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Note

Poly(styrene–divinylbenzene) as reversed-phase adsorbent for the high-performance liquid chromatographic analysis of thiochrome derivatives of thiamine and phosphorylated esters

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Using derivatized silica, the possibilities for the regulation of retention and selectivity by ion suppression and complexation are restricted in reversed-phase separations, as degradation of the solid phase occurs outside the pH range 2–8. This applies to the analysis of thiochrome derivatives of thiamine (T) and its phosphorylated esters, monophosphate (TMP). If high sensitivity is required, an alkaline mobile phase has to be used for this separation. The fluorescence intensity of these oxidized thiamines or thiochromes (Thc, ThcMP, ThcPP and ThcTP) is maximal at pH 9.0[1]. Using mixtures of phosphate buffer (pH 8.4) and methanol, a detection limit of 50 fmol could be achieved[2,3]. However, a limited column lifetime using silica-based material was previously reported[3].

In this paper, it is shown that a column packed with 10-μm poly(styrene–divinylbenzene) beads is well suited for the analysis of thiamine derivatives after pre-column chemical oxidation. The pre-column oxidation may be performed with either CNBr[2] or with $K_3Fe(CN)_6$[3] in 15% NaOH, the latter reaction being safer and simpler. Consequently, the oxidized sample is also alkaline, which is a second factor leading to instability of a C_{18} column. Moreover, with mixtures of phosphate buffer and methanol, peak deformations were observed for thiochromes at an organic modifier concentration lower than 15%, and therefore the possibilities of increasing the retention were limited[3]. None of these shortcomings occurred when using poly(styrene–divinylbenzene), and both higher retentions and a much longer column lifetime could be achieved. An identical elution order, *i.e.*, ThcTP, ThcPP, ThcMP and Thc, was observed with methanol as the organic modifier. The phosphorylated esters could be resolved isocratically in 8 min, while a linear gradient was used for the simultaneous analysis of the four compounds within 15 min.

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EXPERIMENTAL

Reagents, solvents and pre-column derivatization procedure

T, TMP and TPP were obtained from Sigma (St. Louis, MO, U.S.A.). TTP was prepared and re-purified by ion-exchange chromatography according to refs. 4 and 5. The oxidation to thiochrome derivatives was achieved by adding 0.10 ml of alkaline potassium hexacyanoferrate(III) solution [0.02% $K_3Fe(CN)_6$ in 15% NaOH] (prepared fresh daily) to 0.16 ml of sample, 1 min before injection. Oxidized samples were found to be stable for several hours when protected from light or for several months when stored frozen.

Solvent A used for high-performance liquid chromatography (HPLC) was a 25 m*M* sodium phosphate buffer (pH 8.4) prepared with Milli-Q water (Millipore, Bedford, MA, U.S.A.) and analytical-reagent grade sodium phosphate (Merck, Darmstadt, F.R.G.). It was filtered on a 0.45-μm ultrafilter before use. Solvent B was pure methanol (analytical-reagent grade) from Merck.

Chromatographic system

Analyses were performed with a Model 334-50 liquid chromatograph from Altex (Berkeley, CA, U.S.A.) composed of two Model 110A pumps, a high-pressure solvent mixer and a Model 421 system controller. Separations were achieved at 1 ml/min at room temperature on a 250 × 4.1 mm I.D. PRP-1 analytical column from Hamilton (Reno, NV, U.S.A.) packed with 10-μm particles. Samples were introduced into the column through a Model 210 injection valve (Altex) with a loop of 20 μl. Solutes were detected fluorimetrically using a Gilson Spectra-Glo apparatus (Middleton, U.S.A.) equipped with 330–400 and 460–600 nm excitation and emission filters and a 15-μl flow cell. Chromatograms were registered on a Houston Omniscribe recorder set at 10 mV or analysed on a C-R1A Chromatopac integrator from Shimadzu (Kyoto, Japan).

RESULTS AND DISCUSSION

Retention, selectivity and peak symmetry

The capacity factors of the four derivatized thiamine compounds were measured isocratically at different methanol percentages (Fig. 1). As previously observed on C_{18} columns[3], Thc is more strongly retained than its esters and the selectivity decreases drastically with increase in the number of phosphate groups. A k' value of 3 is reached at 4% of organic modifier for ThcTP, the least reatained derivative. For Thc–ThcMP, a high value of the selectivity factor, $\alpha = 28$, is obtained at 30% methanol. The groups ThcMP–ThcPP and ThcPP–ThcTT give constant values of 1.75 and 1.25, respectively, below 20% methanol.

A comparison with the capacity factors observed on C_{18} columns shows that much higher k' values are reached with the polymeric phase. As illustrated in Fig. 1, the retention is enhanced by a factor of 2.8 for Thc. The enhancement is found to be lower (1.6) for ThcMP and ThcPP. Similar differences in retention between a PRP-1 and C_{18} column were reported by Greyson and Patch[6] in the chromatography of gibberellins.

At low methanol percentages, the retention of the esters on PRP-1 increases

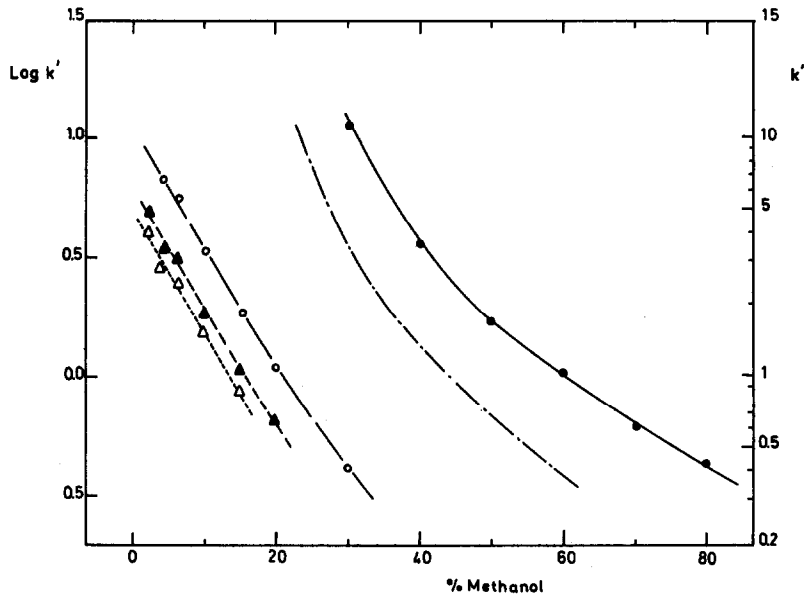

Fig. 1. Effect of organic modifier concentration on capacity factors. Stationary phase: 10 μm PRP-[(250 × 4.1 mm I.D. column). Mobile phase: 25 m*M* phosphate buffer (pH 8.4) as solvent A and pure methanol as solvent B. Flow-rate: 1 ml/min. Temperature: 20°C. Sample: 20 μl of 10^{-5} *M* Thc (●), ThcMP (○), ThcPP (▲) or ThcTP (△). Corresponding capacity factors obtained with a 5-μm Ultrasphere-ODS column (150 × 4.6 mm I.D.) are given for Thc (- · - · -).

linearly with increasing ionic strength of the buffer, as observed by Sanemori *et al.*[2] using a C_{18} column and dimethylformamide as the organic modifier. Such opposing effects of the concentration of the organic solvent and of the ionic strength on retention can be expected when the interactions between the solutes and stationary phase are predominantly hydrophobic[7,8].

With the PRP-1 column, it should be emphasized that, within the concentration range of methanol investigated, the peaks were of normal gaussian shape. In contrast, with the C_{18} column, badly tailing peaks were recorded as the methanol percentage was decreased below the critical value of 15%[3].

Column efficiency and stability

Typical behaviour of the polystyrene column is depicted in Fig. 2A, where the plate height, *H*, is plotted as a function of the capacity ratio, *k'*. Constant values of 0.38 and 0.16 mm are observed for Thc and the phosphorylated esters, respectively, at a linear speed of 0.125 cm/sec. This difference in plate height remains at different linear velocities, as shown in Fig. 2B. The increase in plate height with increasing linear speed is limited, the *H* values being doubled for a 10-fold change in flow-rate.

The column efficiences reported here remained unchanged during a 6-month period of daily use, under both isocratic and gradient elution conditions. Of particular interest was the inertness of this phase towards the strong alkalinity of the injected sample, *i.e.*, 1.44 *M* NaOH. Such a situation is often encountered in pre-column derivatization techniques (biological amines).

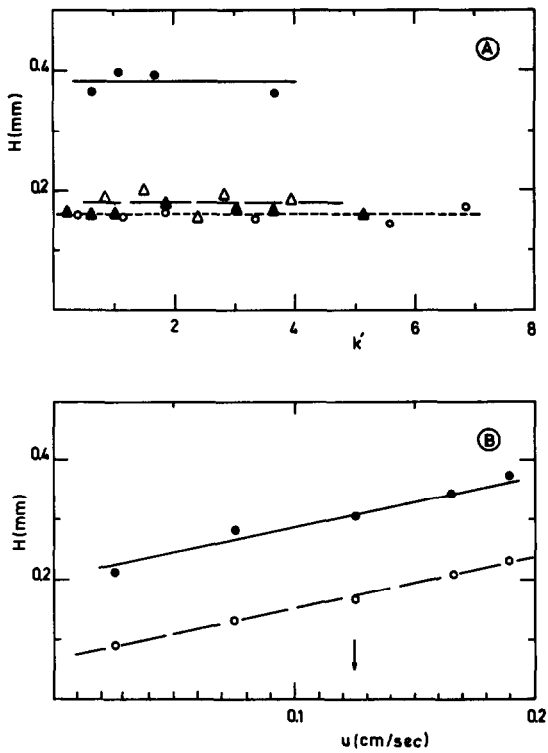

Fig. 2. (A) Dependence of plate height on the capacity factor. Symbols and experimental conditions as in Fig. 1. (B) *H versus u* curves for Thc (●) and ThcMP (○) at the same k' value of 2. The arrow indicates the usual flow-rate of 1 ml/min.

Resolution

It is necessary to obtain a high resolution for the analysis of thiamines in biological samples, owing to the difference in the relative abundances of the four derivatives. In rat tissues, TPP is the major component (85.7–90.0%) and TTP, the derivative of which elutes just before that of TPP, is a minor constituent (0.7–1.6%)[9]. Moreover, in reversed-phase separations, several non-thiochrome peaks appear before the first eluting peak of interest, *i.e.*, ThcTP[2,3]. Consequently, high k' values are also needed. The data in Table I indicate that adequate resolution (higher than 1) can be obtained for the three esters on the PRP-1 column at methanol concentrations not higher than 15%. Under these conditions, the differences in resolution are due mainly to changes in retention as the selectivity and efficiency are fairly constant in this concentration range (Figs. 1 and 2A).

At methanol concentration higher than 15%, ThcTP and ThcPP have too low retentions to be resolved. The higher resolution obtained with the C_{18} column in this range is due to the higher efficiency of this column ($N \approx 3000$ for a 15-cm length and 5-μm particles) compared with that of the PRP-1 column ($N \approx 1510$ for a 25-cm length and 10-μm particles). The difference between the two columns cancels if R_S values are divided by $\sqrt{N}$, the contribution of the efficiency to the resolution factor.

TABLE I

INFLUENCE OF METHANOL CONCENTRATION ON RESOLUTION FOR THE POLY(STYRENE–DIVINYLBENZENE) AND AN ULTRASPHERE-ODS COLUMN, CALCULATED FROM RETENTION TIMES AND BAND WIDTHS AT HALF-HEIGHT

Methanol (%, v/v)	*10-μm PRP-1 column (250 × 4.1 mm I.D.)*		*5-μm Ultrasphere-ODS column (150 × 4.6 mm I.D.):*
	ThcTP–ThcPP	*ThcPP–ThcMP*	*ThcPP–ThcMP*
2	2.07	–	–
4	1.81	4.93	–
6	1.81	4.92	–
10	1.19	4.01	–
15*	1.03	3.14	3.52
20	–	2.40	2.90
25	–	–	1.75
30	–	1.21	1.42

* Limiting condition for obtaining symmetrical peaks with the Ultrasphere-ODS column.

Analysis of a TTP preparation

The purification of TTP synthetized according to refs. 5 and 6 was monitored using the PRP column. A pure fraction was assayed for its phosphorus content and compared with TMP and TPP at equal concentrations. It was stored for 2 months at −20°C and pH 5.0 and its possible degradation was checked by HPLC. Fig. 3 compares the chromatograms obtained by gradient elution with the thawed TTP solution (A) and with an equimolar mixture of TPP, TMP and T (B). After storage,

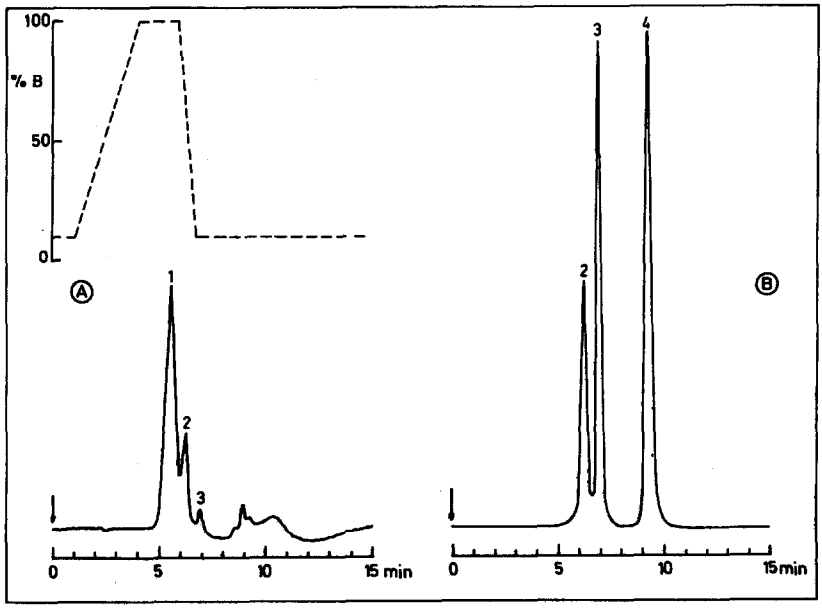

Fig. 3. Analysis of a sample of TTP (A) and an equimolar mixture of TPP, TMP and T (B) under gradient conditions (---). Peaks 1–4 refer to ThcTP, ThcPP, ThcMP and Thc, respectively.

the TTP solution is partially contaminated by TPP (peak 2) and TMP (peak 3), whereas no trace of T is visible.

Instead of a gradient run with an injection-to-injection time of 15 min, an isocratic separation of the three phosphorylated esters can be achieved isocratically within 8 min at 15% methanol. At 50% methanol, thiamine elutes after 6.1 min; under these conditions, the phosphorus-containing compounds appear unseparated and close to the frontal peak.

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