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The simultaneous assay of riboflavin 5-phosphate sodium and other water-soluble vitamins in liquid multivitamin formulations by liquid chromatography

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Abstract: A simple liquid chromatographic method for the simultaneous assay of niacinamide, pyridoxine, thiamine and riboflavin 5′-phosphate sodium in liquid multivitamin formulations is described. A novel aspect of the procedure is the conversion of the riboflavin 5′-phosphate sodium to riboflavin (vitamin B_2) by reaction with alkaline phosphatase prior to quantitative HPLC. Excellent recovery, linearity and accuracy are obtained by the proposed method.

Keywords: *Liquid chromatographic assay; riboflavin 5′-phosphate sodium; water soluble vitamins; enzymatic hydrolysis.*

Introduction

The simultaneous liquid chromatographic assay of water-soluble vitamins in multivitamin formulations using ion-pairing conditions is a well established [1] analytical procedure. However, the separation of the vitamin B group does not usually include riboflavin 5′-phosphate sodium which is a component of most liquid formulations. At present, riboflavin 5′-phosphate in multivitamin preparations is assayed by microbiological or autoanalyzer methods [2]. Both methods, however, are tedious and prone to poor reproducibility. The use of riboflavin 5′-phosphate sodium in liquid formulation has created a difficult separation problem using established LC methods. In contrast to riboflavin, the sodium salt of riboflavin 5′-phosphate is normally very rapidly eluted and is poorly resolved from other vitamins [2, 3], for example, pyridoxine. Furthermore there is no official reference standard due to the unavailability of any pure riboflavin 5′-phosphate sodium salt. The successful use of alkaline phosphatase for the conversion of the riboflavin 5′-phosphate sodium salt to riboflavin in the assay preparation prior to analysis provides a solution to the problem of obtaining a pure reference standard and the liquid chromatographic separation difficulties.

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Experimental

Reagents and materials

The reference standards are prepared using USP reference materials, niacinamide, pyridoxine hydrochloride, thiamine hydrochloride and riboflavin. The reagents required are triethylamine (Matheson, Coleman and Bell), 2-monothioglycerol, *n*-heptanesulphonic acid sodium salt (LC grade, Fisher Scientific), methanol, glacial acetic acid, Tris buffer (J. T. Baker), EDTA, analytical grade and phosphatase enzyme, alkaline Type I, (from calf intestine — activity 1–3 units mg^{-1}), obtained from Sigma Chemical Co. (No. P-3877).

The mobile phase is acetonitrile : water (10 : 90 v/v) containing 0.005 M heptanesulphonic acid sodium salt, 50 mg l^{-1} EDTA, 0.3% triethylamine and adjusted to pH 3.6 with (v/v) glacial acetic acid. Depending on the age and condition of the HPLC column used for the separation, it may be necessary to vary the triethylamine concentration from 0.25 to 0.5% to achieve a good separation of the thiamine and riboflavin components. The acetic acid concentration must then be readjusted to give pH 3.6.

To prepare the mobile phase 2.2 g of 1-heptanesulphonic acid sodium salt and 100 mg EDTA are weighed into a large beaker containing 1760 ml of distilled water and followed by 28 ml of glacial acetic acid and 6 ml of triethylamine with thorough mixing. The pH of the solution is adjusted to 3.6 ± 0.05 by the addition of glacial acetic acid or triethylamine. Then 200 ml of HPLC grade acetonitrile is added with mixing. The solution is filtered through a 0.45 μm nylon filter and degassed under vacuum prior to use.

A 0.1 M Tris solution is prepared by weighing 12.1 g Tris buffer, tris-(hydroxymethyl)aminomethane and transferring to a 1000 ml volumetric flask containing about 500 ml distilled water. Swirl to dissolve and dilute to volume with distilled water.

The working buffer solution (pH 8.0) is prepared by transferring 250 ml of the above 0.1 M Tris buffer solution to a 500 ml volumetric flask, adding 146 ml 0.1 M hydrochloric acid and diluting to volume with distilled water. The buffer is filtered through a 0.45 m nylon filter prior to use.

To prepare the pH 3.6 buffer solution add 48 ml glacial acetic acid and 10 ml triethylamine to 1940 ml of distilled water. Mix well and measure the pH. If a pH of 3.6 ± 0.05 is not obtained, adjust accordingly with acetic acid or triethylamine.

Assay preparation

Transfer 15 ml Tris buffer solution (pH 8) into a 50 ml low actinic volumetric flask pipette 2.0 ml of liquid vitamin sample into the flask. Using a Pasteur capillary pipette rinse the T/C pipette with a few millilitres of Tris buffer directly into the flask. Transfer 25 mg of alkaline phosphatase enzyme into the flask, place the flask in a mechanical shaker water bath at 37°C and shake gently for 30 min. Immediately add one drop of monothioglycerol to the flask, dilute to volume with pH 3.6 buffer solution and mix well. Monothioglycerol is required to assure the stability of the assay preparation. Filter the solution through Whatman GF/C glass microfiber filter paper prior to chromatography.

Standard preparation

Transfer appropriate amounts of USP reference standards (niacinamide, pyridoxine hydrochloride, thiamine hydrochloride and riboflavin), accurately weighed, equivalent to the concentration of the assay preparation into a 500 ml low actinic volumetric flask.

Add 400 ml pH 3.6 buffer solution and mix with a magnetic stirrer until all components are in solution. Add 1 ml of thioglycerol, dilute to volume with pH 3.6 buffer and mix well. The resulting solution is used as the working reference solution and should be kept in the refrigerator where it should remain stable for at least 24 h.

Apparatus

Analyses were performed using a Hewlett–Packard 1090 liquid chromatograph equipped with a 1040 A Diode Array Detector. The analytes were monitored at 280 nm. An Altex Ultrasphere reversed-phase octadecylsilane (C_{18}), (15 cm × 4.6 mm i.d., particle size 5 μm) column was used and peak areas were integrated by means of a Hewlett–Packard Model 3357 Computer.

Procedure and calculations

Equilibrate the liquid chromatograph for 1–2 h with mobile phase flowing at 1.0 ml min^{-1}. Prior to the HPLC assay inject a reference standard of riboflavin into the chromatograph to ascertain the retention time and order of elution between thiamine and riboflavin. This procedure should be followed when a new column is used and each time a new mobile phase solution is prepared.

Inject 10 ml of the standard preparation followed by duplicate injections of the sample preparation into the stabilized liquid chromatographic system. Measure the individual peak areas of the preparation and use the average peak area for calculation.

$$\text{Analyte, mg ml}^{-1}\text{ of liquid formulation} = \frac{A_u}{A_s} \times \frac{C}{500} \times \frac{50}{2} = 0.05\ C \times \frac{H_u}{A_s},$$

where C is the weight in mg of the reference standard material in the standard preparation; A_u and A_s are the average peak areas of assay preparation and standard, respectively.

Raw material (riboflavin 5'-phosphate sodium) assay

Prepare a reference preparation by accurately weighing about 10 mg of riboflavin USP reference standard (or a suitable secondary standard) and transfer to a 500 ml low actinic volumetric flask containing 150 ml of Tris buffer solution. Dilute to volume with distilled water and mix well.

The sample preparation is made by accurately weighing about 15 mg of riboflavin 5-phosphate sodium and transferring to a 500 ml low actinic volumetric flask containing 150 ml of Tris buffer solution. Add 100 mg of alkaline phosphatase enzyme to the solution and agitate for 1/2 h at 37°C in a water bath-mechanical shaker. Dilute to volume with pH 3.6 buffer and mix well. Filter a portion of the sample through GF/C filter paper.

Calculation:

$$\%\ \text{riboflavin} = W_{RS} \times \frac{1}{500} \times \frac{500}{W_s} \times \frac{A_u}{A_s} \times 100 = \frac{W_{RS}}{W_s} \times \frac{A_u}{A_s} \times 100$$

where W_{RS} is the weight of riboflavin USP-RS in mg, W_s is the weight of sample riboflavin phosphate sodium, in mg, after correction for moisture content. A_u and A_s are peak heights of sample preparation and standard preparation, respectively.

Results and Discussion

Due to the solubility problems, riboflavin 5′-phosphate sodium is generally used in liquid multivitamin formulations instead of riboflavin. The use of riboflavin 5′-phosphate sodium, however, raised two major problems for development of a liquid chromatographic analytical method. Namely, the unavailability of a reference standard as well as the lack of separation of the phosphate salt from other vitamins or excipients by the established LC methods.

A typical chromatogram of 70% raw material riboflavin 5′-phosphate sodium salt shows a mixture of riboflavin (10%) and phosphate salt (60%) (see Fig. 1). A novel

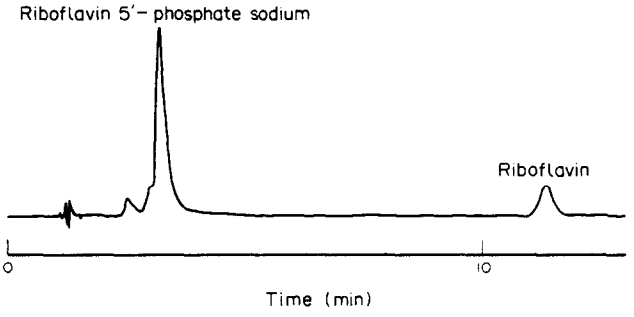


Figure 1
Chromatogram of raw material of riboflavin 5′-phosphate sodium.

approach to the solution of the assay problem is to convert the raw material to riboflavin by removing its phosphate group. Hydrolysis by strong acid or base is ruled out due to the instability of many vitamins under drastic hydrolysis conditions. It is well known that the terminal 5′-phosphate group in nucleosides can be removed by enzymatic reactions utilizing either alkaline or acidic phosphatases under very mild conditions [4]. Because of the structural similarity of riboflavin 5′-phosphate sodium to nucleosides such as adenosine 5′-phosphate (Fig. 2), hydrolytic removal of the 5′-phosphate group by either acidic or alkaline phosphatases seemed to be a logical choice. Vitamin B complex is known to be stable in a mildly acidic medium with a pH in a range of 3–6 [5]. While degradation occurs at increasing pH values. The reaction of riboflavin 5′-phosphate with acidic enzyme at pH 5.0 failed to remove the phosphate group in the moiety of the raw material. When alkaline phosphatase and riboflavin 5′-phosphate are mixed in a Tris buffer (pH 8.0) at 37°C, hydrolysis is complete in less than one hour (Fig. 3). A kinetic experiment was performed to confirm the rapidity of the enzymatic reaction (Fig. 4).

The successful hydrolysis of the raw material of riboflavin 5′-phosphate sodium led us to study the possibility of developing a new analytical procedure which would enable the simultaneous assay of riboflavin 5′-phosphate as well as other vitamin B group in most multivitamin liquid formulations.

Sample pretreatment

The complete removal of the terminal 5′-phosphate group in riboflavin 5′-phosphate sodium without destroying other vitamins is the most critical step for the assay. Since the

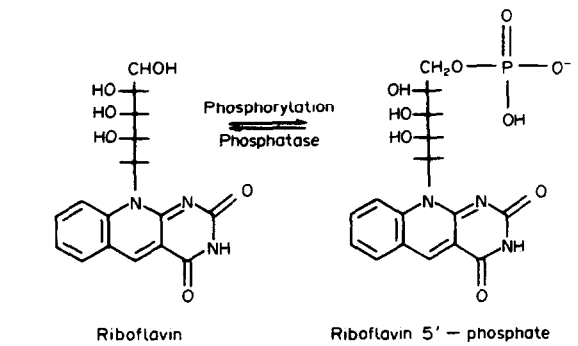
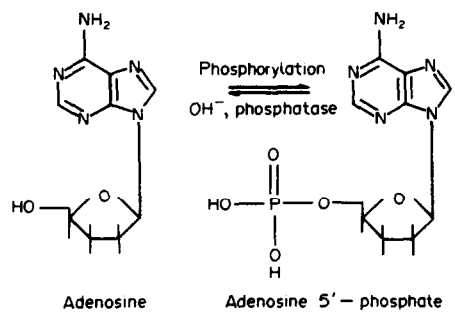


Figure 2



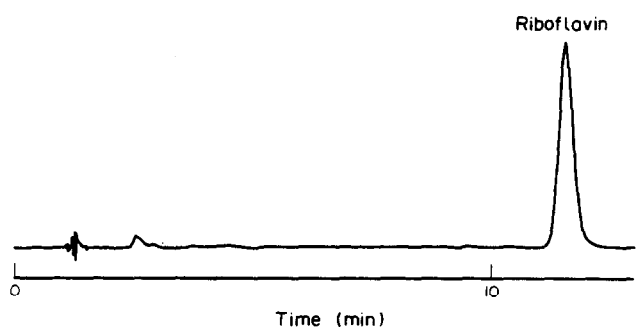


Figure 3
Chromatogram of raw material of riboflavin 5'-phosphate after treatment with alkaline phosphatase.

vitamin B group is known to be more stable in slightly acidic conditions, the reaction mixture is immediately diluted to desired volume with pH 3.6 buffer as soon as the enzymatic reaction is completed. The reaction normally requires 30 min for completion and under these conditions the other B vitamins are quite stable. The assay preparation should not be allowed to stand more than 30 min before injection into the liquid chromatograph (Figs 5 and 6) otherwise degradation of riboflavin may occur, giving low assay results. It is imperative that all steps be performed as quickly as possible. Enzymatic hydrolysis by alkaline phosphatase is inhibited if inorganic phosphate salt is present in the vitamin formulations.

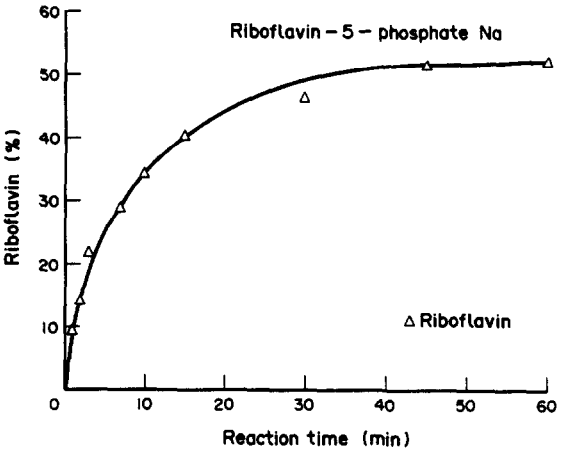


Figure 4

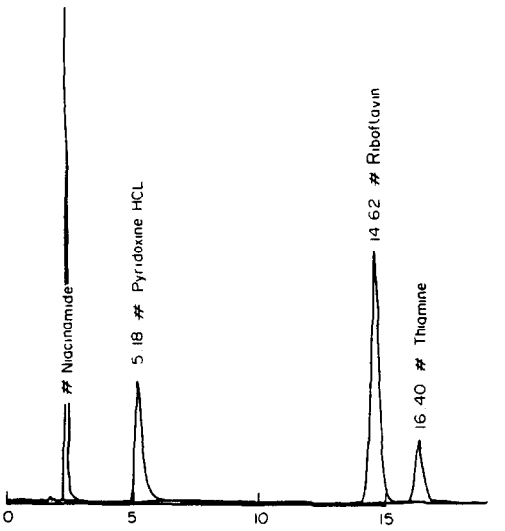


Figure 5
Chromatogram of reference standards.

Column and mobile phase effects

The amount of amine modifier in the mobile phase is very critical for the separation of thiamine and B_2 riboflavin. The optimal amount of triethylamine varies between 0.25 and 0.5% v/v depending on the condition of the column. The order of elution of the thiamine and riboflavin peaks was observed to reverse when varying amounts of triethylamine were added to the mobile phase. The pH of the mobile phase is kept at 3.6 by adjusting the amount of acetic acid accordingly.

Linearity

Concentrations of 25–125% of input were run using the proposed procedure. Plots of peak height versus concentration were linear. The coefficients of determination (r^2) of

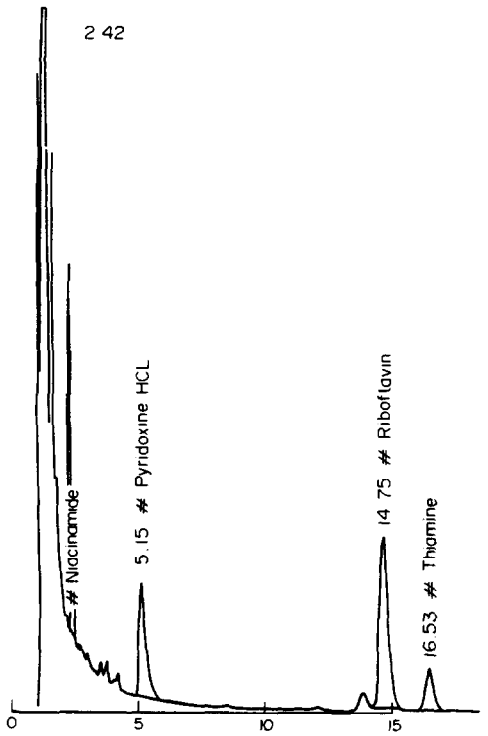


Figure 6
Chromatogram of assay preparation.

four vitamins (niacinamide, B_6, B_1, and B_2) were calculated and found to be in the range of 1.000–0.999.

Recovery and accuracy study

An accuracy and recovery study from a vitamin formulation placebo was completed over the 80–120% input range (Table 1). The average recovery for niacinamide was 99.9% with a relative standard deviation (RSD) of ±1.23% and 99% confidence limit (3 s) of ±3.69%. Average recovery for pyridoxine HCl was 100.6% with a RSD ±1.05% and 99% confidence limit (3 s) of ±3.15%. For thiamine HCl the average recovery was 97.4% with a RSD ±2.05% and 99% confidence limit of ±6.00%. Riboflavin average recovery was 100.2% with a RSD of ±0.56% and 99% confidence limit (3 s) of ±1.68%.

Stability of the chromatographic system

Eight replicate injections of the reference standard were made over a period of 3 h. Peak height data showed a relative standard deviation of 0.35% for niacinamide, 0.53% for pyridoxine hydrochloride, 0.37% for thiamine and 0.81% for riboflavin.

Production lots

The method has been applied to two different liquid formulations and their stability samples. The data are tabulated in Table 2. In general, good agreement was obtained between LC and autoanalyzer methods.

Table 1
Accuracy and recovery study of the liquid chromatographic assay of niacinamide, pyridoxine, hydrochloride, thiamine HCl and riboflavin 5-phosphate

Sample No.	% Input	Niacinamide % Recovery	pyridoxine HCl % Recovery	Thiamine HCl % Recovery	Riboflavin 5′-phosphate % Recovery
1	80	100.3	99.9	95.6	100.0
2	85	101.8	99.1	95.8	100.8
3	90	99.1	99.7	97.8	100.0
4	95	101.2	100.1	96.8	100.7
5	100	100.8	101.3	95.5	100.7
6	105	100.2	100.1	95.7	100.6
7	110	98.7	101.4	100.5	99.4
8	115	99.0	101.3	99.6	100.6
9	120	98.2	102.4	99.6	99.4
Average		99.9	100.6	97.4	100.2
Range		98.2–101.8	99.1–102.4	95.5–100.5	99.4–100.8
% RSD		1.23	1.05	2.05	0.56
s		1.23	1.05	2.00	0.56
Confidence limits (99%) 3 s		±3.69	3.15	±6.00	±1.68

Table 2
Comparison of the liquid chromatographic assay with the autoanalyzer assay of water-soluble vitamins in commercial multivitamin liquid formulations

Formulation	Niacinamide (mg)		Pyridoxine HCl (mg)		Thiamine HCl (mg)		Riboflavin (mg)	
	LC	*AA*	*LC*	*AA*	*LC*	*AA*	*LC*	*AA*
1	8.4	8.3	0.60	0.59	0.5	0.67	0.6	0.7
2	9.3	8.2	0.60	0.59	0.5	0.14	0.6	0.7

Conclusion

The liquid chromatographic method for niacinamide, pyridoxine HCl, thiamine HCl and riboflavin in liquid multivitamin formulations has been validated. Good recovery, linearity and accuracy were obtained by the proposed procedure. The assay method for the four water soluble vitamins involves a single sample preparation and a single injection into the liquid chromatograph. A novel aspect of the recommended procedure is the conversion of the riboflavin 5′-phosphate sodium to riboflavin by reaction with alkaline phosphatase enzyme prior to quantitative measurement by LC. The use of the alkaline phosphatase enzyme for the conversion of the phosphate sodium salt to riboflavin prior to analysis results in two specific advantages: (i) there is no official reference standard for riboflavin 5′-phosphate sodium, and (ii) elimination of the multiple peak retention time problem caused when the riboflavin 5′-phosphate sodium and pyridoxine HCl elute too close to each other early in the chromatographic separation. In addition, the proposed method is by far superior to any other methods for raw material determination of the sodium salt of riboflavin phosphate because of its simplicity, specificity and reproducibility. The enzymatic reaction should also be very useful for the analysis of riboflavin and its phosphate in many food products.

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