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Screening approach for chiral separation of pharmaceuticals Part I. Normal-phase liquid chromatography

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Abstract

A strategy for rapid screening for the separation of chiral molecules of pharmaceutical interest by normal-phase liquid chromatography using three cellulose/amylose stationary phases is proposed. In a first step, the most important parameters for the separations were determined and studied for their effects by means of experimental designs. Results showed that the cellulose tris-(3,5-dimethylphenylcarbamate), the amylose tris-(3,5-dimethylphenylcarbamate) and the cellulose tris-(4-methylbenzoate) stationary phases have very broad and complementary enantiorecognition properties. The type of organic modifier used in the mobile phase appeared to have a dramatic influence on the quality of the separation. Based on the results of the preliminary study, a screening strategy was developed and successfully applied to a set of 36 diverse drugs. Enantiomeric separation was observed in 89% of cases and the analysis times were usually shorter than 20 min.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Pharmaceuticals

1. Introduction

The development of chiral drugs requires the screening of both enantiomers as well as the racemic mixture from the early stages of drug development [1–3]. When stereoselective synthesis is used, the starting materials also need to be screened to ensure their enantiomeric purity. Due to the increasing number of new compounds produced daily, there is a requirement for rapid method development based on very simple protocols.

Thus, screening strategies for the separation of enantiomers have been developed in our laboratories using different separation techniques. The following four techniques, commonly used in the pharmaceutical industry, were considered: normal-phase liquid chromatography (NPLC), reversed-phase liquid chromatography (RPLC), capillary electrophoresis (CE) and supercritical fluid chromatography (SFC).

The aim of these screening strategies is to be able to analyse quickly a series of molecules with diverse structures and chemical properties using a minimal set of experimental conditions. The aim is not to achieve optimal separations, since this is only re-

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quired in the later stages of method development, but to determine rapidly whether a technique can achieve an acceptable separation that will constitute a good starting point for further optimisation. The key point for developing rapid screening schemes is to include a reduced set of chiral selectors with a broad application range. Indeed, more than 100 different chiral selectors have been developed for chromatographic and electrophoretic techniques, the enantioseparation performances of which are quite different. Chiral discrimination is a very complex phenomenon and sometimes depends on little known properties, which makes the prediction of a suitable selector very difficult. Thus, a small set of chiral selectors with broad enantiorecognition properties was selected for each separation technique based on the literature and experience from our laboratories.

A screening approach for chiral separation by CE was proposed in a previous publication [4]. This article is the first of a series dealing with the development of screening strategies for chiral separation using chromatographic techniques. In this article, a screening strategy for NPLC is presented.

Different approaches, direct and indirect, are possible to achieve chiral separations in LC [3,5]. The use of chiral stationary phases (CSPs) was preferred for the development of a screening strategy because of their ease of use and the efficiency of some bonded chiral selectors. Polysaccharide-based stationary phases (cellulose and amylose derivatives), developed by Okamoto's group [6], appear to be the most useful in the organic, bioorganic and pharmaceutical fields [1,7–10]. Several derivatives are available, but three of them, namely cellulose tris-(3,5-dimethylphenylcarbamate), amylose tris-(3,5-dimethylphenylcarbamate) and cellulose tris-(4-methylbenzoate) (Fig. 1), have very complementary properties and numerous publications have demonstrated that they are able to achieve a chiral resolution of more than 80% for drugs currently available on the market [9–17]. These CSPs are known under their commercial names, Chiralcel OD, Chiralpak AD and Chiralcel OJ, respectively. Experience acquired in our laboratories confirmed the choice of these CSPs for the development of a rapid screening strategy in NPLC.

To develop the screening strategy, the most important parameters for the separations were first identified and carefully studied for their effects by means of experimental designs. Experimental designs are very efficient tools for the development of analytical methods, since they allow the simultaneous study of different analytical parameters with a reduced number of experiments. Several studies have demonstrated their usefulness for the development or the optimisation of enantiomeric separations [18–

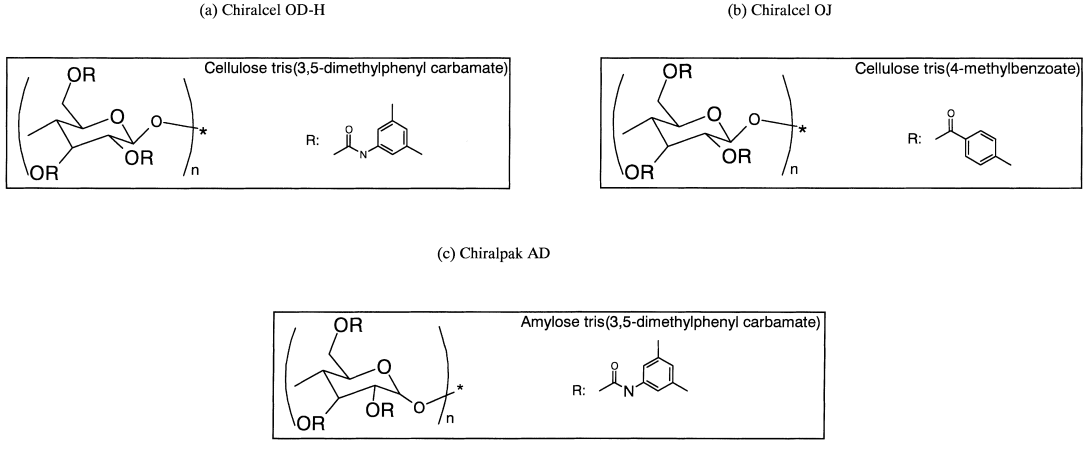

Fig. 1. Structure of the three CSPs selected for the screening strategy.

25]. From the results of this preliminary study, the screening strategy was set up and applied to a wide range of chiral pharmaceutical compounds.

2. Experimental

2.1. Chromatography

In the preliminary study, the chromatographic system comprised an Alltech on-line degassing system (Deerfield, IL, USA), a Shimadzu LC-10AD pump (Kyoto, Japan), a Rheodyne 7725i injector (Cotati, CA, USA) with a 20 μl sample loop and a Shimadzu SPD-M10A diode-array detector.

For the final screening of pharmaceuticals, a chromatographic HP 1050 system (Agilent Technologies, Palo Alto, CA, USA) equipped with an automatic injector and a UV detector was used.

A Chiralcel OD-H column (25 cm×4.6 mm I.D.) packed with cellulose tris(3,5-dimethylphenyl carbamate) coated on 5 μm silica-gel substrate particles (Daicel, Tokyo, Japan), a Chiralpak AD column (25 cm×4.6 mm I.D.) packed with amylose tris(3,5-dimethylphenyl carbamate) and a Chiralcel OJ column (25 cm×4.6 mm I.D.) packed with cellulose tris(4-methylbenzoate) coated on 10 μm silica-gel substrate particles (Daicel) were used.

Mobile-phase compositions and other chromatographic conditions are given in the Results and discussion section.

2.2. Chemicals

n-Hexane, HPLC grade, was purchased from BDH (Poole, UK). Ethanol absolute extra pure and 2-propanol HPLC grade were purchased from Merck (Darmstadt, Germany). Diethylamine (DEA) was obtained from UCB (Brussel, Belgium). Trifluoroacetic acid (TFA) was purchased from Sigma (Steinheim, Germany).

Alprenolol, flurbiprofen, ibuprofen, ketoprofen, metoprolol, praziquantel, sulpiride, suprofen and warfarin were purchased from Sigma and verapamil hydrochloride from Fluka (Buchs, Switzerland). Pindolol and acenocoumarol were obtained from Novartis (Basel, Switzerland), acebutolol from Rhone-Poulenc (Vitry Sur Seine, France), propranolol hydrochloride from Certa (Braine-l'Alleud, Belgium), atenolol, bisoprolol and oxprenolol from Ciba-Geigy (Barcelona, Spain), leucovorin from Cyanamid (Benelux), nadolol from Bristo-Myers-Squibb (Barcelona, Spain), fluoxetine hydrochloride from Lilly (Mont Saint Guibert, Belgium), and dilthiazem, hexobarbital, lorazepam, lormetazepam, mianserin, naproxen, oxazepam, propiomazine, temazepam, sotalol and phenobarbital were gifts from diverse sources.

Samples were dissolved at an approximate concentration of 0.1 mg/ml either in ethanol or 2-propanol depending on the mobile phase used.

2.3. Data processing

Analytical data were acquired and processed with a Shimadzu workstation Class-M10A 1.6D or with a HP Chemstation for LC (Agilent Technologies).

Resolutions (R_S) were calculated according to the United States Pharmacopeia (USP) [26]:

$$R_S = \frac{2(t_R(b) - t_R(a))}{W_B(b) + W_B(a)} \text{ (Tangent method)} \quad (1)$$

where $t_R(b)$ and $t_R(a)$ are the retention times of the last- and first-eluting peaks (in min), respectively, and $W_B(b)$ and $W_B(a)$ are the base widths of peaks *b* and *a* (in min), respectively.

Experimental designs were obtained from the program Trial Run 1.0 (SPSS, IL, USA).

2.4. Calculations

Two different fractional factorial designs were applied: (i) a seven-factor, eight-experiment Plackett–Burman design and (ii) a 3×2^2 design (12 experiments).

The effect of each factor was calculated as follows:

$$E_{x[+,-]} = \frac{\sum Y(+)}{n} - \frac{\sum Y(-)}{n} \quad (2)$$

where $\Sigma Y(-)$ and $\Sigma Y(+)$ represent the sum of the responses when the factor is at a low or high level, respectively, and n is the number of experiments in

the design when the factor is at a low or high level, respectively.

3. Results and discussion

Cellulose and amylose derivative CSPs are mostly used, in the normal-phase mode, with *n*-hexane-based mobile phases containing some alcohol as modifier. Chromatographic performance, retention and selectivity are reported to be affected by the composition of the mobile phase and the structure of the alcohol present in the mobile phase [5,27]. 2-Propanol and ethanol (EtOH) are the most commonly used modifiers and, according to the literature and our own experience, should allow the separation of most drug enantiomers on the Chiralcel OD-H, Chiralcel OJ and Chiralpak AD columns. Basic and acidic mobile-phase additives are often required to improve separations and peak shapes [28]. Diethylamine (DEA) is often added to the mobile phase when the analytes contain an amino basic function, to reduce peak tailing by masking the residual silanol groups of the CSP [29]. Trifluoroacetic acid (TFA) is usually added to the mobile phase to attenuate the binding of acidic analytes, which are often excessively retained under normal-phase conditions with polysaccharide CSPs [30]. A significant enhancement in selectivity and resolution on the addition of these additives has been reported in the literature [29–34].

Inversion of the elution order of the enantiomers may occur from one column to another or when changing the type of organic modifier used [5,42]. However, although it is of great interest in the later stages of method development (i.e. for the detection of impurities), the elution order was not considered in this study since, at the screening stage, only the feasibility of the separation is considered.

The selection of the minimal number of experiments to define a screening strategy was our main interest. In the first instance, we determined the most important parameters for the separation. Then, the values of those parameters that would lead to the separation of enantiomers within an acceptable analysis time for a wide range of compounds were determined. Three different groups of compounds (basic, bifunctional and acidic) were selected as test compounds. Experimental designs were used to map the experimental domain in an efficient way.

3.1. Analysis of basic compounds

A first study of the influence of different factors on the separation of enantiomers was performed using a set of 10 β-blockers. Four factors, namely the type of organic modifier, its concentration, the concentration of DEA and the flow-rate, were studied for each of the three columns. Temperature can also have an important effect on the separation. Indeed, it has been shown that sub-ambient temperature often leads to an improvement in selectivity and resolution [3,5]. However, not many laboratories are equipped with a chromatographic system allowing sub-ambient analysis. As a consequence, this factor was not considered in our screening approach.

A seven-factor, eight-experiment Plackett–Burman design was used to perform the experiments in an efficient way (Table 1). Plackett–Burman designs are used to screen a large number of factors with a small number of experiments [35]. They are described for a number of experiments *N*, where *N* is a multiple of four [36]. Up to $N-1$ factors can be examined with an *N*-experiment Plackett–Burman design. In Plackett–Burman designs, the main effect of each factor is estimated independently, but two-factor and higher-order interactions are confounded with the main effects [36]. Therefore, when using Plackett–Burman designs to estimate the main effect of factors, it is assumed that interaction effects are negligible. Plackett–Burman designs have already been used successfully for the identification of important factors in analytical methods [23,37–39]. Since four factors were examined in this study, the smallest Plackett–Burman design that could be used contains eight experiments. As fewer than the maximum possible number of factors (i.e. seven) are studied, three columns are left in the design. These columns represent imaginary factors, called dummy factors. The effect of these factors has no physical meaning and is considered to be due to experimental error. Therefore, the dummy factors can be used for the statistical interpretation of the effect of the real factors (i.e. to determine whether the effect of a factor is significant or not) [35]. However, in this study, only the relative importance of the factors was

Table 1
The seven-factor, eight-experiment Plackett–Burman design

Exp. No.	Factor						
	A Type of organic modifier	*B* % of organic modifier (v/v)	*C* % of DEA (v/v)	*D* Flow-rate (ml/min)	*E* Dummy 1	*F* Dummy 2	*G* Dummy 3
1	+1	+1	+1	−1	+1	−1	−1
2	+1	−1	+1	+1	−1	−1	+1
3	+1	+1	−1	−1	−1	+1	+1
4	−1	−1	+1	−1	+1	+1	+1
5	+1	−1	−1	+1	+1	+1	−1
6	−1	+1	−1	+1	+1	−1	+1
7	−1	+1	+1	+1	−1	+1	−1
8	−1	−1	−1	−1	−1	−1	−1

considered and thus such calculations were not performed. The factors and their levels, as investigated in the experimental design, are shown in Table 2. The levels of the factors were chosen based on results published in the literature. The design was applied for each column.

Resolution values obtained in the design are given in Table 3. Baseline separation was observed for all β-blockers except sotalol on the Chiralcel OD-H column. For nadolol, only the first pair of enantiomers could be resolved. For six compounds, the best resolution was obtained with 2-propanol at a concentration of 5% and a flow-rate of 0.5 ml/min (experiments 4 and 8 of the design). In the case of atenolol, nadolol and pindolol, the retention times were too long to detect peaks under these conditions. The best separation of these enantiomers was obtained with 20% 2-propanol and a flow-rate of 0.8 ml/min (experiment 6 of the design).

Baseline separation was observed for nine compounds out of 10 on the Chiralpak AD column. Most compounds were better resolved with ethanol as organic modifier at a concentration of 5% and a flow-rate of 0.8 ml/min (experiments 2 and 5 of the design). No peaks were detected for nadolol and sotalol under these conditions. They were better resolved under the conditions of experiment 1 (20% ethanol, 0.5 ml/min).

Enantiomeric separation was only observed for four compounds with the Chiralcel OJ column. No general tendency can be observed due to the lack of selectivity of the column towards this set of compounds. From the few results obtained for Chiralcel OJ, one can see that separations are achieved for different experimental conditions. For instance, separation of the enantiomers of metoprolol and sotalol is achieved with 2-propanol, while ethanol is required for pindolol and propranolol.

The effects of the factors on the resolution are shown in Fig. 2. Calculations were only performed for compounds for which all data were available (i.e. the resolution could be calculated for each experiment). No effects of the factors could be calculated for Chiralcel OJ due to the few separations obtained.

Table 2
Factors and their levels as examined in the seven-factor, eight-experiment Plackett–Burman design

Factor	Level	
	(−)	(+)
A: Type of organic modifier	2-Propanol	Ethanol
B: % of DEA (v/v)	0.1%	0.4%
C: % of organic modifier (v/v)	5%	15% on Chiralpak AD[a] 20% on Chiralcel OD-H and OJ
D: Flow-rate (ml/min)	0.5	0.8

[a] An unstable baseline is obtained when 20% (v/v) organic modifier is used with the Chiralpak AD column.

Table 3
Chiral resolutions and analysis times (AT) obtained in the seven-factor, eight-experiment Plackett–Burman design on (a) a Chiralcel OD-H column, (b) a Chiralpak AD column and (c) a Chiralcel OJ column

Exp. No.	Compound																			
	Acebutolol		Alprenolol		Atenolol		Bisoprolol		Metoprolol		Nadolol		Oxprenolol		Pindolol		Propranolol		Sotalol	
	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT
(*a*) *Chiralcel OD-H*																				
1	0.38	10.95	1.19	9.59	2.33	22.01	0.67	9.69	2.04	10.52	2.98/1.59/0.00	15.48	7.99	16.26	22.34	68.58	3.86	15.45	0.00	10.33
2	1.26	47.92	2.63	10.05	–[a]	–	2.59	13.68	3.57	14.23	–	–	16.03	31.86	0.00	71.32	7.84	27.26	–	–
3	0.00	9.75	0.91	8.65	2.63	20.41	1.07	9.37	2.16	10.38	3.02/1.57/0.00	14.68	8.26	16.13	21.35	64.79	3.31	14.31	0.00	10.79
4	2.35	204.26	12.82	31.79	–	–	9.03	37.01	13.32	38.43	–	–	31.36	206.20	–	–	20.65	115.96	–	–
5	2.02	45.15	3.12	9.59	–	–	2.71	13.51	3.87	14.18	–	–	16.11	32.34	0.00	74.96	7.65	26.67	–	–
6	0.61	9.29	4.78	8.96	6.26	27.19	3.56	8.33	6.70	10.29	10.46/1.33/0.00	21.47	19.10	27.30	22.55	126.33	7.69	15.44	0.00	9.72
7	0.72	9.04	3.19	7.64	6.21	24.33	2.96	8.01	3.63	9.54	10.88/2.65/0.00	24.16	18.66	24.34	24.54	133.18	8.47	16.08	0.00	9.76
8	1.95	41.01	16.75	41.01	–	–	11.64	45.78	16.80	48.40	–	–	31.88	202.36	–	–	22.86	126.49	–	–
(*b*) *Chiralpak AD*																				
1	3.53	27.89	1.38	9.48	0.00	37.98	1.61	13.23	2.83	16.21	3.05/8.06/8.59	77.14	2.90	12.71	1.94	18.30	1.25	10.84	8.51	41.28
2	4.83	154.22	2.65	9.63	–	–	2.41	21.97	4.45	26.67	–	–	6.00	17.31	3.57	62.25	3.18	13.57	–	–
3	3.21	26.92	1.39	9.61	0.00	37.08	1.45	12.72	2.73	15.68	1.61/5.88/7.27	80.12	3.46	13.29	1.99	18.67	1.36	10.76	8.68	40.54
4	0.53	158.43	2.66	17.20	–	–	1.22	28.73	1.50	29.95	–	–	3.64	27.48	1.37	93.55	0.00	18.87	–	–
5	4.59	161.31	2.07	8.75	–	–	2.74	22.88	4.61	28.15	–	–	4.61	15.03	3.99	74.25	4.11	15.29	–	–
6	0.00	10.48	2.53	6.28	0.00	15.04	0.91	7.16	1.22	7.89	0.91/4.79/3.22	24.89	2.45	7.73	0.71	11.01	0.00	6.23	2.42	20.80
7	0.00	10.28	2.31	6.27	0.00	14.75	0.90	7.13	1.21	7.84	0.93/4.47/3.08	24.46	2.28	7.75	0.70	10.49	0.00	6.17	3.03	19.99
8	0.81	166.02	2.75	17.45	–	–	2.00	29.58	1.80	31.13	–	–	4.58	28.32	1.31	106.73	0.00	19.12	–	–
(*c*) *Chiracel OJ*																				
1	0.00	9.64	0.00	6.81	0.00	14.53	0.00	9.19	0.00	10.70	0.00	8.39	0.00	7.36	1.09	20.14	0.00	10.40	0.00	20.66
2	0.00	71.83	0.00	8.92	–	–	0.00	24.65	0.00	29.88	–	–	–	11.72	–	–	0.99	22.19	–	–
3	0.00	9.25	0.00	7.01	0.00	14.22	0.00	9.24	0.00	10.81	0.00	8.35	0.00	7.38	1.12	20.28	0.00	10.42	0.00	20.58
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	0.00	75.23	0.00	8.71	–	–	0.00	25.03	0.00	29.76	–	–	–	11.51	–	–	1.01	22.43	–	–
6	0.00	13.37	0.00	6.87	0.00	20.20	0.00	10.39	0.89	12.16	0.00	9.57	0.00	7.96	0.00	28.51	0.00	11.07	0.99	31.10
7	0.00	13.17	0.00	6.74	0.00	20.32	0.00	10.41	0.86	12.09	0.00	9.59	0.00	8.03	0.00	28.57	0.00	11.31	1.05	32.03
8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] –, no peak observed within 240 min.

The calculated effects show an important influence of the type of organic modifier and its concentration on the resolution for both columns. 2-Propanol leads to better separations on Chiralcel OD-H, while ethanol is more effective on Chiralpak AD. The effect of the concentration of DEA on the resolution is negligible. In certain cases, the flow-rate appears to have quite an important influence on resolution. The calculated effects suggest that an increase of the flow-rate leads to a decrease in the resolution on Chiralcel OD-H and an increase of resolution on Chiralpak AD. This latter result was quite unexpected, since an increase in flow-rate is often reported to reduce retention [27] due to reduced interactions between the chiral analyte and the CSP. Furthermore, when looking at the experiments individually, this tendency is not observed. Therefore, the effect calculated for the flow-rate was suspected of being wrongly estimated due to possible confounding interaction effects. Indeed, as explained previously, main factor effects are confounded with two-factor and higher-degree effects in a Plackett–Burman design. An interaction effect means that the effect of one factor on the considered response is influenced by the level of one or several other factors. These interactions are assumed to be negligible, but, in the case where two factors are strongly significant, their interaction may also be negligible. With the design used in this study, the flow-rate is confounded with the interaction organic modifier/

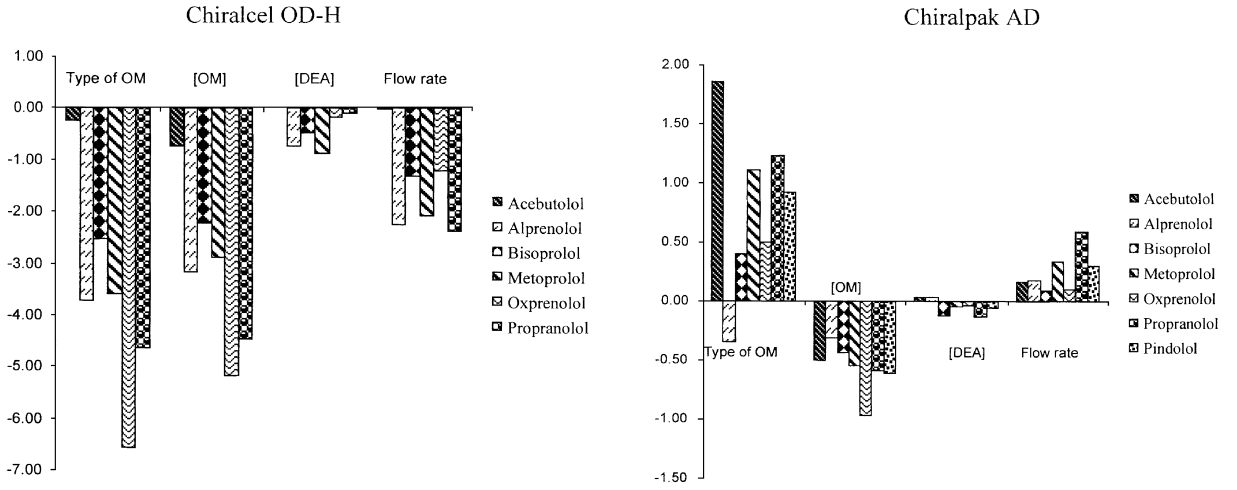

Fig. 2. Effects of the factors on the resolution of β-blockers on the Chiralcel OD-H (left) and the Chiralpak AD (right) columns.

percent of organic modifier. From the results of the design, it appears that these two factors have a very significant effect on the separation. In order to distinguish between the effect of the flow-rate and the effect of the organic modifier/percentage of modifier interaction, extra experiments need to be performed [40]. The effect of the interaction was studied by means of a full factorial design for two factors (i.e. type of organic modifier and percentage of organic modifier) [41]. The results showed that the effect observed in the Plackett–Burman design is mainly due to the interaction ''type of organic modifier/concentration of organic modifier'', while the effect of the flow-rate is rather small and always negative for both columns.

Analysis time is an important criterion in a screening strategy and should be considered (Table 3). The calculated effects of the factors are displayed in Fig. 3. As expected, an increase in the concentration of the organic modifier and of the flow-rate leads to a decrease in the analysis time. The type of organic modifier can also strongly influence the retention. The use of ethanol with Chiralcel OD-H results in a significant decrease of the retention time

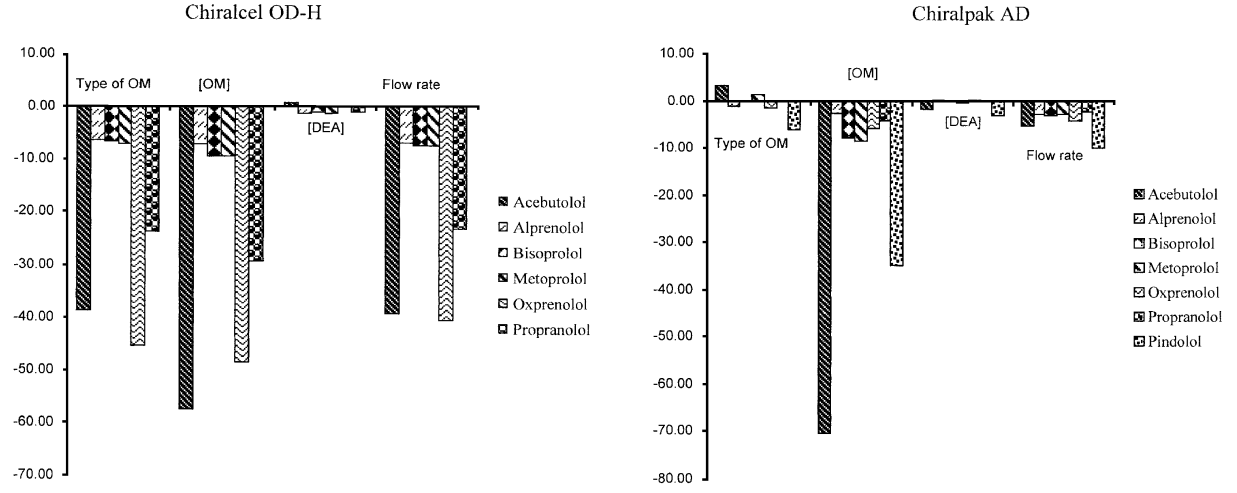

Fig. 3. Effects of the factors on the analysis time of β-blockers on the Chiralcel OD-H (left) and the Chiralpak AD (right) columns.

compared to 2-propanol. This may explain the better separation obtained with 2-propanol on this column due to stronger interactions with the CSP. Although no effect could be calculated, the same tendency can be observed for the Chiralcel OJ column. The effect of the type of organic modifier on retention is less important for Chiralpak AD.

This first study of basic molecules led to the conclusion that the factors with most influence on the separation were the type of column, the type of organic modifier and its concentration. Chiralcel OD-H and Chiralpak AD were found to be complementary columns for the above series of compounds, where sotalol and nadolol could only be resolved on Chiralpak AD, while atenolol enantiomers were only separated on Chiralcel OD-H. The Chiralcel OJ column appeared to be much less efficient for this series of compounds. Better resolutions were achieved on Chiralcel OD-H with 2-propanol and with ethanol on Chiralpak AD. Significant differences in analysis time were observed depending on the type of organic modifier.

However, only one family of compounds was tested, which does not allow us to draw final conclusions. Consequently, it was decided to continue testing both types of organic modifiers on each column in the following investigations. For similar reasons, the factor ''concentration of organic modifier'' was also investigated further. The concentration of DEA, having little effect on the separation, and the flow-rate, having a minor effect compared to the others, were kept at a fixed level [i.e. 0.1% (v/v) and 0.5 ml/min, respectively] in the subsequent investigations.

3.2. Analysis of bifunctional compounds

In this study, four benzodiazepins were used as test compounds.

Based on the previous results, only the type of CSP, the type of organic modifier and its concentration in the mobile phase were studied using an experimental design strategy. The type of organic modifier and its concentration were studied at two levels, while the factor ''type of column'' needed to be studied at three levels. Therefore, a 3×2^2 mixed-level design was used (Tables 4 and 5). This design is in fact a combination of two 2^2 full factorial designs. With full factorial designs, not only can the main effects of the factors be evaluated, but also all the interaction effects of the factors [35]. However, this type of design can only be used with a limited number of factors, since the total number of experiments is equal to 2^f with f being the number of factors. The two-factor interactions were evaluated from the design. The interaction effect of two factors A and B is calculated as half the difference between the effect of factor A when factor B is at a high level and the effect of factor A when factor B is at a low level [43]:

$$\text{Interaction effect (AB)} = 0.5(E_{\text{A,B}(+)} - E_{\text{A,B}(-)}) \quad (3)$$

Resolution values obtained in the design are given in Table 6. Baseline resolution of the four benzodiazepins was achieved on both Chiralcel OD-H and Chiralpak AD, while a beginning of separation was observed for oxazepam and temazepam with Chiralcel OJ. The effects of the factors on the resolution were calculated for lorazepam and oxazepam on Chiralcel OD-H and Chiralpak AD (Fig. 4). They show that better resolution values are obtained on Chiralpak AD. The same tendency is also observed for lormetazepam and temazepam. The resolution improves for both CSPs when 2-propanol is used. The effect of the type of OM depends on the type of column, as shown by the significant column/type of

Table 4
The 3×2^2 experimental design used to screen benzodiazepins

Exp. No.	Factor		
	Type of column	Type of organic modifier	% of organic modifier (v/v)
1	−1	−1	−1
2	+1	−1	−1
3	−1	+1	−1
4	+1	+1	−1
5	−1	−1	+1
6	+1	−1	+1
7	−1	+1	+1
8	+1	+1	+1
9	0	−1	−1
10	0	−1	+1
11	0	+1	−1
12	0	+1	+1

Table 5
Factors and their levels as studied in the 3×2^2 design used to screen benzodiazepins

Factor	Level		
	(−1)	(0)	(+1)
Type of column	Chiralcel OD-H	Chiralcel OJ	Chiralpak AD
Type of organic modifier	Isopropranol		Ethanol
Percentage of organic modifier (v/v)	5%		15% for Chiralpak AD 20% for Chiralcel OD-H and OJ

An unstable baseline is obtained when 20% (v/v) organic modifier is used with the Chiralpak AD column.

OM interaction. The effect of this factor is more important for the Chiralpak AD column. The use of 2-propanol dramatically improves the separation of lorazepam and oxazepam enantiomers, while the enantiomers of lormetazepam are better resolved with ethanol on this column. For Chiralcel OD-H, the enantiomers of oxazepam and temazepam are separated better with ethanol, while higher resolution is achieved with 2-propanol for lorazepam and lormetazepam.

Analysis times obtained in the design (Table 6) show that longer retention times occur on Chiralpak AD, which may explain the higher resolution obtained on this column due to the stronger interactions of the compounds with this CSP. Longer retention times are observed for lorazepam and oxazepam when 2-propanol is used on both columns. In general, resolution increases with increasing retention. However, although temazepam is less retained on Chiralcel OD-H when ethanol is used, its enantiomers are better resolved with this organic modifier. As expected, higher resolutions are obtained when lower concentrations of organic modifier are used, but unacceptable analysis times are then obtained.

The results confirm the strong influence of the type of organic modifier and its concentration on the separation. However, while for the analysis of β-blockers it was found that 2-propanol systematically leads to better separations on Chiralcel OD-H and ethanol on Chiralpak AD, this tendency is not so

Table 6
Chiral resolutions and analysis times (AT) obtained in the 3×2^2 design used to screen the benzodiazepins

Exp. No.	Compound							
	Lorazepam		Lormetazepam		Oxazepam		Temazepam	
	R_S	AT	R_S	AT	R_s	AT	R_S	AT
1	4.38	224.19	3.48	183.36	3.94	204.43	0.53	117.48
2	8.77	278.62	[a]	[a]	13.95	332.59	[a]	[a]
3	4.00	127.63	3.29	104.2	8.03	144.31	1.04	77.52
4	2.74	184.39	[a]	[a]	1.99	390.14	5.91	131.70
5	3.17	34.02	3.14	43.45	4.16	36.34	[a]	[a]
6	7.49	58.74	17.65	153.19	12.85	74.36	[a]	[a]
7	2.25	20.57	2.20	24.76	5.82	25.53	0.89	20.62
8	2.59	40.13	19.66	168.49	0.97	59.55	7.14	109.20
9	0.00	158.42	0.00	173.28	[a]	[a]	[a]	[a]
10	0.00	31.41	0.00	61.47	1.39	39.69	0.34	56.93
11	0.00	140.89	[b]	[b]	2.91	162.37	0.00	136.65
12	0.00	22.18	0.00	35.40	1.60	26.19	0.00	36.39

[a] Only one peak observed within 240 min.
[b] No peak observed within 240 min.

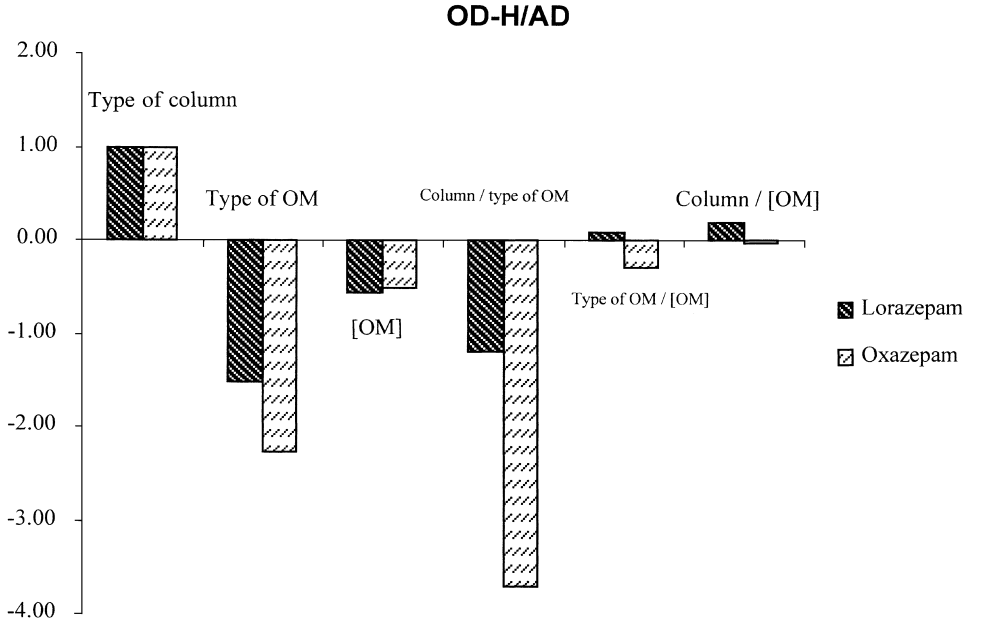

Fig. 4. Effects of the factors on the resolution of benzodiazepins on the Chiralcel OD-H and Chiralpak AD columns.

evident for the benzodiazepins, where the optimal type of organic modifier also appears to depend on the analyte.

3.3. Analysis of acidic compounds

The same design and factors as for the analysis of benzodiazepins were used to screen acidic drugs. The levels of the factors were also the same. TFA was added to the mobile phase instead of DEA at a concentration of 0.1% (v/v).

Resolution values obtained in the design are shown in Table 7. Enantiomeric separations could be observed for all compounds except phenobarbital on at least one of the columns. The effects of the factors were calculated for some compounds (Fig. 5). As the factor type of column was studied at three levels, the columns were compared two by two. Globally, Chiralpak AD and Chiralcel OJ performed better than Chiralcel OD-H for this series of compounds. The optimal type of organic modifier depends both on the type of column and the type of analyte. Anti-inflammatory drugs are better separated with ethanol on Chiralpak AD, while warfarin and acenocoumarol enantiomers are better resolved with 2-propanol. On the contrary, anti-inflammatory drugs are better separated with 2-propanol on Chiralcel OJ.

Longer retention times (Table 7) usually occur with Chiralcel OJ. However, this phenomenon does not always result in higher resolution compared with Chiralpak AD, for instance. Very long analysis times occur with a low concentration of organic modifier, which is not acceptable for a screening strategy. However, when 20% organic modifier is used, a significant decrease in analyte retention is observed, resulting in a significant loss of resolution, which could mask some possible separation.

The results show that Chiralcel OJ, although not giving satisfying results for the other two sets of compounds, could be an interesting CSP for acidic compounds.

The optimal type of organic modifier depends both on the type of column and the analyte. However, within a drug family, one organic modifier seems to be more suitable (i.e. ethanol for anti-inflammatories on Chiralpak AD).

Neither of the two concentrations of organic modifier tested in the design (i.e. 5 and 20%) appeared to be suitable for future screening, since, in several cases, too long (5% organic modifier used) or

Table 7
Chiral resolutions and analysis times (AT) obtained in the 3×2^2 design used to screen acidic drugs

Exp. No.	Compound															
	Ibuprofen		Ketoprofen		Flurbiprofen		Suprofen		Hexobarbital		Phenobarbital		Acenocoumarol		Warfarin	
	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT
1	0.00	9.07	0.00	19.14	0.00	12.01	0.69	31.64	3.83	73.21	0.00	59.86	5.29	155.36	4.21	104.61
2	0.00	11.54	1.66	50.82	2.43	21.28	1.88	84.40	6.00	48.16	[b]	[b]	[b]	[b]	21.77	219.28
3	0.00	0.00	0.00	18.56	0.00	12.01	0.56	28.18	3.54	65.59	4.74	59.34	4.74	107.11	4.60	104.17
4	0.00	10.93	3.82	51.41	4.73	25.22	3.30	102.59	[a]	[a]	[b]	138.35	[b]	[b]	16.62	196.22
5	0.00	7.59	0.00	9.58	0.00	8.30	0.00	11.49	2.29	23.26	2.14	14.24	2.14	23.32	1.47	31.34
6	0.00	8.65	2.08	19.11	2.25	12.14	1.91	22.12	3.48	17.73	18.56	24.57	18.56	114.37	12.41	43.43
7	0.00	10.34	0.00	12.18	0.00	11.22	0.00	10.06	1.62	27.43	2.68	19.13	2.68	25.64	2.07	32.37
8	0.00	8.04	2.04	16.69	2.37	12.18	3.80	27.68	20.24	44.88	13.53	[b]	13.53	95.87	10.51	29.70
9	0.00	6.94	2.16	55.16	0.00	38.00	3.43	161.87	0.77	123.57	[b]	[b]	[b]	[b]	[b]	[b]
10	0.00	8.12	1.61	18.26	0.00	12.80	2.47	29.22	0.90	37.41	[b]	[b]	1.32	83.15	[b]	[b]
11	0.31	13.22	1.56	65.67	0.88	49.17	3.36	161.42	0.95	130.33	[b]	[b]	[b]	[b]	[b]	[b]
12	0.00	9.17	0.84	19.59	0.60	17.90	2.37	33.26	0.77	37.90	[b]	[b]	1.54	55.77	15.76	185.69

[a] Only one peak observed in 240 min.
[b] No peak observed in 240 min.

too short (20% organic modifier used) retention occurred.

3.4. Use of DEA/TFA mixtures

In the previous experiments, DEA was added to the mobile phase when basic and bifunctional compounds were analysed, while TFA was added for the analysis of acidic compounds. However, from the perspective of a screening strategy, a single set of mobile phases independent of the chemical properties of the compounds would be more suitable. Some studies [28,31] have shown that the simultaneous addition of DEA and TFA to the mobile phase can lead, for some compounds, to an improvement in selectivity with polysaccharide CSPs. Therefore, we studied whether DEA/TFA mixtures could be used systematically in the screening strategy. For this, DEA/TFA in different ratios were added to the mobile phase. The influence on the resolution is shown for some compounds in Table 8. For basic compounds, the presence of TFA in the mobile phase

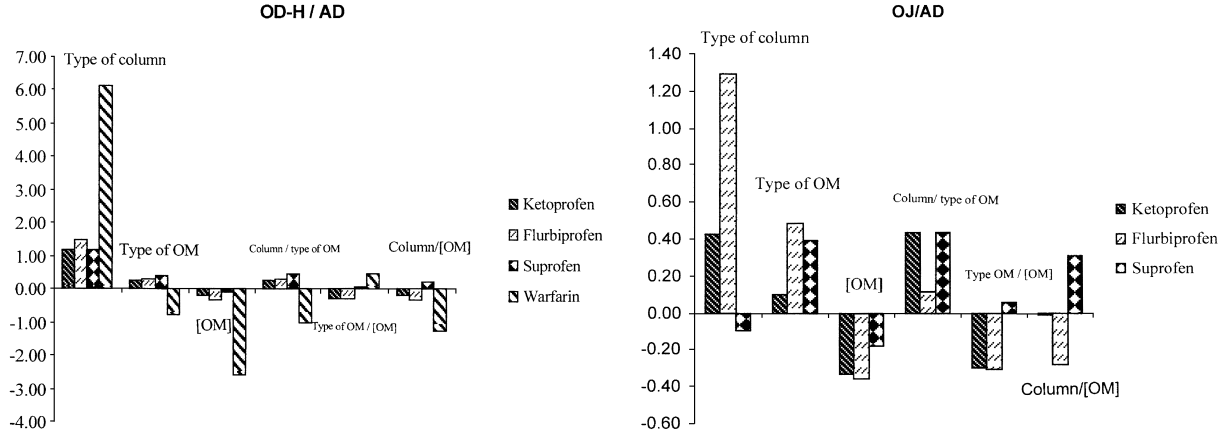

Fig. 5. Effects of the factors on the resolution of acidic compounds. Left: Chiracel OD-H column compared with Chiralpak AD column. Right: Chiralcel OJ column compared with Chiralpak AD column.

Table 8
Influence of TFA/DEA mixtures on the resolution of chiral drugs

Compound	TFA/DEA ratio (%, v/v)									
	0.1:0	0.3:0.1	0.5:0.1	0.5:0.3	0.1:0.1	0.3:0.3	0.5:0.5	0.1:0.3	0.1:0.5	0:0.1
Basic drugs										
Bisoprolol[a]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.53	1.62	1.86
Metoprolol[b]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.80	1.15	1.39
Pindolol[a]	0.00	0.45	0.69	0.00	2.20	0.48	0.00	1.63	1.81	2.65
Sotalol[b]	0.00	1.16	0.91	0.87	1.20	0.99	0.00	2.17	2.24	2.31
Acidic drugs										
Ketoprofen[b]	1.78	1.90	1.98	1.92	1.93	1.89	2.03	1.29	1.44	ND
Suprofen[b]	1.63	1.66	1.68	1.66	1.55	1.59	1.60	0.00	0.00	ND
Bifunctional drug										
Lorazepam[a]	2.31	2.13	1.91	2.15	2.30	1.93	1.77	2.15	2.19	2.37

Experimental conditions: Chiralpak AD column; flow-rate, 0.8 ml/min; temperature, 20 °C; mobile phase, [a]*n*-hexane/EtOH (85:15, v/v), [b]*n*-hexane/2-propanol (85:15, v/v).

always resulted in a decrease in the resolution. This decrease in resolution can mainly be attributed to a decrease in retention resulting from the addition of TFA [32]. When the concentration of TFA was higher than or equal to the concentration of DEA, the resolution of bisoprolol and metoprolol became zero. For acidic compounds, the resolution remained constant when the concentration of TFA was higher than or equal to the concentration of DEA. However, when the concentration of DEA was higher, the resolution decreased and became zero for some compounds. Bifunctional compounds were not affected very much by changes in the concentration of either TFA or DEA, as already observed by Blackwell et al. [32].

With regard to these results, the use of a single mobile phase for the analysis of basic and acidic compounds to which DEA and TFA are added simultaneously does not seem feasible. Indeed, a significant decrease in resolution is observed for both acidic and basic compounds in this study, which may lead the analyst to a wrong conclusion about the feasibility of a separation. Therefore, the use of DEA/TFA mixtures appears to be useful in specific cases and cannot be applied in a screening strategy. Consequently, it was decided that different mobile phases would be used in the screening strategy depending on the acid/basic properties of the analytes. Bifunctional or neutral compounds can be analysed with any of these mobile phases.

3.5. Screening strategy

This study has shown that the selected set of columns was appropriate for the chiral separation of the drugs, since all test compounds, except phenobarbital, could be separated on at least one column. Chiralcel OD-H and Chiralpak AD appear to be useful columns for any type of compound, while Chiralcel OJ should be used for the analysis of acidic compounds or when no separation is observed on the other two columns. The nature of the alcohol used as organic modifier in the mobile phase plays an important role in the enantiorecognition process. No optimal organic modifier was found for a given columnn, as its effects also depend on the analyte. It is important to note that not only is the chiral environment important in the enantiorecognition process in NPLC, but also achiral factors such as the structure of the alcohol used in the mobile phase. Two concentrations of organic modifiers were tested. As expected, in most cases, better separation was obtained at a lower concentration. However, very long analysis times were obtained, especially for bifunctional and acidic compounds, which is not acceptable in a screening strategy. On the other hand, 20% organic modifier usually leads to short analysis times, but a significant decrease in selectivity can occur.

A screening strategy was defined with regard to these results. For each compound, each column was

tested with two different mobile phases, i.e. *n*-hexane–2-propanol and *n*-hexane–ethanol. Due to the wide range of polarity of the drugs, the concentrations of organic modifier tested were not suitable, as either too long or too little retention occurred. Consequently, a compromise of 10% (v/v) appears to be adequate for the analysis of a wide range of compounds. To avoid too long analysis times, it is recommended to increase the flow-rate to 1 ml/min. This should not affect the separation too much as

Table 9
Results of the screening of chiral drugs by NPLC according to the proposed strategy

	Chiralcel OD-H				Chiralpak AD				Chiralpak OJ			
	EtOH		IPA		EtOH		IPA		EtOH		IPA	
	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT	R_S	AT
Basic compounds												
Acebutolol	0.30	9.74	0.41	18.93	1.59	28.72	0.00	17.62				
Alprenolol	1.88	5.64	5.17	9.75	1.53	8.82	2.68	6.89				
Atropine	3.28	9.17	0.62	14.02	1.79	16.98	0.64	10.99				
Clenbuterol	0.00	4.56	0.58	4.73	0.00	5.71	0.00	7.63				
Dilthiazem	0.28	7.46	1.62	12.21	0.00	9.47	0.00	13.53				
Ephedrine	0.70	5.05	0.00	5.45	0.00	8.42	0.00	6.47				
Fluoxetine	0.29	5.05	0.00	6.18	1.38	8.43	0.00	5.24				
Ketamine	0.00	5.52	0.59	6.83	0.00	6.43	0.00	6.63				
Leucovorin	0.00	8.11	0.00	9.50	0.00	9.15	0.00	8.48	0.00	11.23	0.00	13.24
Methadone	0.00	4.08	0.00	4.12	0.00	3.83	0.00	3.99	1.58	6.30	0.00	3.97
Metoprolol	2.44	10.23	12.30	29.56	3.48	19.64	1.41	6.83				
Mianserin	0.00	5.02	0.43	5.46	0.87	5.44	0.00	6.14				
Morphine	0.00	8.54	0.00	10.22	0.00	12.25	0.00	19.67	0.00	14.31	0.00	15.62
Nadolol	1.51/2.24/ 0.00	19.21	0.43/4.63/ 0.37	48.07	1.47/3.01/ 5.49	24.98	0.7/2.03/ 1.57	34.24				
Oxprenolol	8.63	14.05	21.16	43.77	4.13	11.22	2.91	9.01				
Pindolol	28.51	44.50	32.40	49.34	2.45	38.12	0.82	53.67				
Promethazine	0.00	4.72	0.00	4.14	4.05	9.90	8.45	18.88				
Propiomazine	1.68	7.78	1.96	9.03	0.62	8.34	0.99	8.54				
Propanolol	3.03	11.81	5.69	22.17	2.84	7.77	0.00	6.54				
Sulpride	0.00	30.80	0.00	33.95	0.79	37.21	0.00	16.24				
Tetramisol	1.01	11.96	0.83	8.53	1.54	14.34	1.51	14.65				
Verapamil	0.00	7.64	0.00	10.59	0.45	8.29	1.54	9.80				
Acidic compounds												
Acenocoumarol	1.64	18.60	1.84	34.03	7.64	116.02	20.34	144.56	1.22	55.33	1.07	54.18
Fenoprofen	0.00	5.28	0.00	6.07	0.00	5.96	0.67	6.99	0.00	15.88	1.24	9.23
Flurbiprofen	0.00	5.06	0.00	4.92	1.67	7.42	1.57	7.34	0.49	13.14	0.00	8.27
Hexobarbital	0.83	14.64	1.55	17.30	0.47	4.07	3.57	12.38	0.55	32.30	0.49	28.76
Ibuprofen	0.00	5.34	0.00	6.88	0.00	4.75	0.00	4.43	0.00	4.65	0.00	5.12
Ketoprofen	0.00	6.21	0.00	6.55	1.46	12.96	1.56	14.07	0.92	22.15	1.95	17.93
Mandelic acid	0.89	8.13	0.41	9.10	0.00	11.11	1.42	13.67	1.64	25.98	0.74	13.40
Naproxen	0.00	6.70	0.00	6.80	0.00	9.67	0.00	11.05	0.00	34.13	0.00	21.26
Suprofen	0.00	8.51	0.00	9.54	1.29	21.07	1.44	16.08	2.02	36.95	1.74	18.48
Warfarin	1.05	10.67	1.73	38.13	6.14	28.33	6.59	41.00	18.31	240.47	[a]	[a]
Bifunctional, neutral compounds												
Cyclothiazide	1.99	36.16	[a]	[a]	0.32	3.41	0.00	7.51				
Oxazepam	6.68	48.31	4.28	52.13	1.12	72.86	13.01	90.32	2.44	86.42	[a]	[a]
Paziquantel	2.69	25.97	1.82	37.07	0.00	29.45	1.96	31.52	0.67	5.23	0.00	8.62
trans-Stilbene oxide	1.97	6.06	2.14	7.26	2.92	8.56	6.77	9.18	2.39	12.60	0.96	11.20

[a] No peak observed within 240 min.

experiments showed that this factor had a small influence compared to the others. The addition of DEA or TFA to the mobile phase at a concentration of 0.1% (v/v) is required for the analysis of basic or acidic compounds, respectively. In the case of basic compounds, it is recommended to test the Chiralcel OD and the Chiralpak AD columns first. The Chiralcel OJ column should only be tested when no separation is obtained on these two columns, since few basic compounds could be resolved on this column. Thus, in the majority of cases, only four experiments will be required for the analysis of a basic compound. For acidic compounds, the three columns should be tested.

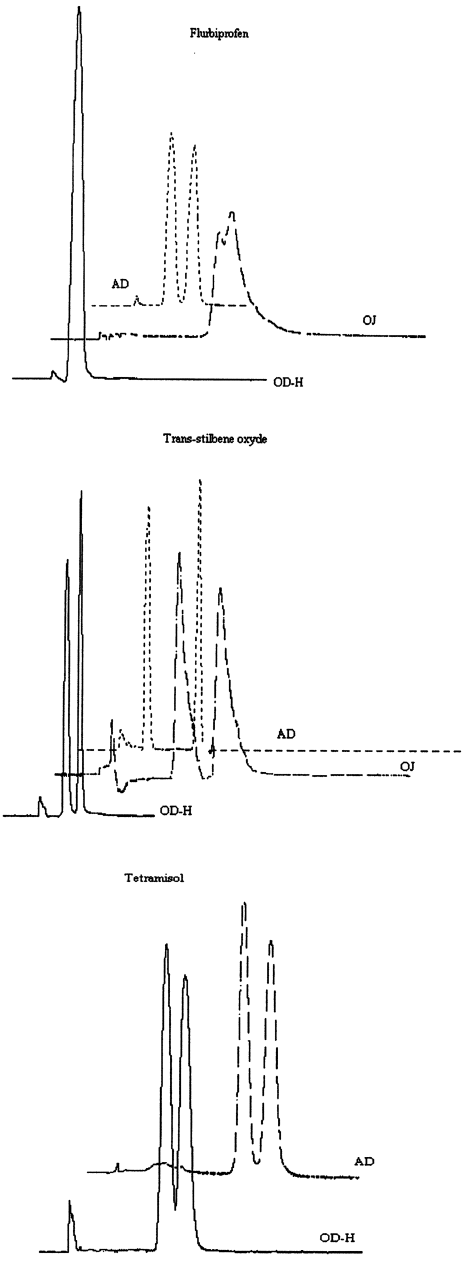

Fig. 6. Examples of separations obtained during screening on the three columns (analytical conditions as in Table 9 and described in Section 3.5).

This strategy was applied to a set of 36 compounds with very different structures and physical/chemical properties. The results are shown in Table 9. The results are satisfying in the sense that separation of the enantiomers is observed for 32 compounds out of the 36 analysed. Furthermore, 24 are baseline resolved after this first set of experiments. Reasonable analysis times (usually <20 min) were achieved, which makes this strategy suitable for the rapid screening of chiral drugs. Some examples of the chromatograms obtained in the screening are shown in Fig. 6.

4. Conclusion

Three columns based on cellulose/amylose derivatives (Chiralcel OD-H, Chiralpak AD and Chiralcel OJ) were evaluated for rapid screening for the separation of chiral drugs. The three columns appear to be complementary and to have broad enantiorecognition capabilities for a wide range of pharmaceutical compounds. This study has shown that not only has the chiral environment (i.e. the chiral selector) an important influence on the quality of the separation, but also achiral parameters such as the alcohol contained in the mobile phase. Knowledge of the acidic–basic properties of the molecules to be analysed is required, as the addition of DEA for basic compounds or TFA for acidic compounds is necessary to achieve good separations. The case studies performed allowed us to define a rapid screening strategy. In this strategy, the compounds are screened on each column using two different mobile phases, i.e. *n*-hexane–2-propanol and *n*-hexane–ethanol (six experiments in total). In the case of basic compounds, screening on the Chiralcel OD-H and Chiralpak AD columns is usually sufficient to

achieve separation. This strategy was applied successfully to a set of 36 different drugs. Resolution of the enantiomers was observed for 32 compounds on at least one column. Short analysis times (i.e. 20 min or less) were usually achieved.

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