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A STUDY OF THE SEPARATION OF ENANTIOMERS OF SOME AROMATIC CARBOXYLIC ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A β-CYCLODEXTRIN-BONDED STATIONARY PHASE

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SUMMARY

The enantiomers of some aromatic carboxylic acids of biomedical interest were separated by high-performance liquid chromatography on a β-cyclodextrin-bonded stationary phase. Evaluation of the system shows that the structure of the acids plays an important role in the possibility for separation of the enantiomers. Moreover, the pH as well as the buffer components of the mobile phase have a pronounced effect on the retention times and the selectivity of the system.

In order to reduce tailing, the column temperature was increased; this resulted in better peak resolution. Anomalous behaviour was seen in the relation between retention time and sample concentration. At low amounts of injected sample the retention times increased, but this could not be ascribed to adsorption processes. This phenomenon caused great problems in the quantitative analysis of the two enantiomers with this system.

INTRODUCTION

It is known that the enantiomers of a compound can show different properties in living systems. In drug therapy, great differences in pharmacological behaviour have been seen for the optical antipodes of drugs. This can be explained by the fact that the interactions with, for example, receptors, plasma proteins, metabolizing enzymes and carriers take place in an asymmetrical environment, which results in different binding affinities to the enantiomers.

There are only a few cases in which one of the enantiomers instead of the racemic compound is used in drug therapy (*e.g.* levodopa, timolol, dextromethorphan, dextromoramide). However, striking examples of different biological activities of optical antipodes of drugs are known; *e.g.* in the case of the hypnotic drug thalidomide only the levo antipode has teratogenic properties, whereas the dextro-antipode is a good hypnotic, but devoid of teratogenic properties[1].

For anticholinergics it has been known for a long time that *l*- and *d*-forms have different parasymphaticolytic activities[2,3]. Recently, differences in excretion rate be-

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tween *l*- and *d*-oxyphenonium, a potent anticholinergic compound that has been on the market for 35 years, were shown[4]. For a good evaluation of the processes in living systems it is necessary that assays are developed, allowing the determination of both enantiomers separately in the same sample. In this paper some attempts to fulfil this aim are described.

oxyphenonium bromide

Oxyphenonium bromide, a quaternary compound, has a positively charged nitrogen atom situated at a relatively long distance from the chiral centre (four atoms). This nitrogen is the most reactive place in the molecule and will be involved into nearly all the interactions with other molecules. For enantiomer separation, however, interactions should take place at or near the chiral centre. Owing to the arrangement of atoms in the molecule it has been so far impossible to separate the enantiomers of the drug by high-performance liquid chromatography (HPLC). Therefore we decided to try to separate the enantiomers of the acid part of the molecule, cyclohexylphenylglycolic acid (CHPGA), which can be obtained very rapidly by hydrolysis of oxyphenonium. Some other aromatic carboxylic acids with similar structures were also investigated in our studies.

The potential of cyclodextrins to separate optical antipodes has been known for a long time[5]. However, utilization of this phenomenon started only a few years ago, when the cyclodextrins became available in larger amounts. We selected β-cyclodextrin for our studies of the enantiomers of oxyphenonium and related compounds, because separations with β-cyclodextrin in the mobile phase have been reported for mandelic acid[6], which is structurally related to cyclohexylphenylglycolic acid. However, we preferred to use a chiral stationary phase, obtained by bonding of β-cyclodextrin covalently to silica. Some preliminary results were reported previously[7], and this paper presents a further evaluation of the system and a discussion of some anomalous observations.

EXPERIMENTAL

Chemicals

Acetonitrile, methanol, orthophosphoric acid (85% w/v), dipotassium hydrogen phosphate 3-hydrate and potassium dihydrogen phosphate were of analytical grade and obtained from E. Merck (Darmstadt, F.R.G.). Tropic acid (synthetic grade) was also obtained from E. Merck. Racemic CHPGA was a gift from Ciba-Geigy (Basle, Switzerland). Cyclohexylphenylacetic acid (CHPAA) and mandelic acid were from Janssen (Beerse, Belgium). They were used as received. Enantiomers of CHPGA were obtained by crystallization from the racemic acid[4]. Baclofen was a gift from E. Wuis, Department of Clinical Pharmacy, Radboud Hospital, Nijmegen, The Netherlands.

For the synthesis of the stationary phase we used LiChrosorb Si 60, particle size 5 μm (E. Merck) and N-[3-(trimethoxysilyl)]propylethylenediamine, technical

grade (Janssen). The cyclodextrin used was a generous gift from Avebe, Veendam, The Netherlands.

Apparatus

The experiments were performed with a Waters M45 solvent delivery system or a Perkin-Elmer Series 10 HPLC-pump, a Rheodyne 7125 sample injector, equipped with a 20-μl loop, a Spectra Physics 770 UV detector operating at 205 nm and a RDK B-161 recorder or a Kipp BD40 recorder.

Chromatographic conditions

The chiral stationary phase containing β-cyclodextrin was synthesized according to Fujimura *et al.*[8]. The silica silanol groups were derivatized with $(CH_3O)_3$–Si–$(CH_2)_3$–NH–CH_2–CH_2–NH_2 and the product was coupled to the β-cyclodextrin. The columns (175 × 4.6 mm I.D. or 250 × 4.6 mm I.D.) were packed with this material by a balanced-density slurry method[9].

The column was thermostatted by means of a water jacket, connected to a water-bath kept at a constant temperature.

Mobile phases were mixtures of potassium phosphate buffer (0.1 *M*), acetonitrile and methanol. The flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

We investigated the effects of compound structure, mobile phase composition, column temperature and sample concentration on retention time and selectivity. Some quantitative aspects were also studied.

Compound structure and mobile phase composition

The aromatic acids investigated were CHPGA, CHPAA, mandelic acid, tropic acid and baclofen (Table I). Fig. 1a shows some chromatograms obtained with our chiral HPLC system, consisting of a β-cyclodextrin-bonded stationary phase and an aqueous mobile phase. CHPGA and CHPAA can be separated into enantiomers with this system, whereas mandelic acid has a very small selectivity factor. Tropic acid is not separated at all, neither is baclofen (not shown in this figure). Obviously, the molecular structure determines the possibility for resolution of a racemate. The interaction of a compound with cyclodextrin is an inclusion process; β-cyclodextrin consists of a hydrophobic cavity and a hydrophilic exterior, hydroxy groups being on the rim of the cavity. The observation of enantioselectivity was explained by Hinze *et al.*[10], according to the "three-point-attachment concept"[11].

CHPGA and CHPAA, which both have a cyclohexyl group attached to the chiral centre, gave good separations and we assume that this ring structure fits well into the cavity of the cyclodextrin molecule. Various possibilities for hydrogen-bonding at the rim of the cavity are left. The phenyl group is somewhat smaller than the cyclohexyl group, which may result in a less tight fit in the cyclodextrin cavity. This may explain the lack of separation of tropic acid and the very small separation of mandelic acid.

Para-substituted benzene derivatives have also been assumed to fit into the cavity[8,12,13]. However, the overall interactions of baclofen are far less than those of

TABLE I

MOLECULAR STRUCTURES OF SOME AROMATIC CARBOXYLIC ACIDS

Structure	Name
OH, C-COOH	Cyclohexylphenylglycolic acid (CHPGA)
H, C-COOH	Cyclohexylphenylacetic acid (CHPAA)
H, C-COOH, CH_2OH	Tropic acid
H, C-COOH, OH	Mandelic acid
Cl, H, C-CH_2COOH, CH_2NH_2	Baclofen

the first two acids, resulting in very small capacity factors ($k' < 0.5$), and no separation is seen at all.

The influence of pH is demonstrated in Fig. 1a and b. The small separation of mandelic acid obtained at pH 4.2, has disappeared at pH 6.5. For CHPGA retention times are longer at pH 4.2, and for CHPAA they are longer at pH 6.5. These observations are difficult to explain: we may hypothesize that differences in dissociation occur at pH 4.2 and pH 6.5, resulting in other types of hydrogen-bonding at the rim of the cavity that give stronger interactions for mandelic acid and CHPGA at pH 4.2, but weaker interactions for CHPAA.

Instead of phosphate buffer, we also used an acetate buffer, a solution of sodium nitrate with comparable ionic strength and water. These changes resulted in very long retention times and in some cases the compounds were not eluted at all. Tanaka *et al.*[12] observed related phenomena. For the elution of aminobenzoic and nitrobenzoic acids on β-cyclodextrin-bonded stationary phases, they had to use mixtures of methanol and phosphate buffer instead of methanol and water. It is likely that this phenomenon is caused by masking of underivatized silanol groups or –NH– groups of the spacer arm by phosphate ions. These findings make it clear that the concentration of the phosphate buffer also influences the analysis time of these carboxylic acids.

Recently, Hinze *et al.*[10] published results obtained with a new, commercially available β-cyclodextrin column (Cyclobond, Astec). On this column the cyclodextrin is bonded to silica through a non-nitrogen-containing spacer arm. No problems related to very long retention times were reported, and the solution to this phenomenon may lie in the synthesis and structure of the stationary phase material, especially of the spacer arm.

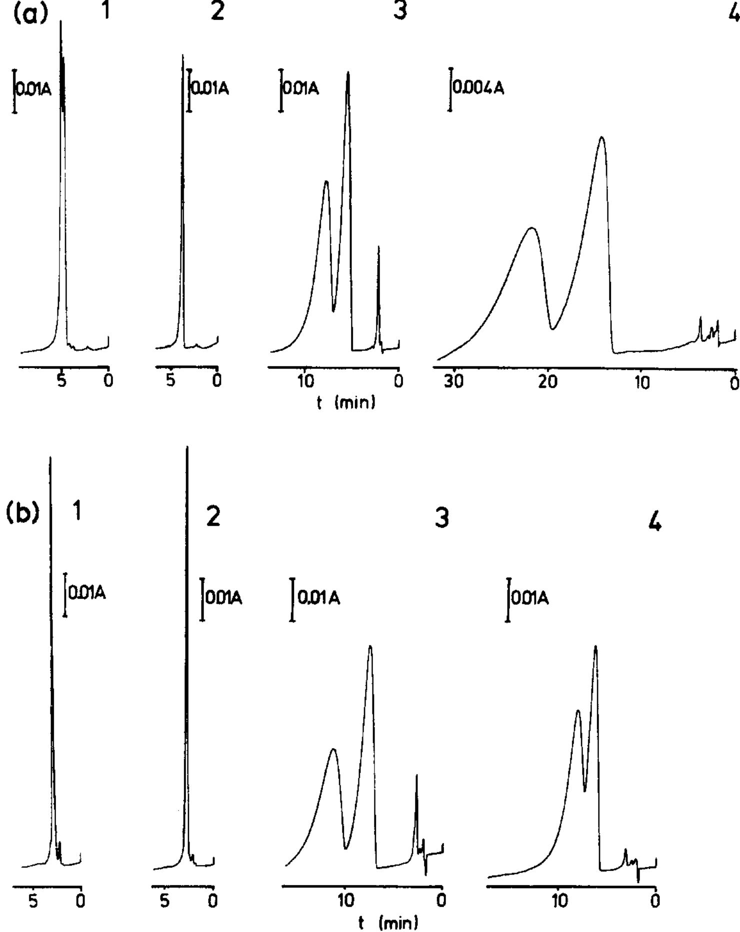

Fig. 1. Influence of pH on separation of the optical antipodes of aromatics acids. (a) At pH 4.2; 1 = 0.6 μg mandelic acid; 2 = 0.3 μg tropic acid; 3 = 3 μg CHPAA; 4 = 3 μg CHPGA. (b) At pH 6.5; 1 = 0.3 μg mandelic acid; 2 = 0.3 μg tropic acid; 3 = 3 μg CHPAA; 4 = 3 μg CHPGA. Column, 175 × 4.6 mm I.D.; mobile phase, acetonitrile–potassium phosphate buffer 0.1 *M* (35:65).

It was stated before that our main interest is focused on CHPGA, the acid moiety of the anticholinergic drug oxyphenonium. Therefore, we applied a modified simplex procedure[14] to obtain the optimum mobile phase composition for separating the enantiomers of this acid. Three variables were chosen: acetonitrile concentration, methanol concentration and pH, and the following optimum composition resulted: 62.5% potassium phosphate buffer (pH 4.2, 0.1 *M*), 31.5% acetonitrile, 6.0% methanol. Further experiments were carried out with this eluent.

Influence of temperature

With the system described above we still observed severe tailing. This may be explained by the presence of non-derivatized silanol groups on the silica gel and/or

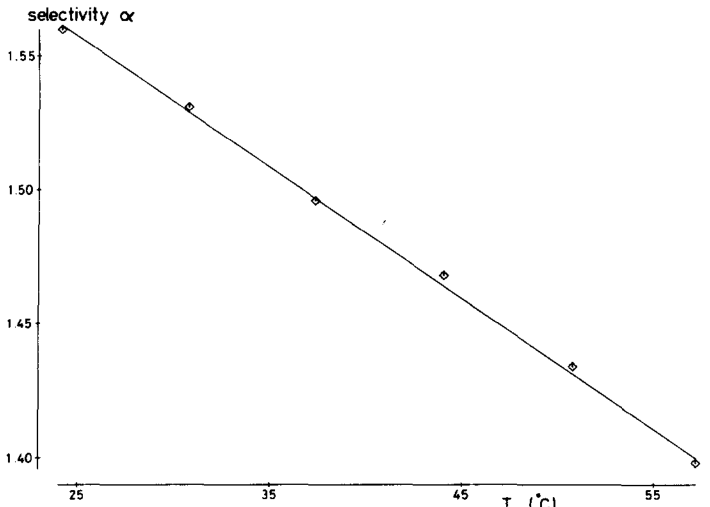

Fig. 2. Relation between selectivity and column temperature for CHPGA. Column, 175 × 4.6 mm I.D.; mobile phase, acetonitrile–methanol–potassium phosphate buffer, pH 4.2, 0.1 *M* (31.5:6.0:62.5); sample, 3 μg CHPGA.

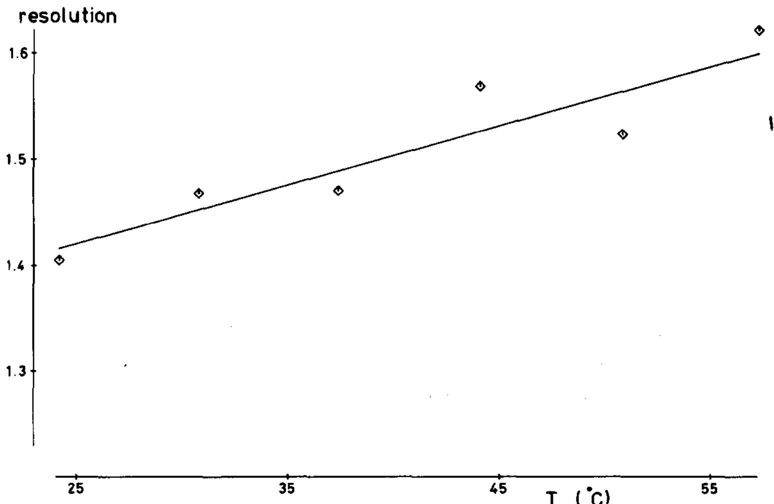

Fig. 3. Relation between resolution and column temperature for enantiomers of CHPGA. Conditions as in Fig. 2.

the presence of an amine function in the spacer arm. However, another factor can result in severe tailing as well: slow mass transfer. The interaction of the molecules with the cavity of the β-cyclodextrin molecule will be slow. We expected that tailing could be reduced by increasing the temperature. Higher temperatures resulted in shorter retention times, this behaviour being consistent with the observation that the stability of various inclusion complexes in solution decreases significantly as the temperature increases[15].

Fig. 2 shows the influence of temperature on separation factor α ($\alpha = k'_l/k'_d$), whereas Fig. 3 gives the relation between temperature and resolution. Resolution is being defined as $R_s = (t_{R,l} - t_{R,d})/2(\sigma_l + \sigma_d)$. Fig. 4 shows there is a linear relationship between ln k' and $1/T$, as can be explained by a thermodynamic approach of liquid chromatography[16]. These plots illustrate that it is preferable to work at higher temperatures. Although the separation factor decreases, the resolution increases, which is important for quantitative applications.

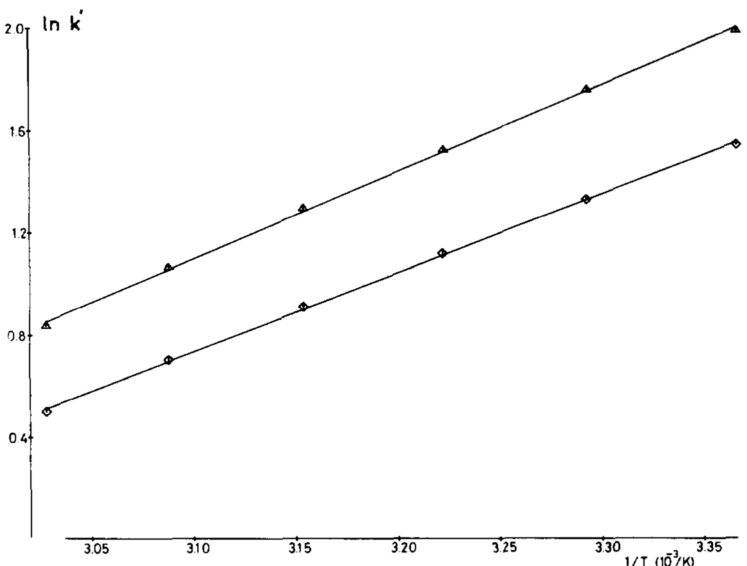

Fig. 4. Relation between ln k' and reciprocal temperature for enantiomers of CHPGA. Conditions as in Fig. 2. Data points: △ = *l*-CHPGA, ◇ = *d*-CHPGA.

However, at temperatures above 50°C the stationary phase began to decompose, as indicated by a yellow colour of the column eluent. So we decided to do further work with this column at temperatures just below 50°C. Fig. 5 shows chromatograms obtained at two different temperatures.

Relation between sample concentration and retention time

In preliminary studies, we observed that the retention time varied with the concentration of the injected sample, so we decided to look at this phenomenon in more detail. Different amounts of *l*- and/or *d*-CHPGA were injected into the system at constant temperature. Fig. 6 shows how the retention time depends on the concentration of the injected sample.

In HPLC theory attention has been paid to the dependency of retention time on the concentration in the sample. In the ideal case a linear isotherm is observed and the retention is independent of the sample concentration. Variations of retention time with changes in injected sample amounts are explained by the occurrence of non-linear distribution curves. A convex isotherm causes a decreasing retention with

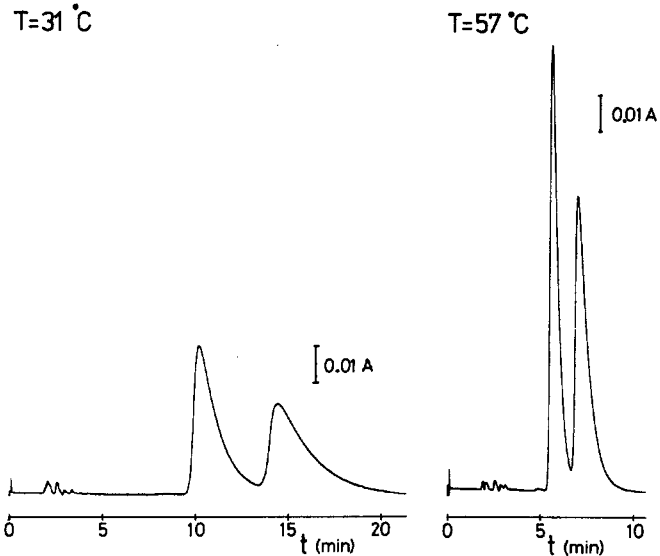

Fig. 5. Chromatograms of CHPGA at different temperatures. Conditions as in Fig. 2.

increasing concentration in the sample, and a concave isotherm results in increasing retention with decreasing concentration of sample. In both cases, the retention time should be nearly constant at low sample concentrations[17]. However, the occurrence of very long retention times at low sample concentrations cannot be explained by this theory. One may think of the irreversible adsorption of small amounts of sample,

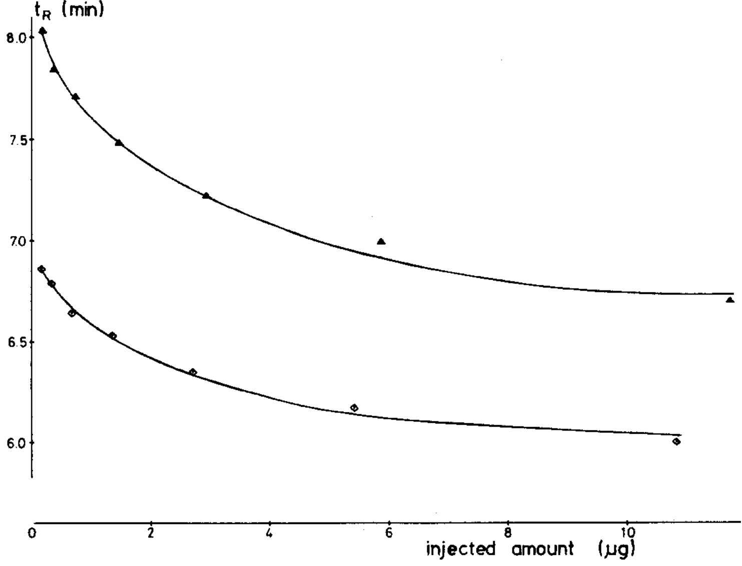

Fig. 6. Relation between retention time and amount of CHPGA injected. Column, 250 × 4.6 mm I.D.; mobile phase, as in Fig. 2; sample, *l*-CHPGA (△) or *d*-CHPGA (◇); injection volume, 20 µl.

but if this were true, the phenomenon should disappear after some injections of large amounts of sample, and this has not been observed. On the other hand, if competitive, reversible processes were involved one would anticipate that a large excess of the slower moving *l*-CHPGA would shorten the retention time of small amounts of *d*-CHPGA. However, this was not observed with a mixture of 90% *l*-CHPGA and 10% *d*-CHPGA; in fact, the separation disappeared altogether. The explanation of these phenomena is still lacking.

Quantitative evaluation

We are especially interested in the possibility of analysing the enantiomers side by side for bioanalytical applications in order to be able to investigate stereoselective phenomena in pharmacotherapeutics.

Typical calibration plots for *l*- and *d*-CHPGA are show in Fig. 7. These plots

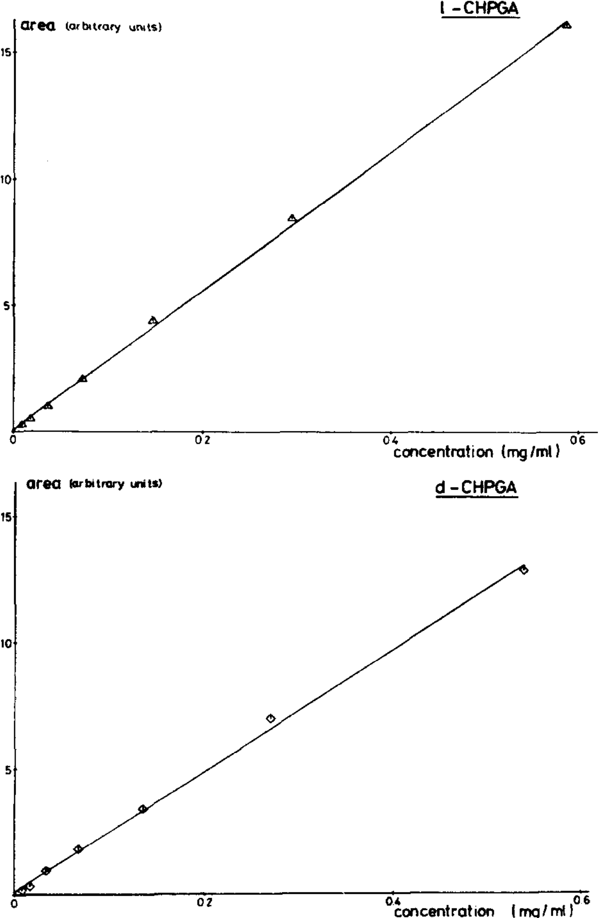

Fig. 7. Calibration plots for *l*- and *d*-CHPGA. Conditions as in Fig. 7. Sample, *l*-CHPGA (△) or *d*-CHPGA (◇) in different concentrations.

show good linearity. However, problems arise when both enantiomers are present in the sample, especially if the ratio of *l*- to *d*-acid in the sample starts to differ from 1, when differences in regression coefficients are observed. As we saw in Fig. 5, resolution of the two enantiomers is still not complete. For quantitative determinations, this is not necessarily a problem, as long as the overlap of peaks is constant in the range of the calibration curve used.

However, as we have seen above, peak overlap will be dependent on the ratio of the enantiomers present. Therefore, it will be clear that quantitative determinations will become very difficult, if not impossible, when our very aim is to establish whether differences exist in the ratio between the *l*- and the *d*-acid in biological samples. This would be the case if pharmacokinetic processes are indeed stereoselective.

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