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Determination of bis(amidinohydrazones) by micellar electrokinetic capillary chromatography

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Abstract: A micellar electrokinetic capillary chromatography method was developed for the separation and determination of aliphatic congeners of bis(amidinohydrazones) in standard solution. Eight bis(amidinohydrazones) could be determined in less than 15 min at an applied voltage of 22 kV, using 0.05 M sodium phosphate as buffer (pH 7.0) together with 1 mM *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide. Hydrostatic sample injection was employed. The method exhibited good repeatability and a linear range of 2.5–100 μg ml^{-1}. A detection limit of 1 μg ml^{-1} was achieved. The method also allows the determination of bis(amidinohydrazones) in human serum samples.

Keywords: *Bis(amidinohydrazones); bis(guanylhydrazones); micellar electrokinetic capillary chromatography; CTAB surfactant.*

Introduction

Electrophoresis in open tubes (0.2–0.5 mm i.d.) was described by Virtanen in 1974 [1]. The more powerful technique of capillary zone electrophoresis (CZE) has developed rapidly since Jorgenson and Lukacs [2, 3] first realized the advantages of using fused silica capillary tubes of less than 100 μm i.d. In CZE, charged species can be efficiently separated on the basis of differences in electrophoretic mobilities (μ_{ep}).

In an electric field, electro-osmosis occurs in an electrolyte-filled fused-silica capillary. This electro-osmotic flow (EOF) has essentially a flat flow profile and a mobility μ_{eo}. Typically $\mu_{eo} > \mu_{ep}$ and this forces all solutes (anionic, cationic and neutral) to the cathode end of the capillary. Anionic compounds move to the cathode because the electro-osmotic flow is much stronger than the electrophoretic mobility of the charged particles. Under the influence of EOF, neutral particles migrate with the same velocity as the electrolyte.

In 1984 Terabe *et al.* [4] introduced micellar electrokinetic capillary chromatography (MECC) an adaptation of CZE where the addition of an ionic surfactant to the electrolyte facilitates the separation of neutral particles. It is thought that chromatographic distribution principles are involved. Although MECC is useful for the separation of electrolytically neutral compounds, it is also effective for separating ionic compounds that cannot be adequately resolved by CZE [5, 6] aiming to similar electrophoretic mobilities. In MECC, the migration time of an ionic substance is a function of four factors: (1) the electrophoretic mobility of the solute; (2) the distribution ratio of the solute between the micellar phase and the aqueous phase; (3) the reactions between the solute molecules and the micelles; and (4) the magnitude of EOF (i.e. pH, ionic strength and viscosity).

The synthesis of glyoxal bis(amidinohydrazone) (GBG) has been reported by Thiele and Dralle [7] at the end of the 19th century. Sixty years later Freedlander and French [8] synthesized its methylglyoxal analogue (MGBG), which they showed to have strong anti-leukemic activity against L1210 leukemia in mice. This discovery was the starting point for a sudden increase in literature publication [8–11] on the properties of bis(amidinohydrazones). These compounds are of great interest because many of them inhibit adenosylmethionine decarboxylase (AdoMetDC), a key enzyme of polyamine biosynthesis. Gly-

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oxal bis(amidinohydrazone) (GBG) and its methyl analogue (MGBG) are both potent anti-leukemic agents [8].

In this work an MECC method was developed for the separation and determination of eight bis(amidinohydrazones) in standard solution and the factors affecting the separation and elution order were evaluated. The method was also applied to the determination of GBG and MGBG in spiked human serum samples.

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 (Milipore Corporation, Waters Chromatography Division, Milford, MA, USA) was employed. Detection was at 280 nm. All experiments were carried out at ambient temperature. Injections were carried out hydrostatically for 12 s and the running voltage was −22 kV. The data (peak area) were collected with an HP 3392A integrator (Hewlett–Packard, Avondale, PA, USA).

Materials

The synthesis of the free bases of GBG, MGBG and their analogues were carried out as previously described [12]. Sodium dihydrogenphosphate monohydrate ($NaH_2PH_4{}^*H_2O$), disodium hydrogenphosphate dihydrate ($Na_2HPO_4{}^*2H_2O$) and *N*-cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) were purchased from Merck (Darmstadt, Germany) and 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 Inc. (Ann Arbor, MI, USA). All the micellar buffer solutions were filtered using 0.45 μm a 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 (Nihon Millipore, Kogyo, K.K. Yonezawa, Japan).

Procedure

To ensure a reproducible separation, the capillary was treated according to the following procedure prior to each buffer change: the capillary was purged for 15 min with 0.5 M KOH and then for 2 min with the new buffer solution. The capillary was also purged for 2 min with the working buffer before each injection.

Preparation of the spiked serum samples

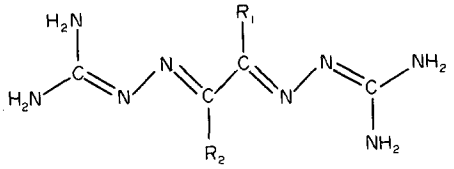
Human blood serum (0.9 ml) was spiked with 0.1 ml of GBG or MGBG solution. The serum samples were filtered into Centrisart I SM 13229E ultrafiltration tubes (5000 molecular cut off) (Sartorius GmbH, Göttingen, Germany) and centrifuged (2800 rpm) for 1 h. A 0.4 ml volume of the filtrate (0.5 ml) was then analysed.

Results and Discussion

Earlier study of the protonation equilibria and species distribution of bis(amidinohydrazones) [12] suggested the possible utilization of CE for congener separation. 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 dialkylglyoxal analogues exist almost exclusively in the mono- and dicationic forms with the proportion of the free base being less than 0.5%. Reports of the separation of some bis(amidinohydrazones) (MGBG) [13–17] by HPLC have appeared in the literature.

To optimize the conditions for the separation of eight bis(amidinohydrazone) congeners in a single MECC run, we studied the effects of CTAB concentration, applied voltage, buffer type and buffer concentration (0.01–0.05 M) at pH 7.0. CTAB concentration was 0.001 M (the critical micellization concentration, CMC [18]). CTAB concentrations of 0.002, 0.01 and 0.05 M were also tested. When the concentration was greater than that of the CMC, the μ_{eo} was increased and the compounds were eluted faster. However, the separation was not improved. A −22 kV voltage was found to give the best separation with acceptable current levels. Ammonium, potassium and sodium phosphate buffer salts were tested. Sodium phosphate buffer (0.05 M) with 0.001 M of CTAB gave the most acceptable results (the best separation with the lowest currents). The operating conditions were then

Table 1
Structures of the bis(amidinohydrazones) studied



Compound	R_1	R_2
GBG	H	H
MGBG	CH_3	H
DMGBG	CH_3	CH_3
EMGBG	CH_2CH_3	CH_3
DEGBG	CH_2CH_3	CH_2CH_3
MPGBG	CH_3	$(CH_2)_2CH_3$
MBGBG	CH_3	$(CH_2)_3CH_3$
DPGBG	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$

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

optimized to achieve good resolution within a reasonable time. The tested solutes are listed in Table 1.

Figure 1 shows three electropherograms of seven bis(amidinohydrazones) at different sodium phosphate buffer concentrations. Solutes were identified via spiking. The resolution improved as the concentration was increased from 0.01 to 0.05 M, but time for separation was increased from 13 to 15 min.

Figure 2 shows the separation of eight bis(amidinohydrazones) which were also identified via spiking. The elution order was appeared to be according to the cationic nature and structure of these compounds under the separation conditions (pH 7.0). GBG and MGBG have more free basic form than the other congeners and therefore they elute first. The dicationic molecules with shorter alkyl

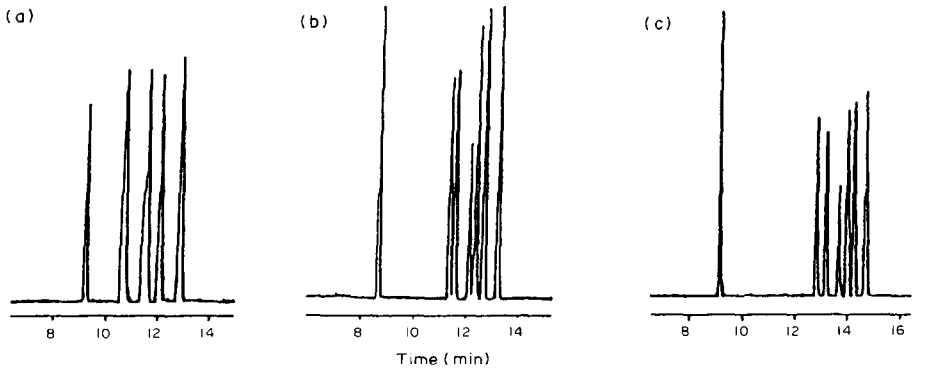


Figure 1
Electropherograms of seven aliphatic bis(amidinohydrazone) congeners (25 μg ml^{-1} of solute). Capillary: 68 cm × 75 μm i.d.; buffer: sodium phosphate with 1 mM CTAB; pH 7.0; hydrodynamic injection mode: 12 s at 10 cm height; detector: UV at 280 nm; applied voltage −22kV; temperature: ambient. Elution order GBG, MBGBG, DPGBG, MPGBG, DEGBG, EMGBG and DMGBG. Solute symbols are given in Table 1. Buffer concentration: (a) 0.01, (b) 0.03 and (c) 0.05 M.

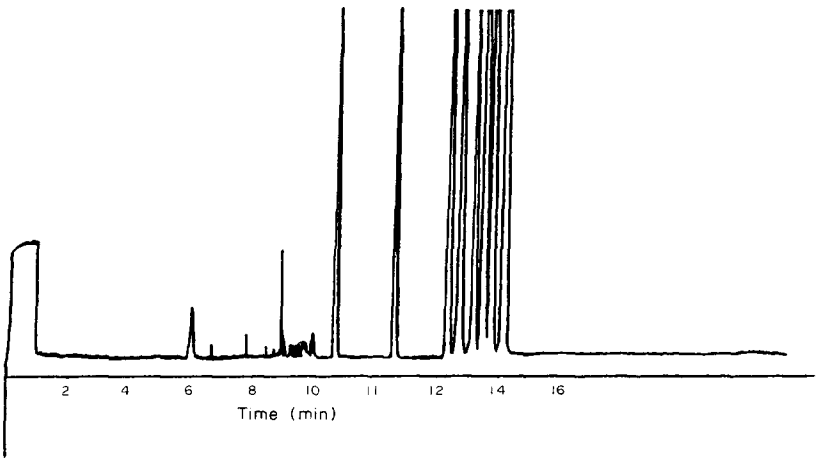


Figure 2
Electropherogram of eight bis(amidinohydrazones) (25 μg ml^{-1} of solute). Experimental conditions as in Fig. 1(c). Elution order: GBG, MGBG, MBGBG, DPGBG, MPGBG, DEGBG, EMGBG and DMGBG.

chain elute more slowly than the molecules with longer alkyl chain, because of their stronger electrophoretic mobility and more intense interaction with the micelles. Symmetric molecules appear to have stronger interaction with the micelles and elute more slowly than asymmetric molecules. The concentration of the buffers and CTAB did not affect the elution order of bis(amidinohydrazones).

Linearity was tested over the range 2.5–100 μg ml^{-1} ($n = 6$). The correlation coefficients of the linearity curves varied from 0.998 to 1.000. The limit of detection was 1 μg ml^{-1} (was determined i.e. $3 \times S/N$). Repeatability of the method was determined at two concentration levels, namely, at 5 and 35 μg ml^{-1}. Relative standard deviations varied from 5.8 to 14.5% ($n = 6$) at the 5 μg ml^{-1} level and from 1.7 to 8.1% ($n = 6$) at the 35 μg ml^{-1} level. DPGBG (35 μg ml^{-1}) was included in these samples as internal standard. Quantitative data for a standard mixture of bis(amidinohydrazones) is shown in Table 2. The

Table 2
Quantification data for the standard samples

	GBG	MGBG	DMGBG	EMGBG	DEGBG	MPGBG	MBGBG
Linearity: 2.5–100 μg ml^{-1}							
r	0.998	0.998	1.000	0.999	0.999	1.000	—
a	−0.043	−0.010	0.045	0.052	0.044	0.026	—
b	0.032	0.013	0.038	0.033	0.032	0.018	—
Repeatability of the method at concentration 5 μg ml^{-1}							
n	6	6	6	6	6	6	6
X	0.159	0.078	0.261	0.227	0.226	0.130	—
SD	0.019	0.008	0.038	0.018	0.018	0.007	—
RSD (%)	12.1	10.6	14.5	8.1	8.1	5.8	—
Repeatability of the method at concentration 35 μg ml^{-1}							
n	6	6	6	6	6	6	6
X	0.936	0.435	1.333	1.179	1.160	0.689	0.989
SD	0.076	0.034	0.070	0.041	0.025	0.012	0.037
RSD (%)	8.1	7.7	5.3	3.5	2.2	1.7	3.7
Repeatability of the injection at concentration 35 μg ml^{-1}							
n	6	6	6	6	6	6	6
X	0.808	0.393	1.518	1.287	1.2325	0.710	0.956
SD	0.060	0.031	0.038	0.040	0.037	0.025	0.013
RSD (%)	7.4	7.9	2.5	3.1	3.0	3.5	1.4

In every analysis 35 μg ml^{-1} DPGBG was included as internal standard.
The equation for the straight line is $Y = aX + b$. *r* is the correlation coefficient, *a* the point at which the line crosses the *Y* axis and *b* the slope, *n* is the number of repetitions, *X* the mean of (peak area of analyte)/(peak area of DPGBG).

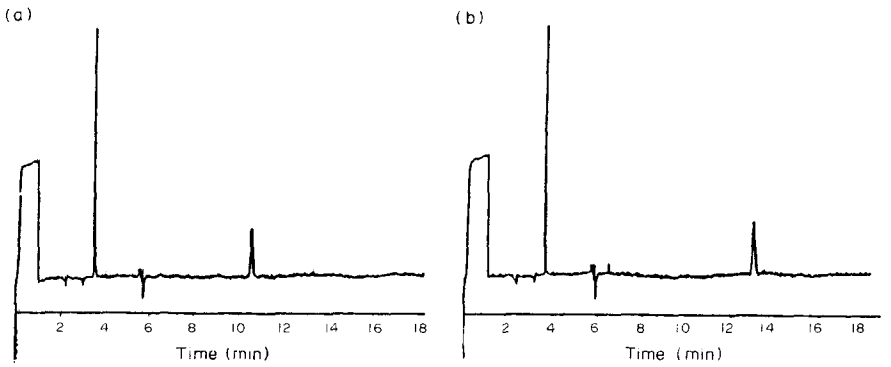


Figure 3
Electropherograms of spiked serum samples. (a) 10 μg ml^{-1} of GBG in human serum. (b) 10 μg ml^{-1} of MGBG in human serum. Experimental conditions as in Fig. 1(c).

high RSDs are thought due the injection technique.

Spiked serum samples were also analysed by the MECC method. Figure 3 shows two electropherograms of GBG and MGBG (i.e. the only aliphatic congeners that have been shown to possess antileukemic activity *in vivo*) [12] present at a concentration of 10 μg ml^{-1} in human serum. The separation conditions were the same as in Fig. 1(c). The first sharp peak and the negative peak of the electropherograms are attributable to the serum effects.

Although the RSD values for the repeatability of hydrostatic injection were high, the method itself has been shown to be linear over the range 2.5–100 μg ml^{-1}. Use of shorter injection time (12 s) can improve repeatabilities, however, the limit of detection suffers. If it should be necessary to improve the quantification further, DPGBG is not a good choice as internal standard for GBG and MGBG because it differs considerably from them in the species distribution.

Conclusions

MECC has been shown to be an effective method for the separation and determination of bis(amidinohydrazones) in standard mixtures and GBG and MGBG in spiked human serum samples. Under the conditions described, eight aliphatic congeners of bis(amidinohydrazones) can be separated in less than 15 min. The method is repeatable and linear over the range 2.5–100 μm ml^{-1}. A detection limit of 1 μg ml^{-1} was achieved.

The repeatabilities of hydrostatic injections are not satisfactory but can be improved with shorter injection time when sensitivity is not important.

The elution order of bis(amidinohydrazones) is determined by the cationic nature and structure of these compounds under separation conditions. Other factors affecting the elution order of bis(amidinohydrazones) will be studied in the authors' laboratory.

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