Chiral Separations in Capillary Electrophoresis

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I. Introduction

Chiral separations comprise one of the more attractive tasks of modern day chemistry because of the quintessential nature of life. Capillary zone electrophoresis (CZE) is classified often as an instrumental technique tailored for the needs of both biochemical and biological analysis. Hence, its utilization for chiral separations is highly attractive.

Systematic research of chiral separations using modern electrophoretic methods, capillary isotachophoresis, and CZE all started approximately in the mid-1980s (Table 1). Until around the mid-1990s, the main thrust of the research consisted of establishing the mathematical description and theoretical background of the chiral separation process by capillary methods. It was found that interactions between separated enantiomers and a chiral selector are the same for electrophoresis as in chromatography and that, as a consequence of this, identical chiral selectors are effective in both separation techniques. Although routine utilization of CZE for chiral separations had been possible for some years, CZE has now become the electrophoretic technique of choice in chiral separations. Communications on isotachophoretic chiral separations have virtually disappeared since 1994.

Table 1. Early Publication Activity on Chiral Separations Using Capillary Electrophoretic Techniques[a]

year	number of communications
1985	2
1986	0
1987	3
1988	5
1989	10
1990	11
1991	15
1992	31
1993	50
1994	45
1995	66
1996	84

[a] Data taken from reference journals.

Now that the initial period of fundamental research of chiral separations by capillary electrophoretic techniques appears to be finished, it seems pertinent to review the whole period critically. Thus, the present status of both the theory of chiral separations and their practical utilization, as well as their research requirements in the field, can be identified. Trends and prospects can be estimated and desirable on expected results outlined.

There is a substantial wealth of knowledge concerning this early period, a substantial amount of which may be found conveniently in review articles[1−30] as well as in a monograph.[31] These reviews deal with either the partial time periods[1−10] or the special aspects of electrophoretic chiral separations.[11−30] An almost complete list of relevant original papers published up until the end of 1996 is given in two of these reviews.[3,10] The increased possibility and attractiveness of using chiral separations practically enhance the frequency with which additional review articles have appeared in recent years. Unfortu-

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Professor Boček was born December 25, 1941, in Brno, Czechoslovakia. He graduated in chemistry in 1964 at Masaryk University, Brno, Czechoslovakia, and received his Dr. rer. nat. degree in Analytical Chemistry at the same university in 1967. In 1966 he began his work in gas chromatography at the Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, in Brno and in 1969 he obtained Ph.D. degree in this field. He then spent one year at the University of Eindhoven working in column technology for GC. In 1972 he became engaged in analytical isotoachophoresis, and after a decade of research, he received his D.Sc. degree in this field in 1983. He is currently head of a group working on all aspects of analytical electrophoresis at the Institute of Analytical Chemistry in Brno, and since 1993 he has also been head of this institute. He spent two long-term scientific stays at NIH, Bethesda, MD, working in the separation of DNA fragments by capillary electrophoresis in 1991 and 1993. In 1996 he was appointed Professor of Analytical Chemistry at University of Pardubice, Czech Republic. He is the author or co-author of more than 200 scientific papers, chapters, and, books on Analytical Capillary Isotachophoresis and Capillary Zone Electrophoresis. He is Deputy Editor of the international journal *Electrophoresis*, Associate Editor of the *Journal of Microcolumn Separations*, and member of the Editorial Board of *Journal of Chromatography*.

nately, the increase in the number of review articles published per year since 1997 and dealing with chiral separations at least partly appears somewhat unwarranted with respect to the increase in relevant knowledge.

II. Principles of Chiral Separations

Compounds where chirality is caused by the presence of asymmetric carbon atoms are the dominating type of chiral compounds of pharmaceutical, biochemical, and biological interest. Consequently, they are the subject of principal analytical interest also. The methodological approaches and related analytical methods effective in the chiral analysis of compounds containing asymmetric carbon atoms are effective in the analysis of chiral compounds of other types too. Chirality caused by other asymmetric atoms such as S, N, P, and B, chiralities of particular molecular structures, e.g., allene chirality, chirality of metal−alkene complexes, chirality of substituted biaryls, and of other compounds with the sterically hindered rotation of a σ-bond, chiralities of helical or sterically crowded molecules are all potential substrates. The discussion on chiral separations may, therefore, be limited to those of chiral compounds containing asymmetric carbon atoms.

The principal task of chiral separations is the creation of the selectivity essential for the separation of the sterically different forms of compounds such as enantiomers. Theoretical concepts presented below have been developed for free-solution electrophoresis. Processes vital for the creation of chiral discrimination in it work in electrophoretic techniques with a collaborating chromatographic process, micellar electrokinetic chromatography (MEKC) and capillary electrochromatography (CEC) (section V), too. Thus, theoretical concepts developed for free-solution electrophoresis are applicable adequately to MEKC and CEC.

A. Separation Selectivity

A difference in the apparent macroscopic migration velocities of the zones of the species A and B, $v_{A,app}$ and $v_{B,app}$, respectively, is the general prerequisite for their electrophoretic separation in free-solution electrophoresis. It is necessary to stress that it is the relative difference in apparent macroscopic migration velocities of species A and B which is the measure of how easily we can separate them. This is called the separation selectivity, S.[32] Let us have two enantiomers, A and B, with their apparent velocities $v_{A,app} > v_{B,app}$. Then the selectivity of their separation, S, can be expressed as

$$S = \frac{\Delta v_{app}}{\bar{v}_{app}} = \frac{v_{A,app} - v_{B,app}}{\bar{v}_{app}} \quad (1)$$

$$\bar{v}_{app} = \frac{1}{2}(v_{A,app} + v_{B,app}) \quad (2)$$

The macroscopic migration velocity of a zone is directly proportional to the electric field strength (voltage gradient), E, which induces the electromigration.

$$v_{app} = \mu_{app} E \quad (3)$$

The magnitude and the physical meaning of the proportionality constant, μ_{app}, called the apparent mobility, results from the mechanisms participating

in the macroscopic migration of a zone. The electric field strength, E, is a vector which is positive in the direction from the anode (+) to the cathode (−). Hence, if a species migrates from the anode to the cathode (cathodic migration), its velocity is positive and vice versa for the anodic migration. The apparent mobility also has a sign, corresponding to the sign of the charge of the migrating species. It is positive (+) ($\mu_{app} > 0$) for cations and negative (−) ($\mu_{app} < 0$) for anions. In the absence of the hydrodynamic flow of the liquid through the capillary, which is a standard situation in free-solution electrophoresis, the electrophoretic transport and the electroosmosis may contribute to μ_{app} only.

$$\mu_{app} = \mu + \mu_{eo} \tag{4}$$

where μ is the electrophoretic mobility of the migrating ion in question and μ_{eo} is the electroosmotic mobility. If the migrating ion is liable to fast and reversible chemical equilibria (see section III.A), the effective mobility of the ion,[33−35] μ_{eff}, is substituted into eq 4 instead of μ. For species A and B migrating as single ions, it holds for selectivity

$$S = \frac{\Delta\mu_{app}}{\bar{\mu} + \mu_{eo}} = \frac{\mu_A - \mu_B}{\bar{\mu} + \mu_{eo}} \tag{5}$$

with respect to eqs 2−4; $\bar{\mu} = {}^1/_2(\mu_A + \mu_B)$. Equation 5 reveals that a difference in either the mobilities or effective mobilities of A and B is the precondition for a nonzero selectivity. If A and B are sterically different forms of a chiral compound, either the a priori natural existence of a mobility difference for A and B or its creation are the preconditions for a nonzero selectivity. With respect to eqs 3 and 4, the same holds for apparent macroscopic velocities of zones of A and B.

The mobility of a species migrating as a single ion depends on (i) the electrophoretically effective charge of the ion, (ii) the volume and shape of the ion, (iii) the volume and shape of the solvation shell of the ion, and (iv) the ionic strength of the solution. The sterically different forms A and B of any chiral compound have identical charges in their common background electrolyte (BGE).[32,34,35] The standard charge control by means of pH[34,35] serves therefore for the electrophoretic mobilizing of A and B only.

The properties and interactions of enantiomers are identical in any achiral medium. Differences in their physicochemical and analytical properties may be evoked only by their interactions with a chiral medium. A species or compound capable of interacting differently with the enantiomers of the compound A, (*R*)A and (*S*)A, is called a chiral selector. In analytical practice, the chiral selector is a constituent of the separation system. It interacts with the enantiomers to be separated in such a way that a difference in the magnitude of their analytical properties results. Electrophoretic mobility is the analytical property of interest in electrophoretic separations.

The interaction between an enantiomer and a chiral selector may always be considered as the formation of a complex. Studies on the structure of a complex formed by the interaction of an enantiomer, e.g., of (*R*)A, with a chiral selector C, e.g., ref 36, prove that the simplest possible scheme of the complex formation

$$(R)\mathrm{A} + \mathrm{C} \rightleftarrows (R)\mathrm{AC} \tag{6}$$

is standard for interactions between enantiomers with a single asymmetric carbon atom and chiral selectors. This type of complexation is assumed in all theoretical studies on the chiral discrimination process. Different stoichiometries, e.g., those of complexation of naproxen[37] and binaphthyl derivatives[38] with cyclodextrins, are exceptions.

Two types of interactions compatible with eq 6 are of analytical interest: (i) the formation of the kinetically labile complex between the separated enantiomer and the chiral selector and (ii) the chemical reaction of the separated enantiomer with the chiral selector on forming a stable compound AC. The transient formation of kinetically labile complexes of the enantiomers during the separation process does not permanently change the chemical forms of the enantiomers. The enantiomers are separated directly in the form they have in the sample. Consequently, this kind of separation is called direct chiral separation.

Chiral compounds with two chiral centers (with two asymmetric carbon atoms) that differ in the sterical structure on one of the chiral centers are diastereoisomers. They have different shapes and, strictly speaking, have to have different mobilities. Consequently, in principle, they are separable in common achiral background electrolytes. The transformation of the separated pair of enantiomers into a pair of diastereoisomers is the trick of approach ii. Hence, the enantiomers are not separated directly in the form they have in the sample. The term indirect chiral separation is, therefore, used for this procedure. Direct chiral separations are preferable to indirect ones both in electrophoresis and in chromatography for reasons detailed below (section II.C.1). At least 95% of published chiral separations in electrophoresis are direct separations.

Compounds with three or more chiral centers, differing by sterical arrangement on one of the centers, may be separated by one of the two principal ways outlined above. Sterically different compounds with two centers of chirality close enough to each to other behave analytically as diastereoisomers. If the asymmetric centers are distant from each other, from an analytical point of view their steric effects are independent of each other and the compounds differing by sterical arrangement on one of the centers may be treated like enantiomers.

B. Separation of Diastereoisomers

Molecular shape differences of diastereoisomers are the principal cause for a difference in their mobilities. However, the principal existence of this difference does not guarantee that it is great enough to permit separation. There are several reasons for this. The magnitude of the shape difference between two diastereoisomeric ions depends strongly on the mutual distance of the chiral atoms.[39] In the most

favorable case, evoking maximum possible shape difference, the centers of chirality are bound directly to each other. With a given distance of the chiral atoms, the more pronounced the final difference will be the greater difference in volume and shape of the groups bonded to the chiral centers.

The mobilities of ionized diastereoisomers in solution are affected by both the volumes and shapes of their solvation shells. Different volumes of the solvation shells may result from the different accessibility of various structural and functional groups of the diastereoisomers to the solvent and from different affinities of the solvent (or its constituents if it mixture) to these groups. Solubility is the overall measure of the affinity of the solvent to the solute. However, as far as we are aware, significant differences in solubilities of diastereoisomers in the same solvent are not known. Therefore, the difference in the solvation shells of the diastereoisomers are unlikely to contribute to their overall shape difference in solution. Rather, the shape difference of diastereoisomers is partially masked by their solvation shells. As a result, one cannot expect that analytically useful differences in diastereoisomer mobilities occur frequently.

This conclusion is supported by the small number of satisfactory electrophoretic separations of diastereoisomers in standard achiral separation systems. To enhance the separation selectivity of diastereoisomers, various means were used. Addition of polymers to the BGE,[40] exploitation of the acidity difference found for diastereoisomers of a particular structure,[41] or addition of chiral selectors effective in separation of enantiomers[42,43] are some examples.

C. Separation of Enantiomers

1. Indirect Separations

A substance and its complex are stable from an analytical separation point of view if, once formed, they do not decompose during the separation. Both the stable compounds and complexes form discrete, well-shaped zones migrating with their own mobilities. The formation of stable reaction products of enantiomers with a chiral selector is the basis of indirect chiral separations (see section II.A). If a pair of enantiomers (*R*)A and (*S*)A is present in the sample, their chemical reactions with the chiral selector that must be pure enantiomer, e.g., (*S*)C, take place as follows

$$(R)\mathrm{A} + (S)\mathrm{A} + (S)\mathrm{C} \rightarrow (R)\mathrm{A}(S)\mathrm{C} + (S)\mathrm{A}(S)\mathrm{C} \qquad (7)$$

Different mobilities of the products (*R*)A(*S*)C and (*S*)A(*S*)C are the prerequisite for the separation of the enantiomers (*R*)A and (*S*)A.

Charges and volumes of diastereoisomeric complexes (*R*)A(*S*)C and (*S*)A(*S*)C resulting from the reaction are identical. The differences in their shapes and volumes and the shape of their solvation layers are the potential reasons for the difference in (*R*)AC and (*S*)AC mobilities. Their mobility difference is greatest if the chirality centers are directly bound to each other[39](section II.B). The enantiomeric derivatization reagents used in electrophoresis are given elsewhere.[7,25] However, the family of reagents recommended for indirect separations by liquid chromatography[39,44,45] may also be of use.

Unfortunately, it is seldom possible to choose a chiral selector and arrange its reaction with the appropriate enantiomers in such a way as to ensure the mutual bonding of chiral atoms. Consequently, indirect separations usually offer limited selectivity in electrophoresis. Moreover, serious drawbacks have to be considered in connection with the derivatization reaction. If the chiral selector is not perfectly pure from the chiral point of view, such that it contains two enantiomers, (*R*)C and (*S*)C, two reaction products are formed even if the analyte is a pure enantiomer.

$$(R)\mathrm{A} + (R)\mathrm{C} + (S)\mathrm{C} \rightarrow (R)\mathrm{A}(R)\mathrm{C} + (R)\mathrm{A}(S)\mathrm{C} \qquad (8)$$

Hence, four reaction products result from the reaction of an impure chiral selector, (*R*,*S*)C, with an analyte (*R*,*S*)A:

$$(R,S)\mathrm{A} + (R,S)\mathrm{C} \rightarrow (R)\mathrm{A}(R)\mathrm{C} + (R)\mathrm{A}(S)\mathrm{C} + (S)\mathrm{A}(R)\mathrm{C} + (S)\mathrm{A}(S)\mathrm{C} \qquad (9)$$

The symmetrical reaction products, (*R*)A(*R*)C and (*S*)A(*S*)C, will have identical analytical properties.[39] Thus, they are separated in the mixed zone and quantified as the sum. The same is true for the asymmetrical products (*R*)A(*S*)C and (*S*)A(*R*)C. Consequently, both the qualitative and quantitative aspects of the analysis deteriorate seriously as a result of the enantiomerically impure chiral selector. Typical problems encountered in achiral derivatization reactions are also relevant in the reactions 7−9.[39]

The possibility of analyzing chiral analytes in achiral separation systems and increasing the optical detection sensitivity by using a chiral selector with a suitable chromophore are the main advantages of indirect chiral separations.[7] However, these advantages are largely outweighed by their inherent drawbacks. In conclusion, indirect chiral separations are seldom used in electrophoresis, and the frequency of their use is decreasing.

2. Direct Separations

Prerequisite for good direct separation of enantiomers is that the equilibrium according to eq 6 is reversible and fast.[7,35] This means that the rates of formation and dissociation of the complex AC are much higher than the rate of the separation process. In such a case, the coexisting forms of the analyte are transported within one zone,[33−35,46] which in properly designed electrophoretic systems is narrow and symmetrical.[34,35]

The formation of the complex is usually visualized by the "three-point interaction" model proposed by Dalgliesh[47] (Figure 1) and revised by Pirkle.[48] The analyte is bonded to the selector at a specific position called the binding site in the model. The binding site consists of three interaction points realized by three mutually independent interactions between the functional or structural groups of the analyte and the selector. The requirement of three mutually independent interactions does not exclude independent

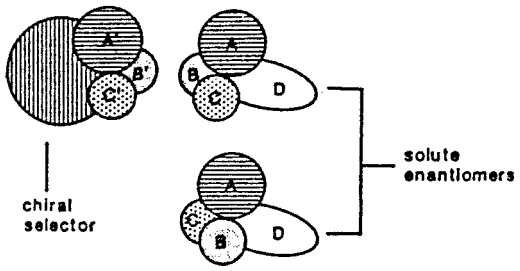


Figure 1. Model of the three-point interaction by Dalgliesh according to ref 39.

participation of two or even three qualitatively identical interactions. These interactions, determined by the chemical compositions of the analyte and the selector, may be either attractive or repulsive. The model allows two interactions for both enantiomers. The third interaction, dependent on the stereochemistry of the enantiomers,[49] is possible for one of them only. The preponderance of attractive interactions is necessary for binding the enantiomer to the chiral selector, and the difference in the sums of interactions is prerequisite for mutual separation of the enantiomers. Any intermolecular interaction, e.g., Coulombic attraction, Coulombic repulsion, hydrogen bonding, hydrophobic interactions, sterical hindrance, inclusion in the cavity, charge transfer, $\pi-\pi$ electron interaction, and dipole−dipole interactions, may participate in the chiral discrimination process. The allowance of the repulsive interaction is characteristic of the "three-point interaction rule". It should not be confused with the "three-point binding rule" (Ogstons's theory),[50] proposed for enzyme−substrate interactions. Ogstons's theory requires binding at least at three points.

From additional models visualizing the chiral discrimination process, the following two are relevant to electrophoretic separations. The model of the chiral cavity (Figure 2a) explains the discrimination process by adsorbents prepared by the surface-imprinting technique introduced by Dickey.[51] In this technique, the adsorbent is prepared in the presence of the molecules leaving "footprints" or imprints of their shape in it. Adsorbents of this type are of use in electrochromatographic separations (section V.B.). The model of a chiral cavity with interaction points (Figure 2b) is relevant in affinity chiral separations characterized by very strong interactions between the selector and the analyte.

The majority of enantiomeric compounds of pharmaceutical, medical, biochemical, or biological interest has complicated structures and bears various functional groups. These groups are potential interaction points. Thus, the greater the variety and number of groups in a chiral compound, the greater the probability that the sterical arrangement of three groups in one of the enantiomers will fit the binding site of a selector. In other words, the more complicated the molecule of the chiral analyte, the greater the chance of separating its enantiomers. The simpler the compound, the lower the probability that the sterical arrangement of three groups in one of the enantiomers will fit the binding site of a selector and, therefore, the more difficult the separation of its

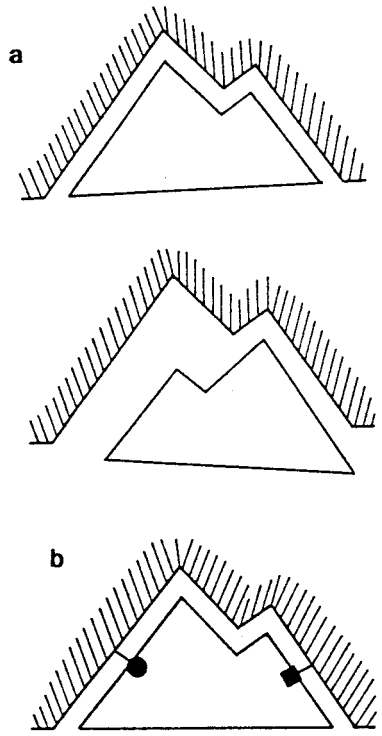


Figure 2. Models of (a) interaction of enantiomers with chiral cavity and (b) interaction of an enantiomer and a chiral cavity with two binding sites. Arranged using Wulff, G.; Minárik, M. In *Chiral Liquid Chromatography*; Lough W. J., Ed.; Blackie: Glasgow, 1989; pp 17 and 18.

enantiomers. For example,[7,21] ibuprofen was separated using β-cyclodextrin, methyl-β-cyclodextrin, avidin, bovine serum albumin, maltodextrin, maltooligosaccharides, and vancomycin. Norepinephrine was resolved using β-cyclodextrin, dimethyl-β-cyclodextrin, hydroxypropyl-β-cyclodextrin, β-cyclodextrin polymer, and rifamycin B. Taurodeoxycholate, dextran sulfate, and chondroitin sulfate were all effective in the separation of diltiazem. Contrary to such successes, to our knowledge the direct separation of simple compound 1,2-diaminocyclohexane, an important raw material in chiral synthesis, has not yet been reported. The prefect fit of three functional groups in one enantiomer with the selector binding site is necessary for the chiral discrimination. It also explains the well-known experimental fact that any difference in the structure of racemic compounds may cause a drastic difference in the enantioselectivity of separation of closely related racemic compounds with a particular selector or even its absence.[7]

In free-solution electrophoresis, as a rule the chiral selector is dissolved in the aqueous solution of the BGE. The composition, pH, and concentration of the BGE itself have to be such that the solubility of the analyte and of the chiral selector and the ionization of one at least of them, necessary for the different electromigration of the enantiomers, is achieved. Typical BGEs used in CZE of various chemical classes of components may be found, for example, in refs 7, 16, 17, 34, and 35. The choice of chiral selector and its concentration, crucial for the separation, is adapted to the related discrimination process. Sometimes, additional BGE constituents such as indifferent electrolytes and organic solvents may be

added.[17,31,34,35] Chiral separations in nonaqueous solutions have also been described.[52−54] Instrumentation and experimental techniques, including micellar electrokinetic chromatography (MEKC)[13,17,20,27,55,56] and electrochromatography,[19,55,56] suitable for chiral separations are identical with those developed for and used in achiral separations.

III. Tuning of Direct Separations

The tuning of direct chiral separation involves the selection of a chiral selector and its optimum concentration in the BGE in order to reach the necessary separation selectivity and the separation speed. Sometimes the composition of the BGE also has to be modified. There are no rules for the a priori selection of the best or most appropriate chiral selector for a given separation problem. This is due to the absence of theories satisfactorily describing the mechanisms of the interactions of selectors with various chiral compounds. The estimation of the discrimination capability of a selector for the compounds in question, based on separations of closely related compounds, is of limited reliability. Dramatically different discriminations of closely related chiral compounds, even including in some cases the absence of chiral discrimination, are a well-known proved experimental fact.[7]

The existing theories of the chiral discrimination process in electrophoresis can help in the selection of the analytically optimum concentration of the chiral selector if the respective stability constants of the selector−enantiomer interaction are known. In practical separations, the influence of the chiral selector, e.g., a cyclodextrin, on the migration of the achiral constituents of the sample (on the sample matrix) is often important. Unfortunately, the magnitudes of these influences cannot be predicted from existing theoretical concepts and experimental facts for particular achiral constituents.

A. Theory

The simultaneous migration of several coexisting forms of a compound, bound to each other by fast chemical equilibria, is described by the concept of effective mobility, μ_{eff}, introduced by Tiselius[33] in the electrophoretic theory.[34,35]

$$\mu_{A,eff} = \sum \mu_{A,i} x_{A,i} \tag{10}$$

Here, $\mu_{A,i}$ is the mobility of the ith form of the analyte present in the molar fraction $x_{A,i}$ in the migrating zone of the compound. Hence, it holds for A $\sum x_{A,i} = 1$ in the zone.

Let us consider for the sake of simplicity a chiral selector present in the system in one chemical form and interacting with a single charged, fully ionized weak acid or weak base. The interaction, expressed by eq 6, represents the separation of the majority of chiral analytes. Considering this prevailing dynamic equilibrium, the effective mobility of A, $\mu_{A,eff}$, is a function of the ionic mobility of A measured in the absence of chiral selector in the system, μ_A, of the mobility of the complex AC, μ_{AC}, and of the molar fractions of both free and complexed forms of A in the migrating zone, x_A and x_{AC}, respectively. In the zone, $x_A + x_{AC} = 1$.

$$\mu_{A,eff} = \mu_A x_A + \mu_{AC} x_{AC} \tag{11}$$

The stoichiometric stability constant[46] of the complex AC, K_A, defined by the equilibrium concentrations[57] of the analyte A, [A], chiral selector C, [C], and of their complex AC, [AC], is

$$K_A = \frac{[AC]}{[A][C]} \tag{12}$$

for the equilibrium given by eq 6. By combining eq 12 and the relationship $x_A + x_{AC} = 1$, the expression for $\mu_{A,eff}$ becomes

$$\mu_{A,eff} = \frac{\mu_A + \mu_{AC} K_A [C]}{1 + K_A [C]} \tag{13}$$

which is a more appropriate form for the understanding of both the origin of the separation selectivity and ways of its manipulation (see section III.B).

The apparent mobility of a zone, μ_{app}, necessary for the calculation of the separation selectivity (eq 5), may be obtained from the total length of the separation capillary, l_c, its migration distance from the injection inlet to the detector, l_d, the migration time, t, and from the applied voltage, V:[31]

$$\mu_{app} = \frac{l_d}{t} \frac{l_c}{V} \tag{14}$$

Here, V/l_c stands for the vector of electric field strength E, which is positive for the migration from anode (+) to cathode (−), i.e., cathodic migration. In practice, considering l_d, l_c, V, and t as positive, we must then ascribe a sign to the mobility, μ_{app}, in such a way that it is positive (+) for cathodic migration and negative (−) for the anodic one.

In analysis with the capillary producing electroosmosis (e.g., an uncoated fused silica capillary) and in the absence of hydrodynamic flow, it holds that

$$\mu_{app} = \mu_{eff} + \mu_{eo} \tag{15}$$

Let us consider that (*R*)A and (*S*)A are the enantiomers of A. With respect to eq 5, their separation selectivity, S, is

$$S = \frac{\mu_{(R)A,eff} - \mu_{(S)A,eff}}{\bar{\mu}_{eff} + \mu_{eo}} = \frac{\Delta\mu_{eff}}{\bar{\mu}_{eff} + \mu_{eo}} \tag{16}$$

where $\bar{\mu}_{A,eff} = {}^1/_2(\mu_{(R)A,eff} + \mu_{(S)A,eff})$; for small differences in $\mu_{(R)A,eff}$ and $\mu_{(S)A,eff}$, $\mu_{(R)A,eff}$ may be used instead of $\bar{\mu}_{A,eff}$ in eq 16. Despite the prounounced influence of electroosmosis on the selectivity, a difference in the effective mobilities of the enantiomers, $\Delta\mu_{eff} = \mu_{(R)A,eff} - \mu_{(S)A,eff} > 0$, is prerequisite for the separation and detection of both the enantiomers, (*R*)A and (*S*)A. If μ_{eff} and μ_{eo} are equal and have opposite signs, then S equals infinity. However, such a case has no practical significance since one of the enantiomers then goes

to the detector and is detected and the other one goes in the opposite direction and is never detected.

The mobilities of the enantiomers (*R*)A and (*S*)A, $\mu_{(R)A}$ and $\mu_{(S)A}$, respectively, are identical and equal to μ_A. Chiral selectors exploited for direct separations are not enantiomers (section IV). Their molecular weights and volumes exceed those of both common monomeric chiral analytes and chiral selectors utilized in indirect separations (section II.C.1). Therefore, the difference in the shape of complexes of chiral selectors for direct separations with the enantiomers (*R*)A, (*S*)A is negligible. Hence, the difference in mobilities of the considered complexes is also negligible and the equivalence

$$\mu_{(R)AC} \doteq \mu_{(S)AC} = \mu_{AC} \tag{17}$$

holds.

Then eq 13 written for the enantiomers (*R*)A and (*S*)A differs only by the stability constants of their complexes with the chiral selector, $K_{(R)A}$ and $K_{(S)A}$, respectively. Using these equations, the difference in effective mobilities of enantiomers (*R*)A and (*S*)A, $\Delta\mu_{eff}$, is[59,60]

$$\Delta\mu_{eff} = \mu_{(R)A,eff} - \mu_{(S)A,eff} = \frac{(\mu_A - \mu_{AC})(K_{(R)A} - K_{(S)A})[C]}{1 + (K_{(R)A} + K_{(S)A})[C] + K_{(R)A}K_{(S)A}[C]^2} \tag{18}$$

A prerequisite for chiral separation is $\Delta\mu_{eff} > 0$ as pointed out above, and hence, the conditions $K_{(R)A} \neq K_{(S)A}$ and $\mu_A \neq \mu_{AC}$ have to be fulfilled. With respect to $\mu_{(R)A} = \mu_{(S)A} = \mu_A$ and $\mu_{(R)AC} \doteq \mu_{(S)AC} = \mu_{AC}$ (eq 17), it is evident from eq 18 that the difference in stability constants of the enantiomers is the crucial parameter securing their chiral discrimination in electrophoresis. A similar conclusion concerning the primary importance of the difference in stability constants is arrived at if chromatographic chiral separation of enantiomers is explored using established chromatographic theories. This is the reason the experimentally found discovery that the knowledge of the chiral discrimination process in liquid chromatography could be transferred to electrophoresis and vice versa.[61−64] Simultaneously, it explains why capillary electrophoretic techniques are recommended as pilot techniques in the development of chromatographic separations of enantiomers.[65] The decisive role of interactions of the chiral selector with the enantiomers underlies the proposed systematic approach to enantiomeric separations by capillary electrophoresis and liquid chromatography.[62−64] However, contrary to liquid chromatography with a chiral selector in one of the chromatographic phases, there is no unambiguous correlation between the strength of interactions of the selector with the enantiomers (*R*)A and (*S*)A, expressed by stability constants $K_{(R)A}$ and $K_{(S)A}$, on their migration order. The migration order of enantiomers depends, in addition, on their mobilities, the selector mobility, and electroosmosis.[7]

Equation 18 holds only if both the chiral selector and the enantiomers entering into mutual interaction are present in single chemical form in the system. Almost all chiral analytes and many chiral selectors are compounds bearing at least one weakly acidic or weakly basic group. For all tested chiral selectors, the pH was found to be most efficacious in the selectivity tuning. At this point, it is therefore reasonable to briefly explore the possibility of describing the chiral discrimination process, characterized by the $\Delta\mu_{app}$ value, as a function of pH. Let us start the analysis with the simplest possible case—the interaction of a chiral selector free of ionizable groups, e.g., α- or β-cyclodextrin, and a chiral compound bearing one ionizing group.

The effect of pH on the mobility of such a chiral analyte, characterized by its ionization constant, may be introduced into eq 18. Complexation of the chiral selector with the two chemical forms of the enantiomers (*R*)A and (*S*)A, characterized by four stability constants, also has to be included in the equation. Furthermore, mobilities of the complexes of the two chemical forms of the chiral analyte A with the chiral selector and their pH dependences, characterized by the respective dissociation constants, must be included. Thus, in its correct form, the equation expressing the pH dependence has to contain seven stability constants as well as the mobilities of the two complexes (chiral selector with charged form, chiral selector with uncharged form of the analyte). These mobilities are not assessable experimentally[66] (section III.C.2).

Such an equation may serve for computer optimization based on a set of experiments. However, for a priori predictions and other practical purposes, such a highly complicated equation is useless even if the pH dependence for mobilities of the chiral selector−analyte complexes is neglected.[67,68] Of its seven physicochemical constants, at best only the dissociation constant of the analyte may be found in the literature. The other six constants have to be measured and the mobilities of the chiral selector-analyte complexes calculated.[66] Up until now, only two attempts at obtaining the set of constants using computer-fitting procedures have been published.[67,68] In all other cases, e.g., if the chiral selector and the chiral analyte vary their chemical forms with pH because of ionization of a single functional group, a quantitative description of the pH effect on the separation selectivity is even more complicated.

If eq 18 is applied formally to a separation in which either the uncomplexed enantiomers or the chiral selector or both are present in two equilibrium forms, then the constants $K_{(R)A}$ and $K_{(S)A}$ become apparent stability constants.[47] Despite these deficiencies, eqs 13 and 18 may serve as a convenient aid in a discussion of possible ways for the selectivity tuning. In such a case, it is not the magnitude of the mobility difference but rather the character of its changes with the experimental variables, e.g., pH and chiral selector concentration, that is important.

The published theory[59,60,69−71] of chiral discrimination for separations with cyclodextrins, summarized in the eq 18, generally holds for electrophoresis. Experimental results incompatible with the theory were found only in the study on the influence of various buffer co-ions on the stability constants and on the mobilities of complexes of racemic anionic

sulfonamides with native and modified cyclodextrins.[72]

B. Optimization of the Analysis

Complete and fast separation of the analytes is the standard goal of separation methods. To meet it, both the selectivity and the speed of the process have to be optimized. Unfortunately, in chiral separations the way to increased selectivity and improve separation speeds are often contradictory. The principal goal in all chiral separations is to achieve optimum selectivity. Optimization of the separation speed is of secondary importance and becomes relevant only when sufficient or even excessive selectivity is reached.

The most effective way for both higher selectivity (eq 16) and speed is to increase the difference in effective mobilities of the enantiomers, $\Delta\mu_{eff}$. The drop in the denominator, $\mu_{eff} + \mu_{eo}$, attainable by the manipulation of electroosmosis, is paid for by the increased analysis time (eqs 14 and 15). Simultaneously, a longer analysis time is accompanied by increased peak widths adversely affecting the resolution.[73]

The most straightforward way to affect $\Delta\mu_{eff}$ (eq 18) is to control the concentration of a chiral selector in the system. The dependence of $\Delta\mu_{eff}$ on the equilibrium concentration of the chiral selector in the separation system, [C], is found to pass through a maximum.[59,60,69−71,74] The total analytical concentration of the chiral selector in the separation system, *c*, is used currently as the parameter in experimentally measured dependencies of the effective mobilities of the enantiomers and of its difference (Figure 3a). Recalculation of the total analytical concentration, *c*, to the relative equilibrium concentration, [C], is discussed in section III.C.1.

For systems in which the approximation expressed by eq 17 is valid, the location of the maximum difference in mobilities, $\Delta\mu_{eff,\,max}$, in the scale of the equilibrium concentrations of the chiral selector, [C] (Figure 3b), correlates simply with the stability constants $K_{(R)A}$ and $K_{(S)A}$:[59,69,74,75]

$$[C]_{max} = \frac{1}{\sqrt{K_{(R)A}K_{(S)A}}} \qquad (19)$$

The lower the stability constants of the complexes, the higher the concentration of the chiral selector must be in order to reach the maximum possible difference in mobilities of the enantiomers. It is useless to utilize the chiral selector in concentrations which exceed the c_{max} corresponding to the $[C]_{max}$ in eq 19. The difference in the effective mobilities decreases if $c > c_{max}$ is used[59,60,69−72,76] (Figure 3b). Simultaneously, the analysis time increases in the analysis of charged analytes due to the monotonic decrease of the mobilities of the enantiomers with increasing concentration of the chiral selector (Figure 3a). A long analysis time increases the peak dispersion[73] which, together with the decreased separation selectivity, contributes to the drop in resolution.[34,35] In separations based upon chiral selectors with a high capability of discriminating sterical differences of interacting compounds, e.g., proteins or macrocyclic antibiotics, as a rule concentrations close to c_{max} produce analytically excessive mobility differences (Figure 4). A more viable analysis is the result using much lower concentrations in highly selective, speed-efficient separation systems[77,78] (Figure 5).

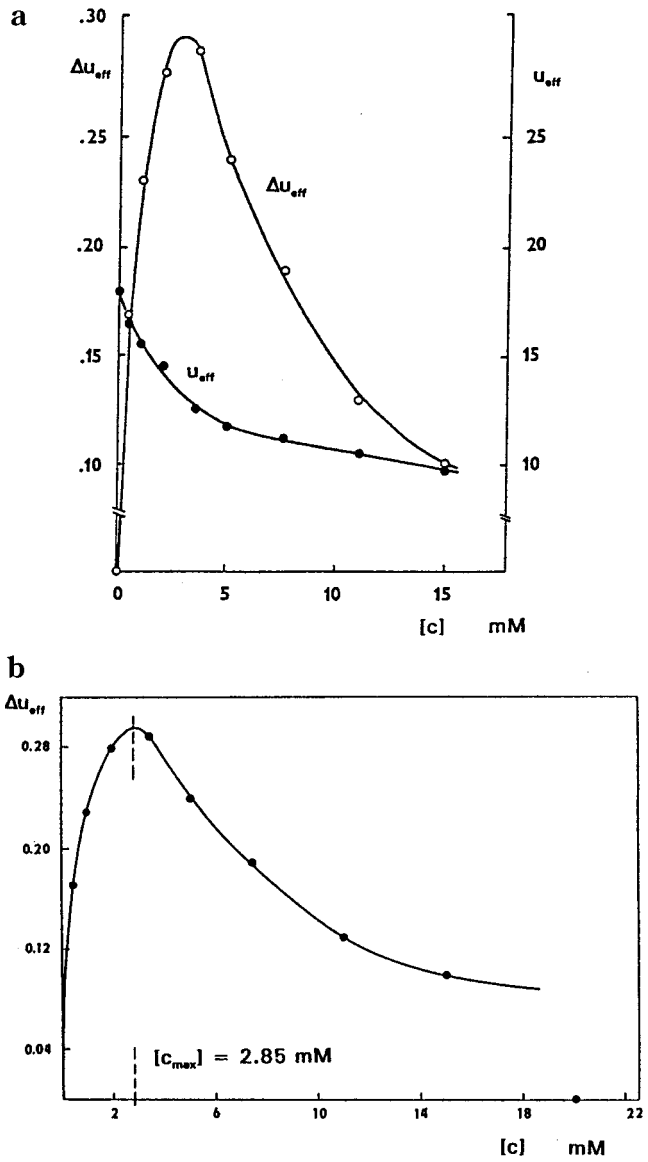


Figure 3. (a) Typical dependencies of effective mobility of an enantiomer, u_{eff}, and the difference in effective mobilites, Δu_{eff}, on total (analytical) concentration of the chiral selector, *c*. (b) Determination of the optimal concentration of the chiral selector in the system from the dependence of difference in effective mobilites, Δu_{eff}, on the concentration of the chiral selector, *c*, in the background electrolyte. Data represent separation of *N*-t-BOC-D,L-tryptophan by β-cyclodextrin in 20 mM α-hydroxyisobutyric acid, adjusted by NaOH to pH 4.5, at 25° C. For other details, see ref 76.

pH is a powerful variable in selectivity tuning. Its complex effect on the final $\Delta\mu_{eff}$ is dependent on both the nature of the chiral selector and the analyte. For example, the ability of bovine serum albumin to discriminate sterical structures of various compounds and compound types is markedly affected by its conformation.[61,79] In separations based on albumin, the selectivity effects caused by the pH-dependent conformation often dominate. The effects resulting

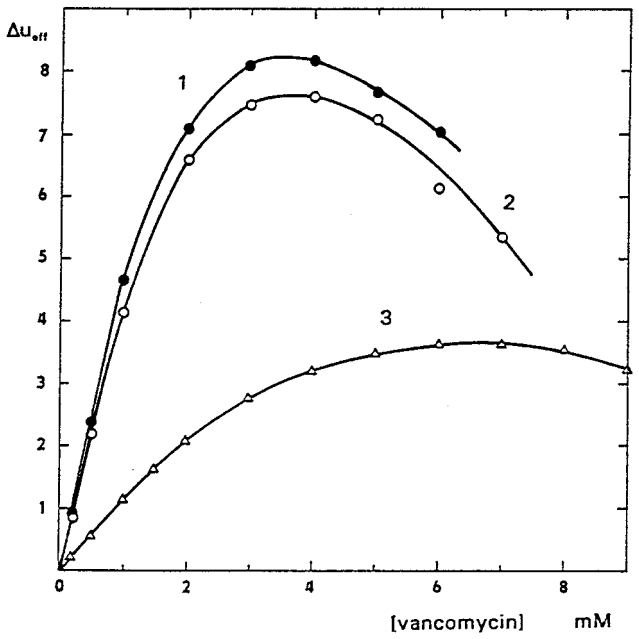


Figure 4. Differences in effective mobilities, given in units 10^{-9} m^2 V^{-1} s^{-1}, of sterically different forms of (1) selenocystine, (2) cystine, and (3) methionine, derivatized with the aminoquinolinecarbamate (AQC) reagent, reached with vancomycin dissolved in 20% mM MOPS-Tris buffer of pH 7 at 25° C. For details, see ref 77.

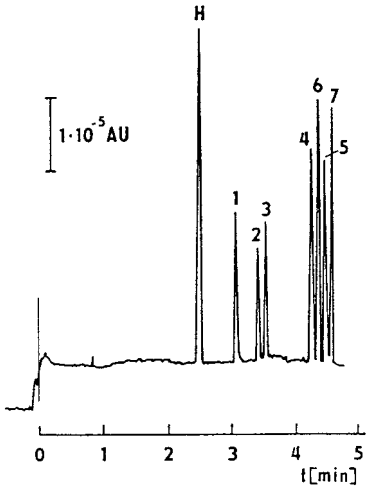


Figure 5. Fast separation of sterically different forms of selenocystine (peaks 1−3), methionine (peaks 4,5), and selenomethionine (peaks 6,7), derivatized with the aminoquinolinecarbamate (AQC) reagent, by 0.7 mM vancomycin dissolved in 20 mM MOPS-Tris buffer, pH 7. For approximate optimal concentrations of vancomycin, relevant to selenocystine and methionine, see Figure 4. Main experimental details: polyacrylamide-coated 50 μm i.d. fused silica capillary, 37.8 cm total length, 30 cm effective length; voltage −25 kV. For other details, see refs 77 and 78. (Reprinted with permission from ref 77. Copyright 1996 Wiley-VCH.)

from the interactions of the various chemical forms of enantiomers and chiral selectors (from the varying number of elementary enantiomer−selector equilibria), discussed in the previous chapter, are general. The possibility of modeling the effect of pH on the resolution via its effect on the separation selectivity was demonstrated using chiral separations with uncharged cyclodextrins.[80] The effect of pH on separation selectivity through its effect on electroosmosis (eq 16) is also general.

Keeping other quantities in eq 18 constant, $\Delta\mu_{eff}$ increases with $(\mu_A - \mu_{AC})$. The mobility of the complex, μ_{AC}, always lies between the mobility of the uncomplexed analyte, μ_A, and of the mobility of the selector, μ_C, $(\mu_A > \mu_{AC} > \mu_C)$. It lies close to μ_C as a rule.[66] Consequently, the selection of a pH which gives the enantiomers and the chiral selector opposite charges is sometimes particularly efficacious in selectivity manipulation.[78] The strong Coulombic attraction of the opposite charges stimulates the complexation. A complete ionization of A together with opposite charges of A and C maximize the difference $(\mu_A - \mu_{AC})$. Moreover, the increased ionization of A promotes the separation speed. Hence, charged chiral selectors capable of discriminating fully ionized enantiomers and bearing opposite charges than enantiomers are preferable to uncharged selectors from both the selectivity and separation speed viewpoints.

Temperature is another variable which can efficiently contribute to selectivity manipulation. However, compared to the changes resulting from pH tuning, the results of temperature changes are usually less pronounced. A decrease in temperature increases the separation selectivity irrespective of the chiral selector used as long as the concentration of the chiral selector is below $[C]_{max}$. An increase in separation selectivity has, to our knowledge, not yet been reported. Therefore, elevated temperatures and the production of Joule heat inside the capillary have to be avoided or at least minimized in chiral separations. The common increase in selectivity with decreasing temperature indicates that the spontaneous complexation of chiral analytes by chiral selectors is an exothermal process.

The addition of modifiers such as polar organic solvents as well as changes in the composition and concentration of the buffering electrolyte may also prove useful in the manipulation of enantioselectivity.[17,52,77,78] In this case, both the additives and the constituents of the separation systems compete with the analytes for the chiral selector. Consequently, the stability constant K (eq 12), which expresses the strength of the selector−analyte interaction, decreases.[60,62] The addition of an organic competitor, e.g., methanol[80] or cyclohexanol,[62] causes, therefore, a weakening in the too strong interactions of the analytes with the chiral selector. This weakening is reflected in lower stability constants.[62,80] Consequently, an increase in the optimum concentration of the chiral selector results (see eq 19). The same effect, a decrease in the stability constants, is observed in nonaqueous solvents as well as mixtures with water.[52−54] A strong increase in the separation selectivity was observed in the separation of derivatized amino acids with β-CD if either urea or its allyl derivatives were added in very high concentrations. However, with other native and derivatized cyclodextrins, the selectivity decreased.[81] Organic solvents affect the solubility of hydrophobic analytes and, if present in high concentrations, the ionization of analytes too.

The efficacy of such auxiliary methods varies widely depending on the nature of the chiral selector as well as the compounds to be separated.[80]

A single chiral selector is usually sufficient for the separation of one or two related compounds. In the separation of a family of chemically different compounds, e.g., a drug and its metabolites, it is often impossible to separate all of them by means of a single chiral selector. In such a case, a combination of various chiral selectors is often of help. Examples are given in sections IV and V. The term "array of selectors" often denotes such a combination of chiral selectors.

C. Calculation of Stability Constants

Equation 6 describing the fast and reversible one-to-one association of a chiral selector with a chiral analyte and eq 12 giving its principal thermodynamic characteristic generally hold for fast and reversible one-to-one association processes in solution.[24] Consequently, the complexation underlying direct chiral separations by electrophoresis is a particular case of fast reversible association in solution. In the literature, different names such as stability constant, association constant, and binding constant are used for this constant. In this review, the term stability constant is preferred.

There are two simple and straightforward possibilities for the calculation of the stability constants offered by the electrophoretic theory. Both are based on simple equations and require a set of experimental data for calculation. Identical data may serve for both calculations.

1. Mean Value of the Constants

For the estimation of approximate values of the stability constants $K_{(R)A}$, $K_{(S)A}$, the dependence $\Delta\mu_{eff} = f([C])$ (Figure 3b) may be used. Equation 19 states that the geometrical mean of the stability constants $K_{(R)A}$ and $K_{(S)A}$, $K_{mean} = (K_{(R)A}. K_{(S)A})^{1/2}$, relates to $[C]_{max}$[59,63,69,74] if proper input data are used. Thus, eq 19 may be rewritten in the form[66]

$$K_{mean} = \frac{1}{[C]_{max}} \qquad (20)$$

The experimental data set only is sufficient for the calculation. The location of the maximum in the difference of the effective mobilities of separated enantiomers, $\Delta\mu_{eff,max}$, in the scales of either [C] or c is important for the calculation result only; no other data are required. The magnitude of the difference, $\Delta\mu_{eff,max}$, as well as the magnitudes of the effective mobilities of the enantiomers, $\mu_{(R)A,eff,max}$ and $\mu_{(S)A,eff,max}$, are unimportant. A constant temperature inside the capillary during the experiments, the elimination of or correction for viscosity effects, and a negligible influence of the chiral selector adsorption on the capillary wall on [C] are the only requirements. Low Joule heat production is the easiest way to regulate the temperature inside the capillary during experiments with variable concentrations of the chiral selector. The microscopic viscosity of the solvent in the immediate surroundings of the dissolved ions is important for ion migration. The viscosity measured using classic viscosimetry is relevant only if cyclodextrins[59,75,82,83] and other low-molecular-weight constituents are in the system. If polymeric constituents such as polymerized cyclodextrins or poly(vinylpyrrolidone)[41] are added, the strongly increasing macroscopic viscosity of the system is not decisive for the electrophoretic transport. This is evidenced by the electromigration of DNA fragments in capillaries filled with rigid cross-linked polacrylamide gel[34,35] and analysis with the chiral selector incorporated into the three-dimensional gel polymerized in the capillary and having infinite macroscopic viscosity.[88]

If a single form of the chiral selector interacts with the single form of the enantiomers (*R*)A and (*S*)A, a single equilibrium corresponding to eq 6 applies for each of the enantiomers. Hence, we obtain true stability constants[46] $K_{(R)A}$, $K_{(S)A}$, defined by eq 12, and the value calculated from eq 20 is their geometrical mean. This mean is independent of pH in the range in which the single forms of the selector and the enantiomers exist. Outside this range, more than two equilibria contribute to the chiral discrimination of each of the enantiomers. For example, if the enantiomers become partially charged, different equilibria for the charged and uncharged forms of each of the enantiomers with the chiral selector, present in a single form, affect the separation result. Obviously, the final mobility difference, $\Delta\mu_{eff}$, is given by four elementary equilibria according to eq 6. From a mathematical point of view, eq 18 also covers the dependence $\Delta\mu_{eff} = f([C])$ in this case. However, the calculated K_{mean} value is the mean of apparent stability constants[46] in this case and, in general, is pH dependent. The shift in the molar fractions of the uncharged and charged forms of the analytes due to pH changes is one of the reasons. In the case where both enantiomers as well as the chiral selector are partially charged under the experimental pH, eight elementary equilibria underlay the separation.

Analytically convenient concentrations of cyclodextrins and other commonly used chiral selectors, not exceeding their optimum relative concentrations $[C]_{max}$, rarely lie outside the concentration range $1-10^2$ mmol/L. Concentrations of chiral analytes are below the concentration limit of 0.1 mmol/L as a rule, and the stability constants, characterizing their interactions with the selectors, seldom exceed $K = 1000$. The fraction of the complexed selector is negligible in such systems, and the numerical value of the total concentration of the selector in the system, c, expressed in mol/L, may be identified with [C]. If the stability constants are of the order of 10^4 and higher, this approximation becomes invalid and, in addition, systematical errors which increase with the magnitude of the stability constant are introduced into the experimental data.[58] Unfortunately, no solution for these difficulties has been proposed yet.

The estimate of stability constants given by eq 20 is reasonable for separations with low and moderate selectivity where $K_{(R)A}$ and $K_{(S)A}$ differ by up to ca. 25%. For markedly higher differences in stability constants, the method becomes unreliable.

2. Individual Constants

The methods for the calculation of individual stability constants described below hold for the systems in which single forms of the enantiomers interact with a single form of a chiral selector. It means that a single elementary equilibrium describes the interaction of the chiral selector with each of the separated enantiomers. All of the published methods for the calculation of individual stability constants,[24] including computer fitting procedures, originate from eq 13.[24,66] Its rearrangement to the form is convenient

$$K = \frac{1}{[\mathrm{C}]} \frac{\mu_{\mathrm{A}} - \mu_{\mathrm{A,eff}}}{\mu_{\mathrm{A,eff}} - \mu_{\mathrm{AC}}} \quad (21)$$

for the numerical calculation. The difference in the effective mobilities of the enantiomers, $\Delta\mu_{\mathrm{eff}} = \mu_{(R)\mathrm{A,eff}} - \mu_{(S)\mathrm{A,eff}}$, is measured easily and accurately experimentally. This difference may be converted to the difference in stability constants $\Delta K = K_{(R)\mathrm{A}} - K_{(S)\mathrm{A}}$ using eq 22.

$$\Delta K = \frac{1}{[\mathrm{C}]} \frac{(\mu_{\mathrm{A}} - \mu_{\mathrm{AC}})(\mu_{(R)\mathrm{A,eff}} - \mu_{(S)\mathrm{A,eff}})}{(\mu_{(R)\mathrm{A,eff}} - \mu_{\mathrm{AC}})(\mu_{(S)\mathrm{A,eff}} - \mu_{\mathrm{AC}})} \quad (22)$$

The ionic mobility of A, μ_{A}, measured in the absence of the chiral selector, the set of mobility data $\mu_{\mathrm{eff}} = f([\mathrm{C}])$, measured for each of the enantiomers, and the mobility of the complex, μ_{AC}, are necessary for point-to-point calculation. However, obtaining the true values of the required data is not an easy task.

Electrophoretic migration of ions in the free solution is a simple process described by simple equations, such as eqs 6, 11−13, and 21. Electrophoretic methods are highly effective in the separation of charged analytes. Thus, the idea arose that representative stability constants are so easy obtained and that routine analytical data may be used. Unfortunately, the idea is incorrect. The experimental data μ_{A} and $\mu_{\mathrm{eff}} = f([\mathrm{C}])$ have to be measured under identical experimental conditions, including the temperature inside the capillary and the ionic strength and viscosity of the solution. The measured changes in μ_{eff}, used in the calculation, must result only from the effect of the variable concentration of the chiral selector on its interactions with the enantiomers. The mean concentration of the selector which interacts with the enantiomers inside the capillary has to be identical with its concentration in the BGE filled into the capillary. Any other effects connected with or caused by the variable concentrations of chiral selectors are side effects that have to be either eliminated experimentally or corrected for. Changes in viscosity and ionic strength of the background electrolyte, Joule heat production, electroosmosis due to adsorption of the selector,[19] analytes and of the complex on the capillary wall, interactions of the enantiomers with the selector adsorbed on the capillary wall, competition of the BGE constituents for the chiral selector, and complexation of analytes or the selector with a BGE constituent are common side effects. Input data have to be measured with the highest possible precision; 1% error in the input mobility data causes up to a 10% error in the calculated stability constants.[66]

In common analytical measurements such as separations, these side effects are unimportant provided that the migration times of the analytes are reproducible, their peaks symmetrical, and the sample constituents of interest are resolved satisfactorily. Therefore, in common analysis, as a rule the fulfilment of the above listed conditions is not checked and until now minor attention was paid to them in determinations of stability constants[75,85] including those summarized in Tables 2 and 3. The absence of proper criteria is one of the reasons.

The mobility of a dissolved species A at an infinitely high concentration of C is the physical meaning of the chiral selector−analyte complex mobility, μ_{AC}.[66] This is why, in principle, μ_{AC} is not directly accessible experimentally and has to be determined in another way. The common methods for the estimation of μ_{AC} are applicable under special conditions only.[66] Otherwise, significant errors may be introduced in μ_{AC} and, with respect to eq 21, also in the stability constant. If the acceptable relative error in μ_{AC} is 5%, the extrapolation of μ_{AC} from the dependence $\mu_{\mathrm{eff}} = f([\mathrm{C}])$ is possible for strong interactions ($K > 10^4$). For large chiral selectors such as polymers with molecular weights greater than 5×10^4, the approximation of μ_{AC} by the mobility of the selector is possible. Correct values of μ_{AC} may be calculated from the effective mobilities of the enantiomers (*R*)A and (*S*)A, measured at the chiral selector concentration $[\mathrm{C}]_{\mathrm{max}}$ corresponding to the maximum mobility difference (Figure 3b), $\mu_{(R)\mathrm{A,eff,\ max}}$ and $\mu_{(S)\mathrm{A,eff,\ max}}$, respectively, and from the mobility of A, μ_{A}:[66]

$$\mu_{\mathrm{AC}} = (\mu_{(R)\mathrm{A,eff,max}} + \mu_{(S)\mathrm{A,eff,max}}) - \mu_{\mathrm{A}} \quad (23)$$

Existing computation techniques can easily treat the formulas and the input sets of experimental data for calculation of the stability constants. Therefore, it is not so much the formula and the calculation procedures used but rather the way in which the input data are obtained that is decisive for the relevance and potential usefulness of the calculated data. Stability constants related to the chiral discrimination process and chiral selectors, calculated from electrophoretic migration data,[19,52,54,59,62−68,72,75,77,80,82,83,85−100] are summarized in Tables 2 and 3. The extent to which the conditions discussed above were met in the measurements of the input data was seldom mentioned by authors. Rather, standard corrections for the viscosity and ionic strength of the background electrolyte were only applied. It is necessary, therefore, to realize that the summarized constants have to be understood as conditional ones[46,58] and relevant to the actual experimental conditions only. Despite that limitation, they are helpful in optimizing separations and in interpreting the experimental results. Information on typical values of stability constants of chiral selector complexes, on experimental variables affecting constant magnitudes, and on the general capability of the selectors to discriminate sterical structure of enantiomers may be also extracted from these data.

Table 2. Stability Constants of Analyte Complexes with Native Cyclodextrins Calculated from Electrophoretic Mobility Data[a]

selector	analyte	K_1[b]	$100\Delta K/K_1$[c]	K_{mean}[d]	comment	ref
α-CD	benzylamine	15			50 mM benzylamine pH 6.0 adjusted by HCl	92
	3-nitrophenol	199			50 mM sodium phosphate pH 11.1	85
	4-nitrophenol	1570			50 mM sodium phosphate pH 11.1	85
	salicylic acid	8			50 mM salicylic acid adjusted by NaOH to pH 6	92
	salicylic acid	15			20 mM phosphate pH 11	92
	tryptophan	15.4	31.2		100 mM phosphoric acid + Tris pH 2.5; 25° C	82
β-CD	L-Ala-L-Phe, protonized	42			70 mM sodium phosphate + 2 M urea	98
	L-Ala-L-Phe, deprotonized	4			70 mM sodium phosphate + 2 M urea	98
	D-Ala-D-Phe, protonized	44			70 mM sodium phosphate + 2 M urea	98
	D-Ala-D-Phe, deprotonized	2			70 mM sodium phosphate + 2 M urea	98
	L-Ala-D-Phe, protonized	60			70 mM sodium phosphate + 2 M urea	98
	L-Ala-D-Phe, deprotonized	23			70 mM sodium phosphate + 2 M urea	98
	D-Ala-L-Phe, protonized	66			70 mM sodium phosphate + 2 M urea	98
	D-Ala-L-Phe, protonized	41			70 mM sodium phosphate + 2 M urea	98
	amphetamine	59	11.9		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	benzylamine	9			5O mM benzylamine pH 6.0 adjusted by HCl	92
	carprofen			630[e]	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	dansyl-glutamate	187	17.6		20 mM phosphate pH 6.8 + 20% methanol	62
	dansyl-leucine	141	20.5		20 mM phosphate pH 6.8 + 20% methanol	62
	L-Leu-L-Phe, protonized	43			70 mM sodium phosphate + 2 M urea	98
	L-Leu-L-Phe, deprotonized	5			70 mM sodium phosphate + 2 M urea	98
	D-Lue-D-Phe, protonized	47			70 mM sodium phosphate + 2 M urea	98
	D-Lue-D-Phe, deprotonized	1			70 mM sodium phosphate + 2 M urea	98
	L-Leu-D-Phe, protonized	90			70 mM sodium phosphate + 2 M urea	98
	L-Leu-D-Phe, deprotonized	10			70 mM sodium phosphate + 2 M urea	98
	D-Leu-L-Phe, protonized	94			70 mM sodium phosphate + 2 M urea	98
	D-Leu-L-Phe, deprotonized	28			70 mM sodium phosphate + 2 M urea	98
	dansyl-serine			~5	10 mM sodium chloride in dimethylformamide	54
	dansyl-threonine			~5	10 mM sodium chloride in dimethylformamide	54
	3,5-dinitrobenzamido-phenylalanine, uncharged	62.7	29.3		100 mM ϵ-amino-*n*-caproic acid + 100 mM methanesulfonic acid; pH adjusted by LiOH	88
	3,5-dinitrobenzamido-phenylalanine, charged	62	61		100 mM ϵ-amino-*n*-caproic acid + 100 mM methanesulfonic acid; pH adjusted by LiOH	88
	fenoprofen, uncharged	608	4.4		200 Mm mes + 0.2% hydroxyethyl cellulose	67
	fenoprofen, charged	325	0.0		200 Mm mes + 0.2% hydroxyethyl cellulose	67
	fluoxetine	1080	6.1		1% triethylammonium acetate pH 5.5 + 10% acetonitrile	63
	fluoxetine			760	50 mM sodium acetate (pH not given explicitly; 5.5 probably)	63
	fluoxetine			110	100 mM triethylammonium acetate (pH not given explicitly; 5.5 probably)	63
	fluoxetine	143	5.9		1% triethylammonium acetate pH 5.5 + 20% acetonitrile	63
	flurbiprofen			3980[e]	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	homatropine, uncharged	1310	3.4		35 mM phosphate + 0.2% hydroxyethyl cellulose	68
	homatropine, charged	88	18.5		35 mM phosphate + 0.2% hydroxyethyl cellulose	68
	ibuprofen, uncharged	1870	4.4		200 mM Mes + 0.2% hydroxyethyl cellulose	67
	ibuprofen charged	1280	0.0	1280	200 mM Mes + 0.2% hydroxyethyl cellulose	67
	indoprofen			200[e]	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	ketoprofen			630	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	metamphetamine	74	16.3		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	3,4-methylenedioxyamphetamine	198	9.1		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	3,4-methylenedioxyethyl-amphetamine	323	10.5		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	3,4-methylenedioxymethyl-amphetamine	235	11.0		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	3,4-methylenedioxymethyl-amphetamine	357	12.1		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	5-methoxy-(di-*n*-propylamino)-3,4-dihydro-2*H*-1-bynzopyran	58	24.0		50 mM phosphate-borate pH 7.0 + 8 M urea; 25° C	89
	5-hydroxy-(di-*n*-propylamino)-3,4-dihydro-2*H*-1-bynzopyran	138	16.8		50 mM phosphate-borate pH 7.0 + 8 M urea; 25° C	89
	mianserin	135	91.7		50 mM citric acid + 25 mM Tris in water	52
	mianserin	23.8	64.7		50 mM citric acid + 25 mM Tris + 6 M urea in water	52
	mianserin	2,20	5.0		50 mM citric acid + 25 mM Tris in formamide pH 5.4	52
	mianserin	0.11	54.5		50 mM citric acid + 25 mM Tris in methylformamide pH 6.3	52
	mianserin	0.025	0.0		200 mM citric acid + Tris in dimethyl-formamide pH 5.8	52
	naproxen			1260[e]	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	3-nitrophenol	109			50 mM phosphate pH 11.1	85

Table 2 (Continued)

selector	analyte	K_1[b]	$100\Delta K/K_1$[c]	K_{mean}[d]	comment	
	4-nitrophenol	1500			50 mM phosphate pH 11.1	85
	norfluoxetine	991	5.0		1% triethylammonium acetate pH 5.5 + 10% acetonitrile	63
	norfluoxetine			840	50 mM sodium acetate (pH not given explicitly; 5.5 probably)	63
	norfluoxetine			1010	100 mM triethylammonim acetate (pH not given explicitly; 5.5 probably)	63
	propranolol			67	40 mM lithium phosphate + 4 M urea pH 3.1	59
	propranolol			160	20 mM phosphate pH 7.4	62
	propranofen			398[e]	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	salbutamol			9.6	20 mM citrate and 40 mM phosphate adjusted by NaOH to pH 7	95
	salicylic acid	82			50 mM salicylic acid adjusted by NaOH to pH 6	92
	salicylic acid	50			20 mM phosphate pH 11	92
	suprofen			1260[e]	formic acid adjusted to pH 4 by NaOH; $I = 0.75$	94
	thioridazin	29 100	21.6		50 mM citric acid + 25 mM Tris in water	52
	thioridazin	2980	28.2		50 mM citric acid + 25 mM Tris + 6 M urea in water pH 3.9	52
	thioridazin	5.39	20.2		50 mM citric acid + 25 mM Tris in formamide pH 5.4	52
	thioridazin	0.59	0.0		50 mM citric acid + 25 mM Tris in methylformamide pH 6.3	52
	thioridazin	0.059	0.0		200 mM citric acid + Tris in dimethylformamide pH 5.8	52
	tioconazole	1320	21.2		20 mM phosphate.citrate buffer pH 4.3	62
	tioconazole	223	16.1		20 mM phosphate.citrate buffer pH 4.3 + 0.1% of cyclohexanol	62
	tioconazole	1670	25.3	1880	100 mM phosphoric acid−triethanolamine pH 3	64
	tioconazole	870	24.0	974	100 mM phosphoric acid−triethanolamine pH 3 + 5% acetonitrile	64
	tioconazole	612	21.2	677	100 mM phosphoric acid−triethanolamine pH 3 + 10% acetonitrile	64
	tioconazole	237	21.8	263	100 mM phosphoric acid−triethanolamine pH 3 + 15% acetonitrile	64
	tioconazole	1320	21	1460	200 mM phosphate-citrate pH 4.3	64
	tioconazole	1120	21.2	1310	200 mM phosphate-citrate pH 4.3 + 1% methanol	64
	tioconazole	1090	23.6	1220	200 mM phosphate-citrate pH 4.3 + 4% methanol	64
	tioconazole	580	20.7	640	200 mM phosphate-citrate pH 4.3 + 10% methanol	64
	tioconazole	240	20.8	265	200 mM phosphate-citrate pH 4.3 + 25% methanol	64
	tioconazole	1320	20.5		5-fold diluted 100 mM citric acid and 200 mM disodium phosphate pH 4.3 ($I = 0.04$)	75
	trimipramin	5020	43.3		50 mM citric acid + 25 mM Tris in water	52
	trimipramin	1170	16.3		50 mM citric acid + 25 mM Tris + 6 M urea in water	52
	trimipramin	8.59	20.2		50 mM citric acid + 25 mM Tris in formamide pH 5.4	52
	trimipramin	0.34	31		50 mM citric acid + 25 mM Tris in methylformamide pH 6.3	52
	trimipramin	0.052	0.0		200 mM citric acid + Tris in dimethylformamide pH 5.8	52
γ-CD	leucovorin	2.21	2.3		sodium phosphate pH 7.1 ($I = 0.1$) + 6 M urea; 40° C	86
	5-methyltetrahydrofolate	3.85	1.6		sodium phosphate pH 7.1 ($I = 0.1$) + 6 M urea; 40° C	86

[a] The constants are rounded to three digits and given without reported uncertainty limits for the sake of simplicity. [b] Either the stability constant for the less interacting enantiomer or the stability constant for the achiral analyte; neither $\Delta K/K_1$ nor K_{mean} are given for the achiral analyte. [c] $\Delta K = K_2 - K_1$; K_2 is the stability constant for the more interacting enantiomer. [d] Either K_{mean} according to eq 19 or the value reported as the common value for the enantiomers.

IV. Chiral Selectors for Direct Separations in Free Solution

The investigation of direct chiral separations is, in fact, the investigation of selected properties of the chiral selectors and their complexation with chiral compounds. The reason for this is of course the principal role of the chiral selector in the sterical discrimination. The presence of a chiral selector in the separation system and its ability to interact in different ways with the enantiomers is the only precondition for chiral separation. Any differences between chromatographic and electrophoretic separation techniques and separation systems really only modify the process.

A range of compounds can interact stereospecifically with chiral compounds, and many of them have been tested as chiral selectors. However, only a few of them were found to be analytically convenient and accepted by the analytical community. The ideal chiral selector should be able to discriminate all enantiomers with a high and tunable selectivity. The discrimination process should not adversely affect

Table 3. Stability Constants of Analyte Complexes with Chemically Modified Cyclodextrins and Other Chiral Selectors, Calculated from Electrophore[a]

selector	analyte	K_1[b]	$100\Delta K/K_1$[c]	K_{mean}[d]	comment	ref
HP-α-CD[f]	4.nitrophenol	1860			50 mM phosphate pH 11.1	85
M-β-CD	atenolol	30	6.1		concentration and pH (2 or 3) of the lithium phosphate buffer not specified unequivocally	90
	fluoxetine	995	2.4		1% triethylammonium acetate + 10% acetonitrile	63
	4-nitrophenol	677			50 mM phosphate pH 11.1	85
	orcipremaline	106	56.1		phosphate buffer pH 3.0 and $I = 0.04$ at 20 °C	99
	propranolol		12	180	40 mM lithium phosphate pH 3.1	59
	propranolol	88	12.5		concentration and pH (2 or 3) of the lithium phosphate buffer not specified unequivocally	90
	salicylic acid	99			20 mM phosphate pH 11.1	92
	tioconazole	1770	12.3		5-fold diluted 100 mM citric acid and 200 mM disodium phosphate pH 4.3 ($I = 0.04$)	75
DM-β-CD	amphetamine	89	6.1		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	amphetamine	71	10.7		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 1:9 (v/v) ratio; 21 °C	93
	amphetamine	57	0.0	57	20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 2:8 (v/v) ratio; 21 °C	93
	amphetamine[i]	93	4.3		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	amphetamine[j]	43	2.5		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	methamphetamine[i]	113	5.3		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	methamphetamine[j]	47	14.9		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-amphetamine[i]	320	6.0		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-amphetamine[j]	139	0.0		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-ethamphetamine[i]	540	7.8		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-ethamphetamine[j]	191	5.3		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-methamphetamine[i]	386	8.1		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-methamphetamine[j]	151	2.5		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-propamphetamine[i]	631	6.6		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	benzylamine	10			50 mM benzylamine pH 6.0 adjusted by HCl	92
	bupivacaine	16	62.5		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	BuPPT[i]	43	46.6		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	deprenyl	142	7.8		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	deprenyl	103	8.9		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 1:9 (v/v) ratio; 21 °C	93
	deprenyl	79	8.1		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 2:8 (v/v) ratio; 21 °C	93
	ephedrine	58	9.1		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	ephedrine	53	6.2		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 1:9 (v/v) ratio; 21 °C	93
	ephedrine	35	4.8		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 2:8 (v/v) ratio; 21 °C	93
	Ψ-ephedrine	54	44.0		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	Ψ-ephedrine	45	42.0		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 1:9 (v/v) ratio; 21 °C	93
	Ψ-ephedrine	47	24.0		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 2:8 (v/v) ratio; 21 °C	93
	p-flourodeprenyl	123	7.4		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	p-fluoromethamphetamine	99	1.3		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	[i]BuPPT[i]	33	48.4		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96

Table 3 (Continued)

selector	analyte	K_1[b]	$100\Delta K/K_1$[c]	K_{mean}[d]	comment	
	^{i}BuPPX[i]	26	42.5		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	^{i}PrPPT[i]	41	19.6		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	^{i}PrPPX[i]	29	10.4		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	mepivacaine	18	33.3		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	metamphetamine	107	4.5		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	metamphetamine	79	6.1		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 1:9 (v/v) ratio; 21 °C	93
	metamphetamine	62	5.2		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 2:8 (v/v) ratio; 21 °C	93
	2-methylbutylsulfonamide			22[i]	100 mM Tris-benzoate pH 8.5	72
	2-methylbutylsulfonamide			25[i]	100 mM Tris-maleate pH 8.2	72
	2-methylbutylsulfonamide			25[i]	100 mM Tris-fumarate	72
	2-methylbutylsulfonamide			22[i]	100 mM Tris-chromate pH 8.2	72
	β-methylphenetylsulfonamide			120[i]	100 mM Tris-benzoate pH 8.5	72
	β-methylphenetylsulfonamide			108[i]	100 mM Tris-fumarate pH 8.2	72
	β-methylphenetylsulfonamide			120[i]	100 mM Tris-chloride pH 8.2	72
	β-methylphenetylsulfonamide			117[i]	100 mM Tris-chromate pH 8.2	72
	norephedrine	44	20.0		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	norephedrine	43	22.8		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 1:9 (v/v) ratio; 21 °C	93
	norephedrine	31	2.2		20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose diluted by methanol in the 2:8 (v/v) ratio; 21 °C	93
	prilocaine	41	51.3		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	propargylamphetamine	152	0.0	152	20 mM Tris-phosphate pH 2.7 + 0.5% hydroxypropylmethyl cellulose; 21 °C	93
	ropivacaine	18	44.3		100 mM phosphoric acid adjusted to pH 3 by triethanolamine	96
	salicylic acid	79			50 mM salicylic acid pH 6 adjusted by NaOH	92
	salicylic acid	57			20 mM phosphate pH 11	92
	tioconazole	6860	9.8		5-fold diluted 100 mM citric acid and 200 mM disodium phosphate pH 4.3 ($I = 0.04$)	75
TM-β-CD	amphetanime	16	12.5		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	methamphetamine	16	6.3		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-amphetamine	15	13.2		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-ethamphetamine	18	11.1		60 mM Tris/phosphate buffer pH 3.04 at 25° C	100
	3,4-methylenedioxy-ethamphetamine	16	6.3		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-propamphetamine	19	15.8		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	salicylic acid	24			50 mM salicylic acid pH 6 adjusted by NaOH	92
	salicylic acid	13			20 mM phosphate pH 11	92
Et-β-CD	salbutamol			153	20 mM citrate and 40 mM phosphate adjusted by NaOH to pH 7	95
	salbutamol			44	10 mM sodim borate oH 11	95
HP-β-CD	amphetamine	20	10		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	methamphetamine	23	12.9		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-amphetamine	57	7.0		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-ethamphetamine	73	6.8		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-methamphetamine	63	9.5		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	3,4-methylenedioxy-propamphetamine	73	5.5		60 mM Tris/phosphate buffer pH 3.04 at 25 °C	100
	fluoxetine	307	5.7		1% triethylammonium acetate pH 5.5 + 10% acetonitrile	63
	naproxen uncharged	985	14.8		200 mM MES + 0.2% hydroxyethyl cellulose	80
	naproxen charged	186	0.0	186	200 mM MES + 0.2% hydroxyethyl cellulose	80
	4-nitrophenol	654			50 mM phosphate pH 11.1	85
	salicylic acid	83			20 mM phosphate pH 11	92

Table 3 (Continued)

selector	analyte	K_1[b]	$100\Delta K/K_1$[c]	K_{mean}[d]	comment	
	tioconazole	1030	30.0		5-fold diluted 100 mM citric acid and 200 mM disodium phosphate pH 4.3 ($I = 0.04$)	75
	tioconazole	201	14.9	231	(100 mM citric acid + 200 mM disodium phosphate pH 4.3):water = 1:4; then diluted by methanol in the 1:3 ratio	83
NEC-β-CD	3,5-DNB-homophenylalanine[k]	43	0.0	43	50 mM disodium phosphate adjusted by phosphoric acid to pH 6.5	87
	3,5-DNB-phenylalanine[k]	161	61.0		50 mM disodium phosphate adjusted by phosphoric acid to pH 6.5	87
	3,5-DNB-phenylglycine[k]	460	2.8		50 mM disodium phosphate adjusted by phosphoric acid to pH 6.5	87
P-β-CD	clorprenaline	240	83		50 mM phosphate pH 5.0	91
	primaquine	170	23.6		50 mM phosphate pH 5.0	91
	sulpirine	217	3.1		50 mM phosphate pH 5.0	91
SB-β-CD	atenolol	276	1.8		concentration and pH (2 or 3) of the lithium phosphate buffer not specified unequivocally	90
	duloxetine	4840	61.5		20 mM phosphate pH 6.0	90
human serum albumin	benzoin			7500	50 mM phosphate pH 7; oven temperature 30 °C, estimated capillary temperature 40 °C	97
	promethazine	6300	23.2		50 mM sodim phosphate adjusted by NaOH to pH 7	19
vancomycin	cystine[l]	130	153	270	20 mM MOPS + Tris to pH 7	77
	selenocystine[l]			290	20 mM MOPS + Tris to pH 7	77
	methionine[l]			150	20 mM MOPS + Tris to pH 7	77

[a] The constants are rounded to three digits and given without reported uncertainty limits for the sake of simplicity. [b] Either the stability constant for the less interacting enantiomer or the stability constant for the achiral analyte; neither $\Delta K/K_1$ nor K_{mean} are given for the achiral analyte. [c] $\Delta K = K_2 - K_1$; K_2 is the stability constant for the more interacting enantiomer. [d] Either K_{mean} according to eq 19 or the value reported as the common value for the enantiomers. [e] Value, given as an example, relates to formic acid adjusted to pH 4 by NaOH to $I = 0.75$. The complete data set was measured with β-CD, HP-β-CD,[f] DM-β-CD, TM-β-CD, and HP-γ-CD at pH 4 and 8 (bicine adjusted by NaOH, $I = 0.075$) and was given as log K_{mean}. The data vary from 1.1 to 4.3; log(ΔK) never exceeded 0.1. [f] Key of abbreviation for the derivatives of cyclodextrins: M = methyl, DM = dimethyl; TM = trimethyl; Et = ethyl; HP = hydroxypropyl; NEC = naphthylethylcarbamoyl; P = phosphate; SB = sulfobutyl. [g] For the identity explanation, see ref 97. [h] Constants for complexation with TM-β-CD, HP-β-CD, α-CD polymer, and β-CD polymer, not given here, are comparable. [i] Isomer with the mean degree of derivatization 2.00, methylated from 0.4% on the pyeanose units 2,3,6, from 99.5% on the pyranose units 2,6, and from 0.1% on the pyranose unit 6.[100] [j] Isomer with the mean degree of derivatization 2.03, methylated from 25.7% on the pyranose units 25.7, from 28.7% on the pyranose units 2,6, from 20.7% on the pyranose units 3,6, from 0.2% on the pyranose units 2,3, from 23.3% on the pyranose unit 6, from 0.6% on the pyranose unit 2, from 0.3% on the pyranose unit 3, and 0.5% of the cyclodextrin was not methylated[100.] [k] DNB = dinitrobenzoyl. [l] As the aminoquinolylcarbamate (AQC) derivative.

either the separation efficiency or the separation speed. The chiral selector should not interfere with the enantiomer detection, must be stable under system conditions, should be free of side interactions, such as with constituents of the BGE and the tube wall, and should not affect the migration of achiral sample constituents. Last but not least, it should be cost effective and environmentally friendly. None of the known chiral selectors meets all these requirements. However, some of the selectors tested nicely approach the main requirements.

A. Cyclodextrins

Cyclodextrins (Figure 6) became the most popular electrophoretic chiral selectors. This is well documented in both specialized[16,29] and general[2,4,5,14,19−21] review articles on the topic as well as in the first monograph on electrophoretic chiral separations.[31] The research effort offered to cyclodextrins has until now not declined. Dissolved in standard BGEs they offer separation systems with high separation efficiency and reasonable selectivity. Cyclodextrins do not interfere with UV-photometric detection. The stability of their aqueous solutions is, however, limited. Thus, fresh solutions prepared daily are necessary for reproducible results. Cyclodextrins do not stick to bare fused silica. However, when polyacrylamide-coated capillaries are used, cyclodextrins cannot be removed completely by rinsing with either water or aqueous solutions.

Cyclodextrins are used in native, chemically modified, and polymerized forms.[31] Their mixtures, so-called arrays of cyclodextrins, have been recommended for the simultaneous separation of several chiral compounds, e.g., cationic drugs of forensic interest,[101] chlorophenoxy acid herbicides,[102] and related binaphthyl derivatives.[103] The resolution of enantiomers of single compounds, e.g., 2-methyltaurine[104] or aminoglutethimide,[105] have also been improved in this way. Reports of the use of mixtures of cyclodextrins with other chiral selectors including those seldom used, e.g., 18-crown-6-tetracarboxylic acid,[106,107] or various metal ions (Na^+, Cu^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+})[108] have also been published. Details of the analytical potential of arrays and mixtures of cyclodextrins are given in previous reviews,[10,16,21] the monograph,[31] and section VI. Separations utilizing cyclodextrins in micellar systems are presented in section V.

All types of chiral compounds of practical interest, both charged and uncharged, were separated using cyclodextrins. The number of compounds separated by native, derivatized, and polymerized cyclodextrins is huge. Many of them were separated repeatedly by various types of cyclodextrins depending either on the accompanying enantiomeric compounds or on the

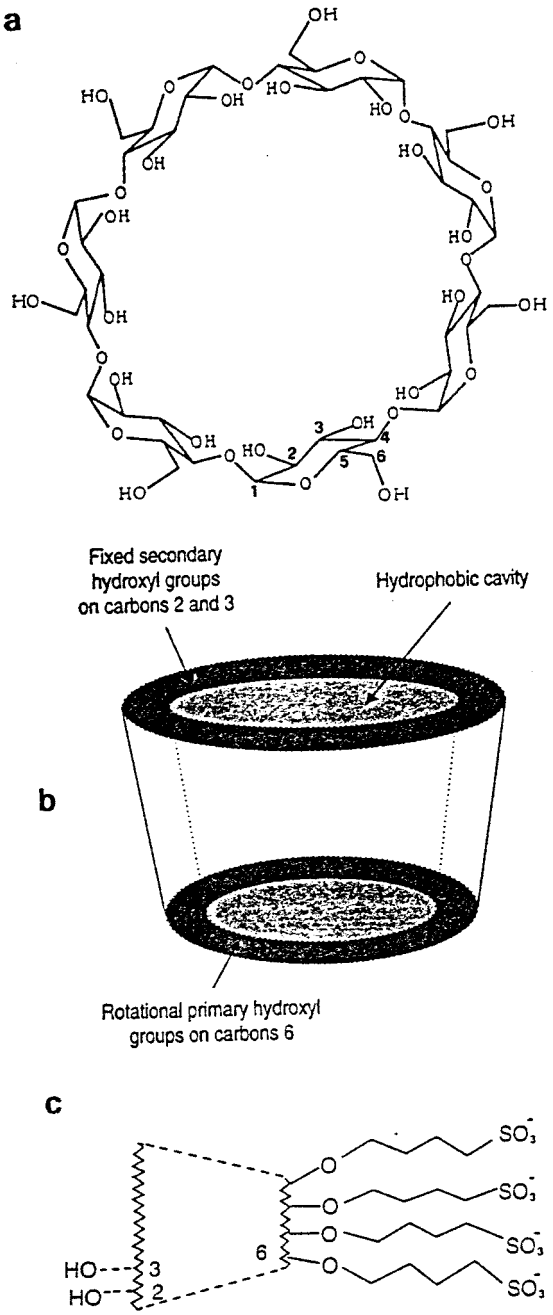


Figure 6. (a) Chemical structure of β-cyclodextrin with numbered carbons in one of the glucopyranose units and approximate shapes of its (b) native and (c) sulfobutylated molecules. Arranged using refs 2, 7, and Fanali, S. *An Introduction to Chiral Analysis by Capillary Electrophoresis* (instruction manual of Bio-Rad).

sample matrix. It is possible to say without exaggeration that the vast majority of chiral compounds may be separated by using these chiral selectors. Unfortunately, some problems either with the selectivity or separation speed have been encountered in these separations. Partial lists of racemic compounds resolved by cyclodextrins have been given in previous review articles.[5,7,9,16,17,20] For the sake of clarity, only the description of cyclodextrins utilized as chiral selectors in classical CZE arrangements is given, therefore, in this section.

Native cyclodextrins are neutral cyclic oligosaccharides prepared by the enzymatic hydrolytic cleavage of starch.[109−111] They consist of 6−13 glucopyranose rings. Products with 6−8 rings, called α-, β-, and γ-cyclodextrin, respectively, are currently used in chiral separations (Table 4). β-Cyclodextrin is usually the most effective agent, while γ-cyclodextrin is seldom used.

Table 4. Selected Characteristics of Native Cyclodextrins[a]

	n^b	RMM	φ_{CD} [g/L]	d_o [nm]	d_c [nm]	w_i
α-CD	6	972	145	1.37	0.57	6
β-CD	7	1135	18.5	1.53	0.78	11
γ-CD	8	1297	233	1.69	0.95	17

[a] According to ref 111. [b] The key of symbols: n = number of the glucopyranose units; RMM = relative molecular mass; φ_{CD} = solubility in water at 25 ° C; d_o = outer diameter of the cyclodextrin molecule; d_c = cavity diameter; w_i = number of water molecules included in the cavity

The shape of the cyclodextrin molecule resembles a truncated cone (Figure 6) with a height of 0.79 nm that is independent of the glucopyranoses number.[110] The diameter of the cavity increases with the number of the glucopyranose units (Table 4). The difference between the outer diameter of the cyclodextrin molecule and the diameter of the cavity drops from 0.80 nm for α-cyclodextrin to 0.74 nm for the γ-form. The increase in the cavity volume with the number of the glucopyranose units is reflected in the number of water molecules filling the cavity. These are replaced by the analyte molecules during the chiral discrimination process. Hydroxyl groups are bonded to the rims of the cone (Figure 6). Secondary 2- and 3-hydroxyls are located at the larger opening, whereas primary 6-hydroxyls, prone to chemical derivatization, are found at the smaller opening.[110−113] The structure of the cyclodextrin ring is rigid due to the formation of intramolecular hydrogen bonds between the 2-hydroxyl and 3-hydroxyl groups of the adjacent glucose units.[112] The solubility of native cyclodextrins depends strongly on the number of glucopyranoses[111,114] (Table 4) and may be increased by the addition of either alcohols[114] or high concentrations of urea.[115] However, the competition of the additives with the analytes for the cyclodextrin affects the separation[81] because of the general capability of cyclodextrins to include any compound.

The hosted analytes slip either completely or by their hydrophobic parts into the relatively hydrophobic cavity of the cyclodextrin[109,110,116] (Figure 7). Because of the two openings of the cone, the guest compound can penetrate into the cavity from either the secondary or the primary hydroxyl side.[117] Compounds containing alkyl groups with at least three carbon atoms as well as single or condensed cycloalkyl and aryl groups are hosted effectively by cyclodextrins. Compounds capable of entering the cavities can be surprisingly large. For example, the electromigration of pyrene substituted by up to four sulfate groups is decelerated markedly by α-cyclodextrin having the smallest cavity (Table 4). Inclusion of a compound into the β-cyclodextrin is inhibited sterically if the effective perimeter of its projection equals the projection of the circle formed by 13 carbon atoms bonded by single bonds, e.g., cyclotridecanone.[109] The fit of the hosted group with the cavity

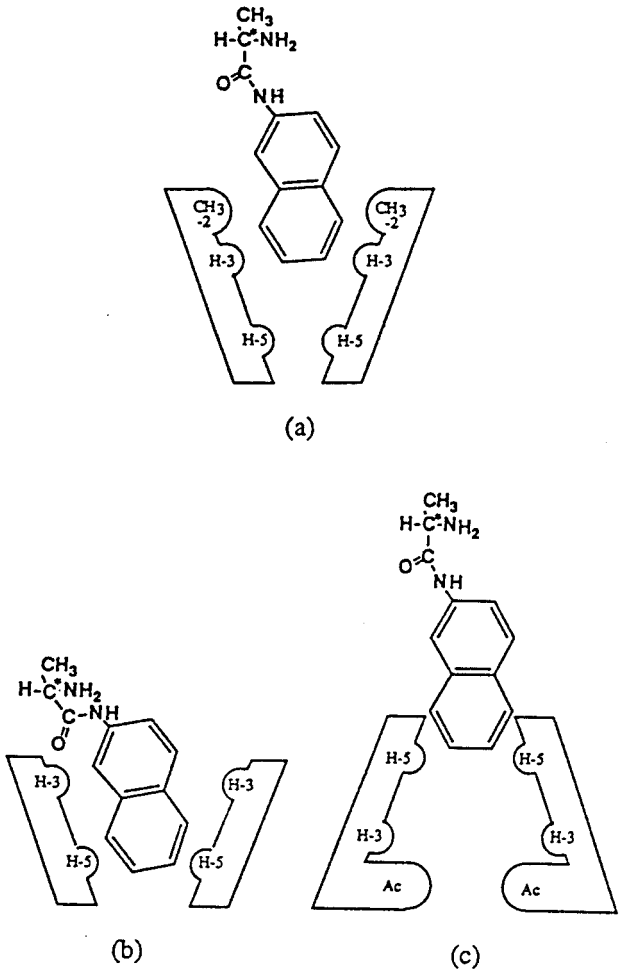


Figure 7. Scheme of complexation between alanine β-naphthylamide and (a) trimethyl-α-cyclodextrin, (b) β-cyclodextrin, and (c) diacetyl-α-cyclodextrin. (Reprinted with permission from ref 119. Copyright 1990 Elsevier Science Ltd.)

dimensions is important for the strength of the inclusion interaction necessary for cyclodextrins chiral discrimination.[109,110] The interactions of separated enantiomers possessing functional groups, present on the rims of the cyclodextrin cone, also contribute to the chiral discrimination. Only hydroxyls participate in the chiral discrimination with native cyclodextrins. The derivatization of the primary 6-hydroxyls at the smaller opening[110−113] introduces different groups capable of participating in the discrimination process. Simultaneously, the diameter of the opening is modified. Nuclear magnetic resonance spectrometry, mass spectrometry, and X-ray crystallography supply data from which information on cyclodextrin inclusion complexes with included analytes may be derived.[31,118]

The chiral discrimination of an enantiomeric compound with cyclodextrins may result from the discrimination of either its uncharged or charged form or both forms (separations of the type I, II, and III, respectively).[67] A theory of separations for type III, called duoselective separations, has been proposed.[88] This theory allows the calculation of stability constants of both the uncharged and charged forms of an enantiomer from one set of mobility data provided that the data were measured using a sufficiently wide pH range.

The absence of chiral discrimination of a pair of enantiomers is no evidence for the absence of an interaction of the compound with the cyclodextrin. The absence of chiral discrimination may result from (i) too low concentration of the chiral selector, (ii) too high concentration of the chiral selector,[62,76,80] and (iii) identical total interactions of the chemical form(s) of the enantiomers present in the system with the cyclodextrin. The relative decrease in the mobility of the compound with increasing concentration of the cyclodextrin which is identical with the relative decrease of either the electroosmotic flow or of a small, highly hydrophilic ion (such as nitrate, sulfate, sodium, or ammonium), not retained by the cyclodextrin, is evidence for such an occurrence.

Relatively weak interactions, such as hydrogen bonding, $\pi-\pi$, and dipole−dipole interactions, are involved in the chiral discrimination process by native cyclodextrins and by cyclodextrins derivatized by uncharged groups. The absence of strong Coulombic interactions helps to explain relatively low enantioselectivity, the absence of the chiral discrimination for ionized analytes, and the low values of stability constants in many separations. Stability constants below 100 are typical of separations with α-cyclodextrin in aqueous solutions (Tables 2 and 3). As a rule, concentrations of α-cyclodextrin in the range 10^1-10^2 mM are therefore necessary to reach the required selectivity. The stability constants of 1 order of magnitude higher, typical of β-cyclodextrin, decrease c_{max} and the location of the maximum mobility difference corresponding to c_{max} to an order of millimolar concentrations. Stability constants of the order of 10^3 or even higher are seldom reported for β-cyclodextrin (Tables 2 and 3). If organic solvents are used in the separations of highly hydrophobic compounds, poorly soluble in water, the stability constants decrease drastically. The drop increases with the hydrophobicity of the solvent.[52] The use of uncharged organic additives of low hydrophilicity may serve, therefore, for decreasing unreasonably high stability constants and for increasing the optimum concentration of cyclodextrin if feasible.[62,80,81] As a rule, the capability of native cyclodextrins to discriminate sterical differences is not high as a rule and is reflected in a small relative difference in the stability constants of the enantiomers. Typically, these lie close to 10−20% (Tables 2 and 3). Stability constants reported for achiral and chiral compounds of comparable magnitudes and structures are similar.

The capability of native cyclodextrins to discriminate sterical differences may be modified and often increased through chemical derivatization. Sometimes the derivatization can change the migration order of enantiomers.[100] β-Cyclodextrin is usually used for the derivatization. Uncharged groups such as methyl, hydroxyethyl, hydroxypropyl, carboxymethyl, and acetyl introduce additional interaction points into the molecule and modify both the depth of the cavity and the free cross section of its smaller opening.[117] For example, the introduction of two methyl groups into β-cyclodextrin increases its normal depth, of 0.79 nm, by approximately 0.2−0.3 nm.[119] The introduction of ionizable groups, like

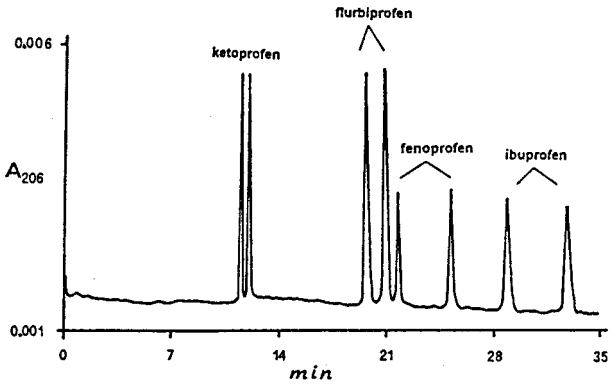


Figure 8. Chiral separation of nonsteroidal antiinflammatory drugs by tri-*O*-methyl-β-cyclodextrin. Selector (30 mM) dissolved in 100 mM MES, pH 5. Polyacrylamide-coated capillary 35 cm (effective length 31.5 cm) × 50 μm i.d.; voltage 20 kV. For other details, see Fanali, S.; Aturki, Z. *J. Chromatogr. A* **1995**, *694*, 297. (Reprinted with permission from Elsevier Science, Ltd.)

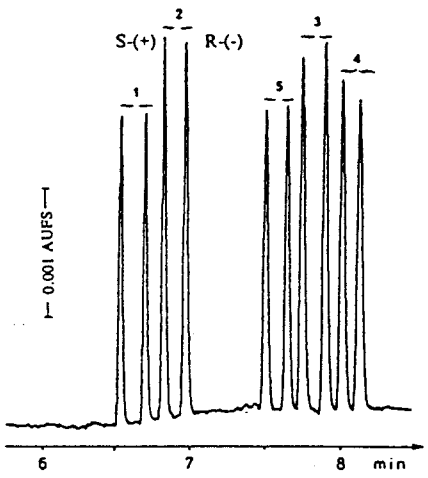


Figure 9. Chiral separation of dimethindene (constituent 2) and four of its possible metabolites (constituents 1, 3, 4, 5) by hydroxypropyl-β-cyclodextrin. A 30 mg/mL sample of the selector dissolved in 50 mM phosphate buffer, pH 3.3. Untreated capillary of 40 cm effective length × 50 μm i.d. at 21 °C; 400 V/cm. For other details, see ref 196. (Reprinted with permission from ref 196. Copyright 1993 Elsevier Science Ltd.)

carboxyl, phosphate, sulfate, alkylsulfate, or methylamino, also changes the dimensions of the cavity. If ionized, these groups change to interaction points capable of Coulombic interactions. The number of available derivatives of native cyclodextrins continues to increase, and still larger groups are being used. For example, the introductions of histamine[120] and of the poly(vinylpyrrolidone) polymer[121] into β-cyclodextrin were reported recently. Charged cyclodextrins allow chiral separations of uncharged compounds[91,122−124] and are highly efficient in the separations of enantiomers bearing opposite charges.[125] The modified enantioselective capabilities of derivatized cyclodextrins, supported by their increased solubility in aqueous solutions, allow separations not normally attainable using native cyclodextrins, e.g., Figures 8 and 9. Consequently, chemically modified cyclodextrins are proving to be highly effective in practical applications (ref 31, section VI).

In addition to the type of substituent, the degree of derivatization is also important in determining the capability of the derivative at separating a chiral compound or chiral compounds of different types and charge.[100,126−128] Therefore, at least the degree of derivatization of the modified cyclodextrin should be declared for commercial products. Unfortunately, a series of compounds differing in degree of substitution results from commonly used derivatization reactions.[126] Current commercial products, supposedly the same although from different sources, are found to differ markedly not only in their mean degree of derivatization, but also in their isomeric compositions and abundance.[100] This is the reason for different chiral capabilities observed with supposedly identical commercial products.

Steric protection of primary 6-hydroxyls is a way to single-isomer products having a defined degree of derivatization.[129−132] Enantioselective capabilities of these single-isomer preparations are being investigated now in both aqueous[133] and nonaqueous media. Using the sodium salt of heptakis(2,3-dimethyl-6-sulfato)-β-cyclodextrin, the enantiomers of 40 basic compounds, mostly of pharmaceutical interest, were resolved in acidic methanol background electrolytes.[134] However, a study demonstrating the advantages of single-isomer derivatives of cyclodextrins over present commercially available derivatives consisting mainly of a single derivatization product[100] remains to be carried out.

Derivatization increases the solubility of native cyclodextrins unless highly hydrophobic substituents are used. For example, the introduction of two methyl groups into the β-cyclodextrin molecule increases its solubility in water from 18.5[110] to 570 g/L.[135] High solubility, exceeding that of β-cyclodextrin by 1 order of magnitude, is therefore the main advantage of polymerized cyclodextrins.[136] Concentrations of the polymer in the BGE exceeding 100 mM of the monomer may be easily reached in this way. Polymerization reaction introduces a binding agent into the polymer, and thus, the enantioselective capability of the polymer is close to but not identical with that of the cyclodextrin monomer.

The increase in viscosity of the BGE brought about by the dissolved cyclodextrins[75,85,87] (Figure 10) is negligible if their concentrations are below 5 mmol/L. From the cyclodextrin concentrations of ca. 10 mmol/L, decreases in the analysis speed, caused by the increased viscosity of BGE, are measured easily.[75] Both these effects are highly pronounced in analysis using cyclodextrin polymers when present in the separation systems in concentrations corresponding to 100 mmol/L of the monomer or even higher. The increase in viscosity evoked by uncharged cyclodextrins may be visualized by a drop in the electric current passing through the capillary at constant voltage analysis.[75,85] Charged cyclodextrins increase the ionic strength of the BGE, its conductivity, and, consequently, the current passing through the capillary.

The complexation of analytes with cyclodextrins is usually reflected by longer migration times. Thus, fast separations using cyclodextrins are uncommon and ultrafast separations such as that of metaprotenerol[137] (Figure 11) are exceptional.

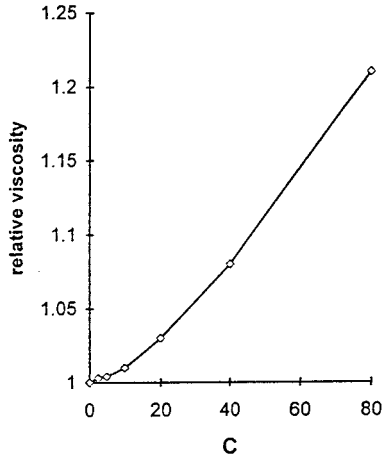


Figure 10. Dependence of the relative viscosity of the running buffer on α-cyclodextrin concentration. The selector concentration is in millimolar, relative viscosity = μ_c/μ_o. (Reprinted with permission from ref 82. Copyright 1996 Wiley-VCH.)

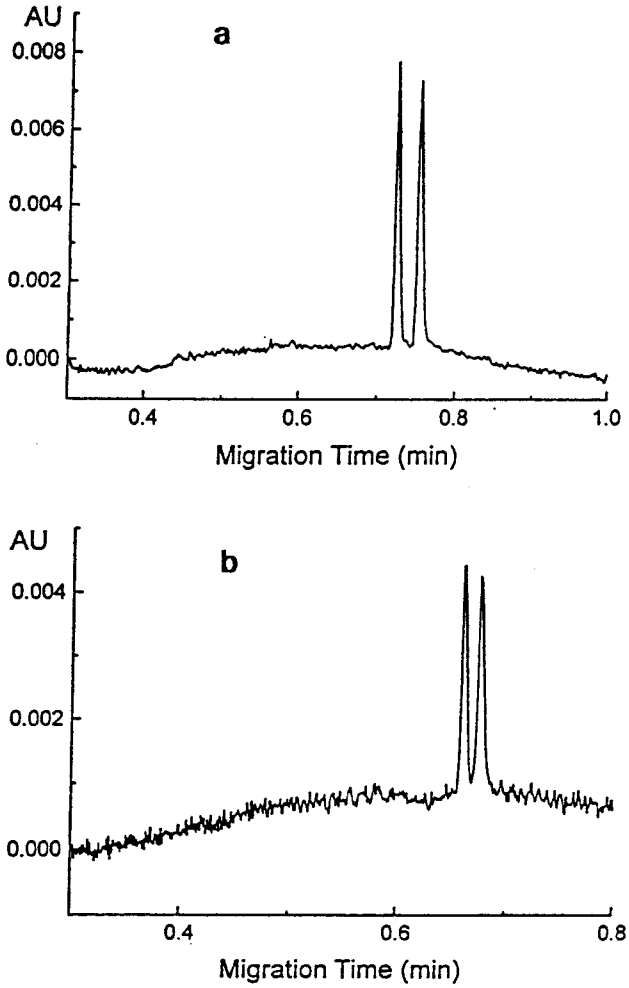


Figure 11. Ultrafast separations of *R*- and *S*-enantiomers of (a) metaprotenerol and (b) isoprotenerol in 30 mM dimethyl-β-cyclodextrin using the neutrally coated capillary of 7 cm migration distance. Background electrolyte: 25 mM phosphate pH 2.5; intensity of electric field $E = 1111$ kV/cm. For other details, see ref 137. (Reprinted with permission from ref 137. Copyright 1995 Elsevier Sceince Ltd.)

The capability of cyclodextrins to discriminate differences in the sterical structure of compounds of various types is linked with their general capability

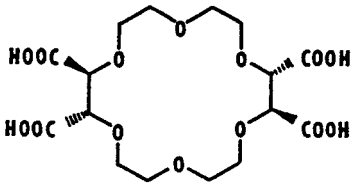


Figure 12. 18-Crown-6-tetracarboxylic acid ether (18C6).

to host any compound which can enter the cyclodextrin cavity either completely or by at least some of its groups.[109,110] Thus, it is a difficult task to find an organic compound incapable of interacting with a cyclodextrin. The utilization of cyclodextrins as carriers of loosely soluble drugs, as agents for improving the solubility of hydrophobic compounds, or as additives tuning the mobilities of achiral compounds[109,110,138] all illustrate it. Consequently, a general shift in the mobilities of achiral sample constituents, dependent on both the cyclodextrin type and concentration, is to be expected in practical separations.

B. Crown Ethers

Crown ethers are synthetic macrocyclic polyethers.[139] Ethylene groups bonded by ether bridges form corresponding cyclic structures. The ability of crown ethers to host ions was originally found for alkaline metal, alkali earth metal, and ammonium ions as well as with organic cations derived from primary amines.[139−141] Therefore, strongly complexed potassium and ammonium ions have to be avoided from the background electrolytes in order to eliminate their competition with the analytes for the crown ether cavity.[106]

The chiral capability of substituted 18-crown-6-ether was recognized in HPLC using its binaphthyl derivative.[142,143] In CZE, 18-crown-6-tetracarboxylic acid ether (Figure 12) was introduced as the chiral selector.[144] Its carboxylic groups, located perpendicularly to the plain of the macrocyclic moiety, have pK_as 2.1, 2.8, 4.3, and 4.9.[145] It makes separations with this selector highly pH sensitive in the acidic range. The development of robust separations in this range is therefore extremely difficult. Even slight shifts in the buffer pH change the mobility of the selector and, in addition, may affect its interactions with the separated enantiomers. The enantioselective capability of crown ethers is not explained unequivocally yet.[145] It is ascribed usually to the inclusion of analytes in the macrocyclic ring combined with interactions of the functional groups of the included compound with the elements forming the ether bridges (O, S, N)[138,139] as well as with the carboxylic groups.[145] Dipole−ionic interactions participate in the complex formation too.[106] Since the initial period of research interest in this area,[106,145−150] only separations dealing with the determination of the enantiomeric purity of 5,6-dihydroxy-2-aminotetralin[151] and the chiral separations of amino acids[152] using this selector were reported within the last three years.

Two interesting effects were observed with 18-crown-6-tetracarboxylic acid ether. The synergic effect of its mixtures with α-cyclodextrin on the separation selectivity of D,L-tryptophan was found to be

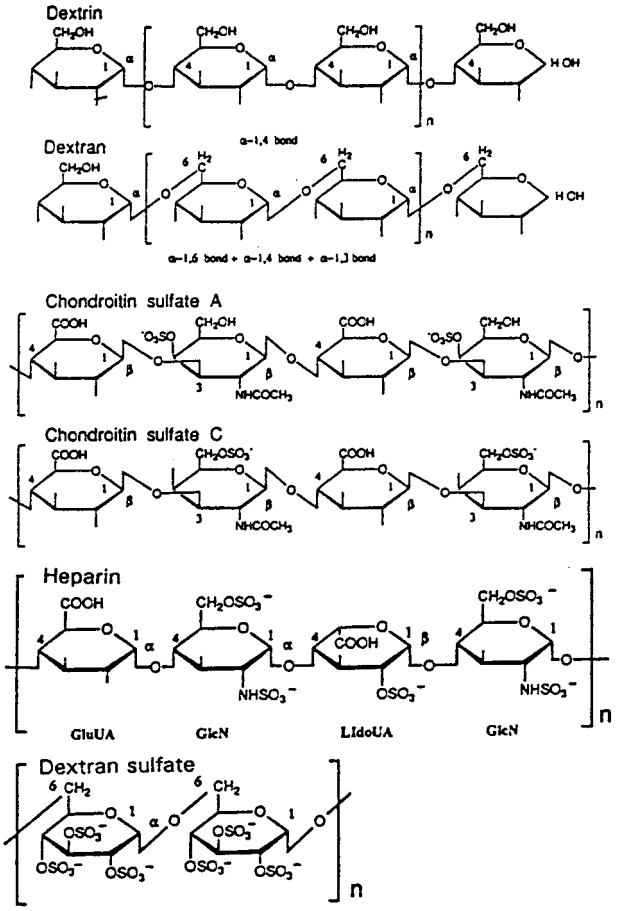


Figure 13. Schemes of structures of linear polysaccharides utilized as chiral selectors: dextrin, dextran, chondroitin sulfate A, chondroitin sulfate C, heparin, and dextran sulfate. Arranged using refs 172 and 173.

very pronounced and dependent on the ratio of the selectors.[146] Using this synergic effect, the primary amines which were inseparable using single chiral selectors were finally resolved.[148] In the simultaneous chiral analysis of *o*-, *m*-, and *p*-fluoro-DL-phenylalanines, a mixture of *β*-cyclodextrin and 18-crown-6-tetracarboxylic acid ether proved effective.[107]

In the separation of D,L-dihydroxyphenylalanine and some other amino acids, a solution of 18-crown-6-tetracarboxylic acid ether at pH 2.2, free of buffering compounds, was used as the background electrolyte.[106] Resolution was improved by increasing the concentration of the chiral selector, but in contrast to chiral separations with cyclodextrins, the migration times of the enantiomers were not as greatly influenced by the pronounced mobility decreases. As a result, the analysis time was shorter by almost 50% with the crown ether than with α-cyclodextrin.

Crown ethers are much less popular compared to cyclodextrins due to similar enantioselective capabilities and almost identical separation efficiency of their separation systems.[106]

C. Linear Polysaccharides

Linear oligo- and polysaccharides, both charged and uncharged, consist of cyclic hexoses bound to each other to form linear chains (Figure 13) of variable length. Obviously only water-soluble oligo- and polysaccharides are suitable chiral selectors for free-solution electrophoresis. Their separation systems usually show low stability constants, comparable with those of α-cyclodextrins, and high separation efficiencies increasing with the number of hexose units in the chain.[153] The probable cause is the chemical affinity of building blocks of both types of chiral selectors. The selectivity of linear oligo- and polysaccharides for cationic drugs and for some other physiologically active compounds resembles that of cyclodextrins. However, several nice separations with linear oligo- and polysaccharides, where analogy with cyclodextrins is impossible, have been reported.[30] This is especially true for enantioseparations of monosaccharides which are generally difficult to separate.

From the vast family of uncharged water-soluble saccharides, maltodextrin mixtures, corn syrups, pure maltooligosaccharides, linear non-α-(1−4)-linked glucose polymers, and low-molecular-weight galactose−glucose−fructose polymers were all tested as potential chiral selectors for the separation of 2-arylpropionic acids, nonsteroidal inflammatory drugs, coumarinic anticoagulant drugs, and cefalosporin antibiotics. However, only the maltodextrins have been found to be effective in enantiomeric separations.[149] Maltodextrins are defined by the U.S. Food and Drug Administration "as saccharide polymers consisting of D-glucose units linked by α-(1−4) bonds". Prepared by the partial acid and/or enzymatic hydrolysis of corn starch, they are commercially available as mixtures of maltooligosaccharides. Their enantioselectivity depends on a combination of so-far unidentified variables. Dextrin, dextran, laminarin, alginate, and maltotriose have been tested for the chiral separation of monosaccharides (ribose, xylose, glucose, arabinose, and mannose) derivatized by the 5-amino-2-naphthalenesulfonate fluorophore. Only the dextrin having glycosidic bonds, characteristic of cyclodextrins, displayed enantioselectivity. Furthermore, the migration order of the enantiomers was not the same for all tested monosaccharides.[154] In addition, dextrins dissolved in borate buffer were found[155] to be able to separate enantiomers of monosaccharides. Optimum separation conditions were found at low borate pH. The reaction of the OH groups of the hexose units with the tetrahydroxyborate anion or with the trihydroxyboronate anion converts the uncharged chiral selectors to the charged ones,[156−162] thus permitting separation of the uncharged analytes.

Uncharged linear oligosaccharides also proved to be effective in the separation of drugs. For example, maltodextrins can separate coumarinic anticoagulant drugs[163,164] and a wide range of racemic basic drugs belonging to different pharmacological groups (antiarrythmic, anticholinegic, antifungal, antihistaminic, antidepressant, antipsychotic)[165] as well as some other cationic and anionic drugs[164] (Figure 14). A different interaction mechanism of the chiral analytes with the maltodextrin helical entities emerges as the basis of such enantioselectivity.[164] Dextrans and dextrins were both tested for the chiral separation of the anionic 1,1′-binaphthyl-2,2′-diyl-hydrogenphos-

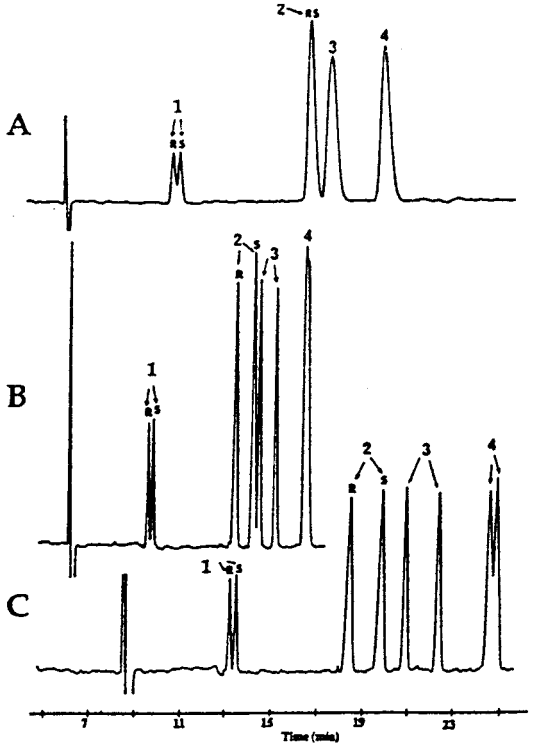


Figure 14. Separation of acidic chiral compounds with dextrin 10 as the chiral selector, utilized in concentrations of (A) 5%, (B) 10%, and (C) 15%: (1) = simendan, (2) = ibuprofen; (3) = warfarin; (4) = ketoprofen. Selector dissolved in 20 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS)/6.5 mM tris(hydroxymethyl)-aminomethane (Tris)/4% (v/v) ethanol, pH 7.7; 60 cm (44 cm effective length) × 50 μm i.d.; 26 kV; UV detection at 220 nm. (Reprinted with permission from ref 164. Copyright 1994 American Chemical Society.)

phate, for the separation of anionic drugs (ibuprofen, naproxen, warfarin), and for the chiral purity evaluation of drug substances.[166]

The naturally occurring charged linear polysaccharides are also effective as chiral selectors. Negatively charged compounds have been the principal forms of attention. Heparin was introduced in CZE for the separation of antimalarial compounds (chloroquinone, hydroxychloroquinone, primaquine, mefloquine, and epiroline), antihistamines (pheniramine, chlorpheniramine, brompheniramine, carbinox amine, doxylamine, dimethindene), as well as of some other compounds (tetramisole, tryptophan methylester, anabazine, nornicotine).[167] Later on it was used for the separation of oxaminique enantiomers[168] and antihistamines[169] and, together with dextran sulfate, for the resolution of the chloroquine and chlorpheniramine enantiomers.[170] Dextran sulfate was effective in the simultaneous separation of enantiomers of trimetoquinol and of its positional isomer.[171] Chondroitin sulfate C[172] and chondroitin sulfate A[173] were effective in the separation of sterically different forms of ceratin cationic compounds, namely, diltiazem, trimetoquinol and related compounds, chlordiltiazem, chlorpheniramine, clentiazem, verapamil, sulfaconazole, propranolol, and primaquine. Curdlan, a linear β-1,3-glucan which easily transforms into a stable gel when heated, resolves N-substituted aromatic amino acids.[174] Dextrin sulfopropyl ether was recommended

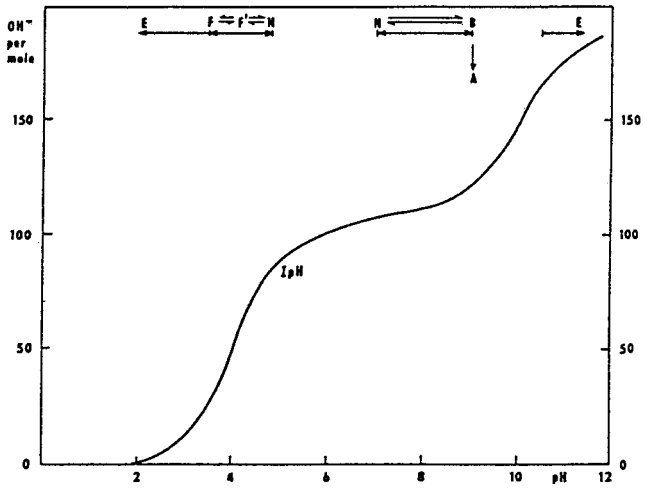


Figure 15. Titration curve of human serum albumin, completed with the pH diagram of its conformations.[162] The letters A, B, E, F, F′, N denote albumin conformations stable at different pH. (Reprinted with permission from ref 79. Copyright 1994 Elsevier Science, Ltd.) The titration curve is from ref 178.

for enantiomer separations using micellar electokinetic chromatography (MEKC).[175]

Aminoglycosides such as kanamycin, fradiomycin, and streptomycin consist of pentoses and hexoses and bear positive charges of the protonated primary amine groups in the form of sulfate salts. They were investigated as chiral additives for the separation of intermediates of diltiazem and clentiazem and of acid derivatives of 1,1′-binaphthyl.[176] These basic polysaccharides, classified as antibiotics because of their pharmaceutical activity, are linear. They have to be strictly differentiated from macrocyclic antibiotics described in section IV.E.

D. Affinity Chiral Selectors

This family of chiral selectors consists of compounds such as polypeptides, proteins, glycoproteins, and related biopolymers. Biochemically, they represent a heterogeneous group of compounds. Acidic and basic amino acids in their protein chains contain functional groups not engaged in peptidic bonds. In these biomacromolecules these are the groups that produce the pH-dependent charge which varies continuously over a wide pH range from exclusively positive in strongly acidic medium to exclusively negative in strongly alkaline medium. The titration curve expresses the shape of this pH-dependent charge shift (Figure 15). The electrophoretic charge of the biomacromolecule at a particular pH is given by the balance of its positive and negative charges. This balance is zero if the solution pH is equal to the macromolecule pK_I (Table 5).

The strong and highly selective interactions of polypeptides, proteins, and glycoproteins with other compounds are called affinity interactions. The complexed compounds are usually bonded at particular selector binding sites with high selectivity. Results so far strongly imply that the existence of particular binding sites responsible for the enantioselective capability depends on the conformation of the respective affinity selector (see below). Stability constants

Table 5. Selected Characteristics of Biopolymers as Affinity Chiral Selectors[a]

	relative molecular mass	pI	carbohydrate(%)	disulfide bridges
α-$_1$ acid glycoprotein	44 000	2.7	45	2
avidin	70 000	10−10.5	20.5	-
β-lactoglobulin (as monomer)	18 000	5.2	-	2
bovine serum albumin[b]	67 000	4.7	-	17
casein a (70%)	26 200	4.7	0.38	-
casein b (27%)	24 400	4.0−4.5	-	-
casein g (3%)	30 000	5.8−6.0	-	-
cellobiohydrolase	64 000	3.9	6	-
conalbumin	70 000−78 000	6.1.6.6	25	17
fungal cellulase	60 000−70 000	3.9	6	12
human serum trasferrin	76 500	5.5	5.7	19
human serum albumin	66 500 (68 000)[b]	4.9 (4.7)[b]	-	17
ovomucoid	28 800	3.9−4.5	30	8
riboflavin binding protein	32 000	3.9−4.1	14	9

[a] According to ref 179. [b] According to ref 9.

characterizing the interactions[177,178] are on the order of 10^3-10^6. High selectivity and low separation efficiency are typical of separations based on affinity chiral selectors in free-solution electrophoresis if purely aqueous background electrolytes are used.

Proteins and glycoproteins have been studied as chiral selectors since the start of systematic research into chiral separation. Chromatographic methods were used exclusively at the beginning. The possibility of transferring knowledge on affinity chiral selectors from liquid chromatography to CZE and vice versa has been repeatedly demonstrated,[61,79,179] and it was shown that the interactions causing chiral discrimination in electrophoresis and in chromatography are the same. The fact that this is so is to be expected from the existing chiral discrimination process theory. Simultaneously, it makes it possible to treat affinity chiral selectors in a rather general way regardless of the relatively low number of communications on electrophoretic chiral separations with affinity chiral selectors. These are summarized below.

The enantioselective capabilities and other analytical properties of proteins and glycoproteins are similar.[180,181] Albumins, bovine serum albumin (BSA) as well as human serum albumin (HSA) in particular, are the selectors most intensively and systematically studied, and it was found that the differences in their behaviors and capabilities as chiral selectors are in fact small. This is probably due to the close analogies in their compositions and structures.[178,182] Therefore, BSA and HSA may be considered from a qualitative point of view as identical selectors and may serve as typical representatives of affinity chiral selectors.

There are two principle characteristics by which the family of macromolecular chiral selectors differs from other chiral selector types, e.g., cyclodextrins, whose sterical structure is rigid and independent of the composition of the background electrolyte and of the experimental conditions used. The highly complicated sterical structure of proteins and related compounds, called conformation, is strongly dependent on the composition and pH of the medium, on temperature, and on their preparation methods from raw biological material. The capability of any affinity chiral selector to discriminate enantiomers is strongly dependent on its conformation. Thus, a particular affinity chiral selector may mimic the enantioselective capabilities of an array of structurally rigid chiral selectors provided its conformation is manipulated properly.

In separations using dissolved albumin, the flexibility of the polymer chain as well as its conformational variability, known from biochemical studies, are perfectly retained. Both features remain effectively unaltered if BSA is chemically bonded at only a few points of the macromolecule to a solid carrier with sufficiently wide pores.[179,183] Electrophoretic separations using BSA incorporated into a gel, which disable the conformational variability of the BSA, e.g., ref 84, are good analogies to separations using BSA bonded to a solid matrix in such a way that conformational changes are eliminated.[184] However, in practice, electrophoretic separations using dissolved albumin are preferred due to their experimental simplicity.

BSA always exhibits an ability to discriminate differences in the sterical structure of enantiomers. This general capability is independent of its conformation. Thus, the conformation of BSA, which is stable under conditions characteristic of the living organism, called the native conformation, is not required when BSA is used as a chiral selector. However, the ability of BSA to discriminate the enantiomers of a particular compound or of a particular compound type is substantially affected by its conformation. This was demonstrated in separations of amino acids, monocarboxylic acids, and dicarboxylic acids using both liquid chromatography[183] and electrophoresis.[61] The total number, sign, and distribution of charges in the protein macromolecule play an important role in BSA conformation because of the strong dependence of the BSA conformation on the Coulombic interactions of these charges.[182] Coulombic interactions proved to be substantial also in the enantioselective discrimination of various compound types.[183] Consequently, the number and sign of the charged groups of the analyte are also important in determining the resulting chiral selectivity (section II.A). The charges of the BSA and often in the chiral analytes are pH-dependent. Thus, pH is the main tool used for selectivity tuning[39,61,183] and the optimization of the separation efficiency.[42] The BSA titration curve, from which the pH dependence

of a charge of the chiral selector may be derived, and the pH diagram of its conformations (Figure 15) are very advantageous in the chiral selectivity tuning of the selector for various compounds.

Generally hydrophobic interactions and hydrogen bonding are the other important interactions for chiral discrimination using affinity selectors. These interactions are strongly affected by the solvent. Therefore, organic solvents added to the system may serve as a highly effective tool in tuning the separation selectivity.[39,181] Hydrogen bonding and hydrophobic interactions also play an important and sometimes even decisive role in the conformation of proteinic biopolymers.[185] Thus, the overall selectivity effect, brought about by the addition of high concentrations of an organic solvent to the separation system, is complex. The following aspects must be considered: (i) hydrogen bonding, (ii) hydrophobic interactions, (iii) charge effects, and (iv) chiral selector conformation effects. As demonstrated by the example of tryptophan separation,[186] the effect of a preliminary treatment of the selector by a pure organic solvent may also be of importance.

The conformation of a protein or of a glycoprotein is affected by both the temperature and its history. The isolation history of a selector from raw biological material has a significant effect on its conformation. The conformation adopted during the preparation and isolation need not be necessarily identical with the stable conformation in solution under experimental conditions.[61,184] Consequently, the conformation of the selector may need to be adjusted to the needs of a particular separation or even in some cases be restored if needed.[186]

The mechanism theory for chiral discrimination by albumins[79] utilizes knowledge from both liquid chromatography and electrophoresis. The conformational variability of albumins is considered the most significant factor in their ability to discriminate specific compounds and classes of compounds. The theory is formulated in such a general way that it may be applied to any affinity chiral selector.

By using human serum albumin,[61] BSA, orosomucoid (acid α-glycoprotein), ovomucoid, and fungal cellulase[180] as the chiral selectors in electrophoresis, the effects of pH and other experimental variables have been found to be qualitatively identical with those found in liquid chromatography.[187,188] Human serum transferin in both its iron-free and di-iron forms,[189] cellulase,[190] avidin,[191] a basic protein isolated from egg white, and quail egg white riboflavin binding protein[192] were introduced as affinity chiral selectors for free-solution electrophoresis. Due to the impact of the biochemical aspects of chiral separations with affinity selectors, attempts were made to investigate these chiral separations under approximately biological conditions (pH 7.4, low ionic strength of a weak electrolyte). The separation of warfarin and tryptophan using dissolved albumin was studied under these conditions.[193] Displacement interactions and the enantioselective ligand−protein binding[194] as well as the effect of the competitive adsorption of low-molecular-weight analytes on BSA in chiral discrimination were all investigated.[195] Unfortunately, the reports did not specify whether the chiral selectors used in these studies were in their native conformations or not.

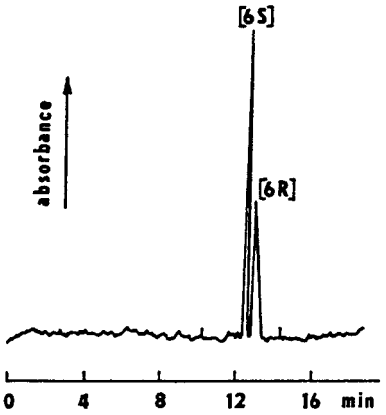


Figure 16. Separation of leucovorin with 1 mg/mL bovine serum albumin dissolved in 20 mM phosphate buffer pH 7.2. 95 cm × 75 μm i.d. fused silica capillary coated by poly(ethylene glycol); E = 285 V/cm; UV detection at 280 nm. (Reprinted with permission from ref 42. Copyright 1992 American Chemical Society.)

The separation of leucovorin using BSA[42] (Figure 16) is an example of the use of an affinity chiral selector for the improved resolution of sterically different forms of a diastereoisomeric compound with distant centers of chirality. Using data from this separation, the possibility of calculating the thermodynamic data on the analyte−selector interaction from the electrophoretic measurements was demonstrated. The addition of dextran to the BSA solution affects its mobility and, in this way, increases the resolution of analytes having an electrophoretic mobility close to that of albumin and interacting weakly with albumin.[196] The effect of dextran as coadditive on the binding of some ligands to HSA was also shown.[197] A comparison of the separability of dinitrophenyl derivatives of six amino acids (phenylalanine, glutamic acid, proline, alanine, lysine, and ornithine) with α-, β-, and γ-cyclodextrins and with several affinity chiral selectors (BSA, α-acid glycoprotein, ovomucoid, and mixture of α-, β-, and γ-caseins) revealed that only BSA resolves all of them.[161] If BSA is bonded to a glutaraldehyde gel inside the capillary, its concentrations in the gel exceeding concentrations possible in the free solution by 1 order of magnitude may be reached.[84] The α_1-acid glycoprotein bonded to the wall of the 50 μm i.d. fused silica capillary offers a separation system whose separation efficiency (5800 plates per meter of capillary for benzoin) exceeds the separation efficiency of liquid chromatography systems with chemically bonded α_1-acid glycoprotein.[198]

Polypeptides, proteins, and glycoproteins absorb UV light due to the presence of the UV-light-absorbing amino acids such as tryptophan in their chains. This light absorption causes the UV-detection sensitivity for enantiomers below approximately 300 nm to deteriorate. Two experimental techniques based on the elimination of the light-absorbing affinity chiral selectors from the detection window of the separation capillary were proposed. The first applies to the analytes and the selector bearing opposite

Table 6. Selected Properties of Macrocyclic Antibiotics as Chiral Selectors[a]

substance	relative molecular mass	purity[b]	number of macrocycles	p*I*[b]
rifamycin B	756	1	1	
rifamycin SV	720[c]	1	1	
vancomycin	1449	1	3	7.2−7.8
ristocetin A	2066[d]	2	4	7.5
teicoplanin	1877[b]	5	4	4.2;6.5
avoparcin	1980[e]	2	3	7.5
	1942[f]			

[a] According to refs 26 and 77. [b] Given as the number of compounds in commercial preparations, according to ref 26 approximate value, slightly dependent on the used buffer. [c] Sodium salt. [d] Main constituent. [e] α-form. [f] β-form with one chloro-substituent on one of the phenolic rings.

charges. The selector is present in the capillary only and not in the running buffer in the electrode chamber. Thus, it could migrate away from the detection window before detection occurs.[190] The second, a partial filling technique, called the partial separation zone technique[199] by its authors, is more generally applicable. In this technique, only a fraction of the separation capillary is filled with the selector. This guarantees that the UV-absorbing selector will not reach the detection window during the experimental run.

Despite the limited number of original papers on affinity chiral separations and the absence of systematic studies on the topic, both general and specialized[19,28,177] review articles have often appeared.

E. Macrocyclic Antibiotics

None of the chiral selectors described above simultaneously fulfill the two basic requirements for the ideal chiral selector: high selectivity for a sufficient number of compounds and a high efficiency of the separation systems. Of all the compounds proposed as chiral selectors for free-solution electrophoresis and liquid chromatography during recent years, macrocyclic antibiotics[26,200] have proven to be the most promising.

Macrocyclic antibiotics (Table 6) are compounds of medium molecular weight and complex structure (Figure 17). They are of biological origin and were originally used as drugs. Various functional groups including ionizable ones and at least one macrocyclic ring are the potential interaction points for the analytes. The rigid structures of macrocyclic antibiotics with several chiral atoms ensure a fixed sterical arrangement of the interaction points and, consequently, the discrimination of the sterical arrangement of the interacting species.

Antibiotics used as chiral selectors belong chemically to two families of compounds. The characteristic of the closely related selectors rifamycin SV (Figure 17a) and rifamycin B, classified as ansamycins, is one macrocyclic ring. The *ansa* compounds possess an aromatic chromophore system spanned by an aliphatic bridge which gives a particular shape to the resulting molecule (Figure 17b). Rifamycin B differs from rifamycin SV by the presence of a carboxylic group bonded via a methylene group to the naphthohydroquinone ring in position 9. Rifamycin B with two ionizable groups (pK_a of approximately 2.8 and 6.7)[26] effectively resolves basic, amine-containing compounds.[26,201,202] Neutral rifamycin SV resolves

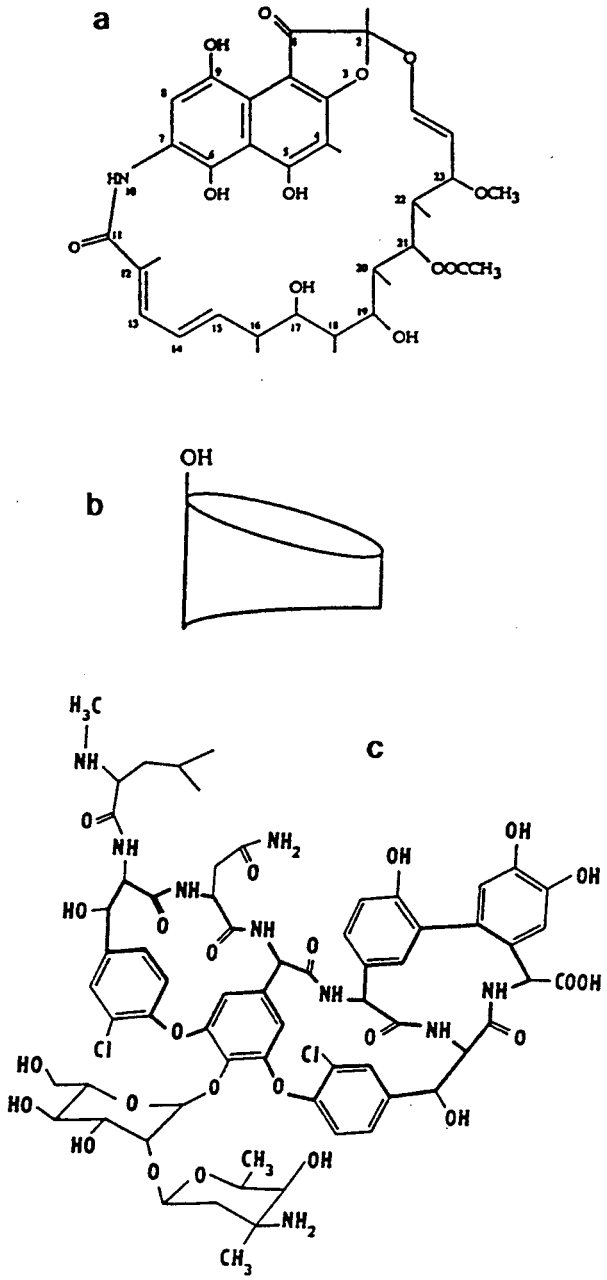


Figure 17. (a) Structural formula of rifamycin SV. (b) Shape schematics of its molecule, and (c) structural formula of vancomycin with marked macrocycles.

negatively charged enantiomers[202] and is also adept in separating some neutral molecules.[26,202] Addition of 10−40% (v/v) of short-chain alcohols or some other organic modifier to the running buffer is necessary. Without modifier, chiral discrimination is not ob-

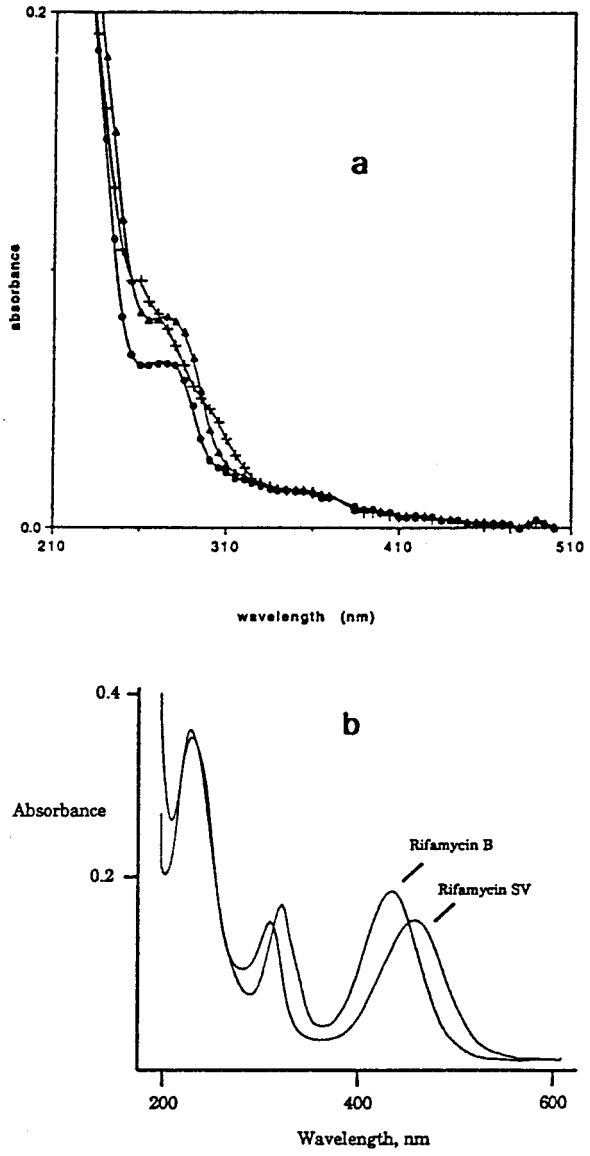


Figure 18. Comparison of the light-absorption spectra of (a) 3.4×10^{-6} M vancomycin at pH 4 (●), pH 7 (▲), and pH 10 (+) in 100 mM phosphate buffer[203] and (b) of 25 mM rifamycins disolved in 100 mM phosphate buffer pH 7 mixed with 2-propanol in the ratio 7:3 (v/v).[202] (Figure 18a is reprinted with permission from ref 203. Copyright 1994 Wiley-Liss, Inc, a subsidiary of John Wiley & Sons, Inc. Figure 18b is reprinted with permission from ref 202. Copyright 1995 Elsevier Science, Ltd.)

tained. Because of the large chromophore system in their molecules, these compounds are yellow in the solid state and measurably absorb as far as 500 nm in aqueous solutions[201,202] (Figure 18). Thus, either indirect detection or detection at high wavelengths (above 350 nm)[26] or the partial separation zone technique[199] are necessary with these selectors if common UV-photometric detection is used.

Glycopeptides (Figure 17c), another group of macrocyclic antibiotics, consist of up to four fused macrocyclic rings, formed by linked amino acids and substituted phenols with various sugars or saccharide moieties bound to them. The rigid structure of these glycopeptides containing numbers of chiral carbon atoms, several macrocyclic rings, aromatic moieties, and hydrophobic clefts as well as various functional

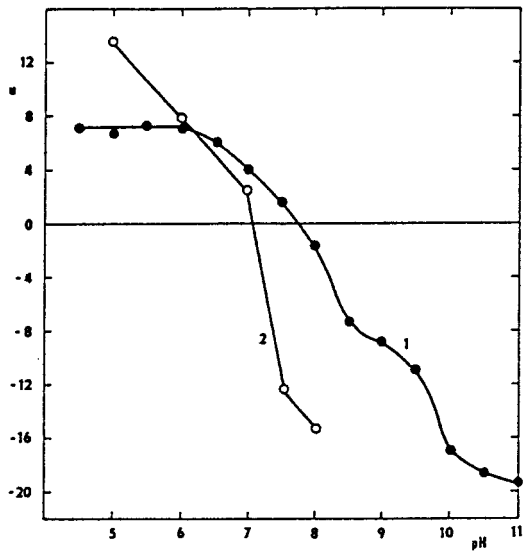


Figure 19. Dependences of the vancomycin mobility on pH, given in units 10^{-9} m^2 V^{-1} s^{-1}, in (1) 15 mM uniunivalent buffers[80] and (2) in the 100 mM sodium phosphate buffer.[182] (Reprinted with permission from ref 77. Copyright 1996 Elsevier Science, Ltd.)

and chargeable groups in close proximity to one another are highly favorable for the sterical discrimination of interacting species. Several acidic and basic ionizable groups provide the charge in macrocyclic antibiotics, and consequently, they cause an electrophoretic mobility which is variable in the broad pH range (Figure 19). The UV-light absorption of glycopeptide antibiotics, caused by small aromatic groups, drops strongly with increasing wavelength contrary to the ansa compounds (Figure 18).

Glycopeptide macrocyclic antibiotics are highly effective in the sterical discrimination of anionic racemates. The enantioselective capabilities of vancomycin,[202] ristocetin,[204] and teicoplanin[205] dissolved in 100 mM phosphate buffer in the pH range 4−7 and of avoparcin dissolved in a 50 mM phosphate buffer of pH 6[206] were demonstrated with over of 100 compounds in each case. Slight variations in the enantioselectivities of these glycopeptides[206,207] reflect morphological differences in their aglycon macrocyclic portions, structural differences, and different aggregational properties. Ristocetin A is considered to be the selector of potentially the greatest applicability in capillary electrophoresis. In the comparative study,[207] teicoplanin is classified as the most distinct of the three compounds because of its surface activity and low solubility in aqueous solutions of salts. Avoparcin[206] and A35512B,[207] the antibiotic produced by *Streptomyces candidus*, complete the family of naturally occurring macrocyclic glycopeptides tested as chiral selectors. The effect of the addition of methoxyethanol was substantial in the optimization of the separation conditions with A53312B.[207] The studies[203−208] are highly informative and inspirational as far as the enantioselective capabilities of the glycopeptide macrocyclic antibiotics to various compound types are concerned.

Of the four chiral selectors mentioned above, vancomycin and teicoplanin are commercially accessible at reasonable prices. Ristocetin A is, roughly, 20

times more expensive; at the time of writing avoparcin is not still commercially available. Vancomycin is the only one produced as pure a compound.[26] Vancomycin resolves enantiomeric anions in the absence of organic modifiers in the system,[203] dissolves easily in aqueous solutions of common electrophoretic buffers, and its solutions are more stable[26,78] than those of cyclodextrins. Moreover, vancomycin resolves fully ionized anionic enantiomers. These properties make vancomycin analytically very attractive.

Vancomycin has been applied to the separation of quinolinecarboxylic acid enantiomers,[209] nonsteroidal anti-inflammatory drugs, dansyl amino acids,[210] di- and tripeptides derivatized with 9-fluorenylmethyl chloroformate,[211] the separation of sulfur- and selenium-containing amino acids (methionine, ethionine, cystine, and their selenium analogues) derivatized with aminoquinolylcarbamate[77,78,212] as well as a variety other compounds.[77,212] Recently, vancomycin was evaluated as the chiral selector for the quantitation of loxiglumide enantiomers,[213] acidic herbicides in groundwater and river water,[214] and for the hyphenation of electrophoretic chiral separations with electrospray mass spectrometry.[215,216] In MEKC with sodium dodecyl sulfate as the micelle-forming constituent, vancomycin resolves a variety of uncharged hydrophobic drugs and pesticides.[192,193]

Positively charged vancomycin, recommended for chiral separations,[78,203] adsorbs onto bare fused silica. In this way, vancomycin strongly affects the electroosmotic flow, greatly increases the run times, and restricts its range of concentrations in the background electrolyte which are convenient for separations.[77,212] Polyacrylamide-coated capillaries proved to be more appropriate for such separations.[77,78,212]

Separation selectivity increases with the number of negatively charged groups of the analyte and with the number and volume of aromatic groups.[77,203] For a given analyte, the separation selectivity depends on the chemical composition of the buffer used and decreases with increasing concentration of the buffer.[78] The unique enantioselective power of vancomycin for many compounds is reflected in the difference in stability constants of the separated enantiomers which reaches the same order of magnitude as the stability constants themselves (Tables 2 and 3). The enantioselective power of positively charged vancomycin for anionic racemates is so high that the separation selectivity for multiply charged analytes is more than sufficient even at submillimolar vancomycin concentrations in the BGE (Figure 20). At such low chiral selector concentrations, the faster migrating enantiomer is decelerated negligibly and only a small drop in the effective mobility of the strongly interacting enantiomer is sufficient for a good separation. Stability constants, either calculated for the vancomycin separations or estimated from measured mobility data, are on the order of 10^2. Such medium value constants support the high efficiency of these separation systems. The combination of high separation efficiency and high separation selectivity makes it possible to attain fast chiral separations (Figure 5).

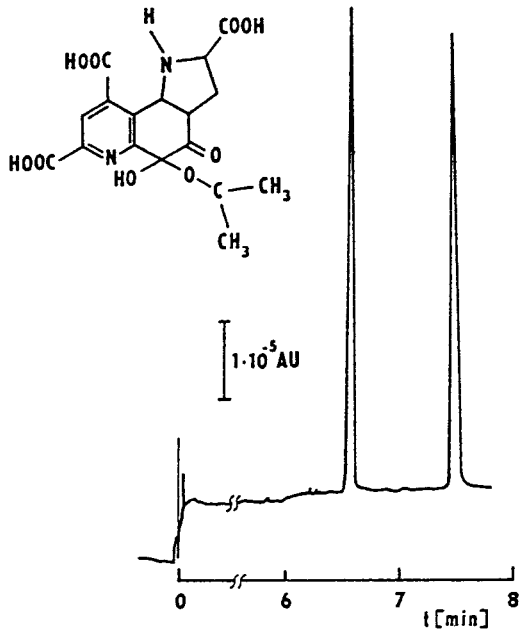


Figure 20. Chiral separation of the acetone adduct of pyroquinoline quinone in 0.7 mM vancomycin dissolved in the 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)−tris(hydroxymethyl)aminomethane (Tris) buffer pH 7; $R_s = 10$. Polyacrylamide-coated 50 μm i.d. fused silica capillary, 37.8 cm total length, 30 cm migration distance; voltage −8.5 kV. For other details, see ref 77. (Reprinted with permission from ref 77. Copyright 1996 Wiley-VCH.)

Previous results reveal that at least some macrocyclic antibiotics adequately fulfill the requirements for both high separation selectivity and high separation efficiency of their separation systems. However, a thorough understanding of the analytically important properties of macrocyclic antibiotics is necessary for adequate exploitation of their analytical potential. A good starting point is the specialized review given in ref 26.

F. Other Chiral Selectors

Many other compounds have been tested as chiral selectors for free-solution electrophoresis. Among them, the ligand-exchange chiral selectors which were highly popular in the past will be mentioned here. These selectors are prepared by the complexation of transition metal cations, namely, Cu(II), Zn(II), Ni(II), and Co(II), with chiral compounds containing either a hydroxyl or an amine group in addition to the carboxylic group (by hydroxycarboxylic acids or amino acids) working as the primary ligands.[7,219] The primary complex formed in this way is chiral, and two of its ligand positions are occupied by water molecules which may be easily replaced (exchanged) by another organic molecule, effective as the secondary ligand. If the secondary ligand is a racemate, the stabilities of the secondary complexes with the separated enantiomers are different. Ligand-exchange selectors have been used for separations of amino acids and dipeptides as well as their derivatives. The limited stability of the ligand-exchange selectors and detection difficulties resulting from their UV-light absorption are the main practical disadvantages of ligand-exchange selectors. Only a

Table 7. Selected Properties of Surfactants Commonly Used in Chiral MEKC Separations[a]

surfactant	CMC[b] [mM]	*n*	ESC	charge
sodium dodecyl sulfate (SDS)	8.1	62	no	−
sodium *N*-dodecanoyl-L-valinate (SDVal)	5.7 (40°C)	-	yes	−
sodium cholate (SC)	13−15	2−4	yes	−
sodium deoxycholate (SCD)	4−6	4−10	yes	−
sodium taurocholate (STC)	10−15	5	yes	−
sodium taurodeoxycholate (STDC)	2−6		yes	−
dodecyltrimethylammonium bromide (DTBA)	15	56	no	+
tetradecyltrimethylammonium bromide (TTAB)	3.5	75	no	+
cetyltrimethylammonium bromide (CTAB)	0.92	61	no	+
rifamycin B[c]	23		yes	−

[a] From ref 228. [b] The key of symbols: CMC = critical micellar concentration at 25 °C; *n* = aggregation number; ESC = enantioselective capability. [c] From ref 26.

single[220] communication on their use over the past several years documents that these selectors have lost their practical importance in free-solution separations. In micellar separation systems they are used more frequently.[221−224] However, compared with the number of chiral micellar separations, they are of minor importance.

A positively charged ergot alkaloid (+)-(5*R*,8*S*,-10*R*)-1-allylterguride was used for the separation of the negatively charged racemic compounds mandelic, *p*-hydroxymandelic, 3,4-dihydroxymandelic, 3-methoxy-4-hydroxymandelic, and tropic acids.[225] The natural polymeric compound chitosan, partially deacetylated chitin, in the form of a positively charged film deposited on the surface of fused silica has been used for the chiral separation of basic drugs and proteins.[226] Quinine was used as a positively charged chiral additive for the separation of *N*-3,5-dinitrobenzoylated amino acids, (±)-1,1′-binaphthyl-2,2′-diylhydrogenphosphate and *N*-[1-(1-naphthyl)ethyl]phthalamic acid in nonaqueous CZE.[227] As negatively charged chiral additives for this CZE mode, enantiomerically pure (+)- or (−)-camphorsulfonates were recommended for the separation of basic drugs that are not very soluble in water.[228]

V. Direct Separations in Two-Phase Systems

The previous section dealt only with the separations in one phase. However, remarkable separations may be realized if the electrophoretic separation process is combined with the distribution of the analytes between two immiscible phases. This family of electrophoretic chiral separations consists of micellar systems containing the chiral selector both in solution and in the micelles and systems with a chiral selector bound either to the wall of the electrophoretic capillary or to its packing.

A. Micellar Systems

Micelles are created by the spontaneous self-association of a low-molecular-weight compound having the character of a detergent when the concentration of the compound in solution exceeds its critical micellar concentration (CMC) (Table 7). In standard aqueous electrophoresis solutions, the hydrophilic heads of the detergent remain in contact with the water while the hydrophobic tails form the interior of the micelles (Figure 21). Hydrophobicity is therefore the property of the analyte which determines its

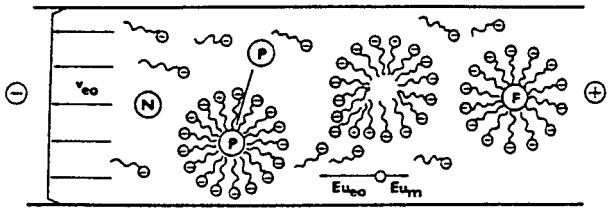
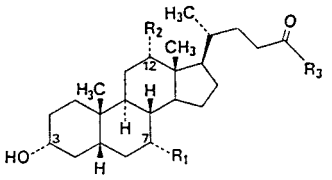


Figure 21. Scheme of MEKC separation of nonsolubilized (N), partially solubilized (P), and fully solubilized (F) analytes. (Reprinted with permission from ref 35. Copyright 1993 Weinheim.) For the explanation of the scheme, see text.

distribution between its aqueous solution and the interior of the micelles. This distribution, which is in fact a chromatographic separation mechanism, combines with the electroosmotic migration of the analyte during separation. The term micellar electrokinetic chromatography (MEKC) was therefore adopted for this technique. In MEKC experiments, fused silica capillaries, usually uncoated ones, with pronounced electroosmotic flow of the background electrolyte and charged detergents having their own characteristic mobility are preferred as a rule. The exchange of analytes between the solution and the interior of the micelles is fast. Thus, separation efficiency in the range of 100 000−500 000 theoretical plates per meter of capillary is typical of MEKC separations.[229] Because of the combination of two qualitatively different separation processes that permit unusual separation selectivities with a high separation efficiency, MEKC is an extraordinarily powerful separation technique. For a more detailed description of micellar separation systems including their theory, see the specialized reviews, e.g., refs 2, 14, 16, 19, and 229.

In chiral separations, micelles may be formed by chiral selectors having either a detergent nature, such as in the bile acids or by using preferably charged achiral detergents, e.g., sodium dodecyl sulfate or cetyltrimethylammonium bromide. In the case of the latter, chiral selectors either participate in the micelle formation, e.g., rifamycin B[26] and bile acids, or are highly soluble in the micelles, e.g., vancomycin[217,218] and cyclodextrins, and provoke the chiral discrimination.

Bile acids and their salts (Figure 22) are the most popular chiral detergents. Their micelles separate dansylamino acids,[230] basic compounds, such as 1-naphthylethylamine,[231] basic drugs, such as dilt-



Bile salt	R_1	R_2	R_3
cholate	OH	OH	ONa
taurocholate	OH	OH	$NHCH_2COONa$
deoxycholate	H	OH	ONa
taurodeoxycholate	H	OH	$NHCH_2COONa$

Figure 22. Structure of sodium salts of bile acids utilized as electrophoretic chiral selectors.

iazem and trimetoquinol,[231−233] local anesthetic drugs,[234] anionic[235] and uncharged solutes,[235] as well as the aza aromatic ligand compounds of iron(II).[236] With the latter, the influence of organic solvents miscible with water on the chiral resolution was pronounced. The demonstrated[237] influence of the temperature on chiral discrimination with bile salts was qualitatively the same as the temperature influence on any other chiral discrimination in free-solution electrophoresis and in chromatography: an increased temperature diminishes the chiral discrimination.

The synergistic effects of mixed chiral selectors, observed in free-solution electrophoresis, also occur in micellar systems. For example, the improved separation of diastereomers was observed if the sodium taurodeoxycholate forming the micelles was mixed with β-cyclodextrin.[238] A similar synergetic effect of these two chiral selectors was also demonstrated with the dansylated amino acids.[239] It is therefore not surprising that mixtures of sodium taurodeoxycholate with hydroxypropyl-β-cyclodextrin were effective in the enantiomeric differentiation of a wide range of pharmacologically active substances. Using either this mixture of chiral selectors or by the addition of sodium sulfobutyl ether-β-cyclodextrin to sodium dodecyl sulfate micelles, 22 compounds, mostly cationic, were optically resolved.[240] The discrimination ability of micelles containing cyclodextrins for various classes of compounds is demonstrated in detail in the addendum of the review.[7] The effect of organic modifiers on resolution and elution order, for mixed micelles formed by sodium dodecyl sulfate and β- and γ-cyclodextrins as chiral selectors, was studied for amino acids derivatized with 2-(9-anthryl)ethyl chloroformate.[241] The observed analytical effects of the organic solvents result both from competition of the solvent and the analytes for the chiral selector, as described above, and from the influence of the solvent on the micelles, see, e.g., ref 200.

However, the achiral, micelle-forming detergent can also compete with analytes for the chiral selector. Naturally, this competition acts against the chiral discrimination. The competition of sodium dodecyl sulfate molecules and of chiral analytes for the cyclodextrin cavity acts against the chiral selector−analyte interaction and, thus, against the chiral discrimination. This was proven experimentally in the chiral separation of dihydropyridine calcium antagonists in nonmicellar systems with native and substituted cyclodextrins and in micellar systems of sodium dodecyl sulfate acting as the achiral micelle-forming agent and the same chiral selectors.[242] The advantageous synergetic effect of the polymerized chiral micelle of poly(sodium *N*-undecylene-D-valinate) in conjunction with γ-cyclodextrin was observed.[243] The reason is the absence of polymer inclusion into the γ-cyclodextrin molecule, in contrast to monomeric sodium *N*-undecylene-D-valinate. The resolution of D,L-laudanosine enantiomers and verapamil and binaphthyl derivatives is far superior to that obtained using either polymerized micelles or γ-cyclodextrin alone. Polymerized β-cyclodextrin mixed with sodium taurodeoxycholate was found to be superior to mixtures of monomeric β-cyclodextrin and its derivatives with sodium taurodeoxycholate in the chiral analysis of a variety of drugs.[136]

Mixed micelles consisting of an achiral micelle-forming agent and of one or more chiral selectors proved to have high analytical potential. The combination of sodium cholate and sodium deoxycholate with sodium dodecyl sulfate was used for the enantiomeric separation of three binaphthyl compounds.[244] Twelve polychlorinated biphenyls were individually separated into their sterically different forms using γ-cyclodextrin as the chiral additive for sodium dodecyl sulfate micelles in the CHES (2-(*N*-cyclohexylamino)ethane sulfonic acid) buffer. The simultaneous separation of nine of these compounds has been reached in ca. 35 min[245] (Figure 23). The ligand-exchange mechanism is effective in the separation of dansyl derivatives of amino acids using a running buffer containing the Cu(II) complex of *N*,*N*-didecyl-D-alanine together with sodium dodecyl sulfate micelles[221] as well as in other micellar separations with the participation of the ligand-exchange mechanism.[222−224]

Additional effects of the structure of bile acids on chiral separations were studied using mixed micelles of bile salts (sodium salts of cholic, deoxycholic, chenodeoxycholic, and ursudeoxycholic acids) and of polyoxyethylene ether. Methanol was the organic solvent additive, and verapamil, norverapamil, gallopamil, and bi-2-naphthol were the chiral analytes in this study.[246] Using the data from this study, simultaneous baseline separation of the enantiomers of verapamil, norverapamil, and gallopamil was possible in the separation system using sodium deoxycholate, polyoxyethylene ether, and methanol.[247]

In addition to the approaches described above, several other ways to chiral discrimination in MEKC were found. Neutral saponins such as glycyrrhizic acid at pH 7, β-escin,[123] and digitonin,[125] an uncharged glycoside of digitogenin, may be mixed with sodium dodecyl sulfate. Their charged micelles are effective in the separation of amino acids derivatized with phenylthiohydantoin. The neutral steroidal glucoside surfactants *N*,*N*-bis-(3-D-gluconamidopropyl)-cholamine and *N*,*N*-bis-(3-D-gluconamidopropyl)-deoxycholamine were readily charged via borate

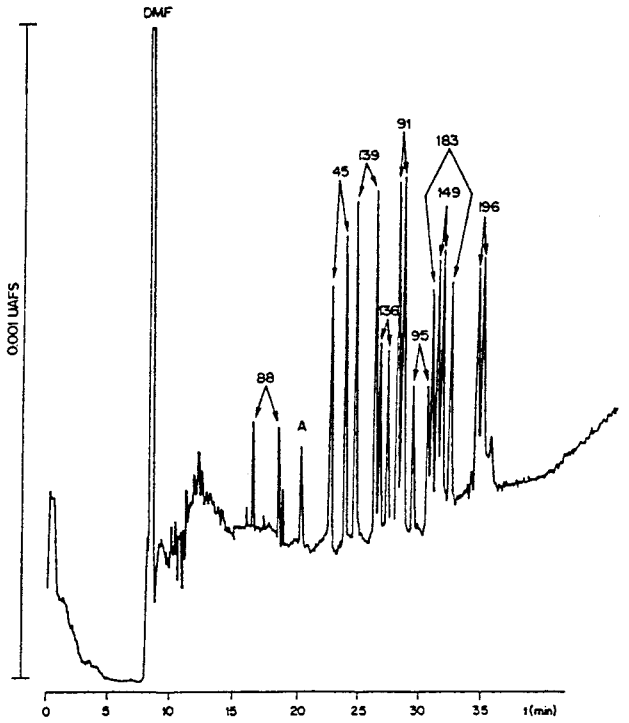


Figure 23. Separation of sterically different forms of nine polychlorinated biphenyls, identified by their numbers, by micellar electrokinetic chromatography using γ-cyclodextrin as the selector. Background electrolyte: 100 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) pH 10.0, 2 M urea, 110 mM sodium dodecyl sulfate, and 5 mM γ-cyclodextrin; 45° C. For other details, see ref 245. (Reprinted with permission from ref 245. Copyright 1996 Vieweg Publishing.)

complexation.[156−162] Consequently, the surface charge density of the corresponding micelles could be conveniently adjusted by varying the borate concentration and the pH of the micellar system.[248]

The use of tailormade surfactants obtained by introducing hydrophobic chains into amino acids is another effective approach. Derivatives of valine,[249−251] alanine,[249] cysteine,[252] and proline[251] forming negatively charged micelles were synthesized and applied with success to the analysis of compounds of pharmaceutical interest. The same approach was applied in the preparation of surfactants derived from (*R,R*)-tartaric acid[253] and with glucopyranoside-based surfactants bearing long alkyl chains with phosphate or sulfate-charged groups.[254] The latter exhibits very low critical micellar concentrations in the range of 0.5−1.0 mM. Of the *n*-alkyl-β-D-glucopyranosides used in the first exploratory study of neutral micelles in MEKC separations, the nonyl homolog proved the best for resolving the carbamate derivatives of amino acids.[255] Chondroitin sulfate and heparin, negatively charged linear mucopolysaccharides of biological origin, have been recommended for drug analysis.[256] The utilization of monomeric and polymeric chiral surfactants as pseudostationary phases in micellar systems has been reviewed.[22] MEKC with laser-induced fluorescence (LIF) detection offers the analysis of fluorenyl derivatives of amino acids with a detection limit at the micromolar level.[257] For the on-column derivatization of amino acids, a zone-mixing technique was developed.[258] A concept of migration indices was proposed for the better identification of peaks in chiral MEKC separations.[259]

B. Electrochromatographic Systems

It is evident from the previous section that the simultaneous utilization of two qualitatively different separation mechanisms in a single separation process proved to be very effective in separation selectivities even in chiral separations. If the elementary steps of these mechanisms are fast enough and if the processes causing the spreading of analyte bands are minimized, a fast and highly effective analysis with sufficient analyte resolution may be designed. However, separation systems that utilize micelles for the interfacial distribution of analytes retain one substantial drawback of free-solution CZE analysis, namely, low sample capacity. Chromatographic separation systems are capable of treating much larger samples. Thus, research aimed at replacing the micelles by a standard chromatographic packing in order to retain analytes transported through the capillary has been going on for several years. If the electrophoretic velocities of analytes are combined with the electroosmotic flow of the liquid inside a capillary, the advantage of the flat concentration profiles of the migrating species is preserved. The electroosmotic transport of dissolved analytes with respect to the stationary phase also allows the analysis of uncharged analytes.[260] Moreover, the chemical bonding of the chiral selector to either the solid packing of the capillary, which usually has a particle diameter in the 3−5 μm range, or to the capillary wall eliminates the selector from the solution. Detection difficulties, caused by the UV-light absorption of many chiral selectors, are avoided in this way.

The advantages of electrochromatography are to some extent paid for by the practical difficulties encountered, as is common with any new instrumental technique. The sensitivity of the electroosmotic flow to the compositions of both the background electrolyte and the chromatographic stationary phase appears to be the main problem. In the analysis of complex samples, the electroosmotic flow may also be affected by the irreversible adsorption of constituents of the sample matrix. Nevertheless, electrochromatography, as a hybrid separation technique, has attracted strongly increasing attention in recent years, e.g., refs 55, 56, 261, and 262.

The possibility of performing chiral separations by electrochromatography in wall-coated open-tubular capillaries of 50 μm i.d. has been theoretically proposed.[234] However, computer simulations revealed that wall-coated capillary work at suboptimal efficiency when compared with micellar electrokinetic chromatography and other electrodriven separation methods. This fact is due to the deteriorating influence of slow mass transfer in the liquid mobile phase in the relatively wide-bore capillaries which are standard in electrophoresis. The use of capillaries with an inner diameter below 50 μm was recommended as a way of reaching high efficiency in such separation systems.[255]

In addition to the proteins bonded to the capillary wall,[198] β-cyclodextrin bonded to a solid support

packed in the electrophoretic capillary was investigated for the separation of both uncharged and anionic chiral analytes.[260] The application of this approach for the separation of chlorthalidone enantiomers has been published.[264] Immobilized polysiloxane-containing derivatized β- and γ-cyclodextrins were studied and compared with the same derivatives dissolved in the background electrolyte.[265] An experimental comparative study of capillary electrochromatography with selected chromatographic techniques was carried out by using 3,5-dimethylphenylcarbamoyl cellulose and *p*-methylbenzoyl cellulose as chiral agents bonded to the capillary wall.[266] Effective separations of several pharmaceuticals were reached due to the high enantioselectivity of the selectors. For electrochromatographic chiral separations in nonaqueous medium, the helically chiral poly(diphenyl-2-pyridylmethylmethacrylate) served as the stationary phase.[267] Using pure methanol as the liquid phase, benzoin, *trans*-stilbene oxide, and 1,1′-binaphthyl-2,2′-diol were separated. For the separation of methylbenzoin and Tröger's base, a methanolic solution of ammonium acetate, adjusted by glacial acetic acid to pH* 4.5, was used. In pressure-supported electrochromatography, permethyl-β-cyclodextrin covalently linked via an octamethylene spacer to dimethylpolysiloxane and immobilized on silica (Chirasil-Dex silica) has been employed for the separation of 14 compounds including mephobarbital, hexobarbital, 1-(2-naphthyl)ethanol, and the methylthiohydantoin derivative of proline.[268] For the separation of chlorinated alkyl phenoxypropanoates, 20 M% of bare silica was added to the Chirasil-Dex. The column efficiencies ranged from approximately 66 000 to 32 000 theoretical plates per meter of capillary with an the analysis time from 10 to 40 min.

A review on the use of modified cyclodextrins as stationary phases for electrochromatographic separations[269] may be summarized as follows. The family of stationary phases, suitable for either coating or immobilization on the capillary wall, consists of polysiloxane-linked permethylated β-cyclodextrin (Chirasil Dex 1) and related selectors. Open-tubular columns are prepared in this way. Sorbents for the packing of the capillary are prepared by the chemical bonding of native β-cyclodextrin, permethylated-β-cyclodextrin, or hydroxypropyl-β-cyclodextrin onto the silica. The packed capillary is closed by a frit at both ends. Both the open-tubular columns and the packed columns can be operated using any chromatographic mobile phase (gas, liquid, and supercritical fluid). The flow of the liquid phase through the capillary may be secured by pressure only, by the combination of pressure and electroosmotic transport, or by electroosmotic transport only. The lowest separation efficiencies result from the pressure-driven mode. The adverse effect of the hydrodynamic parabolic profile of the flowing liquid is evidently responsible for this. The highest separation efficiencies are in the electroosmosis-driven mode. Here, the contribution of the parabolic profile is absent. Moreover, the separation efficiency increases with the voltage used. The reason is the shorter time of the broadening of the solute bands by the longitudinal diffusion at higher voltages. For example, in the separation of phenylethanol, driven by electroosmosis only, separation efficiencies increasing from 1×10^5 to 5×10^5 theoretical plates per meter of open-tubular capillary are reported with an increase in the separation voltage from 10 to 30 kV, respectively.[270] Chiral systems with dual recognition are obtained if another chiral selector is added to the liquid phase.[269] The overall enantioselectivity of the separation system for a given compound increases if both of the selectors cooperate in the mutual separation of its enantiomers. If not, the overall enantioselectivity drops down.

VI. Practical Applications

The dramatic increase in the number of communications devoted to the practical use of electrophoretic chiral separations within the last three years proves that the research knowledge accumulated satisfies the needs of analytical practice. The ability of chiral separations to meet, or at least to approach, the requirements standard in routine achiral analyses is the prerequisite. An intercompany cross-validation project on the chiral analysis of clenbuterol using 30 mM hydroxypropyl-β-cyclodextrin in 50 mM disodium tetraborate adjusted by H_3PO_4 to pH 2.2[271] is the first published evidence of this kind. Validation of capillary electrophoretic methods for the testing of the enantiomeric purity of fluparoxan[272] and ropivacaine,[273] the development and validation of the combined potency assay and the enantiomeric purity method for a new chiral pharmaceutical compound,[274] as well as the validation of the enantiomeric separation of ephedrine using MEKC[275] belong to this class of communications. A review on the quantitative applications of chiral capillary electrophoresis which gives indications of the performance of these methods and deals with aspects of their routine use has been published.[276]

The penetration of electrophoretic chiral separation into analytical practice is best documented by the monograph[277] that describes the current state of the art analysis of pharmaceuticals using capillary electrophoresis. However, special chapters on electrophoretic chiral separations may now be found in practically oriented review articles dealing with electrophoretic techniques[278] for the analysis of particular classes of compounds, e.g., peptides,[279] amino acids,[280] pesticides,[281] 1,4-benzodiazepines,[282] drugs,[283] as well as with the development of methods for their analysis.[284] In addition, reviews written from a particular point of view with a single compound, such as isoprotenerol[285] or cefadroxil,[286] may also be found.

The tendency to include electrophoretic chiral separations as an integral part of the topic discussed is frequent and reflects a pronounced increase in both the number and diversity of practical applications. Examples of practical applications presented below are intended to be understood as an inspiring illustration of the possibilities offered by the practical utilization of electrophoretic chiral separations and the difficulties linked with them rather than a complete up-to-date list.

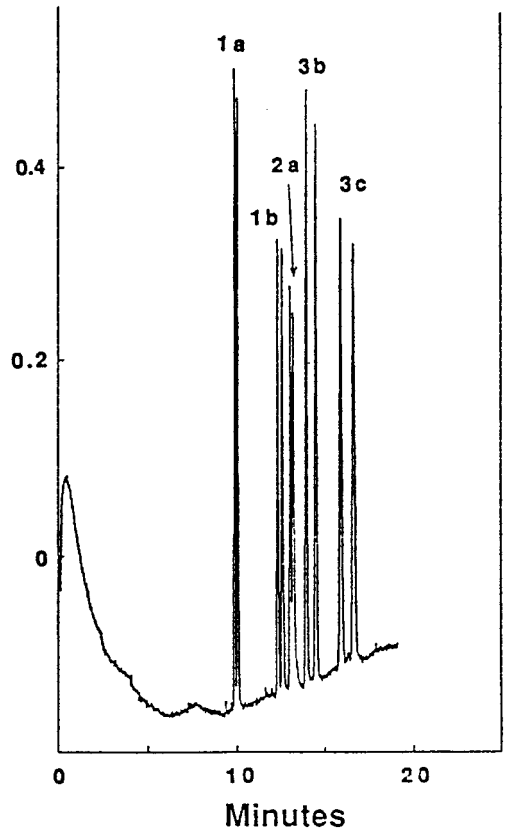


Figure 24. Subzero chiral separation of five biogenic amines (1a) β-hydroxyphenethylamine, (1b) norephedrine, (2a) octopamine, (3b) norepinephrine, and (3c) isoprotenerol at −16° C using dimethyl-β-cyclodextrin as the chiral selector. For details, see ref 295. (Reprinted with permission from ref 295. Copyright 1997 Wiley-VCH.)

In practice, the optimization of separations is of principal importance. Unfortunately, simple optimizations such as that of propranolol with hydroxypropyl-β-cyclodextrin[287] are not common. If the complex interaction mechanisms of the analyte with the chiral selector, e.g., such as in the separation of disopyramide with cyclodextrins,[288] takes place, then the selection of the optimum selector requires testing of an extended family of candidates and sophisticated approaches are often necessary. Mixtures of neutral and charged cyclodextrins enhance the CZE separations of acylated dipeptide stereoisomers[289] or nonsteroidal anti-inflammatory drugs.[290] Histamine-modified β-cyclodextrins were used for the separation of dansyl derivatives of amino acids,[291] and zwitterionic cyclodextrin served as the chiral agent the in separation of chlorthalidone.[292] Anionic and cationic derivatives of β-cyclodextrin were used in micellar separations of neutral barbiturates, chorthalidone, terbutaline, warfarin, salbutamol, and brompheniramide.[293] Three different approaches were used in the preparation of the chiral stationary phases, based on β-cyclodextrin and its derivatives, for the electrochromatographic separations of the β-andregenic antagonists (acebutolol, alprenolol, atenolol, metoprolol, pindolol, prenalterol, and propranolol).[294] Subambient and subzero temperatures improved the separation of biogenic amines[295] (Figure 24).

In addition to experimental studies, optimizations based on the application of chemometric approaches have also been proposed. A simple approach was demonstrated in the optimization of the chiral separation of newly developed pharmaceuticals with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin and native cyclodextrins.[296] However, a systematic chemometric approach in the development of methods for chiral separations has been described.[297] The application of the complex multivariate method for optimization of analysis conditions, called the Plackett−Burman experimental design, was tested using cyclodextrins as the chiral selectors.[298] Five interacting parameters, namely, the buffer concentration, pH, the chiral selector concentration, temperature, and applied voltage, were used as variables in the development of a chemometric model for the determination of the most favorable conditions for the chiral separation of amphetamines.[299] The chiral screening method proposed by this procedure is fast and robust with a typical analysis time below 20 min. The migration order of enantiomers may be changed by tuning the chiral selector charge and by judicious selection of both the capillary coating and buffer pH.[300]

Ionic mobilities of analytes and of the background electrolyte co-ion are important for maximum separation efficiency in chiral separations.[301,302] The possibility of tuning the chiral analyte resolution via manipulation of the electroosmosis (section II.A) has also been utilized in practically oriented studies. The gradient of the electric potential applied in a radial direction across the capillary wall,[303] tetraalkylammonium ions added to the background electrolyte,[304,305] and coating the capillary wall with linear polyacrylamide[306] were all used for the purpose.

The limited transparency of some chiral selectors, namely, proteins[307] and macrocyclic antibiotics,[26,78,203] in the short-range UV-light region causes sensitivity problems in photomeric detection. The so-called partial filling technique,[199] described in section IV.D, is one possibility of how to solve the problem.[308] A substantial decrease in the consumption of the chiral selector per run and, thus, a more economic analysis are additional advantages of the partial filling technique.[309] To divide the whole analysis into two subsequent but separated steps in an on-line arrangement, separation and detection, is another way of solving problems caused by light-absorbing selectors. Each step uses its own capillary which is filled with an electrolyte optimum for that particular step.[310]

Labeling analytes with a fluorescent tag substantially enhances the detection sensitivity compared to photometric detection. For example, by using 7-aminonaphthalene-1,3-disulfonic acid as the derivatization agent, herbicide enantiomers in 0.2 ppb concentrations may be detected using the laser-induced fluorescence (LIF) detection technique.[311] With fluorescein isothiocyanate as the derivatization agent, formoterol enantiomers in concentrations of 1 μg/L may be detected provided that chiral selectors are absent in the detection cell.[312] A *o*-phthaldialdehyde reagent was used for on-column labeling of D- and L-amino acids separated in a micellar system.[313]

The optical purity control was developed for 5,6-dihydro-2-aminotetralin,[151] for acetylcysteine,[314] for

the basic drugs denopamine, timepidium, and trimetoquinol,[242] for the chiral reagent 1-(9-fluorenyl)ethyl chloroformate,[315] and for *N*-propionyl-6,7-dimethoxy-2-aminotetralin.[316] Procedures using cyclodextrins as chiral selectors[102,317,318] were proposed for the drug screening.

A continuously growing family of methods is being developed and successfully tested for various biological, pharmaceutical, and medical problems. The methods deal with the enantiomeric determination of aminophosphoric acids effective in the medication of osteoporosis,[319] for the determination of amphetamines and amphetamine analogues in urine, and for the determination of their enantiomeric composition.[320] Enantiomeric separations of D,L-carnitine,[321,322] 2′-deoxo-3′-thiacytidine,[323] deprenyl and its major metabolites,[324] the capillary electrophoretic enantioseparation of ephedrine, selegiline, and methamphetamine,[325] the determination of ephedrine compounds in nutritional supplements,[326] the determination of fenfluramide enantiomers in pharmaceutical formulations,[327] the quantitation of l-L-folinic acid in the presence of its pharmaceutically inactive D,L-form in commercial pharmaceutical formulations,[328] the separation of the enantiomers and diastereoisomers of 4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one derivatives,[329] the optimized separation and detection of the enantiomers of isoprotenerol in microdialysis samples,[330] the chiral separation of lobeline and metoxamine[331] and of local anesthetics,[96] the ultrafast chiral separations of basic drugs isoprotenerol and metaprotenerol,[137] the separation of enantiomers of mianserine analogues,[332] muscarinic antagonists,[333] as well as the drugs norverapamil and verapamil[334] are important examples of such analyses. The analysis of pilocarpine and its trans epimer isopilocarpine was proposed for the routine control of eye drops,[293] the chiral differentiation of optical isomers of racemorphan in urine serves for the separation of synthetic analogues of morphine in forensic control.[336] The enantiomeric separation of salbutamol and related impurities serves as a step in drug control.[337] The determination of warfarin enantiomers[338] was developed and applied to human plasma samples. Zopiclone and its metabolites were determined in urine.[339] Methadone and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone were analyzed in serum, urine, and hair[340] (Figure 25). 3,4-Methylenedioxymethamphetamine and two of its metabolites were analyzed in human urine.[341] The usability of capillary electrophoretic methods in drug development was investigated.[65] The fully automated analysis of amino acid enantiomers including their derivatization was proposed.[342] Two comparative studies on drug analysis using capillary electrophoresis and HPLC,[343,344] based on the exploitation of cyclodextrins as chiral selectors, proved that the results obtained by both methods are comparable. The attractiveness of electrophoretic chiral separations in biochemistry, pharmacy, and medicine are all illustrated by review articles[283,345] containing lists of successfully separated compounds.

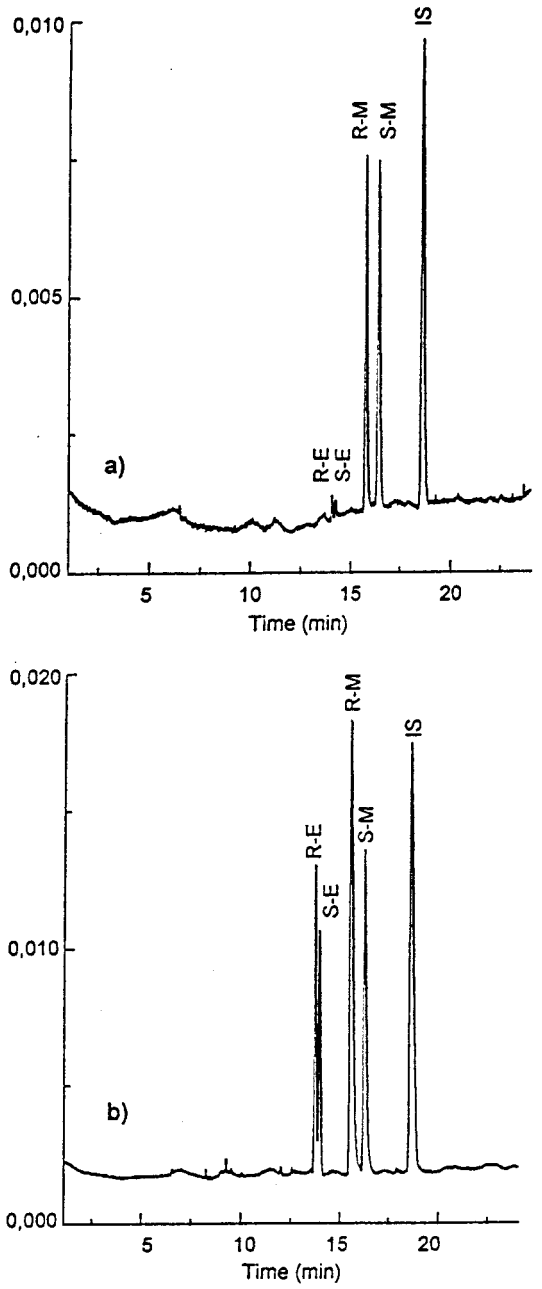


Figure 25. Concentrations of *R*-methadone (*R*-M), *S*-methadone (*S*-M), and their main metabolites, *R*- and *S*-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidone (*R*-E and *S*-E, respectively), in (a) serum and (b) urine of the patient treated by *rac*-methadone. IS = internal standard. For details, see ref 340. (Reprinted with permission from ref 340. Copyright 1997 Wiley-VCH.)

The separation of enantiomers of phenoxy acid herbicides[343] is oriented toward environmental studies.

VII. Conclusions

The fact that chiral separations using modern capillary electrophoretic techniques has been routine over recent years is of principal importance in both analytical practice and research. The speed with which this goal was reached documents both the enormous practical importance of chiral analysis and the enormous research effort devoted to them. As a rule, it is possible to separate a chiral compound of

Table 8. Typical Characteristics of Chiral Separations with Main Selectors Types in the Free-solution Electrophoresis[a]

selector type	separation efficiency [N/m]	$\Delta\mu^a/\mu$	K	$100\ \Delta K/K$
cyclodextrins	150−250 000	0.01−0.2	10^1-10^3	5−25
linear polysaccharides	150−250 000	0.01−0.1	$10^1-10^{2\ b}$	5−25[b]
affinity selectors	10−25 000	0.05−0.5	10^4-10^6	>25
macrocyclic antibiotics	>250 000	0.05−1[c]	$10^{2\ d}$	50−150[d]

[a] The separation efficiency never drops below 250 000 theoretical plates per meter of capillary in reasonably designed micellar systems. In electrochromatography, it depends on the extent of the pressure support to the electroosmotic flow and on the speeds of processes that participate in the chromatographic distribution of analytes between the mobile and stationary phases. The separation selectivity of micellar and electrochromatographic systems cannot be derived from the differences in effective mobilities of separated analytes only. [b] Attainable relative mobility differences either calculated from reported mobility differences or estimated from published data. [c] Estimated from published separations. [d] Estimated from refs 203 and 210. [e] Reference 77, and estimation from unpublished data, measured in addition to those published in refs 77, 78, and 202.

interest using either different chiral selectors or, using a given selector, in different separation systems.[7,16,283,345,347,348] Some separations approach a speed typical of achiral electrophoretic separations. Thus, the possibility of meeting the required standards in both selectivity and separation speed, which are common in achiral separations, becomes the present challenge for research in chiral separations. Fast chiral separations require a combination of high separation efficiency with a reasonable difference in the effective mobilities of the chiral analytes. In free-solution electrophoresis, both goals have to be reached for fully ionized enantiomers and at the cost of minimum retardation of the more interacting enantiomer. From characteristics relating to the "ideal" chiral selector (section IV), those affecting the separation selectivity and the analysis speed are therefore of primary importance.

It is evident from section VI that presently only cyclodextrins may be classified as chiral selectors established in analytical practice. The hydroxypropyl and methyl derivatives of β-cyclodextrin proved to be highly effective in practical analysis in the past. Now, the practical attractiveness of charged derivatives has increased markedly. Cyclodextrins have been investigated since the beginnings of electrophoretic chiral separation, and their analytical properties such as chiral selectors have been so thoroughly investigated that no substantial increased knowledge has occurred in recent years.[29] The fact that each chemically modified cyclodextrin, prepared without the protection of derivatizable hydroxyls, is a mixture of reaction products[100,126] is one drawback in the use of chemically modified cyclodextrins because of the reproducibility of the analysis. However, the contribution of single-enantiomer derivatives of cyclodextrins[129−132] to separation selectivity and its reproducibility needs evaluation with respect to increased cost.

There is generally at least one native or chemically modified cyclodextrin which is capable of separating a selected chiral compound if a proper separation system, including a micellar one, is used. This is only true, however, for compounds with sufficient number of structural and functional groups that can interact with the selector. The higher the number and the greater the variety of the groups, the higher the probability that three of them in one of the enantiomers fit the interaction points of the selector. Fortunately, almost all chiral compounds of pharmaceutical, medical, and biological interest have complicated structures that fulfill the criterion. The chiral analysis of simple compounds is more difficult.

Cyclodextrins are able to host most organic compounds in the cavity. However, irrespective of its strength, the hosting itself does not cause the formation of analytically significant differences in the interaction between the enantiomers to be analyzed and the cyclodextrin. Two additional interactions are necessary for chiral discrimination. Sometimes only the uncharged forms of the enantiomers exhibit a difference in their interactions with the cyclodextrins.[67,88] In this case, the price of the creation of a separation selectivity is paid for by the markedly decreased speed of the analysis.

Of the other chiral selectors, macrocyclic antibiotics of the glycosidic type are very promising from the viewpoint of enantioselective capability, separation efficiency, and, in particular, separation speed. The glycosidic antibiotics exhibit a high discriminating power with negatively charged chiral analytes. In this respect, they are complementary to cyclodextrins which proved effective in the separation of chiral cations. While the efficiencies of separation systems with glycosidic macrocyclic antibiotics are not lower than those of the cyclodextrin systems, their discriminating power with anionic chiral analytes markedly exceeds that of the cyclodextrins (Table 8). With respect to the purity of commercially available preparations, their price, and their ability to discriminate chiral analytes in systems free of organic solvents, vancomycin may be considered as the selector with the highest potential.

Crown ethers and linear polysaccharides have enantioselectivities resembling those of cyclodextrins. They are becoming increasingly useful in special separations, e.g., linear polysaccharides in the analysis of monosaccharides.[154,155]

Affinity chiral selectors provide separation systems marked by good separation selectivity. Unfortunately, when purely aqueous background electrolytes are used, the separation efficiency of such systems is low. This is analogous to corresponding systems in liquid chromatography. The reasons for it is not yet explained. The principal difference between affinity chiral selectors and other categories is the conformational dependence of their enantioselective capabilities. Due to this conformationally conditioned variability, a single affinity selector may produce separation systems with different selectivities not

only for various compounds, but also for different compound types such as monocarboxylic acids, dicarboxylic acids, and amino acids. In this way, a single affinity chiral selector may be substituted for a family of related selectors, as long as its conformation is correctly controlled. Without such a control, analytical results become poorly reproducible or even irreproducible. It should be stressed that the requisite conformation cannot be guaranteed by the composition of the background electrolyte alone. The preparation of the selector and the conditions under which previous separations were realized may also be of importance.

Interest in compounds effective as affinity chiral selectors continues because of their important role in living organisms. Knowledge of their behavior as chiral selectors is helpful in gaining an understanding of the mechanism of molecular recognition of these compounds. Therefore, there is no doubt that such research will continue. An increased knowledge may make it possible to discover selectors suitable for some routine chiral separations. An explanation of the generally low separation efficiency of affinity selectors will be surely a significant contribution despite their current low routine use.

In addition to the selectivity and separation efficiency of chiral systems, there is another analytically important factor in the evaluation of chiral selectors, namely, their UV-light absorption property. The reason is that the photometric detection in the UV region is the dramatically prevailing technique. In this respect, cyclodextrins, crown ethers, and linear polysaccharides are all excellent. The introduction of another powerful detection techniques, namely, mass spectrometry, into the practical CZE could very well make it possible to avoid the drawbacks of this UV-light absorption in the near future. This may increase the attraction of macrocyclic antibiotics and other UV-light-absorbing chiral selectors. The elimination of light absorption as an important characteristic will also make the search for new chiral selectors easier.

It is well-known from both gas and liquid chromatography that the number of chromatographic phases, acceptable in routine practice, is much less than the number of phases that have been investigated and recommended for use. First, in practice there are a few established phases which set standards that then have to be exceeded by any new candidate. It is reasonable to expect the same procedure in electrophoretic chiral separations. Thus, with respect to the preference for cyclodextrins in practice, the properties and analytical characteristics of their separation systems (Table 8) may be considered as the standards. Extending the existing family of chiral selectors by the addition of members of identical or even lower analytical prospects does not make sense. Slight modification of established selectors that do not markedly improve either their enantioselective capabilities or the separation system efficiency cannot be considered as a meaningful contribution for practice. The same criteria should be applied in the evaluation of newly introduced selectors.

Further investigation of established selectors should aim at completing the existing knowledge which is somewhat fragmentary. The creation of databases, such as the Chirbase database in liquid chromatography, and expert systems on electrophoretic chiral separations may be a reasonable way to achieve this. A systematic compilation of the existing literature on chiral electrophoretic separations is recommendable as a first step. The identity of the interactions that cause chiral discrimination in both electrophoresis and liquid chromatography transformed both the Chirbase and the chromatographic literature on chiral discrimination into rich sources of relevant information. Investigations of both the mechanisms and physicochemical aspects of interactions between chiral selectors and analytes should be included in this research. Only then will it be possible to construct models that allow predictions for the recognition of chiral analytes with individual chiral selectors. Models would have to include molecular recognition principles as an integral part for the construction of such expert systems.

The need for routine chiral separations was the decisive stimulus for electrophoretic research in the field. Thus, the results of the research discussed in this review are evaluated from the viewpoint of their contribution for analytical practice. However, the impact of the instrumental techniques developed, the methods using these techniques, as well as the accumulated knowledge in the field far exceeds the framework of analytical chemistry. There are two aspects outside the analytical framework that cannot be ignored.

The mechanism of molecular recognition is the very process that creates the possible analytical discrimination of chiral analytes in direct separations. The investigation of chiral separations is, therefore, the investigation of the processes and mechanisms of the molecular recognition of chiral compounds by another compounds termed chiral selectors. The investigation of methods for the control of separation selectivity in chiral separations is, in fact, the investigation of methods for the tuning of molecular recognition. The fact that it has been clearly shown that knowledge on chiral separations and the relevant physicochemical data can be transferred from electrophoresis to liquid chromatography and vice versa[61−64,179] is the best evidence. Thus, every experiment on chiral separation is, simultaneously, a chiral recognition experiment. The application of both experimental and theoretical approaches from the field of molecular recognition research is therefore highly attractive for both the theory and practice of chiral separations. On the other hand, instrumental techniques and methods for chiral separations based on them as well as relevant accumulated knowledge may be of help in the study of molecular recognition mechanisms.

The interaction of a chiral selector with an analyte is a particular case of a more general process in solution, i.e., the reversible and fast complexation of dissolved species. Such interactions are of great importance in biochemistry, biology, pharmacy, and medicine, to mention only a few selected fields. The possibility of investigating these processes, including

their physicochemical aspects, using CZE techniques and methods is extraordinarily attractive and challenging, see, e.g., refs 349−354.

VIII. Acknowledgments

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(57) Alternatively, the constant defined by eq 12 is called the stability, assocation, binding, or complexation constant. Its magnitude depends on the temperature, pressure, and ionic strength of the solution. Obviously, such constants hold for the particular experimental conditions only. A dimension is ascribed to the constant depending on the concentration units used. The molar scale is recommended for the sake of comparison with other stability constants. Because of its dimension, neither decadic nor the natural logarithm of K_A may be calculated. To transform the stoichiometric stability constant into a dimensionless value, the equilibrium concentrations [A], [C], and [AC] may be replaced by the relative concentrations $[A]_r = [A]/c^0_A$, $[C]_r = [C]/c^0_C$, and $[AC]_r = [AC]/c^0_{AC}$, where c^0 is 1 M/L for each of the species. Equation 12 assumes that the complex constituents do not participate in any other equilibrium. This condition is barely, if at all, met in electrophoretic separation systems. Interactions of the selector or of the analytes with constituents of the background electrolyte or with the added organic modifier are the most probable side interactions. In this case, the so-called "conditional stability constants", relevant for the composition of the separation system only, are obtained.[46] For more detailed discussion of the topic, see ref 58.
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