Determination of Vitamin B₆ (pyridoxine hydrochloride) in Pharmaceutical Preparations Using High Performance Liquid Chromatography

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Abstract
Determination of vitamin B₆ (pyridoxine hydrochloride) was described using high performance liquid chromatographic method. The analysis was achieved by cosmos IL 5C₁₈-MS-II column (250 mm x 4.6 mm i. d., 5µm particle size) at room temperature. The mobile phase used was Acetonitrile, buffer solution (Citric acid, Na₂HPO₄ pH4) buffer solution in the ratio (70:30) (V: V), the flow rate was set to 1.25 mL.min⁻¹ and the retention time 1.82 min with UV-detection at 282 nm. Beer's law was obeyed over the concentration range 10-1250 µg.mL⁻¹. The method was accurate (relative error % less than 0.05%), precise (RSD better than ±1.05%), average recovery 100.05%, with a limit of detection and quantification of 2.2 μg.ml⁻¹, and 7.34 µg.mL⁻¹ respectively. The proposed method was successfully applied to determine the pyridoxine hydrochloride in pharmaceutical preparations in both forms of tablet and injection.

Keywords: vitamin B₆ (pyridoxine hydrochloride), HPLC, pharmaceutical preparations.

Introduction
Pyridoxine hydrochloride (vitamin B₆) is a 5-hydroxy-6-methyl-3, 4-pyridinedimethanol hydrochloride with the chemical formula C₈H₁₁NO₃.HCl [1, 2]. It is a white powder, soluble in water, slightly soluble in acetone, and insoluble in chloroform and ether [3]. The determination of vitamin content in food and in nutritional supplements is important in quality, labeling, and marketing [4].

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Pyridoxine is used to prevent low levels of vitamin B₆ which plays an important role in the health of nerves, skin and red blood cells [5].

Many spectrophotometric methods for the determination of pyridoxine hydrochloride were described, using diazotization reaction [6, 7], or using oxidation reduction reaction [8]; such methods were leaked to sensitivity [9-11], others were proposed derivative spectrophotometric procedures [12-14]. On the other side, such chromatographic methods need a special detection tools like fluorometer [15, 16], or mass spectrometer [17].

In this paper, pyridoxine hydrochloride (vitamin B₆) was determined by chromatographic method using cosmosil 5C₁₈-MS-column and UV-detection at 282nm with high sensitivity and wide applicability.

Experimental Apparatus

A (shimadzue, LC-2010A, Japan) HPLC instrument was used with a pump (model LC-2010, high flow rate mode) and auto injector sampler. The detector was UV LC-2010 with D₂ lamp (800mv minimum energy). Separation was achieved using a cosmosil 5C₁₈-MS-II column (4.6 mm I.D. x 250 mm, 5 µm particle size, Japan).

A shimadzue UV-1650 PC double beam spectrophotometer with 1cm quartz cells have been used for scanning spectra. pH measurement has beam done by pH-meter (HANNA pH 211, microprocessor pH meter, Mauritius), balance (Sartorius BL 20 S, Germany) has been used for weight measurements.

Materials

All chemicals and reagents are of analytical grade. Water was double distilled and filtered. Acetonitril (ACN), dichloromethane (DCM), methanol, and ethanol (Loba. Chemie) are of HPLC.

Pyridoxine hydrochloride (2000 µg.mL⁻¹): this solution was prepared by dissolving 0.10 gm of pure pyridoxine hydrochloride (from BDH) in double distilled water and filtered using 0.45 µm filter, the volume was completed to 50 ml in a volumetric flask, further dilution was followed to prepare 500 µg.mL⁻¹ working solution.

Citric acid (0.1 M) solution: this solution was prepared by dissolving 1.92 g of citric acid (C₆H₈O₇) (from BDH) in 100 mL of double distilled water and filtered using 0.45 µm filter.

Disodium hydrogen phosphate (0.2 M) solution: this solution was prepared by dissolving 1.42 g of (Na₂HPO₄) (from Sigma) in 50 mL of double distilled water and filtered using 0.45 µm filter.

Buffer (pH 4) solution: this solution was prepared by mixing 61.9 mL of citric acid (0.1 M) with 38.1 mL of (0.2 M) Na₂HPO₄.

Pharmaceutical preparations

Pyridoxine hydrochloride (Smamvit-B6 tablet) 1000 µg.mL⁻¹ solution: this solution was prepared by weighing 5 tablets (40 mg SDI), grinding to fine particles and mixing; a weight of this pharmaceutical powder (0.0457 g) equivalent to 0.01 g of pure pyridoxine hydrochloric acid was dissolved in solvent mixture (CAN: citric acid, Na₂HPO₄) (70: 30), then filtered using 0.45 µm filter, the volume was completed to 10 mL with the same solvent in a volumetric flask.

Pyridoxine hydrochloride (Meho B6 tablet) 1000 µg.mL⁻¹ solution: this solution was prepared by weighing 5 tablets (40 mg Bijing company), grinding to fine particles and mixing; a weight of this pharmaceutical powder (0.042 g) equivalent to 0.01 g of pure pyridoxine hydrochloric acid was dissolved in solvent mixture (CAN: citric acid, Na₂HPO₄) (70: 30), then filtered using 0.45 µm filter, the volume was completed to 10 mL with the same solvent in a volumetric flask.

Pyridoxine hydrochloride (Mason natural-B6 tablet) 1000 µg.mL⁻¹ solution: this solution was prepared by weighing 5 tablets (50 mg mason natural company), grinding to fine particles and mixing; a weight of this pharmaceutical powder (0.070 g) equivalent to 0.01 g of pure pyridoxine hydrochloric acid was dissolved in solvent mixture (CAN: citric acid, Na₂HPO₄) (70: 30), then filtered using 0.45 µm filter, the volume was completed to 10 ml with the same solvent in a volumetric flask.

Pyridoxine hydrochloride (Mefron injection) 1000 µg.mL⁻¹ solution: this solution was prepared by taking 0.5 mL of injection solution (100mg pyridoxine hydrochloric acid /2ml) (Shanohai) and diluted to 25 mL using solvent mixture (CAN: citric acid, Na₂HPO₄) (70: 30), then filtered using 0.45 µm filter.

Recommended Procedure and Calibration Graph

20 µL of pyridoxine hydrochloride in the concentration range between (10 to 1250 µg.mL⁻¹) was injected under the optimum condition of analysis acetonitrile, buffer solution (citric acid, Na₂HPO₄ pH4) in the ratio (70:30)(V:V). The flow rate was set to 1.25 mL. min⁻¹, which exhibits a retention
time of 1.82 min using UV-detection at 282 nm. The sample was isocratically eluted through cosmosil 5C-18-MS-II column (4.6 mm I.D. x 250 mm, 5 µm particle size). Figure-1 shows the calibration graph of pyridoxine hydrochloride, and according to Figure-1 Beer's law is obeyed over the concentration range 10-1250 µg.mL⁻¹.

Figure 1-Calibration graph of pyridoxine hydrochloride

Figure-1 shows the linear relation between the area under the curve with the concentration of the pyridoxine hydrochloride with 0.998 as a determination coefficient and 0.999 as a correlation coefficient.

Optimization of conditions
Selection of dissolution solvent and analytical wavelength

Figure-2 shows the absorption spectrum (at the range 190-400 nm) of 100 µg.mL⁻¹ of pyridoxine hydrochloride in different dissolution solvents against blank solution (dissolution solvent). The Figure exhibits higher absorption intensity at 282 ±2 nm., and acetonitrile: buffer gave the maximum one.

Figure 2-Absorption spectra (at the range 190-400 nm) of 100 µg.mL⁻¹ of pyridoxine hydrochloride in different dissolution solvents
Selection of mobile phase
Many solvents in many compositions and ratios were used as a mobile phase for isocratically elution of sample after injection of 20 µl of 500 µg.ml⁻¹ of the standard solution. The measurements were done at 282 nm at room temperature and using 1 ml min⁻¹ as a flow rate.

| No. | Mobile phase | % Ratio | tᵣ (min) | k' | As. | Notes |
|-----|--------------|---------|----------|----|-----|-------|
| 1   | Methanol: Water | 90:10 | - | - | - | N0 peak |
| 2   | Methanol: Water | 70:30 | 2.21 | 1.30 | 3.78 | Tailing |
| 3   | Methanol: Water | 50:50 | 2.25 | 1.43 | 2.41 | Tailing |
| 4   | Ethanol: Water | 90:10 | - | - | - | N0 peak |
| 5   | Ethanol: Water | 70:30 | 2.72 | 2.62 | 2.93 | Tailing |
| 6   | Ethanol: Water | 50:50 | 2.70 | 2.38 | 3.16 | Two peaks |
| 7   | Ethanol: Water | 40:60 | 2.69 | 3.11 | 2.51 | Two peaks |
| 8   | ACN: Water | 90:10 | 2.40 | 1.43 | 1.66 | Tailing |
| 9   | ACN: Water | 70:30 | 2.34 | 1.64 | 1.22 | Sharp peak |
| 10  | ACN: Water | 50:50 | 2.33 | 1.55 | 1.56 | Sharp peak |
| 11  | ACN: Water | 40:60 | 2.32 | 2.30 | 1.84 | Tailing |
| 12  | ACN: Water:DCM | 70:20:10 | 2.41 | 1.19 | 2.37 | Tailing |
| 13  | ACN:Water:DCM | 60:30:20 | 2.38 | 1.22 | 3.14 | Tailing |

From Table-1 ACN: water (70:30)(V:V) was selected as a mobile phase of pyridoxine hydrochloride because of the symmetric chromatogram and the ideal capacity factor (k'); therefore, it was selected in subsequent experiments.

Selection of the analysis media
Many different weak acidic and weak basic media have been used to enhance the symmetry and to get a specific chromatogram of 20 µl of 500 µg.ml⁻¹ of standard solutions; the measurement was done at 282 nm at room temperature and using 1 mL min⁻¹ as a flow rate.

| No. | Mobile phase | % Ratio | pH | tᵣ (min) | k' | As. |
|-----|--------------|---------|----|----------|----|-----|
| 1   | ACN: Water: 1% Sod. bicarbonate | 70:25:5 | 11 | 2.34 | 3.43 | 2.35 |
| 2   | ACN: Water: 1% K₂HPO₄ | 70:25:5 | 9.5 | 2.41 | 4.38 | 2.78 |
| 3   | ACN: Water: 1% Sod.acetate | 70:25:5 | 8.6 | 2.35 | 0.65 | 3.51 |
| 4   | ACN: Water: Phosphate buffer | 70:15:15 | 7 | 2.24 | 3.85 | 1.65 |
| 5   | ACN: Water: 1% Ammonium acetate | 70:25:5 | 6.5 | 2.46 | 3.31 | 2.51 |
| 6   | ACN: Water: Phosphate buffer | 70:25:5 | 6 | 2.22 | 2.43 | 1.91 |
| 7   | ACN: Water: KH₂PO₄ | 70:25:5 | 5.0 | 2.27 | 4.21 | 2.83 |
| 8   | ACN: Water: 1% KHphthalate | 70:25:5 | 4.1 | 2.25 | 2.07 | 2.25 |
| 9   | ACN: Buffer solution (Sodium acetate, acetic acid) | 70:30 | 4.0 | 2.20 | 0.98 | 2.41 |
| 10  | ACN: Buffer solution (Citric acid, Na₃HPO₄) | 70:30 | 4.0 | 2.24 | 2.29 | 1.18 |
| 11  | ACN: Buffer solution(Tartaric acid, NaOH) | 70:30 | 3.8 | 2.26 | 2.95 | 2.84 |
| 12  | ACN: Buffer solution(Glycine, HCl) | 70:30 | 3.8 | 2.23 | 2.17 | 2.42 |
| 13  | ACN:H₃PO₄ | 70:30 | 3.5 | 2.24 | 3.55 | 1.88 |
Table-2 The best symmetry resulting from using ACN: buffer pH 4 solution (Citric acid, Na₂HPO₄) in the ratio (70:30) with the retention time 2.24 min, Figure-3.

**Figure 3**-Chromatogram of pyridoxine hydrochloride (20 µl of 500 µg.mL⁻¹) using ACN:bufferpH 4 (Citric acid, Na₂HPO₄) (70:30) as a selected medium for the analysis

**Effect of flow rate**

Between 0.5 to 1.5 mL.min⁻¹ a flow rate for the elution of pyridoxine hydrochloride has been followed. Table-3 shows that 1.25 mL.min⁻¹ gave a good result in which it reduces the time of analysis and gave the best symmetry; therefore, it was used in subsequent experiments.

| No. | Flow rate (mL.min⁻¹) | t_R (min) | k’ | As. | Pressure (Mpa) |
|-----|---------------------|----------|----|-----|---------------|
| 1   | 0.5                 | 4.46     | 1.89 | 1.22 | 4.8           |
| 2   | 0.75                | 2.99     | 1.49 | 1.19 | 6.8           |
| 3   | 1.0                 | 2.24     | 2.29 | 1.20 | 9.5           |
| 4   | 1.25                | 1.82     | 1.16 | 1.10 | 11.5          |
| 5   | 1.5                 | 1.51     | 2.31 | 1.22 | 13.5          |

**Effect of temperature**

15, room temperature, 30, and 35 ºC as a temperature of the column has been adjusted to follow its effect on the performance of analysis. Table-4 indicates good results at all temperatures, the room temperature was selected. Figure-4 shows the chromatogram at room temperature.

| No. | Temperature (ºC) | t_R (min) | k’ | As. |
|-----|-----------------|-----------|----|-----|
| 1   | 15              | 1.78      | 2.12 | 1.18 |
| 2   | 25              | 1.82      | 1.16 | 1.10 |
| 3   | 30              | 1.85      | 1.31 | 1.18 |
| 4   | 35              | 1.85      | 1.36 | 1.17 |
Figure 4-The final chromatogram of pyridoxine hydrochloride (20 µL of 500 µg.mL⁻¹) using ACN: buffer pH 4(Citric acid, Na₂HPO₄) (70:30) as a mobile phase and 1.25 ml.min⁻¹ as a flow rate at room temperature

Accuracy and precision
Table-5 shows the accuracy and precision of three different concentrations of pyridoxine hydrochloride (50, 250, and 500 µg.mL⁻¹) in the form of recovery %, relative error RE %, and relative standard deviation RSD %.

Table 5-Accuracy and precision of the calibration graph

| Pyridoxine Hydrochloride (µg.ml⁻¹) | Recovery %* | Relative error,% | Relative standard deviation,% |
|-----------------------------------|-------------|------------------|-----------------------------|
| 50                                | 100.15      | 0.15             | ±1.35                       |
| 250                               | 100         | 0                | ±0.96                       |
| 500                               | 100         | 0                | ±0.84                       |
| Average                           | 100.05      | 0.05             | ±1.05                       |

*Average of five determinations

Table-5 shows that the proposed method provides a good accuracy (average R.E. % is 0.05) and a good precision (average of RSD% is better than ±1.05).

Effect of ingredients
The recovery % of 10 µg.ml⁻¹ of pyridoxine hydrochloride in the presence of the same amount of expected ingredient has been followed under the optimum analysis conditions. Table-6 shows the results.

Table 6-Effect of ingredients

| Ingredient  | Recovery % of 10µg Pyridoxine HCl/10 µg ingredient |
|-------------|--------------------------------------------------|
| Starch      | 102                                              |
| Fructose    | 101                                              |
| CaSO₄       | 104                                              |
| Sucrose     | 107                                              |
| Glucose     | 109                                              |
| Arabic Gum  | 122                                              |
Table-6 shows that only Arabic gum is seriously and positively interfered in the analysis of pyridoxine hydrochloride.

**Application of the method**

Using the proposed chromatographic method, assay of pyridoxine hydrochloride in its pharmaceutical preparations, (Samavit B6 (Tablet) 40mg SDI-Iraq), (Mason natural B6 (Tablet) 50mg USA),(Meheco B6 (Tablet) 40mg Beijing-China), and (MefronB6 (Injection) 100mg/2ml Shanohai-China) has been followed under the optimum analysis conditions. The results are listed in Table-7.

**Table 7-Application of the method**

| Pharmaceutical preparations | Pyridoxine HCl(µg.mL⁻¹) | tR (min) | Peak area | Recovery % |
|-----------------------------|------------------------|---------|-----------|------------|
|                             | Drug       | Standard | Drug | Standard |           |
| Samavit B6 (Tablet) 40mg SDI-Iraq | 25 | 1.83 | 1.84 | 11851223 | 12632548 | 93.81 |
|                            | 50         | 1.83 | 1.84 | 21955184 | 23704571 | 92.62 |
|                            | 750        | 1.84 | 1.84 | 31626701 | 34560441 | 91.51 |
|                            | 1000       | 1.84 | 1.84 | 41716705 | 45414788 | 91.85 |
| Mason natural B6 (Tablet) 50mg USA | 25 | 1.87 | 1.83 | 13185678 | 12632548 | 104.37 |
|                            | 50         | 1.87 | 1.83 | 24535660 | 23704571 | 103.50 |
|                            | 750        | 1.88 | 1.84 | 36323016 | 34560441 | 105.09 |
|                            | 1000       | 1.89 | 1.84 | 46800306 | 45414788 | 103.30 |
| MehecoB6 (Tablet) 40mg Beijing –China | 25 | 1.83 | 1.83 | 13046867 | 12632548 | 103.27 |
|                            | 50         | 1.83 | 1.83 | 24257703 | 23704571 | 102.33 |
|                            | 750        | 1.84 | 1.84 | 33745565 | 34560441 | 97.64 |
|                            | 1000       | 1.84 | 1.84 | 43906323 | 45414788 | 96.67 |
| MefronB6 (Injection) 100mg/2ml Shanohai – China | 25 | 1.82 | 1.84 | 12671315 | 12632548 | 100.30 |
|                            | 50         | 1.82 | 1.84 | 22909665 | 23704571 | 96.64 |
|                            | 750        | 1.82 | 1.84 | 34034303 | 34560441 | 98.47 |
|                            | 1000       | 1.83 | 1.84 | 44410757 | 45414788 | 97.78 |

Table-7 shows a good applicability of pyridoxine hydrochloride in its pharmaceutical preparations in the both forms tablets and injections.

**Experimental t-test**

The table of t-test (at 95% confidence and for four degrees of freedom [18]) shows good trustability, according to Table (8) there is no significant difference between the proposed method and the literature method [19].

**Table 8-Experimental t-test**

| Pharmaceutical preparations | Recovery % * | t-exp |
|-----------------------------|--------------|------|
|                            | Present method | Literature method [19] |
| Samavit B6 (Tablet) 40mg SDI – Iraq | 92.44 | 91.84 | 1.37 |
| Mason natural B6 (Tablet) 50 mg – USA | 104.06 | 103.41 | 1.64 |
| Meheco (Tablet) 40 mg Beijing – China | 99.97 | 98.54 | 1.11 |
| MefronB6 (Injection) 100 mg/2ml Shanohai – China | 98.29 | 97.57 | 1.01 |

*Average of five determinations*
Comparison of the method
A comparison of the proposed method with the literature methods [15,16] (Table-9) shows that the three methods are applicable. Moreover, the present method is more rapid, simple, and precise.

Table 9-Comparison of the method

| Parameter          | Present method                        | Literature method [15] | Literature method [16] |
|--------------------|---------------------------------------|------------------------|------------------------|
| **Column**         | Cosmosil 5C18-MS-II (250 mm x 4.6 mm i.d., 5µm particle size) | Spherisort OSD C18(250 mm x 4.6 mm i.d., 5µm particle size m) | Phenomenex OSD C18 (250 mm x 3.2 mm i.d., 5µm particle size m) |
| **Mobile phase**   | ACN: buffer solution (Citric acid, Na2HPO4) (70:30)(V:V) | Methanol: Sodium acetate : water (50:40:50)(V:V:V) | ACN: 0.05 M TEA and phosphate buffer (12:88) (V:V) |
| **Temperature**    | Room temperature                       | Room temperature       | Room temperature       |
| **Detection**      | UV-detection at 282 nm                 | UV-detection           | Fluorescence λEx = 330, λEm = 420 |
| **Retention time(min)** | 1.82                                   | 3.03                   | 7.35                   |
| **Flow rate (ml.min⁻¹)** | 1.25                                   | 1.0                    | 0.4                    |
| **Linearity(µg.ml⁻¹)** | 0.2-22                                 | 0.4-150                | -                     |
| **Recovery %**     | 100.05                                | 102.52                 | 105-97.8               |
| **RSD %**          | 1.05                                  | 3.0                    | -                     |
| **Application**    | Pharmaceutical Preparations           | Pharmaceutical Preparations | Cinchona bark |

Conclusion
A simple, precise, and accurate HPLC method for the determination of vitamin B6 (pyridoxine hydrochloride) in different pharmaceutical preparations using the proposed procedure without pre separation steps and/or adjustment of temperature has been reported in this paper.

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