VALIDATION OF HPLC METHOD FOR DETERMINATION OF
THIAMINE HYDROCHLORIDE, RIBOFLAVIN, NICOTINAMIDE,
AND PYRIDOXINE HYDROCHLORIDE IN SYRUP PREPARATION

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ABSTRACT

Multivitamin syrup containing various substances of varying characteristics may have a
problem in quantitative analysis. This research has developed and validated of HPLC method
for determination of four vitamin components, that is thiamine hydrochloride, riboflavin,
nicotinamide, and pyridoxine hydrochloride in syrup multivitamin-mineral. The
chromatographic separation was achieved by using a C18 column with dimension of
3.9x300mm and particle size of 10µm. A mixture of methanol – 1% acetic acid by using 7mM
1-hexane sulphonic acid sodium salt (20:80) as mobile phase with flow rate of 1mL/min. The
effluent was monitored at 280 nm. Effective separation and quantification was achieved in less
than 20 min. That method was simple, accurate, precise, and could be successfully applied for
the analysis of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride
syrup multivitamin-mineral.

Keywords: Thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride,
syrup, HPLC.
INTRODUCTION

Syrup multivitamin and mineral preparations containing various substances of varying characteristics may have many problems in quantitative analysis. High performance liquid chromatography (HPLC) due to its high capacity in separating a mixture of substances was applicable in determining each component in multivitamin preparations simultaneously.

Several investigator have reported the used of HPLC methods for the determination of water-soluble vitamins, such as thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride in pharmaceuticals preparations [1, 2, 3]. Thomas et al. [1] investigated thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride in syrup preparation by reverse–phase (RP)-HPLC with gradien eluation.

This research was aimed to develop and validate an ion-pair RP-HPLC with isocratic eluation for simultaneous determination of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride in multivitamin-mineral simulation syrup. It would be advantageous in the routine analyzed the fourth water-soluble vitamins in tablet preparations, if they could be determined simultaneously in a single chromatographic run.

EXPERIMENTAL METHOD

1. Apparatus

A Waters µBondapak C18 10µm, 125 Å (3.9 x 300mm) was used as column in Shimadzu UFLC 20AB with setting of UV-detector at 275nm. pH meter (Radiomater Analytical PHM 201) was used to determine of pH.

2. Chemicals and Reagents

All chemicals and reagents were of analytical grade and water was distilled and filtered
through a membrane filter (0.45 µm). Thiamine hydrochloride, nicotinamide, and pyridoxine hydrochloride (Brataco chemica), and riboflavin (Hubei) were used as working standard. Methanol and acetonitrile (HPLC grade, Mallinckrodt), 1-hexane sulphonylic acid sodium salt (Merck), bi-distilled water (Brataco chemica), and acetic acid (Merck) were used to prepare the dilute solution and mobile phase. Triethylamine (TEA, Merck) for adjusting the pH.

3. Method

3.1 Preparation of Diluting Solution

A mixture of air– acetonitril – asam asetat glasial (94:5:1) was used for diluting solution.

3.2 Preparation of Mobil Phase

Prepared 7mM hexane sulphonylic acid sodium salt solution was in water and acetic acid. The mixture was set the pH value of 2.9 with TEA and than filtered with Whatman filter paper (0.45µm). The mobile phase was sonicated. The HPLC column was washed with the mobile phase of methanol-the mixture (20:80) which was mixed automatically at the flow rate 1mL/min before injected.

3.3 Preparation of Standard Solution

*Standard Stock Solution Preparation*

Riboflavin standard solution was prepared from the weight 10mg in base solution. The solution was added acetic acid solution to set pH of 5-6 and transferred to a 50mL volumetric flask. Accurately weighed amounts, 30mg thiamine hydrochloride, 100mg nicotinamide, and 10mg pyridoxine hydrochloride were taken to that 50.0mL volumetric flask and were diluted in water. The volume was made up to the mark with water.

*Standard Solution Preparation*

Stock solution (1, 2, 3, 4, and 5mL), were transferred to a 10mL volumetric flask and than were
made up to the mark with diluting solution. After shaking well, each standard solution was filtered with Whatman filter paper (0.45µm).

3.4 Preparation of Sample Solution

For validation of method, amount of volume of the sample which contained 40% of the analyt was transfers into three of 50mL nessler tube. At the nessler tube, standards of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride were added to made the sample that containing analyts, 80, 100, and 120%, respectively. Each samples was mixed by using a vortex-mixer for 30 seconds. The tube were immersed in a hot water bath maintained at 65 – 70°C for 5 minutes and mixed on a vortex-mixer for 30 seconds. A portion of the solution was filtered by filter paper of 0.2µm, cooled to room temperature, and the clear filtrate was used. The filtrate can be used within 3 hours of filtration. The steps above were repeated three times.

RESULTS AND DISCUSSION

The best result of RP-HPLC method for the simultaneous determination of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride was obtained by using a C18 column with dimension of 3.9x300mm and particle size of 10µm. A mixture of methanol–1% acetic acid by using 7mM 1-hexane sulphonic acid sodium salt (20:80) as mobile phase with flow rate of 1mL/min. The effluent was monitored at 280 nm. Under the described experimental conditions, the fourth water-soluble vitamins were selectively separated (Figure 1), no significant interfering peaks were observed at the retention times of the vitamins, except pyridoxine hydrochloride (Figure 2). Resolution of pyridoxine hydrochloride and unknown compound was 1.19.
**Figure 1.** Chromatograms of the fourth water-soluble vitamins (nicotinamide (1), pyridoxine hydrochloride (2), thiamine hydrochloride (3), and riboflavin (4)), in standard solution

**Figure 2.** Chromatograms of the fourth water-soluble vitamins, (nicotinamide (1), pyridoxine hydrochloride (2), thiamine hydrochloride (3), and riboflavin (4)), in syrup

Effective separation and quantification of the fourth water-soluble vitamins was achieved in less than 20 min (Figure 1 and 2). Thomas et al. reported that the running time was about 60 minutes by using a C18 column (46x250mm) with 5 µm of particel size and with gradien elution of mobile phase methanol–15mM 1-hexane sulphonic acid sodium salt solution pH
3.00 for determination of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride [1].

Syrup preparation has various characteristics, there were acids, bases, or neutral compounds under certain circumstances. At the RP-HPLC, the neutral compounds would be retained on column depend of their polarity, but the ionic compounds would be eluated spontaneously. A mixture of ionic and neutral compounds could be seperated by RP–ion-pair chromatography. The ionic compounds were pairing with counter ion and distributed between the mobile and stationary phase as a non-ionic molecule. By using an alkylsulphonate as a counter ion, the cation such as thiamine could be a non-ionic molecule and retained on column because of the lipophylicity of the alkyl chain. Solution of 1-hexane sulphonic acid sodium salt was used to decrease the retention time. However, the concentration of the counter ion and the pH value of mobil phase influenced the retention time. At the pH 3.5 [2], the operational time was shorter than pH 2.9. In the other side, the running time was higher when the counter ion concentration was increased [1].

The validation method, according to the International Conference on Harmonization (ICH) [4], was performed to ensure that an analytical methodology were system suitability, accurate, specific, reproducible, and rugged over the specified range that an analyte would be analyzed [5]. The performance qualification of HPLC was determined with the system suitability to verify system performance under actual running conditions with a well-characterized analyte mixture, column, and mobile phase.

<table>
<thead>
<tr>
<th>Table 1. Concentration, Retention Time, and Response Instrument at the System Suitability Test</th>
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</thead>
<tbody>
<tr>
<td><strong>Vitamins</strong></td>
</tr>
<tr>
<td>thiamine hydrochloride</td>
</tr>
</tbody>
</table>
The system suitability was showed with the coefficient of variation (CV) of < 2% for all parameter determined (the retention time and response) [6]. The coefficient of variation from three times of injecting for each vitamins were less 2 % (Table 1).

Table 2. The Presented of the Regression Equations, Correlation Coefficients, and the Range of Concentration Vitamins

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Concentration range (ug/mL)</th>
<th>Y = ax + b</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide</td>
<td>200–1000</td>
<td>Y = 2015x + 51903</td>
<td>0.9995</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>20–100</td>
<td>Y = 16038x + 17121</td>
<td>0.9996</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>60–300</td>
<td>Y = 9144.1x + 3785.1</td>
<td>0.9999</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>20–100</td>
<td>Y = 32406x – 14335</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Note: a: Slope; b: Intercept, r : Correlation coefficient

The linearity of the method was determined of the sample solution of concentration between 33 % and 166 % of the expected concentration. The best linearity of the vitamins was obtained with correlation coefficients above 0.99[7] (Table 2).

Table 3. The Recovery of Vitamins by Standard Addition Method
The accuracy and precision of the method was indicated by the values of recovery and CV of less than 2%. The accuracy was carried out by standard addition method (Table 3). The values obtained for recovery (79.23–102.27%) and the CV (below 2 %) showed the accuracy and reproducibility of the method (Table 3).

Table 4. Assay of Vitamins from Simulated Syrup

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Amount in syrup (mg/5mL)</th>
<th>Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinamide</td>
<td>20.0</td>
<td>94.99 ± 1.02</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>2.5</td>
<td>100.56 ± 1.90</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>5.0</td>
<td>97.86 ± 0.38</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>2.0</td>
<td>99.99 ± 0.38</td>
</tr>
</tbody>
</table>

The result of determination of the fourth water-soluble vitamins in syrup simulated were given in Table 4. Simulated syrup was used to ensure the quality of measurement, especially the influent of matrix. Besides the fourth vitamins, simulated syrup contained panthotenic calcium,
cyanocobalamine, folic acid, ferric (II) fumarate, copper (II) sulphate dehydrate, and sucrose. The method has applied successfully to assay the fourth vitamins.

CONCLUSIONS

The simultaneous determination of the fourth water-soluble vitamins was performed on a C18 column of (3.9x300mm) dimension and 10µm of particle size. A mixture of methanol–1% acetic acid by using 7mM 1-hexane sulphonic acid sodium salt solution (20:80) as mobile phase with flow rate of 1mL/min. The effluent was monitored at 280 nm. That method was simple, accurate, precise, and could be successfully applied for the analysis of thiamine hydrochloride, riboflavin, nicotinamide, and pyridoxine hydrochloride syrup multivitamin-mineral.

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REFERENCES


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