturer were the only type involved in the study. Only aqueous based commercial products were selected. Food coloring within the particular product was permitted, but not any product having visible suspensions or particulate matter.

No commercial products generated precipitates or viscous material prior to analysis or during analysis. Disposal of eluent containing eluted commercial contents and ethanol was sufficiently low as not to require special disposition. However, the acetic acid in the column solvent (see Methodology) required neutralization with sodium bicarbonate prior to disposal.

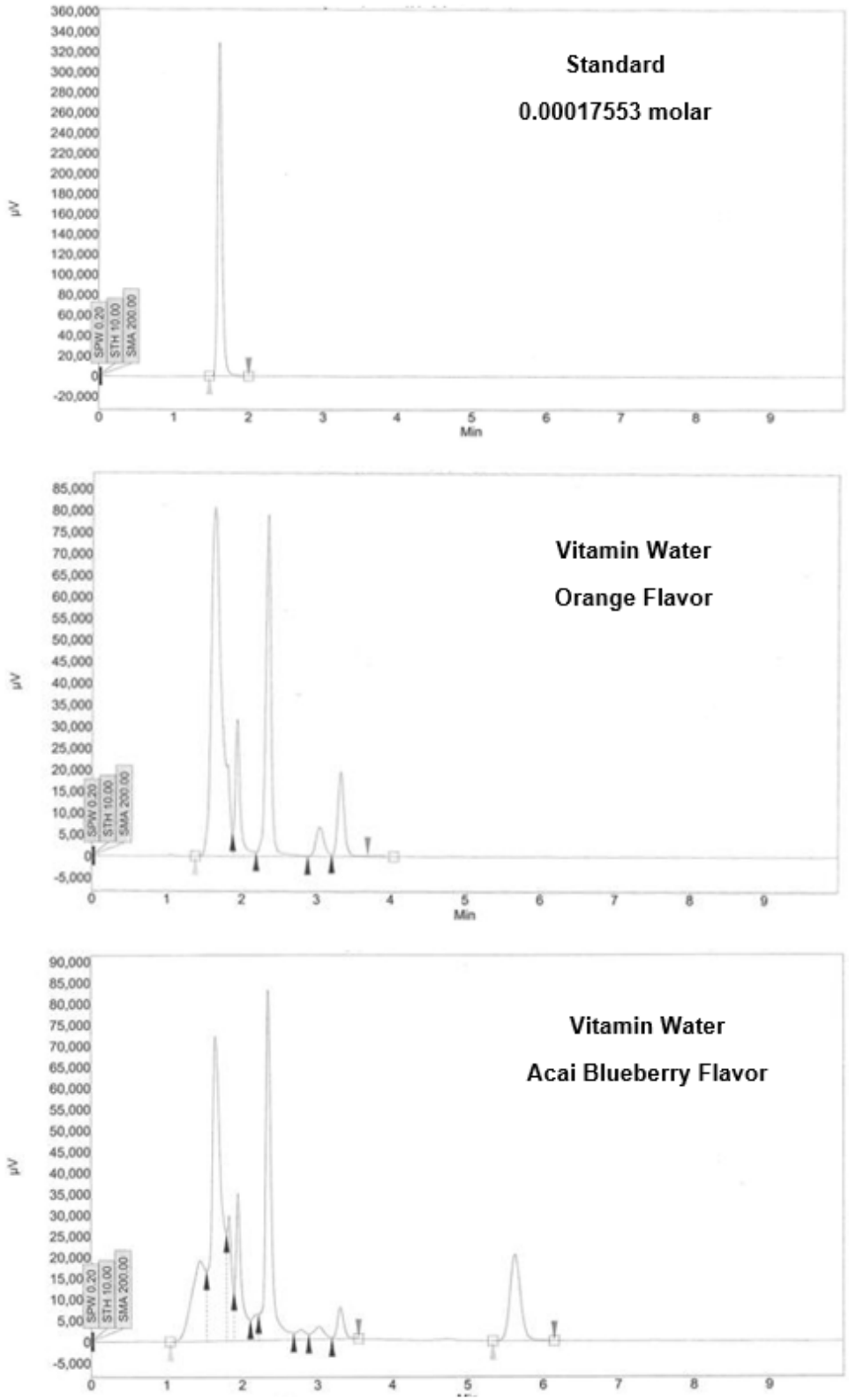

Fig. 6. Chromatograms of a standard sample and various commercial products

For commercial tablet pills the amount of vitamin B6 found was above the manufacturer claim accompanying the container. The product obtained from a single manufacturer was analyzed and found to contain an amount of vitamin B6 to be consistent, as follows (milligrams): 124, 125, 126, 116, 123, and 131. These findings were consistent with this manufactured product, having no outliers by Grubb's test (two-sided). The mean of this outcome is 124 milligrams, having a standard deviation of 4.9 milligrams.

For a further evaluation of HPLC based detection, a popular sports drink having no vitamin content (merely electrolytes), was spiked with pyridoxine hydrochloride at known concentrations. Then treated to a known volume of ethanol, followed by injection into HPLC according to parameters previously described. The recovery of vitamin B6 was found to be consistent, by this approach, to be as follows: 89.5%, 91%, 91.9%, 97.1%, 93.7%, 92.7%, and 90.9%. This recovery was consistent, having no outliers according to Grubb's test and mean of 92.4% with standard deviation 2.5%.

The amounts of vitamin B6 found in soft drinks prepared for commercial usage varied according to manufacturer. Different amounts are found from brand to brand. Examples observed in this study are presented in Table 2. So manufacturers vary in the amounts of vitamin B6 placed into commercial products. Range of amounts in molar value is from 9.122×10^{-6} molar to 8.675×10^{-5} molar. Range of amounts in grams per liter value is from 1.8759×10^{-3} grams/Liter to 1.7838×10^{-2} grams/Liter.

Table 2. Example amounts of vitamin B6 found in commercial products

Sample number	Amount (Molar)	Amount (grams/Liter)
1	1.593×10^{-5}	3.2748×10^{-3}
2	1.205×10^{-5}	2.4787×10^{-3}
3	1.083×10^{-5}	2.2263×10^{-3}
4	1.033×10^{-5}	2.1234×10^{-3}
5	1.172×10^{-5}	2.4105×10^{-3}
6	1.087×10^{-5}	2.22353×10^{-3}
7	9.122×10^{-6}	1.8759×10^{-3}
8	1.034×10^{-5}	2.1257×10^{-3}
9	4.085×10^{-5}	8.400×10^{-3}
10	1.928×10^{-5}	3.9640×10^{-3}
11	8.675×10^{-5}	1.7838×10^{-2}

The approach presented in this study is simple to employ and requires ethanol which is a common solvent utilized for HPLC. The run time on the HPLC is within 10 minutes. This is a simple and comparatively facile method of vitamin B6 assay compared to other methodologies requiring expensive instrumentation (i.e. fluorimeters, computer interface for derivative analysis).

Utilizing the same conditions for analysis for vitamin B6, this methodology can detect 2-thiobarbituric acid (see Fig. 7). The elution of 2-thiobarbituric acid at 1.5 minutes, occurs earlier than for vitamin B6, and shows that the beneficial combination of water with ethanol as the solvent. An ethanol and water solvent system is versatile and effective.

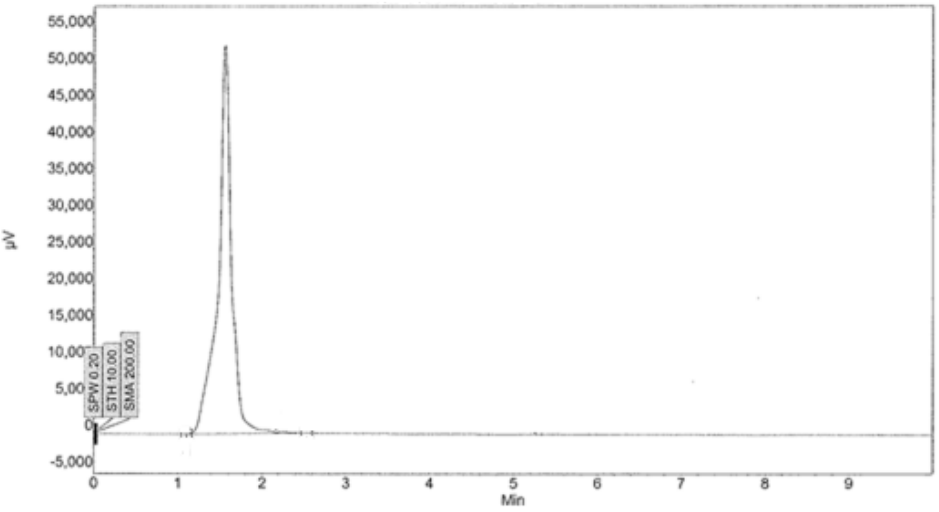

Fig. 7. Elution of 2-thiobarbituric acid at 1.5 minutes. Same conditions as vitamin B6 analysis (concentration of 2-thiobarbituric acid is 0.0005351 molar)

The instrumental analysis of commercial products meant for human consumption is an important function of analytical chemistry. The various methodologies employed have become powerful tools for identifying adulterants (substitutes in products purposefully introduced by manufacturer), contaminants, and quality control management. The types of instrumentation available and numerous methodologies available allow investigators a very broad level of choices. The analysis of commercial products by HPLC continues to be a vigorous area of application, due to high specificity and sensitivity of detection. Analysis by HPLC will continue to permit confidence in the type and range of commercial products available to consumers.

4. CONCLUSION

Vitamin B6 has been shown to be protective against nasopharyngeal carcinoma, in addition to its nutritional properties. Pyridoxine hydrochloride is assayed effectively by isocratic reversed-phase high performance liquid chromatography. Detection of vitamin B6 by UV absorbance was accomplished at 290 nm. A standard curve enabled detection of amounts from 4.4029×10^{-5} molar to more than 7.500×10^{-4} molar, with an $R^2 = 0.9948$ accounting for 99.48% of variance in the dependent variable. Percent recovery of vitamin averaged 101% with a standard deviation of 2.4%. Pyridoxine hydrochloride was effectively assayed from aqueous samples, vitamin preparations, and vitamin tablets. The methodology presented in this study will be useful for quality control analysis for commercial production. Analysis methods for vitamin assay are a necessary objective to ensure quality control of commercial products and medicinal applications.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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