

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

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ABSTRACT

Aims: To demonstrate an analysis for vitamin B₆ 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 B₆ 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% 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

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 60015-1899USA). 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 nonretained species is 1.5 minutes and calculated based on relationship, $dead\ time = t_0 = volume/flow\ rate = 1.5\text{mL}/1.0\text{ mL/min.}$

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, 25mm, 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

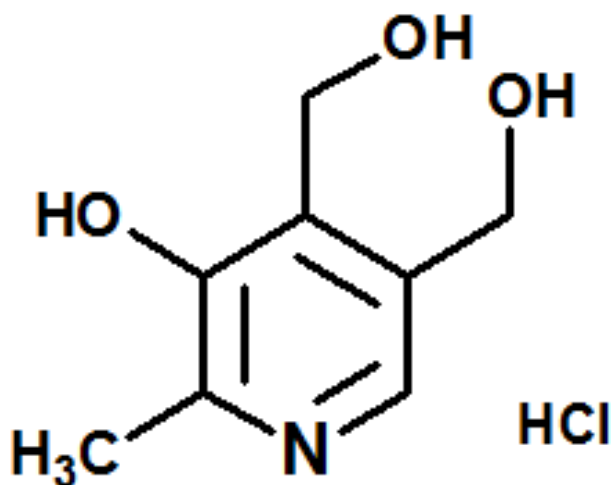
The molecular structure of pyridoxine hydrochloride is shown in Figure 1. It consists of a pyridine ring (a heterocyclic organic compound) with one methyl group substituent and two hydroxymethyl substituents. The Log P value of -0.55 indicates the compound is hydrophilic (i.e. higher values of Log P 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 (-CH₃) (Log P = 0.75). However, addition of a hydroxyl group (-OH) and two hydroxymethyl (-CH₂OH) substituents increases hydrophilic tendency to Log = -0.55 (see Figure 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.

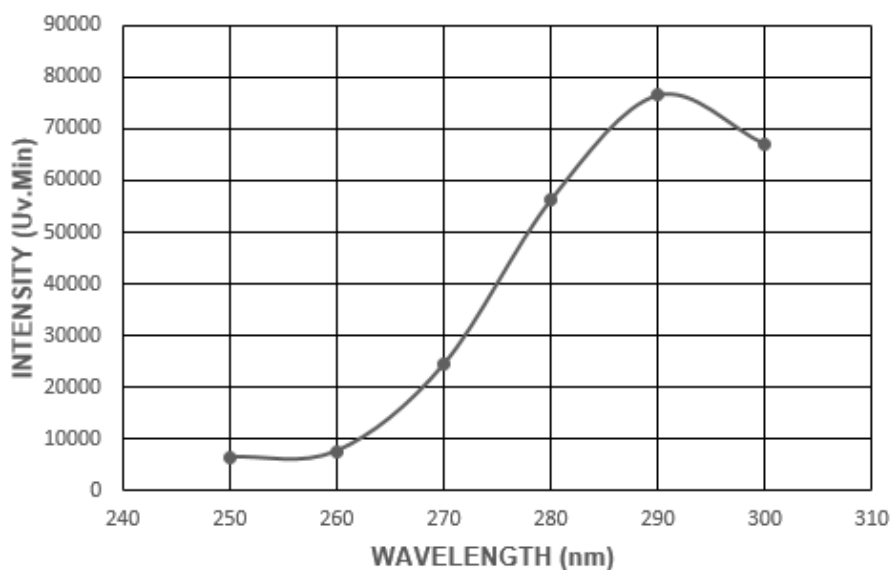
Another facet that is important to determine is the wavelength setting of the UV-Vis detector to signal the elution of vitamin B₆ from the instrument. Ideally, the wavelength of detection is set to the wavelength by which the maximum absorbance of the desired analyte occurs in the solvent mixture chosen 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} molar) 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.

182 The outcome of this determination is plotted and shown in Figure 2. The maximum
 183 absorbance is clearly shown to be at 290 nm. This is the wavelength setting for the detector
 184 in this determination of vitamin B₆. Absorbance at wavelengths below 290 nm quickly drop
 185 lower and approach zero intensity at 250 nm and 260 nm. Formation of standard curves and
 186 subsequent analysis of test samples was conducted with detection wavelength at 290 nm.
 187 From this plot of absorbance and intensity (see Figure 2) the wavelength with the highest
 188 absorbance is again the wavelength of the absorption peak, λ_{max} . At this wavelength the
 189 spectrophotometric method is most sensitive for the analyte vitamin B₆.
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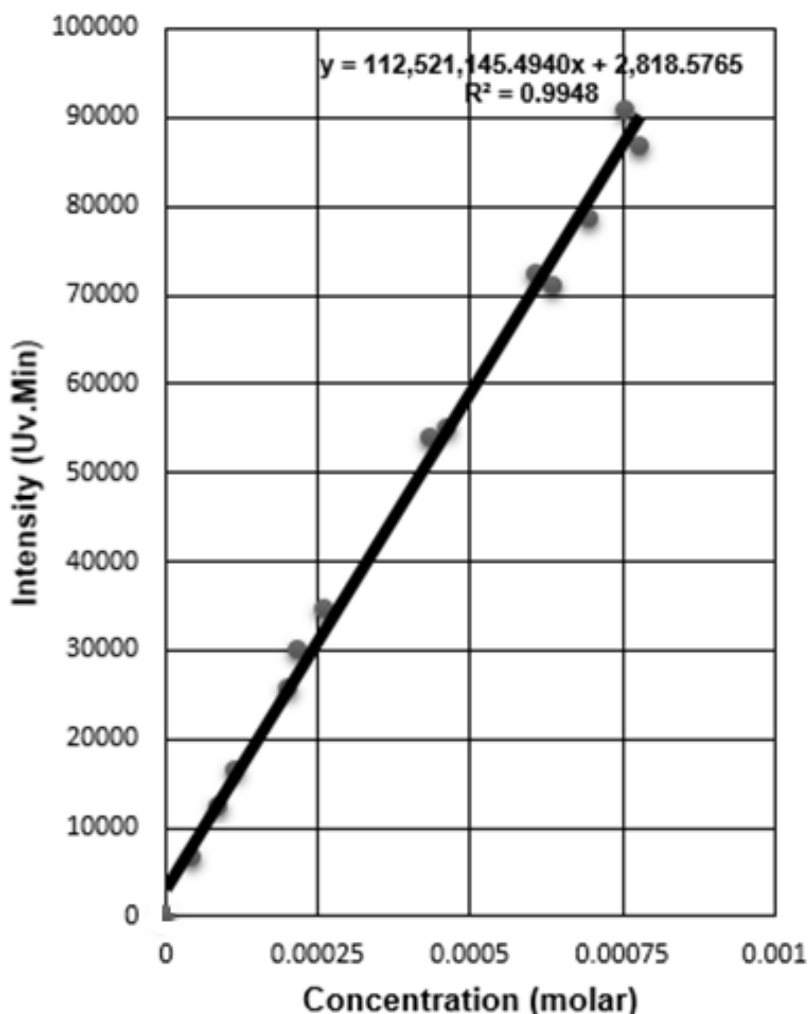
Fig. 1. The molecular structure of pyridoxine hydrochloride (4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol hydrochloride), which has hydroxyl groups (-OH). The formula weight is 205.64 grams per mole, Log P = -0.55, and chemical formula of C₈H₁₁NO₃⁻ HCl.



200

201 **Fig. 2. Determination of wavelength for detection with HPLC analysis. Mixtures in**
 202 **19% ethanol and 81% distilled water were prepared with identical concentrations of**
 203 **vitamin B₆ at 7.4416×10^{-4} molar concentration. The intensity of vitamin B₆ detection**
 204 **examined from 300 nm to 250 nm registered a maximum absorbance intensity at 290**
 205 **nm. Formation of standard curves and subsequent analysis of test samples was**
 206 **conducted at 290 nm.**

207
 208 A standard curve acquired for the assay of vitamin B₆ is shown in Figure 3. The relationship
 209 is highly linear having a correlation coefficient of 0.9974, indicating a very strong positive
 210 relationship. The range of detection runs from 4.4029×10^{-5} molar to 7.8081×10^{-4} molar.
 211 The equation of the line is determined to be: $y = 112,521,145.5x + 2,818.6$. The limit of
 212 detection is taken to be here at 4.4029×10^{-5} molar. This concentration provides a signal to
 213 noise ratio of 2.688 (S/N = 6720.5 uv.Min/2500 uv.Min). The limit of quantification (LOQ) is
 214 1.9606×10^{-4} molar (from LOQ = 10(standard deviation of y-residuals/slope of line). The
 215 coefficient of determination, $R^2 = 0.9948$, or 99.48% of the variance in the dependent
 216 variable (Intensity) is predictable from the independent variable (Concentration). Vitamin B₆
 217 consistently eluted at 1.6 minutes.
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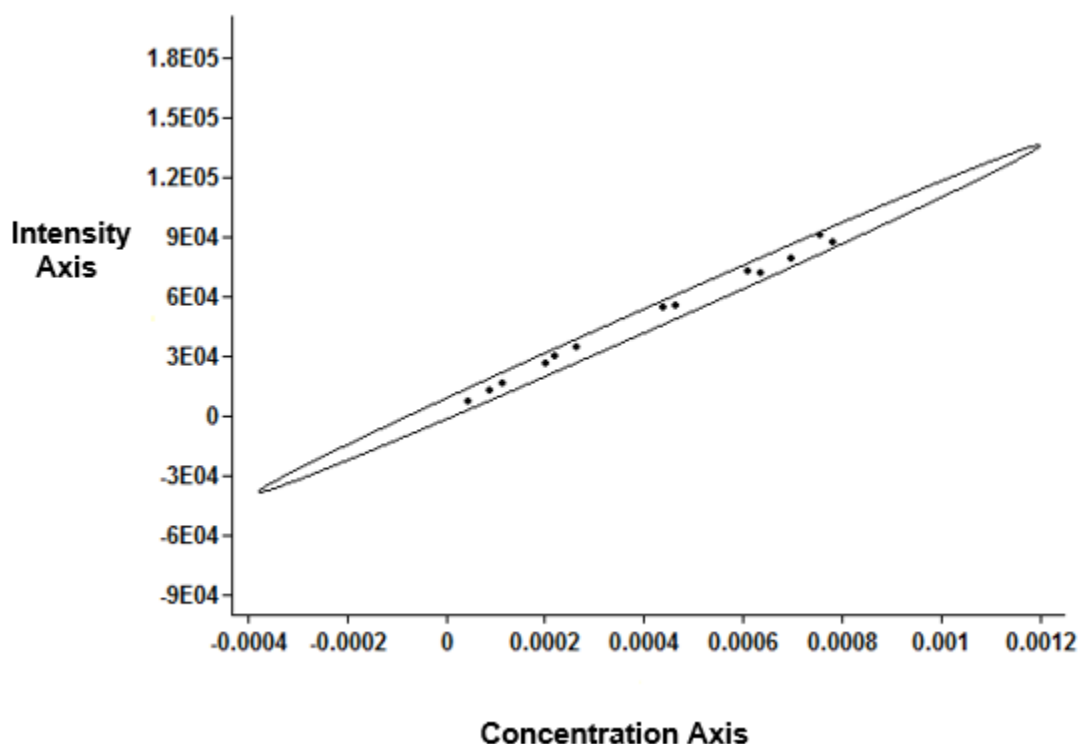
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220 **Fig. 3. A standard curve for analysis of vitamin B₆ by HPLC. The coefficient of**
 221 **determination is $R^2 = 0.9948$, indicating 99.48% of the variance in the dependent**

222 variable is predictable from the independent variable. The correlation coefficient is
223 0.9974 (very strong positive relationship). Vitamin B₆ (pyridoxine hydrochloride)
224 elutes at 1.6 minutes.

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

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



232

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

237 Recovery of the analyte need not to be 100%, but the extent of recovery of the analyte
238 should be consistent, precise and reproducible. This was accomplished in this study. The
239 record of recovery rate for vitamin B₆ is presented in Table 1. The values of calculated
240 molar values is determined from the molarity of the mother stock solution of a known amount
241 of vitamin B₆ dissolved in aqueous solution that is 19% ethanol (v/v). These are compared
242 to the measured molar value that is obtained from injection into the HPLC instrumentation.
243 The Grubbs' test, also known as extreme studentized deviate test, is a statistical test used to
244 detect outliers in a univariate data set [17, 18]. For all 42 values of calculated molar values
245 (see Table 1) the Grubb's test indicated no outliers ($P = .05$, two sided). The mean value of
246 calculated molar values is $5.2184 \times 10^{-4} \pm 0.00017$ 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 $5.2605 \times 10^{-4} \pm 0.00017$ 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%.

Table 1. Percent Recovery of Vitamin B₆

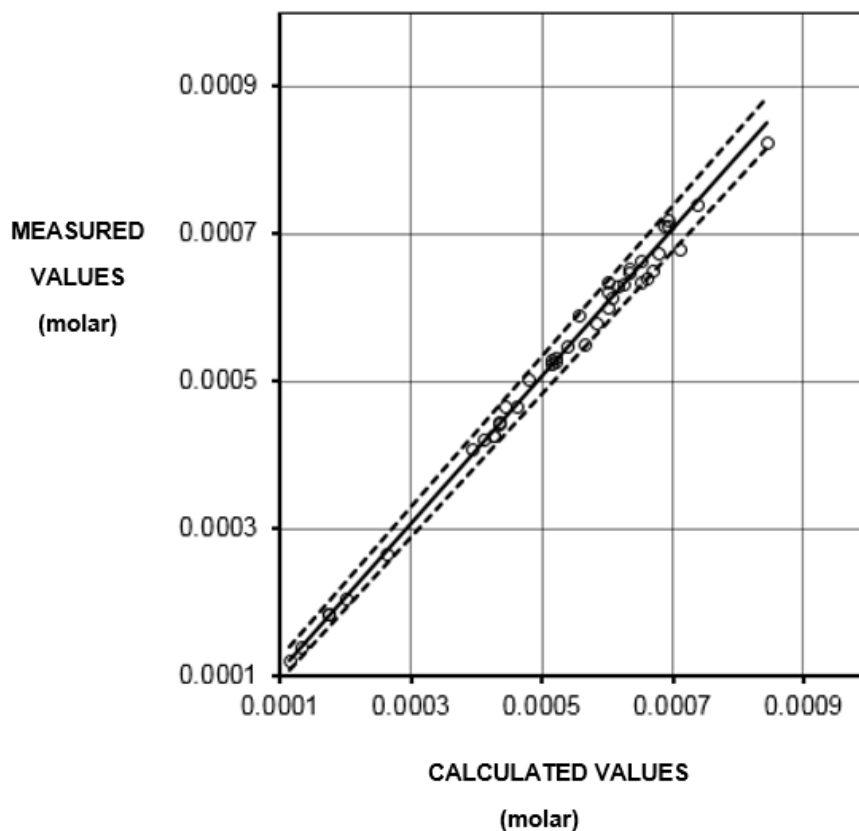
Number	Calculated Molar Value	Measured Molar Value	Percent Recovery
1	4.3665×10^{-4}	4.4077×10^{-4}	101
2	3.9331×10^{-4}	4.0555×10^{-4}	103
3	8.4522×10^{-4}	8.2323×10^{-4}	97.4
4	6.9520×10^{-4}	7.0901×10^{-4}	102
5	6.5228×10^{-4}	6.6255×10^{-4}	102
6	6.0930×10^{-4}	6.1316×10^{-4}	101
7	5.2312×10^{-4}	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

255

256 Comparison of calculated molar values and measured molar values statistically gave a linear
 257 correlation r value of 0.9967, indicating a very strong positive relationship [17, 19]. Analysis
 258 of calculated molar values and measured molar values by Kurskal-Wallis test indicates the
 259 two sets of values have equal medians ($P = .85$, with actual medians of 0.00056194 and
 260 0.00056302 for calculated and measured molar values, respectively). The F and T tests for
 261 these two groups of data indicated the mean of any numerical difference is zero ($P = .91$).
 262 The Mann-Whitney test for equal medians, also indicated the populations have equal
 263 medians ($P = .86$). The one-way ANOVA analysis of the two groups indicates clearly that all
 264 group means are the same ($P = .93$).

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



274

275 **Fig. 5. Passing-Bablok regression analysis of calculated values versus measured**
276 **values (see Table 1). The 95% confidence interval for the y-axis intercept includes**
277 **zero and the 95% confidence interval for the slope includes 1. Therefore there are no**
278 **constant differences between the calculated and measured values, and these values**
279 **can be used interchangeably. The slope is 0.9966 with 95% confidence interval of**
280 **0.9670 to 1.0191.**

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

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

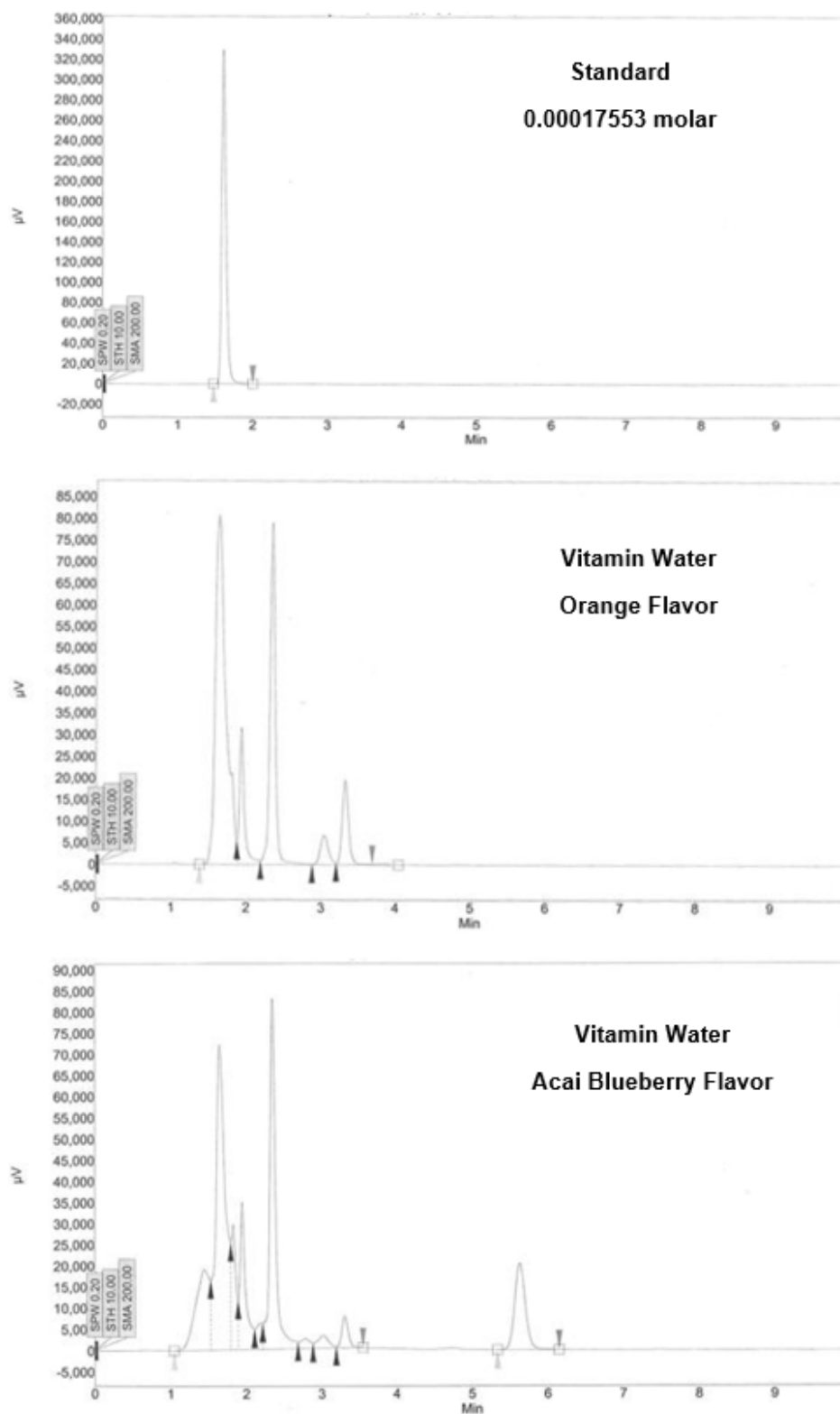
293 The pyridoxine hydrochloride eluted consistently at 1.6 minutes. This elution time at 1.6
294 minutes for vitamin B₆ was consistent for all commercial vitamin containing drinks and
295 commercial vitamin tablets. Examples of chromatograms obtained after elution from HPLC
296 are shown in Figure 6.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

303 Presented in Figure 6, is an example of a chromatogram for a standard sample (top). Also
304 presented are chromatograms of various commercial drinks analyzed in this study. Coming
305 from the identical manufacturer, visible are numerous peaks appearing generally from 1
306 minute elution time to 3.5 minutes elution time. Acai blueberry product has an additional
307 peak at 5.6 minutes. Nevertheless, the peak of vitamin B₆ elution remains adhering to 1.6
308 minutes. Any commercial product containing food coloring, eluted the food coloring without
309 causing effects on the rate of column flow or elution time of vitamin B₆.

310 All commercial samples (pre-cleared by the manufacturer) were prepared the same day of
311 opening to avoid any potential microbial contamination. Commercial samples were made to
312 be ethanol 19% (v/v) by addition of 95% ethanol to a suitable volume. Upon addition of
313 ethanol no precipitation of contents within commercial products occurred. At no time during
314 the study did blockage of column or perturbation of instrument settings occur.

315 Commercial products that were pre-cleared by the manufacturer were only type involved in
316 the study. Only aqueous based commercial products were selected. Food coloring within
317 the particular product was permitted, but not any product having visible suspensions or
318 particulate matter.

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



323

324 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). 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

357 detection. Analysis by HPLC will continue to permit confidence in the type and range of
358 commercial products available to consumers.

359 **4. CONCLUSION**

360
361 Vitamin B6 has been shown to be protective against nasopharyngeal carcinoma, in addition
362 to its nutritional properties. Pyridoxine hydrochloride is assayed effectively by isocratic
363 reversed-phase high performance liquid chromatography. Detection of vitamin B6 by UV
364 absorbance was accomplished at 290 nm. A standard curve enabled detection of amounts
365 from 4.4029×10^{-5} molar to more than 0.0007500 molar, with an $R^2 = 0.9948$ accounting for
366 99.48% of variance in the dependent variable. Percent recovery of vitamin averaged 101%
367 with a standard deviation of 2.4%. Pyridoxine hydrochloride was effectively assayed from
368 aqueous samples, vitamin preparations, and vitamin tablets. The methodology presented in
369 this study will be useful for quality control analysis for commercial production. Analysis
370 methods for vitamin assay are a necessary objective to ensure quality control of commercial
371 products and medicinal applications.

372

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374

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377

378 **COMPETING INTERESTS**

379

380 Authors declare they have no competing interests.

381

382 **AUTHORS' CONTRIBUTIONS**

383

384 Ronald Bartzatt designed the study, performed the statistical analysis, wrote the protocol,
385 and wrote the first draft of the manuscript. Purnima Gajmer, Cassandra Nguyen, and
386 Alexandra Tran managed the analyses of the study and operated the instruments. All
387 authors read and approved the final manuscript.

388

389 **CONSENT**

390

391 Not applicable.

392

393 **ETHICAL APPROVAL**

394

395 Not applicable.

396

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460

461 **DEFINITIONS, ACRONYMS, ABBREVIATIONS**

462

463 **Term:** HPLC, high performance liquid chromatography; UV, ultraviolet.

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