The Optimization of HPLC–UV Conditions for Use with FTIR Detection in the Analysis of B Vitamins

Ying Li and **Phyllis R. Brown***
Department of Chemistry, University of Rhode Island, Kingston, RI, 02881

Abstract

The water-soluble vitamins thiamine (B_1), riboflavin (B_2), pantothenic acid (B_5), and pyridoxine (B_6) are separated by high-performance liquid chromatography. The mobile phase, column temperature, and flow rate are optimized so that the chromatograph can be used with a Fourier-transform infrared (FTIR) detector. Reproducibility, linearity, and detection limits are evaluated for method validation. Finally, this method is successfully transferred to liquid chromatography–FTIR with a standard mixture.

Introduction

Vitamins are micronutrients that are essential for human health. They are categorized in two groups: (*a*) fat-soluble and (*b*) water-soluble vitamins. Water-soluble vitamins consist of vitamin C (ascorbic acid) and the vitamin B group, which includes thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin, folic acid, and cyanocobalamin (B_{12}). These vitamins are chemically inhomogeneous and therefore display different chemical and physiological properties. The B-group vitamins, in most cases, act as coenzymes and participate in a number of enzymatic reactions to produce energy.

The standard and official methods for the analysis of the water-soluble vitamins include spectrophotometric, polarographic, fluorimetric, enzymatic, and microbiological procedures. These methods are sometimes tedious, nonspecific, and time-consuming. Recently, high-performance liquid chromatography (HPLC) and capillary electrophoresis have been widely used.

Currently, the reversed-phase mode of HPLC (RPLC) has become the most popular analytical technique for the analysis of water-soluble vitamins in food, biological samples, and pharmaceuticals. It has the advantages of speed, selectivity, sensitivity, and versatility. Many detectors have been used to detect water-soluble vitamins separated by RPLC including UV and photodiode array (1,2), fluorescence (3,4), electrochemical (5), and mass spectrometry (MS) (6–8).

* Author to whom correspondence should be addressed: email pbrown@chm.uri.edu.

Recently, Fourier-transform infrared detection (FTIR) has become available as a detector for HPLC (9,10); the coupled technique is referred to as LC–FTIR in this paper. The infrared (IR) spectrum can be matched with a standard or a known compound from spectral libraries; thus unequivocal identification or purity analysis or both can be obtained. LC–FTIR can also aid in structural elucidation. Because LC–FTIR is nondestructive and both sample recovery and purification are feasible, the method is suitable for pharmaceutical analysis (11). However, the critical requirement for the solvent (which can either be volatile for the solvent elimination mode or easy to subtract the solvent for the flow cell mode) has limited the use of LC–FTIR, and analyses of water-soluble vitamins using LC–FTIR have not yet been reported in the literature.

We report an LC–FTIR method for the analysis of four water-soluble vitamins: (*a*) thiamine (B_1), (*b*) riboflavin (B_2), (*c*) pantothenic acid (B_5), and (*d*) pyridoxine (B_6). The HPLC method was first developed with a UV detector and then adapted for use with an FTIR detector.

Experimental

Chemicals and reagents

The vitamin standards of thiamine hydrochloride (B_1), riboflavin (B_2), pyridoxine monohydrochloride (B_6), and D-pantothenic acid (B_5) semicalcium salt were all obtained from Sigma-Aldrich (Milwaukee, WI). Ammonium acetate, ammonium formate, formic acid, methanol, acetonitrile, and HPLC-grade water were obtained from Fisher Scientific (Suwanee, GA). Liquid nitrogen and helium gas were obtained from Medical-Technical Gases (Medford, MA).

LC–FTIR

The LC–FTIR instrument used was from Bourne Scientific (Acton, MA). In this instrument the interface between the HPLC and the FTIR detector consists of an ultrasonic nebulizer and a heated drift tube (12). The effluent from the HPLC column is split into a UV detector and the FTIR detector (Figure 1). The effluent that flows into the FTIR detector is first nebulized ultrasonically

to break the solvent into a mist. The gaseous solvent is then carried in helium through the drift tube into a vacuum chamber where it is evaporated and removed by a vacuum pump. The solute is deposited on a moving zinc selenide plate under the drift tube. Consequently, each analyte occupies a different position on the plate. Meanwhile, the IR beam scans the plate (transmission mode) generating the band chromatograms.

LC–FTIR instrumentation

The HPLC pump was a Constametric 4100 from Thermo Separations (Austin, TX) and was used in conjunction with an LDC analytical membrane degasser (Austin, TX). An Eppendorf CH-30 column heater (Brinkmann Instruments, Westbury, NY) was used to maintain column temperature. A Sonics Vibracell (Sonic and Materials, Newton, CT) provided ultrasonic nebulization. The interface and the FTIR were integral parts of the instrument from Bourne Scientific (Acton, MA). The FTIR detector was from Midac (Irvine, CA) and required cooling with liquid nitrogen. Midac Grams software was used to collect the data. Separations were on a C18 microsorb column (250- × 4.6-mm i.d., 5 µm) from Rainin Instrument Company (Woburn, MA).

LC–FTIR operating conditions

In the HPLC, the flow rate was 0.75 mL/min. The flow rate of the HPLC effluent to the FTIR was 0.13 mL/min. The column temperature was 30ºC and the temperature of the drift tube was 130ºC. The nebulizer was operated at an amplitude of 40% of total power. A pressure of 1 atm of helium was used. The scan rate of the IR beam was 10 times per second. The data were coadded to produce an FTIR spectrum every 2 s with a resolution of 8 cm^{-1}.

UV detection

The UV detector was a 3200 UV spectrometer (Thermo Separations). An SP4270 Spectra-Physics integrator (Thermo Separations) recorded the UV data. A wavelength of 254 nm was selected for monitoring riboflavin, pyridoxine, and thiamine. Because pantothenic acid did not absorb at 254 nm, it was monitored at 210 nm.

Results and Discussion

Method development for LC–FTIR

In order to develop a method that can be easily transferred to LC–FTIR, certain criteria must be met. Because the optimal flow rate for the FTIR detector is 0.1 mL/min, the flow from HPLC was split. Although a method developed at a low flow rate is preferred, a short analysis time is desired for the analysis of water-soluble vitamins. Therefore, a flow rate of 0.75 mL/min was used as a compromise.

Because FTIR detection is not as sensitive as UV, the standards were diluted to and run at concentrations of at least 1 mg/mL. However, the concentration of the riboflavin, which has lower solubility, was 0.37 mg/mL.

Higher resolution of the peaks is required with the FTIR detector than with the UV detector. First, because a higher concentration of samples must be used with the LC–FTIR, peaks may be coeluted or have poorer resolution than when lower concentrations are analyzed. Second, with the LC–FTIR the analytes are deposited on the plate and band broadening is common; thus the resolution must be as large as possible within a relatively short elution time.

Variations in column temperature affected the separation of pyridoxine and thiamine. Therefore, a column at 30ºC was used.

Mobile phase

Normally, acidic or neutral mobile phases are used to separate water-soluble vitamins. Because the mobile phase must be volatile in order to obtain IR spectra of pure solid-state analytes, a volatile mobile phase (such as ammonium formate or acetate) was used instead of the alkali phosphate, which is commonly used for the HPLC separations of the vitamins. To evaporate off the solvent easily, the maximum concentration of buffer was 0.02M. An acetate mobile phase was used when a pH higher than 4 was required. In addition to the formate or acetate, organic solvents such as methanol and acetonitrile were used. In FTIR spectra,

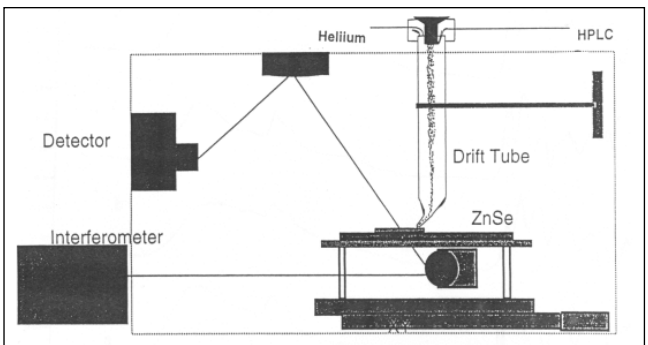

Figure 1. Schematic of HPLC–FTIR.

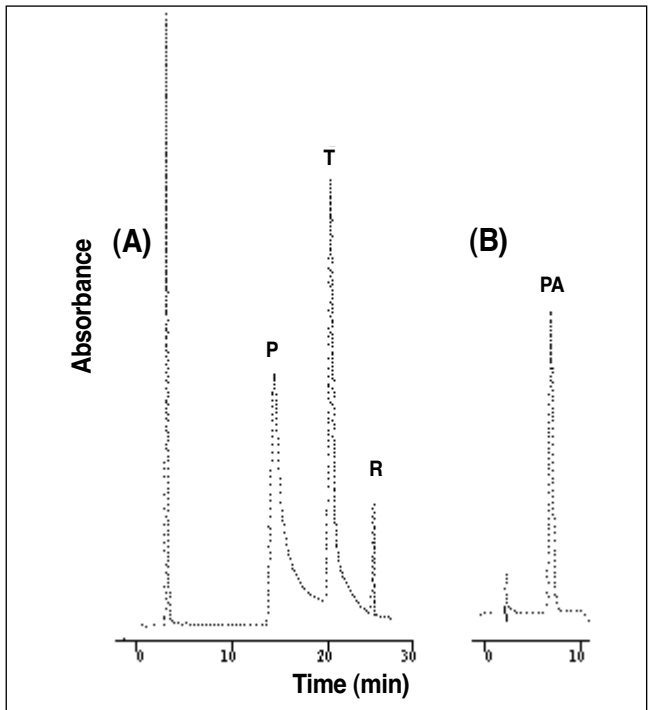

Figure 2. The separation of vitamin standard mixture at (A) λ = 254 nm and (B) λ = 210 nm for pantothenic acid (PA), pyricloxime (P), thiamine (T), and riboflavin (R).

HPLC-grade acetonitrile contained less contamination than methanol. However, methanol combined with the ammonium salt gave better separations of the vitamins of interest.

Mobile phases at pH of 2.5, 3.8, 4.8, 5.2, and 6.7 were investigated. The retention times (RT) of the standards increased with a higher pH in the mobile phase. The two compounds with the poorest resolution (pyridoxine and thiamine) were best separated at a pH of 6.7. There is another advantage to a pH of 6.7 in the mobile phase. The pantothenic acid has an amide group, which is only stable in neutral solutions; otherwise either acidic or basic hydrolysis may destroy this vitamin during the separation process.

The best separation was obtained when the mobile phase consisted of methanol and 0.01M ammonium acetate at pH 6.7. Riboflavin is the least polar compound in the vitamins of interest. Therefore, it was eluted last from the C18 column. To decrease total elution time, a gradient [consisting of 0–15 min, 85% 0.01M ammonium acetate (solvent A)–15% methanol (solvent B); 15–20 min, linear gradient from 85% solvent A to 50% solvent A; and 20–30 min, 50% solvent A–50% solvent B] was used.

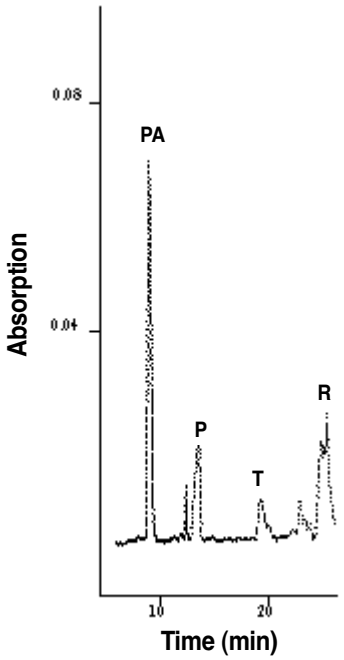

Figure 3. The band chromatogram of components in the vitamin standard mixture obtained using LC–FTIR at 1550 cm^{-1} for pantothenie acid (PA), pyridoxine (P), thiamine (T), and riboflavin (R).

Table I. Linearity, Regression of Calibration Graph, and LOD for the HPLC Analysis with UV Detector

	Repeatability RSD (%)	Linearity range (mg/mL)	R^2	LOD (mg/mL)
Pyridoxine (B_6)*	4.05	0.28–1.50	0.9988	0.056
Pantothenic acid (B_5)†	3.19	0.0041–2.59	0.9978	0.0033
Thiamine (B_1)*	6.69	0.0031–1.93	0.9983	0.0025
Riboflavin (B_2)*	1.25	0.00043–0.27	0.9985	0.00035

* The analyses was performed at λ = 254 nm.
† The analysis was performed at λ = 210 nm.

At a wavelength of 254 nm, pyridoxine was detected at 14.74 min, thiamine at 20.16 min, and riboflavin at 26.67 min (Figure 2A). Pantothenic acid, which was not detected at a wavelength of 254 nm was detected at a wavelength of 210 nm at 8.88 min (Figure 2B). The total elution time was 28 min.

LC–FTIR chromatogram

The standard mixture was analyzed by LC–FTIR with the optimized HPLC conditions. Figure 3 shows the band chromatogram generated at 1550 cm^{-1} for the vitamin standards. The elution order of the standards was pantothenic acid, pyridoxine, thiamine, and riboflavin. Because the vitamins were not deposited smoothly on the zinc selenide plate, the shapes of peaks were not as well-formed as the UV peaks. The reproducibility of peak area of these vitamins was poor. Therefore, quantitative measurements were not reliable. However, because the stream is split, the peaks in a sample are obtained by UV and FTIR simultaneously; thus the UV detector can be used for quantitation and the FTIR for the qualitative detection and identification of components in a mixture.

HPLC method validation

Using the UV detector, triplicate analyses of thiamine, pyridoxine, pantothenic acid, and riboflavin were performed. The relative standard deviations (RSD) obtained in the analysis of the vitamins are shown in Table I. Thiamine has the poorest reproducibility (6.69%) because it is more sensitive to heat during the analytical procedure.

Linearity was obtained for each vitamin. Standards were analyzed three times and the regression was calculated by the method of least-squares (Table I).

The limits of detection (LOD) were obtained by repeated injection of diluted solutions at a signal-to-noise ratio of 3. The results are shown in Table I. Riboflavin had the lowest LOD (3.5×10^{-4} mg/mL), and pyridoxine had the highest LOD(5.6×10^{-2} mg/mL).

Conclusion

An HPLC–UV method that could be adapted for use with an FTIR detector was optimized for the separation of the water-soluble vitamins thiamine, riboflavin, pyridoxine, and pantothenic acid. A gradient elution method using a mobile phase of methanol and 0.01M ammonium acetate with a C18 column gave the best separations. The overall RT was 28 min. Constant temperature of 30°C was necessary to obtain reproducible separations. The method was validated and the linearity, reproducibility, and LOD were obtained. This method was successfully used with an FTIR detector. In the interface of this detector, the HPLC solvent was evaporated and the FTIR spectra of the vitamins were obtained as solids. Band chromatograms were generated as well as IR spectra of the vitamins. The analytes were easy to recognize based on the IR spectra. Therefore, this LC–FTIR method could be applied to different food or biological samples to detect the vitamins of interest.

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