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Note

Separation of vitamin B_6 by reversed-phase ion-pair high-performance liquid chromatography

EMIKO MORITA and NOBUYASU MIZUNO*
Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Hyogo 663 (Japan)
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In animals, vitamin B_6 occurs in the form of pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), their corresponding phosphates and their metabolite 4'-pyridoxic acid (PIC). The determination of these different forms in biological materials is not only important in connection with nutritional problems[1], but also for problems of clinical chemistry[2]. Recently, high-performance liquid chromatography (HPLC) has been used for the analysis of B_6 compounds, and several workers[3–6] have reported the use of an ion-exchange column for the separation of such compounds. Ion-pair chromatography can, in many cases, be used instead of ion-exchange for ionic or ionizable compounds and has some advantages such as high selectivity, column performance and simplicity of the mobile phase[7]. PN, PL and PM can be separated by this technique using 1-heptanesulphonic acid as the pairing agent[8].

The present study employed ion-pair chromatography with cetyltrimethylammonium bromide (CTAB) for the separation of B_6 compounds, particularly phosphates and PIC, and for the determination of PIC in urine.

EXPERIMENTAL

Chemicals

Pyridoxamine 5'-phosphate (PMP), pyridoxal 5'-phosphate (PLP), PN, PL, PM and CTAB were purchased from Nakarai Chemicals (Tokyo, Japan) and PIC from Sigma (St. Louis, MO, U.S.A.). Pyridoxine 5'-phosphate (PNP) was synthesized by the procedure of Kuroda[9]. 4'-Propoxypyridoxine 5'-phosphate (4'-PPNP) used as the internal standard, was synthesized by phosphorylating 4'-propoxypyridoxine[10] with a H_3PO_4–P_2O_5 mixture. All other chemicals were of analytical grade.

Chromatography

A Hitachi Model 635-T high-performance liquid chromatograph was used for the analyses. The wavelength of the UV detector was set at 292 nm. The sensitivity was kept on 0.08 a.u.f.s. throughout.

All mobile phases contained 0.002 *M* CTAB in methanol–phosphate buffer solution (1:1). CTAB was used as the pairing agent. Phosphate buffer solution was prepared from 0.1% NaH_2PO_4 solution and the pH values (3.4–4.2) were adjusted with 0.1% H_3PO_4 solution. The mobile phases were filtered through a 0.45-μm

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membrane filter (Toyo Roshi, Tokyo, Japan) and degassed in an ultrasonic bath prior to use. The analyses were carried out at room temperature on a stainless-steel column (15 cm × 4 mm I.D.) packed with LiChrosorb RP-8 (5-μm particles) (E. Merck, Darmstadt, G.F.R.). The flow-rate was 0.5 ml/min.

Sample preparation

Standard solutions of B_6 compounds were prepared in distilled water and stored at 5°C. Methanol–water (1:1) was used as the diluent. Urine samples were filtered with a 0.45-μm membrane filter, diluted with methanol–water (1:1) and a 20-μl sample was injected onto the column.

Calibration

Peak height ratios were calculated by dividing the heights of the PNP, PIC and PLP peaks by the height of the internal standard and plotted against the amounts of PNP, PIC and PLP.

RESULTS AND DISCUSSION

In order to find the optimal chromatographic conditions, the influence of CTAB concentration and of the pH of the mobile phase on the retention time was investigated. The influence of the pH of the mobile phase was studied using phosphate-buffered mobile phases of different pH values (range 3.4–4.2) (Fig. 1). The retention

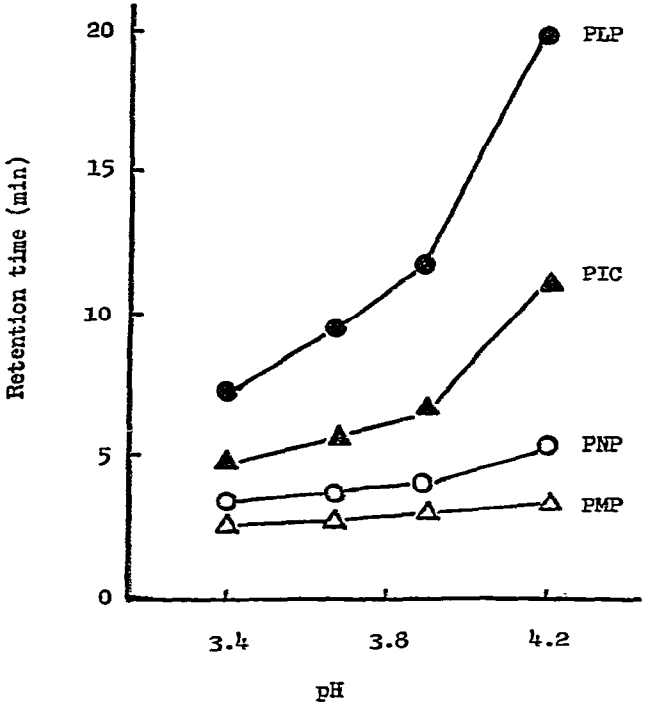


Fig. 1. Relationship between retention times and pH of the mobile phase for B_6 compounds.

times of B_6 compounds increased and could be resolved better as the pH was increased. However, at pH 4.2, poor reproducibility of the retention time was observed. Therefore, the optimal pH seemed to be 3.89. PLP and PNP showed slight peak tailing when the pH of the mobile phase was adjusted with acetate buffer solution instead of phosphate buffer solution. The retention times of B_6 compounds were little affected by the CTAB concentration ($5 \cdot 10^{-4}$–$5 \cdot 10^{-3}$ *M*).

A typical chromatogram is shown in Fig. 2. Separation of PNP, PIC and PLP was complete in about 12 min. PMP was eluted with the solvent front and not retained under any of the conditions.

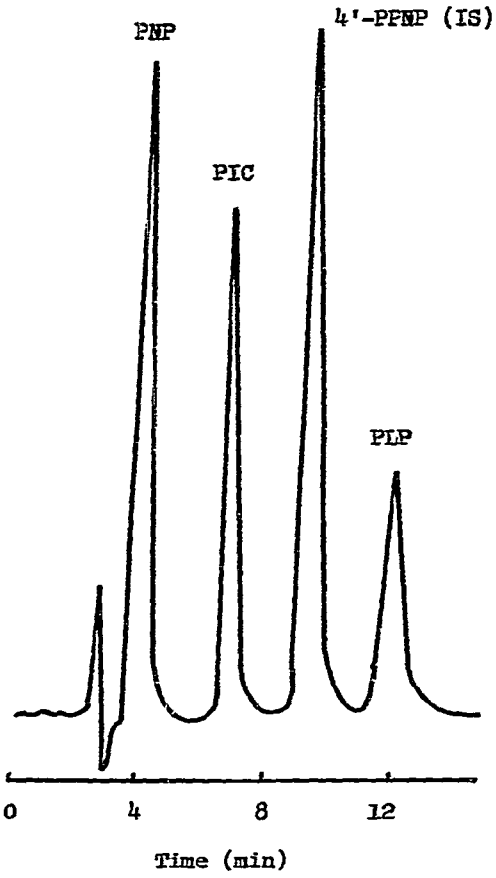


Fig. 2. Chromatogram of B_6 compounds. Mobile phase: phosphate buffer solution–methanol (1:1) with CTAB (0.002 *M*), pH 3.89.

The calibration curves were linear over the range of 0.2–1.5 nmoles of PNP and 0.2–4.0 nmoles of PIC and PLP (Fig. 3). The correlation coefficient for each B_6 compound was greater than 0.99. In the range of 1.0–4.0 nmoles, the mean PIC recovery from control urine was $98.7 \pm 7\%$ ($n = 6$).

The main metabolite of vitamin B_6 in human urine is PIC and its determination is important in studies of the bioavailability[11] of vitamin B_6 in various dosage

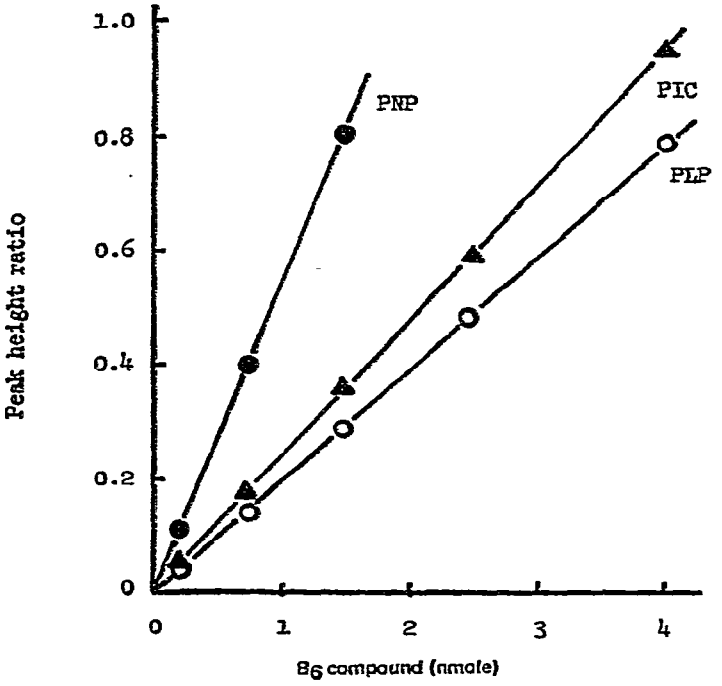
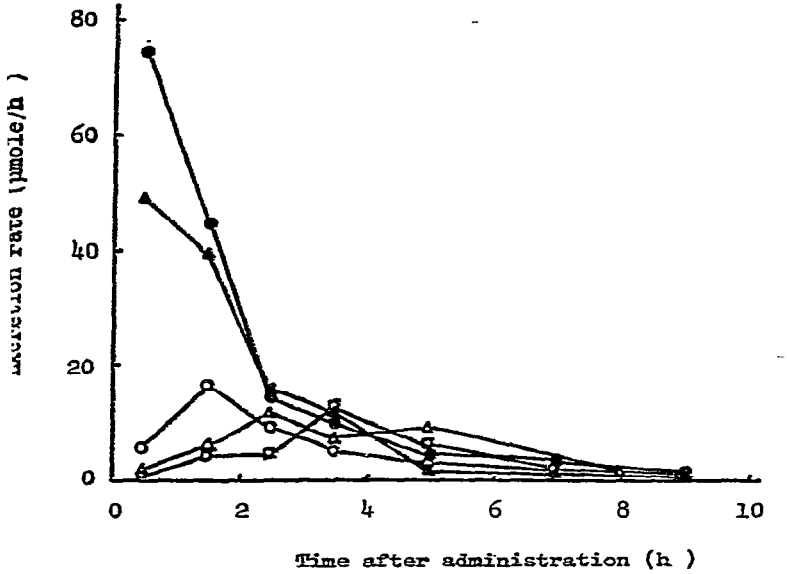


Fig. 3. Calibration curves of B_6 compounds.



ig. 4. Urinary excretion of PIC following oral administration of various forms of vitamin B_6:)—○, PN; ●—●, PL; △—△, PM; ▲—▲, PLP; □—□, PMP.

forms and its metabolism in humans[1,12]. We used the procedure described above to determine the PIC content in human urine. Urinary excretion of PIC for an orally administered dose of 243.14 μmoles of B_6 compounds was determined (Fig. 4). Following oral administration of PL and PLP, the urinary excretion rate of PIC was rapid compared with the other B_6 compounds. The reason for this is that PL[13] and PLP are more easily metabolized to PIC in the human body than other B_6 compounds. The data obtained were similar to the results published by Rabinowitz and Snell[13].

Major advantages of our chromatographic technique are that PNP, PLP and PIC can be assayed at the same time in a simple manner, and the degree of accuracy and reproducibility are excellent. However, the sensitivity is not adequate for blood determination. We are now studying more sensitive methods which use other detection techniques, such as the fluorometric method.

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