New Column Packing Materials for Separation of Water-Soluble Vitamins by High-Performance Liquid Chromatography

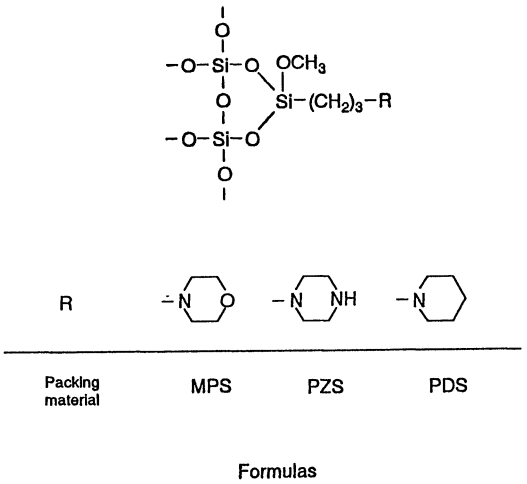
Shuzo AKIYAMA,* Kenichiro NAKASHIMA, Nanako SHIRAKAWA, and Kyoko YAMADA
School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852
(Received April 26, 1990)

Three types of high-performance liquid chromatographic column packing materials ((3-morpholinopropyl)-, [3-(1-piperazinyl)propyl]-, and (3-piperidinopropyl)silyl silica gels) were newly prepared and examined for their ability to separate water-soluble vitamins. Six vitamins (ascorbic acid, nicotinic acid, thiamine hydrochloride, pyridoxine hydrochloride, hydroxocobalamin acetate, and flavin adenine dinucleotide) in sample solutions could be completely separated within less than 12 min by UV detection on a column packed with morpholinopropylsilyl silica gel by using a phosphate buffer [0.1 M (1 M=1 mol dm^{-3}) H_3PO_4–0.1 M Na_2HPO_4 (pH 6.0) containing 10 mM tetrabutylammonium bromide] as an eluent. This efficient gel was successfully applied to vitamin analyses of commercially available healthful beverages. The other column packing materials also showed characteristic for specific water-soluble vitamin analysis. Typical separations are presented.

Reversed-phase chromatography using, typically, an octadecylsilylated silica gel (ODS) column is now widely applied to various fields and continues to dominate all other high-performance liquid chromatographic (HPLC) modes;[1] in parallel, over the past several years much effort has aimed toward increasing the efficiency of HPLC. Recent trends have been directed toward the development of specialty phases for the realization of selectivity e.g., the direct optical resolution of enantiomeric carboxylic acids[2] or aliphatic alcohols.[3]

In this paper we report on the development of new HPLC column packing materials for separation of water-soluble vitamins. Quite recent HPLC methods for the determination of water-soluble vitamins are mainly reported regarding analyses of various pharmaceutical preparations,[4–9] foods,[10–12] and biomedical samples.[13,14]

We have found that newly developed silica gels (MPS, PZS, and PDS) for HPLC having a (3-morpholinopropyl)-, [3-(1-piperazinyl)propyl]-, or (3-piperidinopropyl)-silyl group (as shown in Formulas) are suitable for the simultaneous separation of several vitamin components in a single analysis by a single buffer elution with UV detection, in addition to shortening the analysis time. The preparation of these stationary phases is easy and the mobile phase used in the analyses is consistently water [a phosphate buffer containing an ion-pair reagent (pH 5—6)]. These columns have a reversed-phase type character accompanying on ion-exchange behavior like a zwitterionic stationary phase. The aim of this study was to demonstrate the usefulness of the columns and their applicability to water-soluble vitamin analysis.



Formulas

Experimental

Materials. Silica gel for HPLC, Daiso gel SP120 (particle size 5 μm, spherical; nominal pore diameter, 146.7 Å; specific surface, 284 m^2 g^{-1}; pore volume, 1.0426 mL g^{-1}) was supplied from Daiso Co., Ltd. (3-Morpholinopropyl)-, [3-(1-piperazinyl)propyl]-, and (3-piperidinopropyl)trimethoxysilane were purchased from Shin-Etsu Chemical Industry Co., Ltd. All other reagents (Wako Pure Chemicals Co., Ltd.) used were of analytical grade.

Apparatus. The chromatography system comprised a TOSOH CCPD pump with a Rheodyne-type injector with a 100 μL loop, a Hitachi 638 variable-wavelength UV monitor and a Hitachi Model 561 recorder. The eluates were monitored at 270 nm.

General Synthesis of Bonded Phases. Chemically bonded stationary phases were prepared in the following manner: silica gel (10.0 g) was suspended in toluene (300 mL) and the mixture was refluxed for 24 h to remove water. In the case of MPS as a representative, trimethoxy-(3-morpholinopropyl)silane (12 g) was added to the reaction vessel and the mixture was refluxed for 24 h. After the treatment, the chemically bonded gel was collected and then rinsed with toluene, acetone, and ether, successively; it was then dried under reduced pressure at 100 °C for 4 h. On the other hand, trimethylsilylation to MPS by treatment with chlorotrimethylsilane (3.76 g) and hexamethyldisilazane (5.43 g) was carried out in situ to remove as many of the residual free silanol functions as possible (so-called end-capping). The end-capped MPS was washed with toluene, acetone, water, acetone, and ether, successively, and then

dried in vacuo at 80 °C.

Elemental analyses data of MPS, PZS, PDS, and end-capped MPS and grafted values (*G*) estimated from the value of nitrogen analysis were as follows: MPS, Found: C, 7.89; H, 1.54; N, 1.20%; *G*, 0.86 mmol g^{-1}; PZS, C, 7.92; H, 1.64; N, 2.42%; *G*, 0.86 mmol g^{-1}; PDS, C, 8.47; H, 1.68, N, 1.19%; *G*, 0.86 mmol g^{-1}; end-capped MPD, C, 8.50; H, 1.65; N, 1.36%.

Preparation of Column for HPLC. The gels (MPS, PZS, PDS, and end-capped MPS) were packed into (6.0 mm i.d.×150 mm) stainless-steel tubes by a slurry-packing technique [glycerole: methanol, 4:6 (v/v)] and applied to the separation of water-soluble vitamins at a flow rate of 1 mL min^{-1}.

Preparation of Solution of Water-Soluble Vitamins. Aqueous solutions of the vitamins were prepared daily under light shielding; aliquots (100 μL) were injected onto the HPLC.

Results and Discussion

Evaluation of MPS. As a preliminary experiment, a phosphate buffer [0.1 M (1 M=1 mol dm^{-3}) H_3PO_4-0.1 M Na_2HPO_4 (pH 6.0)] was used to separate six water-soluble vitamins (ascorbic acid (AA), thiamine hydrochloride (TA), nicotinic acid (NA), hydroxocobalamin acetate (HC), pyridoxine hydrochloride (P), flavin adenine dinucleotide (FAD)), by MPS. Consequently, the phosphate buffer was found to be suitable since some of the vitamins showed good separations from each other. For the separation of ionic compounds, ion-pair reagents have often been used. Thus, tetrabutylammonium bromide (Bu_4NBr) and sodium 1-octanesulfonate were tried for the separation of vitamins. Here, two eluent systems ((1), 0.1 M H_3PO_4-0.1 M Na_2HPO_4 containing 10 mM Bu_4NBr and (2), 0.1 M H_3PO_4-0.1 M Na_2HPO_4 containing 10 mM sodium 1-octanesulfonate) were selected and examined in order to determine the degree of separation of the vitamin components on the column. First of all, the effect of the eluent pH was examined (Fig. 1). From Fig. 1 is apparent that the pH greatly influences the retention times of TA, HC, and FAD, but not AA, NA, and P. pH values of about 6 and 4 were found to be optimum in the separation of these vitamins on the column. The effects of the concentration of Bu_4NBr and sodium 1-octanesulfonate at pH 6 and pH 4, respectively, were examined within the range 2—30 mM. The best separation was obtained at 10 mM in the both cases. Under the above HPLC conditions, it was demonstrated that the six water-soluble vitamins (AA, P, NA, FAD, HC, and TA) can be separated within 12 min in a single analysis [Fig. 2(a) and (b)].

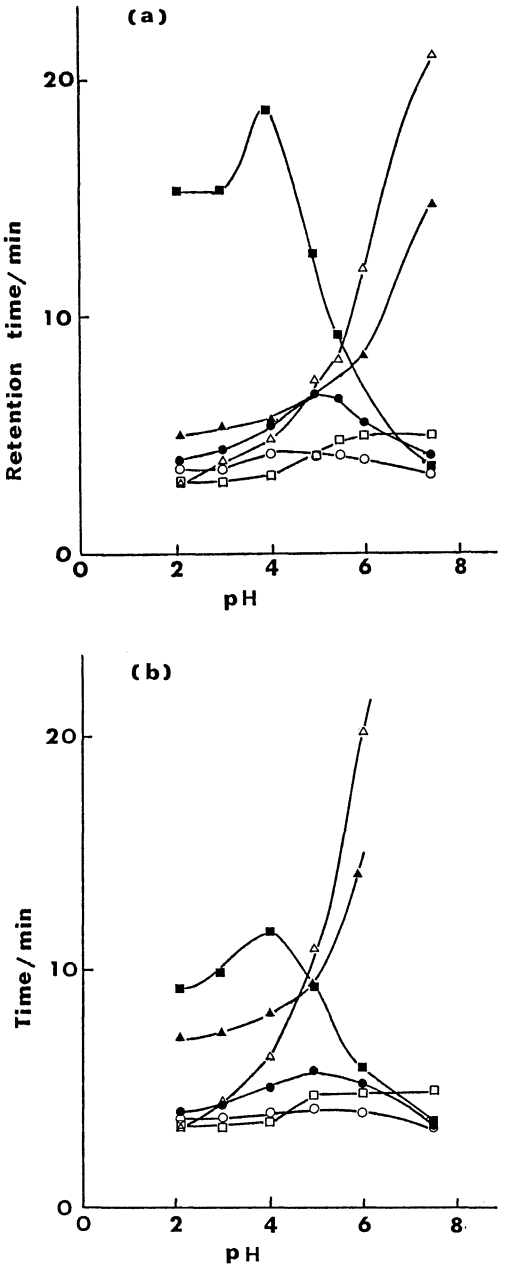


Fig. 1. Effect of pH on separation of vitamins by MPS. (a) Mobile phase: 0.1 M H_3PO_4-0.1 M Na_2HPO_4 buffer (pH 2.1—7.5) containing 10 mM tetrabutylammonium bromide. Sample: Ascorbic acid (AA, ○), Nicotinic acid (NA, ●), Thyamine hydrochloride (TA, △), Pyridoxine hydrochloride (P, □), Hydroxocobalamin acetate (HC, ▲), and Flavin adenine dinucleotide (FAD, ■). (b) Mobile phase: 0.1 M H_3PO_4-0.1 M Na_2HPO_4 buffer (pH 2.1—7.5) containing 10 mM sodium 1-octanesulfonate.

Evaluation of PZS and PDS. The conditions established above-mentioned were applied to PZS and PDS in order to separate the same vitamin mixture. As shown in Fig. 3, a good peak separation was realized. In Fig. 3(a) the peak shapes of NA, TA, and FAD were improved compared to the case of MPS. NA and FAD were completely separated from the other vitamins on PDS [Fig. 3(b)]. In eluent system (1), the effect of the pH (5.0—7.5) was tested regarding the retention times of the vitamins by PZS and PDS. In both cases, the change in their retention times was larger than that of MPS.

Evaluation of End-Capped MPS. Figure 4(a) illustrates a separation profile of six vitamins on a column

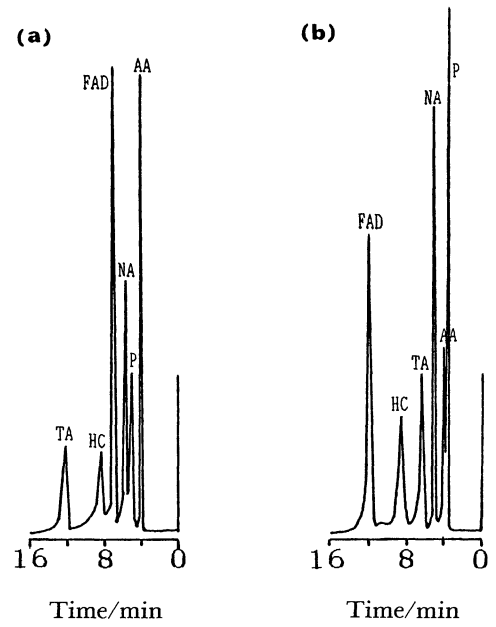


Fig. 2. Chromatograms of water-soluble vitamins obtained by MPS. (a) Mobile phase: 0.1 M H_3PO_4 buffer (pH 6) containing 10 mM tetrabutylammonium bromide. (b) Mobile phase: 0.1 M H_3PO_4–0.1 M Na_2HPO_4 buffer (pH 4.0) containing 10 mM sodium 1-octanesulfonate. Sample concentration (nmol/injection): AA (8.3), NA (5.5), TA (1.3), HC (3.3), P (1.0), FAD (8.3); flow rate, 1.0 mL min^{-1}; detection, 270 nm with 0.16 AUFS. Abbreviations are the same as those given in Fig. 1.

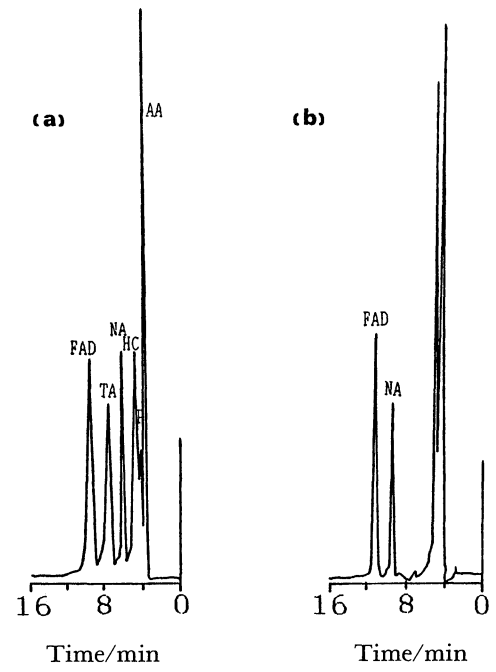
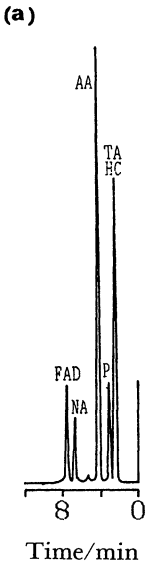


Fig. 3. Chromatograms of water-soluble vitamins obtained by PZS and PDS. (a) Chromatogram by PZS and (b) by PDS: mobile phase; 0.1 M H_3PO_4–0.1 M Na_2HPO_4 buffer (pH 6.0) containing 10 mM tetrabutylammonium bromide. Other conditions are the same as those given in Fig. 2.

of end-capped MPS with the same conditions as in Fig. 1(a). Although the separation was achieved within 8 min, peak separations were not realized for TA and HC. The end-capping treatment remarkably influenced both the elution order of the analytes and the retention times-pH profiles [Fig. 4 (b)]. The retention times of P, TA, and HC are shorter, conversely, those of NA and FAD are longer than the case of MPS. Though such behavior cannot be clearly elucidated, it seems to be related to both an increase in the hydro-



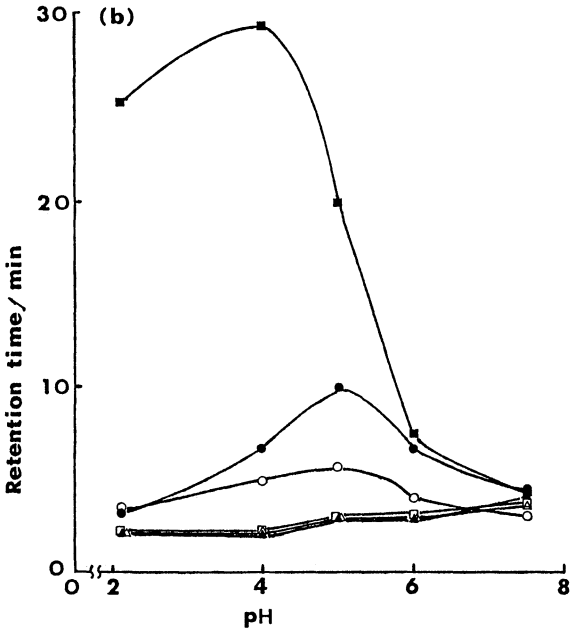


Fig. 4. (a) Chromatogram and (b) effect of pH on separation of vitamins by end-capped MPS. (a) Mobile phase: 0.1 M H_3PO_4–0.1 M Na_2HPO_4 buffer (pH 6.0) containing 10 mM tetrabutylammonium bromide. (b) Mobile phase: 0.1 M H_3PO_4–0.1 M Na_2HPO_4 buffer (pH 2.1—7.5) containing 10 mM tetrabutylammonium bromide. Other conditions are the same as those given in Fig. 2.

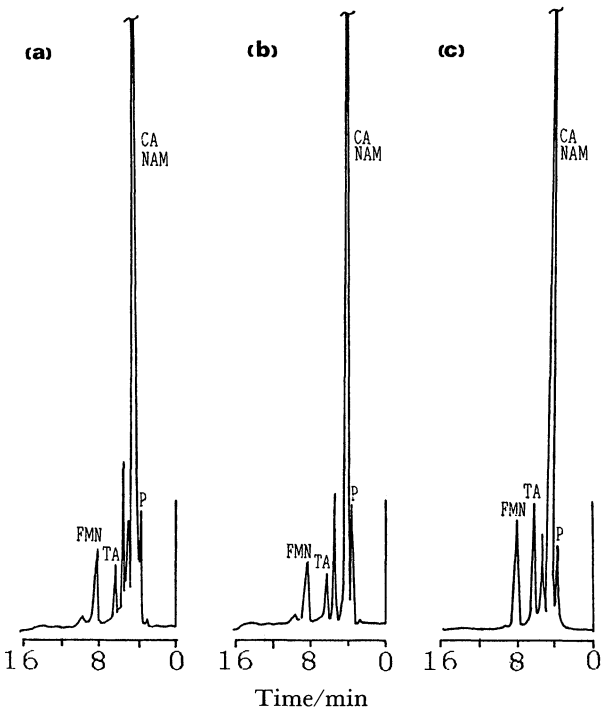


Fig. 5. Chromatograms of water-soluble vitamins in healthful beverages. Beverage samples obtained from A, B, and C companies were diluted 10 folds with water. 100 μL aliquot of each diluted sample was injected HPLC column. Mobile phase: 0.1 M H_3PO_4-0.1 M Na_2HPO_4 buffer (pH 5.0) containing 10 mM tetrabutylammonium bromide; nicotinamide (NAM), flavin mononucleotide (FMN); other abbreviations: see Fig. 1. AUFS: (a) and (b), 0.64; (c), 1.28.

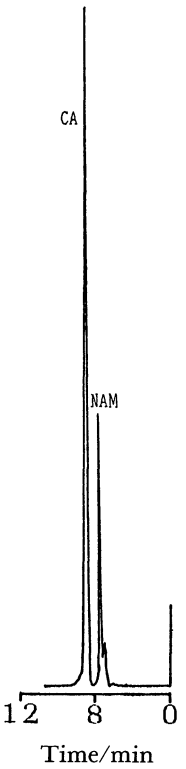


Fig. 6. Separation of NA and caffeine. Mobile phase: 0.1 M H_3PO_4-0.1 M Na_2HPO_4 buffer (pH 3.0) containing 10 mM tetrabutylammonium bromide, caffeine (CA, 1.25×10^{-8} mol/injection), and NAM (2.5×10^{-8} mol/injection); flow rate 1.0 mL min^{-1}; detection with 270 nm with 1.28 AUFS.

phobic area by end-capping and a polarity change of the eluent by pH.

Application of MPS to Water-Soluble Vitamin Analysis in Beverages. As shown in Fig. 5, MPS can quite easily separate water-soluble vitamins contained in commercially available healthful beverages. This showed that the three representative beverages ((a)—(c)) contain four or five water-soluble vitamins [pyridoxine, thyamine, nicotinamide (NAM), flavin mononucleotide (FMN), or/and cyanocobalamin], caffeine or/and extract components, as listed on the labels. By pH change experiments, separation of the combined peak of nicotinamide with caffeine can be realized at pH 3.0, though at the sacrifice of the analysis of the other vitamins (Fig. 6). This result brings about the possibility of caffeine analysis.

In conclusion, special-purpose column packing materials (MPS, PZS, PDS, and end-capped MPS) for water-soluble vitamin analysis were developed, examined and applied to the analysis of three kinds of beverages. In particular, MPS is suitable for the analysis of a water-soluble vitamin solution. On the other hand, PZS and PDS have characteristics capable of analyzing specific vitamins. Since linear calibration curves corresponding to the six vitamins were obtained, the determination of these vitamins in pharmaceutical preparations, beverages, food is now in progress.

One of the major advantages over the traditional reversed-column packing material, such as ODS,[1] is that a favorable separation within a short retention time can be achieved with a buffer solution as an eluent throughout the analysis. These column packing materials can be used more than ca. 1000 times in analyses. As a plausible explanation for the separation of vitamins by these packing materials, we speculate that the columns might have both a partition action and a weak ion-exchange ability due to a tertiary or/and a secondary amino group and, further, a free silanol group in the eluent systems used. Consequently, the separation of a complex mixture like vitamins would be realized by these column packing materials having composite functions depending on the pH, which should be elucidated in the near future.

We thank Emeritus Professor Masazumi Nakagawa of Osaka University for valuable advice and discussions and Daiso Co., Ltd., for providing Daiso gel.

References

1) R. E. Majors, *LC-GC*, **7**, 212 (1989).

2) Y. Okamoto, R. Aburatani, Y. Kaida, and K. Hatada, *Chem. Lett.*, **1988**, 1125.
3) S. Oi, M. Shijo, J. Yamashita, and S. Miyano, *Chem. Lett.*, **1988**, 1545.
4) Y. Maeda, M. Yamamoto, K. Owada, S. Sato, T. Masui, and H. Nakazawa, *J. Assoc. Off. Anal. Chem.*, **72**, 244 (1989).
5) M. Amin and J. Reusch, *Pharm. Ind.*, **50**, 1307 (1988).
6) M. Suzuki, K. Yamasaki, and S. Kawabata, *Kanzei Chuo Bunsekishoho*, **28**, 85 (1988).
7) F. L. Lam and A. Lowande, *J. Pharm. Biomed. Anal.*, **6**, 87 (1988).
8) M. Amin and J. Reusch, *Analyst (London)*, **112**, 989 (1987).
9) M. Amin and J. Reusch, *J. Chromatogr.*, **390**, 448 (1987).
10) Y. Kitada, M. Sasaki, Y. Yamazoe, Y. Maeda, M. Yamamoto, and H. Nakazawa, *Bunseki Kagaku*, **37**, 561 (1989).
11) P. Nandhasri and S. Suksangpleng, *J. Sci. Soc. Thailand*, **12**, 111 (1986).
12) A. Polesello and A. Rizzolo, *J. Micronutr. Anal.*, **2**, 153 (1986).
13) M. W. Dong, J. Lepore, and T. Tarumoto, *J. Chromatogr.*, **442**, 81 (1988).
14) E. Wang and W. Hou, *J. Chromatogr.*, **447**, 256 (1988).