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Enantiomeric separations and their application in pharmaceutical analysis using chiral eluents*

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Abstract: The most important problems of enantiomeric separations using chiral eluents are discussed. The methods have been compared with respect to enantiomeric purity of reagents, reagent selection and separation variables. The most important considerations for methods based on inclusion-complexation with different CDs and on using chiral counter ions are summarised and compared. A new possibility for the separation of enantiomeric compounds with the aid of a combination of ion-pair and inclusion-complex formation has been introduced. As a consequence of this investigation, the selectivity of the separation can be significantly improved; those ionic isomers and enantiomers which cannot be separated or are unsatisfactorily separated by ion-pair chromatography or by inclusion-complex formation, can be separated by the combined technique. Comparison of methods applicable for enantiomeric separations is also given with respect to solving specific analytical tasks in the field of pharmaceutical analysis.

Keywords: *Chiral separations; cyclodextrins; ion-pair formation; inclusion-complexation; liquid chromatography.*

Introduction

Several chromatographic methods are now available for the chiral separation of various kinds of compounds [1–3]. The methods are mainly based on transformation of enantiomers to diastereomeric complexes or to derivatives — direct and indirect resolution of enantiomers. Inclusion-complex formation is a different possibility, when the lipophilic portions of molecules penetrate into the cavity of cyclodextrins (CDs), and come into contact with the hydrophobic surface of the cavity. The other substituents attached to the chiral carbon can interact with the substituents on the rim of CDs to give primary and secondary interactions.

Direct resolution by diastereomeric complex or inclusion-complex formation can be performed on chiral stationary phases and by using chiral eluents. Both types of method possess advantages and limitations, as shown below.

In the present paper, the most important problems of enantiomeric separations using chiral eluents are discussed. A new possibility for the separation of enantiomeric compounds by using a combination of ion-pair and inclusion-complex chromatography

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will be introduced. A comparison of the principal methods applicable to enantiomeric separations is also given.

Experimental

A Liquochrom 2010 liquid chromatograph equipped with variable wavelength UV-detector, loop injector and recorder (Labor MIM, Esztergom-Budapest, Hungary) was used for the experiments. The separations were performed on Nucleosil-10-CN and Hypersil-ODS columns (250 × 4.6 mm, i.d.) obtained from Chrompack BV (Middleburg, The Netherlands).

Eluents were prepared with HPLC-grade solvents (E. Merck, Darmstadt, G.F.R.) and were filtered prior to use. Reagents were of analytical grade (Reanal, Budapest, Hungary); the cyclodextrins were obtained from Chinoin (Budapest, Hungary) and were used without further purification. The compounds to be tested were prepared at Chemical Works of Gedeon Richter (Budapest, Hungary) and their quality checked by HPLC. Their structures are shown in Fig. 1.

Results and Discussion

In the Authors' recently published papers [4–8], the use of 10-camphorsulphonic acid (CSA) as well as that of different CDs for the separation of enantiomeric compounds have been reported. Based on recent experiments, as well as on literature data, the most important problems of enantiomeric separations using chiral eluents are discussed.

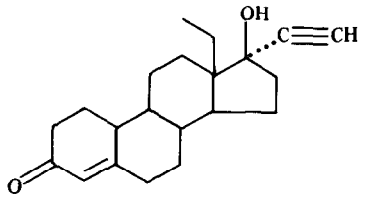
Diastereomeric complex formation using chiral counter ions (CCI)

(a) Enantiomeric purity of chiral counter ions. The application of several different chiral counter ions has been reported [9–15]. From recent experiments published in the literature, it can be concluded that the most important properties of the reagents are the relatively strong acid–base characteristics and simultaneously, their relatively low polarity.

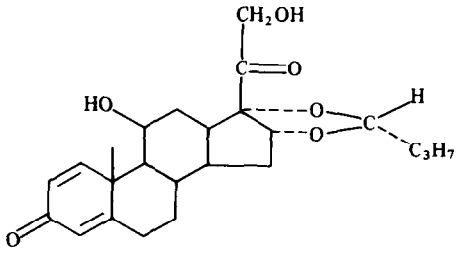
Both properties are needed together for diastereomeric complex formation due to the desired ionic interaction (primary interaction) between the compounds to be tested and the reagent. This also due to the hydrophobic interaction (secondary interaction) between the lipophilic part of the reagent and sample molecule. Regarding the enantiomeric purity of the chiral reagent, the situation is similar, but not the same, to the chiral derivatisation technique as shown in Fig. 2.

As can be seen in Fig. 2 in the case of derivatisation the enantiomeric impurity appears as an impurity in the sample (Fig. 2A), resulting in false impurity data. One of the main advantages of the CCI technique is that the optical purity of the chiral reagent is limited by the selectivity of the separations. At low impurity content, the analytical results are not affected by the reagent purity if the separation is sufficient (Fig. 2B). With increasing impurity content, the selectivity of the separation decreases.

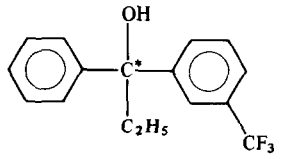
(b) Chiral reagent selection. Three important features of a chiral reagent have been mentioned: its acid–base characteristics; lipophilic nature; and optical purity. Besides the commercial availability, which is also important, the applicability of chiral reagents is limited by two additional factors. The first is the low UV-absorbance in the spectral range applicable for detection, which can exclude the use of several generally applied



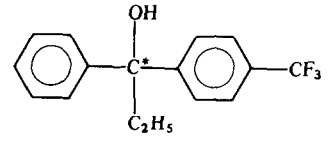
D,L-norgestrel



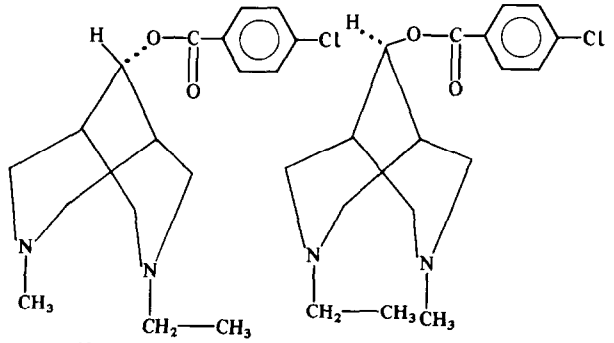
R,S-budesonide



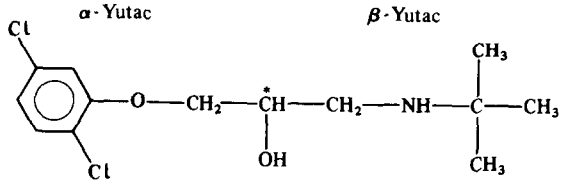
(±)-Flumecinol



para isomer



α-Yutac β-Yutac



(±) Tobanum

Figure 1
Structures of compounds investigated.

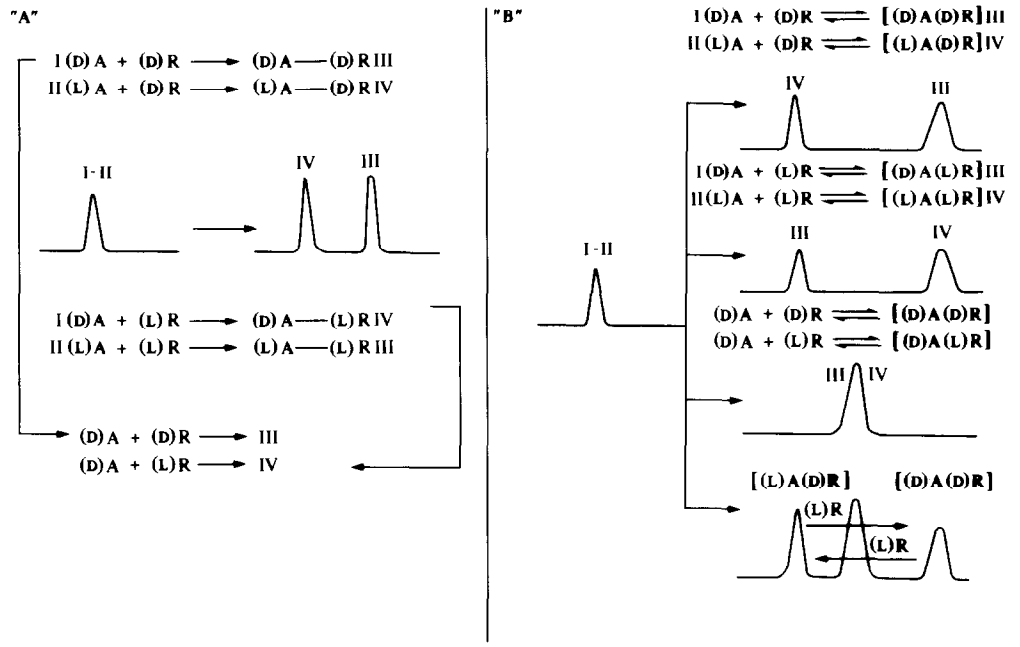


Figure 2
Effect of enantiomeric purity of the chiral reagent on the separation of enantiomers. "A": Chiral derivatisation. "B": Using chiral counter ions in the eluent (D)-A, (L)-A: Sample, (D)-R, (L)-R: reagent.

reagents of synthetic chemistry. The second is the solubility of the reagents in the mobile phase. Most of the methods applied for enantiomeric separations by CCI use straight-phase systems. Thus several reagents satisfying the above mentioned criteria are excluded due to their low solubility in less polar eluents. Contrary to these limitations the different chemical nature of chiral counter ions offer an excellent possibility to improve the stereo-selectivity of the separation methods.

(c) Separation variables. As already mentioned, straight-phase chromatography using polar (silica, diol) [9, 16], medium polarity (cyano-propyl-silica) [4, 5] stationary phases and less polar eluents have been applied. In the Authors' experiments the eluents were prepared from solvents having different polarities (polar, medium polarity and apolar solvents). According to these findings, the retention can be controlled by the ratio of apolar and medium polar solvents. The selectivity is influenced by the nature of medium polarity solvents. The efficiency of the separation is dependent on the nature of polar constituents, as well as on the concentration of the additive binding to the free silanols on the stationary phase surface. As a consequence of the several variables, the optimisation of these separations requires more skill and expertise, as well as more time, than other techniques.

One point should also be mentioned concerning the CCI technique, namely the effect of the water content of the eluent. Equilibria other than diastereomeric formation and the distribution of the diastereomeric complexes formed between the stationary and

mobile phases can be minimised, due to the detrimental effect of water on the separation experienced by several authors [9–11, 12]. In the Authors' experience, using three-component eluent mixtures and cyano-propyl-silica stationary phase, less than 1% water does not affect the separation [4, 5].

Inclusion complex formation

(a) Purity of cyclodextrins. This problem has not been investigated. However, when α-, β- and γ-CD have been used in a mixture [8], the CD will react only with the guest molecule which can fit into its cavity; the other CDs have no significant effect on retention. It means that the purity of a CD according to cavity size is not so important a factor in inclusion complex formation as is the enantiomeric purity of counter ions in diastereomeric complex formation.

(b) Reagent selection. Presently α-, β- and γ-CD and their methylated and acylated derivatives are available for experiments. It means that only guest molecules which conform in size should be separated — this is the first limitation. The second limitation is that only those guest molecules can react, which possess the necessary lipophilic portion, hence the size of the hydrophobic surface of the molecules to be tested is also an important factor. The third limitation relates to the location of the chiral carbon, because the substituents attached to the chiral carbon should interact with the rim of the CDs; the molecules should be involved in a repulsive (steric hindrance) or attractive fashion (hydrogen bonding with the hydroxyls on the rim). The fourth limitation is connected with the polarity of the molecules. Very polar compounds cannot be investigated owing to their small retention in reversed-phase systems. Apolar components cannot be investigated due to the high organic solvent concentration in the eluent required to obtain acceptable retention. The organic solvents in the eluent can compete with the guest molecules for the inner surface of CD cavity and can avoid interaction. In a recent study [8], the best situation exists when α-, β- and γ-CD are used in mixture in the eluent. The guest molecule itself can select that CD which forms the strongest complex. It is an easy way for phase system optimisation.

(c) Separation variables. The phase system optimisation procedure developed in the Authors' laboratory has been recently reported [8]. The selectivity and efficiency of a separation can be influenced by the following parameters: cavity size of CDs; concentration of CDs in the eluent; type of stationary phases; nature and concentration of organic solvents in the eluent; pH and ionic strength in the case of ionic compounds.

Comparing the possibilities for phase system optimisation when the separation is performed on chemically bonded CD columns, or when CD-containing eluent is used, two differences are worth mentioning. The first, difference in separation variables can provide a better chance for improvement in separation when CD-containing eluent is used. The second is that when chemically bonded CD columns are used, the retention will increase due to the interaction between the guest molecules and the CD cavity bonded to the stationary phase surface; the elution order of the isomers will not change — less polar compounds form stronger complexes resulting in higher retention than polar compounds. In CD-containing eluent the elution order should be reversed, as shown in Fig. 3, because the less polar compounds form stronger complexes in the mobile phase; if the complexes are more polar than those formed with polar compounds, the elution order will be altered.

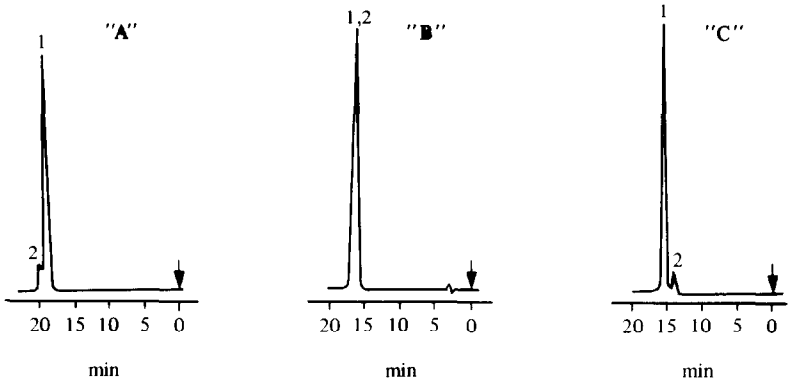


Figure 3
Dependence of elution order on γ-CD concentration. Conditions: column, Hypersil ODS; eluent, methanol–water (1:1) containing: "A" no CD; "B": 10^{-3} mol dm^{-3} γ-CD; "C": 10^{-2} mol dm^{-3} γ-CD; flow rate: 1 cm^3 min^{-1}; detection: 254 nm. Compounds: "1": R-Budesonide, "A": S-Budesonide.

Combination of ion-pair chromatography and inclusion-complex formation

For further improvement of selectivity of enantiomeric separations, the combination of ion-pair (IP) and inclusion-complex (IC) formation has been studied. In most cases, ion-pair chromatography itself in reversed-phase systems is not sufficient, due to the dissociation and solvation effect of water present in the eluent. Inclusion-complexation is also carried out in aqueous eluent, because of the unfavourable solubility of CDs as well as the disadvantageous effect of organic solvents on the process of inclusion-complex formation. The influence of ion-pair and inclusion-complex formation on the retention of the compounds to be tested is reversed. In reversed-phase systems ion-pair chromatography increases, while inclusion-complexation decreases the retention. This principle is shown in Figs 4–6 in the example of the separation of α- and β-isomers of Yutac (cf. Fig. 1).

When experiments are started with IP (Fig. 4A), the retention of the two isomers changes to give a maximum in the curve. Using constant CSA concentration and increasing CD concentration, the retention rapidly decreases (Fig. 4B). This means that the ion-pairs formed react with CD, to form more polar inclusion-complexes than the ion-pairs or unreacted isomers themselves. When CD-containing eluent is used (Fig. 5A), firstly the two isomers cannot form inclusion-complexes. With increased CD concentration only a slight change in retention has been observed.

Using constant CD concentration and increasing CSA concentration the retention will increase (Fig. 5B), indicating the formation of ion-pairs. In Fig. 6, this principle is demonstrated by reference to chromatograms. As can be seen, a significant improvement in separation efficiency has been achieved by IC + IP technique. From the experimental data shown in Figs 4–6, it can be concluded that the two isomers firstly react with CSA. In the presence of CDs, the ion-pairs formed can be transformed to inclusion-complexes in the second step.

The experimental data for the compounds investigated indicated in Fig. 1 using CSA as ion-pair reagent are summarised in Tables 1–3.

The relative retention values indicated in Tables 1–3 were used for evaluation of the results. In Table 1, the relative retention (r_1) obtained when only CDs (k'_{CD}) *and CSA*

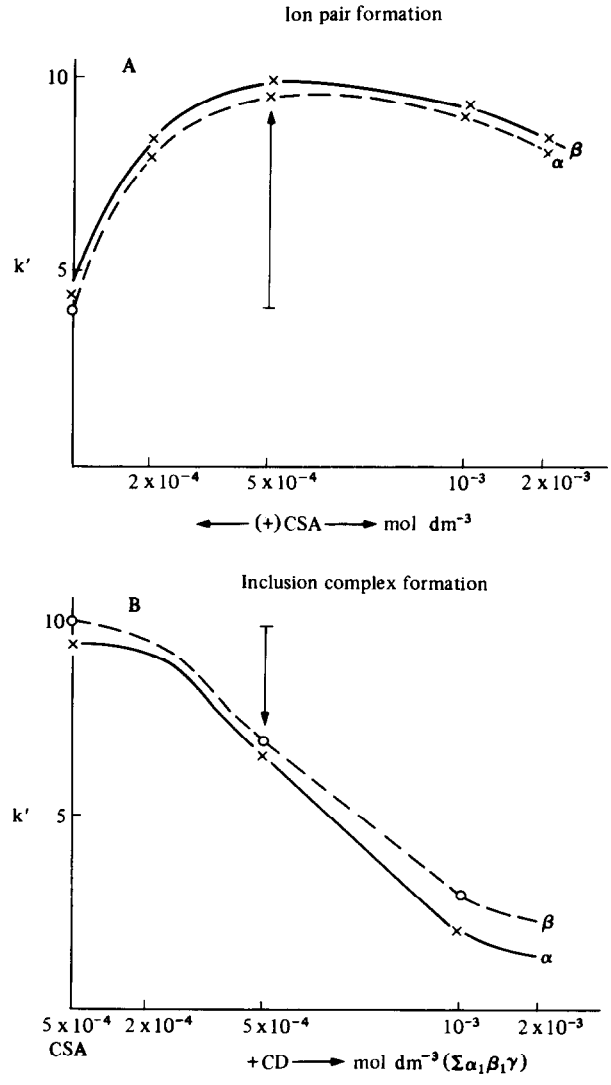


Figure 4
Dependence of capacity factors of Yutac isomers on the concentration of CSA and CD (when CSA concentration is constant.) Conditions: as in Table 1.

(k'_{CSA}) was added to the eluent, are collected. In the case of Yutac isomers, no inclusion-complex formation has been observed. The value of r_1 is dependent only on CSA concentration. In the case of norgestrel, budesonide and flumecinol isomers, it is evident that only inclusion-complex formation occurs. A negligible effect of CSA concentration on retention has been observed.

In the case of Tobanum isomers (Fig. 1) the decreasing r_1-values indicate the importance of ion-pair formation, while tryptophan does not react with either CSA or CDs.

In Table 2, r_2-values calculated from the capacity ratios obtained in the presence of CSA and CSA + CDs, respectively, are shown. Comparing the data collected in Tables

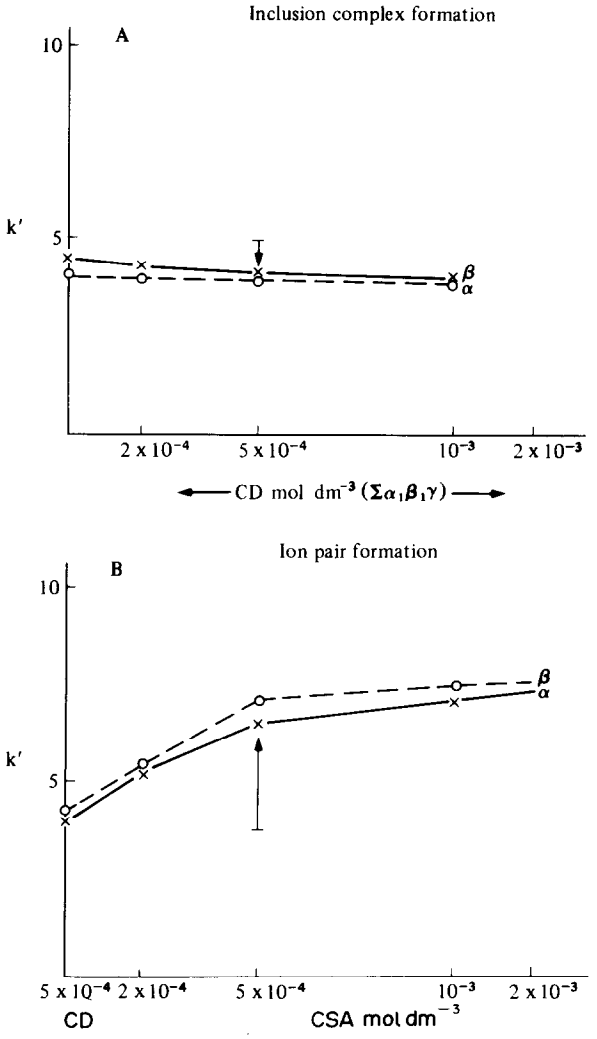


Figure 5
Dependence of capacity factors of Yutac isomers on the concentration of CSA and CD (CD concentration is constant). Conditions: as in Table 1.

1 and 2, it can be concluded, that those compounds having no ionisable functional group possess similar r_1- and r_2-values. Different values for Yutac and Tobanum isomers were obtained. This can be explained by a higher contribution of IP to retention, than by the decreasing effect of IC on retention.

Table 3 shows the effect of CSA on the separation. The values of r_3 calculated from the capacity factors obtained in the presence of CD and CD + CSA, respectively, clearly indicate the significance of ion-pair formation in inclusion-complex formation. In the case of Yutac isomers, increasing values of r_3 with increasing CSA concentration can be traced to the higher retention of inclusion-complexes of the ion-pairs formed (see Fig. 5B). In this case ion-pair formation determines the chemical equilibrium; inclusion-complexation is easily performed by the ion-pairs. In the case of Tobanum isomers the situation is different. As the r_2-values are similar to r_3 (tendency of CSA concentration

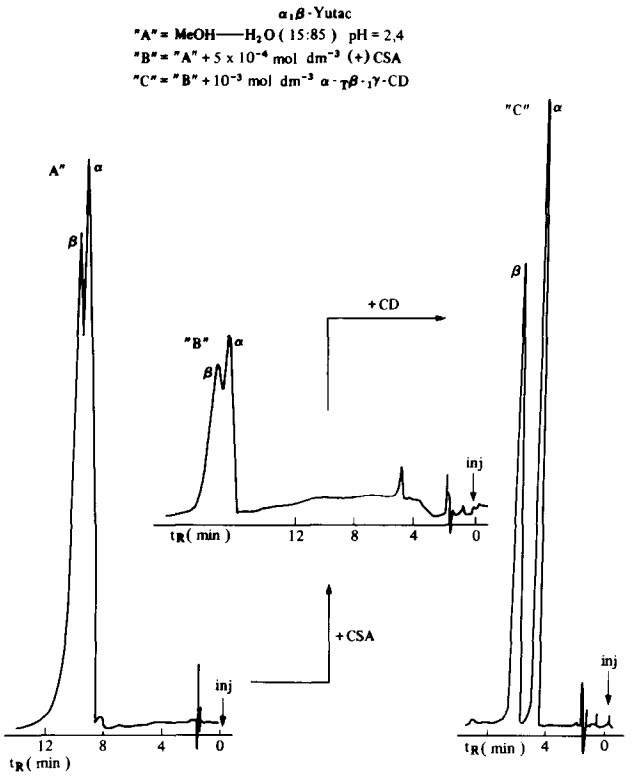


Figure 6
Dependence of the selectivity and efficiency of the separation on the eluent composition. Conditions: as in Table 1.

dependence is the same), it suggests the priority of inclusion-complexation through the easily formed ion-pairs.

In Table 4 the dependence of selectivity factors on the presence of CSA and CD is shown.

From the data presented in Table 4 it can be concluded, that the presence of ion-pairing reagent in the eluent does not cause an unfavourable effect on the separation of compounds having no ionisable functional group. Practically the same selectivity can be achieved. The separation of Tobanum and Yutac isomers should be improved by the combination of IC and IP.

Similar experiments were carried out by using sodium dodecyl sulphate (SDS) as reagent. In Table 5, the changes in selectivity factors when the eluents contain CD and SDS + CD, respectively, are demonstrated.

In Fig. 7, the dependence of capacity ratios using SDS + β-CD-containing eluent on the pH is shown.

From the data shown in Fig. 7, it can be concluded, that the pH of the eluent has a significant influence on the retention only in the case of ionic compounds (Yutac, Tobanum). At high pH no ion-pair formation, and evidently no inclusion-complexation occur. The retention values of other compounds show slight changes depending on the eluent pH.

Table 1
Dependence of relative retention (r_1) on CSA concentration. Conditions: column: Nucleosil-10-CN, eluent: methanol–water (15:85) pH 2.4 containing 5×10^{-4} mol dm^{-3} α-, β- and γ-CD each (k'_{CD}) and CSA/k'_{CSA}); flow rate: 1 cm^3 min^{-1}, detection: 254 nm. Compounds: see Fig. 1

$$r_1 = \frac{k'_{CD}}{k'_{CSA}} = \frac{r_2}{r_3}$$

Model compounds	2×10^{-4} mol dm^{-3} CSA	5×10^{-4} mol dm^{-3} CSA	10^{-3} mol dm^{-3} CSA
α-Yutac	0.49	0.41	0.43
β-Yutac	0.49	0.41	0.43
D-Norgestrel	0.13	0.14	0.15
L-Norgestrel	0.16	0.17	0.18
R-Budesonide	0.65	0.67	0.71
S-Budesonide	0.45	0.47	0.50
(+) Flumecinol	0.83	0.82	0.83
(−) Flumecinol	0.83	0.82	0.80
p-Flumecinol	0.56	0.57	0.57
(+) Tobanum	1.07	0.97	0.88
(−) Tobanum	1.08	0.98	0.88
Tryptophan	1.0	1.0	1.0

Table 2
Dependence of relative retention (r_2) on CSA concentration. Conditions: as in Table 1

$$r_2 = \frac{k'_{(CD\ +\ CSA)}}{k'_{CSA}}$$

Model compounds	2×10^{-4} mol dm^{-3} CSA	5×10^{-4} mol dm^{-3} CSA	10^{-3} mol dm^{-3} CSA
α-Yutac	0.68	0.73	0.82
β-Yutac	0.63	0.66	0.74
D-Norgestrel	0.13	0.15	0.17
L-Norgestrel	0.16	0.18	0.20
R-Budesonide	0.62	0.66	0.71
S-Budesonide	0.43	0.47	0.51
(+) Flumecinol	0.80	0.81	0.78
(−) Flumecinol	0.80	0.78	0.76
p-Flumecinol	0.54	0.56	0.57
(+) Tobanum	1.83	1.07	0.83
(−) Tobanum	1.77	1.05	0.83
Tryptophan	1.0	1.0	1.0

Table 3
Dependence of relative retention (r_3) on CSA concentration. Conditions: as in Table 1

$$r_3 = \frac{k'_{(CD + CSA)}}{k'_{CD}}$$

Model compounds	2×10^{-4} mol dm^{-3} CSA	5×10^{-4} mol dm^{-3} CSA	10^{-3} mol dm^{-3} CSA
α-Yutac	1.39	1.79	1.89
β-Yutac	1.28	1.59	1.71
D-Norgestrel	1.0	1.07	1.14
L-Norgestrel	1.03	1.07	1.13
R-Budesonide	0.96	0.98	1.00
S-Budesonide	0.96	0.99	1.03
(+) Flumecinol	0.96	0.99	0.94
(−) Flumecinol	0.96	0.95	0.95
p-Flumecinol	0.96	0.99	1.00
(+) Tobanum	1.71	1.10	0.94
(−) Tobanum	1.64	1.07	0.94
Tryptophan	1.0	1.0	1.0

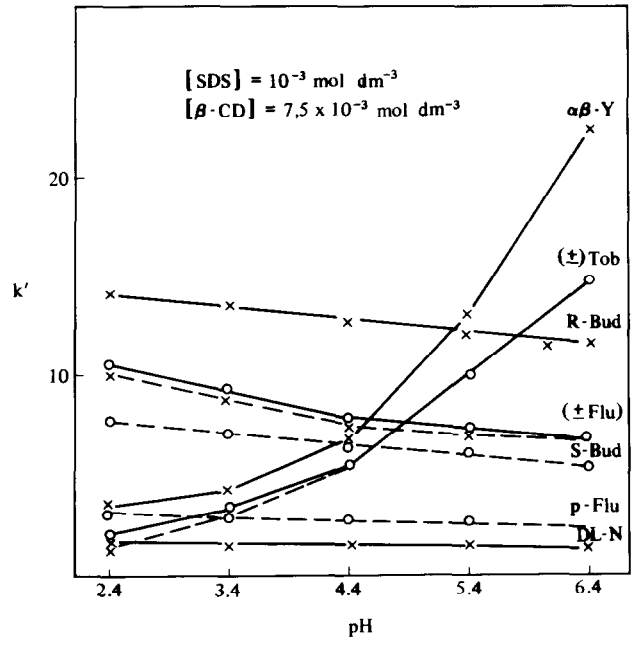


Figure 7
Dependence of capacity ratios on the pH of the eluent. Conditions: as in Table 1, except that β-CD was used in the eluent.

Table 4
Dependence of selectivity factors on the eluent composition. Conditions: as in Table 1

$$\alpha = \frac{k_1'}{k_2'} \text{ (ratio of capacity factors of two isomers)}$$

CSA*	—	—	2×10^{-4}	2×10^{-4}	5×10^{-4}	5×10^{-4}	10^{-3}	10^{-3}
α, β, γ-CD*	—	1.5×10^{-3}	—	1.5×10^{-3}	—	1.5×10^{-3}	—	1.5×10^{-3}
α-Yutac β-Yutac	1.15	1.08	1.05	1.03	1.04	1.07	1.03	1.16
D-Norgestrel L-Norgestrel	1.0	1.16	1.0	1.18	1.0	1.16	1.0	1.17
R-Budesonide S-Budesonide	1.08	1.33	1.09	1.32	1.09	1.32	1.09	1.30
(±) Flumecinol	1.0	1.02	1.0	1.05	1.0	1.07	1.0	1.08
(±) Tobanum	1.0	1.0	1.0	1.02	1.0	1.05	1.0	1.06

*mol dm^{-3}.

Table 5
Dependence of selectivity factors on the eluent composition using SDS as a pairing reagent. Conditions: as in Table 1, except for the ion-pair reagent which is SDS

$$\alpha = \frac{k_1'}{k_2'} \text{ (ratio of capacity factors of two isomers)}$$

SDS*	—	10^{-3}	—	10^{-3}	—	10^{-3}	—	10^{-3}
α, β, γ-CD*	5×10^{-4}	5×10^{-4}	10^{-3}	10^{-3}	2×10^{-3}	2×10^{-3}	3×10^{-3}	3×10^{-3}
α-Yutac β-Yutac	1.08	1.09	1.04	1.04	1.02	1.03	1.0	1.05
D-Norgestrel L-Norgestrel	1.16	1.18	1.16	1.17	1.16	1.10	1.15	1.08
R-Budesonide S-Budesonide	1.33	1.26	1.30	1.37	1.27	1.42	1.25	1.46
(±) Flumecinol	1.02	1.01	1.02	1.04	1.04	1.07	1.01	1.06
(±) Tobanum	1.0	1.12	1.0	1.08	1.0	1.04	1.0	1.02

*mol dm^{-3}.

Table 6
The most important characteristics of relevant methods

		Direct methods			
			Chiral eluents		
	Chiral derivatisation	CCI	IC	IC + IP	Chiral stationary phase
I. Method validation					
Sample preparation	difficult	easy	easy	easy	easy
Purity of reagent	important	less important	no problem	no problem	—
Solubility:					
reagent	no problem	important	no problem	no problem	—
sample	no problem	important	no problem	no problem	no problem
Selection of					
reagent	not limited	slightly limited	limited	limited	—
stationary phase	easy	slightly limited	easy	easy	difficult
mobile phase	easy	limited	limited	limited	limited
Column efficiency	good	problematic	good	good	problematic
Phase system sensitivity	not	for water	not	not	not
Accuracy and precision	problematic	no problem	no problem	no problem	no problem
II. Separation variables					
Number of variables	few	several	several	many	few
Nature and concentration of					
organic solvents	+	++	+	++	++
reagents	−	++	+	+++	−
pH and ionic strength	+	−	+	++	+
Change in elution order	−	++	++	+++	+

III. Applicability					
According to					
Type of components					
apolar	−	−	+	+	+
medium polarity	+	−	++	++	++
polar	++	−	+	+	++
ionic	+	++	+	+++	+
Size of compounds					
small	+	−	−	++	+
medium large	+	++	++	++	++
large	−	−	+	−	+
Chemical structure of compounds	+	++	++	++	++
IV. Selectivity of the method					
For:					
Type of compounds	−	++	+	+++	+
Size of compounds	−	−	++	++	−
Chemical structure of compounds					
functional groups	−	+	−	+	++
place of chiral carbon	−	++	+	++	++
size of lyphophilic portion	−	+	++	+++	+
steric effects	++	−	++	++	+

Signs: − none; + weak; ++ strong; +++ very strong (or equivalent to express the size of the given parameters); CCI = chiral counter ion; IC = inclusion — complex formation; IC + IP = combination of ion-pair and inclusion-complex formation.

From these experiments the following general conclusion can be drawn: the most important advantage of this combined technique is derived from the extended possibilities to improve the selectivity and efficiency of enantiomeric separations through the increased number of separation variables, such as: the nature and concentration of ion-pair reagent; the nature and concentration of CDs; the ratio between the ion-pair reagent and CD; the stationary phase selection; the nature and concentration of organic solvents in the eluent; pH and ionic strength.

This technique enables the simultaneous investigation of ionic and non-ionic compounds also. In the Authors' opinion the combination of ion-pair chromatography with inclusion-complex formation can possibly be exploited on chemically bonded CD columns. Work in this field is in progress.

Conclusions

Regarding the tasks in the field of pharmaceutical analysis as discussed in a previously published paper [17], when enantiomeric separations are necessary, they are concentrated on four main fields: in-process control of asymmetrical synthesis; control of optical purity of intermediates and active ingredients; determination of eudismic ratio (ratio of activities of different enantiomers in biological media); preparation of pure enantiomers.

If the problem is simple, direct and indirect methods should be applicable for enantiomeric separation. These should preferably be used for solving a given analytical task, considering the power and performance of these methods. In Table 6, the most important characteristics of the relevant methods are collected.

Considering the advantages and limitations of the methods shown in Table 6, the following conclusions can be drawn: (a) For the control of optical purity of intermediates and active ingredients, as well as for in-process control of asymmetric synthesis, chiral eluents seem to be the most applicable, because of the greater freedom for phase system optimisation to achieve the necessary selectivity. The methods belonging to this group are especially attractive when a low amount of enantiomeric impurity is to be determined.

(b) When the enantiomers can be separated and determined in biological media (pharmaco-kinetic and metabolic studies) the methods based on chiral derivatisation seem to be most advantageously applied. Because derivatisation is attended with a certain degree of purification, the interference of unknown background materials can more easily be avoided. By using a suitable chiral reagent, the detectability of the compounds to be tested can be improved.

(c) When pure enantiomers should be prepared, derivatisation should not be considered. Given the limitations associated with the use of chiral eluents (solubility problems, column loadability and additional steps necessary for re-transformation of pure enantiomers from the formed complexes after separation) separation on chemically bonded chiral stationary phases is the most applicable method.

As can be seen the situation is complex: in addition to the non-universality of the methods, the different analytical objectives require the use of methods that basically differ in their separation principles.

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