Analysis of Riboflavin in Commercial Multivitamin Preparations by High-Speed Liquid Chromatography

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Abstract □ A high-speed liquid chromatographic procedure for analysis of riboflavin in multivitamin preparations was developed and evaluated. The method utilizes 2,2′-diphenic acid as the internal standard and 254-nm detection of separated constituents. This procedure was compared with that of the USP with respect to accuracy in the analysis of riboflavin in the presence of its photolytic degradation products. In addition, the procedure was applied to the analysis of several commercial multivitamin products containing riboflavin, with reproducible results ranging from 27.23 to 103.45% of the labeled amount of riboflavin.

Keyphrases □ Riboflavin—high-speed liquid chromatographic analysis in commercial multivitamin preparations, compared to official method □ High-speed liquid chromatography—analysis of riboflavin in commercial multivitamin preparations, compared to official method □ Vitamins—high-speed liquid chromatographic analysis of riboflavin in commercial multivitamin preparations

Determination of riboflavin by chemical methods has been of considerable interest to the pharmaceutical analyst. While microbiological methods of analysis have been available for some time (1), it was not until fluorometric analyses of riboflavin were developed (2) that chemical methods approached microbiological methods in sensitivity and selectivity. The fluorometric procedure has now been refined to such an apparently acceptable point that it has been adopted, in about the same form, by the USP (3), the BP (4), and the Association of Official Analytical Chemists (5).

Recently, however, Ismaiel and Yassa (6) published evidence indicating that fluorometric procedures are unsuitable for determination of riboflavin, since the degradation products of riboflavin appear to interfere with the analysis. With most official drug products, the possibility that degradation products interfere with assay results would not be critical, since other monograph requirements (description, identification, *etc.*) would probably suffice to eliminate those products having significant degradation. The complex nature of such products as decavitamin capsules USP (7), however, prohibits use of such supplementary tests, and the reliability of the assay procedure is particularly important in establishing drug quality.

As a result, a procedure was developed for the analysis of riboflavin using high-speed liquid chromatography. The effectiveness of this procedure was evaluated in determining riboflavin in the presence of its degradation products and other vitamins. Finally, this procedure was applied to the determination of riboflavin in several commercial multivitamin products and the results were compared to those obtained using a spectrophotofluorometric modification of the currently official procedure (3).

Table I—Analysis of Contrived Mixtures Containing Riboflavin

Sample	Riboflavin Added, mg	Riboflavin Found, mg	Error, %	SD^a, %
1	0.80	0.814	1.72	2.14
2	1.50	1.473	1.83	2.02
3	2.50	2.480	0.83	1.74
4	3.50	3.42	2.33	2.46
5	7.50[b]	7.41	1.20	1.47
6	10.00[b]	9.81	1.93	2.36
Mean			1.64	2.03

[a] $n = 10$. [b] High potency modification.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a 254-nm detector was used in the study. A stainless steel column [0.31 cm (0.12 in.) i.d. and 1.22 m (4 ft) long] was packed with liquid chromatography grade silicic acid[2]. A hot-block attached to a circulating water bath heated the column. Peak areas were determined using an electronic digital integrator[3] and a planimeter. Injections into the pressurized system were made with syringes[4] (10 μl) fitted with special plunger tips. A spectrophotofluorometer[5] with excitation and emission wavelengths of 442 and 564 nm, respectively, and slit width of 0.5 mm was used for supporting studies.

Chromatographic Conditions—The chromatographic solvent was a mixture of chloroform, methanol, and acetate buffer, pH 4 (60:28:4.5 v/v). The column temperature was 42°, and the rate of flow was 0.95 ml/min. A pressure of 105.43 kg/cm^2 (1500 lb/in.^{2}) was necessary to maintain this rate of flow.

Materials and Reagents—The chromatographic internal standard was 2,2′-diphenic acid[6]. USP Riboflavin Reference Standard[7] was treated according to official procedures (3). All other reagents and solvents were of the best grade commercially available and were used without further purification.

Solutions—*Acetate Buffer Solution, pH 4*—Dissolve sodium acetate (25.0 g) in distilled water (400 ml) and adjust to pH 4 with acetic acid. Dilute the solution with distilled water to a final volume of 500 ml.

Riboflavin Standard Solution—Place 50.0 mg of USP Riboflavin Reference Standard in a light-resistant volumetric flask (100 ml) containing acetate buffer, pH 4 (60 ml). Heat the mixture until the riboflavin has dissolved, cool to room temperature, and dilute to 100 ml with acetate buffer, pH 4.

Internal Standard Solution—Dissolve the internal standard (375.0 mg) in methanol and dilute with methanol to a final volume of 250 ml.

Preparation of Standard Curve—Add internal standard solution (10.0 ml) to each of seven light-resistant containers (100 ml). To each container add the riboflavin standard solution in amounts from 1.0 to 7.0 ml in 1.0-ml increments. Then add chloroform (50 ml) and methanol (15 ml), shake the solution, and inject 4–8 μl into the chromatographic system. The ratio of areas

[1] ALC 202, Waters Associates.
[2] LiChrosorb SI 60, E. Merck.
[3] Varian model 475.
[4] Pierce Chemical Co., Rockford, Ill.
[5] SPF 125, Aminco-Bowman.
[6] Baker-Analyzed, J. T. Baker Co.
[7] USP Reference Standards, Rockville, MD 20852

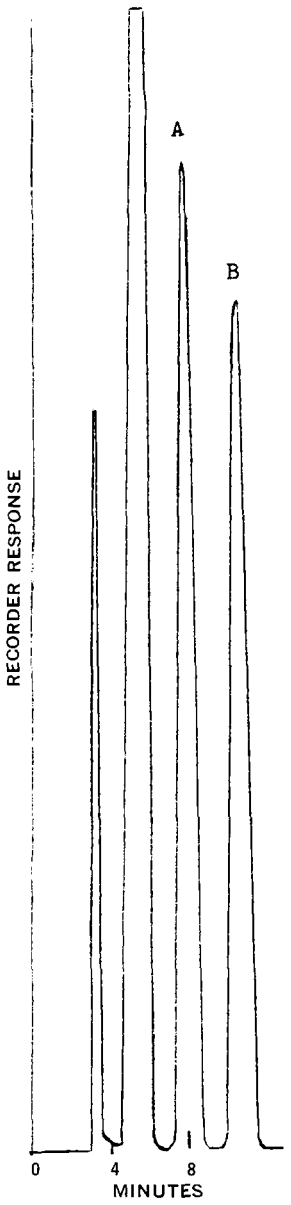

Figure 1—*Chromatographic trace of a multivitamin preparation. Key: A, riboflavin; and B, internal standard.*

under the peaks corresponding to riboflavin and the internal standard is plotted against the quantity of riboflavin in the solution.

Analysis of Riboflavin in Vitamin Products—Place 1 dosage unit in a light-resistant container containing acetate buffer, pH 4 (5 ml). Heat the mixture to boiling for 1 min, shake, and allow to cool to room temperature. If the dosage unit does not appear to be homogeneously distributed, repeat the procedure. Add internal standard solution (10.0 ml), chloroform (50 ml), and methanol (15 ml). Shake the resulting mixture and inject 4–8 μl into the chromatographic system. Calculate the ratio of the area under the peaks corresponding to riboflavin and the internal standard and determine the quantity of riboflavin in the sample graphically.

For high potency preparations (those containing more than 5 mg of riboflavin per dosage unit), the procedure is modified by the use of an additional 15.0 ml of internal standard solution and the omission of methanol.

In addition, vitamin products were analyzed for riboflavin content according to the USP procedure (3) using a spectrophotofluorometer.

Analysis of Riboflavin in Presence of Its Photolytic Degradation Products—Riboflavin standard solution (40 ml) was placed in a clear Pyrex volumetric flask and exposed to normal laboratory artificial lighting. Aliquots were taken for analysis at 20-min intervals, and the apparent riboflavin content was determined by the chromatographic method and the fluorometric method.

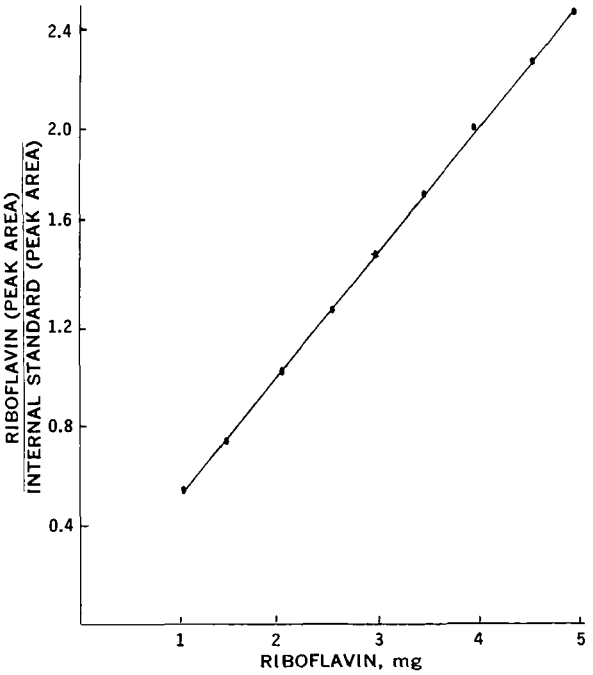

Figure 2—*Standard curve for the chromatographic analysis of riboflavin.*

Separation of Riboflavin Degradation Products by TLC—Riboflavin solutions that had been exposed to light were applied to the system developed by Ismaiel and Yassa (6). Spots on the developed chromatogram were eluted with acetate buffer, pH 4.

RESULTS AND DISCUSSION

The solvent system used in this study is a modification of one used previously in TLC studies of riboflavin (6). With this solvent system, riboflavin elutes after 8 min with a column efficiency of 750 theoretical plates. It is well resolved from other vitamins and from common constituents of vitamin preparations (Fig. 1). In the products studied, thiamine elutes closest to riboflavin (relative retention volume 0.69), but the thiamine content must exceed the riboflavin content by 60-fold before the two peaks are incompletely resolved.

Linearity of the response over the range studied is shown in Fig. 2. Standard curves were prepared daily for 10 days, and the standard deviation of the slope of the curve was found to be 1.41%. The average correlation coefficient was 0.993, and the average y-intercept value was −0.012. This indicates that the procedure is amenable to use of a single-point standard.

Accuracy and precision of the procedure were studied using contrived mixtures (Table I). Each value represents the mean of two determinations on each of 5 consecutive days. In contrast to the precision shown here, the coefficient of variation of the fluoro-

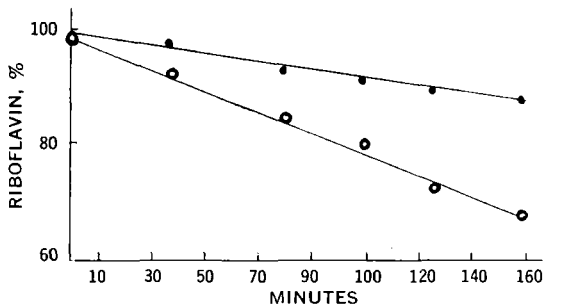

Figure 3—*Apparent percent riboflavin remaining after photolysis as determined by fluorometric procedure* (●) *and chromatographic procedure* (○).

Table II—Analysis of Riboflavin in Multivitamin Products

Sample	Dosage Form	Labeled Components	Labeled Riboflavin Content, mg	Riboflavin Found, %	
				Chromatographic Method	Fluorometric Method
1	Tablet	Iron, 10 vitamins, artificial colorings, flavorings, and sweeteners	1.5	97.64[a] (9.16)	98.47[a] (9.74)
2	Tablet	11 vitamins, natural sweeteners, artificial coloring, and flavoring	1.2	95.26 (3.70)	97.93 (4.33)
3	Tablet	10 vitamins and sodium fluoride	1.5	98.34 (5.15)	104.42 (4.86)
4	Capsule	Nine vitamins	3.0	101.18 (4.77)	109.31 (5.11)
5	Capsule	Six vitamins	10.0	103.45[b] (3.94)	105.62 (4.72)
6	Liquid	Nine vitamins, natural sweeteners, and artificial flavoring	0.7	27.23 (2.14)	89.47 (3.51)
7	Liquid	Nine vitamins and natural sweeteners	1.2	93.61 (3.14)	106.29 (4.12)

[a] Percent of labeled amount (% *SD*) with $n = 10$. [b] High potency modification.

metric procedure was shown to be as high as 15.1% in a collaborative study (8).

The results of experiments comparing the effectiveness of this procedure with that of the official procedure in determining riboflavin in the presence of its photolytic degradation products are shown in Fig. 3. Since apparent riboflavin content as determined by the chromatographic procedure drops more rapidly than that determined by the fluorometric procedure, it would appear that one or more degradation products are contributing to fluorescence at the analytical wavelengths. If a fluorometer had been used in place of the spectrophotofluorometer, it is quite possible that the broader range of excitation and emission wavelengths would have resulted in an even larger discrepancy between results of the two procedures.

In light of the number of isolated riboflavin degradation products (9), it is not surprising that one or more possess fluorescence characteristics similar to the parent compound. By using the described system, it was possible to isolate four peaks from riboflavin solutions exposed to artificial light in pH 4 buffer. At a flow rate of 0.95 ml/min, two peaks with retention volumes of 0.25 and 0.56 (relative to riboflavin) appeared after photolysis. The more rapidly eluting peak was, however, resolvable into three components when the flow rate was lowered to 0.25 ml/min.

To investigate the selectivity of the procedure further, photolytic degradation products of riboflavin were isolated by the method of Ismaiel and Yassa, eluted, and injected into the chromatographic system. Spot 3 (6) on the thin-layer chromatogram corresponds to the peak at 0.56 relative retention volume, and Spots 4, 5, and 6 correspond to the peak with relative retention volume 0.25. Spot 1 does not emerge from the chromatograph.

Results of the analysis of riboflavin in multivitamin preparations using the official and chromatographic procedures are presented in Table II. Placebo preparations were subjected to analysis where these were available (Samples 1, 2, 4, and 7, Table II), and no constituent interfered with the analysis.

The chromatographic procedure gives significantly lower values for riboflavin content in several cases, especially the liquid preparations. However, results from the two methods with respect to the riboflavin content of Liquid 1 are so disparate as to require comment. Two different lots of the same preparations were tested with approximately the same results. There are two possible explanations for this disparity. A derivative of riboflavin having similar fluorescence characteristics but different chromatographic characteristics may have been used to prepare the liquid. Information regarding this possibility was not available, but riboflavin-5′-phosphate, a likely candidate for such a substitution, does not appear in the chromatogram (relative retention volume 1.86). On the other hand, degradation of riboflavin may have occurred in the solution, generating one or a combination of products which account for the apparent riboflavin level as determined by the USP procedure. The latter possibility is supported by the fact that the chromatogram of this product has a peak at 0.25 relative retention volume, corresponding to that from riboflavin degradation products. Additionally, DeMerre and Brown (9) showed that, in certain solvent systems, photolytic degradation of riboflavin appears to cease when fluorometry indicates 60–80% of the riboflavin still remains. They accounted for these findings by theorizing that photolytic degradation products formed in these solvents systems possess similar fluorescence characteristics to the parent compound.

Results from the analysis of Tablet 1 (Table II) have relatively high standard deviations with both methods. However, when a larger number of tablets was analyzed and the results were grouped according to tablet color, the red tablets were found to contain 91.34% (2.74 *SD*) of the labeled amount of riboflavin, the blue tablets contained 104.21% (3.37 *SD*), and the green tablets contained 97.60% (3.14 *SD*). These results indicate that tablet color influences degradation or, more likely, that different formulation batches are used to make the different colored tablets.

The chromatographic method itself appears to be practical. It is possible to determine riboflavin content of seven units, each of three different products, in a 4-hr period. The life of the column under the assay conditions is at least 3 months, with no perceptible change in retention characteristics or results.

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