Journal of Chromatography, 559 (1991) 537–545
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 469

Separation of Dns-amino acids and vitamins by micellar electrokinetic chromatography

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ABSTRACT

Fifteen Dns-amino acids were analysed by micellar electrokinetic chromatography (MEKC). All the amino acids were satisfactorily separated within *ca.* 26 min with 40 m*M* sodium dodecyl sulphate in phosphate–borate buffer (pH 7.56). The migration behaviour of the amino acids at different pH values and SDS concentrations was examined. In addition, attempts were made to separate a mixture of amino acids and vitamins simultaneously in a single run by MEKC. Detection for this mixture was carried out with an on-column fluorescence detection system with the capability of performing multi-wavelength programming.

INTRODUCTION

Interest in the use of high-performance capillary electrophoresis (CE) as a separation technique in chromatography has been rapidly increasing over the years. Its popularity has been attributed largely to its high separation efficiency with a relatively simple instrumental set-up [1–3].

Micellar electrokinetic chromatography (MEKC), one of the many modes of CE, was first initiated by Terabe *et al.* [3] in 1984. This mode of separation involves the addition of a surfactant to the carrier electrolyte solution. The introduction of MEKC has since expanded the scope of applications in CE. The usefulness and versatility of MEKC have been demonstrated by the numerous papers published since [4—7]. The main advantage of this technique is largely due to the extra partition mechanism for solutes to distribute between the aqueous and the micellar phases in the electrophoretic media. As a result, higher selectivity is often observed, particularly for non-ionic species.

The analysis of vitamins and amino acids has been carried out using a wide range of chromatographic techniques, such as ion-exchange chromatography for amino acids [8] and normal-phase high-performance liquid chromatography (HPLC) for vitamins [9]. To date the simultaneous analysis of this two groups of compounds by HPLC has rarely been explored. One of the reasons could be that each group requires a different HPLC mode for optimum separation and therefore sequential analysis would be needed if HPLC is used. In this work, attempts were made to analyse these two groups of compounds simultaneously using MEKC.

0021-9673/91/$03.50

The first part of the paper reports the use of an MEKC system with on-column fluorescence detection for the separation of fifteen Dns-amino acids (Dns = dansyl = 5-dimethylaminonaphthalene-1-sulphonyl). The separation of the amino acids is achieved by optimizing the pH and the concentration of the sodium dodecyl sulphate (SDS) in the electrophoretic media. The retention characteristics of the amino acids at various SDS concentrations and pH conditions were also examined. In the second part of the investigation, the separation of a mixture of amino acids and vitamins simultaneously in a single analysis using MEKC was studied. An on-column fluorescence detection system which permits programming of the excitation and emission wavelengths during the analysis was used for this investigation.

EXPERIMENTAL

A Spellman (Plainview, NY, USA) Model RM15P10KD power supply capable of delivering up to 15 kV was used. A fused-silica capillary (50 μm I.D. $\times$ 60 cm effective length) obtained from Polymicro Technologies (Phoenix, AZ, USA) was used as the separation tube. Detection of the Dns-amino acids was carried out on a Shimadzu (Kyoto, Japan) Model RF-535 fluorescence detector. For the detection of mixtures containing Dns-amino acids and vitamins, a Shimadzu Model RF-551 fluorescence detector capable of multi-wavelength programming was used. In both instances, the detector cells were modified as described elsewhere [10]. Briefly, the on-column micro fluorescence flow cell was fabricated by replacing the quartz cell with a separating capillary tube. A section of the polyimide coating on the capillary *ca.* 5 mm was removed to form the detection window. A slit was made for the incident light to improve the background signal. A Linear Instruments (Irvine, CA, USA) Model 252A/MM chart recorder was used to record the chromatograms.

All chemicals were of analytical-reagent grade or better. The buffer solution was prepared by dissolving sodium dihydrogenphosphate dihydrate and sodium tetraborate in water purified with a Milli-Q system (Millipore, Bedford, MA, USA). The electrophoretic medium containing sodium dodecyl sulphate (SDS) micelles was prepared as described previously [3]. Standard solutions of Dns-amino acids and Dns-amino acids–vitamins mixtures were prepared in HPLC-grade methanol (J. T. Baker, Phillipsburg, NJ, USA) at a concentration of 500 ppm for each of the species. The Dns-amino acids were supplied by Sigma (St. Louis, MO, USA) and the other chemicals by Fluka (Buchs, Switzerland).

Sample solution was introduced manually by gravity feed. This was carried out by placing the tip of the capillary at the high-potential end in a sample vial at a height 10 cm higher than the buffer reservoir. The time for each injection was 5 s.

RESULTS AND DISCUSSION

In previous investigations using MEKC, Sudan III has been the most commonly used marker to obtain the migration time of the micelles, which is used for calculating the capacity factors of the solutes. In this work, as Sudan III could not be detected at the wavelength at which the system was operating, migration times, t_r, rather than the capacity factors were used throughout. The two parameters used for the optimization of the separation of the Dns-amino acids were pH and SDS concentration. For

each of these parameters, a series of experiments were conducted to study their effect on the separation of the amino acids.

All the Dns-amino acids possess, in addition to their original ionizable groups, two other groups, namely the dimethylamino group and the α-carboxyl group from the Dns group. As all the amino acids investigated have the same Dns group attached to them, its effect on the migration behaviour would be expected to be the same. Therefore, the major factor that influences the migration order of these amino acids would be largely due to the differences in their main structural parent chains.

Effect of SDS

The results obtained for the migration times at different SDS concentrations are shown in Fig. 1. There was no apparent change in the migration order of any of the Dns-amino acid in all three sets of experiments. This observation is in agreement with the fact that as these experiments were carried out at the same pH, the extent of ionization of the Dns-amino acids would be the same in all three sets of experiments. In Table I, the log *P* (partition coefficient) values for some amino acids are listed according to the migration order observed. Even though not all the log *P* values are available, a distinct migration pattern could be observed. It can be seen that there is a good correlation between the migration order and the log *P* values. This relationship strongly suggests that the separation is primarily due to differences in hydrophobicity rather than differences in charge for these amino acids. This observation is typically observed in many MEKC applications in which the presence of micelles in the electrophoretic media influences the elution of the solutes. The extent of solubilization of the compounds into the neutral cavity of the micelles would be largely dependent on the hydrophobicity of the solutes. The more hydrophobic species would tend to be strongly associated with the micelles. As the anionic micelles are attracted by the

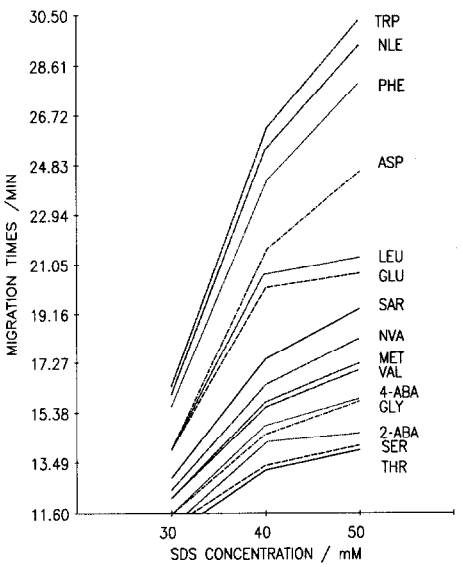

Fig. 1. Plot of migration times against SDS concentration. Experiments were carried out at pH 7.56.

TABLE I

LOG *P* [11] AND pK_a [12] VALUES FOR SOME AMINO ACIDS

Migration order	Amino acid	Log $P^{a,b}$	pK_a [b]
1	Thr	–	–
2	Ser	–	9.15
3	α-Aba	−1.81	–
4	Gly	−1.34	9.78
5	γ-Aba	–	–
6	Val	−1.41	9.72
7	Met	–	9.21
8	Nva	−0.98	–
9	Sar	–	–
10	Glu	–	4.07
11	Leu	−0.74	9.70
12	Asp	–	3.86
13	Phe	−0.59	–
14	Nle	−0.51	–
15	Trp	0.30	9.39

[a] Measured using *n*-butanol.
[b] – = Not available.

negative electrophorctic attraction towards the positive electrode, the solubilized species would tend to migrate more slowly than the hydrophilic species. For example, with Dns-Trp, its high log *P* value (0.3) would favour stronger interaction with the micelles in comparison with the other amino acids. Consequently, it would be highly incorporated in the micelles and thus would have a longer migration time, as observed in Fig. 1. On the other hand, Gly was one of the first few amino acids to migrate as it has the smallest log *P* values (−1.81). The trend observed is in good agreement with the results previously obtained by reversed-phase HPLC [8].

The migration behaviour of the other Dns-amino acids listed in Table I (*i.e.*, those amino acids for which the log *P* values are not available) can be categorized into the following groups: group I, consisting of Dns-Thr, Dns-Ser and Dns-Met; group II, consisting of Dns-γ-Aba and Dns-Sar; and group III, consisting of Dns-Glu and Dns-Asp.

The characteristic feature of the amino acids in group I is that each possesses at least one polar substituent group, which makes them hydrophilic. Consequently, these amino acids would tend to be solvated more by the aqueous phase and effective solubilization by the micelles would be inhibited. Therefore, their migration times were shorter than those of most of the other amino acids. For example, for Dns-Met, even though it has the same number of methylene groups in its alkyl chain as Dns-Nva, a shorter migration time was observed. The difference in the migration times between these two species could be largely due to the presence of the polar sulphur atom in the alkyl chain in Dns-Met, which makes it more hydrophilic. Similar results were obtained for Dns-Thr and Dns-Ser. Further, it was noted that the extent of solvation of these two amino acids by the aqueous phase seems more pronounced than for Dns-Met. The fact that their hydroxyl groups are at the terminus enables

these amino acids to form hydrogen bonds more readily with the aqueous phase. Subsequently, these two amino acids were found to have the shortest migration times.

An interesting trend was observed for the amino acids in group II. Both Dns-γ-Aba and Dns-Sar were found to have a longer migration times than expected. For example, for Dns-Sar, the increase in hydrophobicity due to the additional methyl group could not have resulted in such a large increase in migration time when compared with Dns-Gly. A longer migration time was also observed for Dns-γ-Aba when compared with Dns-α-Aba. A possible reason for the longer migration times observed could be that these amino acids are generally more basic than the others and therefore they would be susceptible to protonation. These positively charged species would be very prone to ion-pair formation with the micelles. Consequently, owing to the negative electrophoretic attraction towards the anode, longer migration times would be expected for these amino acids. Among the two amino acids in group II, this effect seems more pronounced for Dns-Sar. The reason is probably its secondary amino group, which makes it more basic and therefore protonation would be more favorable for Dns-Sar. As a result, Dns-Sar was found to migrate more slowly. For Dns-γ-Aba, unlike the other of amino acids, its carboxylate group is not attached to α-carbon. This would make its amino group more basic than that in Dns-α-Aba. Consequently, it is possible for Dns-γ-Aba to undergo preferential protonation, and therefore it would have a longer migration time than Dns-α-Aba.

Dns-Glu and Dns-Asp are the two diacid amino acids in group III. In view of the extra methylene group in the alkyl chain of the more hydrophobic Dns-Glu, it would be reasonable to expect it to migrate more slowly than Dns-Asp. However, the opposite was observed. Further, on comparing their migration order with that of some of the amino acids having the same number of carbons in the alkyl chain, it was observed that both Dns-Asp and Dns-Glu have longer migration times. In addition, because of the presence of the additional carboxylic group in these two species, which could have made them more hydrophilic, these species would be expected to be less solubilized by the micelles and therefore they should have shorter migration times. However, the results obtained contradict this expectation. This anomalous behaviour can be explained by the differences in pK_a values. From Table I, it can be seen that the pK_a values of these two amino acids are significantly smaller than those of the other amino acids. Therefore, dissociation of the carboxylic group in these compounds is possible and these negatively charged, dissociated species would be electrostatically attracted to the anode. Consequently, because of this negative electrophoretic attraction, they would tend to migrate more slowly. As the pK_a value of Asp (3.86) is lower than that of Glu (4.07), the extent of ionization in Asp would be expected to be higher. Therefore, Asp would experience a stronger interaction with the anode and hence a longer migration time is expected for Asp. Further, it is worth noting that even under conditions where dissociation of these compounds is not favourable, the migration order for these two "neutral" amino acids would remain the same (*i.e.*, t_r for Asp would be longer than t_r for Glu). The behaviour can be explained by their tendency to form hydrogen bonds. The two carboxylic groups in these species are oriented in such a position that intramolecular hydrogen bonding would be possible. As a result, it is expected that the two polar carboxylic groups in these species would be in a "cage-like" configuration. This caging effect would render the species less hydrophilic. As the two carboxylic acids are in closer proximity in Asp than in Glu

(*i.e.*, they are separated by one less methylene group), stronger intramolecular hydrogen bonding would be expected. Hence Asp would be more hydrophobic than Glu, and therefore would have a longer migration time.

From Fig. 1, it was also noted that with an increase in SDS concentration a corresponding increase in the migration times was observed for all the amino acids in all three sets. This is because an increase in SDS concentration would result in an increase in solubilization of the species by the micelles. Therefore, longer migration times would be expected.

Effect of pH

The results obtained for the migration times at different pH values are shown in Fig. 2. It can be seen that with increase in pH from 6.6 to 7.5, a corresponding decrease in the migration times was observed for all the Dns-amino acids. With a further increase in pH to 8.0, a reversal of this trend was noted. This interesting effect is largely due to the presence of two different types of ionizable amino and carboxylate groups in these compounds. At low pH, it is reasonable to expect the amino group to be protonated. These positively charged protonated species would then form ion pairs with the anionic micelles. As the pH of the electrophoretic medium increases, the extent of protonation would be lowered and in this event the species would no longer be able to effectively form ion pairs with the micelles. Therefore, a decrease in the migration times was observed as the pH increased. On the other hand, at higher pH, ionization of the carboxylate group in these compounds would be favourable. As the negatively charged amino acids would be attracted to the anode via electrophoretic interaction, therefore, at pH 8 the amino acids would elute at longer migration times, as observed in Fig. 2.

It is worth noting that in spite of the reversal in the trend observed with changes

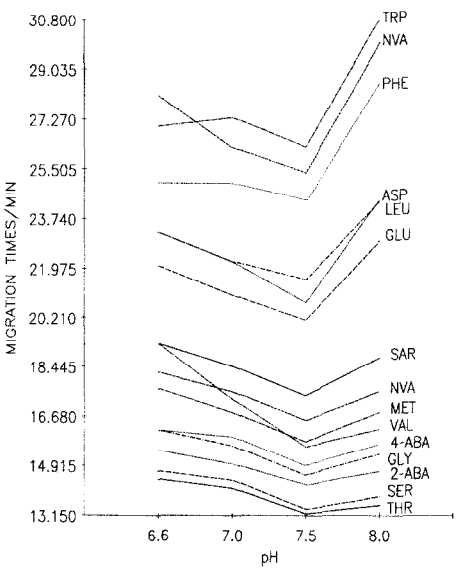

Fig. 2. Plot of migration times against pH. Experiments were carried out at 40 m*M* SDS.

in pH, with the exception of the group consisting of Dns-Met, Dns-Val and Dns-Nva and the pair Dns-Nle and Dns-Trp, the migration order of the amino acids remained unchanged in the pH range investigated. For these pH values hydrophobicity is one of the principal factors dominating the migration order.

For Dns-Met, Dns-Val and Dns-Nva, crossover of peaks was observed with changes in pH. At lower pH, preferential protonation of the amino group in some of the amino acids would be possible. This would subsequently lead to the formation of ion pairs between the positively charged amino acids and the micelles. Hence longer migration times would be expected for these species. With Dns-Val, the highly branched, electon-donating isopropyl group attached to the α-carbon would enhance the basicity of the amino group. This is in contrast to Dns-Val, which has a straight, less electron-donating alkyl chain, and Dns-Met, which contains an electron-withdrawing sulphur group. As a result, protonation of the basic Dns-Val would be more favourable than that of the other two amino acids. Consequently, because of the possibility of ion-pair formation in Dns-Val, a longer migration time was observed. From Fig. 2, it can be seen that this is more apparent at lower pH. On increasing the pH, protonation of Dns-Val would be inhibited. Under such circumstances, the migration would now be governed by hydrophobicity. Therefore, at higher pH, Dns-Val would be expected to migrate faster than Dns-Met and Dns-Val as it has the smallest log *P* among the three amino acids.

A change in migration order was also observed in Dns-Nle and Dns-Trp. It was noted that at lower pH (i.e., pH 6.6), Dns-Trp, in spite of its large log *P* value, was found to have a migration time smaller than that of Dns-Leu. A possible reason could be the basicity of the pyrrole ring, which makes it susceptible to protonation at this pH. Consequently, this would lead to the formation of ion pairs with the micelles, therefore inhibiting effective solubilization by the micelles and giving a longer migration time. However, unlike with Dns-Val, ion-pair formation in Dns-Trp seems to result in a shorter migration time. This discrepancy can be attributed to the fact that as the pyrrole ring in Dns-Trp is more basic than Dns-Val, the extent of protonation would be greater. Consequently the electrophoretic repulsion experienced by the more positively charged Dns-Trp would then be more pronounced. As a result, Dns-Trp would migrate faster, as is observed in Fig. 2.

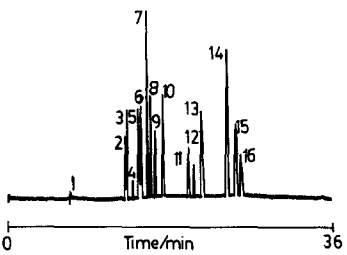

Fig. 3. Electrokinetic chromatogram obtained for the fifteen Dns-amino acids. Peaks: 1 = methanol; 2 = Dns-Thr; 3 = Dns-Ser; 4 = Dns-α-Aba; 5 = Dns-Gly; 6 = Dns-γ-Aba; 7 = Dns-Val; 8 = Dns-Met; 9 = Dns-Nva; 10 = Dns-Sar; 11 = Dns-Glu; 12 = Dns-Leu; 13 = Dns-Asp; 14 Dns -Phe; 15 = Dns-Nle; 16 = Dns-Trp. Electrophoretic conditions: 40 m*M* SDS in 0.1 *M* borate–0.05 *M* phosphate buffer (pH 7.56); separation tube, 60 cm × 50 μm I.D. fused-silica capillary; applied voltage, 15 kV; excitation wavelength, 325 nm; emission wavelength, 550 nm.

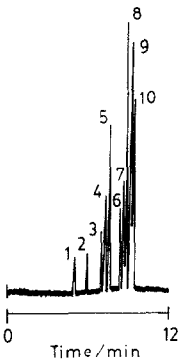

Fig. 4. Electrokinetic chromatogram obtained for a mixture of seven selected Dns-amino acids and two vitamins. Peaks: 1 = methanol; 2 = vitamin B_6; 3 = Dns-Thr; 4 = Vitamin B_2; 5 = Dns-γ-Aba; 6 = Dns-Glu; 7 = Dns-Leu; 8 = Dns-Asp; 9 = Dns-Phe; 10 = Dns-Nle. Electrophoretic conditions: 40 m*M* SDS in 0.1 *M* borate–0.05 *M* phosphate buffer (pH 7.56); separation tube, 60 cm × 50 μm I.D. fused-silica capillary; applied voltage, 15 kV. Wavelength programme: 0.1 min, excitation wavelength 340 nm, emission wavelength 400 nm; 6.1 min, excitation wavelength 325 nm, emission wavelength 550 nm; 7.10 min, excitation wavelength 370 nm, emission wavelength 440 nm; 7.40 min, excitation wavelength 325 nm, emission wavelength 550 nm.

The optimum separation of the fifteen Dns-amino acids was obtained at pH 7.56 with 40 m*M* SDS. The corresponding chromatogram obtained is shown in Fig. 3. All the peaks were satisfactorily separated within 30 min.

Dns-amino acids and vitamins

In an earlier investigation, Nishi *et al.* [13] successfully separated a group of vitamins. In this work, the separation of a mixture of amino acids and water-soluble vitamins in a single analysis was attempted. The unique feature of this investigation is the use of a multi-wavelength programmable fluorescence detector. The excitation and emission wavelengths for detection were programmed to give maximum sensitivity for each of the species in the mixture.

A typical chromatogram obtained is shown in Fig. 4. It can be seen that all the peaks were satisfactorily separated. The conditions used for the optimum separation of the vitamins and Dns-amino acids were similar to those used in Fig. 3 (*i.e.*, 40 m*M* SDS in an electrophoretic medium at pH 7.56). The migration order for the seven selected Dns-amino acid would therefore be expected to be the same as that obtained earlier. It was noted that vitamin B_6 has the shortest migration time. This is largely due to the presence of the three hydroxyl groups, which makes vitamin B_6 even more hydrophilic than Dns-Thr. For vitamin B_2, because of its bulkier substituent group, effective solubilization by the micelles would be hindered. Hence, a shorter migration time was observed vitamin for B_2 compared with the other Dns-amino acids.

The separation of these two groups of compounds in a single analysis by MEKC is, to our knowledge, the first attempt in which a multi-wavelength programmable fluorescence detector has been used. In view of the encouraging results obtained in this investigation, it is believed that the inherently high efficiency of MEKC and the versatility of the wavelength-programmable fluorescence detector can be exploited for the separation of complicated mixtures.

ACKNOWLEDGEMENTS

We thank the National University of Singapore for financial support. We also express our gratitude to Schmidt Scientific (Singapore) and Shimadzu (Asia Pacific) for their generous loan of the Shimadzu RF551 fluorescence detector.

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