Journal of Chromatography, 608 (1992) 317–323
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 256

Effect of the buffer solution on the elution order and separation of bis(amidinohydrazones) by micellar electrokinetic capillary chromatography

P. Lukkari, J. Jumppanen, T. Holma and H. Sirén

Analytical Chemistry Division, Department of Chemistry, University of Helsinki, Vuorikatu 20, SF-00100 Helsinki (Finland)

K. Jinno

Materials Science, Toyohashi University of Technology, Tompaku-chi, Toyohashi 440 (Japan)

H. Elo

Chemistry Division, Department of Pharmacy, University of Helsinki, Fabianinkatu 35, SF-00170 Helsinki (Finland)

M.-L. Riekkola

Analytical Chemistry Division, Department of Chemistry, University of Helsinki, Vuorikatu 20, SF-00100 Helsinki (Finland)

ABSTRACT

The effect of five different buffer solutions on the elution order and separation of bis(amidinohydrazones) by micellar electrokinetic capillary chromatography was studied at pH 7.0. The buffers were sodium phosphate, tris(hydroxymethyl)aminomethane, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid), N,N-dimethylmethylenediamine and N,N-diethylethylenediamine. The factors affecting the elution order of the solutes were: (1) ion-pair formation between the solute and the buffer ion, (2) the cationic nature and structure of the solute, (3) reactions between ion-pair complexes and micelles and (4) the nature of the buffer solution. Sodium phosphate (0.05 *M*) with 1 m*M* N-cetyl-N,N,N-trimethylammonium bromide was the only buffer solution to fully separate eight aliphatic congeners of bis(amidinohydrazone).

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC), which is an adaptation of capillary zone electrophoresis (CZE), extends the enormous power of CZE to the separation of uncharged molecules [1,2]. In MECC the addition of an ionic surfactant to the electrolyte in an amount greater than its critical micelle concentration (CMC) makes possible the separation of neutral particles. Because the micelles provide ionic and hydrophobic sites of interaction simultaneously, MECC is also preferable to CZE for the separation of mixtures of charged and uncharged solutes. Yet another application of MECC is the separation of ionic compounds, such as the bis(amidinohydrazones) studied here, whose electrophoretic mobilities are too similar to be separated by CZE [3,4]. In MECC, the migration time of an ionic substance is a function of three factors: (1) the electrophoretic mobility of the solute, (2) the distri-

Correspondence to: Dr. M.-L. Riekkola, Analytical Chemistry Division, Department of Chemistry, University of Helsinki, Vuorikatu 20, SF-00100 Helsinki, Finland.

0021-9673/92/$05.00

bution ratio of the solute between the micellar phase and the aqueous phase and (3) the chemical reactions between the solute molecules and the micelles.

The synthesis of glyoxal bis(amidinohydrazone) (GBG) was reported by Thiele and Dralle [5] toward the end of the nineteenth century. Sixty years later Freedlander and French [6] synthesized its methylglyoxal analogue (MGBG), which they showed to have strong antileukaemic activity against L1210 leukaemia in mice. These compounds are of great interest because many of them inhibit adenosylmethionine decarboxylase, a key enzyme of polyamine biosynthesis. GBG and MGBG are both potent antileukaemic agents [7,8].

In earlier work we developed a quantitative MECC method for the determination of bis(amidinohydrazones) [9]. At that time we found the elution order of these solutes to differ in inorganic and organic buffer solutions. The quality of the separation was affected by the nature of the buffer solution.

In this work, the suitability of buffer solutions of different strength, with pH adjusted to 7.0, was tested for the separation of eight bis(amidinohydrazones). The buffers were sodium phosphate, tris(hydroxymethyl)aminomethane (Tris), N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES), N,N-dimethylmethylenediamine (DMAEA) and N,N-diethylethylenediamine (DEAEA). N-Cetyl-N,N,N-trimethylammonium bromide (CTAB) was used for coating the inner column and for micelle and ion-pair formation. The separated solutes were detected by UV–VIS method at wavelength 280 nm.

EXPERIMENTAL

Apparatus

MECC was performed in a 680 mm × 0.075 mm I.D. fused-silica capillary tube (SGE, Milton Keynes, UK) where 600 mm was the effective length for separation. A Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) was employed. Detection was at wavelength 280 nm with UV–VIS detection. All experiments were carried out at ambient temperature (*ca.* 24–27°C). Injections were made in hydrostatic mode for 10 or 12 s and the running negative voltage was betweeen 20 and 25 kV. The data were collected with an HP 3392A integrator (Hewlett-Packard, Avondale, USA).

Materials

The synthesis of the free bases of GBG, MGBG and their analogues was carried out as described previously [10]. Sodium dihydrogenphosphate monohydrate, disodium hydrogenphosphate dihydrate, Tris and CTAB were purchased from Merck (Darmstadt, Germany), HEPES was purchased from Sigma (Dorset, UK) and DMAEA and DEAEA were purchased from Fluka (Buchs, Switzerland). All compounds were used as received. Other reagents used in the development of the method were of analytical grade and were used without further purification. Distilled water was purified through a Water-I system from Gelman Sciences (Ann Arbor, MI, USA). All the micellar buffer solutions were filtered using 0.45-μm membrane filters (Millipore, Molsheim, France) and degassed before use. Samples and other solutions were filtered through Millex filters of 0.5-μm pore size from Millipore (Nikon Millipore, Kogyo, Yonezawa, Japan). With Tris, HEPES, DMAEA and DEAEA, the pH was adjusted to 7.0 with 0.1 *M* hydrochloric acid solution.

MECC procedure

To obtain good separation, the capillary was cleaned according to the following procedure each time the buffer solution was changed: the capillary was purged for 15 min with 0.5 *M* potassium hydroxide and then for 2 min with the new buffer solution. In addition, the capillary was purged for 2 min with the working buffer before each injection.

RESULTS AND DISCUSSION

Earlier studies on the protonation equilibria and species distribution of bis(amidinohydrazones) [10] suggested to us that a good separation of these congeners would be achieved by CZE. The species distribution of GBG and MGBG (at pH 7.4 and 37°C) is distinctly different from that of the dialkylglyoxal congeners. Moreover, considerable portions of GBG (*ca.* 10%) and MGBG (*ca.* 4%) exist in the free base form, whereas the diakylglyoxal analogues exist almost exclusively in the mono- and dicationic forms and the proportion of the free base is only

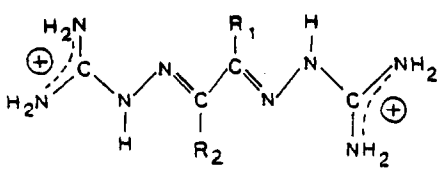
ca. 0.5% or less. The bis(amidinohydrazones) we wished to separate are listed in Table I.

To study the effect of the buffer solution on the elution order and separation, we carried out MECC experiments with five different buffers (sodium phosphate, Tris, HEPES, DMAEA and DEAEA) at pH 7.0. The concentration of the buffers was 0.05, 0.1, 1.2, 0.02 and 0.03 *M*, respectively, and CTAB concentration was 0.001 *M* (CMC [11]). The structures of the buffer solutions are shown in Table II. Sodium phosphate buffer at concentration 0.05 *M* gave the best separation with the lowest currents (Fig. 1). It was also the only buffer in which all eight bis(amidinohydrazone) congeners were fully separated. Accordingly, the operating conditions for this system were optimized to provide good resolution within a reasonable time. Under the optimized conditions (0.05 *M* sodium phosphate buffer with 1 m*M* CTAB, voltage −22 kV and hydrodynamic injection 12 s), the method gave good repeatability and linearity between 2.5 and 100 μg per ml of solute [9].

TABLE I

STRUCTURE OF THE BIS(AMIDINOHYDRAZONES) STUDIED

The *Chemical Abstracts*' systematic name for MGBG is 2,2'-(1-methyl-1,2-ethanediylidine)bis(hydrazinecarboximidamide), and other congeners are named analogously.



Compound	R_1	R_2	M	Cationic form[a]
GBG	H	H	170	MC
MGBG	CH_3	H	184	MC
DMGBG	CH_3	CH_3	198	DC
EMGBG	CH_2CH_3	CH_3	212	DC
DEGBG	CH_2CH_3	CH_2CH_3	226	DC
MPGBG	CH_3	$(CH_2)_2CH_3$	226	DC
MBGBG	CH_3	$(CH_2)_3CH_3$	240	DC
DPGBG	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$	254	DC

[a] The main cationic form of the compound at pH 7.0: MC = monocationic; DC = dicationic.

The elution order of the bis(amidinohydrazone) congeners varied with the buffer. In inorganic buffer solution (sodium phosphate), monocationic solutes eluted first and then the dicationic solutes. Evidently, the symmetric molecules interacted more strongly with the micelles and eluted more slowly than the asymmetric molecules. By contrast, in the organic buffer solutions (Tris, HEPES, DMAEA and DEAEA) the bis(amidinohydrazone) congeners eluted in decreasing size order, except for the monocationic molecules (GBG and MGBG), which behave irregularly. The concentration of the buffer and of CTAB did not affect the elution order of the solutes, only the resolution. The elution orders and relative retention times are listed in Table III.

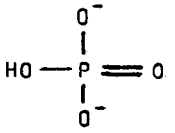
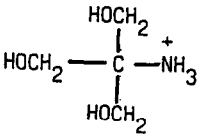
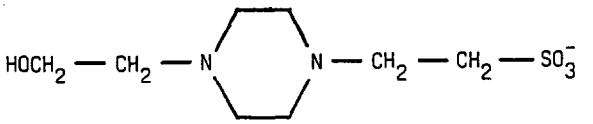
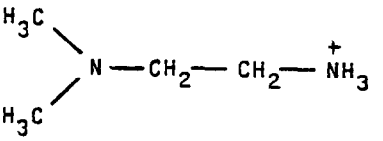
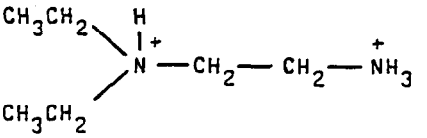
In phosphate buffer the elution order was determined by the cationic nature and structure of the compounds (Fig. 1, Table III). Because GBG and MGBG are more monocationic than the other congeners, they eluted first. The dicationic molecules with an alkyl chain even one carbon atom shorter eluted more slowly than the molecules with longer alkyl chains, because of their stronger electrophoretic mobility and more intense interaction with the micelles. In addition, the symmetric molecules apparently reacted more strongly with the micelles and eluted more slowly than the asymmetric molecules.

The elution order in Tris differed from that in phosphate buffer solution (Fig. 2A, Table III). The Tris molecule is hydrophilic and at pH 7.0 the structure contains three free polar hydroxyl groups and one NH_3^+ group. The dicationic molecules with long alkyl chains eluted more quickly than the molecules with short alkyl chains, because they undergo stronger interactions with the slowly eluting micelles. The two monocationic compounds eluted according to their molecular weight in separation conditions (hydrophilic eluent), because the methyl substituent is more hydrophobic than hydrogen (Table I).

The elution order of bis(amidinohydrazones) was different in HEPES solution (Fig. 2B, Table III). The compounds of high molecular weight eluted before those of low molecular weight. HEPES and bis(amidinohydrazone) molecules can form ion pairs because a HEPES molecule contains one negatively charged group (Table II). There are also hydrogen bonds between these buffer molecules and

TABLE II

STRUCTURES OF THE TESTED BUFFER IONS AT pH 7.0 WITH THEIR pK_a VALUES

Buffer ion	Structure
Sodium phosphate (pK_{a_2} 7.21) [12]	
Tris (pK_{a_1} 8.09) [13]	
HEPES (pK_{a_1} 7.57) [13]	
DMAEA (pK_{a_2} 6.49) [13]	
DEAEA (pK_{a_2} 7.25) [13]	

solutes. The complex is more stable and elutes more slowly the shorter the alkyl chains of the solute, owing to interaction with slowly eluting micelles.

There is only one positively charged group in the DMAEA molecule at pH 7.0 under the separation conditions tested (Table II). Non-polar interactions should occur between DMAEA molecules and bis-(amidinohydrazones) in polar media. Under these

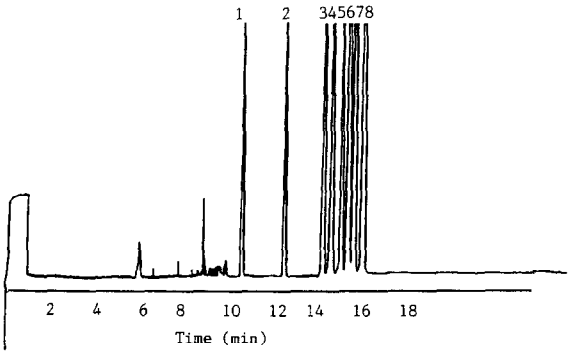


Fig. 1. Electropherogram of eight bis(amidinohydrazones) (25 μg per ml of solute) in 0.05 *M* sodium phosphate buffer with 1 m*M* CTAB. Capillary: 68 cm × 75 μm I.D.; pH 7.0; hydrodynamic injection mode: 12 s at 10 cm height; detector: UV at 280 nm; applied voltage: −22 kV; temperature: ambient. Peaks: 1 = GBG; 2 = MGBG; 3 = MBGBG; 4 = DPGBG; 5 = MPGBG; 6 = DEGBG; 7 = EMGBG; 8 = DMGBG [9].

forces, solutes, buffer ions and micelle molecules form large positively charged complexes. In the case of bis(amidinohydrazones) the charge of the complex is more positive when alkyl chains are long. In polar solutions a complex with large radius elutes before one with a shorter radius. Because monocations have shorter alkyl chains the complexes formed with the monocationic solutes GBG and MGBG are smaller and less stable than those formed with the dicationic solutes, and they eluted late (Fig. 3A, Table III). The dicationic compounds eluted in order of decreasing molecular weight. The monocationic compounds eluted between EMGBG and DMGBG, but in order of increasing molecular weight because in the electro-osmotic flow large particles elute more quickly than small ones.

The DEAEA molecule has two positively charged groups at pH 7.0 under the separation conditions tested (Table II). The non-polar interactions occur in the same way in DEAEA as in DMAEA and the elution order of the bis(amidinohydrazones) is the same (Fig. 3B). In the case of GBG, the micelle and DEAEA molecules are competing for the same bonding place (two carbon atoms and the bond between them in the middle of the GBG molecule, see Table I), and the complex is positively charged. MGBG has room for one more positively charged molecule than GBG, and so the MGBG complex is more positively charged than the GBG complex. DMGBG forms an even more positively charged complex, and the elution order is GBG and then MGBG and DMGBG (Fig. 3B, Table III).

TABLE III

ELUTION ORDER OF BIS(AMIDINOHYDRAZONES) WITH RELATIVE RETENTION TIMES RELATIVE TO DPGBG IN THE DIFFERENT BUFFER SOLUTIONS AT pH 7.0 AND WITH 1 m*M* CONCENTRATION OF CTAB

Elution order	Tested buffer solutions with relative retention times				
	Sodium phosphate (0.05 *M*)	Tris (0.1 *M*)	HEPES (1.2 *M*)	DMAEA (0.02 *M*)	DEAEA (0.03 *M*)
I	GBG, 0.72	DPGBG, 1.00	DPGBG, 1.00	DPGBG, 1.00	DPGBG, 1.00
II	MGBG, 0.85	MBGBG, 1.03	MBGBG, 1.05	MBGBG, 1.05	MBGBG, 1.03
III	MBGBG, 0.97	DEGBG MPGBG, 1.08	DEGBG MPGBG, 1.12	DEGBG MPGBG, 1.13	DEGBG MPGBG, 1.08
IV	DPGBG, 1.00	GBG, 1.14	EMGBG, 1.21	EMGBG, 1.24	EMGBG, 1.12
V	MPGBG, 1.03	EMGBG, 1.16	DMGBG, 1.34	GBG, 1.28	GBG, 1.15
VI	DEGBG, 1.05	MGBG, 1.20	MGBG, 1.38	MGBG, 1.37	MGBG DMGBG, 1.20
VII	EMGBG, 1.07	DMGBG, 1.24	GBG, 1.43	DMGBG, 1.40	
VIII	DMGBG, 1.10				

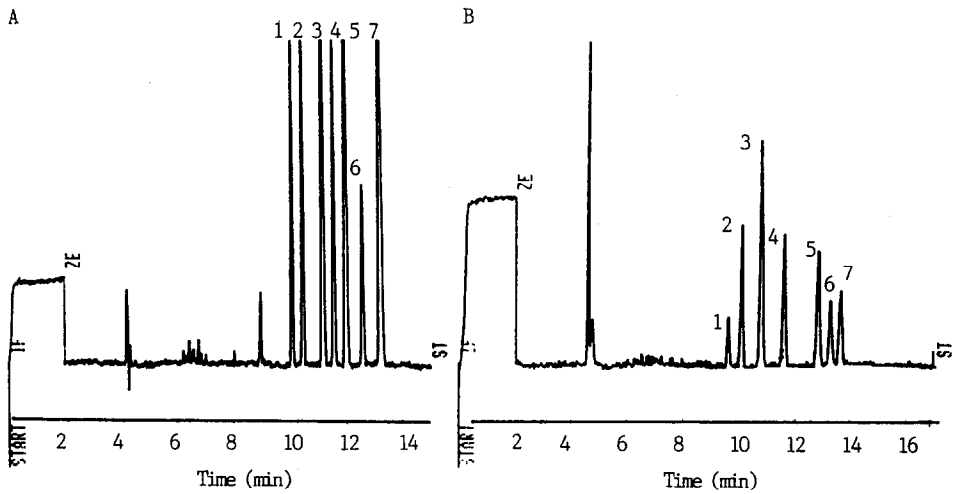


Fig. 2. (A) Electropherogram of eight bis(amidinohydrazones) (25 μg per ml of solute) in 0.1 *M* Tris buffer with 1 m*M* CTAB. Experimental conditions as in Fig. 1. Peaks: 1 = DPGBG; 2 = MBGBG; 3 = DEGBG and MPGBG; 4 = GBG; 5 = EMGBG; 6 = MGBG; 7 = DMGBG. (B) Electropherogram of eight bis(amidinohydrazones) (25 μg per ml of solute) in 1.2 *M* HEPES buffer with 1 m*M* CTAB. Experimental conditions as in Fig. 1. Peaks: 1 = DPGBG; 2 = MBGBG; 3 = DEGBG and MPGBG; 4 = EMBGB; 5 = DMGBG; 6 = MGBG; 7 = GBG.

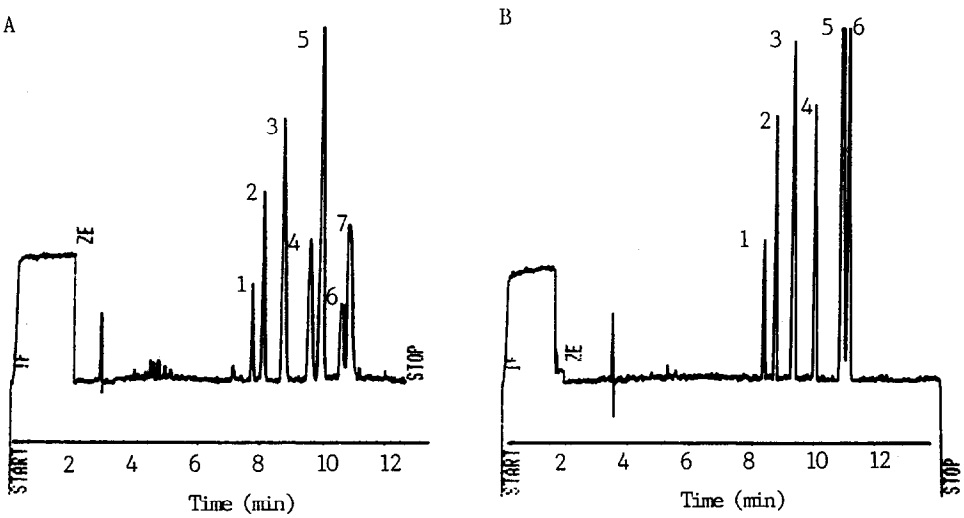


Fig. 3. (A) Electropherogram of eight bis(amidinohydrazones) (25 μg per ml of solute) in 0.02 *M* DMAEA buffer in 1 m*M* CTAB. Experimental conditions as in Fig. 1 except the applied voltage was −25 kV. Peaks: 1 = DPGBG; 2 = MBGBG; 3 = DEGBG and MPGBG; 4 = EMGBG; 5 = GBG; 6 = MGBG; 7 = DMGBG. (B) Electropherogram of eight bis(amidinohydrazones) (25 μg per ml of solute) in 0.03 *M* DEAEA buffer with 1 m*M* CTAB. Experimental conditions as in Fig. 1 except the applied voltage was −20 kV. Peaks: 1 = DPGBG; 2 = MBGBG; 3 = DEGBG and MPGBG; 4 = EMGBG; 5 = GBG; 6 = MGBG and DMGBG.

CONCLUSIONS

MECC is an effective method for the separation of bis(amidinohydrazones). In phosphate buffer solution eight aliphatic congeners were fully separated. Under the other buffer conditions, MPGBG and DEGBG eluted with the same retention time. The elution order of bis(amidinohydrazones) is determined by the cationic nature and structure of the compounds. In inorganic buffer solution adjusted to pH 7.0, monocationic solutes elute first and then the dicationic. The monocationic nature of GBG and MGBG has less effect on the elution order of bis(amidinohydrazones) in organic than in inorganic buffer solutions. Ion-pair formation between solutes and buffer ions has a strong effect on the elution order.

Factors affecting the elution order of bis(amidinohydrazones) in MECC are: (1) ion-pair formation between solute and buffer ion; (2) the cationic nature and structure of the solute; (3) reactions between ion-pair complexes and micelles; and (4) the nature of the buffer solution.

REFERENCES

1 S. Terabe, K. Otsuka, K. Ichikava, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
2 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
3 A. S. Cohen, S. Terabe, J. A. Smith and B. L. Karger, *Anal. Chem.*, 59 (1987) 1021.
4 H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, *J. Chromatogr.*, 465 (1989) 331.
5 J. Thiele and E. Dralle, *Liebig's Ann. Chem.*, 302 (1898) 275.
6 B. L. Freedlander and F. A. French, *Cancer Res.*, 18 (1958) 360.
7 H. G. Williams-Ashman and A. Schenone, *Biochem. Biophys. Res. Commun.*, 46 (1972) 288.
8 L. Alhonen-Hongisto, P. Seppänen, P. Nikula, H. Elo and J. Jänne, *Recent Prog. Polyamine Res.*, (1985) 261.
9 P. Lukkari, J. Jumppanen, K. Jinno, H. Elo and M.-L. Riekkola, *J. Pharm. Biomed. Anal.*, in press.
10 H. Elo, *Ph. D. Thesis*, Helsinki, 1989, and references therein.
11 J. Neugebauer, *A Guide to the Properties and Uses of Detergents in Biology and Biochemistry*, Calbiochem, San Diego, CA, 1988, p. 26.
12 M. Windholz (Editor), *The Merck Index*, Merck & Co., Inc., Rahway, NJ, 1983.
13 A. E. Martell and R. M. Smith, *Critical Stability Constants*, Plenum Press, New York, 1982.