Korean J. of Chem. Eng., **6**(3) (1989) *179-184*

EVALUATION OF MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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(Received 21 June 1988 • accepted 18 January 1989)

Abstract—Micellar Electrokinetic Capillary Chromatography employs electroosmotically pumped micellar mobile phases to achieve very efficient separations. A mixture of 14 modified nucleic acid components can be separated with 0.0081 and 0.075 M sodium dodecyl sulfate in phosphate/borate buffer, 10 kv of separation voltage, and a 68.5 cm × 0.06 mm I.D. fused-silica capillary tubing. Efficiencies in excess of 100,000 plates/m are routinely attained. The comparison between this technique and High Performance Liquid Chromatography is discussed in terms of column efficiency.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC) is a recently introduced separation technique which combines many of the operational principles and advantages of micellar liquid chromatography [1,2] and capillary zone electrophoresis [3]. In capillary zone electrophoresis, charged solutes are efficiently separated in narrow-bore capillary tubes based on differences in their electrophoretic mobilities. The "plug-like" flow profiles for mobile phases in electrophoresis minimize dispersion due to resistance to mass transfer. Electric potentials of tens of kilovolts per meter of column length are employed to drive charged solutes through the capillary columns. When a potential is placed across a column filled with an electrolyte solution, a flow is induced in the column which is known as electroosmotic flow. Electroosmotic flow provides another means of transporting solutes through a column. Differences in the viscous drag of neutral solutes, primarily as a result of size differences, can result in their separation. However, these differences are usually very small and, consequently, capillary zone electrophoresis is not very useful for separating structurally similar neutral compounds.

The addition of a surfactant to the electrophoretic mobile phase, at a concentration above its critical micelle concentration (CMC), affords and effective mechanism for separating neutral compounds. Neutral solutes are separated based on their differential partitioning between an electroosmotically pumped aqueous mobile phase and the hydrophobic interior of the micelles, which are charged and moving at a velocity different than that of the mobile phase due to electrophoretic effects. The separation mechanism is much same as observed in traditional liquid-liquid chromatography, with the micellar phase functioning as "pseudo-stationary phase". But the advantages of the MECC are the selectivity by electrostatic, hydrophobic, and steric interactions, lower cost, and greater safety of micellar solutions, while the sample are limited to an analytical application.

Since this system was first reported by Terabe et al. [4] for the separation of phenolic compounds, the technique has been treated for the various samples including substituted benzenes [5], phenylthiohydantoin amino acids [6], metabolites of vitamin B_6 [7], nitrated polyaromatic hydrocarbons [8], purines [9], amines [10], more recently, modified nucleic acid constituents [11,12].

In this paper, the samples are 14 normal and modified deoxyribonucleosides, deoxyribonucleotide mononucleotides, a ribonucleoside, and a pyrimidine. The purpose of this work is to determine the optimum micellar concentration of Sodium Dodecyl Sulfate (SDS) and to investigate the effects of separation voltage on resolution. The performance data between MECC and HPLC (High Performance Liquid Chromatography) will be compared.

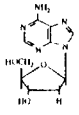
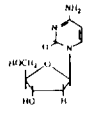
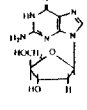
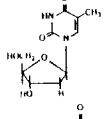
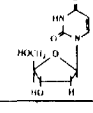
EXPERIMENTAL

1. Experimental materials and apparatus

The normal and modified nucleic acid components

used in the experimental were 2'-deoxyadenosine (2'-dA), 2'-deoxycytidine (2'-dC), 2'-deoxyguanosine (2'-dG), 2'-deoxythymidine (2'-dT), 2'-deoxyuridine (2'-dU), 5-methyl deoxycytidine (MeDC), 5-Bromo-deoxyuridine (BrDU), 5-Chlorouracil (ClU), 8-Bromo Guanosine (BrG), Deoxy Inosine (DI), 2'-deoxyadenosine 5'-monophosphate (dAmp), 2'-deoxycytidine 5'-monophosphate (dCmp), 2'deoxyguanosine 5'monophosphate (dGmp), and thymidine 5'-monophosphate (dTmp). BrG was synthesized in the Oak Ridge National Laboratory (Tennessee, U.S.A.), and the others were obtained from Sigma (St. Louis, MO, U.S.A), Aldrich (Milwaukee, WI, U.S.A.), and Pharmacia Chemical Co.. The characteristics of the normal deoxyribonucleosides are listed in Table 1. SDS and other buffers were from the Aldrich Chemical Co., (Piscataway, NJ, U.S. A.). Acetone was added into the components to measure the relative retention time. The column of 60 μm I.D. was fused-silica capillary tubing (Austin, TX, U.S. A.), and the effective column length to the UV detector was 68.5 cm. High voltage power supply was provided by Hipotronics (Brewster, NY) which could be extended to 40 kv. The two ends of the capillary column put into two reservoirs respectively, and the surface of the small section in the column was removed for UV detector of JASCO (UVIDEC-100-III Spectrophotometric detector, Japan Spectroscopic Co.). Its signals were continuously displayed on the strip chart recorder. Using the duplicate plexiglass boxes, the operator was protected from contact with high voltage.

Table 1. Structures of normal deoxyribonucleosides

Sample	Structure	Formula	Molecular Weight
2'-dA		$C_{10}H_{13}N_5O_3$	251.24
2'-dC		$C_9H_{13}N_3O_4$	227.22
2'-dG		$C_{10}H_{13}N_5O_4$	267.24
2'-dT		$C_{10}H_{14}N_2O_5$	242.23
2'-dU		$C_9H_{12}N_2O_5$	228.20

2. Experimental methods

The column was rinsed with 0.1 M HCl solution for several hours to deactivate the inner surface of the column, and filled with the mobile phase containing Sodium Dodecyl Sulfate (SDS). Four different concentrations of the SDS (0.008, 0.05, 0.075, and 0.10 M) were used with 0.01 M Na_2HPO_4 and 0.006 M $Na_2B_4O_7$. Sample introduction was accomplished by electroinjection [7]. The reservoir at high voltage (positive) end of the column was removed and replaced with a reservoir containing the sample. Voltage was applied for a small amount of time, after which the reservoir containing the mobile phase was replaced and the separation was allowed to proceed. The column was frequently rinsed with the mobile phase to keep the electric current constant. Wavelength in the UV detector was set at 256 nm for all experimental run.

RESULTS AND DISCUSSION

1. Effect of separation voltage

When a SDS is used as the surfactant in a phosphate/borate buffer, electroosmotic flow is toward the negative electrode. The negatively charged micelles are attracted to the positive electrode. The velocity of the electroosmotic flow is greater than the electrophoretic velocity of the micelles, therefore the micelles migrate toward the negative electrode. Since the velocity of the electroosmotic flow is so large, positively charged, neutral, and negatively charged solutes can be separated at the same end of the column. Figure 1 shows the separation of the five mononucleosides. The operational parameters of electroinjection procedures were 5 kv and 5 sec, respectively for injection voltage and duration of the injection process to minimize the contribution of the injection to band broadening. The separation voltage of(a) was 30 kv, and that of (b) 15 kv. By decreasing the voltage alone, 2'-dC and 2'-dA could be separated. In this system, the separation voltage may be deemed as the gas flow rate in the gas chromatography. That is, decreasing the voltage makes longer the retention time of the sample, but it can improve the degree of resolution.

Even in lower voltage of 5 kv, however, the four mononucleotides were not completely separated, and the dAmp was only resolved from the other three mononucleotides. When the mono and modified nucleosides are dissolved in the mobile phase, they show neutral charges, while the mononucleotides have negative charges because of the phosphate group contained. The micelles of SDS are charged and moving at

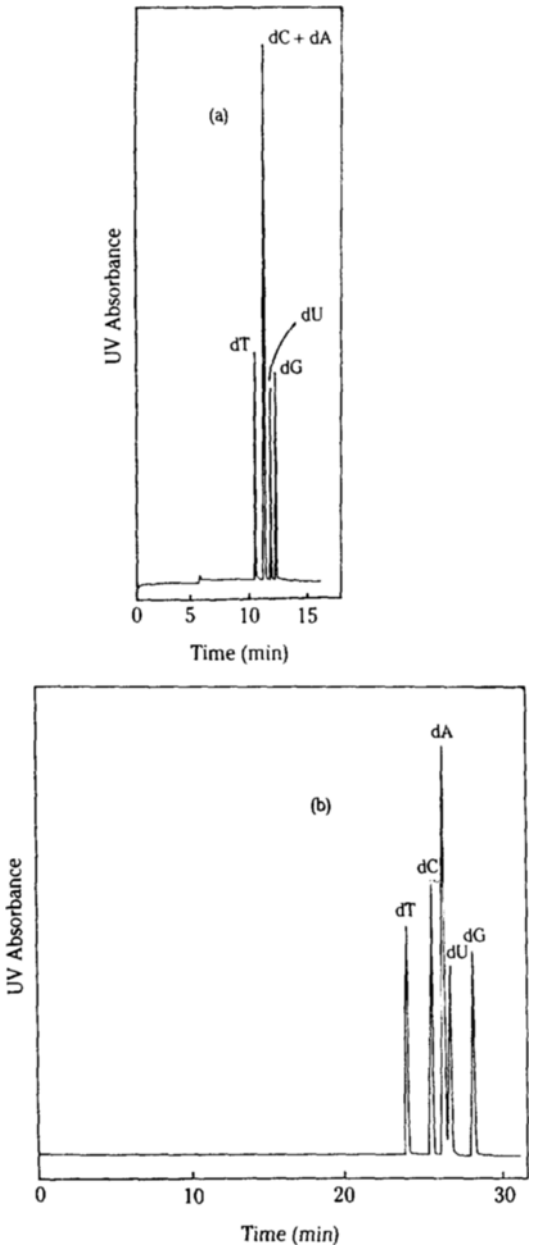


Fig. 1. Separation of mononucleosides by MECC.
(current = (a) 0.04 mA, (b) 0.02 mA, injection time = 30 sec, injection voltage = 5 kv, separation voltage = (a) 30 kv, (b) 15 kv, 0.05 M SDS)

a velocity which is much lower than the mobile phase and opposite in direction due to electrophoretic effects. At the lower separation voltage, the mononucleotides are coeluted, but the higher separation voltage of 40 kv can resolve the components (see Fig. 2). This suggests that the mononucleotides be distributed by their different partition equilibriums between the micelle and the phosphate/borate aqueous phase. However at such high voltage, as the mono and modified nucleosides eluted fast, their resolutions became worse.

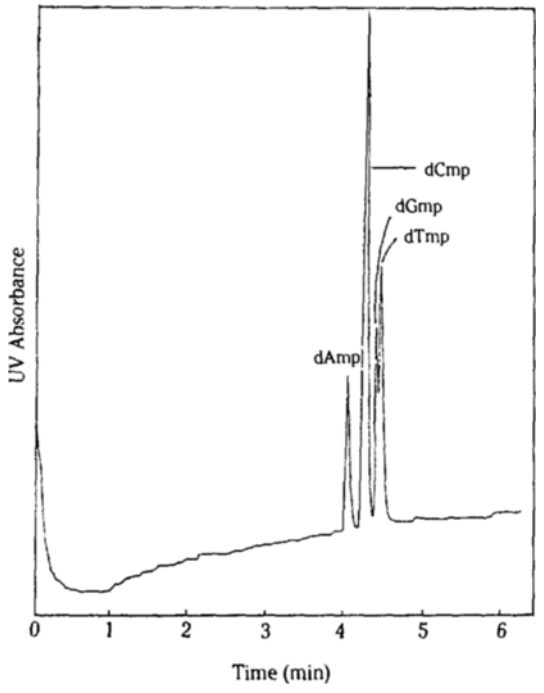


Fig. 2. Separation of mononucleotides by MECC.
(current = 0.11 mA, injection time = 5 sec, injection voltage = 5 kv, separation voltage = 40 kv, 0.05 M SDS)

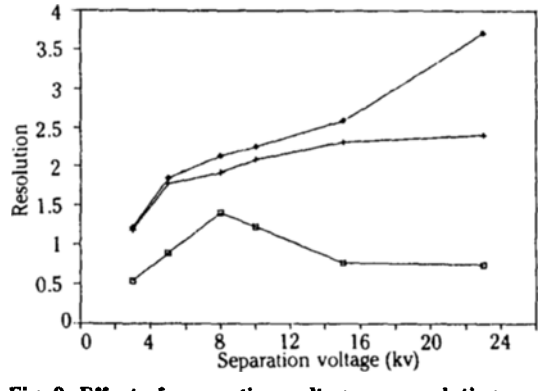


Fig. 3. Effect of separation voltage on resolution.
□ dU/MedC, + dA/dG, ◇ dAMP/dGMP

The resolution of some components is shown in Figure 3, and it is defined by,

$$R_{ij}=2(t_{Rj}-t_{Ri})/(w_j+w_i) \quad (1)$$

where R_{ij} = resolution between components i and j.

For the resolution between dU and MeDC, the optimum value exists in the separation voltage of 8 and 10 kv. But better resolution is achieved with increase in separation voltage for the $R_{2'\text{-}dA,\ 2'\text{-}dG}$ and $R_{dAmp,dGmp}$. The two pairs have the similar structures of the cyclic compound, and it can be said that differences in their electrophoretic mobilities improve the resolution, as the voltage increases to 40 kv. The separation voltage of 8 and 10 kv is recommended to separate a mixture of the normal and modified nucleic constituents.

2. Effect of concentration of SDS

The mononucleotides are negatively charged, and some separation on the basis of electrophoretic mobility differences is possible. Cohen et al.[12] added the metal ions into mobile phase to separate a mixture of six oligonucleotides of eight bases. The selectivity of the solutes is enhanced via differential complexation with metals of Cu, Zn, and Mg added to the SDS mobile phase.

The properties of sodium decylsulfate (STS), docecylrimethyl ammonium chloride (DATC), cetryltrimethylammonium chloride (CATC) micellar solutions as well as SDS were compared as mobile phase [13], and the results of the experiments suggested that the SDS was the most versatile mobile phase for use with MECC. The use of the SDS system resulted in a larger elution range. To take advantage of this range, the conditions of the mobile phase should be optimized for the SDS. Therefore, in this work, the SDS concentration was changed from 0.008 to 0.1 M. In MECC a surfactant is generally added to the mobile phase above its CMC. which is 0.0081 M in the SDS [14]. The

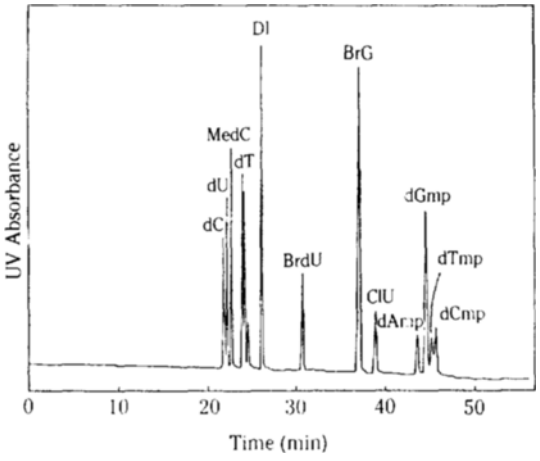


Fig. 4. Separation of modified nucleic acid components by MECC.

(current = 0.028 mA, injection time = 5 sec, injection voltage = 5 kv, separation voltage = 8 kv, 0.008 M SDS)

Table 2. Effect of SDS concentration on column efficiency*

components/SDS conc.	0.008 M	0.05 M	0.075 M	0.1 M
dT	137,600	57,600	130,700	42,800
dG	125,000	57,600	105,000	**
DI	118,400	70,800	99,900	38,900
BrdU	152,100	46,600	100,100	35,300
dAmp	194,000	80,700	107,000	43,541

* : 5 sec and 5 kv of injection procedure, 10 kv separation voltage.

**: The dG was coeluted with dA.

resulting micelles are supposed to provide an effective mechanism for separating neutral and charged solutes. Figure 4 shows the separation of the components in 0.008 M of SDS, which is slightly below the CMC of SDS. The separation was completed about 40 min. The effect of SDS concentration on the column efficiency is listed in Table 2. The number of theoretical plates, N, is calculated by the following equation [15],

$$N = 16\,(t_{Ri}/w_i)^2 \qquad (2)$$

where t_{Ri} = retention time of component i

w_i = peak width of component i.

The 0.008 M SDS was generally the most efficient and reproducible, and the greater efficiency of the sub-CMC implies that mass transfer into the micelles is a limiting factor for the efficiency of the mobile phases with SDS concentration above the CMC. The absolute retention time and peak area reproducibility of the modified nucleic constituents was 1% and 8 to 20%, respectively. As the concentration is increased above the CMC, the electroosmotic flow has the resistance through the column and the retention time of the components which bind to micelle (binding) in the column may be longer, while those which does not bind to micelle (nonbinding) have the same retention behavior in the micellar mobile phase [1]. The 0.05 M SDS had the good reproducibility as in the 0.008 M SDS, but lacked the selectivity to resolve the mononucleotides. The retention time was approximately increased to 18% when the concentration of SDS was increased from 0.05 to 0.075 M. At 0.1 M SDS and separation voltage of 15 kv, the electric current was gradually increased from 0.045 to 0.08 mA. The temperature rise inside the column is caused by the increase in the electric current. It means the temperature increases with the separation voltage. Therefore, excessively high voltages can translate into increased dispersion, since it is affected by the column temperature. Because the current gradually increased during a run, the next experimental run was usually accomplished by rinsing

the corresponding mobile phase. However, in higher concentration of SDS, the retention times were slightly changed. The 0.1 M SDS was the least efficient of the four. The electrophoretic mobility caused by the effect of the increased temperature decreases the retention time. It is important not to exceed the thermal dissipation capabilities of the capillary column by employing high applied voltages and high ionic strength mobile phases which results in band dispersion. These problems resulted from thermal effects arising from joule heating. Narrow-bore capillary tubes dissipate the heat generated in the electrophoretic process very efficiently. Nevertheless, heating effects were observed when power dissipations exceed about 2 watts/m and applied voltages exceed about 30 kv [8]. The heat generated produces a transverse gradient within the column. Since electrophoretic mobility increases with the temperature, the plug-like flow profile is distorted and column efficiency is degraded. Therefore, cooling the column should improve efficiency.

Of the three mobile phases with SDS concentrations above the CMC, the 0.075 M SDS was the most efficient and selective for resolving the nucleic acid constituents. Efficiencies greater than 150,000 theoretical plates were achieved for deoxynucleosides and mononucleotides [11]. However, efficiency was closely coupled to solute concentration, and near the limit of detection, nearly 500,000 theoretical plates were generated for some species. The greater efficiencies for this moderate SDS concentration are attributed to the greater number of micelles per unit volume, analogous to using smaller particle size packings in HPLC.

3. Comparison of MECC and HPLC

In the HPLC by Gehrke et al. [16], an isocratic method was used on a shorter, 15 cm Supelcosil LC-18 column to screen rapidly a large number of mononucleosides and the separation was achieved within 6 min. The lowest concentration that could be detected was 0.8 nmol/ml, which was 80-fold lower than that by the MECC. But in terms of the mass detected, the amounts were 40 pmol much more than 0.027 pmol in the MECC, because the volume of the fused-silica tubing was considerably small. The injection volume of sample was calculated as 1.6 nl for 3 sec using electroinjection [11]. The retention time of unretained component (acetone) in the MECC was 18.5 min, while that was 1.3 min in the HPLC by the extremely fast isocratic method. In Table 3, N/t_{Ri} between the two systems was compared to reduce the difference in the dead volume. The MECC has 2-5 times better efficiency than the HPLC mainly due to the very narrow peak width. The latter is, however, more sensitive technique in terms of the solution concentration of the sample.

Table 3. Comparison of N/t_{Ri} between MECC and HPLC

N/t_{Ri} component	MECC*	HPLC**
2'-dC	5583	2464
2'-dU	5666	3432
MedC	4582	1642
DI	4267	1524
2'-dG	4268	1364
2'-dT	6050	1716
2'-dA	9919	894

* : 0.075 M SDS, separation voltage = 10 kv, injection time = 3 sec, column = fused silica capillary.

**: 0.05 M KH_2PO_4, flow rate = 2.0 ml/min, column = supelcosil LC-18-DBQ2.

MECC separations have primarily employed the SDS and to a lesser degree the CATC as a pseudo stationary phase. In conventional HPLC, the choice of stationary phases is somewhat limited. For majority of analyses, the C-18 reversed-phase stationary phase has been adequate. The range of surfactants available is, however, limited by solubility. The principal strength of the MECC is as a high-resolution microanalytical technique.

CONCLUSION

The effects of the separation voltage and the concentration of SDS on separation of 14 modified nucleic acid components were investigated. At constant injection time and voltage, the separation voltage and the SDS concentration had optimum values to resolve the components. The main utility of this technique fits the high resolution microanalysis, because samples can be detected in the range of sub-pmol.

The MECC is in a developing stage and the theoretical concept are not completely unified. Recently more works on the technique have been reported and show the possibility of further applications for many difficult separation of biological samples.

ACKNOWLEDGEMENT

This work was performed at Oak Ridge National Laboratory (TN, U.S.A.). The author gratefully acknowledges the helpful comments of J.I. Raw and W.H. Griest in preparing this paper.

NOMENCLATURE

N	:	number of theoretical plates
R_{ij}	:	resolution between i and j components
t_{Ri}	:	retention time of i component
w_i	:	peak width of i components

Abbreviations

2'-dA	:	2'-deoxyadenosine
2'-dC	:	2'-deoxycytidine
2'-dG	:	2'-deoxyguanosine
2'-dT	:	2'-deoxythymidine
2'-dU	:	2'-deoxyuridine
BrDU	:	5-Bromo deoxyuridine
BrG	:	8-Bromo Guanosine
CATC	:	cetryltrimethylammonium chloride
ClU	:	5-Chlorouracil
CMC	:	critical micelle concentration
dAmp	:	2'-deoxyadenosine 5'-monophosphate
DATC	:	docecylri-methyl ammonium chloride
dCmp	:	2'-deoxycytidine 5'-monophosphate
dGmp	:	2'-deoxyguanosine 5'-monophosphate
DI	:	Deoxy Inosine
dTmp	:	thymidine 5'-monophosphate
HPLC	:	high performance liquid chromatography
MECC	:	micellar electrokinetic capillary chromatography
MeDC	:	5-methyl deoxycytidine
SDS	:	sodium dodecyl sulfate
STS	:	sodium decylsulfate

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