ANALYSIS OF WATER-SOLUBLE VITAMINS IN SUPPLEMENT MIXTURES BY HPLC/UV

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Summary: The present study describes HPLC/UV method with varying conditions (mobile phases content, flow rates, solvents) for quantitation of water-soluble vitamins (WSV) in supplement mixtures. The method was optimized in respects of analytical and chromatographic parameters such as retention time, symmetry factors, column efficiency as number of theoretical plates, capacity factors, specificity, repeatability, LoD, LoQ and linearity. The performance of these method distinguished with excellent application in the assay tests of WSVs in supplement mixtures containing Thiamine, Riboflavin, Pyridoxine, Niacinamide and Folic acid.

Key words: WSV (water-soluble vitamins); B vitamins, Nicotinic acid, Folic acid, HPLV/UV.

Introduction

The vitamins are an organic group of compounds with diverse biochemical functions. They are essential dietary components, which are needed in relatively small amounts to sustain life and good health. Based on their solubility, vitamins are divided into two main categories: water-soluble vitamins (WSV) and fat soluble vitamins (FSV). WSVs include vitamin C (ascorbic acid), B1 (thiamine), B2 (riboflavine), B3 (niacin, niacinamide), B5 (pentothentic acid), B6 (pyridoxine), B7 (biotin), B9 (folic acid), and B12 (cyanocobalamin).

Vitamins are present in almost all types of foods, food sources, vitamin supplements, drugs and etc. in different preparations such as single- or multi-vitamin tablets, injections and vitamin-enriched beverages. Certain foods are commercially fortified with vitamins and/or other nutritional essentials such as minerals.

The European Union has regulations that define limits of vitamin dosages for their safe use as food supplements. Most vitamins that are sold as food supplements cannot exceed a maximum daily dosage. Vitamin products above these legal limits are not considered food supplements and must be registered as prescription or non-prescription (over-the-counter drugs) due to their potential side effects. All vitamins are pharmacopoeia substances and their quality is defined.

In this connection an accurate quantitative measurements for vitamins are required to ensure product quality and regulatory compliance as well as to monitor vitamin intake. The major analytical challenges are that they present [1,2] and there is a need to quantitate them in a wide range of biological matrices, which include both foods and body fluids (for status indices); [3] the concentrations that are present are usually very low, and the ratio to other components, chemically very similar, is small; [4] they may be present in several or many chemically diverse, but biologically interconvertible, forms; [5] some of them are labile to heat, extremes of pH, degradative enzymes and soon and [6] there is no single analytical approach that can quantify all of them together, within a biological matrix. Established methods for vitamin analysis – from literature and pharmacopoeia include specific chemical titrimetric methods, microbiological methods, which are typically designed for single vitamin analysis and are time consuming [7, 8] and chromatographic methods, including gas chromatography [9] capillary electrophoresis [10, 11] and liquid chromatography (LC) with different detection [12–23]. LC methods are the most commonly used methods for simultaneous determination of multiple vitamins and for establishing vitamin profiles in a variety of matrices with various modes of detection [12–15, 18–20].

The aim of present study is to develop and apply optimized HPLC conditions for simultaneous analysis of water-soluble B vitamins, Niacinamide and Folic acid in supplement mixture with high precision, accuracy and specificity.
Material and Methods

Chromatographic system:
The chromatographic procedure was carried out using:

Liquid chromatograph Shimadzu LC – 10 Advp equipped with 4.6 x 250 mm column RP-18, ODS with particle size 5 μm; Detector SPD 10 AVvp – UV-VIS with fixed analytical wavelengths.

Chromatographic conditions for determination of Thiamine mononitrate, Riboflavine-5-phosphate Sodium and Pyridoxine hydrochloride:

Isocratic mobile phase (1), prepared by mixing of filtered and degassed through filter with pore size 0.45 μm Methanol and 1.36 % solution of Potassium dihydrogen orthophosphate (Monopotassium orthophosphate) in ratio 15:85 v/v respectively;
- 280 nm analytical wavelengths;
- column temperature 25 °C;
- flow rate about 1.5ml/min.

Chromatographic conditions for determination of Niacinamide:

Isocratic mobile phase (2), prepared by mixing of filtered and degassed through filter with pore size 0.22 % solution of Sodium heptansulfonate in solvent mixture of methanol and 1.36 % solution of Potassium dihydrogen orthophosphate (Monopotassium orthophosphate) in ratio 25:75 v/v respectively. pH of final solvent mixture was adjusted to 3.0 pH values with orthophosphoric acid;
- 280 nm analytical wavelengths;
- column temperature 25 °C;
- flow rate about 1.5ml/min.

Chromatographic conditions for determination of Folic acid:

Isocratic mobile phase (3), prepared by mixing of filtered and degassed through filter with pore size 0.45 μm Methanol and 1.36 % solution of Potassium dihydrogen phosphate (Monopotassium phosphate) in ratio 25:75 v/v respectively;
- 280 nm analytical wavelengths;
- column temperature 25 °C;
- flow rate about 1 ml/min.

Reagents:
Water R (Reagents (R), European Pharmacopoeia 7.0), Methanol HPLC grade, Potassium dihydrogen orthophosphate (Monopotassium orthophosphate), Sodium heptansulfonate, 0.01 M Sodium hydroxide; 0.01 M Hydrochloric acid, Thiamine mononitrate CRS, Riboflavine-5-phosphate Sodium CRS, Pyridoxine hydrochloride CRS, Nicotinic acid CRS, Folic acid CRS, Supplement mixture containing Thiamine mononitrate, Riboflavine-5-phosphate Sodium, Pyridoxine hydrochloride, Niacinamide and Folic acid. All reagents are analytical and HPLC grade.

Reference solutions:
Reference solutions of Thiamine mononitrate and Riboflavine-5-phosphate Sodium were prepared by dissolving and mixing of adequate amounts of CR substances in 0.01 M Sodium hydroxide to obtain solutions with concentration about 1 μg/ml.

Reference solution of Pyridoxine hydrochloride was prepared by dissolving and mixing of adequate amounts of CR substance in 0.01 M Hydrochloric acid to obtain solution with concentration about 1 μg/ml.

Reference solution of Niacinamide was prepared by dissolving and mixing of adequate amounts of CR substance in water R to obtain solution with concentration about 10 μg/ml.

Reference solution of Folic acid was prepared by dissolving and mixing of adequate amounts of CR substance in 0.01 M Sodium hydroxide to obtain solution with concentration about 20 μg/ml.

Test solutions:
Vitamin containing samples from supplement mixtures were randomly selected and kept at room temperature until analysis. 0.100 g from supplement mixtures was dispersed using a sonication bath for 30 min in adequate for each vitamin solution shown in “Reference solutions”. The obtained mixture was dissolved to volume of 100.0 ml with the same solution. Aliquot sample of 10.0 ml was worked up through preliminary activated C18 SPE cartridge and dilute to 100.0 ml.

Results and Discussion

Validation of HPLC methods in respect of WSV in Supplement mixture:

Specificity: Specificity in respect of reagents – „Placebo“ solution containing all reagents without active substances was prepared. There no peaks in the chromatogram obtained from this solution with Rt of analyzed WSV.

Repeatability: Six (6) equal solutions from homogenous vitamin containing samples from supplement mixtures were analyzed by HPLC method. Standard deviation (SD) in AU and relative SD (RSD) in % were found to be as follow: for Thiamine mononitrate – 4.3 %; for Riboflavine-5-phosphate Sodium – 7.2 %; for Pyridoxine hydrochloride – 5.0 %; for Niacinamide – 2.5 %; for Folic acid – 7.5 %.

Recruitment in % is found to be in intervals as follow: for Thiamine mononitrate – 95 – 105 %; for Riboflavine-5-phosphate Sodium – 90 – 110 %; for Pyridoxine hydrochloride - 95 – 105 %; for Niacina-
mide – 95 – 105 %; for Folic acid – 90 – 110 %.

Limit of detection – LoD was established for each component separately on the base of ratio noise – signal – 1:3. For B vitamins the method detection limit (MDL) was calculated and applied using 6 equal consecutive injections of a mixture samples with vitamin standards.

Method Detection Limit = S×t

Where, S represents standard deviation (SD) of replicate analysis, t represents Student’s t value for the 99% confidence level with n–1 degrees of freedom (n is number of replicates).

Limit of quantitation – LoQ was established for each component separately on the base of ratio noise – signal – 1:10.

Linearity: The analytical parameter linearity was studied for each component separately in concentration ratio 0.1 μg – 20 mg. The accordance between the Area of peaks, measured in absorption units (AU) and concentrations in g/ml is proportional in the intervals. The correlation coefficients (about 1) was found using linear regression analysis and the external standard method was used to establish the calibration curve and quantitation.

Method performance and quantitation

System suitability test:

For system suitability test determination some chromatographic parameters in different mobile phases (MP) such as retention time - Rt, symmetry factor - S, column efficiency – N as number of theoretical plates and capacity factors – k’ were ap-

| Parameter | Thiamine mononitrate | Riboflavin-5-phosphate Sodium | Pyridoxine hydrochloride | Niacinamide | Folic acid
<table>
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<tbody>
<tr>
<td>MP</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Rt (min)*</td>
<td>3.74</td>
<td>15.58</td>
<td>4.64</td>
<td>4.48</td>
<td>4.42</td>
</tr>
<tr>
<td>S</td>
<td>0.66</td>
<td>0.98</td>
<td>0.60</td>
<td>0.80</td>
<td>1.05</td>
</tr>
<tr>
<td>N</td>
<td>621</td>
<td>6066</td>
<td>538</td>
<td>396</td>
<td>3505</td>
</tr>
<tr>
<td>k’</td>
<td>0.70</td>
<td>14.60</td>
<td>1.10</td>
<td>1.03</td>
<td>1.01</td>
</tr>
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* Preliminary determined SD (+/- 0.02 min) of replicates.

pointed for optimization of conditions. The results are shown on table 2.

The selection of HPLC conditions depends upon the vitamin being analyzed and the matrix content. Some vitamins have features which allow usage of direct methods with isocratic regimens with high specificity and resolution which are very suitable for consecutive analysis in the practice and are cost effective.

In studied and validated HPLC methods water-soluble vitamins were determined using an aqueous mobile phase with low-organic solvent content following their different solubility in water and reversed-phase retention properties. The avoiding of bad resolution and similar capacity factors in mobile phase 1 (MP1) was achieved by prolonging of Rt using phosphate buffer for the separation of B vitamins (Fig. 1, Fig. 2).

Fig 1. Chromatogram of supplement mixture obtained with MP1. Rt of Thiamine mononitrate is 3.74 min and Rt of Pyridoxine hydrochloride is 4.64 min.
Fig 2. Chromatogram of supplement mixture sample containing Riboflavine-5-phosphate Sodium with Rt 15.58 min obtained with MP1.

Fig 3. Chromatogram of supplement mixture sample containing Niacinamide with Rt 4.48 min obtained with MP2.

Fig 4. Chromatogram of supplement mixture sample containing Folic acid with Rt 4.44 min obtained with MP3.
The other way was adjusting pH to appointed pH value with orthophosphoric acid for Niacinamide and adding Sodium heptansulfonate as ion pair (Fig. 3).

Stability of vitamins in supplement matrix solution was another challenge. Instability of the analyte itself can cause substantial variance in the quantitative determination. B vitamins, such as riboflavin, pyridoxine, and thiamin, which are light sensitive, and thiamin (in acid or basic condition), folic acid, and pyridoxine, which are heat labile. To avoid loss of analytes during analysis different solvents were chosen and the samples were prepared at the time of usage. Especially this concerned riboflavin which is easily dissolved in a basic solution but unstable. Thiamine is soluble in water and stable in acidic solution but unstable in light or being heated. Niacinamide is the most stable between all components - in acidic and basic solutions and when exposed to air. Pyridoxine hydrochloride is soluble in several solutions - water, ethanol, methanol, and acetone and stable in acid solution. The substance is unstable in alkali solution. Folic acid is soluble in alkali solution and in opportunity of it category definition insoluble in water. This vitamin is stable when exposed to air and unstable when exposed to light.

The other challenges were differences in concentration of the vitamins present in supplement mixtures. The single standard method for assay test permitted to cover the whole concentration interval and it is in the range of the linear response. Another approach performs several assays for each sample with different dilution factors. This leaded to using of analytical parameters limit of detection (LoD) and method detection limits (MDL) especially for B vitamins.

Conclusion

The present study describes HPLC/UV method with varying conditions (mobile phases content, flow rates, solvents) for quantitation of WSV with precision, accuracy and specificity. The method presented excellent application in the assay tests of WSVs in supplement mixtures containing B vitamins, Niacinamide and Folic acid.

References


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