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Separation of diastereomers by capillary zone electrophoresis in free solution with polymer additive and organic solvent component
Effect of pH and solvent composition ☆

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Abstract

Diastereomeric derivatives of enantiomers generally exhibit differences in their physico-chemical properties. This fact allows the separation of these compounds in non-chiral separation systems. Although charged diastereomers have been reported to be resolved in certain instances by capillary electrophoresis without any additives, a significant enhancement of stereoselectivity can be achieved by means of polymeric pseudophases. The present paper focuses on the influence of the pH and of the organic solvent component acetonitrile on the resolution of diastereomeric compounds in the absence and presence of a polymeric network of polyvinylpyrrolidone. Experimental data are presented for diastereomeric derivatives of α-amino acids as well as for three anticoagulant drugs obtained by reaction with optically pure (+)-O,O′-dibenzoyl-L-tartaric anhydride.

Keywords: Capillary electrophoresis; Diastereomers; Buffer composition; Amino acids; Phenprocoumon; Warfarin; Acenocoumarol

1. Introduction

In a previous paper [1] the electrophoretic separation of diastereomeric derivatives of racemic amino acids obtained by reaction with (+)-O,O′-dibenzoyl-L-tartaric anhydride (DBT-derivatives) has been reported. It was shown there that some, but not all, of the investigated compounds are resolved in their fully dissociated state (at pH 7) in free-solution systems without additives. DBT-derivatives of Val, Leu, Phegly, Met and Gln were separated, at least partially, whereas the DBT-derivatives of Thr, Ser, Phe and Trp, as well as the amino acids derivatized by (+)-O,O′-diacetyl-L-tartaric anhydride (DAT-derivatives) failed to be separated. Re-

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garding α-hydroxy acids, DBT-mandelic acid was partially separated, in contrast to DBT-atrolactic acid. With respect to the high pH value of the electrolyte solution chosen, it is clear that the diastereomeric compounds mentioned were resolved due to differences in their actual mobilities, μ_{act}. Small differences in their shape-dependent migration resistance have thus to be postulated. It has been found that the presence of a network of linear polyvinylpyrrolidone (PVP) in the electrolyte solution significantly increases stereoselectivity and allows the separation of a larger number of diastereomeric analytes [1,2]. This improvement in separation is supposed to be based on intermolecular interactions between the analytes and the polymeric network, which often cause differently strong retardation of the diastereomeric analytes. In this sense, the action of the polymeric pseudophase mirrors to some extent the retardation of analytes by a stationary phase in liquid chromatography.

In the present paper the influence of the pH on separation selectivity is addressed, and how this influence is amplified by the presence of a polymer network. Further, the rather complex mechanism is discussed by which stereoselectivity is affected by solvent components like acetonitrile in electrophoretic systems with and without polymeric pseudophases. The polymer dealt with in this paper is polyvinylpyrrolidone, which as an additive has proven easy to handle, does not cause too high viscosity of the solution and does not suffer from other severe disadvantages such as toxicity. The test analytes selected were racemic α-amino acids as well as three coumarinic drugs used as anticoagulants which were reacted to diastereomeric derivatives by use of (+)-O,O′-dibenzoyl-L-tartaric anhydride. Formulae of these derivatives, indicated as DBT-analytes, are shown in Fig. 1 and Table 1.

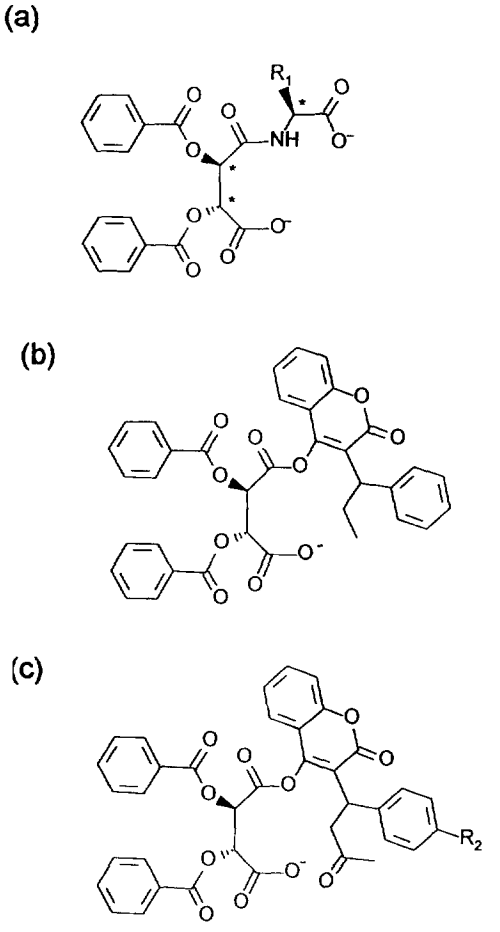

Fig. 1. Chemical structure of DBT-derivatized analytes investigated. R_1 and R_2 as specified in Table 1. (a) α-Amino acids, (b) phenprocoumon, (c) warfarin and acenocoumarol.

2. Experimental

2.1. Apparatus

The experiments were carried out using a laboratory-made apparatus as described in Refs. [1,3]. The dimensions of the fused-silica capillary used (Polymicro Technologies, Phoenix, AZ, USA) were 56 cm × 100 μm I.D., with 39 cm length to the detector (UV absorption at 233 nm). A constant voltage of 12 kV was applied to the capillary for electrophoresis of the anionic analytes. The capillary was coated to suppress the electroosmotic flow; it was kept at ambient temperature (24–25°C) without thermostatting.

Table 1
Chemical structures of R_1 and R_2 in Fig. 1

Analyte	R_1	Analyte	R_2
Valine	$-CH-(CH_3)_2$	Warfarin	$-H$
Leucine	$-CH_2-CH-(CH_3)_2$	Acenocoumarol	$-NO_2$
Serine	$-CH_2OH$		
Threonine	$-CH_2OH-CH_3$		
Phenylglycine	$-C_6H_5$		
Phenylalanine	$-CH_2-C_6H_5$		
Tryptophan	$-CH_2-C_8H_6N$		

Sampling was done by the hydrodynamic method (5 s at a height of 10 cm).

The determination of pK_a values of selected α-amido-carboxylic acids by potentiometric titration with 0.1 *M* sodium hydroxide solution was carried out by using an automatic titrator (Mettler DL67 Titrator, Mettler-Toledo, Schwerzenbach, Switzerland) as described in Ref. [4].

2.2. Chemicals

Optically pure and racemic α-amino acids as well as the reagents, solvents, buffering electrolytes and coating reagents were purchased in the purest obtainable quality from commercial suppliers. Phenprocoumon, acenocoumarol and warfarin were gifts from the Institute of Pharmaceutical Chemistry, University of Vienna. (+)-O,O′-Dibenzoyl-L-tartaric anhydride was synthesized as described in Ref. [1], following the procedure outlined in Ref. [5].

2.3. Sample derivatization and coating

The derivatization of the racemic or optically pure analytes with (+)-O,O′-dibenzoyl-L-tartaric anhydride was performed as given in Ref. [1] and analogous to the procedure described in Ref. [6].

The coating procedure given in Refs. [1,7] was modified in two respects: the concentration of 3-(trimethoxysilyl)propylmethacrylate was 1% v/v in ethanol and the concentration of acrylamide was 4% w/v in water, to which 0.1% v/v TEMED and 0.1% w/w ammonium persulfate were added.

3. Results and discussion

3.1. Stereoselectivity modulation by pH variation

Mono-basic coumarinic drug derivatives

The effective mobilities of the three DBT-derivatized coumarinic drugs which form monovalent anions under the given conditions decrease with decreasing pH as depicted in Fig. 2. From this figure the pK_a values of the three compounds can be obtained as those pH values at which the mobilities are reduced to one-half of the value at full dissociation. These pK_a values (3.8, 4.4 and 4.7) are remarkably different, although they belong to the same α-ester carboxylic group originating from the derivatization agent. Apparently, the acidity of the carboxyl group is considerably influenced by intramolecular interactions, probably involving the carbonyl groups of warfarin and acenocoumarol. In our experiments, however, no stereoseparation was achieved for these compounds in the pH range from 7 to about 4. It will be shown below that differences in pK_a values of diastereomers as small as 0.02 pK units would suffice for their resolution. For these three coumarinic drug derivatives one can conclude that the intramolecular interactions responsible for non-stereoselec-

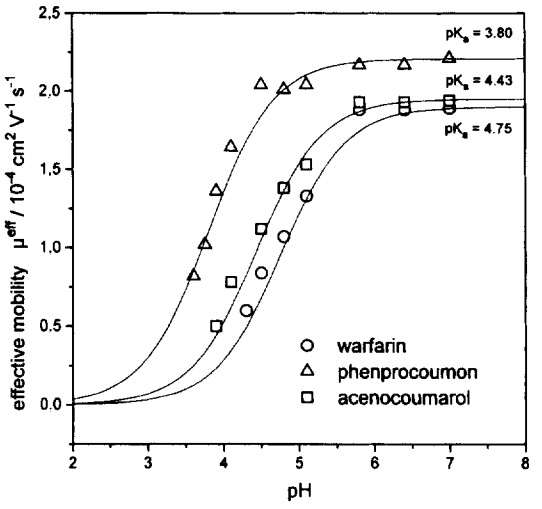

Fig. 2. Effective electrophoretic mobilities of the DBT-derivatives of three coumarinic drugs in dependence on the pH. The symbols represent the experimental data, the curves are simulated using pK_a values of 3.80, 4.43 and 4.75, respectively. Experimental conditions: sodium phosphate buffer 0.030 mol/l, no polymeric additive; coated capillary, total length 56 cm (39 cm to detector); applied voltage 12 kV, current 22 μA. T = 24°C. Analyte concentration (in water) 10^{-4} mol/l. Detection: UV absorbance at 233 nm.

tive pK_a differences of 0.30 to 0.90 pK units are not stereoselective to such an extent that differences in the pK_a values between diastereomers of about 0.02 pK units are caused.

Di-basic amino acid derivatives

Stereoselectivity data of the DBT-amino acids as a function of the pH of the electrolyte solution are given in Table 2, with the effective mobilities of the L-amino acid derivatives in Table 3. The stereoselectivity coefficient, r_{DL}, is defined as the ratio of the measured effective mobilities, $\mu^{D}_{eff}/\mu^{L}_{eff}$, D and L indicating the diastereomeric derivatives of the D- and L-amino acids, respectively. The experimental data exhibit a precision of better than 0.2% in the selectivity coefficient; the precision of pH adjustment, however, is less. Selectivity coefficients between 0.993 and 1.007 could not be distinguished due to the inherent peak dispersion; they are given in the tables as 1.00. With all diastereomeric pairs investigated, separation selectivity is significantly altered when lowering the pH; the mobilities of the D-amino acid derivatives are reduced relative to those of

Table 2
Dependence of the stereoselectivity coefficients, $r_{DL} = \mu^{D}/\mu^{L}$, of DBT-amino acids on the pH value of the electrolyte solution

	Selectivity coefficient, r_{DL}, at pH						
	7	5.8	4.5	4.2	3.9	3.6	3.3
(a) Polymer-free solution							
Val	1.011	1.010	0.987	0.987	0.977	0.971	0.964
Leu	1.020	1.019	1.00	1.00	0.992	0.989	0.987
Ser	1.00	1.00	0.974	0.970	0.957	0.949	0.945
Met	1.014	1.013	1.007	–	–	–	–
Thr	1.00	1.00	0.990	–	–	–	–
Gln	1.011	1.010	1.00	–	–	–	–
Phegly	1.014	1.013	1.015	1.015	1.016	1.017	1.019
Phe	1.00	1.00	1.00	0.990	0.981	0.976	0.970
Trp	1.00	1.00	1.00	1.00	1.00	0.991	0.987
(b) With 0.5% (w/v) of PVP							
Val	1.014	1.014	1.011	1.00	0.991	0.976	0.976
Leu	1.021	1.023	1.019	1.018	1.00	1.00	0.990
Ser	1.00	1.00	1.00	1.00	0.971	0.951	0.949
Phegly	1.014	1.012	1.012	1.011	1.010	1.011	1.00
Phe	1.00	1.00	0.990	0.988	0.982	0.964	0.951
Trp	0.964	0.951	0.948	0.952	0.943	0.922	0.905

Table 3
Dependence of the effective mobilities of the DBT-derivatives of the L-amino acid, μ_{eff}^{L}, on the pH value of the electrolyte solution

	Effective mobility, μ_{eff}^{L} (10^{-4} cm^2 V^{-1} s^{-1}), at pH						
	7	5.8	4.5	4.2	3.9	3.6	3.3
(a) Polymer-free solution							
Val	3.7	3.9	3.4	3.4	3.1	3.0	2.6
Leu	4.3	3.9	3.4	2.9	2.8	2.7	2.3
Ser	3.7	4.0	3.5	3.6	3.4	3.2	2.7
Met	3.9	3.8	3.6	–	–	–	–
Thr	3.4	3.9	3.7	–	–	–	–
Gln	3.6	3.5	3.6	–	–	–	–
Phegly	4.2	3.9	3.8	3.5	3.1	3.3	2.8
Phe	3.6	3.7	3.8	3.4	3.0	2.8	2.6
Trp	3.6	3.8	3.5	3.2	2.9	2.8	2.6
(b) With 0.5% (w/v) of PVP in the background electrolyte							
Val	3.2	2.9	3.5	3.0	3.1	2.7	2.5
Leu	3.3	3.2	3.5	3.4	2.9	2.7	2.1
Ser	3.3	3.3	3.3	3.2	3.6	3.0	2.8
Phegly	3.4	3.6	3.5	3.4	3.3	3.1	2.7
Phe	3.0	3.0	3.1	3.4	3.1	2.8	2.3
Trp	3.0	2.6	2.8	2.9	2.9	2.2	1.9

the L-amino acid derivatives in all instances except for DBT-phenylglycine only. Where no separation could be obtained in the fully dissociated state, a separation L before D is obtained upon decreasing the degree of dissociation. In those instances where the D-enantiomer had the higher actual mobility (DBT-Leu, DBT-Val), the elution order is inverted to L before D upon decreasing the pH. This situation is shown by the electropherograms given in Fig. 3.

With these divalent compounds, selectivity alteration upon pH variation can potentially result from two different sources: first, if slight differences exist in the pK_a values within a pair of diastereomers, and second, if the ratios of the actual mobilities of the diastereomers, $\mu_{act}^{D}/\mu_{act}^{L}$, change when changing from the double to the single charged state. It will be shown in the following section that the experimental data can be fairly well simulated on the basis of both assumptions, either assuming ΔpK_a values unequal to zero or a variation of the mobility ratio of D- and L-isomers with their charge. This means that we have at the moment no firm experimental evidence for the analytes investigated to indicate which of the two mentioned sources is predominantly responsible for the observed effects. The parameter values used in matching

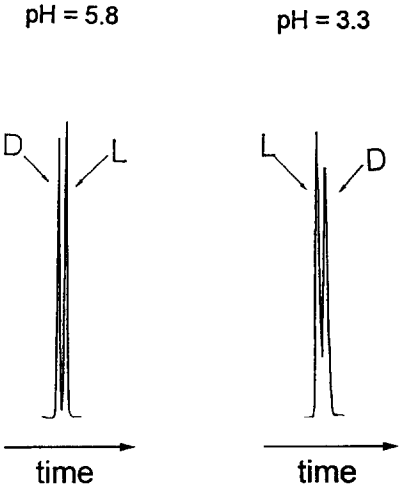

Fig. 3. Electropherograms of racemic DBT-leucine at two different pH values. Experimental conditions as given in Fig. 2. Migration times of the D- and L-isomers, respectively, at pH 5.8: 7.67 and 7.81 min; at pH 3.3: 13.23 and 13.06 min.

simulation curves, however, give some idea about the magnitude of pK_a or actual mobility differences, which potentially might be of relevance.

3.2. Simulation of the pH dependence of selectivity data

The selectivity coefficient, r_{DL}, for a pair of multi-basic acids in dependence on the pH is estimated by means of Eqs. 1 and 2. The mobilities of the analytes are calculated here by adding up the mobility increments contributed by the differently charged species weighted by the probability of finding these species as determined by the degree of dissociation, α_k, [8].

$$r_{DL} = \left[\sum_k (\mu^D_{act,k} - \mu^D_{act,k-1})\alpha^D_k\right] / \left[\sum_k (\mu^L_{act,k} - \mu^L_{act,k-1})\alpha^L_k\right] \quad (1)$$

where k numbers the differently charged species. The α_k values for the single diastereomers, e.g. D, can be evaluated as

$$\alpha^D_k = 1/\left[1 + 10^{(pK^D_{a,k} - pH)}\right] \quad (2)$$

The number of experimental selectivity versus pH data points given in Table 2 does not allow us to determine mobility increments or pK_a values by curve-fitting procedures. The experimental data are thus simulated by means of Eqs. 1 and 2 using reasonable values for (i) the pK_a values of the two carboxylic groups, (ii) the differences in the pK_a values, ΔpK_a, of diastereomeric pairs, and (iii) the mobility increments of the single and double charged species of each diastereomer. For all simulations the pK_a value of the α-benzoylester carboxylic groups was chosen as 4.3 (cf. results of Fig. 2), that for the α-amido carboxylic group as 3.5. This average value could be confirmed by means of titration experiments, yielding pK_a values of N-acetyl amino acids between 3.2 and 3.8, as seen in Table 4.

Simulated curves representing the selectivity coefficients in dependence on the pH according to Eqs. 1 and 2 are shown in Fig. 4 when choosing ΔpK_a values for the two carboxylic groups between 0.02 and 0.04. The following assumptions regarding the mobility increments were made: (i) the mobility increment resulting from the second negative charge, i.e. $\mu_{act,2} - \mu_{act,1}$, is about 0.6 times the mobility of the single charged analyte, $\mu_{act,1}$ [9]; and (ii) the ratio of the actual mobilities of the single charged analytes, $\mu^D_{act,1}/\mu^L_{act,1}$ equals the ratio of the actual mobilities of the double charged analytes, $\mu^D_{act,2}/\mu^L_{act,2}$. Fig. 4 shows that the overall dependence of the experimental r_{DL} versus pH data is adequately represented by the calculated curves. In particular, one can conclude that ΔpK_a values for the diastereomeric analyte pair between 0.01 and 0.05 pK units are sufficient to explain the tendency in stereoselectivity as observed in our experiments.

Fig. 5 shows simulated curves using a different

Table 4
pK_a values of selected α-amido-carboxylic acids at different solvent compositions determined by titration

Titrand	pK_a				
	Acetonitrile				PVP
	0%	20%	40%	50%	2%
Acetic acid	4.70	5.05	5.52	5.76	4.75
N-Acetyl-glycine	3.61	3.93	4.31	4.50	3.74
N-Acetyl-L-glutamine	3.52	3.86	4.22	4.39	3.66
N-Acetyl-L-cysteine	3.24	3.55	3.98	4.18	3.40
N-Acetyl-D,L-tryptophan	3.89	4.07	4.56	4.79	4.36

Ionic strengths in the range of 0.005 to 0.015 mol/l.

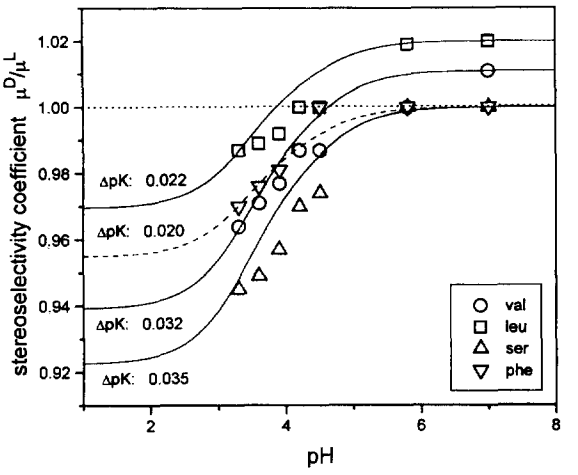

Fig. 4. Stereoselectivity coefficients in dependence on the pH for four selected DBT-amino acids. The symbols represent the experimental data specified in Table 2. The curves are obtained by simulation using Eqs. 1 and 2. Parameter values: pK_{a1}: 3.5, pK_{a2}: 4.3, ΔpK_{a1} and ΔpK_{a2} as given in the figure; $\mu^D_{act,1}/\mu^L_{act,1}$ and $\mu^D_{act,2}/\mu^L_{act,2} = 1.0$.

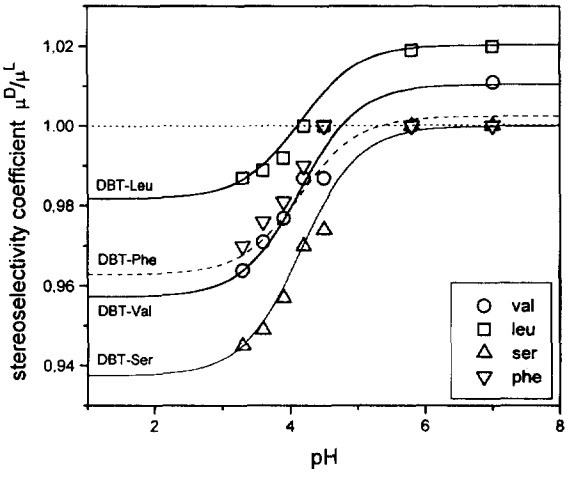

Fig. 5. Stereoselectivity coefficients in dependence on the pH for four selected DBT-amino acids. Experimental data identical to Fig. 4. Parameters for the simulation curves are: pK_{a1}: 3.5, pK_{a2}: 4.3, ΔpK_{a1} and $\Delta pK_{a2} = 0$: $\mu^D_{act,1}/\mu^L_{act,1}$ ratios as follows: 0.970, 0.942, 0.951, and 0.919 for DBT-Leu, DBT-Val, DBT-Phe, and DBT-Ser, respectively. Values for the mobility increments assigned to the second charge, $(\mu^D_{act,2} - \mu^D_{act,1})/(\mu^L_{act,2} - \mu^L_{act,1})$, are 1.110, 1.137, 1.094, and 1.151, respectively.

set of parameters. In these curves it is assumed that the ΔpK_a values for both acidic groups are zero, and unlike for Fig. 4, the ratio of the actual mobilities of the single charged analytes, $\mu^D_{act,1}/\mu^L_{act,1}$, is different from the ratio of the actual mobilities of the double charged analytes, $\mu^D_{act,2}/\mu^L_{act,2}$. This set of parameters allows an even better agreement between the simulated curve and the experimental results than that obtained in Fig. 4, especially for the aliphatic amino acid derivatives.

3.3. pH effects in electrophoretic systems containing polymer networks

Selectivity coefficients in dependence on the pH and in the presence of 0.5% (w/v) PVP are given in Table 2. The selectivity versus pH data points of the aliphatic amino acids are found to be only slightly affected by the polymeric additive: the whole curves are shifted to higher r_{DL} values. With aromatic amino acids, however, a significant influence of the polymer is observed, and the whole curves are shifted to lower r_{DL} values, as seen in Fig. 6. The greatest effect is found with DBT-Trp. With DBT-Phe, the effect of the presence of PVP in a concentration of 0.5% (w/v) is seen at pH values lower than 4

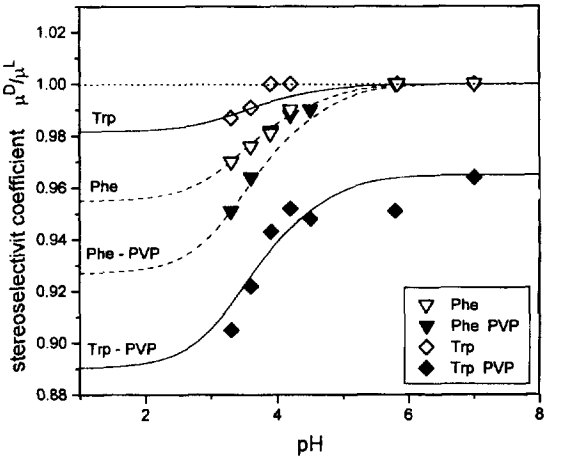

Fig. 6. Stereoselectivity coefficients in dependence on the pH for two aromatic DBT-amino acids in absence and presence of 0.5% (w/v) of PVP. The symbols represent experimental data.

only. It seems that with lower charge number the interactions between analyte and polymer become more selective in the mentioned cases. Again, the experimental data can be fairly well represented by simulation curves similar to those given in Figs. 4 and 5 using slightly different parameter values, either for ΔpK_a or for the actual mobility ratios assigned to the two charged species.

3.4. Influence of organic solvent component on stereoselectivity

Experimental data regarding the influence of the concentration of acetonitrile on the selectivity coefficients of diastereomeric pairs of amino acids are shown in Table 5 (mobilities in Table 6). Two pH values (5.8 and 7) and acetonitrile concentrations up to 50% (v/v) were investigated. Data concern polymer-free electrolyte solution and the presence of 2% (w/v) of PVP. The concentration of acetonitrile was found to have an impact on the stereoselectivity coefficients in all instances. There are some particular findings of note. (i) At 50% acetonitrile the selectivities achieved in the PVP-containing systems become equal to those obtained in the polymer-free systems. At this high concentrations of acetonitrile, the stereoselective effect caused by the polymeric network is apparently fully counteracted by the organic solvent component. (ii) In the polymer-free solution the mobility of the D-derivative was reduced by the addition of acetonitrile relative to the L-derivative in all cases except DBT-Val. This effect of acetonitrile goes in the same direction as that observed upon addition of PVP when dealing with aromatic DBT-amino acids but opposite to that for aliphatic DBT-amino acids. (iii) Stereoselectivity is often observed to be diminished upon addition of the organic solvent. In several instances, however, an enhancement takes place. This is observed with DBT-Ser and

Table 5
Dependence of the stereoselectivity coefficients, $r_{DL} = \mu^D / \mu^L$, of DBT-amino acids on the content of acetonitrile

	Selectivity coefficient, r_{DL}, at % (v/v) of acetonitrile				
	0	20	30	40	50
Polymer-free solution; pH = 5.8					
Val	1.010	1.011	1.009	1.003	1.00
Leu	1.019	1.018	1.012	1.008	1.00
Ser	1.00	1.00	1.00	1.00	0.993
Phegly	1.013	1.010	1.008	1.007	1.00
Trp	1.00	1.00	1.00	0.987	0.983
Polymer-free solution; pH = 7.0					
Val	1.011	1.014		1.008	1.00
Leu	1.020	1.021		1.014	1.008
Ser	1.00	1.00		1.00	1.00
Phegly	1.014	1.010		1.00	1.00
Trp	1.00	1.00		0.989	0.979
With 2% (w/v) of PVP; pH = 5.8					
Val	1.023	1.015	1.015	1.007	1.00
Leu	1.023	1.021	1.017	1.008	1.00
Ser	1.011	1.00	1.00	1.00	0.992
Phegly	1.00	1.00	1.012	1.007	1.00
Trp	0.894	0.973	0.973	0.984	0.980

Table 6
Dependence of the effective mobilities of the DBT-derivatives of the L-amino acids, μ^L_{eff}, on the content of acetonitrile in the background electrolyte

	Effective mobility, μ^L_{eff} (10^{-4} cm^2 V^{-1} s^{-1}), at % (v/v) of acetonitrile				
	0	20	30	40	50
Polymer-free solution; pH = 5.8					
Val	3.9	3.6	3.6	3.5	3.4
Leu	3.9	3.5	3.2	3.3	3.4
Ser	4.0	4.0	3.9	3.5	3.4
Phegly	3.9	3.7	3.5	3.4	3.3
Trp	3.8	3.5	3.7	3.4	2.6
Polymer-free solution; pH = 7.0					
Val	3.7	3.4		3.4	2.8
Leu	4.3	3.5		3.3	2.9
Ser	3.7	3.7		3.6	3.2
Phegly	4.2	3.8		3.3	2.7
Trp	3.6	3.5		3.1	2.9
With 2% (w/v) of PVP; pH = 5.8					
Val	2.9	2.9	2.9	2.6	2.7
Leu	2.9	2.8	2.8	2.5	2.5
Ser	3.2	2.8	2.9	2.9	2.8
Phegly	2.7	2.9	2.9	2.7	2.6
Trp	2.2	2.4	2.4	2.3	2.3

DBT-Trp at pH 5.8 in the polymer-free as well as in the polymer-containing electrolyte systems. (iv) The stereoselectivity data measured for DBT-Val in the polymer-free system exhibit maxima at medium concentrations of acetonitrile. These maxima indicate that the organic solvent acts in an apparently complex way. The maxima observed with DBT-Trp and DBT-Phegly at medium solvent concentrations in the system with 2% of PVP emerge because the effect of PVP is counteracted by the solvent and the specific effect of acetonitrile observed in the polymer-free system is maintained.

For the interpretation of these experimental results, the relevance of the two main effects of acetonitrile in aqueous solution have to be considered, i.e. first, the reduction of the strength of hydrophobic interactions and second, the shift in the pK_a values of the analytes.

(i) Acetonitrile is known to compete with hydrophobic intramolecular interactions. It is likely that in this way the stability of molecule conformations preferred in aqueous solution is affected and, thus, the actual mobility of the compounds. The loss in selectivity upon addition of acetonitrile frequently observed in the polymer-free system and within the investigated concentration range can be explained by this type of action. Similarly, the waiving of the polymer effect by the presence of acetonitrile can be explained by the same argument as far as the polymer effect is predominantly based on hydrophobic interactions.

(ii) The shift of the analyte's pK_a to higher values can reach up to 1 pH unit when increasing the content of acetonitrile up to 50% (v/v), as shown in Table 4. The pK_a of α-amido carboxy groups is shifted from about 3.5 to values between 4.2 and 4.8. At a pH of 7 this shift cannot have any observable influence on the degree of dissociation and thus on selectivity (cf. the results in the previous section), and it will be small even at a pH of 5.8. A pK_a shift effect is thus not of relevance for the data given in Table 5.

No simple explanation can be given for the increase in selectivity for DBT-Trp at acetonitrile concentrations higher than 20% (v/v) at both pH values (5.8 and 7) as well as for the small maxima in stereoselectivity observed reproducibly for DBT-Val at 20% (v/v) acetonitrile. The mechanism of acetonitrile action is obviously rather complicated.

4. Conclusion

The separation of diastereomeric derivatives of enantiomers by capillary electrophoresis in electrolyte solutions free of any solvent additive can attributed to two main factors.

(i) Diastereomeric compounds can exhibit different actual mobilities. This was found for the DBT-derivatives of some of the natural amino acids, but not for other DBT-amino acids and most of the DAT-derivatives. No differences were observed for the diastereomeric DBT-derivatives of three coumarin-related compounds, at least by our system using about 150 000 theoretical plates.

(ii) When selecting pH values near and below the pK_a of the acidic analytes, significantly higher selectivity values can be exploited in the majority of cases. In some instances inversion of migration order is obtained. Under these conditions, however, the migration time is considerably increased due to the lower degree of dissociation, and the efficiency is reduced, as the plate number is dependent on the charge number of the analyte [10,11].

The DBT-derivatized coumarinic drugs investigated have no stereospecific differences in the pK_a values of the diastereomers larger than 0.005 units. For di-basic DBT-amino acids a $\Delta pK_a \neq 0$ cannot be excluded, although it is more likely that the dependence of stereoselectivity on the pH observed results predominantly from the fact that the ratio of the actual mobilities of the D- and L-diastereomers alters when changing from the double charged state to the single charged one. This would imply that the minimum energy conformation adopted by the analytes is altered with the charge number. Following this explanation, the mobilities of the D-analytes in the single charged state are found in all instances to be less than those of the L-analytes, whereas the mobility increments assigned to the second

charge are higher for the D-analyte compared with the L-analyte in all instances. The pH dependence observed in polymer-free solution is not much affected by the presence of a polymeric network when dealing with aliphatic amino acids; it is significantly amplified when dealing with some aromatic amino acids.

(iii) The presence of organic solvent components like acetonitrile affects the separation in polymer-free as well as in polymer-containing solutions. For the investigated systems the counteracting of the shape-forming intramolecular interactions is argued to play the most important role when dealing with polymer-free solutions. In systems containing PVP, acetonitrile strongly counteracts the effect of the polymer on stereoselectivity. Overall, the addition of acetonitrile diminishes the separation in most instances investigated. Due to the manifold mechanisms involved, a rational prediction of optimum separation conditions with respect to this solvent component is difficult.

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