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Note

Capillary tube isotachophoretic separation of niacin derivatives

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Niacin (nicotinic acid is a vitamin widely distributed in biological materials and food and is converted into the coenzyme nicotinamide adenine dinucleotide by biological reactions *in vivo* [1]. Niacin has many structural derivatives, some of which are converted into niacin *in vivo* and others being involved in the biosynthesis of niacin [2]. Some niacin derivatives have been determined by high-performance liquid chromatography or microbioassay [3]. However, a practical method for the simultaneous determination of various niacin derivatives is desirable in order to elucidate the metabolic system of niacine derivatives *in vivo*.

Capillary tube isotachophoresis (CITP) is an excellent separation method for many ionic compounds. Biologically important substances such as vitamins [4], amino acids [5,6] and nucleotides [7,8] have been separated and determined by CITP. Niacin and its derivatives all have a pyridine ring in their structure and act as weak bases. In order for niacin derivatives to migrate and be detected in CITP, it is necessary to protonate the pyridine ring so that they migrate as cationic species (pyridinium ion).

In this paper, the migration behaviour of niacin derivatives is described. Migration systems for some niacin derivatives with small mobilities were investigated. By selecting the optimum leading and terminating electrolytes, the niacin derivatives of weak bases were sufficiently protonated for migration. Six niacin derivatives could be separated using 40 m*M* potassium acetate as the leading electrolyte and 10 m*M* glycine as the terminating electrolyte.

EXPERIMENTAL

Apparatus

A Model IP-3A capillary tube isotachophoretic analyser (Shimadzu, Kyoto, Japan), equipped with a potential gradient detector and a column system consisting of

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a PTFE preseparation capillary (80 × 0.7 mm I.D.) and a fused-silica analytical capillary column (170 × 0.2 mm I.D.), was used. The current was 15–60 μA after migration at 360 μA for 3–10 min. The capillary tube was filled with the leading and terminating electrolytes using a peristaltic pump.

Reagents

Potassium acetate, acetic acid, β-alanine, glycine, hydrochloric acid and Triton X-100 were of analytical-reagent grade from Wako (Osaka, Japan) and used without further purification.

Stock solutions of niacin derivatives were prepared by dissolving pyridine, nicotinamide (Wako), β-picoline, N-methylnicotinamide, 3-hydroxymethylpyridine, 3-acetylpyridine, isonicotinic acid hydrazide (Tokyo Kasei, Tokyo, Japan), thionicotinamide (Nacalai Tesque, Kyoto, Japan) and 6-aminonicotinamide (Sigma, St. Louis, MO, U.S.A.) in water.

Electrolytes

The three operational systems used are given in Table I. The leading electrolyte was prepared by diluting a stock solution of 1 M potassium acetate and 10% Triton X-100 and adjusting the pH by adding acetic acid. The terminating electrolyte for system I was prepared by dissolving β-alanine and adjusting the pH to 1.7 by adding hydrochloric acid. The terminating electrolyte for system II was prepared by dissolving glycine and adjusting the pH with hydrochloric acid to the same value as in system I. The terminating electrolyte for system III was acetic acid. In systems II and III, proton acts as the terminating ion.

TABLE I

OPERATING SYSTEMS

Parameter	Leading electrolyte	Terminating electrolyte		
		System I	System II	System III
Cation	K^+	β-Alanine	H^+	H^+
Counter ion	CH_3COO^-	Cl^-	Cl^-	CH_3COO^-
Concentration	10–50 mM	10 mM	10 mM	10 mM
Additive	0.1% Triton X-100	None	Glycine	None

RESULTS AND DISCUSSION

Niacin derivatives of β-picoline, 3-hydroxymethylpyridine, 6-aminonicotinamide, isonicotinic acid hydrazide, nicotinamide, thionicotinamide, N-methylnicotinamide and 3-acetylpyridine were used as samples and pyridine was added as it has a similar structure. All these substances have a pyridine ring and would be protonated to form cationic species. An acetate buffer of pH 4.0 was chosen as the leading electrolyte for protonation of the pyridine ring. With migration system I using β-alanine as the terminating ion, pyridine, β-picoline, 3-hydroxymethylpyridine and 6-aminonicotinamide migrated and could be separated. The order of migration seems

to depend on the dissociation constants of the substances. The other five substances, however, could not be detected with this migration system, because their effective mobilities are lower than that of the terminating ion. This situation could not be improved even by changing the pH of the leading electrolyte. Niacin could not be detected in a cationic system but could in an anionic system because it exists as an anionic species owing to the dissociation of its carboxyl group.

When glycine was used as the terminating electrolyte (system II), all the niacin derivatives could be detected in front of the terminating zone. Isonicotinic acid hydrazide, nicotinamide, thionicotinamide, N-methylnicotinamide and 3-acetylpyridine, however, were detected with a so-called "enforced zone", the effective mobility of which was lower than that of the terminating ion. A similar isotachopherogram to that with system II was also obtained with system III using acetic acid as the terminating electrolyte. With system I protonated β-alanine would act as the terminating ion, whereas with systems II and III proton itself would act as the terminating ion [9]. It is suggested that weak bases such as some niacin derivatives may be associated with proton in the terminating zone in addition to the leading zone and have a cationic charge. Consequently, they move back to the sample zone, and migrate in front of the terminating zone.

The relationship between the concentration of the leading ion and the R_E value (ratio of the potential gradient of the sample zone to that of the leading zone) of niacin derivatives and proton as terminating ion using system II is shown in Fig. 1. The R_E values of niacin derivatives remained almost unchanged at all concentrations of the leading ion, whereas the R_E value of the terminating ion increased with increasing concentration of the leading ion. The zones of isonicotinic acid hydrazide, nicoti-

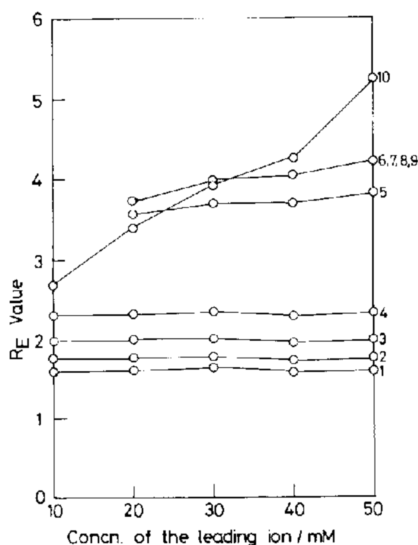

Fig. 1. Effect of the concentration of the leading ion on R_E values of niacin derivatives and the terminating ion. The pH was constant at 4.0. 1 = β-Picoline; 2 = pyridine; 3 = 3-hydroxymethylpyridine; 4 = 6-aminonicotinamide; 5 = isonicotinic acid hydrazide; 6 = nicotinamide; 7 = thionicotinamide; 8 = N-methylnicotinamide; 9 = 3-acetylpyridine; 10 = terminating ion.

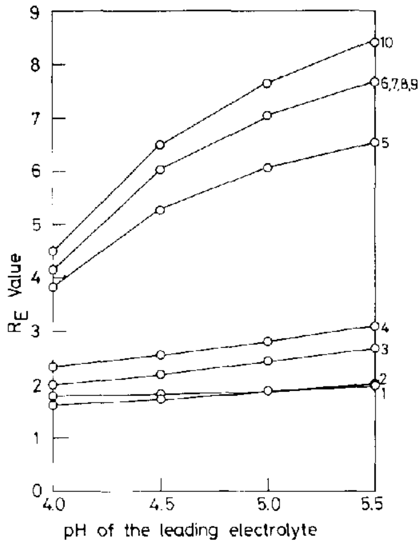

Fig. 2. Effect of pH of the leading electrolyte on R_E values of niacin derivatives and the terminating ion. The concentration of the leading ion was 40 mM. Line numbers as in Fig. 1.

namide, thionicotinamide, N-methylnicotinamide and 3-acetylpyridine were detected as an enforced zone when the concentration of the leading ion was up to 20 mM. However, when the concentration of the leading ion was 40 mM, the effective mobility

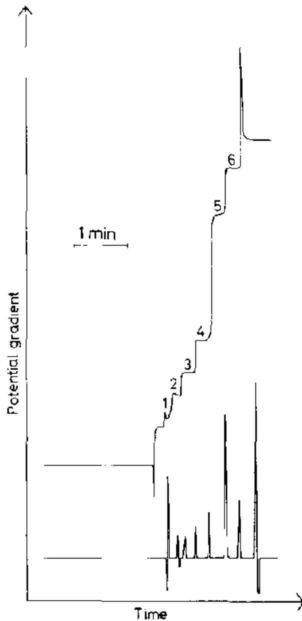

Fig. 3. Isotachopherogram of niacin derivatives. Electrolyte as in Fig. 1, except that the concentration of the leading ion was 40 mM. Numbers as in Fig. 1.

of the terminating ion became lower than that of sample ion and a normal isotachopherogram was obtained. This shows that the effective mobility of proton as terminating ion is controlled by the acid–base equilibrium with acetate ion as the counter ion of the leading electrolyte. Therefore, an increase in the concentration of the leading electrolyte decreases the effective mobility of proton as the terminating ion.

The relationship between the pH of the leading electrolyte and the R_E values of niacin derivatives at a leading ion concentration of 40 mM is shown in Fig. 2. With increasing pH, the R_E values of the niacin derivatives increased. However, the difference in mobility between pyridine and β-picoline decreased. At pH 4.0, β-picoline, pyridine, 3-hydroxymethylpyridine, 6-aminonicotinamide and isonicotinic acid hydrazide could be separated. Nicotinamide, thionicotinamide, N-methylnicotinamide and 3-acetylpyridine could also be detected under these conditions but as a mixed zone, and their separation could not be improved by changing the pH of the leading electrolyte.

An isotachopherogram obtained using 40 mM potassium acetate and acetic acid as the leading electrolyte and 10 mM glycine and hydrochloric acid as the terminating electrolyte is shown in Fig. 3. Six niacin derivatives, of β-picoline, pyridine, 3-hydroxymethylpyridine, 6-aminonicotinamide, isonicotinic acid hydrazide and nicotinamide, could be clearly separated. The calibration graphs were linear over the sample amounts range 1–5 nmol.

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