ELSEVIER

Journal of Chromatography A, 883 (2000) 249–265

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

# Fast development of separation methods for the chiral analysis of amino acid derivatives using capillary electrophoresis and experimental designs

C. Perrin[a], M.G. Vargas[a], Y. Vander Heyden[a], M. Maftouh[b], D.L. Massart[a,*]

[a]*Department of Pharmaceutical and Biomedical Analysis, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium*

[b]*Analytical Research Department, Sanofi-Synthelabo, Centre de Toulouse, 195 Route d'Espagne, 31036 Toulouse, France*

Received 28 December 1999; received in revised form 21 March 2000; accepted 21 March 2000

**Abstract**

The use of experimental design in method development was studied for the chiral separation of several amino acid derivatives with capillary electrophoresis. The aim of this study was to define rapidly experimental conditions under which the enantiomers can be sufficiently separated for quantification and to derive a methodology for the separation of new compounds. Three modified cyclodextrins (CDs) were used as chiral selectors: hydroxypropyl-β-cyclodextrin, carboxymethyl-β-CD and sulfobutylether-β-CD. The following factors were examined: the type of cyclodextrin, the CD concentration, the pH and the % of organic modifier (methanol) of the electrolyte. Two types of fractional factorial design were used depending on the type of analyte and on the number of factors selected: a $3^{4-2}$ fractional factorial design (4 factors studied at 3 different levels) and a $2^{3-1}$ fractional factorial design (3 factors at 2 different levels). From the 14 compounds investigated, 12 could be separated with one or another CD and not more than 9 experiments were required. No generalisation of the best analysis conditions was possible within this family of compounds. Specific analysis conditions must be defined for each analyte. Experimental designs have shown to be very useful to determine rapidly conditions under which each enantiomer can be separated with an acceptable resolution. 

*Keywords:* Method development; Experimental design; Enantiomer separation; Buffer composition; Factorial design; Amino acids; Cyclodextrins

## 1. Introduction

Starting about a decade ago, pharmaceutical companies began to develop technologies to either resolve or selectively synthesize single isomer drugs. The reason is that enantiomers are often readily distinguished by biological systems. As a consequence, different pharmacokinetic properties and pharmacologic or toxicologic effects may occur.

In 1992, the US Food and Drug Administration (FDA) published its policy statement for the development of new stereoisomeric drugs: the stereoisomeric composition of a drug with a chiral center should be known as well as the quantitative isomeric

*Corresponding author. Tel.: +32-2-477-4734; fax: +32-2-477-4735.

*E-mail address:* fabi@vub.vub.ac.be (D.L. Massart)

0021-9673/00/$ – see front matter 
PII: S0021-9673(00)00377-0

composition of the material used in pharmacologic, toxicologic and clinical studies [1]. Applications for enantiomeric and racemic drug substances should also include a stereochemically specific identity test. Thus, the enantiomeric purity of each reagent involved in a synthesis should be controlled in order to reach pure final drug substances.

Fast method development and short analysis times are crucial factors in industry to face the increasing number of analyses. This requirement is becoming all the more important with the rapid development of combinatorial chemistry. There, small chiral molecules such as amino acids and their derivatives are commonly used as building blocks. Some derivatives such as *N-tert.*-butoxycarbonyl (*N*-t-Boc) have the property to be strongly resistant to racemization [2] and are widely used for the synthesis of stereochemically pure compounds in pharmaceutical discovery programs.

The chiral separation of amino acids derivatives by high-performance liquid chromatography (HPLC) [3–6], gas chromatography (GC) [7] and supercritical fluid chromatography (SFC) [8] has been reported, while recently the use of capillary electrophoresis dramatically increased [9,10]. Capillary electrophoresis (CE) is a powerful alternative technique due to its very high efficiency. In comparison with HPLC or GC, it has many advantages such as short analysis times and limited consumption of analytes and buffers, which makes it ideal for the high sample throughput provided by combinatorial chemistry.

Cyclodextrins (CDs) are the most used chiral selectors in CE. Several studies related to the chiral separation of amino acid derivatives by capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) with different types of cyclodextrins have been reported [2,11–13].

In this paper, the development of a methodology for the chiral separation of a series of amino acid derivatives by CE using modified cyclodextrins is proposed. An experimental design approach has been used. Method development is usually carried out with the univariate approach, in which the analyst studies the variables that may affect the separation one by one while the others remain constant. The use of this approach is often limited by the large number of variables that may influence the separation and the size of the experimental domain. If a large number of molecules have to be screened, then the use of this univariate method becomes difficult due to a too important number of experiments that have to be carried out. Experimental designs allow a multivariate approach. They involve the simultaneous change of several variables. Fractional factorial designs are efficient to provide in a few experiments information about the experimental domain where the separation of the enantiomers is possible. CE is especially convenient for the use of experimental design as the experimental conditions can be varied immediately from one experiment to another, unlike chromatographic techniques which require equilibration of the column. Up to now, only few studies report the use of experimental designs in the field of chiral CE [14–21].

The aim of this study was to explore rapidly the experimental domain and to find conditions at which the separation of the enantiomers is sufficiently good for quantification with acceptable analysis time. No modelling of the separation is sought. This strategy is developed for the high throughput screening of samples that have to be analysed rapidly, like in the primary discovery stages of the pharmaceutical programs. This approach can also be considered as a first step for a complete optimisation.

## 2. Experimental

### *2.1. Chemicals and reagents*

DL-Tryptophan methyl ester hydrochloride, DL-tryptophan ethyl ester, DL-tryptophan butyl ester, (*S*)-(+)-2-phenylglycinmethyl ester hydrochloride, (*R*)-(−)-2-phenylglycinmethyl ester hydrochloride, *N*-t-Boc-D-tryptophan, *N*-t-Boc-L-tryptophan, DL-tyrosine methyl ester hydrochloride, *N*-α-carboxybenzyl (*N*-α-CBZ)-L-arginine, *N*-α-CBZ-D-arginine, *N*-α-t-Boc-L-arginine, *N*-t-Boc-D-proline, *N*-t-Boc-L-proline, *N*-t-Boc-D-tyrosine, *N*-t-Boc-L-tyrosine were obtained from Aldrich (Steinheim, Germany), *N*-α-t-Boc-D-arginine hydrochloride was purchased from Acros (NJ, USA), *N*-t-Boc-D-phenylglycine, *N*-t-Boc-L-phenylglycine, *N*-t-Boc-DL-methionine, *N*-CBZ-DL-methionine, D-glutamic acid α-γ-dibenzyl ester

tosylate, L-glutamic acid α-γ-dibenzyl ester tosylate were obtained from Novabiochem (Switzerland).

Hydroxypropyl-β-CD was purchased from Beckman (Fullerton, CA, USA), Carboxymethylated-β-CD was obtained from Cyclolab R&D (Budapest, Hungary), Sulfobutylether-β-CD was purchased from Iris Technologies (Lawrence, KS, USA).

HPLC-grade methanol was obtained from BDH (Poole, UK). Ortho phosphoric acid 85% and triethanolamine were purchased from Merck (Darmstadt, Germany).

Water for preparation of separation buffers and samples was produced in the laboratory by the Milli-Q System (Millipore, Milford, MA, USA).

Three stock buffers consisting of 0.1 *M* phosphate were prepared with orthophosphoric acid 85% and Milli-Q water and were adjusted to the desired pH (2.5; 4.0; 5.5) with triethanolamine. The electrolyte solutions were prepared by dissolving the required cyclodextrin in the appropriate buffer with or without methanol. All sample solutions were prepared in Milli-Q water at a concentration of approximatively 0.24 mg/ml.

### 2.2. Capillary electrophoresis

Experiments were performed using a CE ultra capillary electrophoresis system (TermoQuest, San Jose, CA, USA) equipped with a fast scanning UV–Vis detector. An uncoated fused-silica capillary (Polymicro technologies, Phoenix, AZ, USA) cut to a total length of 40.8 cm with an effective separation length of 34.1 cm and an internal diameter of 50 μm was used.

Before each separation, the capillary was rinsed for 1 min at 60 p.s.i with phosphate buffer of the appropriate pH and then equilibrated for 0.25 min with the electrolyte solution (1 p.s.i.=6894.76 Pa). Sample injections were made using a positive pressure of 0.8 p.s.i for 4 s (6 s for arginine derivatives). Separations were conducted at a field strength of ±613 V/cm (±25 kV overall) for the amino acid alkyl esters or −490 V/cm (−20 kV overall) for the *N*-CBZ and *N*-t-Boc derivatives. The temperature was maintained at 20°C. Detection was achieved by UV absorbance at 220 nm for the tryptophan derivatives or at 200 nm for the other analytes.

### 2.3. Experimental design

Two different fractional factorial designs were applied: (i) a 3-level 4-factor ($3^{4-2}$) design for the amino acid alkyl esters (ii) and a 2-level 3-factor ($2^{3-1}$) design for the *N*-t-Boc and *N*-CBZ amino acids.

Calculation of the effects of the factors was only performed for the $2^{3-1}$ design. The effect of each factor was calculated according to Eq. (1):

$$E_{x[1,-1]} = \frac{\sum Y(+1)}{N/2} - \frac{\sum Y(-1)}{N/2} \quad (1)$$

where $\Sigma\ Y(-1)$ and $\Sigma\ Y(+1)$ represent the sum of the responses when the factor $X$ is at low or high level respectively. $N$ is the total number of experiments in the design.

## 3. Results and discussion

According to several studies, the most important parameters to optimize in a first step in chiral CE are the type of CDs, the concentration of the CD, the pH of the electrolyte and the % of organic modifier in the electrolyte [22–26].

Modified cyclodextrins were chosen as chiral selectors to screen the molecules. They present many advantages compared to the natural ones in terms of solubility, size of the cavity and interactions with the analyte. Furthermore, Lindner et al. [27] investigated the separation of several *N*-amino acid derivatives such as fluorenylmethoxycarbonyl (FMOC) and 5-dimethylaminonaphthylsulfonyl (Dns) and concluded that modified cyclodextrins exhibit better enantioselectivity in most cases. Of the modified CDs available on the market, hydroxypropyl-β-CD (HP-β-CD), carboxymethyl-β-CD (CM-β-CD) and sulfobutylether-β-CD (SBE-β-CD) appear to be very versatile and are recommended to be tested first [28–34]. HP-β-CD is a neutral CD while CM-β-CD and SBE-β-CD are charged. Charged CDs possess their own mobility which makes the separation of neutral compounds possible. Moreover their charge is reported to enhance the chiral recognition [29]. SBE-β-CD possesses sulfonic groups and thus a fully negative charge in the whole pH range avail-

able in CE, while CM-β-CD possesses weakly acidic groups and is therefore negatively charged for pH values above 4.5.

The concentration range for each CD was defined depending on its nature. For the neutral HP-β-CD, a large interval was screened while for the charged ones, low concentrations were selected. The reason is that the charged CDs are known to perform well at relatively low concentration but also because they generate very high current which causes instrumental problems such as heating.

All experiments were conducted at low pH (pH≤ 5.5) with low electroosmotic flow (EOF). Although the importance of EOF on peak resolution has been demonstrated by Williams et al. [24], it is better, in a first approach, to work in the absence of or with low EOF. Indeed, since the EOF varies with the pH and the buffer composition, it would not be controlled in the design. If after the first optimisation step, a too low resolution is found, then the EOF can be varied by changing the nature of the capillary wall at the promising experimental conditions. Low EOF results also in better repeatability of the measurements. In order to reduce the EOF, triethanolamine was added to the electrolyte. Triethanolamine is also useful to improve the separation by increasing the viscosity of the buffer and as a consequence the time of contact between the analyte and the CD.

Several studies deal with the use of methanol to improve resolution and even to allow chiral recognition [2,27]. One effect of methanol is to reduce the EOF but its effect on chiral separation cannot be simply reduced to that. An additional effect via interaction with the selector and/or the analyte must be assumed. This can be explained considering the theory of Wren [26] which says that when the CD is at or below the optimum concentration (maximum of resolution), the addition of organic modifier causes a decrease in resolution due to the change of association constants between the analyte and the CD. In contrast, when the CD concentration is above the optimal one, an increase in the resolution is expected. Wang [35] showed that different recognition mechanisms had to be considered in aqueous media and in the presence of organic solvent. The high complexity of the equilibria involved makes its effect rather unpredictable.

### *3.1. Followed strategies*

Of the 14 chiral amino acid derivatives studied (Fig. 1), six are amino acid alkyl esters and the others *N*-amino acid derivatives, *N*-t-Boc and *N*-CBZ.

At low pH values (pH<6), all the amino acid alkyl esters are positively ionised which confers them their mobility. On the other hand, all the *N*-amino acid derivatives are neutral (pH<4) or weakly negatively charged (pH>4) except the arginine derivatives which are positively charged or neutral. Thus two different analysis strategies (Fig. 2) were defined depending on the nature of the compound:

#### *3.1.1. The compound is charged over the whole pH range (2.5–5.5) (case of amino acid alkyl esters)*

In this case, either neutral or charged cyclodextrins can be used. To have the highest chance to reach good separation conditions for each compound, the three previously discussed CDs, namely HP-β-CD, CM-β-CD and SBE-β-CD are studied in a $3^{4-2}$ fractional factorial design. The factors tested are (i) the type of CD, (ii) the concentration of the CD, (iii) the pH of the electrolyte (BGE), (iv) the % of organic modifier (methanol, MeOH). The description of the factors, the levels and the design are shown in Tables 1 and 2.

If no baseline resolution is achieved after applying the design, an additional experimental design, such as a $2^{3-1}$ (3 factors at 2 levels) fractional factorial design, can be applied with the CD which has shown the greatest enantioselectivity. Levels of the factors (CD concentration, pH and % of methanol) will then be chosen with regard to the results obtained in the previous design. The best point found in the $3^{4-2}$ design is then the central point of the new design.

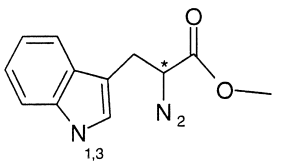
#### *3.1.2. The compound is not charged over the whole pH range (2.5–5.5) (case of N-amino acid derivatives)*

Because of the absence of mobility, neutral CDs are not the most suitable, including CM-β-CD which is neutral below pH 4.5. Thus, from the three selected CDs, SBE-β-CD is considered first choice.

Although one could suppose that the negative charges of the CD and the analyte could lead to bad separation due to electrostatic repulsion, Desiderio et al. [36] obtained very good chiral resolution for negatively charged compounds. They concluded that the coulombic interactions between SBE-β-CD and the analyte were not fundamental for the chiral recognition. Furthermore, Janini et al. [37] reported

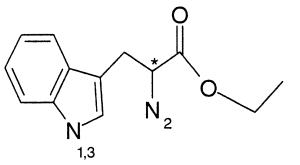
(a) Amino acid alkyl esters

Tryptophan methyl ester



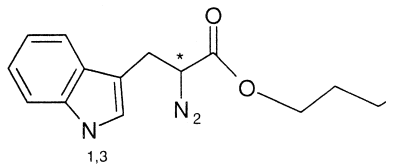
pKa1 = -0.91 ± 0.30
pKa2 = 7.80 ± 0.33
pKa3 = 17.02 ± 0.30

Tryptophan ethyl ester



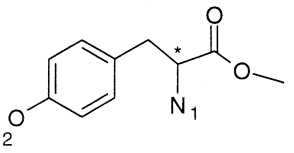
pKa1 = 0.88 ± 0.30
pKa2 = 7.97 ± 0.33
pKa3 = 17.03 ± 0.30

Tryptophan butyl ester



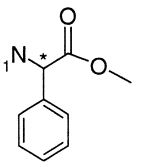
pKa1 = -0.92 ± 0.30
pKa2 = 7.74 ± 0.33
pKa3 = 17.02 ± 0.30

Tyrosine methyl ester



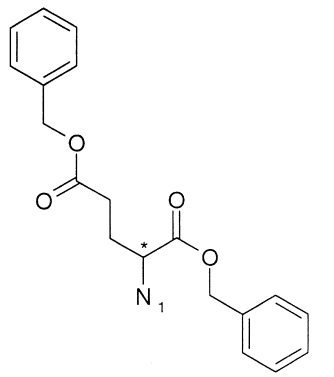
pKa1 = 7.36 ± 0.15
pKa2 = 10.10 ± 0.33

Phenylglycine methyl ester



pKa1 = 6.63 ± 0.29

Glutamic dibenzyl acid ester

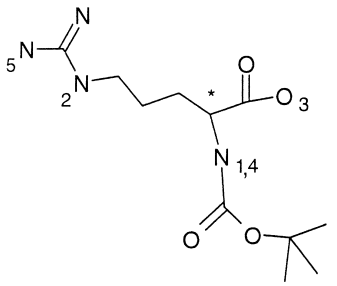


pKa1 = 6.78 ± 0.35

Fig. 1. Structure and estimated p$K_a$ values of amino acid derivatives: (a) Amino acid alkyl esters; (b) *N*-amino acid derivatives.

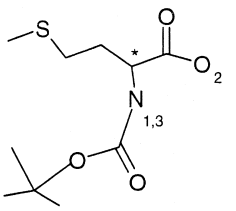
(b) N-amino acid derivatives

N-t-Boc Arginine



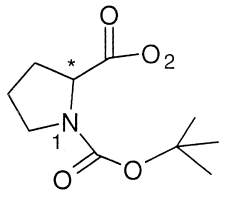
pKa1 = - 1.71 ± 0.36
pKa2 = 0.21 ± 0.50
pKa3 = 3.96 ± 0.21
pKa4 = 13.28 ± 0.46
pKa5 = 13.73 ± 0.36

N-t-Boc Methionine



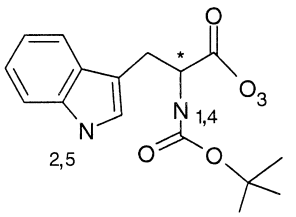
pKa1 = - 1.71 ± 0.7
pKa2 = 3.83 ± 0.1
pKa3 = 13.16 ± 0.46

N-t-Boc Proline



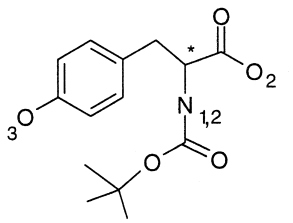
pKa1 = -3.22 ± 0.4
pKa2 = 3.83 ± 0.1

N-t-Boc Tryptophan



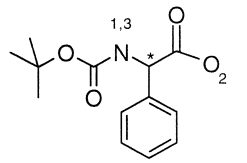
pKa1 = - 1.75 ± 0.70
pKa2 = - 0.23 ± 0.30
pKa3 = 4.00 ± 0.10
pKa4 = 13.34 ± 0.46
pKa5 = 17.48 ± 0.30

N-t-Boc Tyrosine



pKa1 = - 1.70 ± 0.70
pKa2 = 3.97 ± 0.10
pKa3 = 10.32 ± 0.15
pKa4 = 13.32 ± 0.16

N-t-Boc Phenylglycine

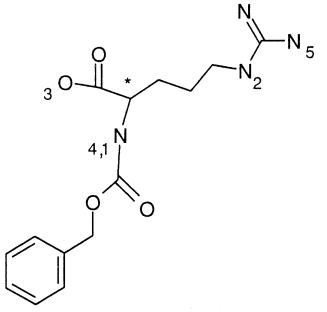


pKa1 = - 1.67 ± 0.70
pKa2 = 3.51 ± 0.10
pKa3 = 12.84 ± 0.46

Fig. 1. (*continued*).

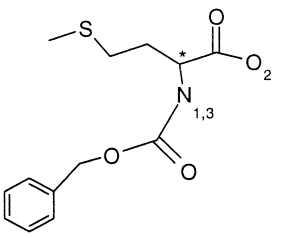
(b) cont'd. N-amino acid derivatives

N-CBZ-Arginine



pKa1 = - 1.76 ± 0.70
pKa2 = 0.21 ± 0.50
pKa3 = 3.94 ± 0.21
pKa4 = 13.07 ± 0.46
pKa5 = 13.73 ± 0.36

N-CBZ-Methionine



pKa1 = - 1.76 ± 0.70
pKa2 = 3.81 ± 0.10
pKa3 = 12.95 ± 0.46

Fig. 1. (*continued*).

the separation of several uncharged or weakly negatively charged dansyl-amino acids with SBE-β-CD at low pH.

As only one CD is studied, the smaller $2^{3-1}$ design is used to screen the compounds. The factors to be tested are: (i) the CD concentration, (ii) the pH of the electrolyte and (iii) the % of methanol in the electrolyte. The description of the factors, the levels and the design are shown in Tables 3 and 4.

If no baseline resolution is achieved after applying the design, an additional experimental design, such as a $2^2$ (2 factors at 2 levels) experimental design can then be applied after evaluation of the effects of the factors. Methanol is not considered as a varying factor anymore but kept at the best level suggested by the first screening. Indeed, it is very useful, in a first approach, to know whether the presence of methanol can improve the separation or not but the determination of its optimal content in the buffer is not necessary. The new CD concentrations and pH levels are determined depending on the calculated effects of the factors. If baseline resolution is still not obtained, the whole procedure applied with SBE-β-CD can be repeated with CM-β-CD and afterwards with HP-β-CD. In order to achieve mobility of the complex, the pH levels must then be adapted so that either the CD or/and the analyte are charged.

### *3.2. Separation of the amino acid alkyl esters*

The resolution values obtained by applying the $3^{4-2}$ fractional factorial design are presented in Table 5.

The best resolution values and the corresponding migration times obtained for each compound and the analysis conditions can be seen in Table 6.

Baseline separation was obtained for each compound (Fig. 3)

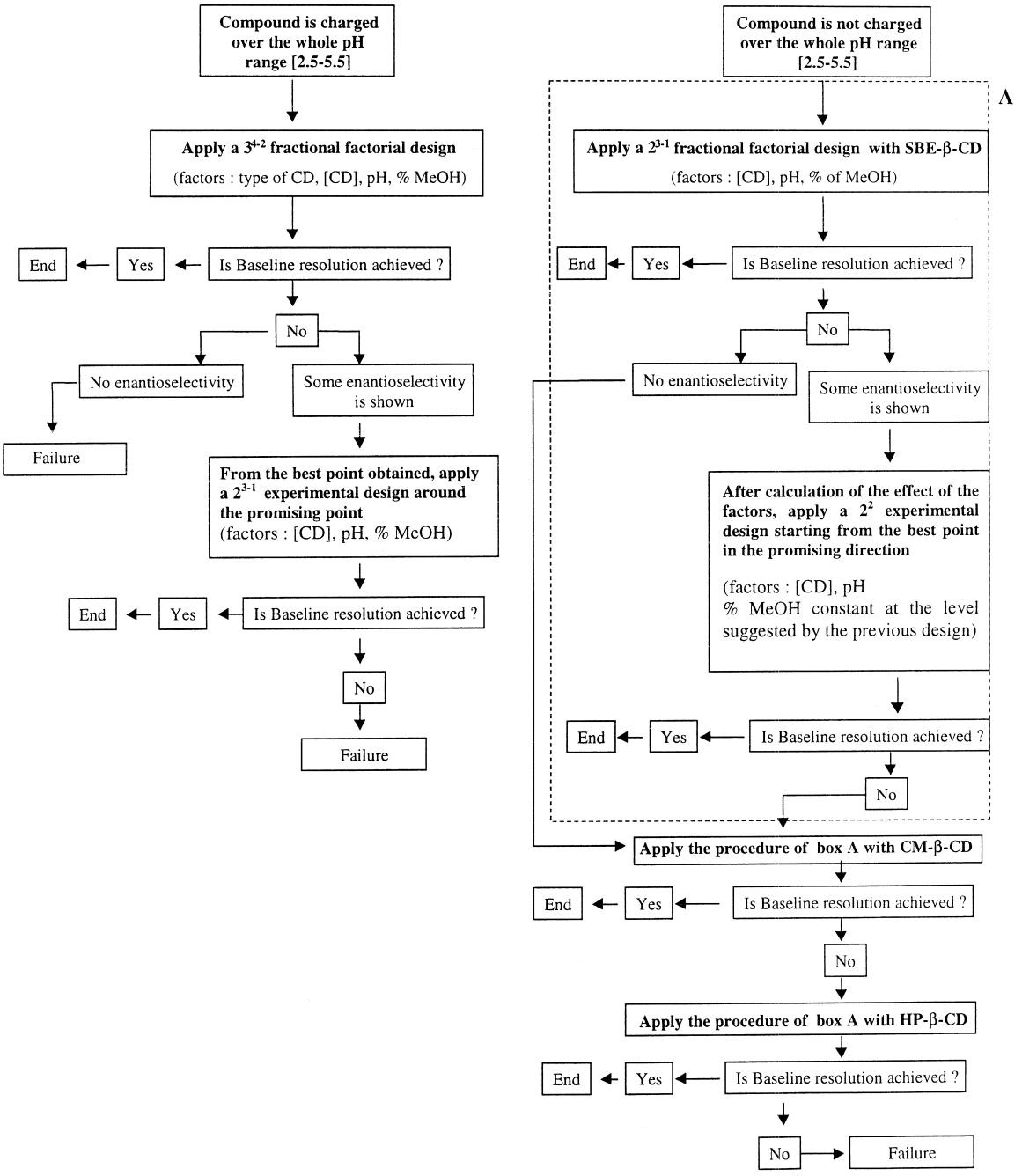



Fig. 2. Summary of method development strategies followed.

The effects of the factors were not computed because they have little physical meaning in our experimental setup since the measured effects of e.g. the cyclodextrin concentration would be an average for the three CDs while, of course, its effect on each CD may be very different. The design was used here

Table 1
Factors and levels of the $3^{4-2}$ fractional factorial design applied to the amino acid alkyl esters

| Factor | Level | | |
|---|---|---|---|
| | −1 | 0 | 1 |
| CD type | SBE-β-CD | CM-β-CD | HP-β-CD |
| [CD] (m*M*) | | | |
| HP-β-CD | 10 | 50 | 120 |
| SBE-β-CD, CM-β-CD | 5 | 15 | 30 |
| pH | 2.5 | 4 | 5.5 |
| MeOH (%) | 0 | 15 | 30 |

Table 2
The $3^{4-2}$ fractional factorial design applied to screen the amino acid alkyl esters

| Experiment | Factor | | | |
|---|---|---|---|---|
| | CD type | [CD] | pH | % MeOH |
| 1 | −1 | 0 | 1 | 1 |
| 2 | 0 | −1 | 0 | 1 |
| 3 | 0 | 0 | −1 | 0 |
| 4 | 1 | 0 | 0 | −1 |
| 5 | −1 | 1 | 0 | 0 |
| 6 | 1 | −1 | 1 | 0 |
| 7 | 1 | 1 | −1 | 1 |
| 8 | 0 | 1 | 1 | −1 |
| 9 | −1 | −1 | −1 | −1 |

Table 3
Factors and levels of the $2^{3-1}$ fractional factorial design applied to the *N*-t-Boc and *N*-CBZ amino acids

| Factor | Level | |
|---|---|---|
| | −1 | 1 |
| [SBE-β-CD] (m*M*) | 5 | 15 |
| pH | 2.5 | 4 |
| MeOH (%) | 0 | 15 |

Table 4
The $2^{3-1}$ fractional factorial design applied to screen the *N*-t-Boc and *N*-CBZ amino acids

| Experiment | Factor | | |
|---|---|---|---|
| | [SBE-β-CD] | pH | % MeOH |
| 1 | −1 | −1 | −1 |
| 2 | 1 | −1 | 1 |
| 3 | −1 | 1 | 1 |
| 4 | 1 | 1 | −1 |

not to make predictions about the factors' effects but to cover the experimental domain maximally in a minimal number of experiments.

Each of the three cyclodextrins allows the separation of all tryptophan alkyl derivatives but the optimal CD differs from one analyte to another. In the domain selected, CM-β-CD performs better than the others to resolve the enantiomers of tryptophan methyl ester and tryptophan ethyl ester while SBE-β-CD allows a better resolution of the tryptophan butyl ester. Best analysis conditions vary from methyl to ethyl derivative: for tryptophan methyl ester, the best resolution occurs with CM-β-CD, 30 m*M* at pH 5.5 without methanol while the enantiomeric resolution of tryptophan ethyl ester is best with CM-β-CD, 15 m*M* at pH 2.5 with 15% of methanol.

No chiral recognition is observed for the tyrosine methyl ester with HP-β-CD. Best separation of the enantiomers is obtained with CM-β-CD, 30 m*M* at pH 5.5 without methanol.

For phenylglycine methyl ester and dibenzyl glutamic acid ester, only the CM-β-CD shows any enantioselectivity. Baseline resolution is obtained for phenylglycine methyl ester with 30 m*M* of CD, pH 5.5 without methanol. Dibenzyl glutamic acid ester enantiomers are best resolved with 15 m*M* of CD at pH 2.5 with 15% of methanol.

From the results, it can be concluded that no general best analysis conditions can be defined. However, the nine experiments performed allowed to define baseline separation conditions for all substances. It was therefore not necessary to perform additional experimental designs, as suggested earlier.

### *3.2.1. Influence of the drug structure on the separation*

Concerning the tryptophan derivatives, we can observe the large influence of the alkyl chain on the chiral recognition. Methyl and ethyl derivatives are better separated with CM-β-CD while the butyl derivative is better separated with SBE-β-CD. We can also see, comparing the results obtained for the methyl and the ethyl derivatives, that the resolution is globally better in the case of the ethyl group than in the case of the methyl group. This is in accordance with the theory of the inclusion complexation which has been developed to explain the chiral recognition using cyclodextrins [38]. The chiral

Table 5
Resolution values found in the screening of the amino acid alkyl esters using the $3^{4-2}$ fractional factorial design

| Experiment | Compound | | | | | |
|---|---|---|---|---|---|---|
| | Tryptophan methyl ester | Tryptophan ethyl ester | Tryptophan butyl ester | Tyrosine methyl ester | Phenylglycine methyl ester | Glutamic acid dibenzyl ester |
| 1 | 0.58 | 2.23 | **10.90**[a] | 0.00 | 0.00 | 0.00 |
| 2 | 1.81 | 6.45 | 0.00 | 2.07 | 0.68 | 1.24 |
| 3 | 2.83 | **11.70** | 6.38 | 0.70 | 1.35 | **8.44** |
| 4 | 2.05 | 3.44 | 2.30 | 0.00 | 0.00 | 0.00 |
| 5 | 1.46[a] | 0.43[a] | 2.51[a] | 1.65[a] | 0.00 | 0.00 |
| 6 | 0.37 | 2.23 | 3.24 | 0.00 | 0.00 | 0.00 |
| 7 | 2.53 | 3.56 | 0.80 | 0.00 | 0.00 | 0.00 |
| 8 | **4.32** | 8.37 | 0.00 | **3.48** | **1.85** | 7.90 |
| 9 | 1.56 | 1.46 | 2.54[a] | 0.00 | 0.00 | 0.00 |

[a] Reverse polarity (−25 kV).

recognition is assumed to be based on the inclusion of a bulky hydrophobic part (generally the aromatic moiety if present) in the hydrophobic cavity of the CD. An additional requirement is that secondary interactions have to occur; these include dipole–dipole interactions or hydrogen bonds between the hydroxyl groups at the edge of the CD and substituents close to the chiral center of the analyte. The influence of the length of non polar substituents on the chiral recognition was studied by Desiderio et al. [36]. They concluded that non-polar substituent groups on the asymmetric carbon enhance in many cases the complexation and the stereoselectivity.

### *3.3. Separation of the N-t-Boc and N-CBZ amino acids*

The resolution values obtained for each compound are presented in Table 7.

The best resolution values obtained, the corresponding analysis times and analysis conditions are described in Table 8. Electropherograms can be seen in Fig. 4.

*N*-t-Boc-Tryptophan, *N*-t-Boc-tyrosine, *N*-t-Boc-methionine and *N*-t-Boc-arginine are baseline separated. *N*-t-Boc-phenylglycine, CBZ-arginine and CBZ-methionine show some enantioselectivity but are not sufficiently resolved. For the *N*-t-Boc-proline, no chiral recognition is observed. Also here, no general best analysis conditions were found. All migration times are short which means that the complexation of the analytes with the SBE-β-CD is high.

As only one CD is tested in the $2^{3-1}$ experimental design, the influence of each factor on the resolution can be calculated (Fig. 5).

The effects suggest that high concentration of CD combined with low pH and high methanol content appear to be the best analysis conditions for the enantiomeric separation of *N*-t-Boc-tryptophan and

Table 6
Separation conditions with the highest resolution values obtained in the screening of the amino acid alkyl esters using the $3^{4-2}$ fractional factorial design

| Compound | CD type | [CD] (m*M*) | pH | MeOH (%) | Resolution | Analysis time (min) |
|---|---|---|---|---|---|---|
| Tryptophan methyl ester | CM-β-CD | 30 | 5.5 | 0 | 4.32 | 15.56 |
| Tryptophan ethyl ester | CM-β-CD | 15 | 2.5 | 15 | 11.70 | 20.66 |
| Tryptophan butyl ester | SBE-β-CD | 15 | 5.5 | 30 | 10.90[a] | 28.56 |
| Tyrosine methyl ester | CM-β-CD | 30 | 5.5 | 0 | 3.48 | 40.19 |
| Phenylglycine methyl ester | CM-β-CD | 30 | 5.5 | 0 | 1.85 | 9.36 |
| Glutamic acid dibenzyl ester | CM-β-CD | 15 | 2.5 | 15 | 8.44 | 25.26 |

[a] Reverse polarity (−25 kV).

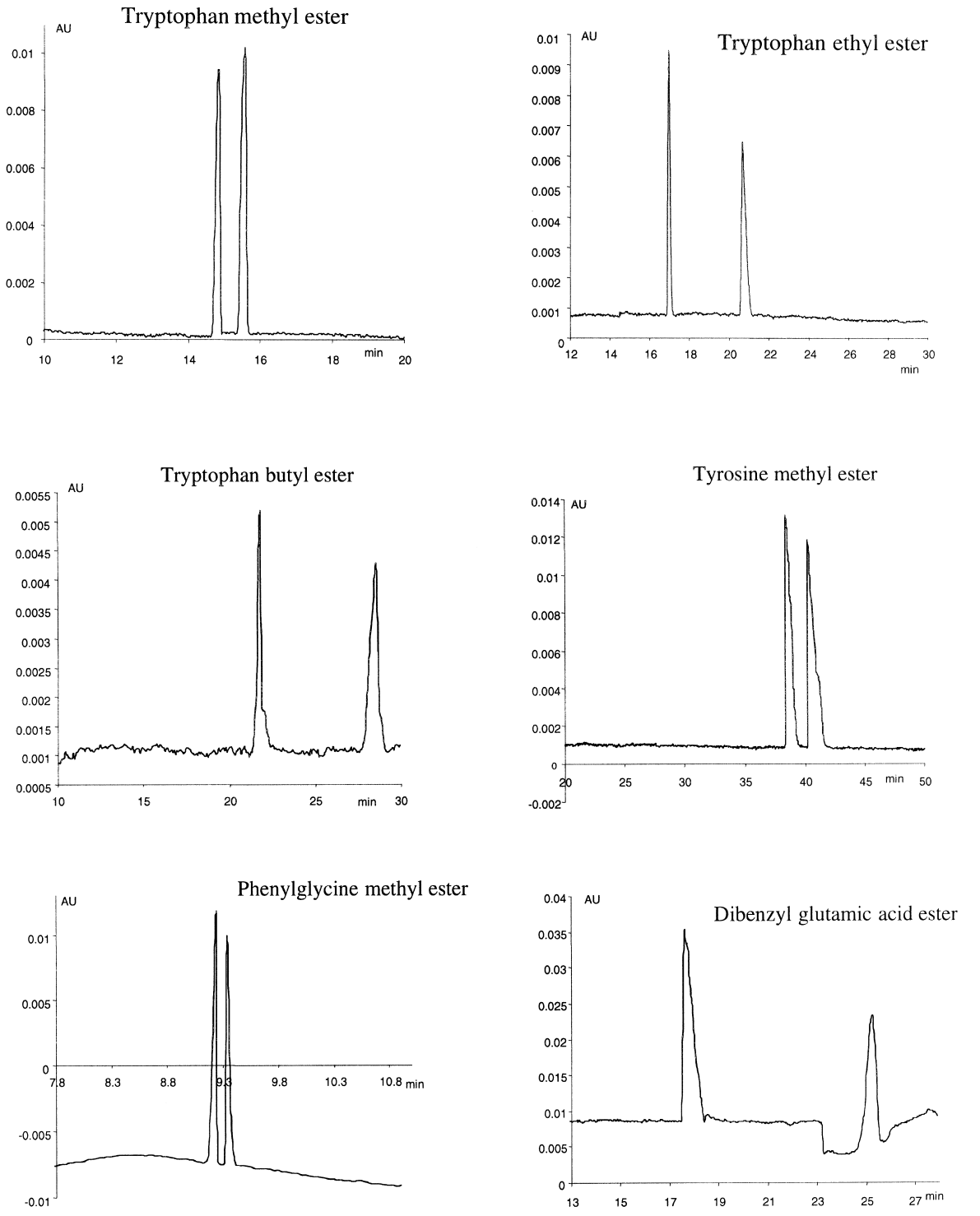



Fig. 3. Electropherograms of the separation of the amino acid alkyl esters obtained under the best analysis conditions (see Table 6) found in the $3^{4-2}$ experimental design.

Table 7
Resolution values found in the screening of the *N*-t-Boc and CBZ amino acids using the $2^{3-1}$ fractional factorial design

| Experiment | Compound | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *N*-t-Boc-Arginine | *N*-t-Boc-Methionine | *N*-t-Boc-Proline | *N*-t-Boc-Tryptophan | *N*-t-Boc-Tyrosine | *N*-t-Boc-Phenylglycine | *N*-CBZ-Arginine | *N*-CBZ-Methionine |
| 1 | 0.30 | 1.80 | 0.00 | 2.81 | 1.56 | 0.70 | **0.88** | **0.68** |
| 2 | 0.71 | **1.94** | 0.00 | **3.73** | **2.36** | 0.68 | 0.44 | 0.54 |
| 3 | 0.54 | 1.71 | 0.00 | 3.20 | 1.78 | 0.81 | 0.32 | 0.56 |
| 4 | **2.37** | 1.74 | 0.00 | 2.62 | 0.79 | **0.95** | 0.00 | 0.00 |

*N*-t-Boc-methionine. Those conditions correspond to Experiment 2 which gives indeed the best resolution value for those two compounds.

Low pH and presence of methanol are favourable for the separation of *N*-t-Boc-tyrosine enantiomers. The influence of the concentration is not important compared to the other factors in the investigated interval. Best resolution is obtained in Experiment 2 with low pH, methanol and high concentration of cyclodextrin.

High concentration of CD combined with high pH and no methanol appears to be the best analysis conditions for the enantiomeric separation of *N*-t-Boc-arginine. Those conditions correspond to Experiment 4 which gives indeed the best resolution value for those two compounds.

In the case of *N*-t-Boc-phenylglycine, no baseline separation was obtained. Effects of the factors suggest that high concentration of cyclodextrin combined with high pH without methanol should be favorable for the separation of its enantiomers. Thus, an additional experimental design was performed (see Fig. 2). Experiment 4, which gives the best resolution value, was taken as a starting point for the new design. A $2^2$ factorial design was constructed in the direction suggested by the effect of the factors. No methanol was added to the buffer. The pH levels were 4 and 5.5 and the concentrations of the CD were varied from 15 m*M* to 30 m*M*. The design and the levels can be seen in Tables 9 and 10.

In order to avoid too high current due to an increase of the CD concentration, the voltage was set to −18 kV instead of −20 kV. Results can be seen in Table 11.

Enantiomers of the *N*-t-Boc phenylglycine are now well resolved (Fig. 6). Calculation of the effect of the factors (Fig. 7) show that low concentration of the CD (15 m*M*) and high pH (5.5) should provide the best separation. Those conditions correspond to Experiment 3 which indeed gives the best resolution value.

From the results of the two experimental designs, we can see that the optimum concentration of the CD for the separation passes through a maximum.

For *N*-t-Boc-proline, no enantioselectivity was observed with SBE-β-CD. Therefore, CM-β-CD was used as selector in another $2^{3-1}$ fractional factorial design (see Fig. 2). Since at low pH, neither the

Table 8
Separation conditions with the highest resolution values found obtained in the screening of the *N*-t-Boc and *N*-CBZ amino acids using the $2^{3-1}$ fractional factorial design

| Compound | [SBE CD] (m*M*) | pH | MeOH (%) | Resolution | Analysis time (min) |
|---|---|---|---|---|---|
| *N*-t-Boc-Arginine | 15 | 4 | 0 | 2.37 | 10.64 |
| *N*-t-Boc-Methionine | 15 | 2.5 | 15 | 1.94 | 7.42 |
| *N*-t-Boc-Proline | | | | 0.00 | |
| *N*-t-Boc-Tryptophan | 15 | 2.5 | 15 | 3.73 | 6.82 |
| *N*-t-Boc-Tyrosine | 15 | 2.5 | 15 | 2.36 | 6.50 |
| *N*-t-Boc-Phenylglycine | 15 | 4 | 0 | 0.95 | 4.98 |
| *N*-CBZ-Arginine | 5 | 2.5 | 0 | 0.88 | 7.01 |
| *N*-CBZ-Methionine | 5 | 2.5 | 0 | 0.68 | 10.28 |

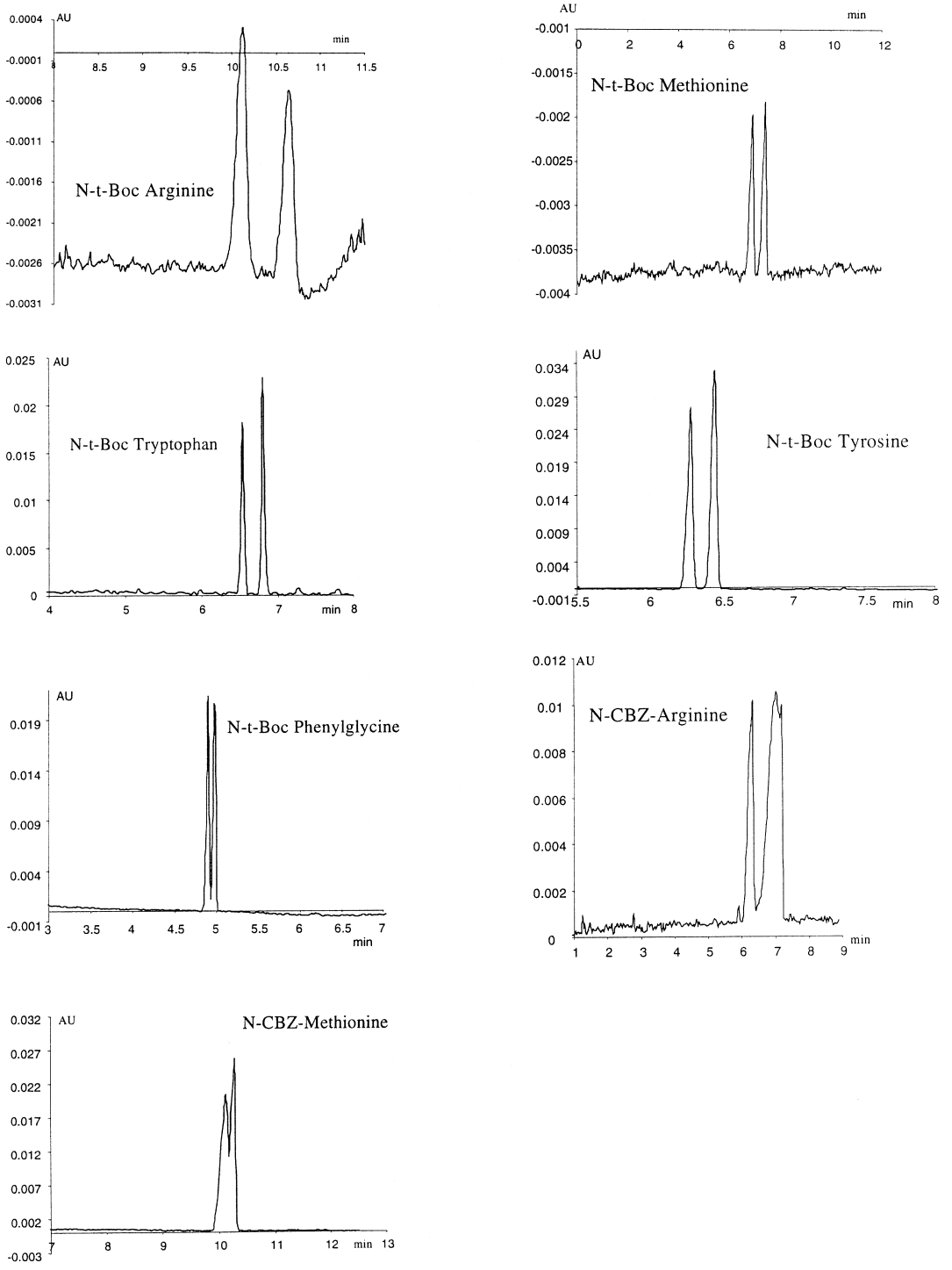



Fig. 4. Electropherograms of the separation of the *N*-t-Boc and *N*-CBZ amino acids obtained under the best analysis conditions (see Table 8) found in the first $2^{3-1}$ experimental design.

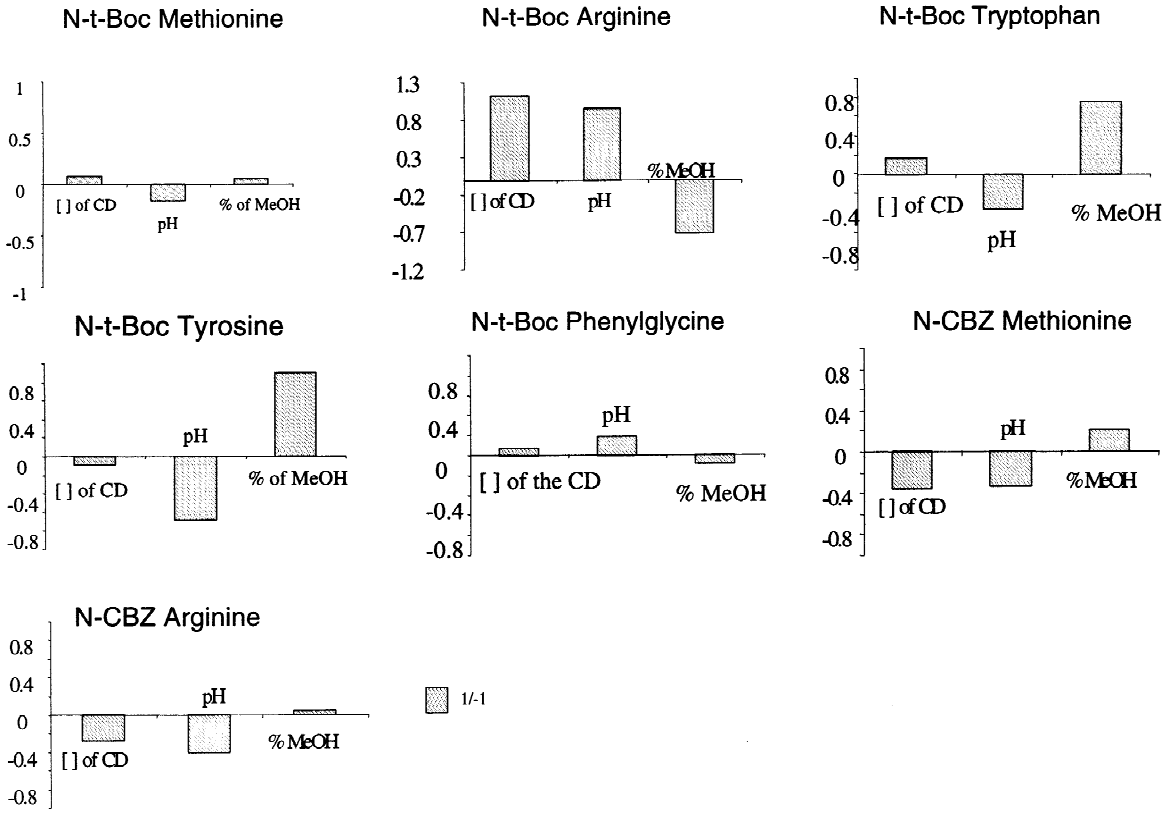



Fig. 5. Calculated effects for the *N*-t-Boc and *N*-CBZ amino acids from the $2^{3-1}$ fractional factorial design.

Table 9
Factors and levels of the factorial designs $2^2$ applied to the *N*-t-Boc phenyglycine

| Factor | Level | |
|---|---|---|
| | −1 | 1 |
| [SBE-β-CD] (m*M*) | 15 | 30 |
| pH | 4 | 5.5 |

Table 10
The $2^2$ fractional factorial design applied to the *N*-t-Boc phenylglycine

| Experimental | Factor | |
|---|---|---|
| | [SBE-β-CD] | pH |
| 1 | −1 | −1 |
| 2 | 1 | −1 |
| 3 | −1 | 1 |
| 4 | 1 | 1 |

Table 11
Resolution values found in the second screening of the *N*-t-Boc–phenylglycine

| Experiment | Resolution | Analysis time (min) |
|---|---|---|
| 1 | 1.80 | 6.70 |
| 2 | 1.64 | 6.91 |
| 3 | **3.28** | 14.67 |
| 4 | 1.77 | 15.00 |

analyte nor the CD is charged, the pH levels were changed from 2.5–4 to 5.5–7.5 (Table 12).

In that pH interval, the analyte and the CD are both negatively charged. At pH 5.5, the analyte–CD complex migrates towards the anode while it migrates towards the cathode at pH 7.5 due to an increase of the EOF (although triethanolamine was added). Baseline separation was achieved at pH 5.5 with 15 m*M* of CM-β-CD and 15% MeOH (Table

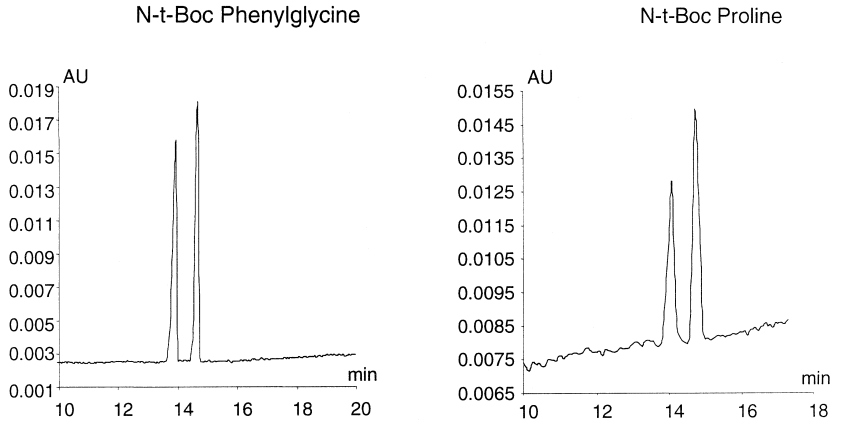



Fig. 6. Electropherogram of *N*-t-Boc-phenylglycine and *N*-t-Boc-proline under best conditions (see Tables 12 and 13) obtained after applying a second experimental design.

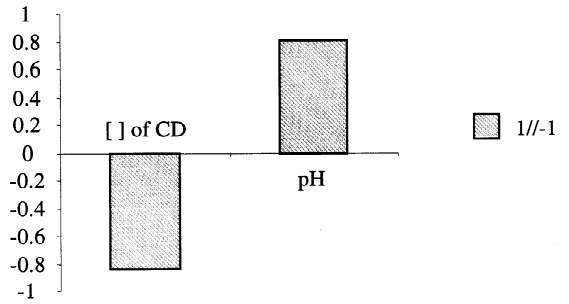



Fig. 7. Calculated effects for the *N*-t-Boc-phenylglycine from the $2^2$ fractional factorial design.

13 and Fig. 6). A too high pH value had a bad effect on the resolution. This effect can be attributed to the high EOF that reduces the separation between the two enantiomers.

Resolution values of only 0.68 and 0.88 were achieved for CBZ-methionine and CBZ-arginine, respectively (Table 8). For the latter, near baseline resolution was obtained but an important deformation of the peak shape was also observed. The effects of the factors (Fig. 5) suggest that a decrease of the CD concentration and the pH should be favourable for the separation of both type of enantiomers. Since the low pH level considered in the design was 2.5, a decrease of this factor was not performed because this would lead to unstable analysis conditions. Therefore, only the CD concentration was gradually decreased from 5 to 1 m*M* with no methanol for the CBZ-arginine and 15% of methanol for CBZ-methionine. With 4 or 3% of CD, no increase or even decrease of the resolution values occurs for both analytes. With 1 or 2%, no peaks are observed within 90 min (both polarities were checked). Additional $2^{3-1}$ experimental designs were performed successively with CM-β-CD and HP-β-CD (see Fig. 2) as chiral selectors, but no enantioselectivity was observed in any situation. Although these experiments do not allow to find a solution, they are useful as rapid means to exclude CM-β-CD and HP-β-CD

Table 12
Factors and levels of the $2^{3-1}$ fractional factorial design applied to the *N*-t-Boc proline

| Factor | Level | |
|---|---|---|
| | −1 | 1 |
| [CM-β-CD) (m*M*) | 5 | 15 |
| pH | 5.5 | 7.5 |
| MeOH (%) | 0 | 15 |

Table 13
Resolution values found in the second screening of the *N*-t-Boc-proline

| Experiment | Resolution | Analysis time (min) |
|---|---|---|
| 1 | 1.12 | 12.55 |
| 2 | **1.39** | 14.74 |
| 3 | 0.10 | 9.45 |
| 4 | 0.20 | 8.22 |

from further consideration, thus avoiding unnecessary lengthy experimentation, which very probably would not lead to results anyway.

*3.3.1. Influence of the drug structure*

From the results, we have seen that the separation of *N*-CBZ derivatives of arginine and methionine was much more difficult to achieve than for the corresponding *N*-t-Boc derivatives.

Supposing that *N*-t-Boc and *N*-CBZ are the moieties which are locked into the CD cavity [2], then it can be seen that the functions directly connected to the chiral carbon and which are responsible for chiral recognition are the same. Thus, we can conclude that the enantioselectivity difference observed is due the difference of distance between the hydrophobic moiety of the molecule and its asymmetric center. This distance is more important in the case of *N*-CBZ derivatives and this must hinder the chiral recognition.

## 4. Conclusion

A scheme for the chiral separation of amino acid derivatives was developed based on an experimental design approach. The three CDs, HP-β-CD, CM-β-CD and SBE-β-CD have shown to be very effective and should lead to the separation of a wide range of analytes. Experimental designs appear to be very efficient to determine in a few experiments experimental conditions where baseline separation of the compounds can be achieved. Of the 14 compounds investigated, 4 were enantiomerically resolved within 4 experiments and 8 within 8 experiments. For the other two, it is made clear in a reasonable number of experiments, that there is no solution to be found with the chiral selectors studied and that different approaches, e.g. with highly sulfated cyclodextrins, are required. The use of experimental design is very useful because, as the results show, no general experimental conditions could be found within this family of compounds.

The strategy defined in this paper will be used in a future work as a base for the development of a more general strategy for the chiral separations of different families of compounds.

## Acknowledgements

Y.V.H. is a postdoctoral fellow of the Fund for Scientific Research (F.W.O. Vlaanderen).

## References

[1] FDA Policy, Statement for the Development of New Stereoisomeric Drugs, Washington, DC, May 1992.
[2] G.G. Yowell, S.D. Fazio, R.V. Vivilecchia, J. Chromatogr. A 745 (1996) 73.
[3] C. Petterson, J. Chromatogr. 316 (1984) 553.
[4] S.C. Chang, L.R. Wang, D.W. Armstrong, J. Liq. Chromatogr. 15 (1992) 1411.
[5] J.B. Esquivel, C. Sanchez, M.J. Fazio, J. Liq. Chromatogr. 21 (1998) 777.
[6] C.C. Chen, C.E. Lin, J. Chromatogr. Sci. 33 (1995) 229.
[7] F. Yasuhara, M. Takeda, Y. Ochiai, Chem. Lett. 2 (1992) 251.
[8] S. Hara, A. Dobashi, K. Kinoshita, T. Hondo, M. Saito, M. Senda, J. High Resolut. Chromatogr. 371 (1986) 153.
[9] S. Fanali, J. Chromatogr. A 792 (1997) 227.
[10] B. Chankvetadze, J. Chromatogr. A 792 (1997) 269.
[11] A. Nardi, L. Ossicini, S. Fanali, Chirality 4 (1992) 56.
[12] S. Terabe, J. Chromatogr. 636 (1993) 47.
[13] T. Ueda, Y. Kitamura, Y. Ishihama, O. Shibata, Anal. Chem. 63 (1991) 2979.
[14] S. Boonkerd, M.R. Detaevernier, Y. Vander Heyden, J. Vindevogel, Y. Michotte, J. Chromatogr. A 736 (1997) 281.
[15] K. Lilley, T.E. Wheat, J. Chromatogr. B 683 (1996) 67.
[16] E. Vaserio, J.Y. Gauvrit, R. Longeray, P. Lantern, J.L. Veuthey, Electrophoresis 18 (1997) 931.
[17] M.M. Rogan, K.D. Altria, Chromatographia 38 (1994) 723.
[18] M.G. Vargas, Y. Vander Heyden, M. Maftouh, D.L. Massart, J. Chromatogr. A 855 (1999) 681.
[19] Y. Daali, S. Cherkaoui, J.L. Veuthey, Electrophoresis 20 (1999) 3424.
[20] S. Fanali, S. Furlanetto, S. Pinzauti, Chromatographia 48 (1998) 395.
[21] L. Mateus, S. Cherkaoui, P. Christen, J.L. Veuthey, J. Chromatogr. A 868 (2000) 285.
[22] A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg, B.L. Karger, J. Chromatogr. 448 (1988) 41.
[23] J. Snopek, I. Jelinek, E. Smolkova-Keulemansova, J. Chromatogr. 438 (1988) 211.
[24] B.A. Williams, G. Vigh, J. Chromatogr. A 777 (1997) 295.
[25] I.E. Valko, H.A.H. Billiet, J. Frank, K.C.A.M. Luyben, Chromatographia 38 (1994) 730.
[26] S.A.C. Wren, J. Chromatogr. A 636 (1993) 57.
[27] W. Lindner, B. Böhs, V. Seidel, J. Chromatogr. A 697 (1995) 549.
[28] C. Dette, S. Ebel, S. Terabe, Electrophoresis 15 (1994) 799.
[29] R.J. Tait, D.O. Thompson, V.J. Stella, J.F. Stobaugh, Anal. Chem. 66 (1994) 4013.

[30] B. Chankvetadze, G. Endresz, G. Blaschke, Electrophoresis 15 (1994) 804.
[31] B. Chankvetadze, G. Endresz, G. Blaschke, J. Cap. Electrophoresis 2 (1995) 235.
[32] M. Fillet, I. Bechet, P. Chiap, P. Hubert, J. Crommen, J. Chromatogr. A 717 (1995) 203.
[33] C. Weinz, G. Blaschke, J. Chromatogr. B 674 (1995) 287.
[34] M.G. Schmidt, K. Wirnsberger, G. Gübitz, Pharmazie 51 (1996) 852.
[35] F. Wang, M.G. Khaledi, Anal. Chem. 68 (1996) 3460.
[36] C. Desiderio, S. Fanali, J. Chromatogr. A. 716 (1995) 183.
[37] G.M. Janini, G.M. Muschik, H.J. Issaq, Electrophoresis 17 (1996) 1575.
[38] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.