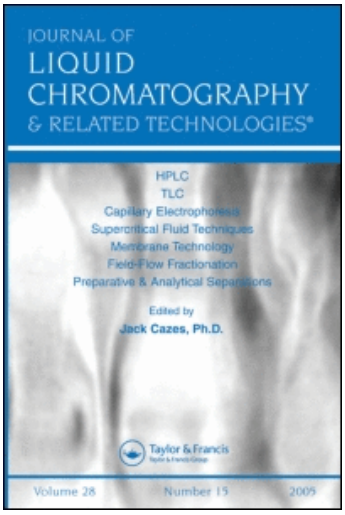

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Ying Li[a]; Phyllis R. Brown[a]
[a] Department of Chemistry, University of Rhode Island, Kingston, Rhode Island, USA

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On-Line Detection for the HPLC Analysis of Water-Soluble Vitamins in Multivitamin Tablets

Ying Li and Phyllis R. Brown*

Department of Chemistry, University of Rhode Island, Kingston, Rhode Island, USA

ABSTRACT

Four water-soluble vitamins, thiamine (B_1), riboflavin (B_2), pantothenic acid (B_5), and pyridoxine (B_6), frequently found in multivitamin tablets were analyzed by LC-FTIR. The samples were run on a C18 column using gradient elution with a mobile phase of 0.01 M ammonium acetate and methanol. The separation was carried out on a LC-FTIR system with a solvent elimination mode. The interface between high performance liquid chromatography (HPLC) and FTIR, which included an ultrasonic nebulizer and a heated drift tube removed the solvent from the HPLC effluent prior to FTIR detection. The analytes were deposited on a moving zinc selenide plate and FTIR spectra of the solid analytes were obtained. Spectral profiles of the vitamins of interest were constructed from the recorded standard spectra. The vitamins of interest in three multivitamin

*Correspondence: Phyllis R. Brown, Department of Chemistry, University of Rhode Island, Kingston, RI 02881, USA; E-mail: prbchrom@netscape.net.

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tablets were separated and identified, based on retention time and infrared spectral matching to those of standards.

INTRODUCTION

Currently, the reverse phase high performance liquid chromatography (HPLC) mode with a variety of detectors is being used for the analysis of water-soluble vitamins in food, biological samples, and pharmaceuticals.[1–6] Water-soluble vitamins are micronutrients, which are important for good health in humans.[7,8]

On-line FTIR detectors are now commercially available for HPLC,[9] providing detection and peak identification. The components separated by HPLC can be identified by specific structural characteristics and/or matched with standards or spectral libraries. Thus, identification and purity analysis can be obtained. Moreover, chemical changes of individual components can be monitored with high sensitivity by FTIR detection.

Once data are collected, spectral manipulations such as spectral subtraction, generation of band chromatograms, and sample rescanning may be performed to get better chromatographic resolution and clear FTIR spectra. In addition, thermal degradation can be monitored and isomers readily distinguished. LC-FTIR is suitable for pharmaceutical analyses[10] because it is non-destructive, and both sample recovery and purification are feasible. LC-FTIR can also aid in the structural elucidation of unknown components.

In this study, LC-FTIR was used for the analysis of the water-soluble vitamins thiamine (B_1), riboflavin (B_2), pantothenic acid (B_5), and pyridoxine (B_6). The deposition and spectral characteristics of each vitamin in a standard mixture were investigated. Finally, three different brands of pharmaceutical tablets were analyzed and the peaks in the FTIR spectra of the vitamins in these three multivitamin tablets were identified.

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) semi-calcium salt were obtained from Sigma Aldrich, Milwaukee, WI. Ammonium acetate, 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 Inc., Medford, Mass.

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Samples of pharmaceutical preparations were: Super B-Complex (Nature Made, Mission Hills, CA), B-100 ultra B-complex (Vitamin World, Oakdale, NY), and mega multivitamins (CVS Pharmacy, Woonsocket, RI). The pharmaceutical content of these three tablets are listed in Table 1.

LC-FTIR Instrumentation

The HPLC pump, a Constametric 4100, was from Thermo Separations (Austin, TX) and was used in conjunction with a LDC (Austin, TX) analytical membrane degasser. An Eppendorf CH-30 column heater (Brinkmann Instruments, Westbury, NY) was used to maintain column temperature. A Sonics Vibracell, (Sonic and Materials Inc, Newton, CT) provided ultrasonic nebulization. The interface from the HPLC to the FTIR and the detector were integral parts of the infrared chromatograph 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 from Rainin Instrument Company (Woburn, MA). The dimensions of the column were 250 mm × 4.6 mm (I.D.) with packing particle size of 5 μm.

Table 1. Pharmaceutical content of a table of each of the three multi-vitamin preparations used in this study.

Sample content	Super B-complex (Nature Made)	Vitamin world (B-100 ultra B-complex)	CVS natural (mega multivitamins)
Vitamin A			5,000 I.U.
Vitamin C	150 mg		300 mg
Vitamin D			400 I.U.
Vitamin E			150 I.U.
Vitamin K			40 mcg
Thiamine	100 mg	100 mg	50 mg
Riboflavin	20 mg	100 mg	50 mg
Niacin	25 mg	100 mg	50 mg
Vitamin B6	2 mg	100 mg	50 mg
Folate		400 mcg	400 mcg
Vitamin B12	15 mcg	100 mcg	50 mcg
Biotin		100 mcg	50 mcg
Pantothenic acid	5.5 mg	100 mg	50 mg
Dried yeasts	100 mg		
Liver concentrate	100 mg		

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LC-FTIR Operating Conditions

In the HPLC, a flow rate of 0.75 mL/min was used. The flow rate of the effluent to the FTIR was decreased to 0.13 mL/min. Column temperature was 30°C. The effluent was nebulized ultrasonically into a mist prior to the drift tube, at an amplitude of 40% of total nebulizer power. A pressure of 1 atm of helium was used. The temperature of the drift tube was 130°C. The scan rate of the infrared beam was 10 times per second with a resolution of 8 cm^{-1}. The data were co-added to produce an FTIR spectrum every 2 seconds.

Ultra-Violet Detection

The UV detector was a 3200 UV spectrometer (Thermo Separations, Austin, TX), which monitored the excess effluent from the HPLC column. A SP4270 Spectra-Physics integrator (Thermo Separations, Austin, TX) recorded the UV data. A wavelength of 254 nm was selected for monitoring riboflavin, pyridoxine and thiamine, and 210 nm for pantothenic acid because this vitamin does not absorb at 254 nm.

Preparation of Standard

All the glassware was meticulously cleaned. The vials containing solutions of standards and samples were tightly capped right after they were prepared and covered with aluminum foils. They were stored in the refrigerator immediately after use. New stock solutions were made every 2–3 days to prevent degradation.

Since riboflavin was less soluble than the other vitamins (0.07 mg/mL at 25°C),[12] it was heated in a water bath for 10 min at a temperature of 70°C. Dilutions of the standards were made using an Eppendorf (0–200 mL) automatic pipette. Standards were filtered through 0.45 μm filter paper (Gelman Inc., Medford, Mass). The structures of the vitamins of interest in this study are shown in Fig. 2.

Preparation of Sample

Different dilutions of the three vitamin samples were prepared in order to have the same concentration of the vitamins of interest. Super B-complex (Nature Made, Mission Hills, CA), sample 1, was prepared by dissolving 1 tablet in 50 mL double deionized distilled water. B-100 ultra B-complex (Vitamin World, Oakdale, NY) and mega multivitamins (CVS Pharmacy, Woonsocket, RI), sample 2 and sample 3, were prepared by dissolving one tablet into 100 mL double deionized distilled water. These sample solutions

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were then heated for 10 min at a temperature of 70°C and filtered through 0.45 μ filter paper. A fresh sample solution was made right before the analysis to prevent degradation.

High Performance Liquid Chromatography Methodology

The mobile phase composition was: 0–15 min, 85% 0.01 M ammonium acetate (solvent A), and 15% methanol (solvent B); 15–20 min, linear gradient from 85% solvent A to 50% solvent A; 20–30 min, 50% solvent A and 50% solvent B. The column was heated to the temperature of 30°C with a column heater.

RESULTS AND DISCUSSION

LC-FTIR

Two methods have been used to couple HPLC and FTIR.[11] The first method is to use a flow cell through which the column effluent passes and the FTIR spectra are continuously recorded. In the second method, the solvent is eliminated from the HPLC effluent prior to FTIR detection. The analytes are deposited on a zinc selenide plate and spectra of the solid analytes are obtained. In this study, the solvent elimination method was used.[9] The interface between the HPLC and the FTIR consists of an ultrasonic nebulizer and a heated drift tube. The effluent from the HPLC column is split into a UV detector and the FTIR detector (Fig. 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 left as a fine powder, which is deposited on a moving zinc selenide plate placed underneath the drift tube. The plate moves at a rate of 2 mm/min; consequently, each constituent separated by the HPLC occupies a different position on the plate. Meanwhile, the infrared beam scans the plate and displays the spectra in real time on the computer screen, along with the chromatogram. After the data are collected, data manipulation is performed. Chromatograms can be generated at a specific infrared band to get better resolution using Grams software. These chromatograms are referred to as band chromatograms. Spectral subtractions and rescanning at higher resolution can also be performed.

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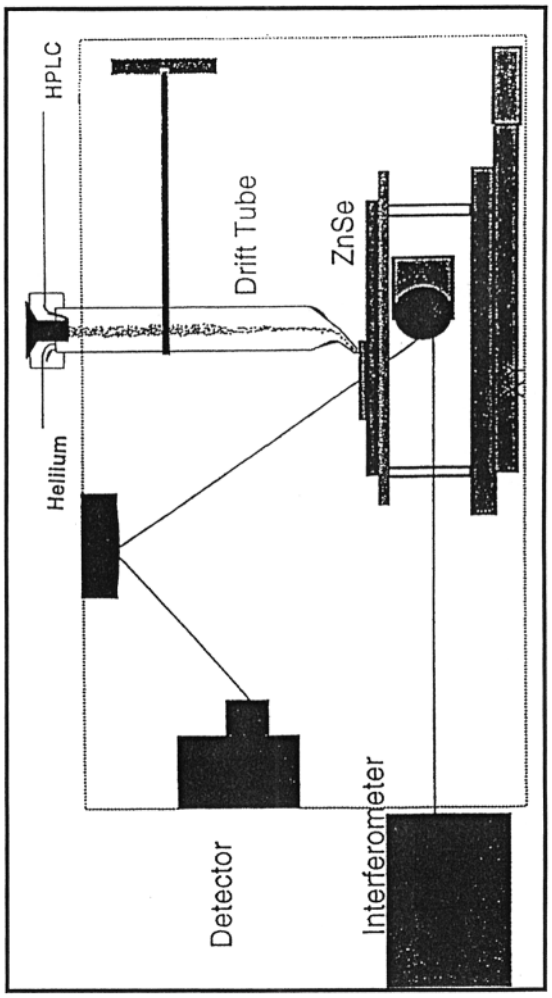

Figure 1. The schematic of LC-FTIR instrument.

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Deposition Characteristics of Water-Soluble Vitamins

The deposition of the analytes on the IR plate plays an important role in LC-FTIR. Only pantothenic acid, which deposited smoothly, had a uniform appearance. The other three vitamins did not stick on the plate firmly, and therefore, did not deposit smoothly. Some of the vitamins were lost in the vacuum chamber, and some vitamins adhered to the side of the drift tube. In order to minimize adhesion of the vitamins to the drift tube, the drift tube was rinsed with methanol before runs and a flow rate of 0.13 mL/min was used to increase the amount of analyte deposited.

LC-FTIR Chromatogram of Standard Mixture

Figure 3 shows the band chromatogram generated at 1550 cm^{-1} by LC-FTIR of the vitamins in the standard mixture. The elution order 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.

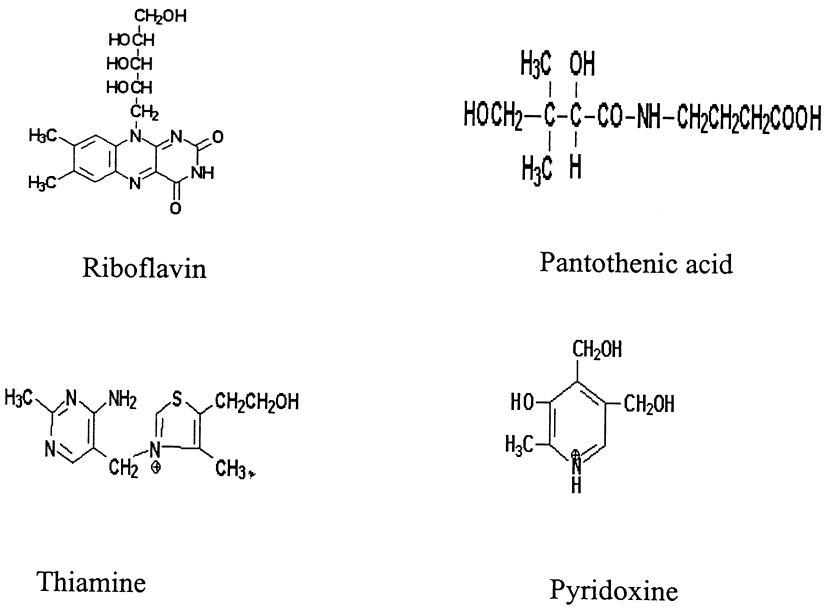

Figure 2. The structures of the vitamins of interest.

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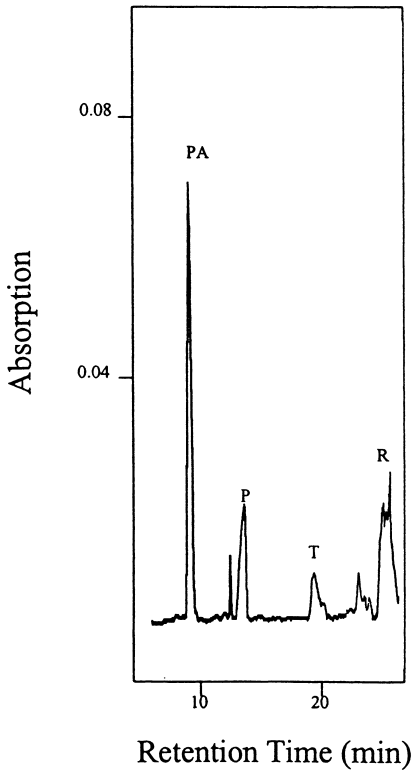

Figure 3. The band chromatogram obtained at $1550\,cm^{-1}$ of components in the vitamin standard mixture using LC–FTIR. *Key:* PA, pantothenic acid; P, pyridoxine; T, thiamine; and R, riboflavin.

Infrared Spectral Characteristics of the B Vitamins

Figure 4 shows the FTIR spectra of four vitamin standards obtained in this study. Figure 4(A) was the FTIR spectrum of pantothenic acid, Fig. 4(B) was the spectrum of riboflavin, Fig. 4(C) was the spectrum of pyridoxine, and Fig. 4(D) was the spectrum of thiamine. Each spectrum was unique because these vitamins have different structures. The spectra can be readily characterized according to their functional groups. Figure 5 shows the IR characteristics of a spectrum of a standard of pantothenic acid based on its structural characteristics. The solid peaks under the standard spectrum of pantothenic acid correspond to one specific functional group in the molecule.

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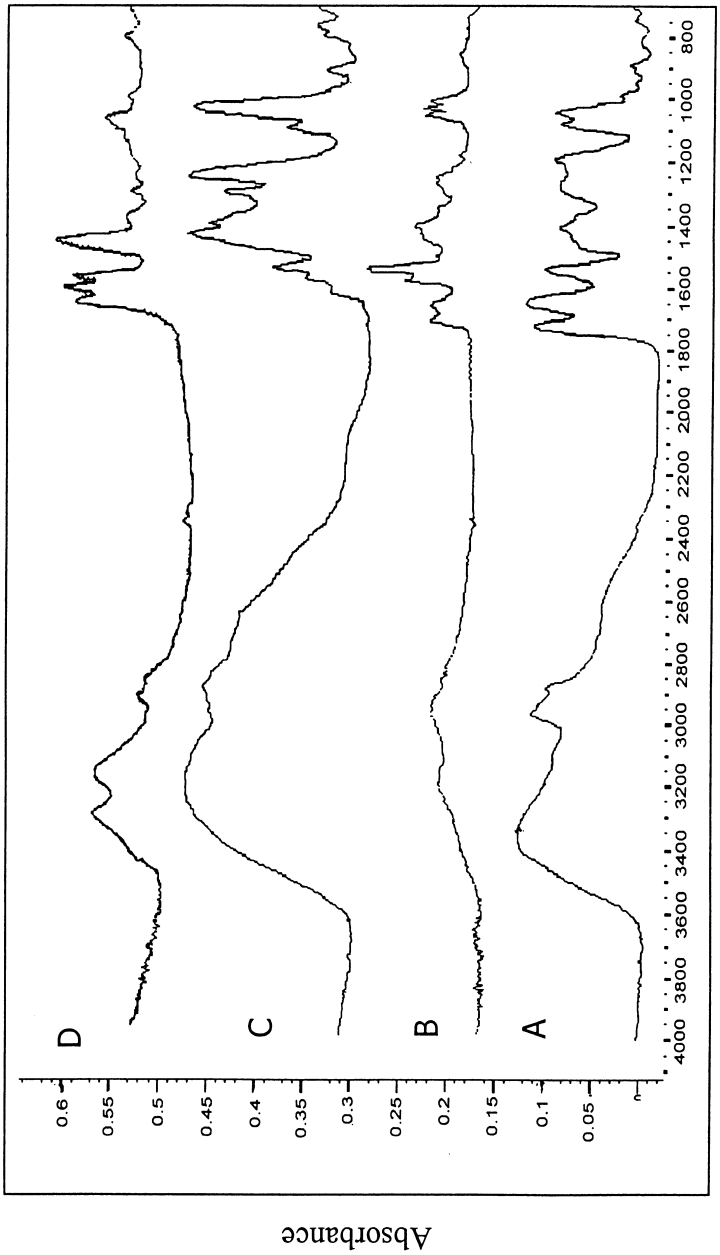

Figure 4. The IR spectra of vitamin standards obtained by LC-FTIR: (A) pantothenic acid; (B) riboflavin; (C) pyridoxine; and (D) thiamine.

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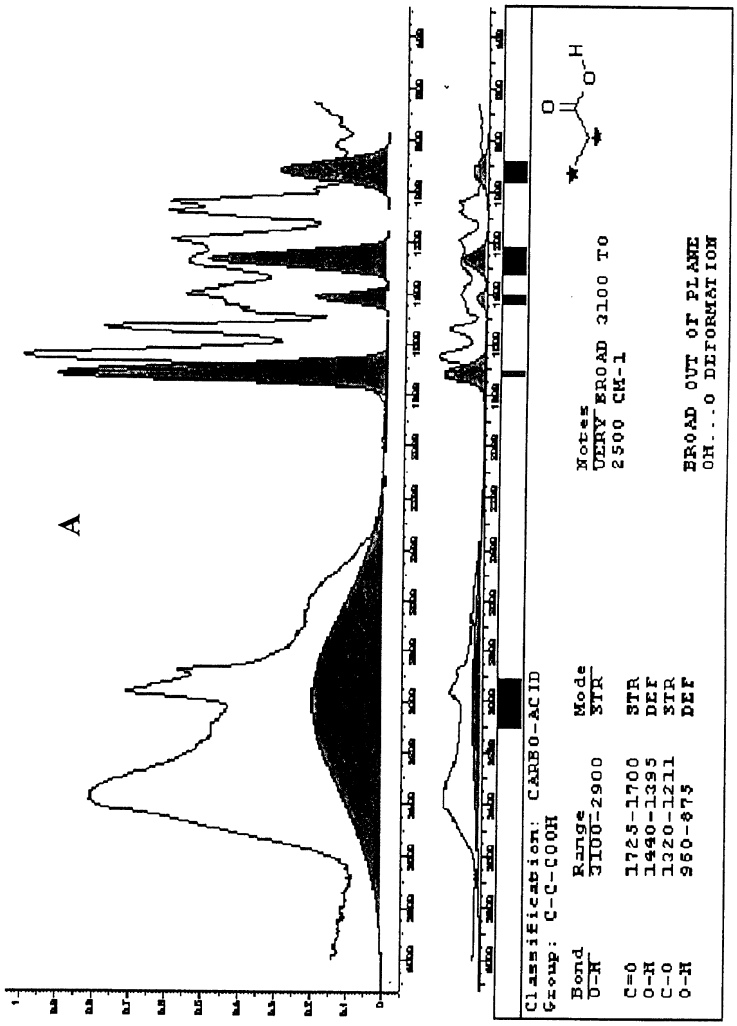

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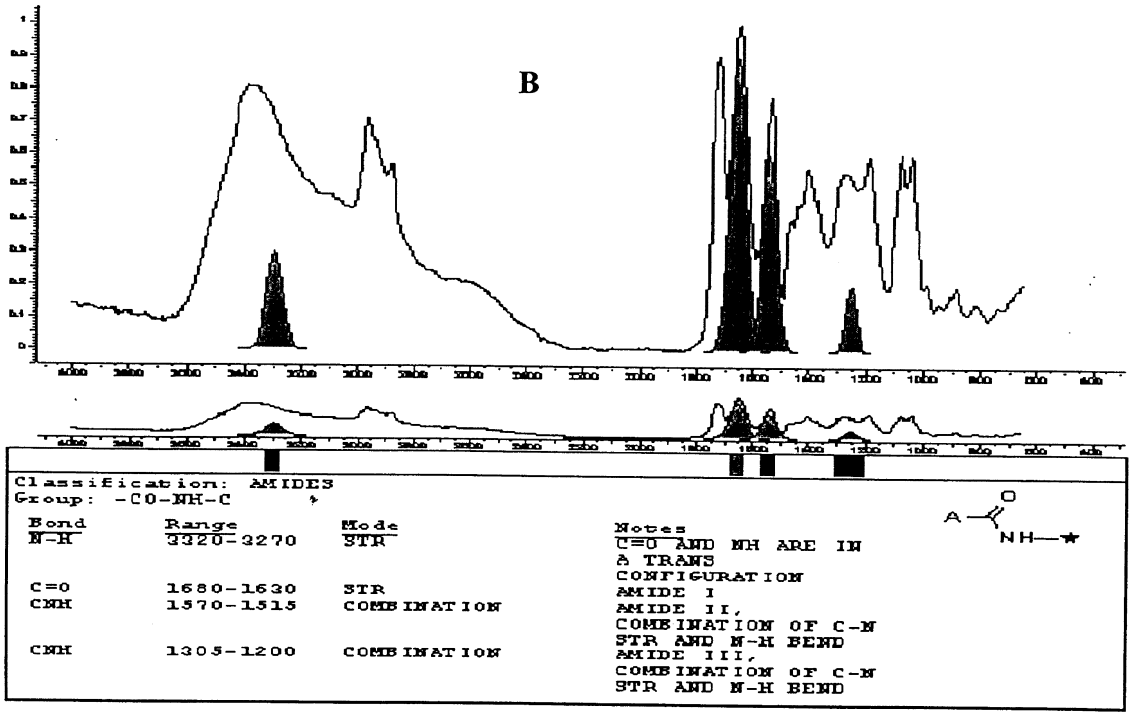

Figure 5. The sets of IR characteristic peaks of standard spectrum of pantothenic acid corresponding to: (A) carboxylic acid functional group; (B) amide group; (C) primary and secondary alcohol groups; and (D) alkane group. *Abbreviation:* STR, stretch; DEF, deformation; ASY, asymmetric, SYM, symmetric.

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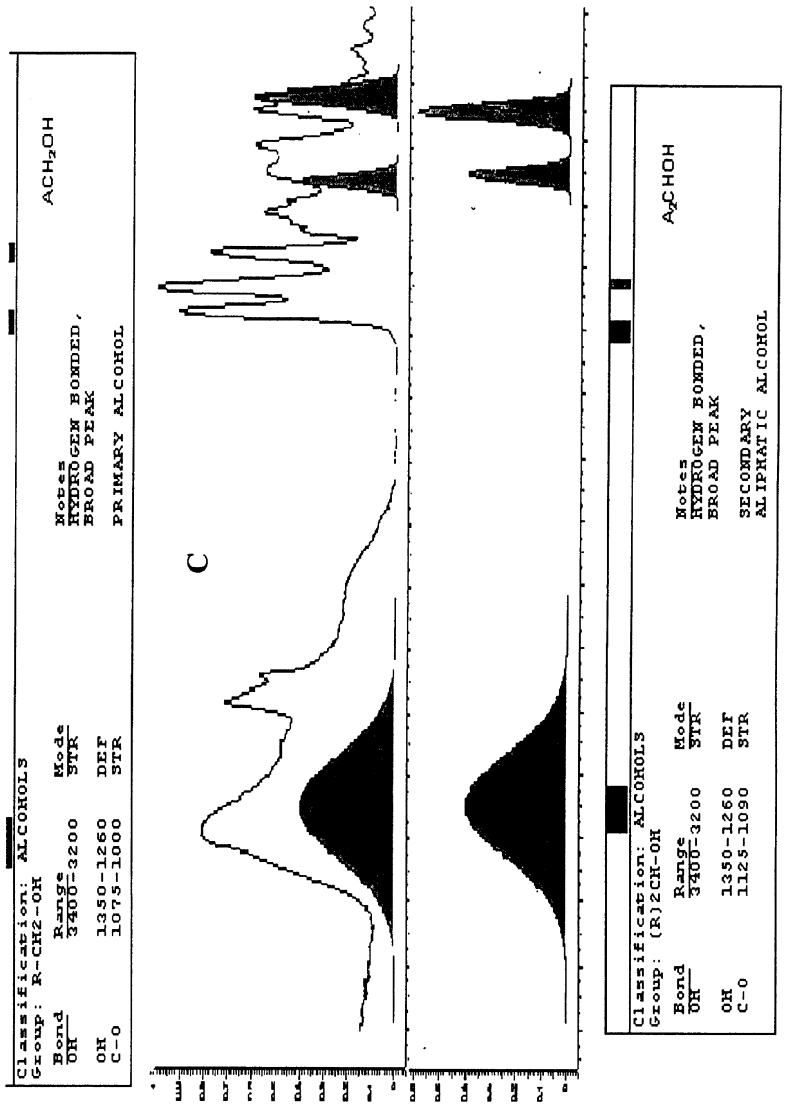

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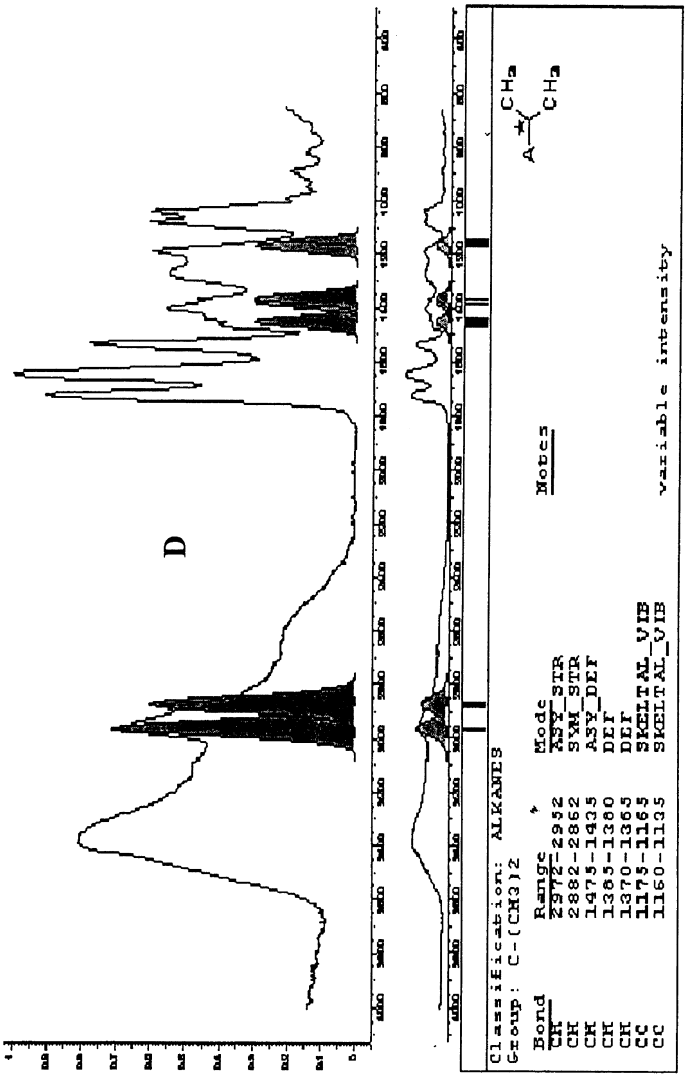

Figure 5. Continued.

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Figure 5(A) shows the vibration modes contributed by the carboxylic acid group in the pantothenic acid molecule. The sharp peak at 1710 cm^{-1} indicates the carbonyl stretch, and the stretch at 1260 cm^{-1} is from the C—O single bond. The very broad band from 3100 cm^{-1} to 2500 cm^{-1} is due to the stretch of O—H bond, which also gives a deformation vibration at 1410 cm^{-1}. Normally, carboxylic acid readily forms a hydrogen-bonded dimer in the condensed state. The characteristic "dimer band" is a strong O—H- - -O hydrogen bonding band deformation at 950 cm^{-1} [shown as the last solid peak in the Fig. 5(A)], which is not present in this spectrum of pantothenic acid. Thus, the dimer of carboxylic acid is not indicated. Instead, the O in the C═O group probably forms intramolecular hydrogen bonding with the—O—H in the same molecule.[13]

In the amide functional group, the C═O and NH are in a trans position; thus, in Fig. 5(B), at 1650 cm^{-1}, there is a strong stretch from the C═O bond, and at 1555 cm^{-1} there is a combination of a C—N stretch and N—H bend vibration from the CNH bond.

Figure 5(C) shows the IR peaks of pantothenic acid corresponding to the primary aliphatic alcohol (the top set) and secondary alcohol (the bottom set). Both alcohols give the broad bands centered at 3300 cm^{-1} from the stretches of hydrogen bonded O—H bonds, which also have the deformation absorption at 1300 cm^{-1}. The two connected peaks at 1100 cm^{-1} and 1030 cm^{-1} correspond to the stretches of C—O single bond in both alcohols: the left peak is from the secondary alcohol, and the right one from the primary alcohol.

Figure 5(D) shows the functional group of C—$(CH_3)_2$ alkane in the molecule. The asymmetric and symmetric stretches of the CH bond of the group are at 2962 cm^{-1} and 2872 cm^{-1}, respectively. From this functional group, the CH bond deformations are at 1450 cm^{-1} and 1380 cm^{-1}.

Multivitamin Tablets

Three different brands of multivitamin pharmaceuticals were analyzed by the LC–FTIR method. The chromatograms are shown in Fig. 6. In the super B-complex, the pyridoxine peak was not present in the chromatogram, indicating that the concentration of pyridoxine in this sample was too low to be detected. For the samples of B-100 ultra B-complex and Mega multivitamins, all four vitamins were detected [Fig. 6(B), (C)]. The latter is the most complicated of the three tablets, as is shown in its chromatogram [Fig. 6(C)]. Although, the retention time of chromatographic peaks were shifted from one sample to another, and there were also some extra peaks in each chromatogram, the chromatographic peaks of the vitamins of interest were readily identified by matching FTIR spectra obtained in the solution of the sample with the spectra of the standards.

Figure 7 shows the comparison of the FTIR spectra of the pantothenic acid (A), pyridoxine (B), thiamine (C), and riboflavin (D) standards to the spectra of

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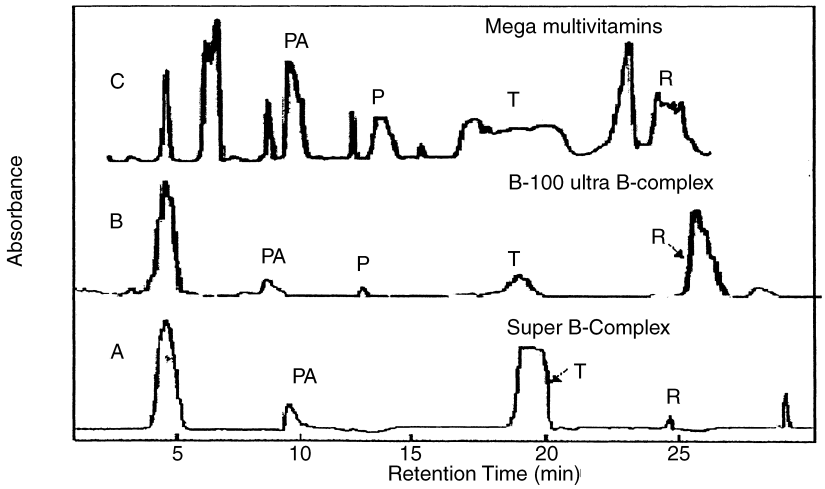

Figure 6. The chromatograms of components in the three samples obtained by LC–FTIR detection. (A) SuperB-Complex; (B) B-100 ultra B-complex; (C) Mega multivitamins. *Key:* PA, pantothenic acid; P, pyridoxine; T, thiamine; and R, riboflavin.

these vitamins in the three multivitamin samples. Although these pharmaceutical tablets have different compositions, there is a good match of the spectra of the components of interest in the samples to the spectra of the standards; thus, the matrix effects are minimal in the analysis of these vitamins by LC–FTIR.

CONCLUSIONS

LC–FTIR is a good technique for the analysis of the water-soluble vitamins thiamine (B_1), riboflavin (B_2), pantothenic acid (B_5), and pyridoxine (B_6) in pharmaceutical tablets. These vitamins were separated by the HPLC method modified for the FTIR detector. The chromatographic peaks were readily identified by specific structural characteristics and comparison of their FTIR spectra to the corresponding spectra of standards of these vitamins. Since the spectra of the standards and samples matched well, the matrix effect in the pharmaceutical tablets were negligible. Therefore, the LC–FTIR has a potential for analyzing other water-soluble vitamins in various matrices. In addition, FTIR spectra obtained in LC–FTIR could be characterized by their functional groups; thus, it is feasible to utilize LC–FTIR to obtain structural information of unknown compounds. Furthermore, it is possible to use the

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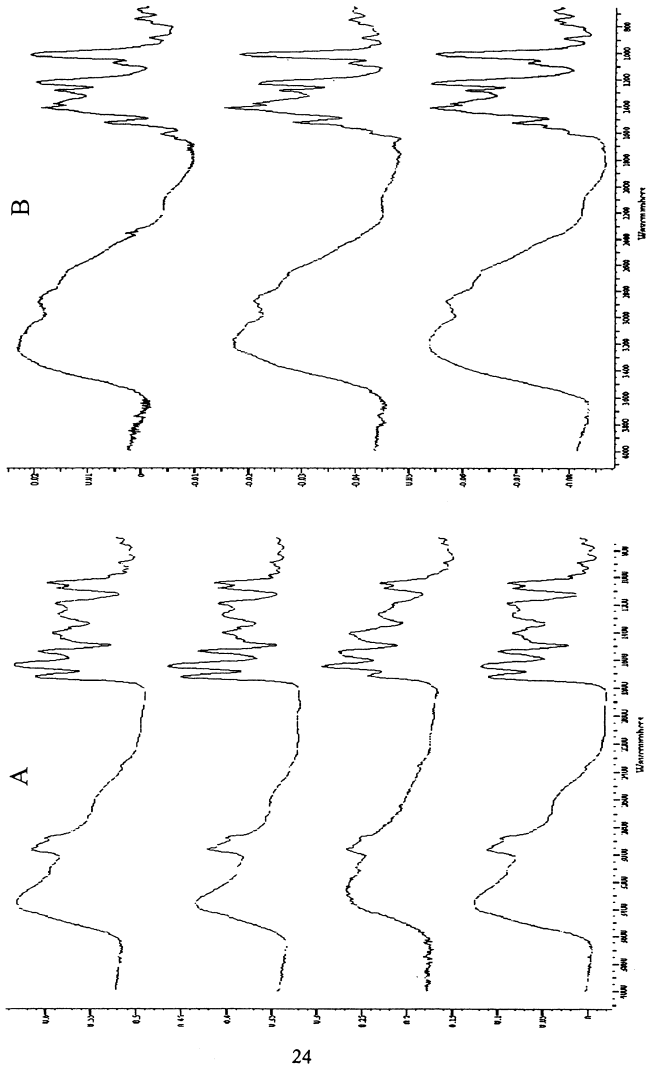

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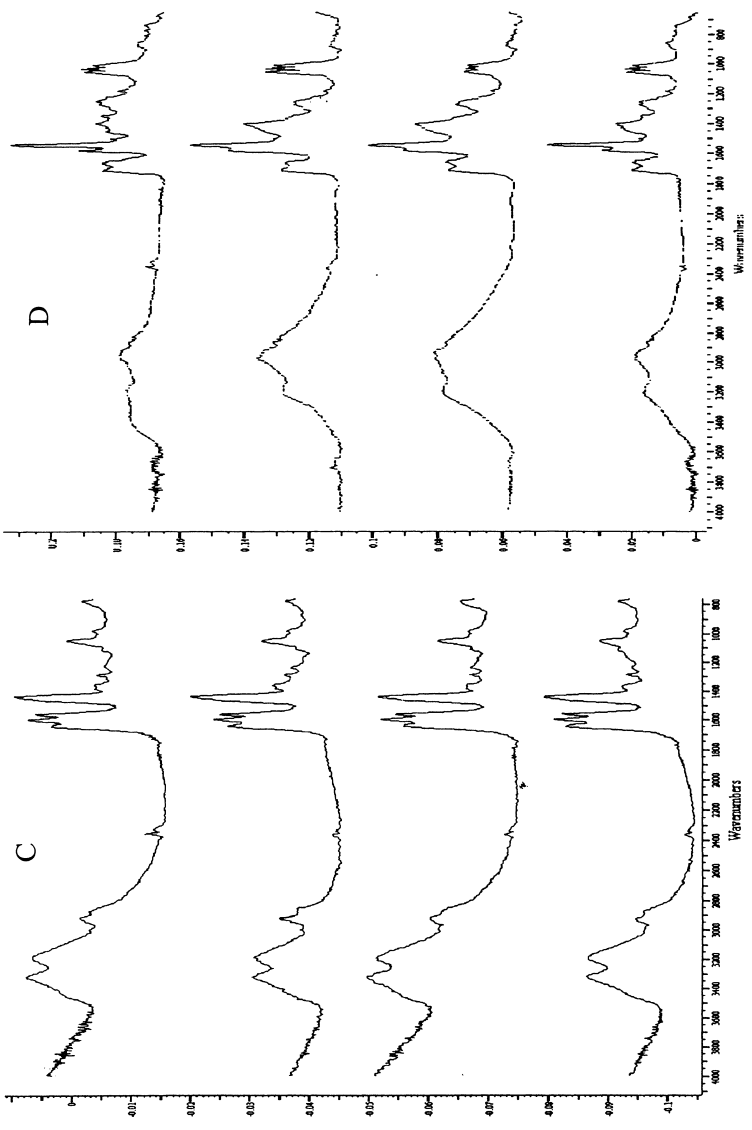

Figure 7. The spectral comparison of (A) pantothenic acid; (B) pyridoxine; (C) thiamine; (D) riboflavin. S: standard; S1: super B-complex; S2: B-100 ultra B-complex; S3: mega multivitamins.

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FTIR detector to monitor the purity and chemical changes of water-soluble vitamins.

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