Journal of Chromatography, 480 (1989) 271–276
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 703

SIMPLE METHOD FOR GENERATION OF A DYNAMIC pH GRADIENT IN CAPILLARY ZONE ELECTROPHORESIS

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SUMMARY

A dynamic pH gradient extends the separation power of zone electrophoresis to mixtures of substances with widely differing pK values. A simple method for the generation of dynamic changes in the pH of the background electrolyte in the separation capillary is described. It is based on a controlled modification of the composition and, thus, of the pH of an electrolyte in the electrode chamber at the injection end of the separation capillary during the analysis. The higher the concentration of H^+ in this electrode chamber, the higher is the electromigration flow of H^+ into the capillary and the lower is the pH of the actual background electrolyte therein. The utilization of this principle is exemplified by the separation of eleven purine and pyrimidine bases with pK values ranging from 6.0 to 1.9.

INTRODUCTION

The main features that contribute to the increasing popularity of capillary zone electrophoresis (CZE) are the simple instrumentation used and the speed and high efficiency of the separation[1]. These have been demonstrated by the separation of both low-molecular-weight substances such as amino acids[2], nucleotides[3] and drugs[4] and biopolymers such as peptides[5] and proteins[6].

The selectivity of the separation of substances is given by the differences in their effective mobilities usually can be optimized by utilizing acid–base equilibria. By the proper selection of the pH of the background electrolyte (BGE), the degree of ionization and hence the effective mobilities of separated substances can easily be manipulated in order to achieve required separation. However, when the sample contains substances having close ionic mobilities and covering a wide range of pK values, there is no possibility of finding a suitable fixed pH that would permit the separation of all of them in one run in a reasonable time.

Recently, dynamic pH gradients[7] were introduced in CZE to solve such problems. For the programming of pH in the separation capillary, a three-pole separation column[8] was used, where the actual operational electrolyte matrix was generated by the simultaneous electromigration of various ionic species of the same polarity from two separate electrode chambers.

0021-9673/89/$03.50

In this paper, a simple means of forming dynamic pH gradients is proposed. It is based on a programmed dynamic change in pH in one electrode chamber during the analysis.

EXPERIMENTAL

Apparatus

Experiments were performed in the set-up shown in Fig. 1. A 40 cm × 130 μm I.D. fused-silica separation capillary (kindly supplied by Dr. Doupovec, Research Laboratory of Silicates, Bratislava, Czechoslovakia) was placed between two electrode chambers equipped with platinum electrodes to connect the high-voltage power supply (delivering up to 13 kV and 200 μA). Injection was performed by hydrodynamic flow with the aid of the swing arm. Rinsing of the capillary was allowed by a refilling block (containing also a conductivity detection cell) connected to the electrode vessel at the detection end of the capillary. A more detailed description of the instrumentation used can be found elsewhere[9].

On-column UV detection at 254 nm was used, employing an LCD-2563 LC detector (Laboratory Instruments, Prague, Czechoslovakia) equipped with a fibre-optic detection cell[10]. A moulded conductivity detection cell[10] placed 5 cm behind the optical cell was connected to the conductivity detector of an Agrofor isotachophoretic analyser (JZD Odra, Krmelín, Czechoslovakia). The modifying electrolyte was pumped by an LD 2 syringe-type linear doser (Research Workshops, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) and was mixed with the background electrolyte in the electrode chamber by bubbling nitrogen from the laboratory supply. The volume of the electrolyte in this electrode chamber was approximately 40 ml.

Chemicals and electrolytes

Purine and pyrimidine derivatives of purum grade were obtained from Lachema (Brno, Czechoslovakia). Trichloroacetic acid (TCA) (Cambrian Chemicals, U.K.) and tris(hydroxymethyl)aminomethane (Tris) (Lachema), both of analytical-reagent grade, and freshly boiled distilled water were used for the preparation of the background electrolyte (BGE). The pH of the BGE, containing 0.01 *M* Tris and TCA, was adjusted to the desired value by adding 0.5 *M* TCA solution. To decrease the electro-

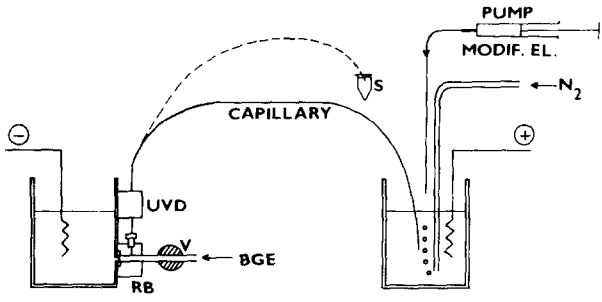

Fig. 1. Schematic diagram of the set-up for electrophoretic analysis with a dynamic pH gradient. S = sample; UVD = UV detector; RB = refilling block; V = valve; BGE = background electrolyte.

TABLE I

PURINE AND PYRIMIDINE DERIVATIVES USED FOR THE PREPARATION OF THE MODEL SAMPLE ANALYSED

c = Concentration of the substance in the sample; pK_a values were taken from ref. 11.

Substance	*Abbreviation*	pK_a	*c (mmol/l)*
4,6-Diaminopyrimidine	4,6-DAP	6.0	0.2
Cytosine	C	4.6	0.2
5-Methylcytosine	5-MC	4.6	0.2
Adenine	A	4.1	0.1
2-Aminopurine	2-AP	3.7	0.2
6-Benzylaminopurine	6-BAP	>4	0.2
Guanine	G	3.3	0.2
5-Aminouracil	5-AU	3.2	0.2
5-Bromocytosine	5-BC	3.0	0.2
Hypoxanthine	HX	1.9	0.2
Guanosine	Guo	1.9	0.2

osmotic flow, 0.25% of polyethylene glycol PEG-6000 (Lachema) was added to the BGE.

TCA solution (0.5 *M*) was used as the modifying electrolyte pumped to the electrode vessel to change the pH of the BGE.

RESULTS AND DISCUSSION

Nucleic bases and their derivatives were used as the model substances covering a wide range of pK_a values from 1.9 to 6. They are listed in Table I together with the abbreviations used and their concentrations in the mixture serving as a sample.

In Fig. 2 the separation of the model mixture in an "isocratic" BGE at a constant pH of 3.5 is shown. At this pH the migration order is controlled mainly by the pK_a of separated substances, as can be seen from the relationship between the effective electrophoretic mobility, $\bar{u}$, acidity constant, pK_a, and pH of the BGE, written in the form

$$\bar{u} = u \cdot \frac{10^{-pH}}{10^{-pH} + 10^{-pK_a}} \quad (1)$$

where u is the ionic mobility of the respective substance corresponding to its mobility in the fully ionized form. Thus, at pH 3.5, 4,6-diaminopyrimidine (4,6-DAP) with pK_a = 6.0 is fully ionized and migrates with the highest velocity and, *e.g.*, guanine (G) is ionized to *ca.* 40% and migrates with an effective mobility that is 40% of its ionic mobility. 6-Benzyl-aminopurine (6-BAP), in spite of its higher pK_a, migrates behind 2-aminopurine (2-AP), probably owing to its much higher molecular mass (225 *vs.* 135). Hypoxanthine (HX) and guanosine (Guo) are ionized to less than 3% at this pH and are not separated at all. These two substances reach the detector mainly by electroosmotic transport, which was not eliminated completely. The magnitude of the contribution of the electroosmotic flow to the velocity of migrating zones was calcu-

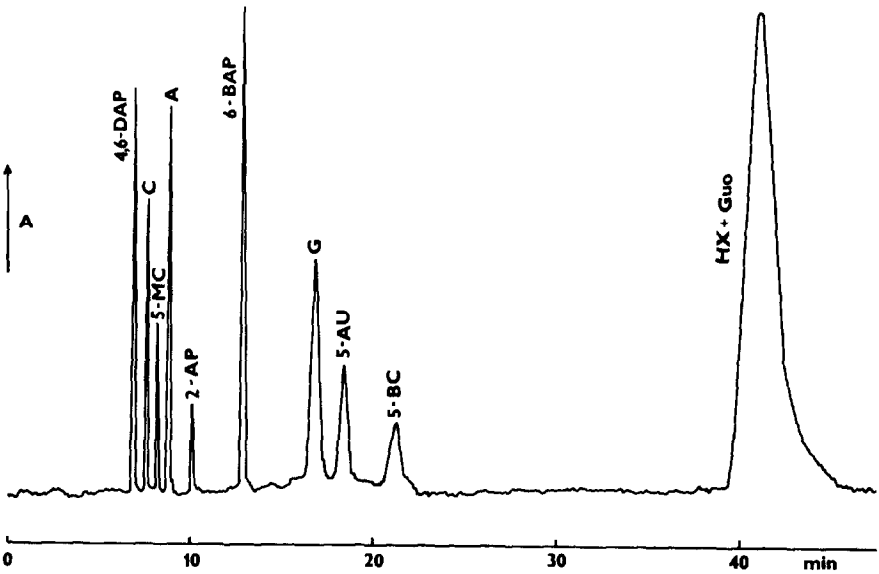

Fig. 2. Electrophoretic analysis at a constant pH of 3.5. Background electrolyte, 0.01 *M* Tris–trichloroacetate. Voltage, 10 kV; current, 30 μA. For abbreviations, see Table I.

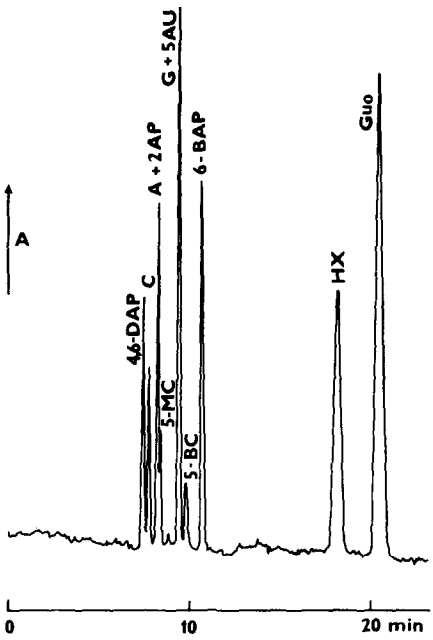

Fig. 3. Electrophoretic analysis at a constant pH of 2.2. Background electrolyte, 0.01 *M* Tris–trichloroacetate. Voltage, 8 kV; current, 130 μA. For abbreviations, see Table I.

lated from the peak with low electrolytic conductivity attributed to the initial sample pulse and followed by the conductivity detector (not shown in Fig. 2). The magnitude of the electroosmosis expressed in terms of mobility was $7 \cdot 10^{-5}$ $cm^2/V \cdot s$ or 10 mm/min in this experiment at pH 3.5.

Hypoxanthine and guanosine can be ionized to a greater extent by lowering the pH of the BGE. In Fig. 3 the separation of the same mixture as in Fig. 2 is shown. The pH of the BGE was set to 2.2, where almost all substances are completely ionized and hypoxanthine and guanosine are ionized to approximately 30%. Their mutual separation is probably again due to differences in molecular mass, 136 for HX *vs.* 319 for Guo. However, at this pH, the high-mobility substances form mixed zones and are not separated.

The practical way to separate all substances in one run is to use a dynamic pH gradient. The record of the separation is shown in Fig. 4. Here the separation started at pH 3.5 and after 9 min of migration the pump delivering the modifying electrolyte was switched on for 5 min at a rate of 0.1 ml/min. During this time the pH of the BGE in the electrode vessel decreased to 2.2, as indicated on the abscissa in Fig. 4. The dynamic pH gradient formed inside the capillary and, changing substantially also the BGE conductivity, can easily be followed by the conductivity detector. The small peak on the conductivity record denoted H^+ was attributed to the H^+ front originated from the sample solution (pH = 3.3) as its magnitude changed in accordance with

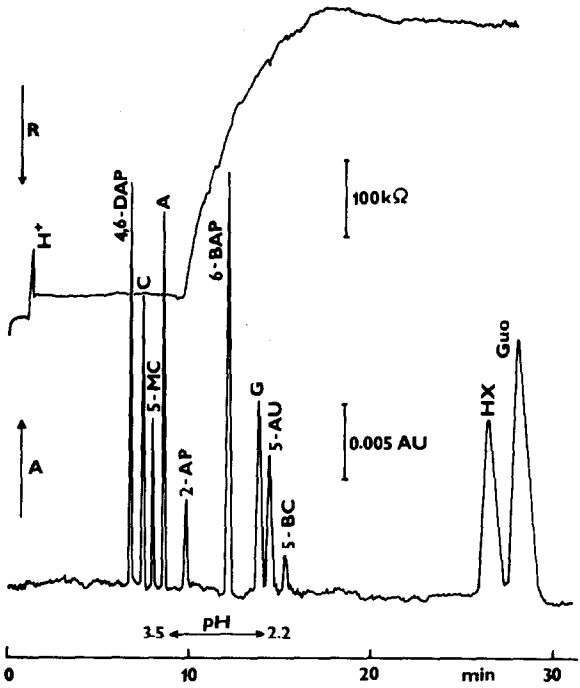

Fig. 4. Electrophoretic analysis in a pH gradient. Background electrolyte, 0.01 *M* Tris–trichloroacetate. Modifying electrolyte, 0.5 *M* trichloroacetic acid, 0.1 ml/min for 5 min (from 9 to 14 min). Voltage, 10 kV (0–15 min), then 8 kV (> 15 min); current, 30–110 μA. For abbreviations, see Table I.

the change in the sample acidity. It should be noted that the conductivity of the BGE at operational pH levels below 3.5 is very high owing to the very high contribution of H^+ and the sensitivity of the conductivity detector does not allow tiny changes in conductivity to be detected within the migrating zones, thus revealing the peaks. The first detected zones were separated at pH 3.5 with a constant voltage of 10 kV; as the current increased during the run, the final voltage decreased to 8 kV after 15 min of migration.

The UV absorbance detector clearly indicates complete separation of all the substances analysed, owing to the favourable pH of 3.5 for separating high-mobility substances and also to the pH of 2.2 later on, which is favourable for separating HX and Guo.

It follows from the results that the use of dynamic pH gradients greatly enhances the selectivity of the separation in CZE and enables one to improve the separation and decrease the migration time of complex samples containing substances with a wide range of pK values. The system proposed for the generation of dynamic pH changes, which is based on changing the composition in a suitable electrode chamber during the analysis, is simple and easy to programme and automate.

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