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Definition and system implementation of strategies for method development of chiral separations in normal- or reversed-phase liquid chromatography using polysaccharide-based stationary phases

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Abstract

This paper proposes strategies in normal- and reversed-phase liquid chromatography (NP-HPLC or NPLC and RP-HPLC or RPLC), which were developed using three polysaccharide-based stationary phases. Those strategies are implemented in a knowledge-based system for the chiral separation of drug enantiomers. Each strategy includes a screening and an optimisation stage. The screening stage allows a fast evaluation of separation possibilities and enantioselectivity for many drugs in a short period of time, while the optimisation stage gives the opportunity to enhance, if needed, the initially obtained separation. Different examples demonstrate the effectiveness of the strategies for fast method development.
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1. Introduction

During the last two decades, chirality and stereoisomerism became very important topics in pharmacology and analytical chemistry. The chiral nature of living systems has evident implications on biologically active compounds interacting with them [1–5]. Nowadays, drug manufactures often try to synthesise enantiomerically pure compounds. As a consequence of the actual techniques of synthesis, e.g. combinatorial chemistry, the speed of method development is rather a more limiting factor than the speed of synthesis. In drug development, analytical methods are required to evaluate the enantiomeric purity of starting materials, reagents and catalysts, because the quality of these compounds limits the enantiomeric purity of the resulting products. Therefore, the goal of the analytical lab in the pharmaceutical industry is to develop as much separations as possible in a minimum of time. In this context, it is useful to have general screening strategies based on, for instance, chromatographic techniques [6–9].

The aim in knowledge technology is to formalise knowledge about a specific domain in such a way that it can be implemented into expert or knowledge-based systems (KBS) [10–27]. Expert systems allow to take intelligent decisions based on the knowledge of an expert. The classical expert systems are programs which are able to solve problems by consulting a reliable database. In general, the program asks the user a question. Based on the answer, the program gains extra information until enough input data are available to make final decisions solving the initial problem. Therefore, different rules are defined and internally combined until a solution is proposed [10–14].

A knowledge-based system has, in principal, the same characteristics as a classical expert system, but its knowledge is more structured and organised. A KBS is a system in

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which the acquired knowledge and experience is organised as a decision tree, and is used to simulate the performance of an expert [6,10,15,16].

In chiral normal- and reversed-phase liquid chromatography (NP-HPLC or NPLC and RP-HPLC or RPLC), polysaccharide-based stationary phases are most popular [28–37]. Among all chiral stationary phases (CSPs), the acetate ester, benzoate ester or phenylcarbamate derivatives of glucose polymers (cellulose and amylose), have shown the have a good and broad performance [38–40]. From those derivatives three of them, i.e. cellulose tris-(3,5-dimethylphenylcarbamate), amylose tris-(3,5-dimethylphenylcarbamate) and cellulose tris-(4-methylbenzoate), have very complementary properties and numerous publications have demonstrated that they were able to achieve the chiral resolution of more than 80% of the drugs currently available on the market [41–49]. Those CSPs are known under the commercial names, Chiralcel® OD, Chiralpak® AD and Chiralcel® OJ, respectively. The effective chiral recognition with these stationary phases in various mobile phases is an important advantage because it allows to resolve many different chiral analytes.

This paper describes the definition of two chiral separation strategies in liquid chromatography, one in NPLC and another in RPLC. Both strategies are implemented in a more elaborated chiral separation knowledge-based system which includes also strategies in other chiral separation techniques such as supercritical fluid chromatography (SFC) and capillary electrophoresis (CE) [6]. The chiral separation strategies, proposed in this paper are developed using only one family of chiral stationary phases, namely the polysaccharide ones. Other CSPs like Pirkle-type, cyclodextrins-, macrocycle- or protein-based columns can give additional selectivity for compounds unable to be separated on the polysaccharide ones. However, prior to the definition of separation strategies we limited ourselves in the number of CSPs to be included.

The main scope of the Chiral KBS and of the strategies included is: (i) to obtain fastly an idea about the selectivity of a given system for the individual substances of large series of compounds; and (ii) to obtain a separation between the enantiomers of many different racemic mixtures in a limited number of experiments.

Therefore, the selection of a restricted number of stationary phases showing a very broad application area is required, and the polysaccharide phases OD, AD and OJ were preferred. This selection is based on our own experience [8,9] and on many published data [29–31,50–69]. Such selection of columns and definition of strategies also involves that for individual compounds better separations might be able on, for instance, the columns mentioned higher which were not considered. This is inherent to our selection of columns and to the strategies proposed, but it is irrelevant in the context of the creation of the Chiral KBS. For those reasons, it is also not our aim to compare the obtained separations with published and optimised results on similar or other chiral systems.

The strategies are defined based on the enantioseparation of large numbers of substances but they are illustrated by applying them on a total of 15 different drug molecules.

2. Experimental

2.1. Materials and reagents

Alprenolol, fenoprofen, atropine, acebutolol, promethazine, tetrimasol, *trans*-stilbene oxide and mandelic acid were obtained from Sigma–Aldrich (Steinheim, Germany). Thiopental is purchased from Abbott (Ottignies, Belgium). Ephedrine hydrochloride was obtained from Vel (Leuven, Belgium). Methadone, hexobarbital and oxazepam were gifts from diverse sources.

Acetonitrile (CH_3CN), methanol (MeOH) and *n*-hexane (all Hypersolv, HPLC grade) were obtained from BDH Laboratory Supplies (Poole, UK). Ethanol (EtOH) absolute extra pure, 2-propanol (IPA; HPLC grade), sodium dihydrogenphosphate monohydrate (analytical reagent grade) was purchased from Merck (Darmstadt, Germany); diethylamine (DEA) was obtained from UCB (Brussels, Belgium).

Trifluoroacetic acid (TFA), sodium tetraborate decahydrate (borax, $Na_2B_4O_7 \cdot 10H_2O$), boric acid (H_3BO_3), potassium hexafluorophosphate (KPF_6) are purchased from Sigma–Aldrich; phosphoric acid (85%) and hydrochloric acid from Carlo Erba (Milan, Italy). Water for the preparation of the mobile phases was produced in house with the Milli-Q system (Millipore, Milford, MA, USA).

2.2. Mobile phase buffers

During method development in RPLC, two buffers are applied, a 50 mM sodium phosphate buffer ($pH = 2 \pm 0.05$) and a borate buffer ($pH = 9 \pm 0.05$). The acidic buffer is prepared by dissolving the required amount of KPF_6 in a mixture of phosphoric acid and sodium dihydrogenphosphate solutions. The pH was adjusted using a diluted HCl solution. A 20 mM borax and a 20 mM boric acid solution were mixed to prepare the basic buffer.

2.3. HPLC instrumentation

Experiments in the normal-phase mode were performed on a Merck–Hitachi system (Hitachi Instruments, Tokyo, Japan) equipped with a D-7000 HPLC System Manager, version 4.1. A L-7400 UV detector is set to 220 nm. A low pressure gradient pump (L-7100) with an L-7612 vacuum degasser, an L-7360 Peltier column oven and an L-7200 autosampler with 100 μl injection loop were used. The mobile phase flow rate and temperature are specified in Section 3.

In reversed-phase chromatography a comparable system is used: an L-7100 HPLC pump, an L-7612 degasser, an

L-7400 UV detector ($\lambda = 220$ nm), an L-7250 autosampler and an L-7350 column oven, linked to the D-7000 HPLC System Manager, version 4.0.

2.4. Chromatography

The chiral stationary phases in NPLC and RPLC were polysaccharide-based. In NPLC, a Chiralcel®OD-H column (25 cm × 4.6 mm i.d.; 5 μm, chiral selector: cellulose tris-(3,5-dimethylphenylcarbamate)), a Chiralpak®AD column (25 cm × 4.6 mm i.d.; 10 μm, amylose tris-(3,5-dimethylphenycarbamate)), and a Chiralcel® OJ column (25 cm × 4.6 mm i.d.; 10 μm, cellulose tris-(4-methylbenzoate)), all from Daicel (Tokyo, Japan) were used with various mobile phases. In reversed-phase conditions, Chiralcel® OD-RH, Chiralpak® AD-RH and Chiralcel® OJ-R columns (Daicel) with the same dimensions were applied. These columns are the reversed-phase versions of the CSPs used in NPLC.

2.5. Data processing

The analytical data were acquired and treated with the software defined in Section 2.3. Resolution (Rs) values were calculated according to the United States Pharmacopeia (USP) [70]:

$$\mathrm{Rs} = \frac{2(t_\mathrm{r}(b) - t_\mathrm{r}(a))}{W_\mathrm{B}(b) + W_\mathrm{B}(a)} \qquad (1)$$

where $t_\mathrm{r}(b)$ and $t_\mathrm{r}(a)$ are the retention times (in min) of the last and the first eluting peak, respectively, and $W_\mathrm{B}(b)$ and $W_\mathrm{B}(a)$ the baseline widths (in min) of these peaks.

3. Results and discussion

3.1. General structure of the Chiral KBS

The "Chiral KBS" is a knowledge-based system, developed in-house, which guides the user through the complete method development of a chiral separation. The Chiral KBS is programmed using a hypermedia tool. Initially, the Chiral KBS was programmed in Toolbook (Asymetrix® Toolbook version 1.5, Bellevue, Washington, DC, USA) but has been re-implemented in a more convenient programming environment, Microsoft® Visual Basic 6.0 (Microsoft® Visual Studio 6.0, © 2000 Microsoft Corporation).

Fig. 1 shows the global structure of the "Chiral KBS". To assist the user step-by-step in the chiral method development, three main levels can be distinguished (technique selection, screening and optimisation). First, a suitable technique out of six included will be selected. These techniques belong to three separation approaches, namely liquid chromatography (NPLC, RPLC, polar organic solvent liquid chromatography (POSC)), supercritical fluid chromatography (SFC) and electromigration-based (CE and CEC) techniques. Selection among them often depends on the availability of instrumentation and analyst skills. When liquid chromatography is selected, NPLC is preferred for speed of method development and short analysis times (because of low viscosity of eluents, faster column equilibration than in RPLC and possibility to use higher flow rates) [50,51]. On the second level, the aim is to achieve very fast a separation, which, if necessary, can be optimised in the third level. The second level includes a screening of the compound(s). The methodology of the Chiral KBS includes that if no separation (Rs = 0) is obtained after screening in the first selected technique, e.g. RPLC, the user is recommended to switch to the screening level of another technique, for instance CE, in which a limited number of cyclodextrins (CDs) are used as selectors. The use of other CSPs (Pirkle-type, etc.), a possible alternative, was a priori excluded, as well as it is the case for the use of other CDs than those included in the CE strategy [6]. The application of complementary techniques and practical restrictions allow justifying from an industrial point of view the use of only a limited number of polysaccharide columns.

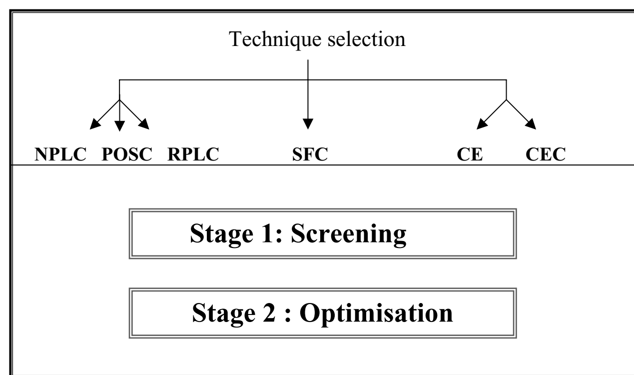


Fig. 1. General structure of the "Chiral KBS".

Within each technique, a screening strategy is proposed (Fig. 1, stage 1). At this stage the KBS makes an initial proposal of the method conditions for the selected technique, e.g. it may propose a given stationary phase to be tried out first, a modifier, its volume fraction, etc. Also several method conditions, defined by an experimental design, can be proposed at this stage. The aim of the screening is to acquire enantioselectivity towards a given compound.

If necessary, e.g. when after screening no baseline separation is obtained, the user can enter an optimisation stage, to enhance the separation (Fig. 1, stage 2). In this stage the KBS should guide the user through an optimisation strategy, which eventually should lead to a separation that allows to determine 0.1% of one enantiomer as impurity in the presence of 99.9% of the other (active compound).

In this paper, the screening and optimisation strategies (stages 1 and 2) for the two liquid chromatography techniques, NPLC and RPLC, are described. The strategies were developed based on the results obtained from our own laboratory experiences [8,9] and on literature data

[29–31,50–69]. Finally, the strategies are illustrated with several examples. During the acquisition of knowledge both in NPLC and RPLC, both 5 and 10 μm cellulose- and amylose-based CSPs were used because the 5 μm versions were not yet available for all columns. Nowadays, the 5 μm CSPs are available for the columns used and are recommended in the KBS because of their higher efficiency.

3.2. Technique selection

Before entering the first stage of the Chiral KBS, a selection among the five techniques must be performed. Preference for either NPLC or RPLC is mainly based on the solubility properties of the compound. NPLC might be preferred as separation method for water insoluble compounds or for preparative chromatography. For the strategies included in the Chiral KBS, only NPLC is intended for preparative chromatography because of the easy elimination of the volatile solvents. Nevertheless, the solubility of the compound may sometimes lead the user to RPLC, or for preparative goals, to polar mode (POSC).

Secondly, the usability and environmental properties are considered. RPLC is, for instance, preferred to NPLC when the toxicity of hexane is of concern. It is suggested to the user of the Chiral KBS, to replace hexane by isohexane or *n*-heptane in preparative approaches [71]. Experiments showed that heptane is a good alternative for *n*-hexane in our strategy, without important loss of selectivity.

In the next sections, the screening and optimisation strategies defined in both techniques are described.

3.3. Normal-phase liquid chromatography

3.3.1. Screening

The general screening and optimisation strategy is presented in Fig. 2. Depending on the nature of the compound (basic on the one hand, acidic, bifunctional or neutral on the other), a different screening strategy is proposed. Acidic, neutral and bifunctional compounds are screened using a 3 × 2 experimental design. This means that the design includes two factors, one tested at three levels and the other at two, and requires six experiments. The factors evaluated are the type of column (three levels) and the type of organic modifier (OM) (two levels). The most popular polysaccharide CSPs are AD, OD, OJ and AS [52–55]. However, study [72], showed that both in NPLC and RPLC, only few separations are obtained on the AS column. Moreover, these separations also are obtained using AD, OD or OJ columns.

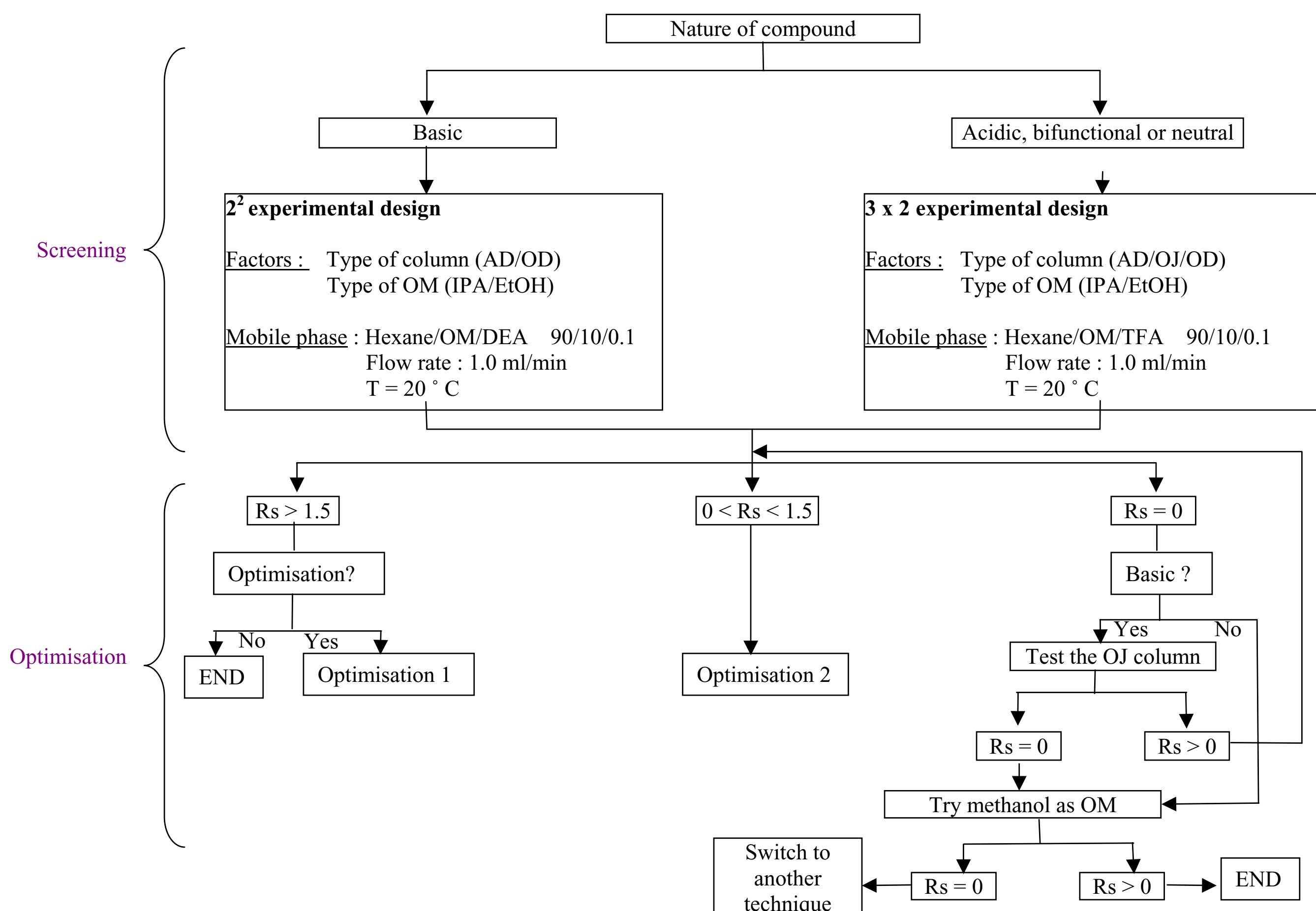


Fig. 2. General strategy in NPLC.

Therefore, the AS column was discarded from the KBS method development strategies. The three other polysaccharide columns (Chiralpak® AD-H, Chiralcel® OJ-H and Chiralcel® OD-H) are evaluated using two modifiers. Isopropanol and ethanol are the most commonly used modifiers on the Chiralcel® OD-H, Chiralcel® OJ and Chiralpak® AD columns and, according to the literature [28–34,56–64] and our own experience [8,9], allow the separation of most drug enantiomers. Two modifiers are screened because it is difficult to predict the optimal organic modifier for a given column and its effect also depends on the analyte [8,56].

For basic compounds, the same factors as for acidic ones are investigated, but here a 2^2 full factorial design is applied because the factors are only examined at two levels. The Chiralcel® OJ-H column is not included in this design because only few separations are observed for basic compounds [8]. This column is scheduled to be tested only when no separation is obtained on the other two (Fig. 2, Rs = 0).

The experimental conditions used in both situations are shown in Fig. 2. A difference for the additive in the mobile phase can be observed. A basic additive, DEA, is added when basic compounds are analysed, while an acidic one, TFA, is added to analyse acidic, neutral or bifunctional compounds. Study showed that simultaneous addition of DEA and TFA, in general, did not lead to improvement of the separations on the applied stationary phases [8,73]. The concentration of DEA or TFA in the mobile phase is set at 0.1% (v/v) [8].

When required, after the initial screening a further optimisation can be performed starting with the best conditions (i.e. giving the highest resolution). Three situations are possible from the screening: (a) good enantioseparation is observed (Rs > 1.5) for at least one of the experiments; (b) a beginning of separation is seen (0 < Rs < 1.5); and (c) no separation occurred in one of the experiments (Rs = 0).

When baseline separation is obtained and the result satisfies the analyst's requirements, it is recommended to end the method development. In some cases, good enantioselectivity is found but further optimisation is preferred, for example: (i) when no baseline separation is achieved because of bad peak shapes; (ii) when analysis time (t_r) is too long; or (iii) when impurity determination is required (i.e. small peak in presence of large one) and Rs is still considered too small. In those cases, an optimisation approach, called optimisation 1 (see Section 3.3.2), is recommended.

In the second situation, when enantioselectivity with limited separation is obtained during screening, it is recommended to perform an optimisation, called optimisation 2 (Section 3.3.2), to enhance retention and separation quality.

When for basic compounds no separation (Rs = 0) occurred in the screening design, the Chiralcel® OJ-H column or an additional alcohol (methanol) is tested (experimental conditions as defined in the screening design) before switching to another technique. When enantioselectivity is shown for this column, the strategy, as explained earlier, is followed.

The above described screening is intended for systems having both eluent and column switching valves. For such systems, the screening will be completely and automatically executed. However, performing the full screening procedure is unpractical and time consuming when no automatic switching device is available. For such systems, a step by step approach is proposed, which is derived from the full screening strategy. Method development, using experimental design, indicates independence of the experiments. However, when performing a sequential approach, the sequence of the experiments (columns, solvents and additives) used, is important and an order is proposed. The most generic conditions, i.e. the most successful column with the best organic modifier, etc. are tested first. The order of column and mobile phase testing is as followed: (1) Chiralpak® AD-H; (2) Chiralcel® OD-H; and (3) Chiralcel® OJ-H. For all columns, isopropanol is preferred to ethanol as organic modifier. As soon as baseline resolution is achieved, the user can stop method development or perform one of the above optimisations. When no baseline result is achieved, the best result is treated as in Fig. 2. The disadvantage of the sequential approach is that experiments with possibly better separation results might be ignored because the screening is stopped at a given point.

3.3.2. *Optimisation stage*

3.3.2.1. Optimisation 1. Optimisation 1 is performed when the initial separation is good and can be considered as an optional fine-tuning of the method. This optimisation (Fig. 3) includes a retention factor (k') or a peak shape optimisation [56–64].

Retention factor optimisation. The factors examined are percentage OM, temperature (T) and flow rate. Increasing the percentage OM has the largest influence on the retention and is performed before optimising temperature and flow rate, which are evaluated using a 2^2 full factorial design. In that design, the factor levels are defined higher than in the screening experiments because the general idea is to shorten analysis time by increasing column temperature and flow rate [50,61]. Limitations for increasing the flow rate are expected because the back pressure should be controlled, which depends on the chromatographic system used. The measured responses are Rs and k'. Since at that moment the most influencing factors are optimised, no further optimisation is proposed. The Pareto Optimal experiments [74] for k' and Rs are determined and proposed as final method conditions. Pareto-Optimality is a multicriteria decision method in which an experiment is considered Pareto Optimal if there is no other experiment which has a better result on one criterion without having a worse result on another [74]. When several experiments are Pareto Optimal, the user selects the one most satisfying, i.e. the one having an acceptable compromise between retention and resolution. Examples applying optimisation 1 are given in Section 3.5.1.

Peak shape optimisation. To optimise peak shape, the concentration of amine (DEA) or acid (TFA), added to the mobile phase, and the temperature (T) are examined

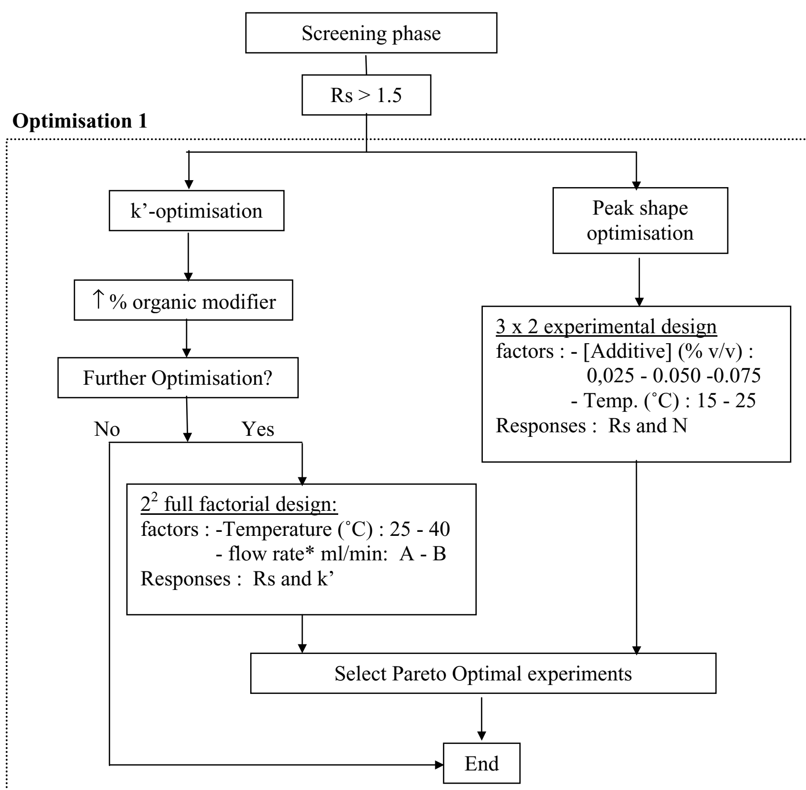


Fig. 3. Optimisation 1 in NPLC.

because both factors influence the efficiency of the separation [61,62]. A 3 × 2 experimental design is used, in which the first factor (additive concentration) is examined at three levels and the second (T) at two. The temperature is important because it can have a large effect on selectivity and efficiency [63]. Temperature variations can also change the ionisation degree of the compound and influence the retention mechanism [38,61–70,72,73]. The concentrations of the additives ([DEA] or [TFA]) are decreased compared to the screening experiment because changing the concentration of the additive can have a positive influence on the peak shape [56–60]. Higher concentrations can also have positive influence [8] but in our cases it gives experimental problems related with stability of the baseline and too high UV absorption. Responses considered are Rs and efficiency (N). The Pareto Optimal experiments of this design are determined before ending the method development. Similar to the retention factor optimisation, the most appropriate Pareto Optimal experiment is selected as final method condition.

3.3.2.2. Optimisation 2. This optimisation (Fig. 4) is proposed for situations where enantioselectivity was observed, but the obtained separation is insufficient. In this optimisation, different pathways are followed depending on the value of the retention factor in the best result from the screening (result with highest resolution). When k' is smaller than 1 or higher than 5, the first step is to change the percentage OM until $1 < k' < 5$. This means decreasing or increasing the percentage OM when $k' < 1$ or $k' > 5$, respectively. When a suitable value cannot be achieved it is proposed to switch to another technique. Changing the percentage OM also can have an influence on the selectivity. Therefore, the next step depends on the obtained resolution. When Rs is higher than 1.5 after adapting the percentage OM and further optimisation (e.g. peak shape) is still required, a slightly adapted version of optimisation 1 is proposed. The only difference is that changing the percentage OM, in the k' optimisation is eliminated because this factor is already considered.

When after changing the percentage OM, the separation is still unacceptable ($0 < \mathrm{Rs} < 1.5$), an optimisation of the [additive] and of the temperature is proposed using a 2^2 full factorial design. Selectivity tuning is then performed by lowering the temperature and changing the [additive]. As the Daicel columns can stand 0–40 °C temperatures, the levels are taken at 5 and 15 °C. The responses for which the Pareto Optimal experiments are determined are Rs and N. No further k' optimisation is proposed after this step since higher flow rates are not allowed at low temperatures (<20 °C) because of viscosity and pressure restrictions. The user now reaches the end of this stage and will either use one of the Pareto Optimal experiments as final method condition; or when the separation result is still unsatisfying, the analyst

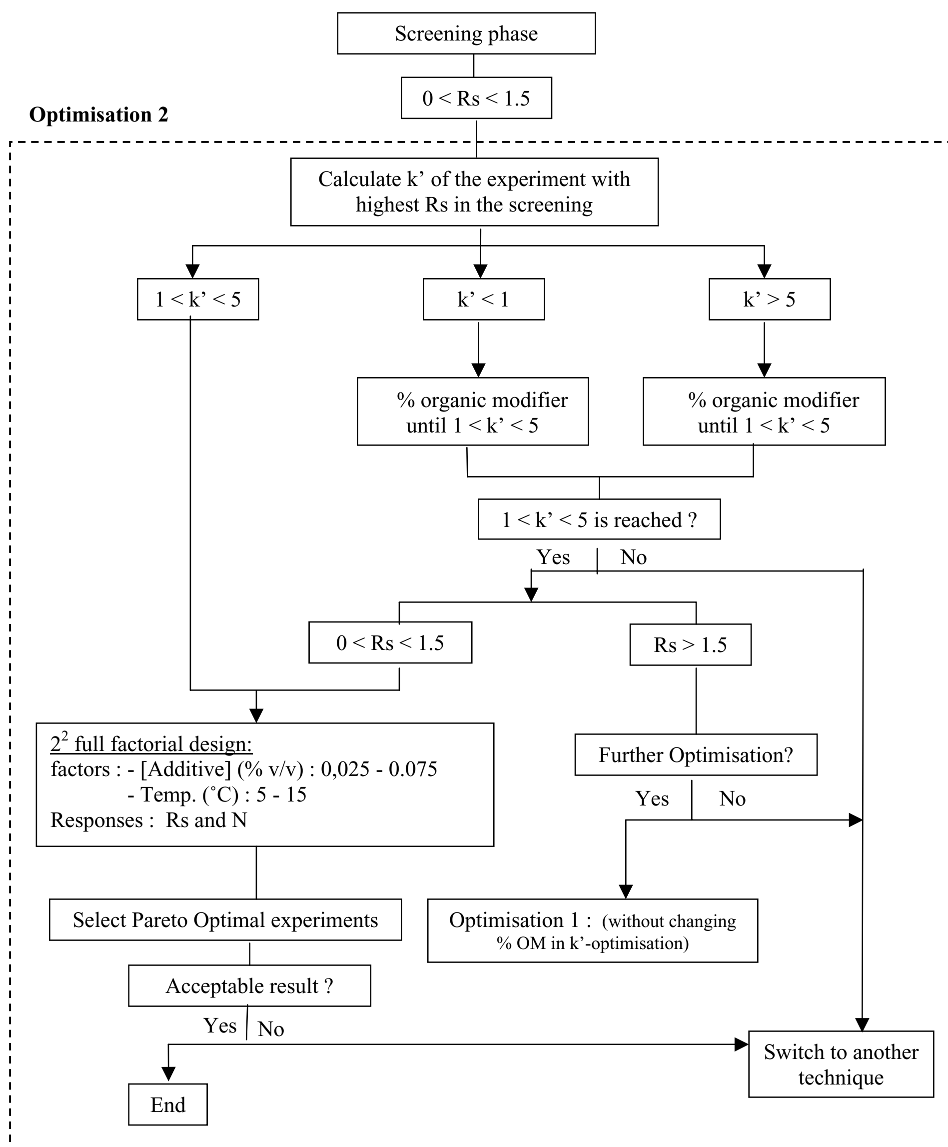


Fig. 4. Optimisation 2 in NPLC.

is recommended to try method development using another technique.

3.4. Reversed-phase liquid chromatography

3.4.1. Screening

Similar to the normal phase screening, also two different approaches (automated–sequential) are proposed depending on the possibilities of the chromatographic system available. The first strategy included is an experimental design based automated screening (Fig. 5). Three polysaccharide stationary phases are included, Chiralcel® OD-RH, Chiralcel® OJ-RH and Chiralpak® AD-RH. These columns are the reversed-phase versions of the columns used in NPLC.

The screening conditions are defined by a 3×2 full factorial design. The factors included are the type of column (three levels) and the buffer composition in the mobile phase (two levels). This means that the three polysaccharide columns are screened with two mobile phases, an acidic and a basic one.

Perrin et al. [9] showed that, in general, on those chiral stationary phases, enantiomers are better separated when they are uncharged. Therefore, acidic compounds are usually better resolved under acidic conditions and basic ones with a basic mobile phase. It was also shown that separation of basic compounds is also possible under acidic conditions on the Chiralcel® OD-RH column when a chaotropic salt, such as potassium hexafluorophosphate, KPF_6, is added to the mobile phase [9,37,65,66]. It is supposed that the hexafluorophosphate anion neutralises the positively charged analyte by forming an ion pair. The optimal concentrations of the phosphate buffer, of KPF_6 and of the amount of OM for screening were defined in a previous study [9]. The conditions of the acidic and basic screening mobile phases

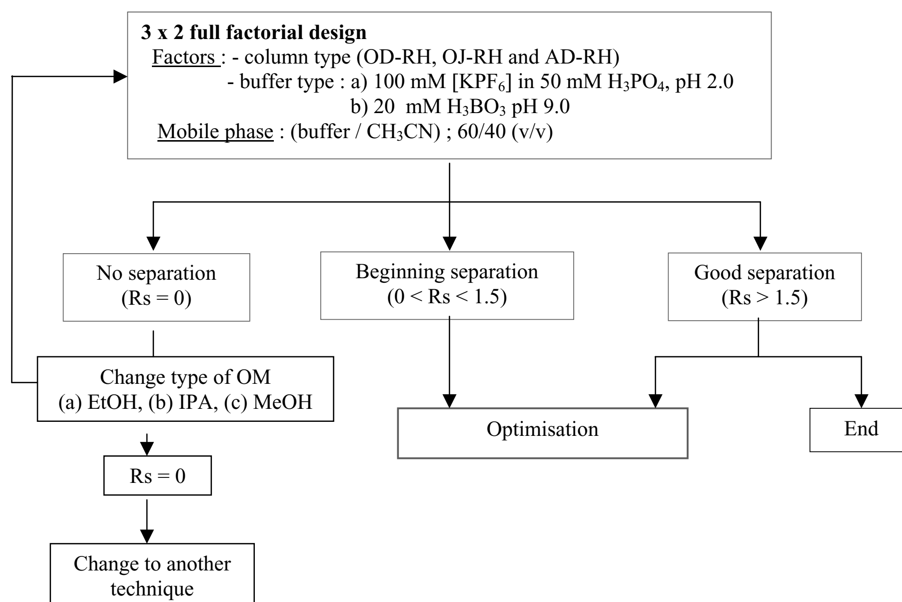


Fig. 5. General RPLC strategy.

were determined as 100 mM KPF_6 in 50 mM phosphate buffer (pH 2.0)/acetonitrile (60/40, v/v) and 20 mM borate buffer (pH 9.0)/acetonitrile (60/40, v/v), respectively. Measurements are performed at room temperature. After executing the design experiments, the experiment with highest resolution is selected for further evaluation. When no enantioseparation is found, it is proposed to change first the type of OM in the mobile phase before switching to another technique. This means that the screening is repeated with another OM, taking into account the solvent strength of the different organic modifiers. It is recommended to test the different OMs in the following sequence: (a) ethanol; (b) isopropanol; and (c) methanol [9]. These OMs might give different enantiorecognition towards a given compound compared to CH_3CN. If still no improvement is observed, it is recommended to change to another technique.

When a beginning of separation is achieved ($0 < Rs < 1.5$) in the best screening experiment, the analyst can enhance the separation in an optimisation stage. When satisfying results are obtained ($Rs > 1.5$), the analyst can end the method development or optimise the retention factor (k') in the optimisation stage.

This automated approach is time consuming and therefore a sequential approach is also proposed. It is similar to the automated strategy, but instead of executing the experimental design, the columns are tested sequentially in the order: (1) Chiralcel® OD-RH; (2) Chiralpak® AD-RH; and (3) Chiralcel® OJ-RH using the acidic mobile phase This means that when an unacceptable separation ($Rs < 1.5$) is obtained with Chiralcel® OD-RH, the second column is tested, occasionally followed by the third. If no separation is seen on any column, the components are tested with the basic mobile phase but only on AD-RH, because the other columns did not show better enantioselectivity at basic conditions [9]. The basic mobile phase is only tested in second order because of an accelerated degradation and ageing of the silica "skeleton" of the stationary phase.

An optimisation is again proposed when a limited separation is achieved from the screening. When a baseline separation is obtained in a reasonable analysis time (e.g. <30 min), method development is terminated. If not, an occasional optimisation of the k' is again possible in the optimisation step.

The automated screening is preferred to the sequential one because the best separation of six different conditions is then selected. With the sequential screening, the analyst stops at the first condition showing a resolution above 1.5, potentially excluding better ones.

3.4.2. Optimisation

An overview is outlined in Fig. 6. In general, when a baseline separation is achieved in the screening, the user will stop the method development. When it is desired to improve analysis time, the KBS proposes a change in the fraction OM in the mobile phase according to the following approach.

In binary reversed-phase systems, retention is described by the relationship [75]:

$$\log(k') = a - m\varphi + d\varphi^2 \quad (2)$$

with φ the organic modifier fraction and k' the retention factor. In practice, Eq. (2) usually can be simplified to Eq. (3). The latter is used in the Chiral KBS to predict the "optimal" fraction OM in the mobile phase when retention is too high after screening:

$$\log(k') = a - m\varphi \quad (3)$$

Fig. 7 shows $\log(k')$ as a function φ for three chiral pharmaceutical compounds to demonstrate that a straight line

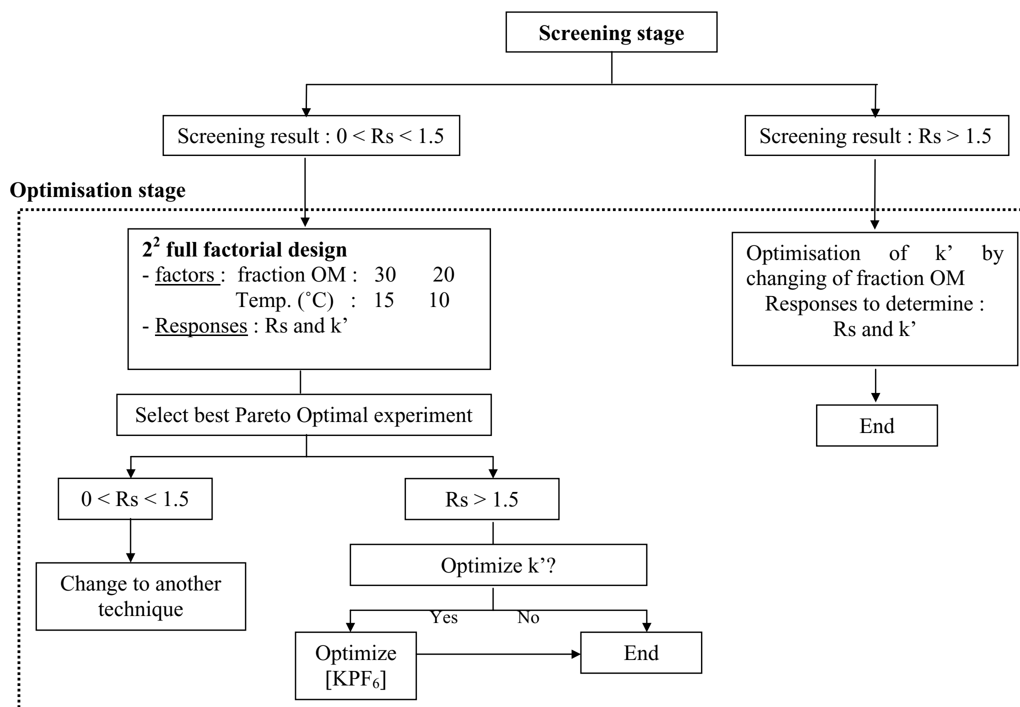


Fig. 6. Optimisation strategy in RPLC.

model is acceptable to predict retention. When a smaller k' value is desired than obtained in the screening, a higher fraction of OM is selected and the retention measured (Fig. 8a). Both results allow estimating the coefficients of Eq. (2). After defining a desired k' value, the required OM fraction is predicted (Fig. 8b). Practical execution at the predicted conditions allows confirming whether the separation is acceptable.

When only a beginning of separation ($0 < \mathrm{Rs} < 1.5$) was achieved in the screening, the enantioselectivity might be improved by optimising temperature and fraction OM. Both factors are examined in a 2^2 full factorial design (Fig. 6). The temperature levels are lowered compared to the screening conditions because it may improve the selectivity (and therefore resolution) due to an increased interaction of the compound with the column [67–69]. The fraction of organic modifier is decreased to 30 and 20% (v/v). Then, the Pareto Optimal experiments for k' and Rs are defined. However, most design experiments will be Pareto Optimal because in many cases both responses react oppositely to the factor changes. In practice, the selected experiment is the one showing an acceptable separation and lowest retention factor. When the separation is satisfying, i.e. $\mathrm{Rs} > 1.5$, in an acceptable analysis time (e.g. <30 min), the analyst will finish. When too high retention times are obtained, the analyst might change the concentration of the chaotropic salt (KPF_6) in the buffer. However, this is only recommended when good separations are achieved because one risks to decrease the selectivity rapidly without much improvement in retention time [9].

When the experimental design does not give satisfying results, no further optimisations are foreseen in our strategy.

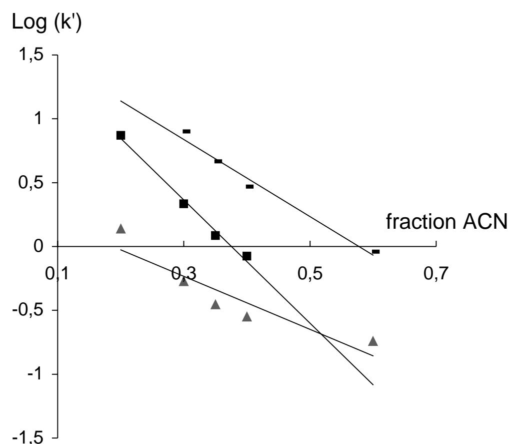
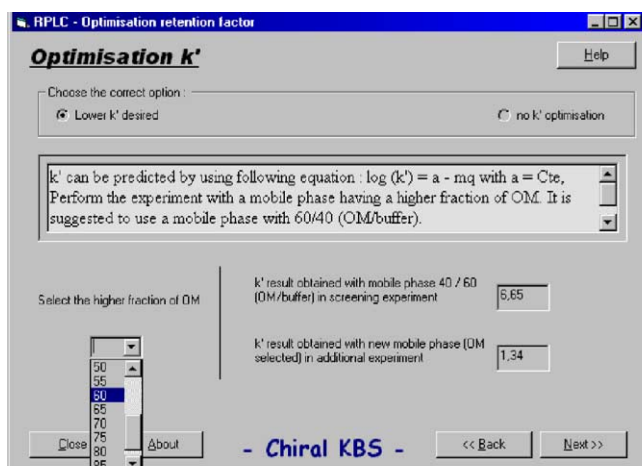


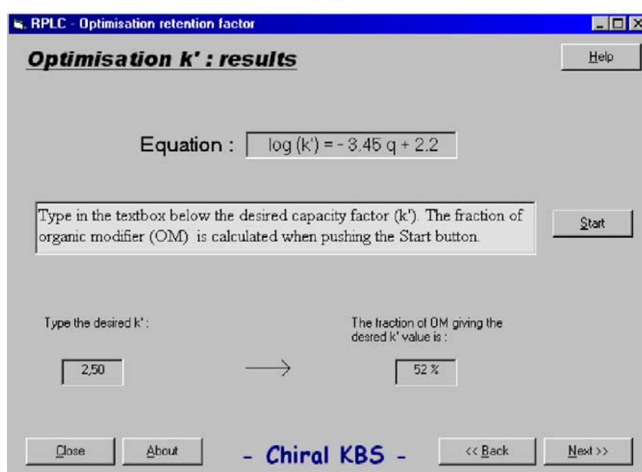
Fig. 7. The log(k') as a function of the fraction organic modifier (ACN) for: (▬) oxprenolol; (■) alprenolol; (▲) metoprolol. Experimental conditions: Chiralcel® OD-RH column; mobile phase, 50 mM $NaClO_4$ in water/CH_3CN; flow rate 0.5 ml/min; room temperature.

3.5. Experimental examples

To demonstrate the performance of the strategies, 15 different pharmaceutical compounds are screened using RPLC (8 compounds) or NPLC (9 compounds). For NPLC, the automatic screening approach is performed, whereas in RPLC the sequential one. Both approaches allowed baseline separation of 87% (13/15) of the investigated compounds.



(a)



(b)

Fig. 8. RPLC optimisation module of the Chiral KBS; optimisation of k'. (a) Selection of a mobile phase with higher OM fraction and entering its measured k'. (b) Determining the coefficient of Eq. (2) and prediction of OM fraction for a desired k'.

An important issue from a practical point of view is the time needed for method development of the compounds. One should take into account that the sequential approach usually will lead to a shorter development time than performing the complete experimental design. Of course, at the other hand, the sequential approach can neglect good separations. For both techniques, equilibration time is defined as 30 min (until stable baseline is obtained) and rinsing times with hexane in NPLC and H_2O/CH_3CN (60/40) in RPLC as 45 min. It is well known that the polysaccharide columns show memory effects on acidic and basic additives [38–40,57–59]. A stringent wash procedure is needed to eliminate those compounds when testing both types on the same column. Because fast method development is our main scope we advise to use different columns when using different additives. Some memory effect of the organic modifier, isopropanol might be seen but its effect was eliminated by washing during 45 min with pure hexane between application of different mobile phases. In RPLC, it is strongly recommended to wash the system properly after finishing experiments (1 h with H_2O/CH_3CN) to eliminate the corrosive KPF_6. Examples of the effective method development times, including rinsing and conditioning times, are established for each compound and reported further.

3.5.1. NPLC

Nine chiral pharmaceutical compounds followed the NPLC screening and optimisation strategy. They include six basic compounds (alprenolol, ephedrine, acebutolol, fluoxetine, sulpiride and methadone and three acidic or bifunctional compounds (fenoprofen, hexobarbital and oxazepam). Following the prescribed screening design (Fig. 2) allowed baseline separation of 67% (6/9) of the compounds. Results are shown in Table 1.

Although method development might already stop here for the baseline separated compounds, a retention factor (acebutolol and oxazepam) or peak shape (alprenolol and ephedrine) optimisation was applied. Two compounds (fluoxetine and sulpiride) were not baseline separated and undergo the optimisations of Fig. 4. One basic compound, methadone, did not show enantioselectivity to the screening columns OD and AD. The user then is proposed to test the OJ column (Fig. 2), which resulted in a successful separation (see Table 1).

Method development was stopped after the screening level for compounds having a baseline separation and a reasonable analysis time, such as fenoprofen and hexobarbital. Final method development time for those compounds is 640 min. Run time is, in generally, set on 30 min during the screening phase and a rinsing time of 45 min is applied when changing from IPA to EtOH. Only in specific cases, when no peak was observed, run time was prolonged until peaks were detected (e.g. 35 min for acebutolol on AD column).

Acebutolol and oxazepam were submitted to optimisation 1 to decrease the retention factor. The percentage OM was increased until reasonable retention was achieved, finally resulting in 50% IPA for oxazepam and 20% EtOH for acebutolol, which allowed shorter retention with still baseline separation (Table 2). For both compounds the next step was also applied, i.e. performing the 2^2 full factorial design. It resulted in shorter runs with even better resolution for acebutolol than from changing the percentage OM. Detailed results are given in Table 2a and b. Optimisation time for oxazepam and acebutolol was 165 and 105 min, respectively, which results in a total method development time of 805 and 585 min, respectively (screening of acebutolol was only 480 min because only two columns were screened).

Ephedrine and alprenolol were submitted to the peak shape optimisation of optimisation 1 (Fig. 3), which allowed improvement of the number of plates and/or peak symmetry with consequently an increased resolution. For ephedrine the optimal result (Rs = 2.49) was achieved at 25 °C and 0.075% DEA (Chiralpak® AD, hexane/EtOH (90/10), flow

Table 1
Results [Rs/$k'(t_r)$] of the screening of chiral drugs by NPLC according to the proposed strategy (analysis time in min)

	Rs/k' (t_r)					
	Chiralcel® OD-H		Chiralpak® AD		Chiralcel® OJ	
	Hex/EtOH (90/10)	Hex/IPA (90/10)	Hex/EtOH (90/10)	Hex/IPA (90/10)	Hex/EtOH (90/10)	Hex/IPA (90/10)
Basic compounds						
Alprenolol	3.89/0.92 (5.63)	**10.76/2.74 (11.30)**	2.33/0.75 (5.41)	2.68/0.91 (5.80)		
Ephedrine	1.12/0.79 (5.27)	1.71/1.12 (6.40)	**1.98/1.54 (7.86)**	0.00/1.34 (7.09)		
Acebutolol	1.08/2.55 (10.31)	1.58/6.54 (22.16)	**3.14/9.65 (33.65)**	0.00/4.73 (17.73)		
Fluoxetine	0.00/0.68 (4.86)	0.00/1.10 (6.19)	**0.616/0.69 (5.31)**	0.00/0.65 (5.11)		
Sulpiride	**0.613/10.17 (32.39)**	n.p.	n.p.	n.p.		
Methadone	0.00/0.61(4.08)	0.00/0.58 (4.12)	0.00/0.49 (3.83)	0.00/0.51 (3.99)	**2.30/1.41 (7.24)**	0.00/0.50 (3.97)
Acidic, bifunctional compounds						
Fenoprofen	0/0.70 (5.05)	0.69/0.81 (5.44)	1.28/1.20 (6.75)	**2.11/1.51 (7.60)**	0.49/3.04 (11.08)	1.26/3.12 (11.36)
Hexobarbital	0.79/3.69 (13.94)	1.47/4.58 (16.57)	0/2.43 (10.55)	**4.93/3.08 (12.35)**	1.95/10.62 (31.85)	0.96/13.24 (39.31)
Oxazepam	5.86/9.20 (30.29)	4.17/15.01 (47.55)	0.60/20.63 (65.77)	**10.41/22.78 (71.81)**	0.61/10.92 (32.67)	1.67/16.82 (49.17)

n.p.: no peak observed within 120 min; t_r: migration time last eluting compound (min). The best result obtained for each compound during NPLC screening is given in bold.

Table 2
Retention factor optimisation of oxazepam (a) and acebutolol (b)

Factors		Responses		
T (°C)	Flow rate (ml/min)	t_r (min)	k'	Rs
(a) 2^2 Full factorial design: MF (50/50) Hex/IPA + TFA 0.1%				
Increasing percentage OM	20% IPA	11.97/28.17	8.58	9.66
	50% IPA	5.53/10.34	2.53	6.93
25	1.25	4.42/8.03	2.46	5.50
25	1.50	3.58/6.53	2.17	5.78
40	1.25	3.93/6.34	1.71	5.92
40	1.50	3.13/5.15	1.67	5.58
(b) 2^2 Full factorial design: MF (80/20) Hex/EtOH + DEA 0.1%				
Increasing percentage OM	20% EtOH	7.41/8.97	1.96	1.83
25	1.25	5.85/7.06	1.91	1.75
25	1.50	4.96/5.97	1.93	1.67
40	1.25	5.37/6.48	1.72	2.64
40	1.50	4.46/5.38	1.69	2.47

Experimental conditions: stationary phase, Chiralpak® AD-H; mobile phase composition, temperature and flow rate are varied during the experiments.

Table 3
Peak shape optimisation of alprenolol using the 3 × 2 experimental design conditions

Factors		Responses				
T (°C)	DEA (%)	t_r (min)	k'	Rs	N	As
15	0.025	5.06/14.10[a]	3.61	12.39	2220/3014[a]	1.58/1.37[a]
15	0.050	4.85/13.06	3.31	12.38	2388/3177	1.40/1.29
15	0.075	4.80/12.57	3.16	12.13	2270/3293	1.56/1.59
25[b]	0.025[b]	4.76/10.89	2.56	12.46	2384/5404	0.99/1.23
25	0.050	4.59/10.31	2.36	11.67	2495/4540	1.42/1.25
25	0.075	4.54/9.97	2.26	11.25	2474/4427	1.36/1.24

Experimental conditions: stationary phase, Chiralpak® OD-H; mobile phase, hexane/IPA/DEA (90/10/varying); flow rate 1.0 ml/min; varying temperature.
[a] Results obtained for first and last eluting enantiomer.
[b] Pareto Optimal experiment.

Table 4
Baseline optimisation (optimisation 2) of fluoxetine

Factors		Responses			
T (°C)	DEA (%)	t_r	k'	Rs	N
2^2 Full factorial design: MP (97.5/2.5) hexane/EtOH + DEA 0.1%					
Decreasing percentage OM	5% EtOH	5.84/6.21	0.96	0.97	2488/2602
	2.5% EtOH	7.80/8.34	1.54	1.01	2963/2826
5[a]	0.025[a]	4.65/8.46	1.58	1.49	2964/3112
5	0.075	13.09/13.92	3.05	1.23	2702/2862
15	0.025	7.53/8.04	1.48	1.02	2940/2960
15	0.075	11.03/11.82	2.47	1.03	2916/2982

Experimental conditions: stationary phase, Chiralpak® AD-H; mobile phase composition and temperature are varied during the experiments; flow rate 1.0 ml/min.

[a] Pareto Optimal experiment (responses = Rs and N).

rate 1.0 ml/min). For alprenolol the design results are given in Table 3. As final method conditions, the Pareto Optimal experiments are selected. These conditions (25 °C and 0.025% DEA, Chiralpak® AD, hexane/EtOH (90/10), flow rate 1.0 ml/min) show the highest Rs and number of plates. Compared to the screening results of Table 1, a better separation with an even shorter retention time is observed. Method development was finished after 775 min (screening 475 min, optimisation 240 min and system rinsing system 60 min).

The compounds, fluoxetine and sulpiride, which were not baseline resolved during screening, were optimised using optimisation 2. First, the retention factors are adjusted to a value between 1 and 5 by changing, i.e. decreasing and increasing, respectively, the percentage OM. This resulted in a k' of 1.54 and 4.51 for fluoxetine and sulpiride at 2.5 and 20% EtOH, respectively. They show an acceptable retention, but the resolution (1.01 and 0.56, respectively) still had to be improved. Therefore, the 2^2 full factorial design, changing additive concentration and temperature, was applied on both compounds. It allowed baseline separation of fluoxetine (Rs = 1.49) after a method development time of 765 min. The design and Pareto Optimal results of fluoxetine are shown in Table 4. Sulpiride was the only compound which could not be completely separated using the strategy (Rs = 0.64, experimental conditions: Chiralcel® OD, heptane/EtOH (80/20) with 0.075% DEA, 5 °C, 1.0 ml/min). The method development ends here and another technique, for instance SFC or RPLC, might be applied.

The strategies and examples given in this paper were produced using *n*-hexane in the mobile phase. As this solvent is not acceptable for use in much industrial domains, certainly when preparative separations are considered, the alternative solvents, isohexane and *n*-heptane, were regarded to replace *n*-hexane.

Table 5 shows the results obtained for the compounds using isohexane or *n*-heptane during screening. Both solvents give similar results to applying *n*-hexane (Table 1). It could be concluded that *n*-heptane is the best alternative for *n*-hexane because of its lower toxicity. Furthermore, *n*-heptane gives equally good or better results than isohexane. Consequently, optimisation is performed using *n*-heptane in the mobile phase. Results and conditions are given in Table 6.

In some specific cases, e.g. fenoprofen on Chiralcel® OD, a loss in selectivity is seen when replacing *n*-hexane but nevertheless the final results after screening and optimisation are not strongly influenced. For all compounds, except fluoxetine, still acceptable Rs are obtained, which confirms the viable use of the replacing solvents, *n*-heptane (first priority) or isohexane. Only in those rare cases where *n*-heptane and isohexane do not give a sufficient separation because of loss in selectivity (e.g. fluoxetine), the use of *n*-hexane might be justified.

3.5.2. RPLC

The sequential screening strategy is applied on eight pharmaceutical compounds (basic compounds: promethazine, atropine, tetramisol and acebutolol; acidic compounds: fenoprofen and mandelic acid; bifunctional/neutral compounds are thiopental and *trans*-stilbene oxide). The results of the first screening step are shown in Table 7. In this step, all compounds are screened using the acidic mobile. Considering the described times needed for equilibration and rinsing, screening took 315 min (runs of 30 min), except for *trans*-stilbene oxide (runs of 60 min). Four compounds (promethazine, thiopental, fenoprofen and *trans*-stilbene oxide) are baseline resolved. Atropine and tetramisol show a beginning of separation, while mandelic acid and acebutolol do not have any enantioselectivity towards any of the three columns. According to the screening strategy (Fig. 5), mandelic acid and acebutolol are first submitted to the basic borate buffer on the Chiralpak® AD-RH column, secondly to different OMs when needed. The first change resulted in a beginning of separation of the acebutolol enantiomers on Chiralpak® AD-RH (Rs = 1.28). Mandelic acid could only be separated using another OM. The best separation was achieved on Chiralpak® AD-RH with MeOH as OM (Rs = 1.69).

Table 5
Results [Rs/k' (t_r)]of the screening of chiral drugs by NPLC, according to the proposed strategy but using isohexane and heptane in the mobile phase (analysis time in min)

	Rs/k' (t_r)											
	Chiralcel® OD-H				Chiralpak® AD				Chiralcel® OJ			
	EtOH		IPA		EtOH		IPA		EtOH		IPA	
	Isohexane	*n*-Heptane	Isohexane	*n*-Heptane	Isohexane	*n*-Heptane	Isohexane	*n*-Heptane	Isohexane	*n*-Heptane	Isohexane	*n*-Heptane
Basic compounds												
Alprenolol	3.22/0.90 (5.69)	1.86/0.68 (4.95)	**6.20/1.32 (7.02)**	**8.99/1.75 (8.35)**	1.72/0.77 (5.23)	1.86/0.62 (5.34)	1.91/0.89 (5.60)	2.78/1.00 (6.67)				
Ephedrine	1.05/0.80 (5.43)	0.83/0.84 (5.44)	1.44/1.18 (6.62)	1.34/1.10 (6.20)	**1.62/1.62 (7.90)**	**1.70/1.40 (8.00)**	0.00/1.31 (6.78)	0.00/1.27 (5.48)				
Acebutolol	0.50/2.86 (11.63)	0.77/3.79 (12.43)	1.22/7.36 (25.25)	1.25/7.17 (25.25)	**3.25/8.23 (28.61)**	**2.79/7.28 (27.67)**	0.00/5.01 (17.89)	0.00/6.98 (27.24)				
Fluoxetine	0.00/0.66 (4.99)	0.00/0.70 (5.02)	0.15/1.12 (6.39)	0.00/0.95 (6.03)	**0.22/0.61 (5.01)**	**0.25/0.50 (4.94)**	0.00/0.73 (5.11)	0.00/0.63 (5.48)				
Sulpiride	**0.55/11.73 (38.21)**	0.60/12.10 (39.03)	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.				
Methadone	0.00/0.44 (3.45)	0.00/0.46 (3.50)	0.00/0.52 (3.62)	0.00/0.52 (3.64)	0.00/0.48 (3.54)	0.00/0.46 (3.50)	0.00/0.54 (3.69)	0.00/0.56 (3.75)	2.09/1.43 (6.75)	2.18/1.79 (6.70)	0.00/0.78 (4.44)	0.00/0.82 (5.39)
Acidic, bifunctional compounds												
Fenoprofen	0.00/0.74 (5.32)	0.00/0.91 (5.33)	0.00/0.82 (5.56)	0.00/0.74 (5.51)	1.05/1.33 (6.76)	1.10/1.08 (6.85)	**1.54/1.68 (7.87)**	**1.73/1.64 (8.23)**	0.00/2.75 (11.24)	0.00/2.80 (11.25)	1.00/3.74 (12.10)	0.77/2.93 (11.79)
Hexobarbital	0.82/4.18 (15.94)	1.05/4.16 (16.16)	2.00/5.52 (19.49)	1.28/4.39 (17.09)	0.00/2.41 (10.59)	0.00/2.58 (11.83)	**4.63/3.17 (12.25)**	**4.85/3.25 (13.27)**	0.33/10.23 (33.59)	0.30/9.91 (31.65)	0.96/12.46 (40.52)	0.94/12.49 (40.33)
Oxazepam	4.96/9.16 (31.50)	5.46/9.08 (31.56)	3.06/11.91 (39.12)	3.88/14.61 (49.49)	0.35/14.38 (47.52)	0.65/17.43 (62.56)	**11.79/21.57 (66.37)**	**10.07/23.62 (78.80)**	1.74/8.32 (27.87)	1.64/9.71 (30.85)	1.45/24.36 (51.23)	1.33/14.75 (46.61)

n.p.: no peak observed within 120 min; t_r: migration time last eluting compound (min). The best result obtained for each compound during NPLC screening is given in bold.

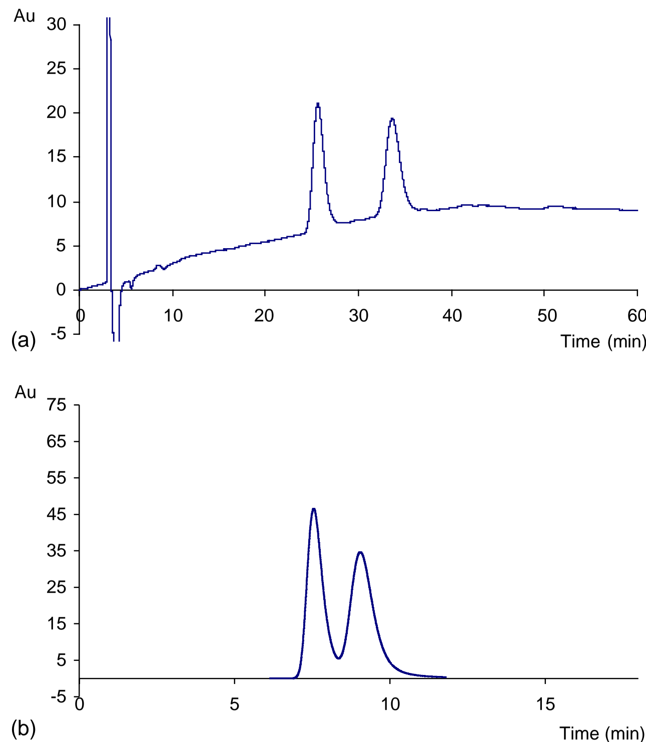


Fig. 9. Screening result of acebutolol using: (a) NPLC; (b) RPLC. Experimental conditions for NPLC: stationary phase, Chiralpak® AD-H; mobile phase composition, hexane/EtOH/DEA (90/10/0.1); temperature 20 °C; flow rate 1.0 ml/min). Experimental conditions for RPLC: Chiralcel® AD-RH; 20 mM borate buffer (pH 9.0)/CH_3CN (60/40, v/v); room temperature; flow rate 0.5 ml/ min.

The example of acebutolol demonstrates the complementary property of the strategies developed in the RPLC and NPLC techniques. Fig. 9 clearly illustrates that no baseline separation could be achieved, following the proposed RPLC strategy while a good separation was obtained in the NPLC screening stage.

The two partially separated compounds (atropine and tetramisol) were optimised according to the optimisation strategy of Fig. 6. Results of the 2^2 full factorial design on atropine are given in Table 8. It shows that both factors, decreasing the percentage of OM and the temperature, have a positive influence on the separation. Because Rs and k', decrease simultaneously, all design experiments are Pareto Optimal. The fastest separation (30% OM, 15 °C) still allows a good baseline separation (Rs = 3.61). Changing the [KPF_6] concentration to 50 mM allowed a further reduction in the retention factor (Table 8). The development time for this compound was 315 min for screening and 300 min for optimisation. It can even be reduced by rinsing the columns on a separate chromatographic pump.

For tetramisol, the design also allowed to increase the resolution but no baseline separation was achieved (Rs = 0.90). Method development is stopped here and occasionally can be restarted using another technique.

Table 6
Results obtained in NPLC at optimised conditions (column, mobile phase, temperature and flow rate) for the chiral drugs using heptane as apolar solvent in the mobile phase

Compound	Optimised conditions	Rs/k' (t_r)
Basic compounds		
Alprenolol	Chiralcel® OD, heptane/IPA (90/10) + 0.025% DEA, 25 °C, 1.0 ml/min	8.25/2.16 (9.17)
Ephedrine	Chiralcel® AD, heptane/EtOH (90/10) + 0.075% DEA, 25 °C, 1.0 ml/min	1.54/1.53 (8.05)
Acebutolol	Chiralcel® AD, heptane/EtOH (80/20) + 0.1% DEA, 40 °C, 1.5 ml/min	1.76/1.67 (6.68)
Fluoxetine	Chiralcel® AD, heptane/EtOH (97.5/2.5) + 0.025% DEA, 5 °C, 1.0 ml/min	1.22/2.37 (11.31)
Sulpiride	Chiralcel® OD, heptane/EtOH (80/20) + 0.075% DEA, 5 °C, 1.0 ml/min	0.24/3.81 (15.45)
Methadone	Idem best screening result	2.18/1.79 (6.70)
Acidic, bifunctional compounds		
Fenoprofen	Idem best screening result	1.73/1.64 (8.23)
Hexobarbital	Idem best screening result	4.85/3.25 (13.27)
Oxazepam	Chiralcel® AD, heptane/IPA (50/50) + 0.1% TFA, 40 °C, 1.5 ml/min	5.46/1.44 (5.53)

Table 7
Best screening results (Rs) obtained after performing the sequential screening strategy in RPLC

		Rs	k' (t_{rmin})	Column
1	Promethazine	1.62	2.50 (13.42)	OJ-R
2	Thiopental	1.61	3.42 (16.15)	OJ-R
3	Fenoprofen	2.45	6.65 (29.83)	OJ-R
4	*trans*-Stilbene oxide	5.06	26.64 (100.35)	OJ-R
5	Atropine	0.67	0.28 (4.92)	OD-RH
6	Tetramisol	0.27	1.90 (11.01)	OD-RH
7	Mandelic acid	0	0.15 (4.39)[a]	**OD-RH**/AD-RH/OJ-R
8	Acebutolol	0	0.25 (4.73)[a]	OD-RH/**AD-RH**/OJ-R

The column on which the result was obtained and highest retention factor of the last eluting enantiomer are also given.
[a] Highest retention factor and time, obtained on the bold column.

Compounds that are baseline resolved but have high k' values (fenoprofen and *trans*-stilbene oxide) are optimised by changing the OM fraction. For fenoprofen, 60% OM gives a k' of 1.34 (t_r = 8.04 min) but resolution was decreased to 1.16. The percentage OM for a desired k' of 2.5 was predicted as 52%. Experimentally, baseline separation (Rs = 1.62) and k' = 1.98 (t_r = 11.66 min) were obtained for fenoprofen. For trans-stilbene oxide, 60% CH_3CN decreased k', while Rs was still acceptable (Rs = 3.69, k' = 3.56 (t_r = 18.05 min). Method development times for the retention factor optimisations are 90 and 50 min for fenoprofen and *trans*-stilbene oxide, respectively. Method development in RPLC was finished at this point. Finally, 87.5% (7/8) of the compounds screened and optimised were baseline resolved using the RPLC strategy.

Table 8
Results for atropine (a) applying the 2^2 full factorial design of Fig. 6

(a) Experimental conditions		Responses	
Percentage OM	Temperature (°C)	Rs	k' ($t_{r,min}$)
20	10	5.90	12.92 (53.16)
20	15	4.96	9.70 (40.87)
30	10	4.29	4.93 (20.59)
30	15	3.61	2.14 (11.61)
(b) Optimisation of [KPF_6] at 15 °C, 30% OM		Rs	k' ($t_{r,min}$)
5 mM		0	1.44 (9.34)
50 mM		2.35	1.52 (9.29)
200 mM		2.34	1.73 (10.66)
300 mM		2.96	2.29 (12.91)
400 mM		2.72	1.99 (11.65)

Chromatographic conditions: Chiralcel® OD-RH; 50 mM phosphate buffer with variable percentage CH_3CN; 100 mM KPF_6; variable temperature; flow rate 0.5 ml/min by changing the [KPF_6] from 5 to 400 mM (b), using a mobile phase with 30% OM cooled to 15 °C.

4. Conclusions

Strategies to develop a separation method for chiral pharmaceutical compounds in normal- and reversed-phase liquid chromatography were defined. They are based on the use of three polysaccharide-based stationary phases. Both strategies are applicable to acid, basic, neutral and bifunctional compounds. They are implemented in a larger knowledge-based system, which includes also strategies in other separation techniques.

The strategies, consisting of screening and optimisation steps and thus also the KBS are structured in such a way that the user is only guided through the information needed, which allows a fast method development and an easy use of the strategy/program.

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