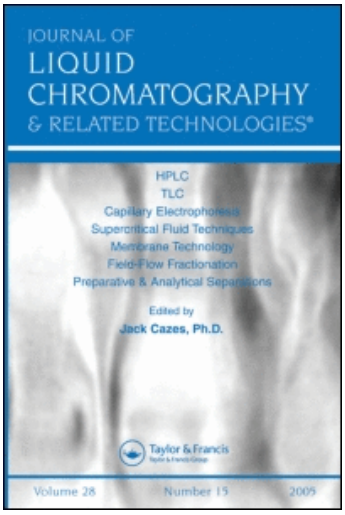

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Separation and Identification of Eight Hydrophilic Vitamins Using a New TLC Method and Raman Spectroscopy

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Abstract: Hydrophilic vitamins play an important role in human health. The analysis of these compounds is indispensable for monitoring their content in pharmaceuticals and food in order to prevent some human diseases. The separation and identification of eight hydrophilic vitamins, i.e., B_1, B_2, B_3, B_5, B_6, B_9, B_{12}, and C, was achieved by thin layer chromatography and Raman spectrometry. After chromatographic experiments, the best results were obtained using programmed multiple development. The unambiguous identification of superimposed spots can be done without using standards of vitamins.

Keywords: Hydrophilic vitamins, TLC, Programmed multiple development, Raman spectroscopy

INTRODUCTION

Vitamins are biologically active organic compounds that are essential nutrients and fundamental for normal health and growth of organisms. They are available in the diet and nutrition supplements. A deficiency of these vitamins in the organism can lead to some human diseases, such as beri-beri, anemia, chellosis, etc.

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Vitamins are ranked in two groups according to their solubility in water and in fats. One group is the lipophilic vitamins, including vitamins A, D, E, and K; excesses of these are stored in the organism for later use. The second group is the hydrophilic vitamins, including vitamin C and B complex vitamins. They pass through the body more quickly than the lipophilic vitamins by circulating in the bloodstream and being excreted in the urine.

The monitoring of hydrophilic vitamins could be considered more important than the lipophilic vitamins because the body does not store them. Therefore, a common method for analyzing all hydrophilic vitamins seems to be necessary, but such a method did not exist until now. Many books and papers report the analysis either of an individual vitamin and its metabolites[1–7] or the analysis of up to five vitamins.[8] Only one paper reports a high performance liquid chromatography (HPLC) method for determination of seven B vitamins.[9]

During recent years, thin-layer chromatography (TLC) was developed as an instrumental technique for identification and determination of compounds in complex mixtures. More characteristic information on the compounds is obtained by coupling TLC with more selective detection techniques, such as Raman spectrometry, infrared spectrometry (IR), or mass spectrometry (MS).[10,11]

Identification of the separated components of a mixture by purely chromatographic characteristics cannot be done unequivocally, thus the demand for spectroscopic fingerprinting in order to increase the reliability of the analysis is highly justified. The vibrational spectrometric techniques provide a useful alternative for compounds separated by TLC.[12] Thus, Raman spectrometry represents one of the most useful techniques for obtaining information about the structure and properties of molecules from their vibrational properties, despite the fact that the direct assignment of the Raman bands of relatively complex molecules is complicated. Raman spectrometry is preferred because TLC sorbents are weak Raman scatters. In the case of IR spectrometry, the sorbent causes strong absorption that can obscure the spectral bands of compounds.[13]

This paper reports the attempt to separate the hydrophilic vitamins by high performance thin layer chromatography (HPTLC) coupled with Raman spectrometry in order to obtain a suitable method for identification of these compounds from different samples.

EXPERIMENTAL

Materials

The solutions of hydrophilic vitamins were prepared in methanol. All solvents were of analytical grade and were obtained from "Reactivul" (Bucharest, Romania). Chromatography was performed on $20 \times 20\,\text{cm}$

plastic-backed HPTLC sheets precoated with silica gel $60\,F_{254}$ (Merck, Darmstadt, Germany).

Chromatography

The solutions (0.2 μL) of mixtures were applied as spots to the plates using a micropipette. The plates were developed at room temperature in a saturated N-chamber by the ascending technique. The development distance was about 180 mm. When programmed multiple development was used, the plate was dried after each development before the next elution. The mobile phases were mixtures of methanol and benzene. The detection was made under UV light at 254 nm, except for vitamin B_5 for which spraying with ninhydrin reagent (2% in ethanol) was used.

Raman Spectroscopy

The Raman spectra of powdered vitamins and of vitamins on a support were recorded on a Bruker (Ettlingen, Germany) EQUINOX 55 spectrometer IFS 66v interferometer equipped with a FT Raman FRA 106 module and OPUS software. The samples were excited by the 1064 nm line of a Nd:YAG laser operating at 380 mW. A total of 500 scans were accumulated for each spectrum. Spectra were recorded at a nominal resolution of $4\,cm^{-1}$.

RESULTS AND DISCUSSION

The analyzed compounds were the hydrophilic vitamins B_1—thiamin, B_2—riboflavin, B_3—nicotinic acid, B_5—pantothenic acid (as the calcium salt), B_6—pyridoxine, B_9—folic acid, B_{12}—cyanocobalamin, and C—ascorbic acid. The chemical structures of these vitamins are presented in Figure 1.

TLC studies were made to find the most effective separation in order to obtain a suitable method for analyses of the hydrophilic vitamins. In the first step, the individual solvents were chosen after preliminary TLC experiments. The preferred solvent was methanol, but the compounds eluted with this mobile phase migrated in the upper part of the plate, except vitamins B_1 and B_{12} (Table 1). For this reason, the solvent strength of methanol was reduced by dilution with benzene, which does not move the components from start (Table 1). In the second step, mixtures of methanol and benzene were tested in order to establish the proper composition of the mobile phase. As it can be seen from Table 1, the separation of all vitamins was not obtained with any combination of solvents. Moreover, the results show that decreasing the methanol proportion or increasing the benzene proportion in the mobile phase did not influence, in the same manner, the retention (R_f) of

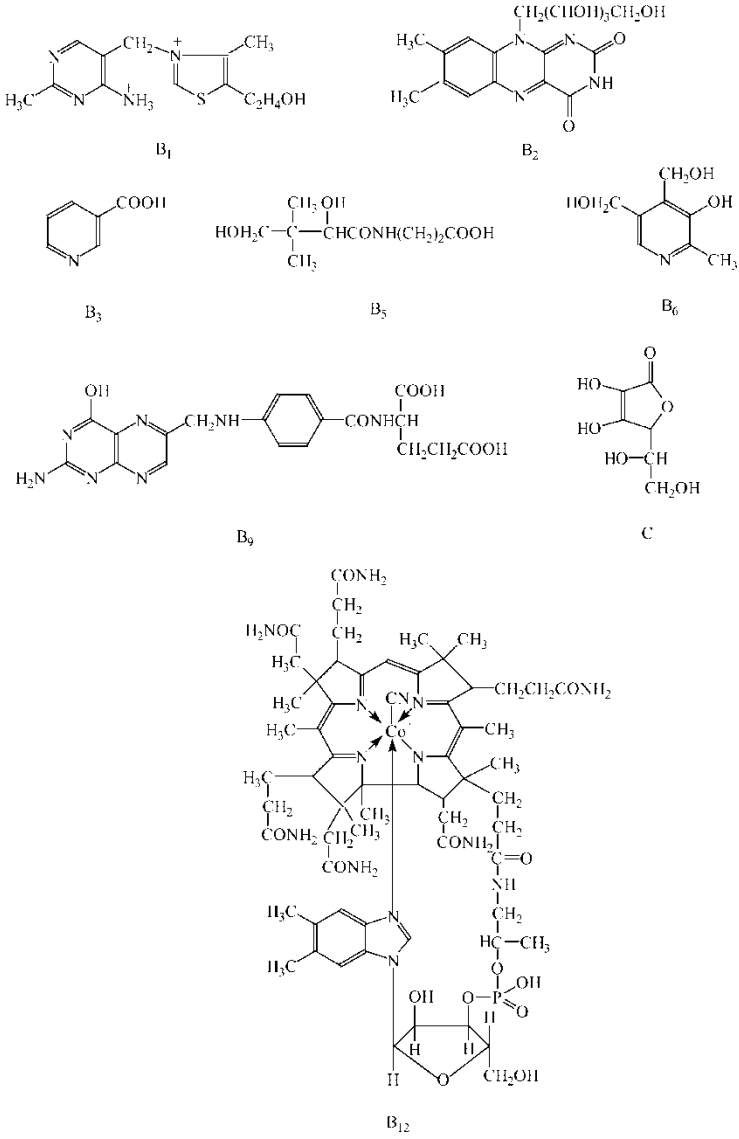

Figure 1. The structures of analyzed hydrophilic vitamins.

individual compounds. In the third step, programmed multiple development was tried in order to optimize the vitamin separation. The solvent composition and the development distance for each step were changed, as presented in Table 2. It can be seen from the experimental results shown in Table 3, that vitamins B_3, B_6, B_9, and C are not separated in the first and second experiment. It is known that two compounds are separated if the difference between their

Table 1. The experimental value of R_f obtained with different compositions of mobile phase (methanol : benzene)

	R_f						
Vitamin	100 : 0	0 : 100	50 : 50	80 : 20	20 : 80	65 : 35	35 : 65
B_1	0.115	0.000	0.041	0.103	0.000	0.072	0.022
B_2	0.892	0.000	0.498	0.829	0.167	0.729	0.221
B_3	0.906	0.000	0.360	0.688	0.136	0.566	0.189
B_5	0.777	0.000	0.365	0.680	0.057	0.571	0.303
B_6	0.766	0.000	0.660	0.719	0.286	0.698	0.478
B_9	0.913	0.000	0.135	0.890	0.000	0.861	0.461
B_{12}	0.312	0.000	0.000	0.122	0.000	0.057	0.026
C	0.699	0.000	0.214	0.628	0.000	0.487	0.110

retention parameters is at least 0.030 ($\Delta R_f \geq 0.030$). As is seen from Table 3, the vitamins are separated in the third experiment, except for B_3 and B_6. The spots of these two vitamins are overlapping ($\Delta R_f \geq 0.028$), and in the case of real samples it can not be always observed if it is one or two spots.

In order to solve this problem, Raman spectrometry of these compounds was applied. This spectroscopic technique was chosen because each molecular species has its own unique set of molecular vibrations, and the Raman spectrum of a particular species will consist of a series of peaks or bands, each shifted by one of the characteristic vibrational frequencies of that molecule. Thus, mixtures of different chemical compounds or mixtures of a drug substance can give characteristic Raman features ("fingerprint"), allowing determination of the nature of each component of a substance from which the molecular composition can be determined.

Table 2. Composition of mobile phases used in programmed multiple development

Experiment I		Experiment II		Experiment III	
Distance (cm)	CH_3OH : C_6H_6 (v/v)	Distance (cm)	CH_3OH : C_6H_6 (v/v)	Distance (cm)	CH_3OH : C_6H_6 (v/v)
0–4	100 : 0	0–3	20 : 80	0–3	100 : 0
0–6	100 : 0	0–6	20 : 80	0–6	80 : 20
0–8	95 : 5	0–9	40 : 60	0–9	60 : 40
0–10	90 : 10	0–12	60 : 40	0–12	40 : 60
0–12	80 : 20	0–15	80 : 20	0–15	20 : 80
0–14	70 : 30	0–18	100 : 0	0–18	20 : 80
0–16	55 : 45				
0–18	40 : 60				

Table 3. The experimental value of R_f obtained with programmed multiple development

	R_f		
Vitamin	Experiment I	Experiment II	Experiment III
B_1	0.039	0.019	0.065
B_2	0.912	0.915	0.688
B_3	0.796	0.885	0.539
B_5	0.231	0.166	0.488
B_6	0.864	0.885	0.567
B_9	0.836	0.845	0.593
B_{12}	0.681	0.316	0.102
C	0.813	0.842	0.635

In order to identify a certain substance on the chromatographic plate, one needs to know the spectral changes brought about by the interactions and the characteristic bands of each chemical compound.[14] For this reason, the Raman spectra of vitamin B_3 and vitamin B_6 were recorded both in the pure solid state (Figure 2) and on the chromatographic plate (Figure 3). It can be

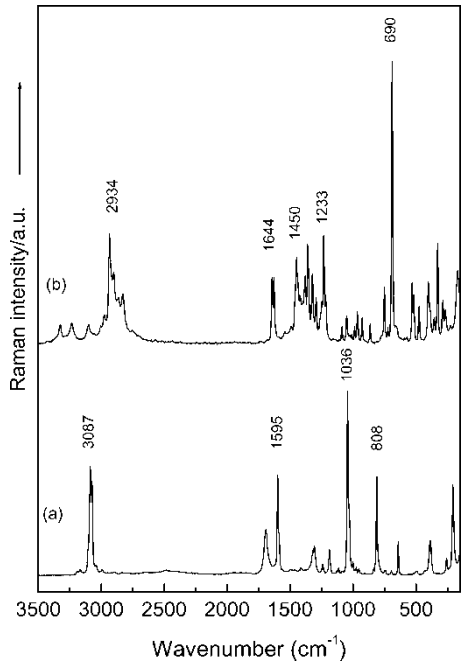

Figure 2. The Raman spectra at 25°C of (a) vitamin B_3 and (b) vitamin B_6 in the pure solid state.

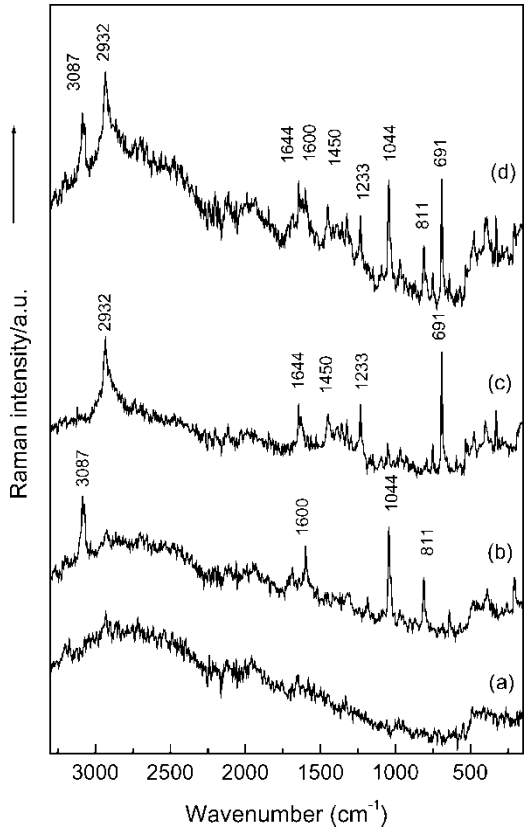

Figure 3. The Raman spectra at 25°C of studied samples (a) chromatographic layer (silica gel), (b) vitamin B_3 on layer, (c) vitamin B_6 on layer, and (d) mixed vitamins B_3 and B_6 on layer.

seen from Figures 2 and 3, that there are no major differences between Raman spectra of pure compounds and Raman spectra of the same compounds adsorbed on silica gel, so the Raman spectra of pure solid compounds can be used for the identification of these vitamins.

The existence of one or more aromatic rings in a structure is normally readily determined from the C–H out of plane bending ring breathing and C═C–C ring-related vibrations. It can be seen from the Raman spectra that these bands were observed at 811 cm^{-1} for vitamin B_3 and at 691 cm^{-1} for pyridoxine. The band at 1044 cm^{-1} in the nicotinic acid, attributed to C–C–C trigonal ring breathing, is more intense than in pyridoxine. The C–H stretching occuring around 3000 cm^{-1} is defined by the number and positions of the C–H bonds around the ring, which in turn are related to the nature and number of other substituents on that ring. Thus, this band is observed at 3087 cm^{-1} for vitamin B_3 and at 2932 cm^{-1} for vitamin B_6.

These bands can be used to identify the existence of vitamins B_3 and B_6 in mixtures. The other set of bands, like the CNC in-plane bending vibration observed at $382\,cm^{-1}$ and $328\,cm^{-1}$ or the C=C stretching at $1600\,cm^{-1}$ and $1644\,cm^{-1}$ for nicotinic acid and pyridoxine, respectively, cannot be used for identification, due to the relatively small difference between the vibration frequencies.

The results demonstrated that this new TLC method can achieve good separation of eight hydrophilic vitamins and can be used for monitoring the hydrophilic vitamins content of real samples. In addition, by coupling the chromatographic method with Raman spectrometry, the uncertainty of compound identification from touching or superimposed spots can be elucidated. Moreover, it is not necessary to use standards of vitamins because identification can be achieved on the basis of Raman spectra of pure compounds, which are found in the spectral library. In the future, research will be focused on quantitative determination by the coupling of HPTLC with Raman spectroscopy.

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