dehyde moiety, with carbethoxymethylenetriphenylphosphorane was reported (17). Thus, the aldehyde (I) was subjected to the reaction with the phosphonate (II) or the phosphorane (III) to yield the ethyl ester (VI) in satisfactory yield. In the reaction of I with II (Method A), 2 equivalents of sodium hydride were used, and aprotic polar solvents such as dimethyl sulfoxide and *N,N*-dimethylformamide were necessary to dissolve the sodium salt of I. The time conversion was monitored by high-performance liquid chromatography (HPLC), and I was converted to VI in a 58% yield when the reaction was carried out with 1.5 equivalents of II at 95° for 3 hr in *N,N*-dimethylformamide. The reaction rate was slower in dimethyl sulfoxide.

On the other hand, I reacted readily with resonance-stabilized ylide III (Method B) in various solvents capable of dissolving I to yield VI. However, the reaction in ethanol resulted in a lower yield than in aprotic solvents, probably due to decomposition of III by a side reaction with ethanol. Of the several solvents used, dimethyl sulfoxide and diglyme were the most suitable for this reaction. The yields of VI for the reaction in dimethyl sulfoxide and in diglyme were 66 and 63%, respectively (molar ratio of III to I of 1.2, 130°, 3 hr).

From the viewpoints of utility and facility, these results indicate that carboalkoxymethylenetriphenylphosphoranes are more appropriate for the preparation of esters of imidazoleacrylic acids containing an acidic imino hydrogen atom. Method B thus was applied to the preparation of long-chain alkyl esters. The aldehyde (I) was allowed to react with the phosphorane (V), and HPLC analysis of the reaction mixture showed the formation of *n*-dodecyl ester (VII) in a 70% yield. Similarly, I was converted, by the reaction with the phosphorane (IV), to *n*-hexadecyl ester (VIII) in 66% yield.

These esters (VI–VIII) were soluble in most oils and had an absorption maximum at 290 nm in 0.75% (w/w) triethylamine in methanol.

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Simultaneous Determination of Niacinamide, Pyridoxine, Riboflavin, and Thiamine in Multivitamin Products by High-Pressure Liquid Chromatography

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Abstract □ A high-pressure liquid chromatographic assay was developed for the determination of four water-soluble vitamins: niacinamide, pyridoxine, thiamine, and riboflavin. The four vitamins are assayed simultaneously in multivitamin products not containing minerals. Thiamine currently is not quantitated in formulations containing minerals because it is not stable under the extraction conditions. The method was applied to the analysis of at least 12 different multivitamin products, including various formulations of sterile products, fluids, compressed tablets, and coated, compressed tablets. The method is stability indicating and is applicable to single-tablet assays.

Keyphrases □ Niacinamide—simultaneous high-pressure liquid chromatographic analysis with pyridoxine, riboflavin, and thiamine in multivitamin products □ Pyridoxine—simultaneous high-pressure liquid chromatographic analysis with niacinamide, riboflavin, and thiamine in multivitamin products □ Riboflavin—simultaneous high-pressure liquid chromatographic analysis with niacinamide, pyridoxine, and thiamine in multivitamin products □ Thiamine—simultaneous high-pressure liquid chromatographic analysis with niacinamide, pyridoxine, and riboflavin in multivitamin products □ Vitamins—niacinamide, pyridoxine, riboflavin, and thiamine, simultaneous high-pressure liquid chromatographic analysis in multivitamin products □ High-pressure liquid chromatography—analysis, niacinamide, pyridoxine, riboflavin, and thiamine in multivitamin products

A substantial amount of information has been published since 1970 on the use of high-pressure liquid chromatography (HPLC) for the analysis of water-soluble vitamins. An excellent review of the subject was prepared by Wittmer and Haney (1). Many of the alternative chemical and microbiological assay methods have time-consuming sample preparations and are not specific. HPLC is the preferred method for vitamin analysis, especially when the sample preparation is simple.

A recent report described the simultaneous determination of niacinamide, pyridoxine, thiamine, and riboflavin (2). Both the previously reported method and the method described in this paper use a paired-ion mobile phase system with a reversed-phase column (3).

The HPLC procedure described in this report has been used for routine analysis for 3 years. The method has been applied to 12 different multivitamin formulations, and work is continuing to add more applications. An average

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Table I—Average Percent Recovery of Vitamins from Various Formulation Placebos

Formulation Placebo	Recovery, %			
	Niacinamide	Pyridoxine	Riboflavin	Thiamine
Compressed tablet	99.7	99.9	100.8	100.1
Coated, compressed tablet	100.0	98.8	101.4	100.2
Coated, compressed tablet with minerals	98.8	99.4	99.1	—

Table II—Precision for the HPLC Determinations of Niacinamide, Pyridoxine, Riboflavin, and Thiamine

Dosage Form	*RSD, %*			
	Niacinamide	Pyridoxine	Riboflavin	Thiamine
Injectable	1.1	1.0	1.7	1.2
Compressed tablet	1.4	1.7	1.8	0.6
Coated, compressed tablet	1.2	2.0	1.5	1.8
Coated, compressed tablet with minerals	1.2	1.9	1.4	—

of 16 assays can be run per day. Sample preparation is minimal and involves only a one-step extraction into an internal standard solution and filtration. The method is stability indicating and is applicable to single-tablet assays.

EXPERIMENTAL

Chromatographic Conditions—The equipment consisted of a suitable liquid chromatographic pump capable of operating at ~1000 psi and 1 ml/min, an automated injector with a fixed-volume 20-μl loop, a dual-wavelength (254 and 280 nm) UV detector, and a reversed-phase C_{18} column[1].

Mobile Phase—The mobile phase contained 25% methanol and 1% acetic acid in water. The hexanesulfonic acid sodium salt concentration was adjusted to $3-8 \times 10^{-3}$ *M* to optimize chromatography and vitamin quantitation.

Internal Standard Solution—The internal standard solution was made up in the mobile phase to contain ~0.05 mg of *p*-hydroxybenzoic acid/ml. For multivitamin–mineral formulations, the internal standard solution also contained ~4 mg/ml of diethylenetriamine pentaacetic acid as the sodium salt.

Reference Standard Solution—All reference standard solutions were prepared using USP reference standards dissolved in the internal standard solution.

Sample Preparation—A representative number of tablets was ground to a fine powder. To an accurately weighed sample was added exactly 25.0 ml of the internal standard solution. The mixture was shaken mechanically for ~1 hr, filtered, and injected onto the column. Samples formulated with coated vitamins were heated, before shaking, at ~70° for 20 min.

RESULTS AND DISCUSSION

To quantitate all four vitamins in one chromatographic run without changing the detector sensitivity, a dual-wavelength detection system was adopted. Figure 1 shows the absorption characteristics of the various vitamins in the mobile phase. Niacinamide, thiamine, and riboflavin usually are monitored at 254 nm; pyridoxine is quantitated at 280 nm. The linear response of the system was determined for the following vitamin concentration ranges: niacinamide, 0.21–0.50 mg/ml; pyridoxine, 0.01–0.06 mg/ml; thiamine, 0.02–0.10 mg/ml; and riboflavin, 0.018–0.042 mg/ml.

The accuracy of the methodology was established from spiked placebo recovery experiments. Results for three representative formulations are given in Table I. The percent recoveries reported for the compressed tablet, a chewable formulation, are averages of six determinations. The corresponding placebo samples were spiked in the range of 80–100% of the theoretical amount. Results for the coated compressed tablet are averages of five different placebo samples spiked with 70–120% of the theoretical amount. Averages of four replicate samples spiked at the theoretical value are reported for the multivitamin–mineral dosage form.

Reproducibility of the assay is excellent. Relative standard deviations for representative dosage forms are given in Table II. The precision data were obtained in a routine assay laboratory by several analysts over a period of at least 3 months and using at least two different columns.

Comparison between the HPLC method and current assay results for seven representative dosage forms are shown in Table III. On the average, the HPLC and available assay results agree within 2%. The HPLC results for niacinamide and pyridoxine are compared to the microbiological

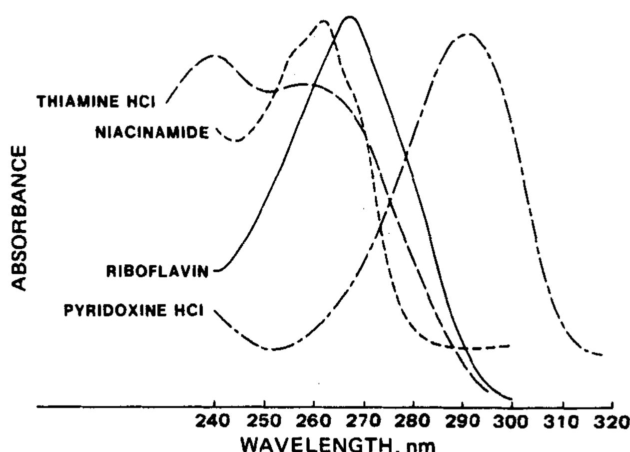

Figure 1—*Absorption characteristics of vitamins in the mobile phase.*

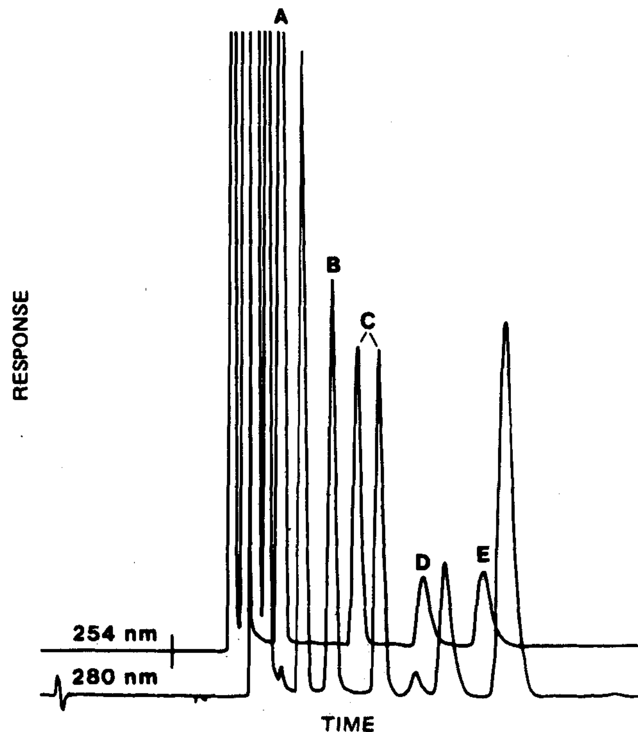

Figure 2—*Chromatogram of a multivitamin–mineral formulation. Niacinamide (A), p-hydroxybenzoic acid (internal standard) (C), thiamine (D), and riboflavin (E) were monitored at 254 nm. Pyridoxine (B) and the internal standard (C) were monitored at 280 nm. The mobile phase contained 25% methanol, 0.1% hexanesulfonic acid sodium salt, and 1% acetic acid.*

[1] μBondapak C_{18} or equivalent, Waters Associates.

Table III—Comparison between HPLC Method and Current Assay Results for Several Commercial Dosage Forms

Dosage Form	Niacinamide HPLC	Niacinamide Microbiological	Pyridoxine HPLC	Pyridoxine Microbiological	Thiamine HPLC	Thiamine Chemical	Riboflavin HPLC	Riboflavin Chemical
Compressed tablet								
Form A lot 1	21.8	22	2.18	2.2	1.94	1.87	1.92	1.95
Form A lot 2	21.9	22	2.26	2.3	1.97	1.90	1.89	1.91
Form A lot 3	21.7	22	2.18	2.1	2.00	1.90	1.94	1.91
Coated compressed tablet								
Form B lot 4	108	107	7.3	7.8	11.9	11.9	10.7	10.9
Form B lot 5	107	112	7.2	7.6	11.5	11.3	10.7	10.7
Form C lot 6	21.7	22.2	2.42	2.30	1.74	1.71	1.83	1.86
Form C lot 7	21.6	21.6	2.47	2.50	1.72	1.74	1.90	1.96
Multivitamin–mineral tablet								
Form D lot 8	21.2	21.1	2.50	2.52	—	—	1.98	1.97
Form D lot 9	21.5	21.9	2.50	2.45	—	—	1.90	1.88
Form E lot 10	101	103	7.1	6.9	—	—	10.2	10.5
Form E lot 11	104	109	7.2	7.0	—	—	10.5	10.7
Form F lot 12	14.6	14.9	2.3	2.4	—	—	1.90	1.90
Form F lot 13	14.9	15.4	2.4	2.5	—	—	1.87	1.85
Form G lot 14	21.3	21.2	2.42	2.55	—	—	1.85	1.80
Form G lot 15	20.8	21.8	2.36	2.50	—	—	1.84	1.87

Amount of Vitamin per Tablet, mg

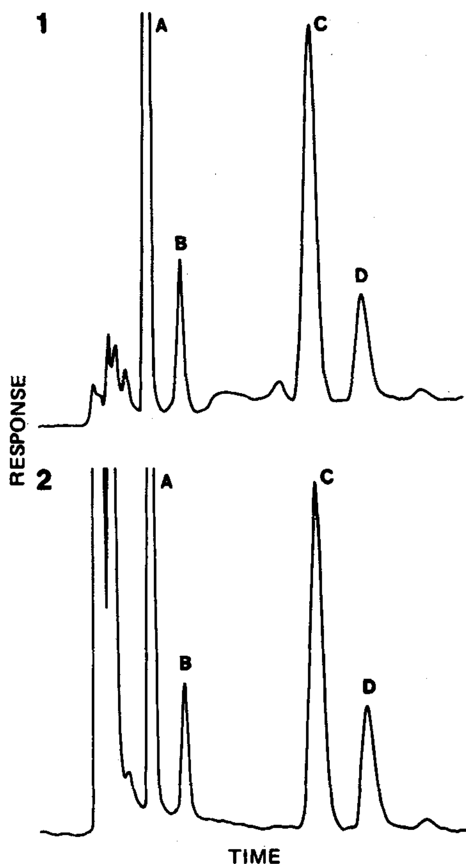

Figure 3—*Chromatogram of a multivitamin-mineral sample immediately after extraction with the mobile phase (1) and with the mobile phase containing 4 mg of diethylenetriamine pentaacetic acid sodium salt/ml (2). Niacinamide (A), pyridoxine (B), riboflavin (C), and thiamine (D) were monitored at 280 nm. The mobile phase contained 27% methanol, 0.14% hexanesulfonic acid sodium salt, and 1% acetic acid.*

procedure results. The current methods for thiamine and riboflavin are fluorometric assays.

For most formulations, a 30–60-min extraction of the ground tablet with the internal standard solution gave excellent recoveries. Samples containing coated vitamins must be warmed to at least the melting point of the coating to extract the vitamins completely from the gelatin matrix.

A stability problem is associated with vitamin–mineral formulations. After extraction, both ascorbic acid and thiamine are unstable in solution in the presence of minerals, especially ferrous and cupric salts. If a metal-chelating agent was not added to the extraction solution, only 60–70% of the thiamine was recovered, and four degradation product peaks appeared, which could interfere with the chromatographic run. The addition of 4 mg of diethylenetriamine pentaacetic acid sodium salt/ml increased thiamine recovery to 95%. Placebo mixtures containing both ascorbic acid and thiamine showed no chromatographic interference from excipients or degradation products. Samples of vitamin–mineral formulations were chromatographed within 24 hr of preparation. Figure 2 shows the chromatogram of a multivitamin–mineral formulation sample.

In the absence of cupric and ferrous salts, the vitamin solutions are stable for at least 2 months when stored in a refrigerator protected from light. To establish that the HPLC assay is stability indicating, vitamin solutions were subjected to accelerated degradation conditions. The decomposition peaks of riboflavin and pyridoxine do not interfere with the chromatography of the vitamins. However, thiamine degradation peaks elute with the internal standard peak. The validity of the assay for these samples was proven without including the internal standard peak in the calculations. Figure 3 shows the chromatogram of a multivitamin–mineral sample immediately after extraction with the mobile phase and with the mobile phase containing 4 mg of diethylenetriamine pentaacetic acid sodium salt/ml.

The HPLC method described here has been implemented for many different multivitamin formulations, such as injectable products, fluids, compressed tablets, and coated, compressed tablets. Work is underway to improve the recovery of thiamine from formulations containing minerals and to increase the number of applications for the assay.

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