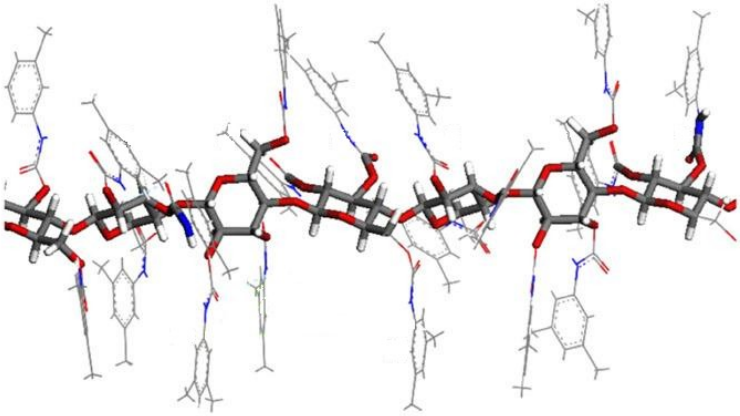
University of Liège

Faculty of Medicine

Laboratory of Analytical Pharmaceutical Chemistry

Professor M. FILLET

LC stereoselective determination of chiral basic molecules using polysaccharide-based chiral stationary phases and non-aqueous polar organic mobile phases



Katina Sourou Sylvestre DOSSOU

Biochemist

DES in Biomedical Sciences option Toxicology

DEA in Pharmaceutical Sciences

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Abstract of the thesis

The increasing number of analytical methods for the enantioseparation of chiral drug molecules is no doubt related to the development of more efficient tools such as chiral stationary phases (CSPs) for direct enantioresolution in liquid chromatography (LC).

In this work, the recognition ability of three new chlorine containing cellulose-based chiral stationary phases, Sepapak-2, and Sepapak-4 and Sepapak-5, towards the enantiomers of thirteen (13) chiral pharmaceuticals with widely different structures and polarities, has been studied in polar organic solvent chromatography (POSC). Since limited information is available in literature about these CSPs used with polar organic mobile phases, the influence of parameters such as temperature, the percentage of polar (methanol) or non-polar (n-hexane) organic modifier, the nature and concentration of the acidic additive, namely trifluoroacetic acid (TFA), formic acid (FA) or acetic acid (AcA), in the mobile phase, was first evaluated on Sepapak-4 using an univariate approach. In all experiments, acetonitrile was selected as the main component of the mobile phase.

The experimental design methodology was then applied for the screening of these factors (except the polar organic modifier), the nature of the basic additive in the mobile phase being included as an additional factor. The application of an optimization design (a face centered central composite design) led to the evaluation of the effects of different factors on enantioresolution for ten of the studied compounds. Furthermore, optimal conditions for the enantioseparation of these chiral molecules could be deduced and two conditions for rapid screening of chiral compounds on Sepapak-4, namely ACN/0.1% DEA/0.1% TFA/5% n-hexane and ACN/0.1% DEA/0.2% FA/5% n-hexane, were retained and tested on other drug molecules at 25°C.

A rapid screening strategy for method development including mobile phases containing TFA (0.1%), FA (0.2%) or AcA (0.2) was proposed and the behavior of the three chlorinated CSPs was compared under these conditions. The following mobile phases were tested at 25°C: ACN/0.1% DEA/0.1%TFA, ACN/0.1% DEA/0.2% FA and ACN/0.1% DEA/0.2% AcA. Sepapak-2 was found to have an intermediate behaviour leading to lower enantioresolution values compared to Sepapak-4 and Sepapak-5. On the other hand, with at maximum four

experiments, it was possible to separate the enantiomers of each of the thirteen studied compounds.

In the part of the thesis devoted to the development of applications, tests were conducted in order to optimize the enantioresolution of ropivacaine, a local anesthetic, the pharmaceutical formulation of which consists of an enantiopure aqueous solution of the S-enantiomer. A reversal of the elution order of the enantiomers, related to the nature of the acidic additive, was observed on both Sepapak-2 and -4. On the contrary, no reversal of the enantiomer elution order was found when Sepapak-5 and Chiralcel OD-H, a CSP with cellulose tris (3,5-dimethylphenylcarbamate) as chiral selector, were used under the same conditions. The best chromatographic conditions for the enantiomeric purity determination of S-ropivacaine were then selected and the developed method was fully validated according to the strategy based on the use of accuracy profiles, which takes into account the total error, i.e. the estimation of the systematic and random errors of measurement results.

The second application that we have developed deals with the enantiomeric purity determination of S-amlodipine in a pharmaceutical formulation. A reversal of the enantiomer elution order caused by changes in the concentration of formic acid (FA) in the mobile phase was pointed out, using Sepapak-4 and Sepapak-2 as CSPs. Studies on the enantioseparation of amlodipine in these chromatographic systems led to the selection of the most suitable CSP and the optimal concentration of formic acid in the mobile phase with respect to the elution order (R-amlodipine eluting first) and the resolution value. The method was then prevalidated using the same approach as for S-ropivacaine.

Résumé de la thèse

Le nombre croissant de méthodes analytiques pour l'énantioséparation de molécules médicamenteuses chirales est sans aucun doute lié au développement d'outils de plus en plus performants tels que de nouvelles phases stationnaires chirales (PSC) pour l'enantiorésolution directe en chromatographie liquide (CL).

Dans ce travail, le pouvoir de résolution de trois nouvelles PSC constituées de dérivés chlorés de phénylcarbamate de cellulose, Sepapak-2, Sepapak-4 et Sepapak-5, vis-à-vis des énantiomères de treize (13) molécules thérapeutiques chirales avec des structures et des polarités très différentes, a été étudié par chromatographie en phase organique polaire (POSC). Ces PSC ayant été peu utilisées jusqu'à présent avec des phases organiques polaires, l'influence des paramètres comme la température, le pourcentage en modificateur organique polaire (méthanol) ou apolaire (n-hexane), la nature et la concentration de l'additif acide, à savoir l'acide trifluoroacétique (TFA), l'acide formique (FA) ou l'acide acétique (AcA), dans la phase mobile, a d'abord été évaluée sur Sepapak-4 au moyen d'une approche univariée. Dans tous les cas, l'acétonitrile a été choisi comme principal composant de la phase mobile.

La méthodologie des plans d'expériences a ensuite été appliquée pour le criblage de ces facteurs (sauf le modificateur organique polaire), la nature de l'additif basique dans la phase mobile étant par ailleurs incluse comme facteur supplémentaire. L'application d'un plan d'optimisation (plan central composite à face centrée) a permis l'évaluation des effets des différents facteurs sur l'énantiorésolution de dix des composés chiraux étudiés. En outre, les conditions optimales pour l'énantiorésolution de ces molécules chirales ont pu être déduites et deux conditions pour le criblage rapide de composés chiraux sur Sepapak-4, à savoir ACN/0,1% DEA/0,1% TFA/5% n-hexane et ACN/0,1% DEA/0,2 % FA/ 5% n-hexane, ont été retenues et testées sur d'autres molécules chirales à 25 °C.

Une stratégie de criblage rapide pour le développement de méthodes, incluant des phases mobiles contenant du TFA (0,1%), du FA (0,2%) ou de l'AcA (0,2%) a été proposée et le pouvoir de résolution de ces trois PSC chlorées a été comparé dans ces conditions. Les phases mobiles suivantes ont été testées à 25°C: ACN/0,1% DEA/0,1% TFA, ACN/0,1% DEA/0,2 % FA et ACN/0,1% DEA/0,2% AcA. Un comportement intermédiaire a été observé dans le cas de Sepapak-2, entraînant des valeurs d'énantiorésolution plus faibles par rapport à celles obtenues avec Sepapak-4 et Sepapak-5. Par ailleurs, il a été possible de séparer, à l'aide

de quatre expériences au maximum, les énantiomères de chacun des treize composés chiraux étudiés.

Dans la partie de la thèse consacrée au développement d'applications, une série de tests ont été réalisés afin d'optimiser l'énantiorésolution de la ropivacaïne, un anesthésique local, dont la formulation pharmaceutique se compose d'une solution aqueuse de S-ropivacaïne. Une inversion de l'ordre d'élution des énantiomères de la ropivacaïne en fonction de la nature de l'additif acide dans la phase mobile a été observée sur les deux PSC, Sepapak-2 et Sepapak-4. Par contre, aucune inversion de l'ordre d'élution des énantiomères de la ropivacaïne n'a été observée lorsque Sepapak-5 et Chiralcel OD-H, dont la cellulose tris (3,5-diméthylphénylcarbamate) constitue le sélecteur chiral, ont été utilisées dans les mêmes conditions. Les meilleures conditions chromatographiques pour la détermination de la pureté énantiomérique de la S-ropivacaïne ont ensuite été sélectionnées et la méthode développée a été entièrement validée au moyen de la stratégie basée sur l'utilisation du profil d'exactitude, qui tient compte de l'erreur totale, c'est-à-dire de l'estimation des erreurs systématiques et aléatoires des résultats de mesure.

La seconde application que nous avons mise au point concerne la détermination de la pureté énantiomérique de la S-amlodipine dans une formulation pharmaceutique. Une inversion de l'ordre d'élution des énantiomères provoquée par le changement de la concentration d'acide formique (FA) dans la phase mobile a été mise en évidence, sur Sepapak-2 et Sepapak-4. L'étude de l'énantiorésolution de l'amlodipine dans ces systèmes chromatographiques a permis la sélection de la PSC la plus adéquate et de la concentration optimale d'acide formique dans la phase mobile en ce qui concerne l'ordre d'élution (la R-amlodipine éluant en premier) et la valeur de résolution. La méthode a ensuite été prévalidée en utilisant la même approche que pour la S-ropivacaïne.

Contents.

Chapter I.

INTRODUCTION

I.1. Background

It is well known that many implications of chirality are due to the fact that the enantiomers of chiral drugs can have different pharmacological activities in relation with their spatial configuration. Indeed, many enantiomerically pure natural compounds such as sugars, amino acids and proteins are involved in the majority of biological processes. Thus, the nature is capable of producing, processing and recognizing chiral compounds with an outstanding specificity. For example, most chemists are familiar with the role of chirality in odorants such as (4S)-(+)-carvone, which has a distinct caraway odor, as compared to (4R)-(-)-carvone, which has a characteristically sweet spearmint odor. We can also distinguish enantiomers of limonene with our sense of smell: R-enantiomer smells orange while the S-enantiomer smells lemon [1]. Although the role of chirality in odor perception is still a rather modern area of interest, it should be noted that more than 285 enantiomeric pairs (570 enantiomers) are known to exhibit either differing odors or odor intensities [2].

Over the last five decades, there has been a particularly great interest in the biological activity, both pharmacological and toxicological, of the enantiomers of chiral drugs since the disaster of the tranquillizer drug called thalidomide. Indeed, thalidomide was manufactured and sold as a racemate of N-phtalylglutamic acid imide, the S-isomer of which was teratogenic and caused serious fetal malformations called phocomelia. For this reason, the control and determination of the enantiomeric composition of chiral drug substances have become key issues for both the pharmaceutical industry and regulatory agencies [3].

Nowadays, most of the top selling drugs around the world are administered as single enantiomers, possessing the desired physiological activity [4]. Thus the development of methods for the production of enantiomerically pure compounds and the assessment of their enantiomeric purity have become more and more important specially in life science applications, such as biochemical, toxicological, forensic and pharmaceutical research. As a result of these news trends, there has been a tremendous impetus to develop separation methods based on chiral LC, both on analytical and preparative scales since an asymmetrical synthesis of enantiomers is very expensive. As the result, a great number of chiral stationary phases (CSPs) were commercialized and among them, polysaccharide based CSPs have been most extensively studied since there are suitable for both analytical and preparative scale enantioseparations.

I.2. Stereochemistry and pharmacology

Earlier in the 18th century, Carl Wilhelm Scheele discovered tartaric acid from tartar (the potassium salt of tartaric acid, deposited on barrels and corks during fermentation of grape juice) while the view of light as a beam of asymmetric particles was discovered. To explain this newly observed light phenomenon associated with reflection, Malus in 1806 claimed that these particles were bipolar. Reflection causes these dipoles to become oriented to produce polarized light and this light has a plane of polarization. In 1815, Biot, a classmate of Malus, noted that certain natural organic compounds (liquids or solutions) rotate plane polarized light, suggesting that this optical activity was a molecular property. In 1838, he discovered tartaric acid from grape juice fermentation to rotate plane polarized light in a clockwise direction while he made the reverse observation for racemic acid.

Ten years later, Pasteur repeated his earlier work on racemic acid, and this time he observed that crystallization of the sodium ammonium salt of this acid produced mirror image crystals that he separated by hand. These two crystalline forms were dissymmetric, non-superimposable mirror images and their equimolar solutions showed equal but opposite optical activity. From these observations, Pasteur concluded that optical activity is based on dissymmetry, not only at the crystalline level but also at the molecular level [5]. Thus, Pasteur's work paved the way for subsequent work of Van't Hoff and Le Bel, who postulated in 1874 the tetrahedral nature of carbon (carbon with 4 attachments is tetrahedral and a molecule having a tetrahedral carbon with 4 different attachments may exist as a pair of isomers) and the basic rules for modern stereochemistry.

Towards the end of the 19th century the role of chirality in biological activity began to receive serious attention. Two lines of investigation were pursued: one focused on the metabolic fate of chiral compounds while the other examined their pharmacological activity. The first report on enantioselectivity in what may be considered a pharmacological effect was in 1886, when (+)-asparagine was found to have a sweet taste while (-)-asparagine was tasteless [6]. Pasteur, aware of the finding, interpreted the results as an indication of the presence of a monochiral compound in the nervous system of taste, suggesting that the interactions of the asparagine enantiomers with the chiral biological mediator were different [8-10].

I.2.1. Stereochemistry: definition, terminology and nomenclature

Stereochemistry is the study of the static and dynamic aspects of the three-dimensional shapes of molecules [11]. It involves studies of chemistry properties of molecules that possess identical constitution**,** but differ in the arrangement of their atoms in space, namely **stereoisomers**. They can have one or more carbon atoms and sometimes another atom bearing 4 different atoms or group of atoms.

One molecule containing one stereocenter (a carbon atom or another atom bearing 4 different atoms or groups of atoms) is a **chiral** molecule and has two stereoisomers which are nonsuperimposable with their mirror image, called **enantiomers**.

Chirality is often illustrated with the idea of left- and right-handedness: a left hand and right hand are mirror images of each other and are not superimposable. The two mirror images of a chiral molecule shown in Figure 1 are enantiomers.

However, a necessary and sufficient criterion for chirality in a rigid molecule is the absence of any improper symmetry elements. Otherwise, atoms other than carbon can exhibit chirality such as sulphur, nitrogen and phosphorus which are the most common atoms in stereocenters after carbon.

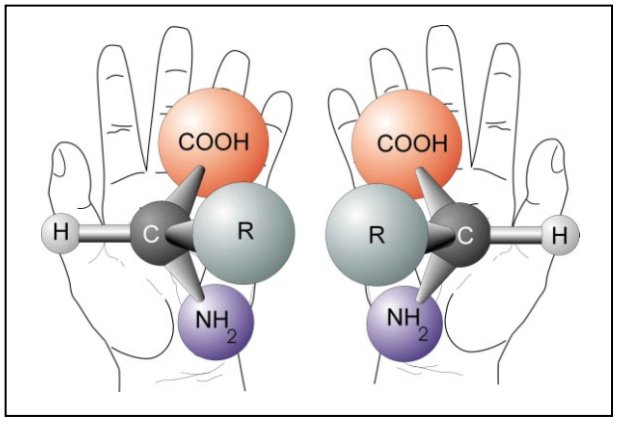

Figure 1. Non-superimposable mirror images are enantiomers.

When a molecule has more than one stereocenter, it can have 2^n stereoisomers (*n* is the number of stereocenters) called **disastereoisomers** which are not nonsuperimposable mirror images but have different chemical and physical properties in any type of environment. Unlike diastereoisomers, enantiomers display the same chemical and physical properties in an achiral environment. They have the ability to rotate plane polarized light and thus are called optically active compounds or sometimes optical antipodes. An enantiomer that provides a clockwise rotation is called **dextrorotatory** [(*d*) or (+)] while that providing an anticlockwise rotation is called levorotatory [(*l*) or (-)]. A mixture of equal quantities of the two enantiomers results in a non-optically active mixture or racemate [(±) or (*d,l*)]. The degree of rotation of plane-polarized light (α) is related to the molecule studied, but also to its concentration, the length of the polarimeter tube, wavelength, temperature and solvent. When it is called specific rotation, $[\alpha]_\lambda^t$ is the rotation observed for a solution containing 1 $g.ml^{-1}$ in 1 dm cell at λ nm and t °C [12, 13].

In biochemistry, the common nomenclature system used is the D (Dextrogyrate) / L (Laevogyrate) based system since this system is used primarily for sugars and amino acids. To name complex carbohydrates or amino acids, one draws a similar Fischer projection where the CH_2OH or R is at the bottom and the carbonyl group (aldehyde, ketone, or carboxylic acid) is at the top. The D descriptor is used when the OH or NH_2 on the penultimate (second from the bottom) carbon points to the right and L is used when the OH or NH_2 on the same carbon points to the left. It is commonly stated that most natural amino acids are L, while natural sugars are D.

For chemists, the *R (rectus) / S (sinister)* system is the most important nomenclature system for notification of absolute configuration of enantiomers. The **Cahn-Ingold-Prelog** (CIP) rule assigns R or S configuration to the enantiomer.

According to the CIP sequencing rules, the four substituents of a tetrahedral chirality center (stereocenter) are ranked in order of decreasing atomic number of the atoms directly bonded to the chirality center. Isotopes of the same chemical element are listed in order of decreasing atomic mass. When two or more atoms bonded to the chirality center have the same atomic number, the second atoms are used to rank the substituents. If the second atoms are also the same, the third are used, and so on. Multiple bonds are counted as the corresponding number of single bonds. The substituent with lowest precedence is visualized as pointing away from the viewer, so that the remaining three substituents are then placed in

order of decreasing precedence, a > b > c > d. If the order is clockwise, then the symbol R is assigned to the enantiomer while symbol S is assigned when the order is anticlockwise [14-18]. The application of these CIP rules to enantiomers of glyceraldehyde (cf. Fig. 2) led to the following order: OH> CHO> CH_2OH> H.

Thus, the absolute configuration of glyceraldehyde enantiomers can be assigned as shown in Figure 2.

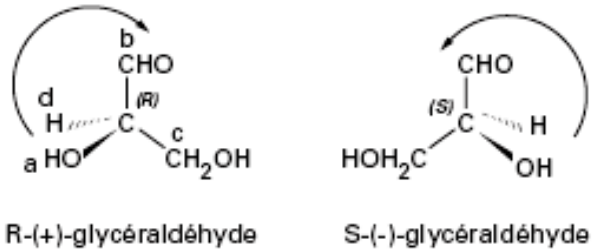

Figure 2. Absolute configuration of enantiomers of glyceraldehydes.

It should be emphasized that the three previous systems of nomenclature [(d, l) or (+, -), (D, L) and (R, S)] are mutually exclusive. R enantiomer of one compound may be either dextrorotatory or levorotatory, while another compound may have its S enantiomer as dextrorotatory. When a molecule contains more than one asymmetric carbon, the absolute configuration of each numbered stereocenter should be identified at the beginning of the name [19].

I.2.2. Pharmacological implication of chirality

The presence of one stereocenter in the absolute structure of a therapeutic molecule can lead to different situations according to toxicological and pharmacological properties. As the two stereoisomers of the drug have different configurations, their complementary binding sites (receptors, enzymes, etc) are also expected to be different. Thus the stereoisomer (commonly referred to as “eutomer”) binding precisely to the target sites could induce the therapeutic activity while the other stereoisomer (distomer) may bind weakly, not at all the relevant site or may bind precisely to other sites that are not the intended targets. In this way, whenever a drug is commercialized as a racemate, the eutomer may be active while the other stereoisomer may have:

- no activity,
- quantitatively and qualitatively the same activity,
- qualitatively the same type of activity but lower intensity,
- a completely separate beneficial activity,
- a completely separate adverse activity.

In the first three cases, the racemic drug is considered to be safe and thus there is no need to perform resolution of enantiomers. Otherwise, the synergistic activity (completely separate beneficial activity) of eutomer and distomer in racemate is also interesting for therapeutic purpose. The enantiomers of cicletanine clearly illustrate this case. Cicletanine is a furopyrine low-ceiling diuretic drug, usually used in the treatment of hypertension. The S-(-) stereoisomer decreases peripheral vascular resistance and in so doing improves the antihypertensive effect of R-(+) cicletanine taken alone [20]. The most uncomfortable situation for drug discovery remains the last case. When the distomer has toxic activity, like S-(-)-enantiomer of thalidomide or D-dopa, the need for production of the single eutomer by means of enantioselective method of synthesis or purification becomes a matter of legal requirements.

However, the phenomenon of "chiral inversion" (conversion of one enantiomer into its mirror image) adds to the complexity. Although S-thalidomide is teratogenic, both R and S enantiomers of thalidomide inverted rapidly to the racemic mixture in the liver [21]. Hence the claims that R-thalidomide could be safer and that the tragedy could have been prevented by using single R-thalidomide are not valid.

Since it is well known that amino acids, carbohydrates and macromolecules like plasma proteins, nuclear and membrane receptors are asymmetric, their interactions with chiral compounds may be enantioselective and lead to different pharmacokinetic parameters for each enantiomer. In the same way, the enzyme system activity could be different from an enantiomer to an other entailing different metabolites in the process. Thus all the pharmacokinetic processes, namely absorption, distribution, metabolism and excretion, may be influenced by chirality. Indeed, active transport processes may discriminate between enantiomers, with implications on biovailability, e.g., esomeprazole is more bioavailable than racemic omeprazole [22]. The volume of distribution of levocetirizine has been shown to be significantly smaller than that of its dextro enantiomer, which is a positive aspect in terms of both safety and efficacy. Glucuronide conjugates and the lateral chain oxidized product of

(S)-(-)-propranolol metabolism are more important than that of its optical antipode, (R)-(+) [23]. Otherwise, enantiomers of drugs are often metabolized at different rates and this could result in accumulation of the inactive enantiomer or rapid elimination of the active one and vice versa. Thus the determination of pharmacological and toxicological activities of each stereoisomer of a chiral compound constitutes an important step in drug development.

I.3. Chirality and interest for pharmaceutical industry

Since single stereoisomers may exhibit quite different bio-activities, pharmacological and toxicological behaviors within the chiral environment of biological systems, drug stereochemistry became an important issue for pharmaceutical industry and the regulatory authorities [24-27]. The development of single enantiomers versus racemates or the introduction of a single enantiomer following the development of the racemic mixture, namely "*chiral switch*", appears to be the new trends either for economic purposes or public health consideration. The single enantiomer can often offer advantages in potency, efficacy and/or safety to patients over the corresponding racemate. The economic interests are obvious and essential driving forces in development of advantageous technology for chiral drugs [28]. Otherwise, if in fact a single enantiomer can demonstrate an actual pharmaceutical advantage, it could have a quicker discovery turnaround time, a more streamlined development, a smoother approval process, and a quicker appearance in the market. This latter consideration is economically interesting since the development of a new drug costs more than 600 million euros and the time between its development and its market approval is over a decade [29]. Moreover, the commercialization of a single enantiomer of a previously approved racemate, may be patentable and therefore, provides additional market exclusivity via trading license. Levosalbutamol, S-ketamine, S-omeprazole (esomeprazole), levobupivacaine and S-amlodipine are some chiral switches that have yielded safer alternatives and provide additional patents for pharmaceutical industries.

It should be emphasized that discovery, development and worldwide sale of enantiomerically pure chiral drugs have been increasingly important over the last two decades. The worldwide sale of chiral drugs in single-enantiomer forms increased annually from 27% (56 billion euros) in 1996, 29% (1998), 32% (1999), 34% (2000), 38% (2001) to an estimated 39% (114 billion euros) in 2002 [30].

Obviously, chirality is of considerable importance in the pharmaceutical industry either for both economic and therapeutic standpoints or for regulatory purposes. Thus, increasing demand for enantiomerically pure chiral compounds has stimulated a rapid development of enantioselective synthesis methodologies and business of enantiopure compounds [31]. Simultaneously, this new trend produces an increase in the demand for stereoselective separation techniques and analytical methods for accurate determination of enantiopure compounds. Over the past two decades, tremendous progress was made in these enantioresolution methods. The development of chiral stationary phases (CSPs) for liquid chromatography (LC), supercritical fluid chromatography (SFC) and gas chromatography (GC) and chiral selectors for capillary electrophoresis, has opened a new dimension in the enantioseparation technologies. An impressive number of CSPs is now available for either analytical or preparative purposes.

I.4. Chiral separations

Many analytical techniques enable the enantioresolution of chiral compounds, namely liquid chromatography (LC), supercritical fluid chromatography (SFC), gas chromatography (GC), capillary electrophoresis (CE), thin layer chromatography (TLC), micellar electrokinetic chromatography (MEKC) and recently capillary electrochromatography (CEC) [32]. Even though some techniques are quite complementary, it should be emphasized that liquid chromatography has grown significantly as a simple and practical method applicable for both analytical and preparative purposes [33-35]. In most techniques, there are two strategies for enantiomeric separation: the direct and the indirect methods [34].

The most important factor in indirect enantioseparation method is the availability of optically pure chiral derivatizing reagents converting racemic mixtures into mixture of diastereoisomeric derivatives differing in physicochemical properties and therefore separated in an achiral environment by chromatographic or electrophoresis techniques. The separation can also be performed by crystallization or distillation [36]. A broad spectrum of monochiral derivatization reagents has been developed for GC, LC and CE [30]. (+)-1-(9-fluorenyl)-ethylchloroformate [(+)-FLEC], (S)-(-)-N-(fluoroacetyl)-prolyl chloride, 1-phenyl-ethylcyanate, (S)-(+)-2-methoxy-2-(1-naphthyl)propionic acid and N-methyl-N-bis(trifluoroacetamide) (MBTFA) are some of the usual monochiral derivatizing reagents [37-

40]. This indirect method appears particularly interesting for the enantiomeric separation of sugars, amino acids, peptides, etc. The sensitivity and the selectivity of the method could be improved through the addition of the chiral derivatization reagent. Fluorescent reagents are specially interesting to enhance detection sensitivity for LC and CE [41,42]. A disadvantage to this approach is the additional step of reaction between the enantiomers and the derivatizing reagent. Moreover, the chiral derivatizing reagent must be controlled so that no racemization has taken place during the reaction.

The direct method involves the separation of enantiomers in a chiral environment and seems to be easiest and most simple. There are two principles that enable one to obtain enantioresolution by this method.

The first principle creates a chiral environment with chiral selector (cyclodextrin, α-amino acid associated with metallic ion, etc.) added to the mobile phase or the background electrolyte in analytical methods such as LC, TLC and CE. In chromatographic techniques achiral stationary phases are used to perform the separation. In many cases, this approach gives good results but the enantioresolution is influenced by pH, temperature, the concentration of organic modifier in the mobile phase, the type and purity of the chiral selector and the nature of the stationary phase for LC and TLC. Otherwise, the high cost of chiral selectors and the compatibility between the detection mode and the chiral reagent make this approach less praticable and expensive in LC. Thus it is mostly used in capillary electrophoresis where the consumption of chiral selector is lower [32, 43-46]. Nowadays, this technique has gained a broad scope of application in the field of enantioseparations with the use of cyclodextrins and derivatives as chiral selectors [13, 47].

The second principle is based on a use of a chiral stationary phase (CSP) where the chiral selector is adsorbed or chemically bonded to a solid phase. These CSPs are mainly used in LC and SFC [48-50]. The method is based on the discriminatory ability of the CSP towards the two enantiomers. According to this recognition ability, the CSP can retain by specific interactions an enantiomer while the second is being eluted or less strongly retained. Even though many models for the requirements to obtain chiral recognition have been described in the literature, the first and most reliable is the three-point model that was proposed by Dalgliesh [51]. This model postulates that three interactions must take place and at least one has to be stereoselective to enable chiral resolution. In other words, if the CSP interacts with three different functional groups (AA', BB', CC') near the stereocenter of the analyte, the

chiral discrimination of its enantiomers can be performed (cf. Fig. 3). On the contrary the chiral recognition cannot be performed in the case where only two functional groups near the stereocenter of the analyte interact with the CSP. The interactions often involve hydrogen bonds and dipole stacking (aromatic interactions) but are sometimes associated with ionic attractions [31, 32, 40, 52].

Others techniques such as capillary electrochromatography (CEC) and capillary liquid chromatography (CLC), also use CSPs packed in a capillary, to perform enantioseparation of chiral compounds [52].

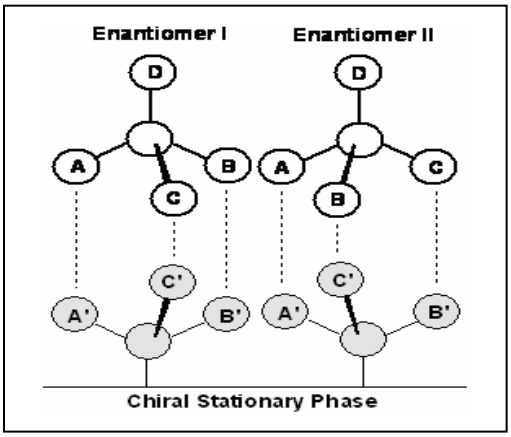

Figure 3. Three points interaction model.

Unlike CLC, capillary electrochromatography (CEC) is a hybrid technique between CE and LC. The driving force allowing the separation in this technique to proceed is the electro-osmotic flow (EOF) obtained by applying a voltage onto the capillary [54-55]. These techniques allow both direct approaches for enantioseparation. However, the comparison of the two techniques revealed significant advantages for CEC [53].

Even though the majority of enantioseparations can be performed on chiral stationary phases in LC, it should be emphasized that there is no universal CSP [34]. Thus, a tremedous number of CSPs have been developed and several hundreds are commercially available. Therefore, the enantioseparation of a target chiral drug depends on the choice of a suitable combination between a CSP and a LC mobile phase.

I.5. Direct LC Enantioseparation: Chiral Stationary Phases (CSPs) and Mobile Phases

I.5.1. CSPs: Classification

The key for the success of enantiomer separations is often based on a proper selection of a suitable chiral stationary phase having a chiral distinction capability for the target solute enantiomers. For this purpose, the knowledge of existing chiral stationary phases and their properties are of major importance in direct LC enantioseparation. Although many CSPs are commercially available, they can be classified according to two main criteria: chiral recognition mechanism and chiral selector nature. However, the classification changes from one author to another because of the rapid development of CSPs and a better understanding of mechanisms underlying.

Earlier in 1987, Wainer's classification of CSPs which was based on the mechanism of chiral recognition depicted in five groups [57]:

Type I. where the solute-CSP complexes are formed by attractive interactions, hydrogen bonding, π-π interactions, dipole stacking, between the solute and CSP;

Type II. where the primary mechanism for formation of the solute-CSP complex is through attractive interactions but where inclusion complexes also play an important role;

Type III. where the solute enters into chiral cavities within the CSP to form inclusion complexes;

Type IV. where the solute is part of a diastereomeric metal complex (chiral ligand exchange chromatography);

Type V. where the CSP is a protein and the solute-CSP complexes are based upon c ombinations of hydrophobic and polar interactions.

Three years later, taking into account the new available CSPs, Gübitz classified the CSPs in five groups based on the major interactions between chiral solute and CSP [59]:

(i) Chiral phases with cavities (cellulose derivatives, cyclodextrins, synthetic chiral polymers, chiral crown ethers and chiral imprinted polymers);

(ii) Chiral affinity phases (bovine serum albumin (BSA), human serum albumin (HSA), α_1-glycoprotein (AGP), ovomucoid and avidin);

(iii) Chiral phases based on multiple hydrogen bonding formation (amino acid amide and tartaric acid amide phases);

(iv) Chiral π-donor and π-acceptor phases (Pirkle phases or brush type phases);

(v) Chiral ligand exchange chromatography phases (polystyrene-divinylbenzene polymers containing amino acid residues complexed with metal ions).

Even though this classification seems clear, Siret *et al.* proposed a restrictive classification with four groups related to both recognition mechanism and chiral selector structure [60]. In their classification, ligand exchange and chiral π-donor and π-acceptor phases were mixed in a group in which the chiral recognition involves superficial complexes formation (Type I). The chiral phases with cavities group without cellulose derivatives, was called Type II. This group comprises two sub-groups: Type IIA, involving formation of inclusion complexes due to the hydrophobic moiety of the solute molecule which must be relatively "tigh fitted" to the hydrophobic cavity of the selector (e.g. cyclodextrin) and Type IIB, involving hydrophilic interactions. A special group was dedicated to protein derived CSPs (Type IV) and the type III was made of natural (Type IIIA) or synthetic polymer-based CSPs (Type IIIB).

Recently, Okamoto *et al*. [51] classified CSPs for LC into two groups while Lämmerhofer [61] proposed three groups. Both authors take into account only the size of the chiral selectors to sort out the presently available CSPs.

In the classification of Okamoto et al., the first type of CSP consists of **optically active small molecules**, which are usually immobilized on silica gel or on organic polymer gel as achiral supports (brush-type CSPs). The authors put into this group ligand-exchange type, crown-ether-based, cyclodextrin-based, donor-acceptor-type, (aglycone)-glycopeptide-based and ion-exchange-type CSPs. The second type of CSP consists of **optically active polymers**, which are further divided into synthetic and natural polymers. Molecular imprinted-type, poly(meth)acrylamide-based, polymethacrylate-based, polyamide-based and tartardiamide-based CSPs were described as synthetic polymer-based CSPs while protein-

based and polysaccharide-based CSPs (including those coated or immobilized on silica gel) are natural polymer-based CSPs.

Lämmerhofer distinguishes CSPs with **macromolecular selectors** (biopolymers like proteins and polysaccharide derivatives, and synthetic polymers such as polytartaramides, poly (meth) acrylamides), those with **macrocyclic selectors** (cyclodextrins, macrocyclic antibiotics and chiral crown ethers) and CSPs with **low molecular mass selectors** (donor-acceptor (Pirkle-type), chiral ion exchange type and ligand (chelating agents) selectors). These different types of CSPs are presented in Table 1 with the corresponding chiral selectors, the recognition mechanisms and some examples of commercialized CSPs.

It should be emphasized that more than 90% of chiral separations by LC are performed using polysaccharide-based CSPs. Futhermore, the newly developed cellulose or amylose based CSPs by immobilizing either cellulose tris (3,5-dimethylphenylcarbamate) or amylose tris (3,5-dimethylphenylcarbamate) onto silica gel, expand the versatility and application range via their extended choice of mobile phases, resulting in an enormous flexibility for method development [62,63]. The recently mixed methyl/chloro or dichloro-substituted polysaccharide derivatives CSPs introduced as Sepapak-2, -3, -4 or -5 by Sepaserve, provide some complementary enantiorecognition profiles and enable multimodal chromatographic use. These chlorine containing polysaccharide-based CSPs will be of main interest in the present thesis.

I.5.2. Interest of non aqueous polar mobile phases in chiral separations

Although chiral stationary phases are dedicated to normal phase and reversed phase systems which cover almost completely the potential applications of CSPs in LC, the use of a polar organic solvent as the main component of the mobile phase has become increasingly popular [57, 65-67]. This system, first called “polar organic mode” or “polar organic solvent chromatography” (POSC) by Amstrong *et al*. consists of the use of only organic solvents (excluding water), usually methanol, ethanol, acetonitrile or their combinations in the mobile phase [68]. Even if this chromatographic mode started with antibiotic and macrocyclic-based CSPs, it is nowadays extended to the other CSPs because of its advantages. The polar organic mode or POSC may offer the advantages of the reversed-phase mode through:

- an increase of solubility of some analytes which are often less soluble in aqueous solvents,
- stronger ionic and polar interactions, allowing alternative chiral recognition mechanisms.

This chromatographic mode may also offer some advantages of normal-phase LC and SFC since the working pressure is lower due to the low viscosity of organic solvents which are easy evaporated, especially in preparative scale applications, and separation efficiency is often higher [66,69]. Futhermore, the use of acidic or basic additives has become of great interest. Indeed, Ye *et al*. demonstrated the effect of acidic and basic (amine) additives on enantioselectivity for phenylalanine analogs using Chiralpak AD as CSP in normal-phase mode [70,71] and then later in POSC. Thus the combination of this polar organic mode with acidic and basic additives may offer alternative possibilities for method development and optimization either by univariate or multivariate techniques (experimental designs).

I.6. Method development: experimental designs

The development of an analytical method usually needs the study of numerous factors. In order to determine initially the factors which could have an impact on the selected response(s), a *screening test* is often performed. It allows reducing potentially the number of factors that need to be investigated in further experimentations. The objective is to eliminate non significant factors before investing time and money in a more elaborate testing. The relevant factors can be *optimized* through a set of experiments that will enable one to get the *optimal conditions* which are the best combination of factors levels that fit the purpose of the study. To achieve these two steps of method development, two approaches can be chosen: the univariate approach and the multivariate approach.

The univariate or classical approach consists of selecting several factors which are varied one-by-one to assess their influence on the response(s) of interest. This sequential approach allows a quite good view of the impact of each factor on the targeted response but there is no possibility to visualize interaction effects which can play major role in the phenomena governing this response. Furthermore the univariate approach sounds less cost-effective and is time-consuming. There may be also a potential loss in evaluating power when

not analyzing the data simultaneously. Nevertheless, this method is adequate for method development when no information is available on factors likely to influence the targeted response(s).

The multivariate approach or experimental design is a core area of study in chemometrics. It is an even better alternative because for a given number of experiments the experimental domain is more completely covered and interaction effects between factors can be evaluated. An experimental design can be considered as a series of experiments that, in general, are defined *a priori* and allow the influence of a predefined number of factors in a predefined number of experiments to be evaluated [72].

Table 1: Classification of CSPs and the mechanism of their recognition [12,52,57-61].

Groups	Sub-groups	Chiral selector structure	Mecanism of chiral recognition	Advantages and drawbacks (e.g.)
CSPs with low molecular mass selectors	*Π-donor-acceptor- type* *(Pirkle type)*	(R)-3,5-dinitrobenzoyl-phénylglycine 1,2-trans-diaminocyclo hexane 1,2-trans-diphenylethylenediamine	The π-π stacking interaction between the electron-deficient 3,5-dinitrobenzoyl-groups and electron-rich aromatic groups plays a significantly important role in discriminating enantiomers of chiral compounds in addition to hydrogen bonding, dipole-dipole and steric interactions.	Compatibility with all conventional mobile phases in LC and SFC; good stability; efficiency equivalent to a classical grafted silica; possibility of use on a preparative scale; less expensive but the scope of application is still limited to compounds of low or medium polarity. (Whelko-01, ULMO and DACH-DNB from Regis; Chirex 3005, 3020 and 3022 from Phenomenex).
	Ligand-exchange-type	amino acid like L-proline, valine or alanine immobilized onto an organic matrix (poly (styrene divinyl-benzene)) or onto silica	Reversible coordination of chelating analyte species from the mobile phase into coordination sphere of a metal ion that is immobilized by complexation with a chelating chiral selector forming mixed ternary metal ion/selector/	Good stability, high selectivity values, less expensive but poor efficiency and the scope of application is limited to bidentate or tridentate ligands (Chiralpak MA+ from Daicel, Nucleosil Chiral-1 from Macherey-Nagel and Chirex 3126

			solute complexes. Depending on the steric and functional properties of the analytes, these diastereomeric ternary chelate complexes show different rates of formation and/or thermodynamic stabilities, giving rise to different retention times of corresponding solute enantiomers.	from Phenomenex).
	Crown ether-based CSPs	Chiral crown ethers bearing optically active binaphthyl and tartric acid units and phenolic pseudo chiral crown ethers.	Generated chiral ammonium ions can bind enantioselectively to macrocyclic crown ether through inclusion complexation driven by triple hydrogen bond formation between the ammonium ion and three oxygens of the crown ether. This CSPs run with strongly acidic aqueous eluents (pH between 1 and 3.5) which ensure full protonation of the solutes amino functionality.	Good stability, high selectivity but application spectrum essentially restricted to primary amines (amino acids, N-acetyl amino acids, amino acid esters and amides, di- and tripeptides, amino alcohols and chiral drugs with free primary amino groups [59] (Crownpak CR from Daicel, .Chirosil RCA (+) and Chirosil SCA (-) from Regis).

	Cyclodextrin-based CSPs (Amstrong and deMond, 1984)	Cyclodextrins (CD) with six (α-CD), sept (β-CD) or eight (γ-CD) glucose units connected via α-1,4-linkages, bonded onto silica.	A two-step mechanism occurs in reversed-phase conditions: the hydrophobic part of the guest molecule penetrates into the CD cavity and leads then to the release of solvent (water) molecules from guest and CD molecules. Further stabilization of the complex may stem from van der Waals interactions inside the cavity while additionally supporting hydrophilic interactions with hydroxyl groups at the upper and lower rim (hydrogen bonding, dipole–dipole interactions) may take place. In polar organic or normal phase mode the inner cavity is blocked by solvent molecules preventing inclusion complexation thus enantioselectivity takes place on the	Good stability, multi-modal in terms of elution conditions (normal and reversed phase, polar organic mode and SFC) but the spectrum of application is limited and their low loading capacity make them useless for preparative separation (Cyclobond from ASTEC; ChiraDex and ChiraDex Gamma, from Merck; Ultron ES-CD, from Shinwa).

			polar surface of the CD through hydrophilic interactions.	
	Glycopeptide (aglycone)-based CSPs *(Macrocyclic antibiotics CSPs)* *(Amstrong et al, 1994)*	Glycopeptide (aglycone) such as: Vancomycin, Ristocetin A, Teicoplanin, Teicoplanin agycone, immobilized onto silica.	The recognition mechanism is not well known but all these selectors have plenty of potential interaction sites closely attached to stereogenic centers including various H-donor/acceptor functionalities, aromatic rings for π-π-interaction, acidic and basic groups that may be involved in electrostatic interactions	These CSPs can be used with a great variety of mobile phases and have a broad spectrum of application (Chirobiotic V, T, R, from ASTEC and TAG from Supelco).
	Ion-exchange-type CSPs (Lindner and Lämmerhofer, 1996)	Cinchona alkaloid derivatives and terguride	With (weakly) acidic mobile phases, the quinuclidine nitrogen becomes protonated and acts as the fixed charge of the chiral anion-exchanger. Acidic analytes are then primarily retained by an ion-exchange process.	Excellent recognition abilities for chiral acidic compounds (anion-exchanger-type), high mass loading capacities but enantioseparation of these compounds preferentially restricted to polar organic and reversed-phase modes (Chiralpak QN-AX and QD-AX from Chiral technologies).

Synthetic polymer-based CSPs	*Molecularly imprinted-type (Wulff and Sarhan, 1972)*	Cross linked polymer gels with enantioselective binding cavities prepared via a molecular imprinting technique.	The size, shape and stereochemical nature of these chiral cavities are complementary to the enantiomer used as the template.	Higher enantioselective affinity for template enantiomer than with the other CSPs. Scope of application with biologically active compounds like amino acids, peptides, hormones and antibiotics.
	Poly(meth)acrylamide-based CSPs (Baschke, 1974 and Okamoto, 1981)	Monomeric optically active acrylamide with acryloyl- or methacryloyl substituted diol silica, Monomers adsorbed on diol silica silica. Monomers adsorbed on silica and subsequent blocking of silanol groups of silica by reaction with dimethyloctylchlorosilane.	The recognition ability on these polymers is significantly dependent on the synthetic methods. The polymers prepared by the radical polymerization of optically active monomers exhibit a much higher chiral recognition than the prepared by the reaction of poly(acryloyl chloride) with the corresponding chiral amines. It has also been found that the tacticity of polymethacrylamides and helicity of the polymers influence their chiral recognition abilities [63].	High selectivity values for atropoisomers and potential preparative applications but efficiency limited. (Chiralpak OT (+) from Daicel, Chiralspher from Merck).

	Polymethacrylate-based CSPs	Optically active poly-methacrylates with one-handed helical structure	Polar or hydrophobic interactions between the triphenylmethyl groups with a chiral propeller structure and racemates are important for the successful chiral recognition.	Efficient resolution of racemates without any functional group.
	Polyamide-based CSPs (Saigo et al, 1985)	Polyamide including poly(α-amino acid) immo-bilized onto cross-linked polystyrene.	Chiral discrimination should involve simultaneous interactions of aromatic stacking, hydrogen-bonding with solute but the high-ordered conformation of the poly (α-amino acid) (which takes an α-helical structure) seems to contribute also to the chiral recognition.	
	Tartardiamide-based CSPs (Allenmark et al, 1995)	Hydrosilylation of C2-symmetric *N,N'*-diallyl-L-tartardiamide derivatives with a multifunctional hydrosilane on an allyl-functionalized silica gel	The retention is mainly caused by the hydrogen-bonding ability of the analyte	A large number of neutral as well as acidic or basic drug racemates can be resolved without derivatization (Kromasil CHI-TBB from Eka Chemicals AB).

Natural polymer-based CSPs	*Protein-based CSPs*	α1-Glycoprotein acid (α1-AGP) Ovomucoid (OVM) Human Serum Albumin (HSA), Bovine Serum Albumin (BSA), Cellobiohydrolase (CBH) or Avidin, immobilized on silica	Their chiral recognition mechanism is not yet well known. However, the immobilized protein should have some regions capable of forming enantioselective cavities for many targeted compounds with acidic, basic amide and hydroxyl groups. The enantioselectivity could involve hydrophobic, hydrophilic, ionic and π-π interactions. Nevertheless, the mechanism depends on the tridimensionnal structure of the immobized protein, which is related to the pH of the mobile phase and the organic modifier (nature and quantity).	Many applications in pharmaceutical enantioseparations and bioanalytical studies but low stability, efficiency and reproductibility and not useful for preparative purposes due to a limited number of recognition sites leading to a lack of sufficient loading capability. (Chiral-HSA, Chiral-AGP and Chiral-CBH from Chiral technologies: Regis, Ultron ES-OVM and Ultron ES-Pepsin from Shinwa Chemicals, Resolvosil BSA from Macherey Nagel).

	Polysaccharide-based CSPs	Cellulose and amylose esters (triacetate, benzoate, cinnamate) or phenyl-carbamates, adsorbed or immobilized onto silica gel,	Chiral recognition often depends on the higher order structure of the chiral polymers and also on the substituents introduced on the phenyl moities. However, enantioseparation may be governed by the π-π, σ-π stacking interactions, but hydrogen bonding and dipole-induced dipole interaction are also essential.	Good stability, use in both LC and SFC modes and recognized as the most powerful CSPs for both analytical and preparative separations, but very large choice (Chiralcel OD, ODH, OD-R, OA, OC, OF, OJ or OK, Chiralpak AD or AS and Chiralpak IA, IB or IC from Daicel; Sepapak-2 and -4 from Sepaserve equivalent to Lux cellulose 2 and 4 from Phenomenex; Cellulose Cel-AC-40XF from Macherey- Nagel).

Otherwise, the objective of an experimental design is to plan and conduct experiments in order to extract the maximum amount of information from the collected data in the presence of noise and the smallest number of experiments. Consequently the main difference with the univariate approach consists in the simultaneous variation of all factors and their levels over a set of planned experiments and the connection of the results by means of a mathematical model [73, 74]. This model is then used for interpretation, prediction and optimization. Furthermore, the development of mathematics and applied statistics with informatics tools leads to more powerful analysis of data. Consequently the new trends in modern research, particularly in analytical chemistry, involve the use of multivariate approaches when the experimental domain and the nature of factors are known. Indeed, such approaches offer many advantages [74]:

- reduction of number of experiments;
- study of numerous factors;
- computer modeling of the results;
- detection of interaction effects between the studied factors;
- better accuracy of the results;
- optimization of the results and detection of the optimums.

The methodology of experimental design in method development is supposed to guarantee *a priori* the best quality of information to be derived from the planned experiments. Therefore, the choice of the experimental design often depends on the purpose of the study, the number of studied factors and their levels and the stage of the method development (screening or optimization). Many experimental designs are described in the literature. Even though some experimental designs can perform both screening and optimization, the large majority can be divided into two groups: screening designs and experimental designs for optimization.

I.6.1. Screening designs

The screening designs involve preliminary experiments the purpose of which is to isolate the most important factors (k) from amongst large number factors that may affect the particularly targeted response(s) [75]. Screening usually involves two level designs, such as fractional factorial or Plackett-Burman designs [72] due to the fact that they are simple and useful in many applications.

Fractional factorial designs are good alternatives to full factorial designs, especially in the initial stages of a project and considered as representative subsets of full factorial designs. The fractional factorial design is based on the principle that interaction effects could be confounded with a main effect. Consequently, the number of experiments (2^{k-p}) is reduced by a number p which represents the number of two-factor interactions confounded with a main effect. However, the result is satisfactory when the interaction effect confounded with a main effect remains negligible [47,74].

The Plackett-Burman design is a part of a full factorial design 2^k which allows estimating a first order model with k quantitative or qualitative factors at two levels. The number of experiments is at least $\geq$ k+1 (a multiple of 4) but in case the number of experiments is a power of 2, the Plackett-Burman design is equal to a fractional factorial design.

The D-optimal design is an alternative design particularly useful when the researcher wants to constrain an experimental region and none of the classical designs is appropriate. There are computer-generated designs in which the determinant of the X'X matrix (an overall measure of the information in X) is maximized. Geometrically, this corresponds to maximize the volume of X in a k-dimensional space. By choosing N experiments from a candidate set that consists of potential experiments, the design can be tailor-made to specific demands regarding the desirability of the researcher (experiments and economy constrains for example) [76]. Full factorial, fractional factorial and Plackett-Burman designs fulfill the D-optimal criteria.

The experimental matrix is built after selecting the levels of each factor which are replaced by (-1), (+1) and possibility (0) for the low level, the high level and the center value, respectively. The design evaluation enables the determination of the significant factors to be optimized. The decision can be made through the pareto plot or the significance of the coefficient of variables defined *a priori* in the given mathematical model (1) of the screening design.

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \varepsilon \quad (1)$$

where y represents the response, β_1, β_2 et β_3 the mains factors, β_{12}, β_{13} et β_{23} the coefficients of possible two-factor interactions, β_0 the intercept et ε the residual error.

The pareto plot is an ordered (largest to smallest) histogram displaying percentages of variables (factors) in the data with a line graph showing cumulative percentages of the factors. Therefore the effects of the factors are visualized and the most important factors can be estimated through the line graph.

The coefficient of a variable should be considered significant when the p-value associated is lower than a value fixed *a priori* (e.g. 5%, value generally admitted). Variables that have non significant coefficients are considered as useless and the corresponding effect should be neglected in further consideration.

I.6.2. Optimization Designs

For the optimization step, an experimental design supporting the estimation of quadratic terms is usually applied in order to further examine the significant factors obtained in the screening step. To achieve this purpose, two approaches are proposed in the literature: the simplex approach and the response surface methodology (RSM).

The simplex approach is a stepwise strategy, which means that experiments are performed one by one. To maximize the response(s), the first step is to run k+1 experiments (k represents the number of variables) to obtain the starting simplex. The yield in each corner of the simplex is then analyzed and the corner showing the least desirable result is mirrored through the geometrical midpoint of the other corners. In this way a new simplex is obtained [76]. The achievement of this approach is to encircle the optimum by means of simplex moving into this direction. This approach could lead to a very great number of experiments and the risk of not coming close to the optimum depends on its size. Moreover, it is not clear at the outset how much resource will be consumed before the purpose is reached. To avoid such risks, most analysts prefer the response surface methodology.

RSMs are multivariate techniques that mathematically fit the experimental domain studied in the theoretical design through a response function [77,78]. Two of the most common designs generally used for response surface modeling in many applications are the central composite and the Box–Behnken designs.

Central composite designs contain imbedded factorial or fractional factorial designs with center points that are augmented with a group of axial (star) points allowing estimation

of curvature (cf. fig. 4) [77,78]. These experiments are at a distance α to the center. The value of α determines the nature of the central composite design (CCD). For example when α=1, the design is called face centered central composite design and the experimental design is cubic. Otherwise, α equal $\sqrt[4]{k}$ and all experiments are situated on a sphere with α as the central radius.

The Box–Behnken design is considered an efficient option in RSM and is an ideal alternative to central composite designs [76]. It has three levels per factor, but avoids the corners of the space, and fills in the combination of center and extreme levels. It combines a fractional factorial design with incomplete block designs in such a way as to avoid the extreme vertices and to present an approximately rotatable design with only three levels per factor. This design is appropriate for situations where the researcher is not interested in predicting response at the extremes.

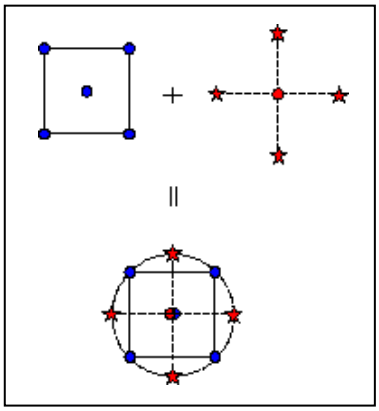

Figure 4. Central composite design (*star).

A less common, but effective method is the Doehlert design. Like the Box–Behnken design, Doehlert designs require a lower number of experiments than the central composite design (76, 78, 79). Moreover, the Doehlert design compared to the central composite approach leads to a higher efficiency value, ultimately determined by dividing the coefficients number of the quadratic equation by the number of experiments required for the design [80]. Otherwise, a neighboring domain is easily explored by just adding a few experiments [77].

Another design for optimization is a three-level full factorial design particularly useful when the number of factors (k) is low (<3) since the number of experiments 3^k grows rapidly with increasing the number of variables. It is made of the combination of the three levels of the k factors that meets the requirement of the maximal determinant. The three-level full factorial design also allows the assessment of the quadratic model.

The final result is a response surface which is a three dimensional view that may provide a clear picture of the response against the most important variables. If the regression model displays only main effects (e.i. first order model), the fitted response will be a plane. If the model contains interaction effects and/or quadratic effects, the contour lines will be curved. The response surface helps the analyst to understand the nature of the relationship between two factors and the response. Otherwise, the goodness of the model is often evaluated through the R^2 (explained variability of response) and Q^2 (explained predicted response variability) coefficients. Both coefficients should be close to the unity to express the goodness of fit.

The last step of an analytical procedure remains the validation which has become necessary according to legal requirements and quality system within a laboratory.

I.7. Analytical method validation

Owing to increasing interdependence among countries during this XXIst century globalization, the results of many analytical methods need to be acceptable by everyone. Therefore, the use of validated methods has increased to guarantee a common level of quality. Actually, in pharmaceutical sciences, the results obtained nowadays with non validated methods are considered as meaningless and will not be recognized by regulatory authorities.

Consequently, method validation procedures become a subject of great interest for analysts and for industrials as well.

The objective of method validation is to prove that a given quantitative method is able to quantify as accurately as possible each of the unknown quantities that the laboratory will have to determine. Otherwise, the analyst expects from the validated method that the difference between the found result and the unknown "true value" of the sample is small or inferior to an acceptance limit (%), *a priori* specified [81]. These specifications are usually admitted by the practice or by the regulatory documents, e.g. 5% for pharmaceutical forms or 15% for biological samples.

Over the past two decades, many conferences were held in USA and Europe to propose a harmonized procedure for analytical method validation with in mind to minimize the risks of accepting a procedure that would not be sufficiently accurate or rejecting a procedure that would be valid [82-84]. Regardless of these international conferences, an association, such as the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP), proposed a powerful guide for the validation of quantitative analytical methods. This guide has significantly contributed to make a progress in the validation of analytical procedures since 1992 [81, 85-89].

The proposed strategy of validation is based on the use of the accuracy profile approach as decision tool, taking into account the new concept of total error (bias + standard deviation for intermediate precision) of the procedure [81]. This approach allows obtaining a graphical representation, e.g. the accuracy profile, offering an easier visual decision tool related to the acceptance limit, for the analyst and other people concerned by the procedure. This approach reflects more directly the performance of individual assays and will result in fewer rejected in-study runs than the current strategies that compare point estimates of observed bias and precision with the targeted acceptance criteria, e.g. 15% for biological methods. Indeed, by considering a risk of 5 %, the analyst is able to guarantee that 95 times out of 100 the future measurements given by the analytical method will be included in the acceptance limits settled according to the requirements.

I.7.1. The validation criteria: definitions [81, 82, 87]

I.7.1.1. Selectivity-specificity

The selectivity of an analytical method is defined as its ability to demonstrate the presence of the targeted analyte in a mixture. It is the property of the analytical method to exclusively meet the characteristic of the analyte, with the guarantee that the result of the analysis is only generated by the analyte.

I.7.1.2. Response function (calibration curve)

The response function of an analytical method is, within the range, the existing relationship between the response (signal) and the concentration (quantity) of the analyte in the sample. The calibration curve is the simplest monotonous response function. Based on the use of the accuracy profile concept, the response function is considered as adequate when the accuracy profile is within the acceptance limits fixed *a priori* over the whole concentration range of interest.

I.7.1.3. Linearity

The linearity of an analytical method is its ability, within a given measurement interval, to obtain results directly proportional to the quantity (e.g. concentration) in analyte within the sample [90]. Therefore, for all series, a regression line was fitted by plotting the back-calculated concentrations as a function of the introduced concentrations. This validation criterion is required for the evaluation of the trueness, but a linear relationship between calculated concentrations and introduced ones does not guarantee the trueness of the analytical method.

I.7.1.4. Trueness (bias)

The trueness of an analytical method expresses the closeness of agreement between the mean value obtained from a series of measurements and the value which is accepted either as a conventional true value or an accepted reference value (international standard, standard

from a pharmacopoeia). Trueness can be expressed in terms of recovery and of absolute bias or relative bias (systematic error).

I.7.1.5. Precision

The precision of an analytical method expresses the closeness of agreement (dispersion level, relative standard deviation (RSD)) between a series of measurements obtained from multiple sampling of the same homogeneous sample (independent assays) under the prescribed conditions. It gives some information on random errors and it can be evaluated at three levels: repeatability, intermediate precision (within the laboratory) and reproducibility (between laboratories).

I.7.1.6. Accuracy

The accuracy takes into account the total error, i.e. systematic and random errors, related to the test results. Therefore, it is the expression of the sum of the trueness and precision. The upper and lower β-expectation tolerance limits expressed as a function of the introduced concentrations should not exceed the acceptance limit, e.g. 5% for pharmaceutical forms or 15% for biological samples, for all concentration levels tested including the lowest concentration level.

I.7.1.7. Limit of detection (LOD)

The limit of detection of an analytical method is the lowest amount of the targeted analyte in the sample which can be detected, but not necessarily quantified under the experimental conditions prescribed.

I.7.1.8. Limit of quantification (LOQ)

Limit of quantification of an analytical method is the lowest amount of the targeted analyte in the sample which can be quantitatively determined under the experimental conditions prescribed with a well defined accuracy.

I.7.2. Validation phases

I.7.2.1. Prevalidation phase

Before beginning a validation, some information is needed concerning the dosage range, the limit(s) of quantification, the variability of the results and the relevance of the regression model retained to be the response function. When experimental designs are applied for method development, this information could be provided and the analyst can directly switch to the validation process. Otherwise, a prevalidation phase should be performed to complete the development of the analytical method. The main objective of this phase is to identify the most appropriate response function (linear, non-linear, weighting, data transformation), to estimate the limit of quantification, to evaluate the range and the number of calibration levels, to determine the analyte recovery and to test method selectivity.

I.7.2.2. Validation phase

The main objective of the validation phase consists in :

- demonstrating the specificity (selectivity);
- validating the response function;
- estimating the precision (repeatability and intermediate precision);
- estimating the trueness;
- estimating the accuracy;
- validating the limit of quantification;
- validating the concentration range and;
- assessing the linearity.

I.7.2.3. Decision: the accuracy profile

The accuracy profile, constructed from the tolerance intervals on expected measurements, allows deciding the capability or not of an analytical method to give results inside the acceptance limits. For a given acceptance limit, the upper and lower β-expectation tolerances for total measurement error at σ% level were calculated at each concentration level

of the validation standards from the mean relative bias and the standard deviation for intermediate precision (cf. Fig.5).

By using different regression models, several accuracy profiles will be obtained. The regression model will be selected according to the accuracy profile which remains within the acceptance limits over a larger concentration range with a lower LOQ. The regression models that involve results outside the acceptance limits should not be selected.

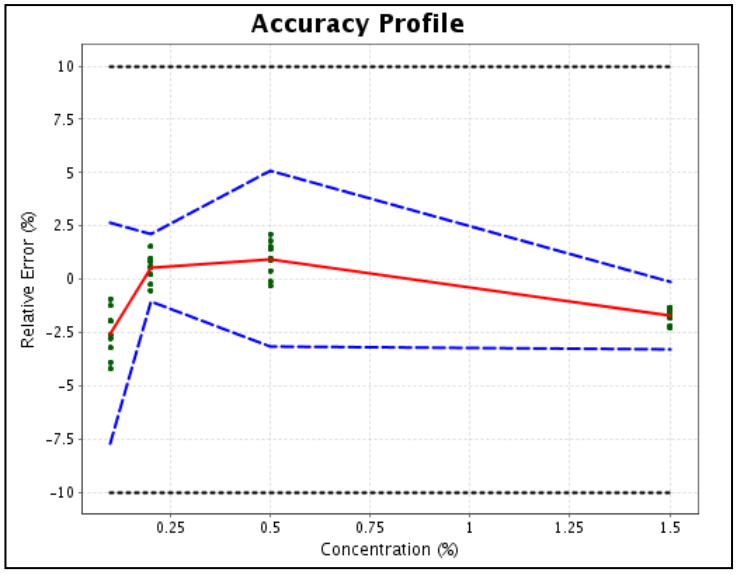

Figure 5. Accuracy profile obtained using a linear regression model after logarithm transformation. The plain line is the relative error (%), the dashed lines correspond to the accuracy profile i.e. to the β-expectation tolerance limits expressed in relative error, and the dotted curves represent the acceptance limits (±10%).

Throughout this thesis, different strategies and concepts described in the present introduction for method development, optimization and validation will be used to fulfill the objectives which are depicted in the following section.

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CHAPTER II.

OBJECTIVES

As a result of an intensive advancement in chiral separation technologies, several hundreds of chiral stationary phases (CSPs) for liquid chromatography (LC) are now commercially available. Among them, polysaccharide-based CSPs have been recognized as the most powerful due to their wide scope of application in the field of enantioseparations, their stability and their high loading capability. Cellulose and amylose are the most commonly used chiral selectors in such polysaccharide-based CSPs. There are the most abundant in the nature and interestingly their ester or phenylcarbamate derivatives have shown high chiral recognition abilities when coated on silica gel. Moreover, the recognition abilities of cellulose phenylcarbamates coated on silica gel can be controlled by the nature and position of the substituents on the phenyl groups. Indeed, the cellulose phenylcarbamates with electron-donating substituents, such as alkyl groups, or/and electron-withdrawing substituents such as halogens, exhibit higher chiral recognition than the non-substituted phenyl groups. Three cellulose-based CSPs, namely Sepapak-2, Sepapak-4 and Sepapak-5, using cellulose tris(3-chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-methylphenylcarbamate) and cellulose tris(3,5-dichlorophenylcarbamate) as chiral selector, respectively, were recently commercialized. Although these CSPs have been studied in LC normal phase systems, no systematic study of these phases has been performed in polar organic solvent chromatography (POSC) which can exhibit alternative recognition mechanisms and some other advantages, such as increased solubility for many drug compounds, particularly useful for preparative purpose.

In this thesis the main objective is to evaluate the chiral recognition abilities of these three chlorine containing cellulose-based CSPs, Sepapak-2; Sepapak-4 and Sepapak-5, in polar organic solvent chromatography. The mobile phase will be made of acetonitrile as the main solvent, with basic (0.1% DEA) and acidic additives. Thirteen basic drugs with widely different structures and polarities will be selected as model compounds: β-blockers (atenolol, betaxolol, celiprolol, metoprolol, oxprenolol, propranolol and sotalol), antimycotics (econazole and miconazole) and local anesthetics (bupivacaine, prilocaine and mepivacaine).

To achieve this goal, we intend first to investigate which factors can influence the retention and enantioresolution of these basic compounds on Sepapak-4 by means of a univariate approach. In this study, the role of the acidic additive and organic modifier added to the mobile phase as well as temperature on retention and enantioresolution will be particularly investigated. Afterwards, multivariate screening and optimization strategy will be applied. A mathematical model able to predict the enantioresolution of the studied compounds

will be developed and a rapid screening strategy for method development using the other chlorine containing CSPs will be proposed.

The second objective is to extend the study of the resolution power of the three CSPs using the rapid screening conditions previously defined and to compare the behavior of these CSPs in such polar organic mobile phases.

The third objective is to develop selective, precise, accurate and reliable LC methods for the determination of R-ropivacaine in a S-ropivacaine pharmaceutical formulation (Naropin®, 10 mg/mL solution) and R-amlodipine in a S-amlodipine pharmaceutical formulation using one of the studied chlorine containing CSPs in the polar organic solvent chromatography mode. Afterwards, these LC methods will be fully validated according to the strategy based on the use of accuracy profiles, which takes into account the total error, i.e. the estimation of systematic and random errors of measurement results.

CHAPTER III.

ENANTIORESOLUTION OF BASIC PHARMACEUTICALS USING CELLULOSE TRIS(4-CHLORO-3-METHYLPHENYLCARBAMATE) AS CHIRAL STATIONARY PHASE AND POLAR ORGANIC MOBILE PHASES

Summary

A polysaccharide-based chiral stationary phase (Sepapak-4), with cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral selector, has been investigated in liquid chromatography (LC). Its enantioresolution power was evaluated towards 13 basic amino-drugs with widely different structures and polarities, using polar organic mobile phases. After preliminary experiments, acetonitrile was selected as the main mobile phase component, to which a low concentration of diethylamine (0.1%) was systematically added in order to obtain efficient and symmetrical peaks. Different organic solvents were first added in small proportions (5–10%) to acetonitrile to modulate analyte retention. Polar organic modifiers were found to decrease retention and enantioresolution while hexane had the opposite effect, indicating normal-phase behaviour under these conditions. The addition of an organic acid (formic, acetic or trifluoroacetic acid) was found to strongly influence the retention of the basic amino drugs in these nonaqueous systems. The nature and proportion of the acidic additive in the mobile phase had also deep impact on enantioresolution. Therefore, the studied compounds could be subdivided in two groups with respect to the acidic additive used. All analytes could be enantioseparated in relatively short analysis times (10-20 min) using these LC conditions.

III.1. Introduction

Over the last decade, many efforts have been focused on the development of original chiral stationary phases (CSPs), either by immobilizing the chiral selector on silica support to extend the choice of modifiers or additives in the mobile phase or by proposing new chiral selectors.

Polysaccharide derivatives are the most commonly used CSPs for direct liquid chromatographic (LC) enantioseparation of chiral compounds. In particular, the phasescomposed of cellulose phenylcarbamate or amylose derivatives as chiral selectors have shown a wide range of applications [1–4]. Their enantioresolution ability often depends on the structure of the chiral polymers but also on the substituents of the phenyl group in the case of phenylcarbamate derivatives [5]. Okamoto and co-workers reported that the derivatives having an electron-donating substituent, such as a methyl group in the 3- or 4-positions of the phenyl moeity show a high chiral recognition due to the higher order structure of the chiral selector adsorbed on silica. Moreover, 3,5-disubstituted phenylcarbamates of cellulose (either bymethyl groups or by chloro groups) also showa high enantioresolutionpower [3]. Nevertheless, the high solubility of the dichlorophenylcarbamate derivative in organic solvents can be problematic. To enhance the chiral recognition, Chankvetadze et al. have developed new polysaccharidic CSPs substituted by methyl and chloro groups in positions 3 and 4 of the phenyl moiety [6–9]. Initially the normal-phase liquid chromatography (NPLC) was proposed for all these CSPs. However, it was shown later that not only reversed-phase LC (RPLC) with aqueous–organic mobile phases [10–18], but also polar organic solvent chromatography (POSC) can be applied [19,20].

Since the development of polysaccharides-based CSPs by Okamoto and co-workers [3,5,21–23] and the commercialization of derivatives, only a few efforts have been focused on the study of the effects of factors, such as the acidic additives, which can play acrucial role in polar organic solvent chromatography with respect to chiral selectivity. To the best of our knowledge, although some previous studies have shown the role of basic and acidic additives for enantioseparation in NPLC [24–26], only few systematic comparative studies have been reported in POSC concerning the use of polysaccharides-based CSPs with different acidic additives for separation of enantiomers [20,27].

In the present study, the chiral recognition ability of a polysaccharide-based CSP with cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral selector (cf. Fig. 1A), namely Sepapak-4, was evaluated for the enantioseparation of 13 basic amino-drugs with widely different structures and polarities (cf. Fig. 1B). Acetonitrile was used as main solvent with basic and acidic additives in POSC. The effect of factors likely to influence the chromatographic parameters such as retention, selectivity and enantioresolution on Sepapak-4, was examined.

III.2. Experimental

III.2.1. Chemicals and reagents

Acebutolol hydrochloride, metoprolol tartrate, oxprenolol hydrochloride, propranolol hydrochloride, econazole nitrate andprilocaine hydrochloride were supplied by Sigma–Aldrich (Saint-Louis, MO, USA). Celiprolol hydrochloride was provided by Rorer (Brussels, Belgium), miconazole nitrate by Janssen Pharmaceutica (Beerse, Belgium), sotalol hydrochloride by Profarmaco Combrex (Milan, Italy), atenolol by Erregierre (Bergamo, Italy), betaxolol by LERS (Paris, France), bupivacaine hydrochloride by Astra Pharmaceutical Products (Södertalje, Sweden) and mepivacaine hydrochloride by Federa (Brussels, Belgium). All samples are racemates used without further purification.

Acetonitrile (ACN), methanol, ethanol and 2-propanol of HPLC grade and glacial acetic acid (AcA) pro analysi were provided by Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), diethylamine (DEA) and formic acid (FA) pro analysi were obtained from Acros Organics (Geel, Belgium) and *n*-hexane from BDH Hypersol (Poole, UK).

III.2.2. Instrumentation

The chromatographic system from Agilent Technologies (Waldbronn, Germany) consisted in a binary pump, a thermostated column compartment, a diode array detector and an automatic injector, all of 1100 series. The Chemstation software was used for system control and data acquisition.

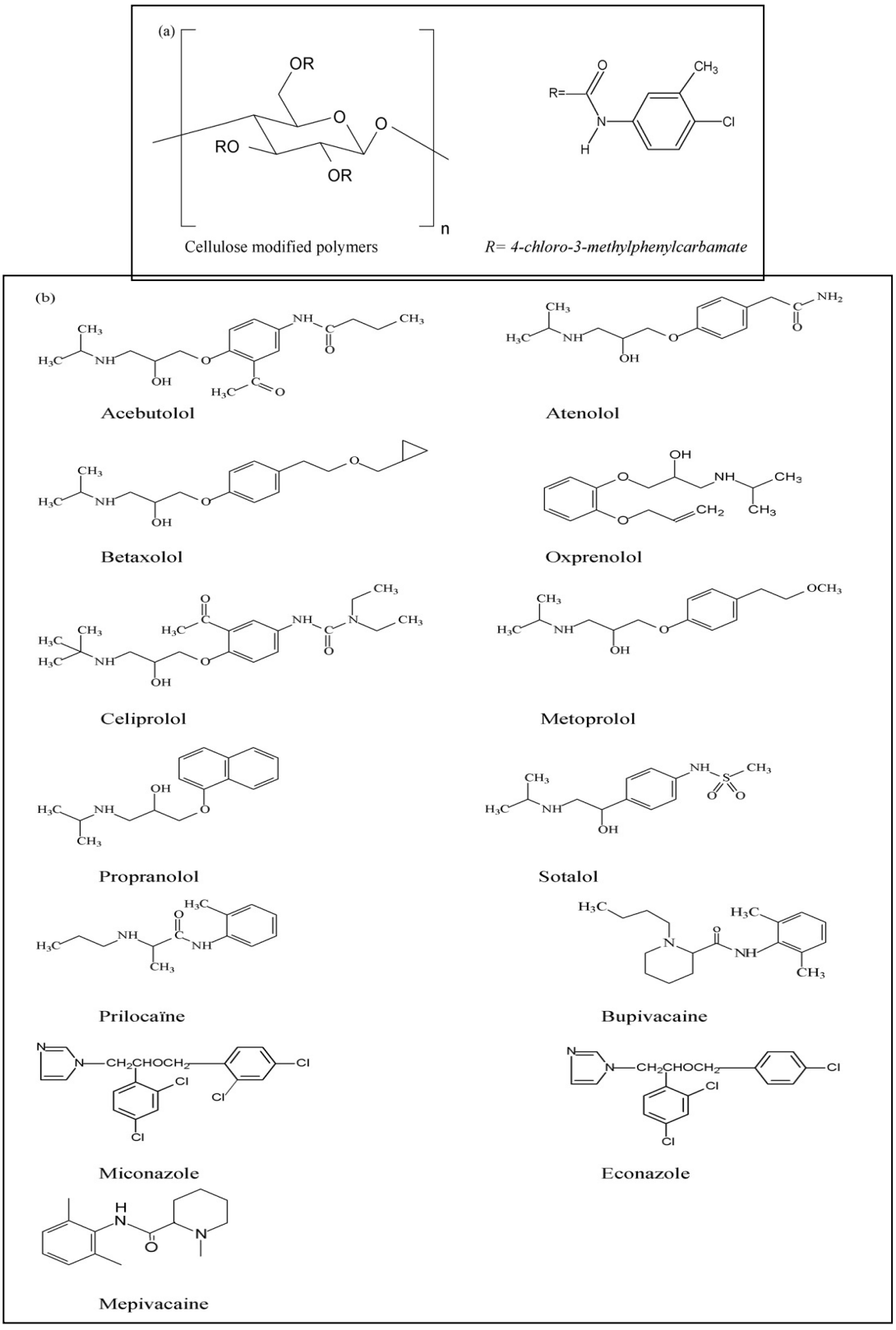

Figure. 1. Structure of Sepapak-4 chiral selector (a) and molecular structures of the studied basic amino drugs (b).

The chiral column Sepapak-4 (250mm×4.6mm I.D.) was kindly provided by Sepaserve (Münster, Germany). The chiral selector adsorbed on aminopropylsilanized silica (nominal particle size 5µm and nominal pore diameter 100 nm) was cellulose tris(4-chloro-3-methylphenylcarbamate) in the amount of 25% (w/w).

III.2.3. Solutions for method development

The mobile phases used of the different experiments were prepared by mixing the required proportions of acetonitrile, organic modifier (methanol or hexane) and acidic additives (TFA; FA; AcA). Then 0.1% of DEA (9.7 mM) was systematically added. Analytical solutions of racemate compounds of nearly 100 µg/ml were prepared by dissolving the appropriate amount of the substance in the required volume of mobile phase.

III.2.4. Chromatographic conditions

The mobile phases consisted in amixture of acetonitrile, organic modifier (methanol or hexane), acidic additive and DEA (v/v) and were pumped at a constant flow-rate of 1.0mL min−1. In the different experiments, the DEA percentage in the mobile phase was settled at 0.1%. The injection volume was 20µL. The analytes were detected photometrically at 220 nm.

III.3. Results and discussion

Originally dedicated for normal- and reversed-phases, polysaccharidic CSPs provided good results in POSC [19, 28–32]. In this system, only polar organic solvents such as acetonitrile, methanol, 2-propanol and their mixtures are used. Methanol and acetonitrile were tested as the main component of the mobile phase and the best results were obtained with acetonitrile. These results confirm observations made by others [29]. Therefore, acetonitrile was selected as the main solvent.

In addition to the classical factors such as temperature, pH and type of organic modifier, which can influence the enantioseparation in LC, several authors demonstrated that the acidic and basic mobile phase additives can also have a significant impact [20,24,33,34]. In these studies mostly carried out in normal-phase, these additives were supposed to minimize the non-specific interactions between the analytes and the free silanol groups of the CSP. Nevertheless, these additives are known to have a strong affinity for the CSP [20,24,33,34]. Therefore, the present study protocol includes a rinsing procedure with neat acetonitrile for 1 h followed by an equilibration of the CSP with the mobile phase containing both acidic and basic additives for 1 h.

Since few information on the behaviour of Sepapak-4 in POSC is available, this screening study was conducted following a classical method development. In accordance with the literature, the studied factors are those commonly investigated, namely the temperature, the nature and the proportion of the organic modifier as well as the acidic additive. It has been checked that the nature of the basic additive, namely diethyamine, triethylamine and butylamine, had no significant influence on enantioresolution, as already observed in the literature for polysaccharide based CSP [20].

III.3.1. Effect of the temperature

The influence of the temperature on the enantioresolution (*R*s) of the studied chiral drugs was investigated (cf. Fig. 2). As can be seen in this figure, the change in enantioresolution with temperature seems to be generally rather limited and very much compound dependent. For those which exhibited an increased enantioresolution with temperature, a significant efficiency enhancement was observed at 35°C (e.g. for celiprolol, plate number (*N*) increases from 3200 at 15°C to 6500 at 35°C). The latter effect seems to be mainly responsible for the observed improvement in resolution. However, for three compounds with low enantioresolution (namely, mepivacaine, oxprenolol and metoprolol (cf. Fig. 2), high temperature is defavourable. Finally, it was decided to select 15°C for further investigations.

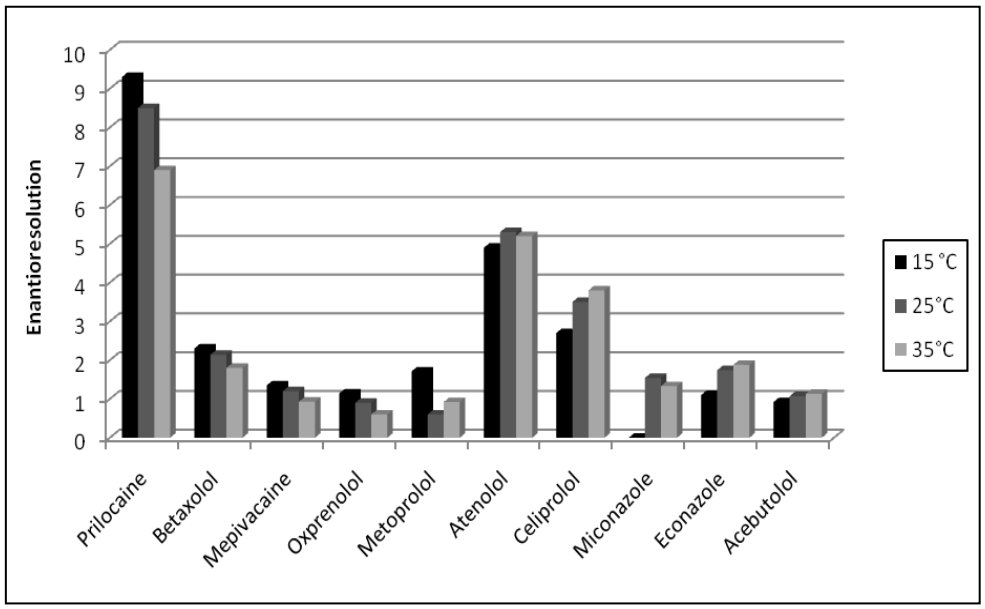

Figure. 2. Influence of the temperature on enantioresolution.
Mobile phase: ACN/0.1%DEA/0.1% TFA; temperature: 15, 25 and 35°C.
Other conditions: see Section 2.

III.3.2. Effect of the addition of an organic modifier

As illustrated in Fig. 3 for atenolol enantiomers, the addition of 10% hexane in the mobile phase increased the retention and the enantioresolution of most of the studied molecules, unlike methanol, confirming the involvement of hydrogen-bond interactions between the analyte and the chiral selector. Therefore, these results clearly show that a polar organic modifier increases the elution capacity of the mobile phase, in accordance with Lyman and Stringham's work [32], indicating a normal-phase behaviour where the hydrogen-bond interactions are regulated by the nature and the proportion of the organic modifier.

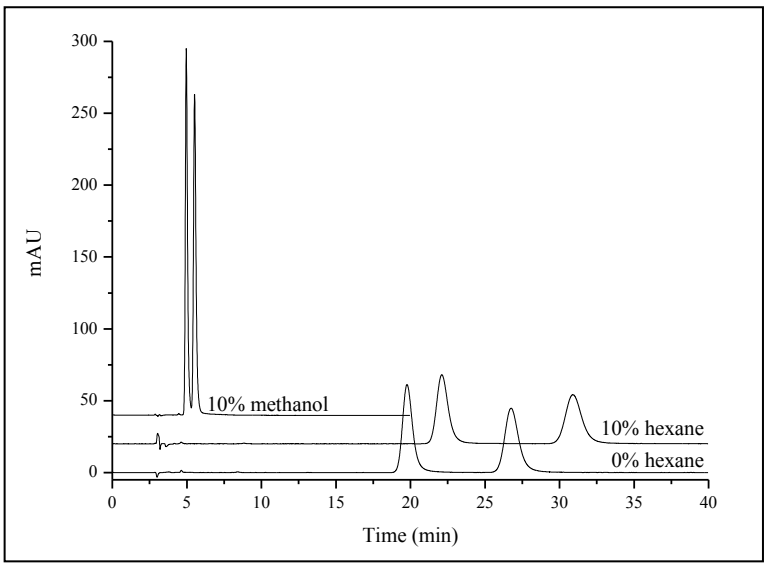

Figure. 3. Chromatograms illustrating the effect of *n*-hexane or methanol addition in the mobile phase on atenolol enantiomers resolution.
Mobile phase: ACN/0.1%DEA/0.1% TFA/*x* (0% or 10%) hexane or methanol; temperature: 15°C. Other conditions: see Section 2.

III.3.3. Effect of the nature of the acidic additive

The importance of the nature of the acidic additive was also demonstrated. Table 1 presents the retention factor, the enantioresolution and selectivity values obtained in the presence of 0.1% TFA (13.5 mM), acetic acid (26.5 mM) and formic acid (17.5 mM). As can be seen in this table, the enantiomers of propranolol, sotalol, miconazole and bupivacaine were not resolved when TFA was used as an acidic modifier, while a complete enantioseparation was observed in the presence of formic acid and/or acetic acid. The best enantioresolution of the β-blockers (except for oxprenolol, propranolol and sotalol) and prilocaine was obtained with TFA while the best results for the imidazole derivatives (econazole and miconazole) and sotalol were observed with acetic acid. As for the other local anaesthetics (bupivacaine and mepivacaine), oxprenolol and propranolol, formic acid gave rise to the highest *R*s values. Moreover, this acid led to the strongest retention for all studied compounds, which indicates that the acidic character of the additive is not the only factor decreasing retention. Indeed, formic acid is stronger than acetic acid but gives rise to higher

retention. Obviously, other factors intervene in the interactions between the analytes and the CSP such as possibly ion-pair formation which is in principle stronger for acetic acid than formic acid. Results from Table 1 clearly show that acidic additives have also an important effect on enantioresolution and selectivity by contrast to data obtained with classical cellulose or amylose tris(3,5-dimethylphenylcarbamate) based CSP [33]. Therefore, it can be assumed that the introduction of chlorine on the phenyl moiety of Sepapak-4 has a deep impact on the ability of acidic additives to enhance enantioresolution. This might be explained by a reduction of non-specific interactions with the CSP due to the presence of acidic additive in the mobile phase [24,33,34].

III.3.4. Effect of the proportion of the acidic additive

Fig. 4 illustrates the influence of the proportion of acetic and formic acids on the retention factor. Since all compounds of the same class exhibited similar behavior, only the results obtained for one compound of each class is presented in Fig. 4. It is worth noting that the percentage of TFA was not investigated since proportions lower or higher than 0.1% led to a very unstable baseline and to a very low retention. As can be seen in Fig. 4, similar effects were observed for the two acidic additives but the influence of acetic acid concentration seems to be less pronounced. A tendency to an increase in retention with the acidic additive content was found for most compounds and seems to be related to their basic character, since only the two analytes with low p*K*a values, namely the two imidazole derivatives, showed an opposite trend.

Tables 2 and 3 present the effect of acetic and formic acids proportion in the mobile phase on the enantioresolution and selectivity values of the studied basic drugs. As can be seen in these tables, the influence of the acidic additive concentration on enantioresolution and selectivity seems to follow the same trend as observed for retention (cf. Fig. 4). In most cases, *R*s and selectivity values were found to increase with acidic additive percentage, except for acetic acid which shows an opposite effect (cf. Tables 1–3). By contrast, a significant decrease in enantioresolution and selectivity was obtained for the two less basic compounds (i.e. the imidazole derivatives).

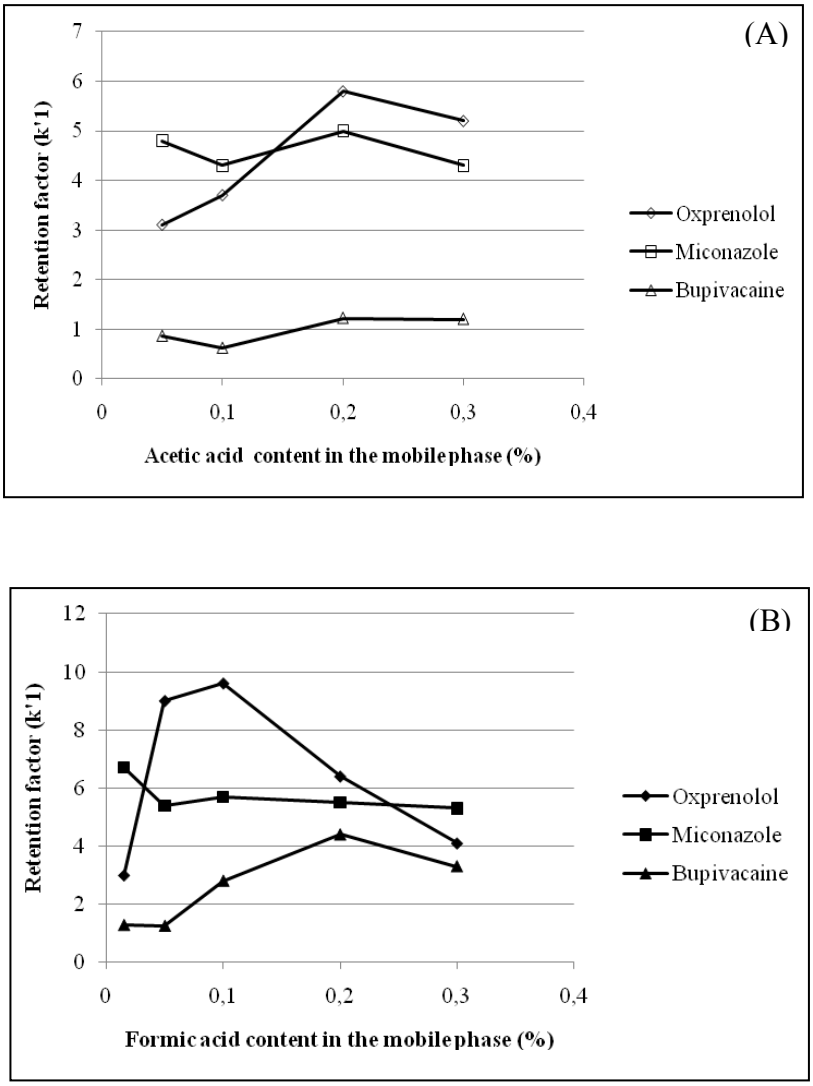

Fig. 4. Influence of the acetic (A) and formic (B) acid proportion in the mobile phase on the retention factor (*k'1*).

Mobile phase: ACN/0.1% DEA/(0.05–0.3%) AcA (A) or ACN/0.1% DEA/(0.015–0.3%) FA (B); temperature: 15°C.

Other conditions: see Section 2

Table 1. Influence of the acidic additive nature in the mobile phase on the retention factor (*k'1*), the enantioresolution (*R*s) and selectivity (α) of the studied basic amino-drugs using Sepapak-4.

	0.1% TFA			0.1% AcA			0.1% FA		
	k'1	*Rs*	α	*k'1*	*Rs*	α	*k'1*	*Rs*	α
Acebutolol	1.50	**0.92**	1.09	/	/	/	/	/	/
Atenolol	5.1	**4.9**	1.42	/	/	/	/	/	/
Betaxolol	0.55	**2.3**	1.34	5.4	1.13	1.09	13.8	-	-
Celiprolol	2.2	**2.7**	1.31	/	/	/	/	/	/
Metoprolol	0.48	**1.71**	1.26	5.0	0.77	1.06	12.4	-	-
Oxprenolol	0.38	1.14	1.20	3.7	**2.2**	1.17	9.6	**2.3**	1.16
Propranolol	0.28	-	-	4.6	1.20	1.08	12.3	**2.2**	1.15
Sotalol	0.16	-	-	4,1	**1.85**	1.16	12.0	1.50	1.11
Econazole	1.31	1.10	1.10	3.0	**6.4**	1.55	4.1	**6.1**	1.48
Miconazole	1.90	-	-	4.3	**3.9**	1.29	5.7	2.7	1.19
Bupivacaine	0.72	-	-	0.62	-	-	2.8	**2.6**	1.19
Mepivacaine	0.64	1.35	1.16	0.65	1.21	1.19	2.6	**2.5**	1.19
Prilocaine	0.55	9.3	2.7	0.29	1.82	1.36	1.85	1.22	1.09

'/' no peak obtained within 60 min. '–' no enantioresolution observed. Mobile phase: ACN/0.1% DEA/0.1% (TFA: 13.5 mM, AcA: 26.5 mM or FA: 17.5 mM); temperature: 15°C; other conditions: see Section 2. Bold values: highest *R*s values obtained for each analyte.

Table 2. Influence of acetic acid proportion in the mobile phase on the enantioresolution (*R*s) and selectivity (α) of the studied basic amino-drugs using Sepapak-4.

	Acetic acid (%)							
	0.05[a]		0.1[b]		0.2[c]		0.3[d]	
	Rs	α	*Rs*	α	*Rs*	α	*Rs*	α
Acebutolol	/	/	/	/	/	/	/	/
Atenolol	/	/	/	/	/	/	/	/
Betaxolol	1.56	1.11	1.31	1.09	1.46	1.10	0.89	1.06
Celiprolol	/	/	/	/	/	/	/	/
Metoprolol	1.08	1.07	0.80	1.06	0.80	1.06	-	-
Oxprenolol	2.3	1.17	2.2	1.17	2.4	1.17	1.98	1.42
Propranolol	0.95	1.07	1.20	1.11	1.54	1.11	2.1	1.15
Sotalol	1.93	1.17	1.85	1.15	1.89	1.15	1.50	1.13
Econazole	6.7	1.53	6.4	1.51	6.1	1.51	5.8	1.50
Miconazole	3.9	1.30	3.9	1.26	3.6	1.26	3.4	1.25
Bupivacaine	-	-	-	-	-	-	-	-
Mepivacaine	1.35	1.14	1.21	1.11	1.20	1.11	1.20	1.12
Prilocaine	2.2	1.35	1.82	1.14	1.38	1.14	-	-

'/' no peak obtained within 60 min. '–' no enantioresolution observed. Mobile phase: ACN/0.1% DEA/(0.05–0.3)% AcA; temperature: 15°C; other conditions: see Section 2.

[a,b,c,d] letters refer to molar concentrations of acidic additives: (a) 8.7 mM; (b) 17.5 mM; (c) 35 mM; (d) 52.5 mM.

Table 3: Influence of formic acid proportion in the mobile phase on the enantioresolution (*Rs*) and selectivity (α) of the studied basic drugs on Sepapak-4.

	Formic acid (%)									
	0.015[a]		0.05[b]		0.1[c]		0.2[d]		0.3[e]	
	Rs	α	*Rs*	α	*Rs*	α	*Rs*	α	*Rs*	α
Acebutolol	-	-	/	/	/	/	/	/	1.32	1.09
Atenolol	/	/	/	/	/	/	/	/	/	/
Betaxolol	-	-	-	-	-	-	0.6	1.05	/	/
Celiprolol	/	/	/	/	/	/	/	/	-	-
Metoprolol	-	-	-	-	-	-	-	1	-	-
Oxprenolol	1.53	1.12	1.68	1.11	2.3	1.16	2.4	1.16	2.4	1.15
Propranolol	1.62	1.12	1.82	1.12	2.2	1.15	2.3	1.16	2.3	1.16
Sotalol	0.77	1.07	1.01	1.07	1.5	1.11	1.6	1.12	1.57	1.10
Econazole	7.7	1.59	7.0	1.58	6.1	1.48	3.7	1.28	2.6	1.16
Miconazole	3.9	1.24	3.2	1.23	2.7	1.19	1.39	1.09	-	-
Bupivacaine	-	-	-	-	2.6	1.19	3.2	1.22	2.9	1.18
Mepivacaine	1.49	1.14	1.28	1.14	2.5	1.19	2.9	1.21	3.3	1.27
Prilocaine	2.9	1.34	1.75	1.21	1.22	1.09	1.09	1.21	2.7	1.27

'/' no peak obtained after 60 min. '–' no enantioresolution observed.

Mobile phase: ACN/0.1% DEA/(0.015–0.3)% FA; temperature: 15°C; other conditions: see Section 2.

[a,b,c,d,e]: letters refer to molar concentration of acidic additives: (a) 4 mM; (b) 13.2 mM; (c) 26.5 mM; (d) 53 mM; (e) 79.5 mM.

The chromatograms illustrated in Fig. 5 show that an increase of the formic acid concentration in the mobile phase gives rise to a decrease of the econazole retention and enantioresolution whereas the opposite trend is observed for the more basic mepivacaine. It is worth noting that higher concentrations (>0.2%) are not recommended because they have a tendency to give rise to noisy baseline.

Therefore, two groups of substances could be distinguished from Tables 1–3, according to the acidic additive used. The first group is constituted of atenolol, celiprolol, acebutolol, betaxolol, metoprolol and prilocaine. For these compounds, TFA gives rise to the best enantioresolution. The second group includes the other compounds, i.e. bupivacaine, mepivacaine, econazole, miconazole oxprenolol, propranolol and sotalol for which a high enantiomeric separation is observed with formic acid.

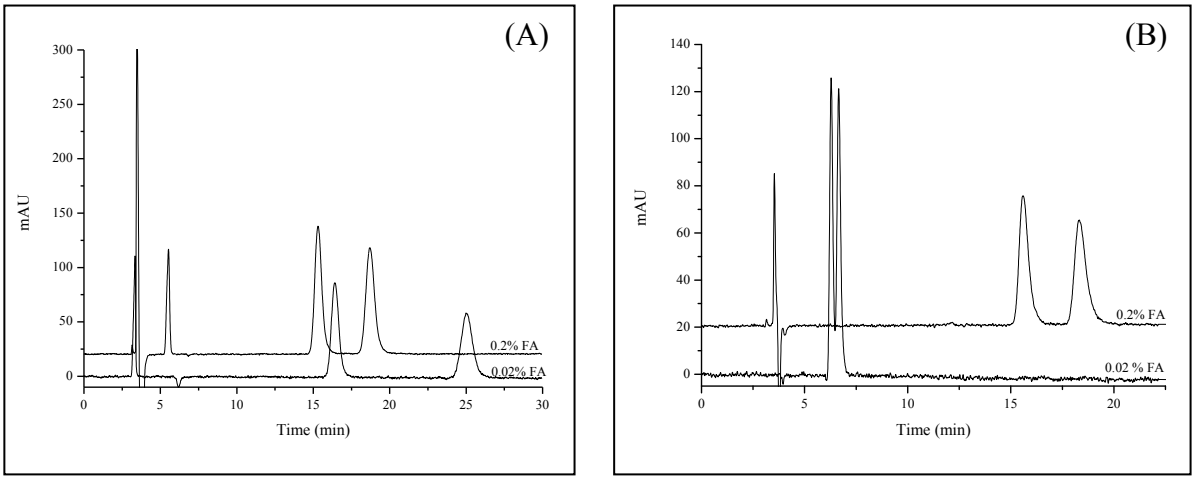

Figure. 5. Chromatograms of econazole (A) and mepivacaine (B) enantiomers illustrating the effect of formic acid proportion on enantioresolution.
Mobile phase: ACN/0.1% DEA/ (0.02–0.2%) FA; temperature: 15°C. Other conditions: see Section 2.

III.4. Conclusion

The newly commercialized chiral stationary phase Sepapak-4 was tested with acetonitrile as main mobile phase component and 0.1% of basic additive (DEA). The results showed a significant effect of acidic additive nature and concentration on retention, selectivity and enantioresolution of the basic chiral compounds. The studied CSP demonstrated good chiral discrimination ability. Temperature, the type and concentration of the acidic additive as well as the presence of an organic modifier in the mobile phase were found to be important parameters to optimize retention and enantioresolution. Furthermore, the tested compounds showed different behaviours and can be classified in two groups according to the acidic additive selected. These results will be exploited to perform multivariate screening and optimization of enantioresolution on this CSP.

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CHAPTER IV.

OPTIMIZATION OF THE LC ENANTIOSEPARATION OF CHIRAL PHARMACEUTICALS USING CELLULOSE TRIS(4-CHLORO-3-METHYLPHENYLCARBAMATE) AS CHIRAL SELECTOR AND POLAR NON-AQUEOUS MOBILE PHASES

Summary

The resolving power of a new commercial polysaccharide-based chiral stationary phase (CSP), Sepapak-4, with cellulose tris(4-chloro-3-methylphenylcarbamate) coated on silica microparticles as chiral selector, was evaluated towards the enantioseparation of ten basic drugs with widely different structures and hydrophobic properties, using acetonitrile as the main component of the mobile phase. A multivariate approach (experimental design) was used to screen the factors (temperature, n-hexane content, acidic and basic additives) likely to influence enantioresolution. Then the optimization was performed using a face-centered central composite design. Complete enantioseparation could be obtained for almost all tested chiral compounds, demonstrating the high chiral discrimination ability of this CSP using polar organic mobile phases made up of acetonitrile and containing an acidic additive (trifluoroacetic or formic acid), 0.1% diethylamine and n-hexane. These results clearly illustrate the key role of the nature of the acidic additive in the mobile phase.

IV.1. Introduction

The use of chiral stationary phases (CSPs) in liquid chromatography (LC) has increased dramatically in the last two decades [1, 2]. This is not only related to the common availability and demonstrated success of LC instrumentation for performing separations in the pharmaceutical industry but also because CSPs can be used in LC both for analytical and preparative chiral separations in a very efficient and flexible way [3].

Among the large number of commercially available CSPs, polysaccharide derivatives belong to the most widely used phases for LC enantioseparations [4, 5]. Many derivatives (acetate, benzoates, carbamates, etc) of polysaccharides such as cellulose [6-9] and amylose [6, 8] have shown particularly high chiral recognition ability for a wide variety of racemates including many drugs [10-16]. This recognition ability not only depends on the nature [17-22] and position of substituents introduced on the phenyl groups of the carbamates derivatives [7] but also on the composition of the mobile phase used and the presence of acidic additives [17; 23-25].

To enhance the chiral recognition ability of polysaccharide based CSPs, new phenylcarbamate derivatives were developed [18-22]. The latter have both an electro-donating methyl group and an electro-withdrawing chloro or fluoro group on the phenyl moiety. These newly commercialised CSPs have shown very high chiral recognition ability [18-22, 26-28], which make them possible alternative to well known CSPs based on 3,5-dimethylphenylcarbamate of cellulose and amylose [25,29-32]. The traditional mobile phase for polysaccharide based CSPs has been an alcohol-alkane combination. Unfortunately, many pharmaceutical compounds have poor solubility in this kind of mobile phase [33]. The reversed phase mode can cover many potential HPLC applications of those polysaccharide based CSPs [34-43]. Nevertheless the polar organic mode has become increasingly popular during last decade [40-45]. In fact, polar organic eluents offer the advantages of alternative chiral recognition mechanisms, higher solubility for many analytes and easier removal from the analytes [44]. These advantages are particularly interesting in preparative scale enantioseparation or simulating moving bed (SMB) chromatography as the loading capacity strictly depends on sample solubility in eluents [44].

In the present work, the resolving power of the recently commercialized polysaccharide based chiral stationary phase (CSP), Sepapak-4, with cellulose tris (4-chloro-

3-methylphenylcarbamate) as chiral selector, has been evaluated toward the enantioseparation of ten basic drugs with widely different structures and hydrophobic properties (Fig.1), using acetonitrile as the main mobile phase component. To achieve this goal, a screening experimental design, namely a fractional factorial design, has been applied to deduce the main factor influencing on enantioresolution in these LC systems. Then, a face-centered central composite design has been used to obtain optimal LC conditions for the enantioresolution of these ten chiral basic drugs. Finally, a strategy for rapid method development has been proposed.

IV.2. Experimental

IV.2.1. Chemicals and reagents

Acebutolol, oxprenolol, propranolol and prilocaine hydrochlorides, metoprolol tartrate, terbutaline hemisulfate, alprenolol, pindolol and econazole, isoconazole and sulconazole nitrates were supplied by Sigma-Aldrich (Saint-Louis, MO, USA). Celiprolol hydrochloride was provided by Rorer (Brussels, Belgium), miconazole nitrate by Janssen Pharmaceutica (Beerse, Belgium), sotalol hydrochloride by Profarmaco Combrex (Milan, Italy), atenolol by Erregierre (Bergamo, Italy), betaxolol by LERS (Paris, France), bupivacaine hydrochloride by Astra Pharmaceutical Products (Södertalje, Sweden) and mepivacaine hydrochloride by Federa (Brussels, Belgium). All samples were racemates used without further purification.

Acetonitrile (ACN) of HPLC grade and glacial acetic acid (AcA) pro analysi were provided by Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), diethylamine (DEA), and formic acid (FA) pro analysi were obtained from Acros Organics (Geel, Belgium). Triethylamine (TEA) pro analysi was provided from Sigma-Aldrich (St Louis, USA), butylamine (BuA) pro analysi from Fluka (Buchs, Switzeland) and *n*-hexane of HPLC grade from BDH Hypersol (Poole, UK).

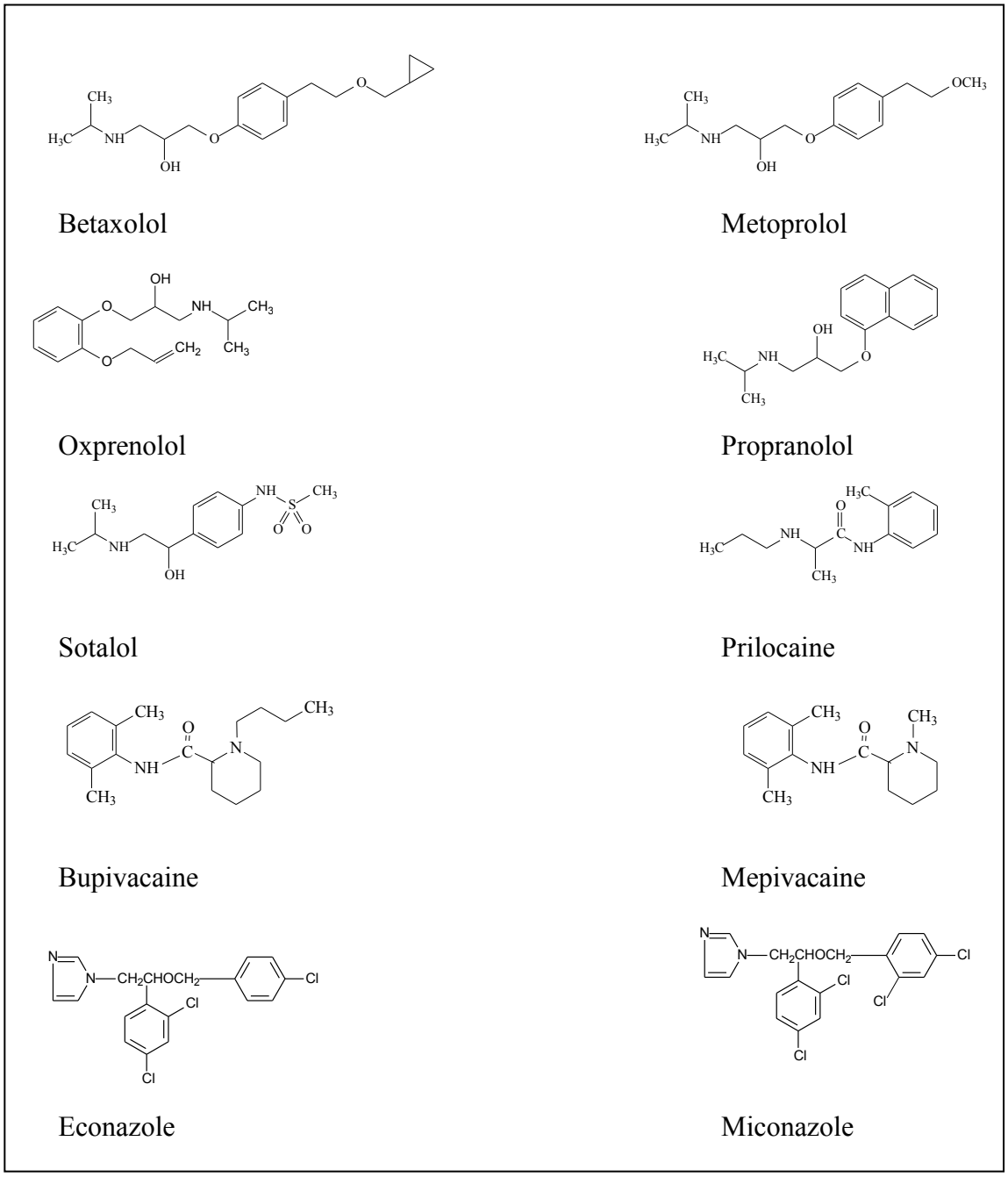

Figure 1. Structure of studied basic drugs.

IV.2.2. Instrumentation

The chromatographic system from Agilent Technologies (Waldbronn, Germany) consisted of a binary pump, a thermostated column compartment, a diode array detector and an automatic injector, all of 1100 series. The Chemstation software was used for system control and data acquisition.

The chiral column Sepapak-4 (250 mm x 4.6 mm I.D.) was kindly provided by Sepaserve GmbH (Münster, Germany). The chiral selector cellulose tris(4-chloro-3-methylphenylcarbamate) was coated on pretreated aminopropylsilanized silica (with nominal particle size 5 μm).

The experimental design and the statistical calculations were performed using the MODDE software version 6.0 from Umetri AB (Umeä, Sweden).

IV.2.3. Solutions for method development

Analytical solutions of racemic compounds of 100 μg/ml were prepared by dissolving the appropriate amount of the substance in the required volume of mobile phase.

IV.2.4. Chromatographic conditions

The mobile phases consisted of a mixture of acetonitrile, organic modifier (hexane), acidic additive and basic additive (*v/v*) and were pumped at a constant flow-rate of 1.0 mL.min-1. In all experiments, the basic additive percentage in the mobile phase was settled at 0.1 %. The injection volume was 20 μL. The analytes were detected photometrically at 220 nm. The repeatability of the main response (enantioresolution), was evaluated using standard deviation (SD), calculated from four independent injections of atenolol. This SD was lower than 0.1.

IV.3. Results and discussion

In our previous work dealing with the screening of the factors likely to influence the enantioresolution of thirteen chiral basic pharmaceuticals, temperature, the nature of the acidic additive and of the organic modifier in the mobile phase were selected as important factors after an univariate screening study [25]. In this previous study, 0.1% DEA was systematically added to the mobile phase as basic additive.

In the present work, we evaluated the effect of three basic additives namely the primary amine butylamine (BuA), the secondary amine diethylamine (DEA) and the tertiary amine triethylamine (TEA). They were added to the previously studied factors for the multivariate optimization. This optimization procedure was divided into two steps: (a) multivariate screening to select the most important factors likely to influence the enantioresolution of the studied compounds; (b) multivariate optimization to obtain chromatographic conditions for maximum enantioresolution. Preliminary experiments were performed to ensure that no reversal in the elution order of enantiomers occurred. Each racemic mixture of propranolol and bupivacaine was spiked with one of the two enantiomers and no reversal of the elution order of enantiomers was observed under variation of temperature or acidic additive concentration.

The screening was carried out with ten chiral drugs, namely betaxolol, metoprolol, oxprenolol, propranolol, sotalol, econazole, miconazole, bupivacaine, mepivacaine and prilocaine.

IV.3.1. Screening design

To achieve the multivariate screening aiming to deduce the most important factors, a fractional factorial design was applied to four factors (two quantitative factors and two qualitative factors), namely the n-hexane proportion (0-10%), the temperature (15-35°C), the nature of the acidic additive (TFA, FA and AcA) and the nature of the basic additive (DEA, TEA and BuA), with three level each. This design involved 9 experimental points with 3 replicates in the center of the domain resulting in 12 randomized runs (Table 1). To define the relationship between the response and the factors, a linear regression model was applied on

the basis of a multiple linear regression (MLR). The selected model included 6 coefficients as indicated in the equation (1)

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \varepsilon \quad (1)$$

where y is the enantiomeric resolution, β_1, β_2, β_3 and β_4 the main effects, β_0 the intercept and ε the error term.

X_1 is the organic modifier percentage (0-10 %; n-hexane); X_2 is the temperature (15-35°C); X_3 is the acidic additive (0.1 % of TFA, AcA or FA); X_4 is the basic additive (0.1% of DEA, TEA or BuA).

Table 2 presents the different coefficients of the screening model and their statistical significance for the ten compounds studied. The effect of a factor is considered as significant if its *p*-value is lower than 0.05. The *p*-value is the probability of getting a result as extreme or more extreme than the one observed if the proposed null hypothese is correct. In this study, only positive effects were taken into account in order to maximize enantioresolution.

As can be seen in this table, temperature has no significant effect on enantioresolution for the studied compounds except sotalol. An increase of hexane proportion in the mobile phase influenced enantioresolution of bupivacaine, econazole and propranolol. Regarding the nature of the acidic additive, it is worth noting that TFA has a significant effect on resolution of betaxolol, metoprolol and prilocaine enantiomers. On the other hand, enantioresolution of bupivacaine, econazole, miconazole oxprenolol, propanolol and sotalol is significatively influenced by FA. Finally, AcA has a significant effect on the enantioresolution of econazole, miconazole and sotalol.

Enantioresolution was slightly influenced by basic additives: the effect of DEA was positive and significant for econazole, prilocaine, and propranolol enantiomers whereas BuA had a significant positive effect on enantioresolution of metoprolol and sotalol. For bupivacaine, the best basic additive was TEA.

Table 1. Screening design.

Trial	Factors			
	X_1	X_2	X_3	X_4
1	0	15	TFA	DEA
2	0	25	AcA	TEA
3	0	35	FA	BuA
4	5	15	AcA	BuA
5	5	25	FA	DEA
6	5	35	TFA	TEA
7	10	15	FA	TEA
8	10	25	TFA	BuA
9	10	35	AcA	DEA
10	5	25	TFA	DEA
11	5	25	TFA	DEA
12	5	25	TFA	DEA

X_1 is hexane proportion in the mobile phase; X_2 is temperature of the CSP; X_3 is the nature of the acidic additive (0.1%) in the mobile phase (TFA: trifluoroacetic acid; FA: formic acid and AcA: acetic acid); X_4 is the nature of the basic additive (0.1%) (BuA: butylamine; DEA: diethylamine and TEA: triethylamine).

Table 2. Coefficients (Coef.) of the model and their *p*-values.

	Betaxolol		Metoprolol		Prilocaine		Propranolol		Oxprenolol	
	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value
$\beta_1$hexan	-0.362	0.1967	-0.238	0.1748	-0.46	0.1013	0.344	**0.0212**	0.318	0.1031
β_2 temp	0.192	0.3620	0.088	0.4867	-0.45	0.1091	0.003	0.9666	0.008	0.9482
β_3 TFA	0.667	**0.0287**	0.47	**0.0189**	4.450	**<0.0001**	-0.92	**0.0003**	-0.52	**0.0148**
β_3 AcA	0.022	0.9405	0.075	0.6801	-1.94	**0.0007**	-0.34	**0.0338**	-0.26	0.2189
β_3 FA	-0.688	**0.0422**	-0.545	**0.0197**	-2.51	**0.0002**	1.269	**0.0002**	0.786	**0.0065**
β^4 DEA	0.007	0.9749	-0.077	0.5680	0.64	**0.0394**	0.241	**0.0391**	0.301	0.0794
β_4 TEA	-0.295	0.3406	-0.335	0.1184	-0.49	0.1184	0.103	0.3992	-0.03	0.8722
β_4 BuA	0.288	0.2849	0.412	**0.0466**	-0.15	0.5788	-0.34	**0.0212**	-0.27	0.1470

	Sotalol		Econazole		Miconazole		Bupivacaine		Mepivacaine	
	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value	Coef.	*p*-value
$\beta_1$hexane	-0.058	**0.0049**	0.4062	**0.0121**	0.1361	0.3687	0.3095	**<0.0001**	0.2674	0.2755
β_2Temp	0.081	**0.0006**	0.0200	0.8013	0.2567	0.0751	-0.3117	**<0.0001**	-0.3800	0.0878
β_3 TFA	-1.2	**<0.0001**	-2.802	**<0.0001**	-1.5886	**0.0002**	-0.7219	**<0.0001**	-0.4538	0.0659
β_3 AcA	0.735	**<0.0001**	1.2014	**0.0004**	1.1348	**0.0019**	-0.7219	**<0.0001**	-0.0788	0.7660
β_3 FA	0.465	**<0.0001**	1.6004	**<0.0001**	0.4538	**0.0279**	1.4438	**<0.0001**	0.5326	0.0658
β_4 DEA	0.023	0.0572	0.2247	**0.0475**	0.0581	0.6393	-0.0086	0.2746	0.1162	0.5552
β_4 TEA	-0.082	**0.0025**	-0.148	0.2441	-0.1086	0.5276	0.3181	**<0.0001**	-0.1955	0.4737
β_4 BuA	0.0583	**0.0049**	-0.076	0.4597	0.0505	0.7266	-0.3095	**<0.0001**	0.0793	0.7272

(a) Values significant at the 5% level are printed in bold

However it should be noted that among these basic additives only DEA had a positive effect in most cases (8/10) even if this effect was significant only for three of the ten compounds studied. Therefore, to simplify the study, DEA was selected as basic additive for further experiments.

From those results, the studied drugs could be divided in two groups: (i) group I, including betaxolol, metoprolol and prilocaine, for which only one factor is significant, i.e. the nature of the acidic additive: TFA being the only additive with a favourable effect on enantioresolution; (ii) group II, containing bupivacaine, econazole , miconazole, oxprenolol, propanolol and sotalol, with two significant factors: the nature of the acidic additive (FA being the most useful additive in this case) and the proportion of *n*-hexane in the mobile phase. On the other hand, no factor was found significant for mepivacaine enantioresolution, even if the best resolution value (2.5) was observed with FA.

Since the nature of acidic additive is the only factor likely to influence significantly the enantioresolution in group I, there is no need to perform further optimization since a proportion lower than 0.1% TFA led to a very unstable baseline while a TFA concentration higher than 0.1% gave rise to a very low retention [25]. Therefore the best conditions for enantioresolution generated from the screening were applied and the observed results were found to be close to those predicted (cf. Table 3). For mepivacaine, 0.1% FA was selected as acid additive, together with the central value of the *n*-hexane proportion (5%). The corresponding chromatograms are presented in Fig.2.

As demonstrated earlier, the proportion of acidic additive in the mobile phase often has a significant influence on the enantioresolution of basic drugs using this kind of polysaccharidic stationary phase. The optimization of enantioresolution for compounds of group II was therefore carried out with two quantitative factors, namely the formic acid (FA) proportion (0.015-0.3%) according to results obtained in our previous study [25] and the *n*-hexane proportion (0-10%) in the mobile phase. The temperature was held at 25°C, central value in the screening design, and the basic additive selected was DEA (0.1%).

Table 3. Highest enantioresolution values (*R*s) obtained from screening experiments.

Analytes	Mobile phase	RT (peak 2) (min)	*Rs*	
			Predicted (confidence interval at 95%)	Observed
Betaxolol	(a)	5.2	2.3 [1.46-3.12]	2.1
Metoprolol		4.9	1.64 [1.12-2.15]	1.5
Prilocaine		7.0	9.2 [8.27-10.1]	8.6
Mepivacaine	(b)	10.3	2.4 [1.32-3.48]	2.4

Mobile phases: (a) *ACN/0.1% DEA/0.1% TFA; (b) ACN/0.1% DEA/0.1% FA/5%* hexane; Temperature: 25°C; Other conditions: see Experimental.

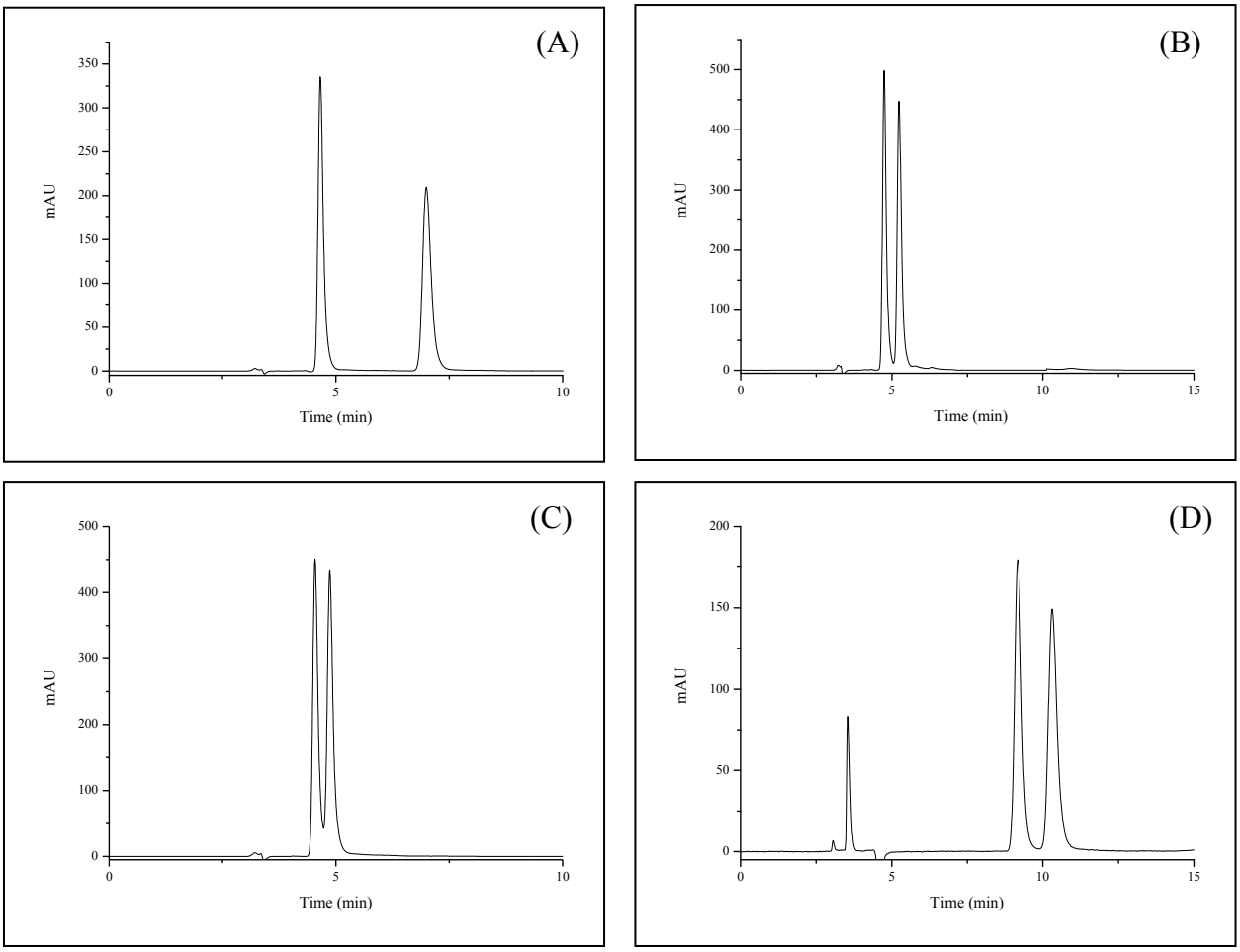

Figure 2. Chromatograms obtained under the best screening conditions.

Prilocaine; B) Betaxolol; C) Metoprolol; D) Mepivacaine.

Mobile phase: Figs A to C: ACN/ 0.1% DEA/0.1%TFA ; Fig D: ACN/ 0.1% DEA/0.1%FA/ 5% hexane; temperature : 25°C; Other conditions: see Experimental and Table 3.

IV.3.2. Optimization

The optimization of the selected quantitative factors was performed using a face-centered central composite design (FCCD) with 8 experimental points and three replicates in the center resulting in 11 randomized runs. It was necessary to select three levels for each factor in order to estimate the quadratic effects. For that purpose a quadratic regression model was applied in order to observe possible quadratic and interactions effects besides the main effects. The model was assumed to be expressed by the following second order polynomial equation:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon$$

where y is enantiomeric resolution; β_1 and β_2 (coefficients of linear effects), β_{11} and β_{22} (coefficients of quadratic effects), β_{12} (coefficient of interaction effect), β_0 (intercept) and ε (error term) are the different coefficients of the model; X_1 and X_2 are the levels of factors.

The quality of fit of the model was assessed by the coefficient of determination (R^2) which is the fraction of response variation explained by the model and the plot of residuals. All values of R^2 obtained were above 0.98, which demonstrated the quality of fit. The fraction of the variation of the resolution that can be predicted by the model, namely Q^2, remained higher than 0.86, meaning that at least 86% of the variability of the resolution could be predicted.

The interpretation of the optimisation results was made from the whole model equation rather than from the analysis of single coefficients. For that purpose, the analysis of the response surface plots was necessary [46, 47]. The response surfaces were obtained by plotting the model response against the two factors. Since econazole and miconazole exhibited a similar behavior, only the results obtained for one compound (miconazole) is presented in Figure 3A. Figure 3B illustrates the response surface of oxprenolol, which is comparable to that of bupivacaine, propranolol and sotalol.

As can be seen in Figure 3A, the effect of the formic acid proportion in the mobile phase on enantioresolution of miconazole is fairly linear. An increase of this acidic additive has a negative effect on enantioresolution whereas the effect of the hexane proportion is very limited so that it seems possible to reach optimum enantioresolution using the lowest proportion of formic acid (0.015%) and no addition of *n*-hexane in the mobile phase.

From Figure 3B, it appears clearly that the formic acid proportion has a quadratic effect on oxprenolol enantioresolution since the response surface passes through a maximum near 0.2% formic acid. A similar effect is observed with bupivacaine, propranolol and sotalol. The *n*-hexane proportion seems to have no significant effect on enantioresolution for oxprenolol and bupivacaine but a positive effect of *n*-hexane addition on propranolol and sotalol enantioresolution is obtained using a high proportion of formic acid (0.15-0.25%). It results from the evalution of these response surfaces that optimum enantioresolution for these four chiral drugs requires a high concentration of formic acid (0.2%) and also a relatively high proportion of hexane in the case of propranolol and sotalol.

These results clearly confirm that the increase of formic acid concentration up to 0.2% has a significant effect on the enantioseparation of the studied drugs, except for the two compounds with lower basic caracter (i.e. the imidazole derivatives) [25]. For most basic analytes, higher concentrations of the acidic additive were needed to improve enantioresolution.

Optimal chromatographic conditions were generated by maximizing enantioresolution. The predicted resolution values and those observed experimentally were compared (Table 4). Very good agreement was obtained in most cases. Even if in the case of bupivacaine, propranolol and sotalol enantioresolution values obtained were slightly lower than those predicted, this is not likely to decrease significantly the quality of the model. The corresponding chromatograms are presented in Fig. 4. If necessary, the retention times of econazole and miconazole enantiomers could be reduced by a slight increase of formic acid concentration in the mobile phase without compromising the baseline separation since the enantioresolution values for these two compounds were sufficiently high. The less good S/N ratios observed in Fig. 4 are related to the additive percentage either lower (Fig 4. A,B) or higher (Fig 4. C-F) than the usual 0.1% level of formic acid in the mobile phases [25].

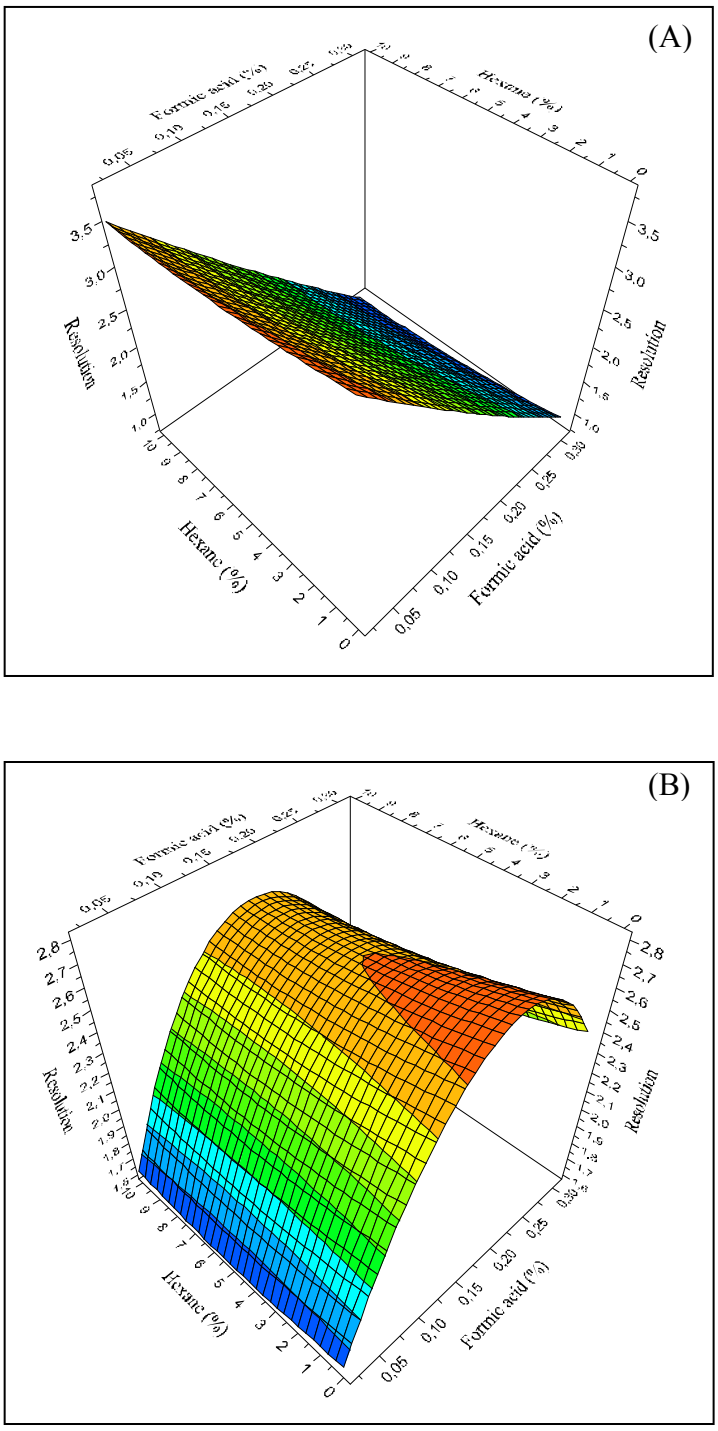

Figure 3. Response surfaces.

(A) Miconazole (B) Oxprenolol.

IV.3.3. Strategy for rapid method development

Based on the optimal conditions and the effect of the factors, a set of experimental conditions can be proposed for rapid method development using Sepapak-4 at 25°C: ACN/ 0.1%DEA /0.1% TFA/5% hexane. A serie of nine basic chiral compounds, namely acebutolol, alprenolol, atenolol, celiprolol, chlorpheniramine, isoconazole, pindolol, sulconazole and terbutaline were tested under these conditions and the results are presented in Table 5. As can be seen in this table the enantiomers of all compounds were at least partially resolved. Baseline enantioseparation was obtained for four compounds, namely atenolol, celiprolol, pindolol and terbutaline. The corresponding chromatograms are presented in Fig.5. These confirm the high chiral discrimination ability of Sepapak-4. An optimization step would probably allow to improve enantioresolution for compounds not completely enantioseparated under these conditions. Alternative conditions using formic acid as mobile phase additive were tested: ACN/0.1% DEA/0.2% FA/5% hexane. Only the enantioresolution of one compound, alprenolol, was improved under these conditions (1.3 instead of 0.6). It is worth nothing that the retention time of the second eluted peak is usually much higher with FA than with TFA, which seems to confirm that the nature of the acidic additive plays an important role in the interaction mechanisms between the analyte enantiomers and CSP as suggested earlier [23,25].

Table 4. Predicted and observed *Rs* values under optimal chromatographic conditions.

Analyte	Mobile phase	RT (peak 2) (min)	*Rs*	
			Predicted (confidence interval at 95%)	Observed
Bupivacaine	ACN/ 0.1% DEA / 0.2% FA	15.6	3.4 [3.16-3.56]	3.1
Oxprenolol		22.1	2.8 [2.65-2.93]	2.8
Econazole	ACN / 0.1% DEA / 0.015% FA	21.7	7.8 [7.46-8.19]	7.5
Miconazole		23.4	3.9 [3.6-4.14]	3.8
Propranolol	ACN / 0.1% DEA / 0.2% FA / 9% hexane	27.2	2.5 [2.40-2.52]	2.3
Sotalol	ACN / 0.1% DEA / 0.2% FA / 7.5% hexane	24.3	2.0 [1.93-2.07]	1.8

Temperature: 25°C; Other conditions: see Experimental.

Table 5. Enantioresolution values obtained using the rapid method development strategy.

Analytes	RT (peak 2) (min)	*Rs*
Acebutolol	7.4	1.0
Atenolol	26.4	4.7
Alprenolol	4.0	0.6 (1.3)
Celiprolol	10.3	3.5
Pindolol	4.3	1.9
Isoconazole	9.2	1.0
Sulconazole	7.1	1.1
Terbutaline	5.9	2.2
Chlorpheniramine	4.2	0.6

Mobile phase: ACN / 0.1% DEA / 0.1% TFA / 5% hexane; temperature: 25°C; Other conditions: see Experimental.

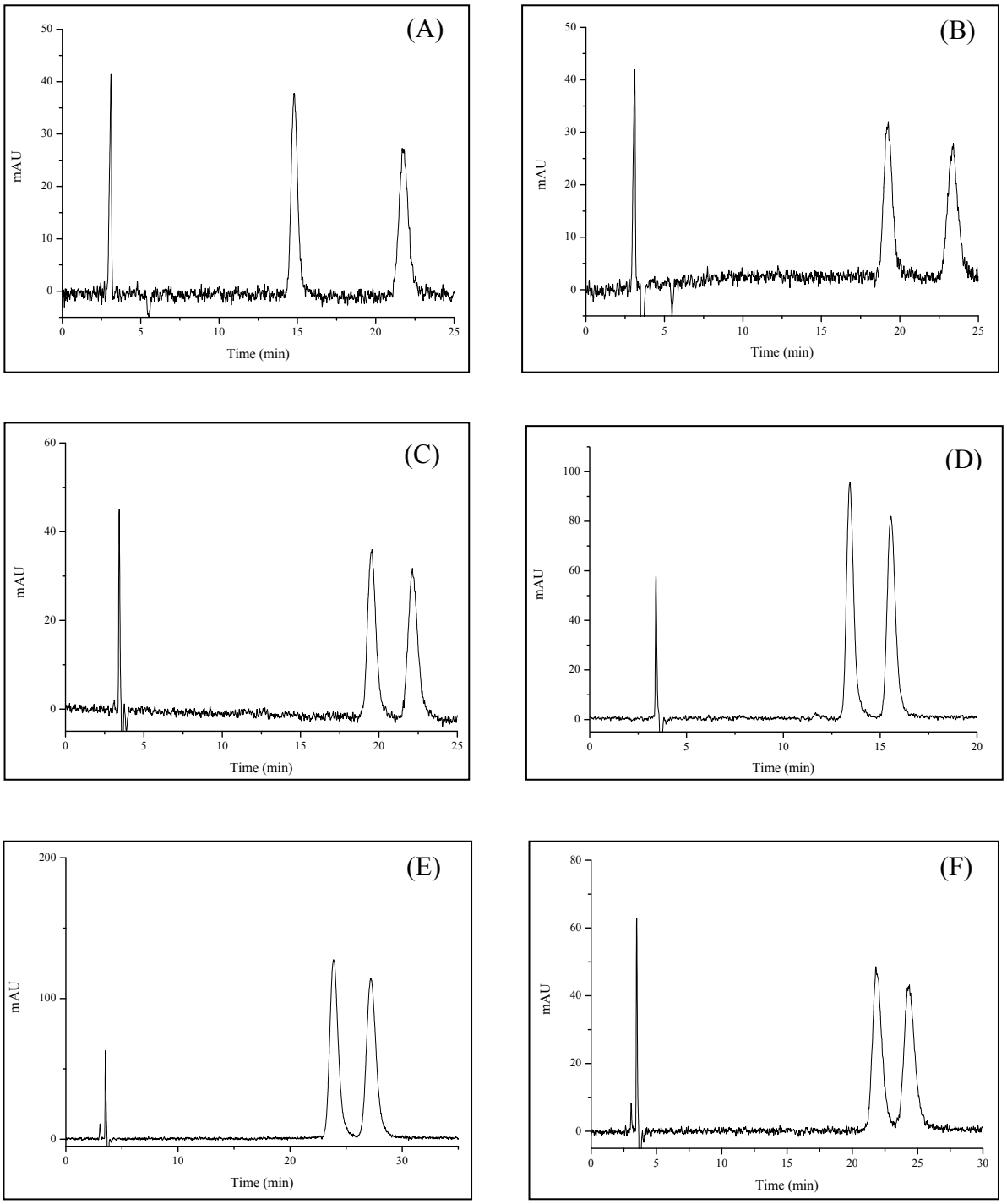

Figure 4 Chromatograms obtained under optimal chromatographic conditions.
(A) Econazole; (B) Miconazole; (C) Oxprenolol; (D) Bupivacaine; (E) Propranolol; (F) Sotalol
Conditions : see Experimental and Table 4.

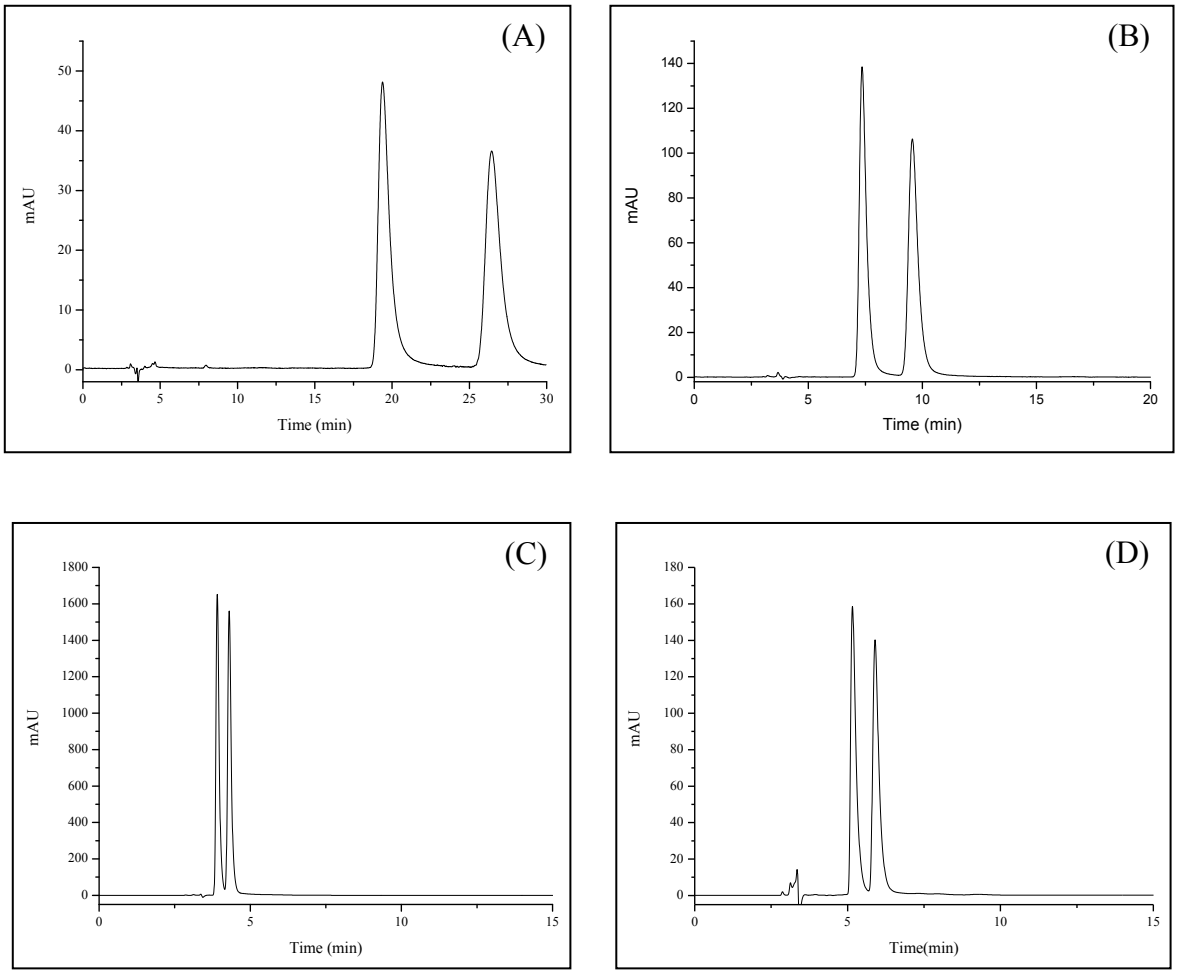

Figure 5. Chromatograms obtained under the proposed strategy for rapid method development.

(A) Atenolol; (B) Celiprolol; (C) Terbutaline ; (D) Pindolol

Mobile phase: ACN/ 0.1% DEA/0.1%TFA/ 5% hexane; temperature: 25°C;

Other conditions: see Experimental.

IV.4. Concluding remarks

The enantioseparation of ten basic drugs were evaluated using the new commercial chiral stationary phase, namely Sepapak-4 in polar organic chromatography mode. These experiments were carried out in two steps: screening and optimisation. The results show a particularly strong interaction and high chiral discrimination for those basic compounds tested using Sepapak-4 as CSP and acetonitrile as the main mobile phase component.

The screening step results, performed with a fractional factorial design, demonstrated good possibilities to regulate significantly enantioresolution by changing the type of the acidic additive and adding n-hexane to the mobile phase. These studies showed a large difference in behaviour between the compounds tested, which could be distributed in two groups according to the acidic additive selected: TFA or FA. Then a multivariate optimisation, using a face-centered central composite design, has confirmed the effect of the formic acid proportion on enantioresolution, high resolution values being obtained for almost all compounds tested in relatively reasonable analysis times.

IV.5. References

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CHAPTER V.

EVALUATION OF CHLORINE CONTAINING CELLULOSE BASED CHIRAL STATIONARY PHASES FOR THE LC ENANTIOSEPARATION OF BASIC PHARMACEUTICALS USING POLAR NON-AQUEOUS MOBILE PHASES

Summary

The discrimination ability of three cellulose-based chiral stationary phases (CSPs) was evaluated towards the enantiomers of basic drugs, using acetonitrile as the main solvent in polar organic mobile phases. The study was focused on CSPs containing cellulose tris(3-chloro-4-methylphenylcarbamate) (3-Cl-4-MePC), cellulose tris(4-chloro-3-methylphenyl-carbamate) (4-Cl-3-MePC) or cellulose tris(3,5-dichlorophenylcarbamate) (3,5-diClPC) as chiral selector. The behaviour of these CSPs was studied systematically in order to investigate the influence of the presence and position of the chlorine substituents on the phenylcarbamate moieties on the retention and resolution of the enantiomers. The evaluation was made with three different generic mobile phases, namely ACN/0.1%DEA/0.1% TFA, ACN/0.1%DEA/ 0.2% FA and ACN/0.1%DEA/0.2%AcA, deduced from previous work. The nature of the acidic additive and of the chiral selector was found to be particularly important for the retention and enantioresolution of these basic compounds. High resolution values could be obtained for most studied enantiomers with these CSPs, clearly demonstrating the interest of using them in combination with polar organic mobile phases. However, significant differences in enantioresolution between the CSPs have been observed for many compounds, indicating that these phases seem to be quite complementary.

V.1. Introduction

Polysaccharide derivatives belong to the most commonly used chiral stationary phases (CSPs) for the direct liquid chromatographic (LC) enantioseparation of chiral compounds. Indeed, more than 90% of the determinations of enantiomeric excess by chiral LC method are performed using the polysaccharide-based CSPs [1,2]. Among these CSPs, those with cellulose or amylose phenylcarbamate derivatives as chiral selectors, have a wide application range [3-8]. The chiral recognition abilities of the cellulose phenylcarbamate derivatives can be controlled by the nature of the substituents on the phenyl groups [2]. Moreover, the cellulose phenylcarbamates with electron-withdrawing substituents such halogens, or electron-donating substituents, such as alkyl groups, exhibit higher chiral recognition than the non-substituted one [2, 5]. These substituents seem to influence the polarity of the carbamate group through an inductive effect and change the interaction mode between the cellulose derivatives and the chiral compound. On the other hand, the chiral recognition on the phenylcarbamate derivatives is also influenced by the position of the substituents on the phenyl group of the polymer [9, 10]. Based on these facts, many possibilities for cellulose phenylcarbamate derivatives chiral selectors can be proposed. Chankvetadze et al. have developed since a decade two cellulose derivatives having both electron-withdrawing (chlorine atom) and electron-donating (methyl group) substituents in positions 3 and/or 4 of the phenyl moiety of the polymer, namely cellulose tris(3-chloro-4-methylphenylcarbamate) (3-Cl-4-MePC) and cellulose tris(4-chloro-3-methylphenylcarbamate) (4-Cl-3-MePC), as well as a cellulose derivative with two electron-withdrawing substituents (chlorine atoms) in position 3 and 5 on the phenyl group of the polymer namely cellulose tris(3,5-dichlorophenylcarbamate) (3,5-diClPC) [11-15].

Since the commercialization of these polysaccharide-based CSPs, little emphasis has been made on the study of the effects of factors, such as the nature and concentration of acidic additives. These factors were found to play a crucial role in polar organic chromatography with respect to retention and enantioresolution using 4-Cl-3-MePC [16,17]. As far as we know, although some previous studies have mentioned their recognition abilities in NPLC [6, 13, 18] and polar organic solvent chromatography (POSC) [19-21], no systematic comparative study has been reported in POSC about the use of these three chlorine containing cellulose-based CSPs with different acidic additives for chiral resolution.

In the present study, the chiral recognition ability of these three CSPs, namely 3-Cl-4-MePC, 4-Cl-3-MePC and 3,5-diClPC, was evaluated towards the enantiomers of thirteen basic drugs using acetonitrile as main solvent with basic and acidic additives in POSC. The purpose of this evaluation is to compare enantioseparation abilities of these CSPs under three rapid screening conditions, ACN/0.1%DEA/0.1% TFA and ACN/0.1%DEA/ 0.2% FA, ACN/0.1%DEA/0.2%AcA deduced from our previous work [16,17].

V.2. Experimental

V.2.1. Chemicals and reagents

Acebutolol hydrochloride, metoprolol tartrate, oxprenolol hydrochloride, propranolol hydrochloride, econazole nitrate and prilocaine hydrochloride were supplied by Sigma–Aldrich (Saint-Louis, MO, USA). Celiprolol hydrochloride was provided by Rorer (Brussels, Belgium), miconazole nitrate by Janssen Pharmaceutica (Beerse, Belgium), sotalol hydrochloride by Profarmaco Combrex (Milan, Italy), atenolol by Erregierre (Bergamo, Italy), betaxolol by LERS (Paris, France), bupivacaine hydrochloride by Astra Pharmaceutical Products (Södertalje, Sweden) and mepivacaine hydrochloride by Federa (Brussels, Belgium). All samples are racemates used without further purification.

Acetonitrile (ACN) of HPLC grade and glacial acetic acid (AcA) pro analysi were provided by Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), diethylamine (DEA) and formic acid (FA) pro analysi were obtained from Acros Organics (Geel, Belgium).

V.2.2. Instrumentation

The chromatographic system from Agilent Technologies (Waldbronn, Germany) consisted in a binary pump, a thermostated column compartment, a diode array detector and an automatic injector, all of 1100 series. The Chemstation software was used for system control and data acquisition. Three chiral columns (250 x 4.6 mm; i.d.), namely Sepapak-2® (3-Cl-4-MePC), Sepapak-4® (4-Cl-3-MePC) and Sepapak-5® (3,5-diClPC), were kindly supplied by Sepaserve (Münster, Germany). The chiral selectors coated on

aminopropylsilanized silica (nominal particle size 5µm) were cellulose tris (3-chloro-4-methylphenylcarbamate), cellulose tris (4-chloro-3-methylphenylcarbamate) and cellulose tris (3,5-dichlorophenylcarbamate) for 3-Cl-4-MePC, 4-Cl-3-MePC and 3,5-diClPC respectively, in the amount of 25% (w/w). Figure 1 presents the chemical structure of these chiral selectors.

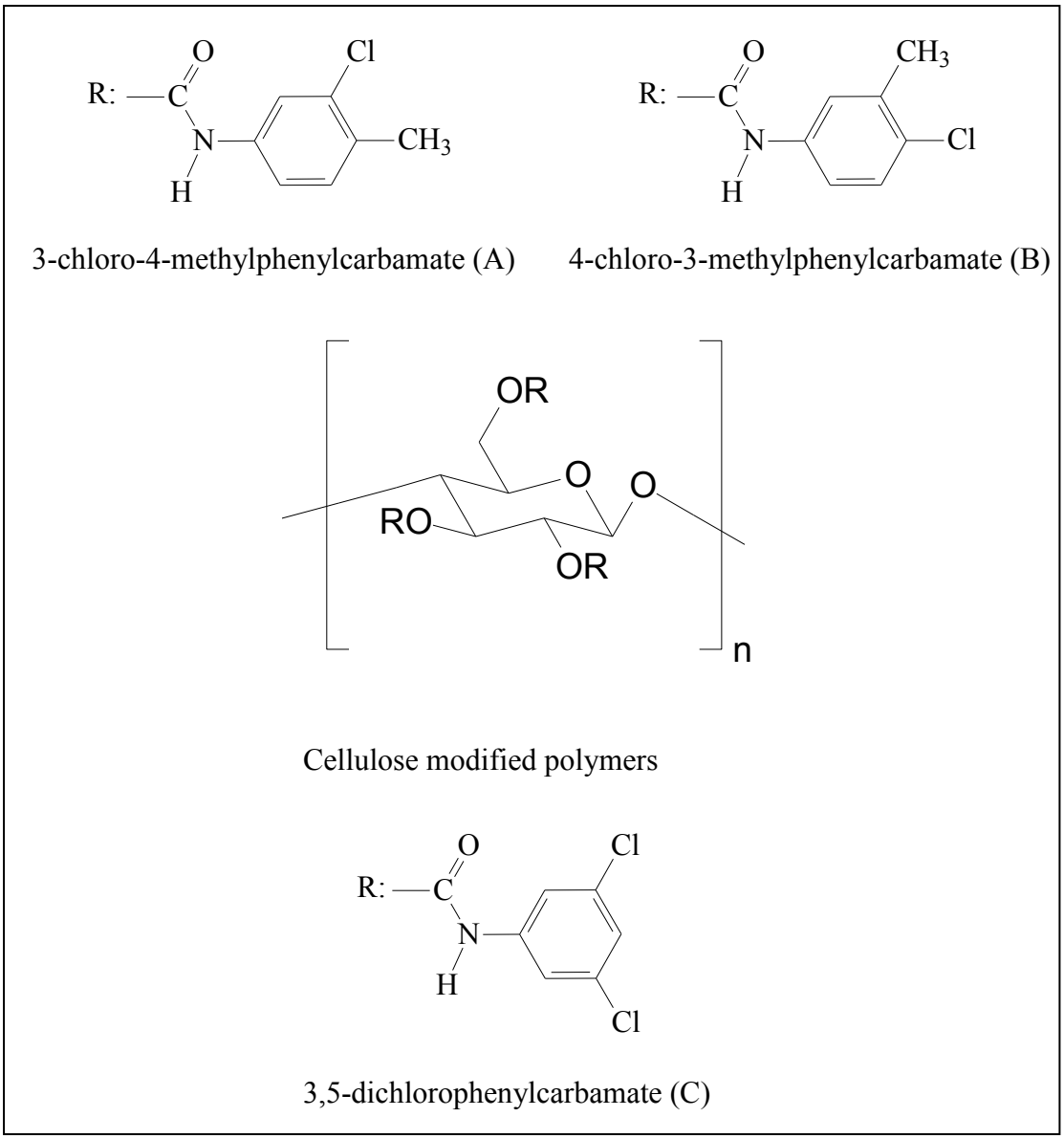

Figure 1. Structure of cellulose derivated chiral selectors of the studied CSPs

3-Cl-4-MePC (A); 4-Cl-3-MePC (B); 3,5-diClPC (C).

V.2.3. Solutions for method development

Solutions of each racemic compound at a concentration of *ca* 100 µg/ml were prepared by dissolving the appropriate amount of the substance in the required volume of mobile phase.

V.2.4. Chromatographic conditions

The mobile phases consisted of a mixture of acetonitrile, acidic additive (TFA, FA or AcA) and diethylamine (DEA) (v/v) and were pumped at a constant flow-rate of 1.0 mL/min. A low concentration of DEA, i.e. 0.1%, was added to the mobile phase in order to obtain efficient and symmetrical peaks. The injection volume was 20 µL. The analytes were detected photometrically at 220 nm and the temperature was settled at 25°C in all experiments.

V.3. Results and discussion

In previous work, the influence of the acidic additive (TFA, AcA or FA) on the enantioresolution and retention of chiral basic pharmaceuticals was demonstrated in LC using ACN and a CSP with cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral selector (4-Cl-3-MePC)) [16,17]. The strategy proposed for rapid screening method development only included TFA (at 0.1%) and FA (at 0.2%) but it is worth noting that AcA was also shown to significantly influence the enantioresolution of some chiral compounds [17]. Therefore, the three acidic additives were included in the present study devoted to the comparison of the behaviour of three chlorine containing cellulose-based CSPs, namely 3-Cl-4-MePC, 4-Cl-3-MePC and 3,5-diClPC.

V.3.1. Effect of the nature of the acidic additive and the chiral selector on retention

Tables 1, 2 and 3 present the retention factors (k'1) of the first eluting enantiomers on the studied CSPs using the three different acidic additives as well as enantioselectivity and enantioresolution values. Using TFA (cf. Table 1), most chiral compounds have low retention factors (k'1< 2), irrespective of the nature of the CSP. It can therefore be assumed that the mobile phase containing TFA (i.e. ACN/0.1%DEA/0.1% TFA) has a very high elution strength. In the presence of this acid in the mobile phase, retention factors obtained on 3,5-diClPC were especially low compared to those observed on 3-Cl-4-MePC and 4-Cl-3-MePC. With AcA as acidic additive instead of TFA (cf. Table 2), a general tendency to an increase in retention can be observed. The retention of most compounds was again the lowest on 3,5-diClPC. However, the three local anesthetics were found to have higher retention on 3,5-diClPC using AcA.

Irrespective of the nature of the CSP, FA led to the highest retention in most cases (cf. Table 3). With this acidic additive, the retention of most compounds was also the lowest on 3,5-diClPC. The higher retention generally observed using 3-Cl-4-MePC and 4-Cl-3-MePC could be explained by the presence of one methyl substituent on the phenylcarbamate groups of these CSPs. Indeed, electron-donating substituents like methyl groups increase the electron density at the carbonyl of the carbamate group and thus the retention time, since hydrogen-bonding interaction between the chiral analyte and the CSP can be promoted [2]. It is noteworthy that econazole and miconazole have an opposite retention behavior, their retention being higher on 3,5-diClPC than on the two other CSPs in the presence of FA in the mobile phase. Sotalol has also a peculiar retention behavior with the same acidic additive since its retention was much lower on 4-Cl-3-MePC (k'1: 1.2) than on 3-Cl-4-MePC (k'1: 8.7) and 3,5-diClPC (k'1: 3.8). These results suggest that in the presence of a weaker acid such as AcA or FA, the retention mechanisms become more complex and analyte-dependent.

V.3.2. Effect of the nature of the acidic additive and the chiral selector on enantioseparation

Tables 1, 2 and 3 also illustrate the influence of the nature of the acidic additive and the CSP on the enantioselectivity and enantioresolution of the studied basic drugs. As can be seen in Table 1, TFA seems to be particularly useful for the enantioseparation of the studied compounds with 4-Cl-3-MePC as CSP compared to 3-Cl-4-MePC. On the contrary, complete enantioseparation could not be obtained for the studied compounds on 3,5-diClPC. This suggests that the use of this acidic additive should be avoided for enantioseparation on 3,5-diClPC, probably related to the very low retention under these conditions (cf. Section 3.1). Even if several compounds such as prilocaine can be fully resolved with 3-Cl-4-MePC, the highest enantioresolution values were obtained with 4-Cl-3-MePC (cf. Fig. 2A).

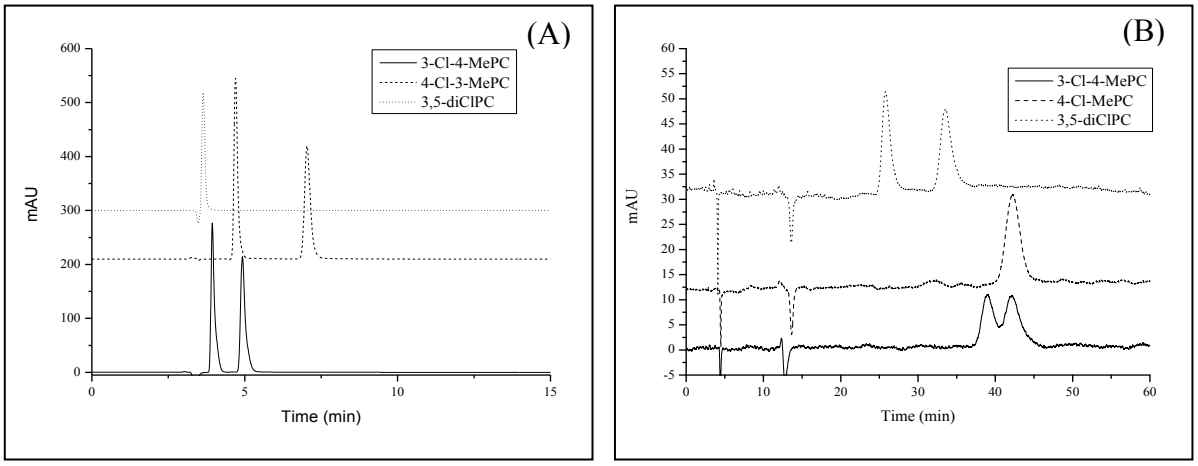

Figure 2. Influence of the nature of the chiral selector on enantioresolution of prilocaine (A) and acebutolol (B).
Mobile phase: ACN/0.1% DEA/0.1% TFA (A) or 0.2% AcA (B).
Other conditions: see Section 2.

With AcA as acidic additive, a completely different behavior was observed: the majority of the compounds show higher enantioresolution values using 3,5-diClPC compared to 4-Cl-3-MePC (cf. Table 2). For example, complete enantioseparation for acebutolol was only obtained on 3,5-diClPC using AcA (cf. Fig. 2B). In this particular case, a partial resolution occurs on 3-Cl-4-MePC while no resolution was observed on 4-Cl-3-MePC.

As can be seen from Table 3, the use of FA as acidic additive for the enantioseparation of beta-blockers gives rise to the highest discrimination ability on 3,5-diClPC On the contrary, for most of the other drug enantiomers, the highest resolution values were obtained on 4-Cl-3-MePC with FA as additive.

As shown in Figure 3, the resolution of sotalol and miconazole enantiomers was higher in the presence of AcA compared to FA using 3,5-diClPC as CSP while no enantioseparation is observed with TFA. Furthermore, for the enantiomers of some drugs like the imidazole derivatives, the use of either FA or AcA was found to be suitable for complete enantioseparation irrespective of the nature of the CSP (cf. Tables 2 and 3).

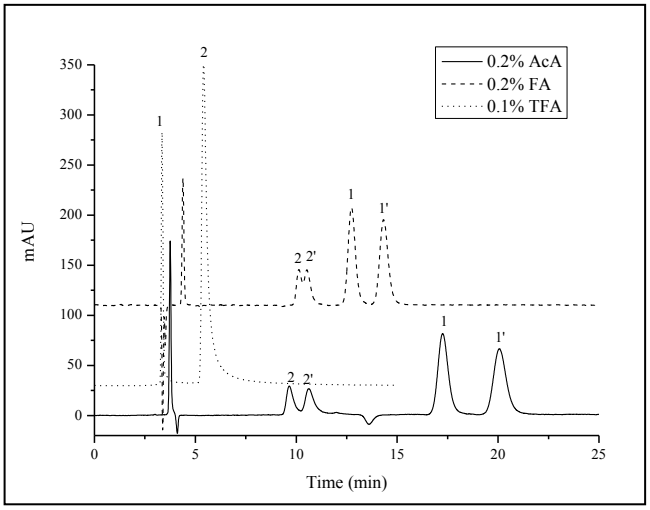

Figure 3. Effect of the nature of the acidic additive on enantioresolution of sotalol and miconazole enantiomers using 3,5-diClPC as CSP.
Mobile phase: ACN/0.1% DEA/0.1%TFA or ACN/0.1% DEA/0.2% (FA or AcA);
Peaks: (1 and 1')- enantiomers of miconazole; (2 and 2')- enantiomers of sotalol.
Other conditions: see Section 2.

Table 1. Influence of the chiral selector nature on the retention factor (*k'1*), the enantioselectivity (α) and enantioresolution (*R*s) of the studied basic amino-drugs enantiomers using TFA as acidic additive.

	3-Cl-4-MePC			4-Cl-3-MePC			3,5-diClPC		
	k'1	α	*Rs*	*k'1*	α	*Rs*	*k'1*	α	*Rs*
Acebutolol	1.90	1.08	0.7	1.50	1.10	1.1	0.64	1.07	1.0
Atenolol	5.0	1.44	4.8	5.1	1.41	5.3	1.28	1.15	1.2
Betaxolol	0.77	1.22	1.4	0.55	1.26	2.1	0.18	1.03	0.7
Celiprolol	2.8	1.27	2.6	2.2	1.36	3.5	1.29	1.09	1.2
Metoprolol	0.71	1.16	0.9	0.48	1.06	0.6	0.16	1.03	0.7
Oxprenolol	0.66	-	-	0.38	1.12	0.9	0.12	1.03	0.7
Propranolol	0.52	-	-	0.28	-	-	0.14	-	-
Sotalol	0.50	-	-	0.16	-	-	0.07	-	-
Econazole	1.60	1.14	1.4	1.31	1.16	1.7	0.63	1.03	0.6
Miconazole	2.3	1.08	0.9	1.90	1.12	1.5	0.91	-	-
Bupivacaine	1.20	1.08	0.6	0.72	-	-	0.38	1.02	0.6
Mepivacaine	0.41	1.28	1.3	0.64	1.13	1.2	0.37	-	-
Prilocaine	0.77	1.56	3.9	0.55	2.47	8.5	0.21	-	-

'–' no enantioresolution observed. Mobile phase: ACN/0.1% DEA/0.1% TFA;.
Other conditions: see Section 2.

Table 2. Influence of the chiral selector nature on the retention factor (*k'1*), the enantioselectivity (α) and enantioresolution (*R*s) of the studied basic amino-drugs enantiomers using AcA as acidic additive.

	3-Cl-4-MePC			4-Cl-3-MePC			3,5-diClPC		
	k'1	α	*Rs*	*k'1'*	α	*Rs*	*k'1*	α	*Rs*
Acebutolol	10.9	1.09	0.9	11.9	-	-	6.9	1.34	3.4
Atenolol	/	/	/	/	/	/	/	/	/
Betaxolol	4.9	1.16	1.8	5.4	1.09	1.3	3.3	1.34	3.6
Celiprolol	15.2	1.15	1.6	15.08	1.15	2.3	12.6	1.32	3.5
Metoprolol	4.8	1.13	1.6	5.0	1.06	0.8	3.1	1.38	4.0
Oxprenolol	3.8	1.18	2.1	3.7	1.14	2.0	1.29	1.46	4.3
Propranolol	4.0	1.07	0.9	4.6	1.12	1.8	2.12	1.18	2.1
Sotalol	4.3	1.26	3.0	4.1	1.15	1.9	3.8	1.15	1.4
Econazole	2.4	1.41	4.4	2.6	1.52	4.7	2.8	1.23	2.7
Miconazole	3.5	1.25	3.1	3.5	1.28	3.9	4.3	1.20	2.7
Bupivacaine	0.9	1.03	0.6	0.62	-	-	1.11	1.08	0.6
Mepivacaine	0.69	1.17	1.3	0.65	1.13	1.3	1.09	1.28	2.5
Prilocaine	0.45	1.27	1.5	0.29	1.30	2.1	0.49	-	-

'/' no peak obtained within 60 min. '–' no enantioresolution observed. Mobile phase: ACN/0.1% DEA/0.2% AcA. Other conditions: see Section 2.

Table 3. Influence of the chiral selector nature on the retention factor (*k'1*), the enantioselectivity (*α*) and enantioresolution (*R*s) of the studied basic amino-drugs enantiomers using FA as acidic additive

	3-Cl-4-MePC			4-Cl-3-MePC			3,5-diClPC		
	k'1	*α*	*Rs*	*k'1*	*α*	*Rs*	*k'1*	*α*	*Rs*
Acebutolol	10.9	1.06	0.7	12.7	1.1	1.5	5.1	1.32	4.0
Atenolol	/	/	/	/	/	/	11.6	1.37	5.3
Betaxolol	4.4	1.12	1.6	6.8	1.06	0.8	2.1	1.19	2.4
Celiprolol	16.2	1.11	1.3	/	/	/	8.4	1.20	2.7
Metoprolol	8.5	1.10	1.4	12.4	-	-	5.1	1.04	2.8
Oxprenolol	6.7	1.17	2.0	9.6	1.16	2.5	3.2	1.19	2.4
Propranolol	9.8	1.08	1.0	12.2	1.15	2.4	5.2	1.06	0.7
Sotalol	8.7	1.06	0.7	1.2	1.14	1.8	3.8	1.06	0.7
Econazole	3.9	1.29	3.4	4.1	1.33	4.7	4.3	1.2	2.0
Miconazole	5.8	1.15	1.9	5.7	1.13	2.0	6.7	1.17	2.2
Bupivacaine	2.4	1.17	2.0	2.8	1.21	3.0	1.69	1.11	1.2
Mepivacaine	1.93	1.19	2.0	2.6	1.2	2.8	1.80	1.23	0.5
Prilocaine	1.69	-	-	1.9	-	-	1.08	-	-

'/' no peak obtained within 60 min. '–' no enantioresolution observed. Mobile phase: ACN/0.1% DEA/0.2% FA. Other conditions: see Section 2.

Table 4 gives the acidic additive leading to the highest Rs value obtained with each CSP. As can be seen in this Table, it appears that all tested compounds could be completely enantioseparated in most cases. Only for enantiomers of prilocaine, no enantioresolution was observed using 3,5-diClPC. 4-Cl-3-MePC seems to have a particularly high resolving power since twelve basic chiral drugs of the thirteen tested were completely resolved and in most cases the Rs values were higher with either FA or TFA, except sotalol and miconazole for which AcA led to the highest enantioresolution. Enantioresolution values were higher with either FA or AcA using 3,5-diClPC, as already observed in Fig.3. In the case of 3-Cl-4-MePC, the three acidic additives seem to be necessary for the enantioseparation of the model compounds.

Moreover, the Rs values observed were generally the lowest on 3-Cl-4-MePC except for sotalol. Therefore, using the appropriate acidic additive, it was possible to resolve the enantiomers of all the basic drugs tested (maximum Rs value: 2.4-8.5), which demonstrates the high resolving power of these CSPs.

Table 4. Conditions for maximum enantioresolution for differents CSPs used

	3-Cl-4-MePC		4-Cl-3-MePC		3.5-diClPC	
	Rs max	Acidic additive	*Rs* max	Acidic additive	*Rs* max	Acidic additive
Acebutolol	0.9	AcA	1.5	FA	4.0	FA
Atenolol	4.8	TFA	5.3	TFA	5.3	FA
Betaxolol	1.8	AcA	2.1	TFA	3.6	AcA
Celiprolol	2.6	TFA	3.5	TFA	3.5	AcA
Metoprolol	1.6	AcA	1.2	TFA	4.0	AcA
Oxprenolol	2.1	AcA	2.5	FA	4.3	AcA
Propranolol	1.0	FA	2.4	FA	2.1	AcA
Sotalol	3.0	AcA	1.9	AcA	1.4	AcA
Econazole	4.4	AcA	4.7	FA	2.7	AcA
Miconazole	3.1	AcA	3.9	AcA	2.7	AcA
Bupivacaine	2.0	FA	3.0	FA	1.2	FA
Mepivacaine	2.0	FA	2.8	FA	2.5	AcA
Prilocaine	3.9	TFA	8.5	TFA	-	-

'–' no enantioresolution observed. Mobile phase: ACN/0.1% DEA/0.1% (TFA) or 0.2% (AcA or FA); Other conditions: see Section 2.

V.4. Concluding remarks

The three chlorine containing cellulose based CSPs tested in polar organic solvent chromatography with different acidic additives, namely TFA, AcA or FA in the mobile phase, have showed quite different enantioseparation capabilities. 3-Cl-4-MePC was found to have an intermediate behavior leading to lower enantioresolution values compared to 4-Cl-3-MePC and 3,5-diClPC. The two latter CSPs seem to be complementary so that the screening strategy can be limited to the combination of 4-Cl-3-MePC with TFA or FA and 3,5-diClPC with FA or AcA. Under these conditions involving at most four experiments, it was possible to enantioresolve each of the thirteen studied compounds. However, 4-Cl-3-MePC can be considered as the most useful of these three CSPs using ACN as main solvent.

V.5-References

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CHAPTER VI

DEVELOPMENT AND VALIDATION OF A LC METHOD FOR THE ENANTIOMERIC PURITY DETERMINATION OF S-ROPIVACAINE IN A PHARMACEUTICAL FORMULATION USING A CHLORINE CONTAINING CELLULOSE-BASED CHIRAL STATIONARY PHASE AND POLAR NON-AQUEOUS MOBILE PHASE

Summary

Ropivacaine is the first enantiomerically pure long-acting local anaesthestic used for surgical anaesthesia and post-operative pain relief.

A liquid chromatographic (LC) method using acetonitrile as the main solvent and cellulose tris(4-chloro-3-methylphenylcarbamate) coated on silica as chiral stationary phase was successfully developed and applied for the enantiomeric purity determination of S-ropivacaine in a pharmaceutical formulation (Naropin®). The key role played by the acidic additive (trifluoroacetic acid or formic acid) in the enantioseparation of basic drugs in these LC systems was demonstrated by the reversal of ropivacaine enantiomers elution order observed when both acids were compared. In order to elute the enantiomeric impurity (R-ropivacaine) before S-ropivacaine, formic acid (FA) was selected. The temperature and the percentages of acidic additive and hexane in the mobile phase were found to significantly influence the retention and resolution of these enantiomers. The optimized mobile phase consisted of ACN/0.1% DEA/0.2% FA/5% hexane (*v/v/v/v*). The temperature was set at 35°C to avoid the interference from a peak system related to the presence of water in the sample on ropivacaine enantiomers.

The LC method was then fully validated applying the strategy based on total measurement error and accuracy profiles. The accuracy profile obtained by linear regression after square root transformation was selected, the acceptance limits being settled at ±10% for the intended use of this analytical method. The relative bias was lower than 1.5%, while the RSD values for repeatability and intermediate precision were both below 1.0%. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were found to be about 0.2 and 1.0 µg/mL, respectively, corresponding to 0.02 and 0.1% of the enantiomeric impurity in S-ropivacaine.

VI.1. Introduction

Chirality has become a very important topic in pharmacology and analytical chemistry since a thorough assessment of potential chiral drug candidates is required in drug development by the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) [1,2]. These guidelines recommend to test the new chiral drug molecules in their racemic form as well as single-isomers in the various stages of development. Both regulatory bodies recommend the use of validated methods to evaluate the enantiomeric purity of single-isomers.

Ropivacaine is the first enantiomerically pure local anesthetic available for therapeutic use. It belongs to the same structural group (pipecoloxylidide) as bupivacaine and mepivacaine used for many decades as racemates. Even though (R)- and (S)-(-)-1-propyl-2',6'-pipecoloxylidide have similar nerve blocking properties, S-ropivacaine has shown less cardiotoxicity than the R-enantiomer as it is also the case for bupivacaine [3, 4, 5]. Ropivacaine has then been developed as a pure enantiomer and is currently used in epidural anesthesia and post operative pain [6].

Sänger-van de Griend *et al*. have developed a capillary electrophoretic method for the enantiomeric purity determination of ropivacaine using heptakis (2,6-di-*O*-methyl)-β-cyclodextrin as chiral selector [7, 8]. The method was found to be specific, linear, robust, accurate with a LOQ of 0.1% for the enantiomeric impurity. Nevertheless, the analysis time (about 25 min) as well as the LOD (0.05%) could be improved.

In a pending monograph of ropivacaine from the United States Pharmacopeia (USP), the enantiomeric purity of ropivacaine is determined by a LC method [9]. The separation was achieved on a chiral-AGP column (150 mm x 4.0 mm i.d., 5 µm). The mobile phase consisted of isopropyl alcohol and monobasic sodium phosphate buffer (1:9, v/v). Under the prescribed experimental conditions, the run time was about 15 minutes and an enantioresolution of 1.8 between S-ropivacaine and its enantiomeric impurity was obtained. Eriksson Möller reported a very similar LC method for which a detection limit of 0.1% for the enantiomeric impurity was observed [10].

The main goal of the present work was to develop and validate a selective, precise, accurate and reliable LC method for the determination of R-ropivacaine in S-ropivacaine

pharmaceutical formulation (Naropin®, 10 mg/mL solution). The method should be suitable for routine quality control of S-ropivacaine and the run time should be less than 15 min. To achieve this purpose, the newly commercialized chiral stationary phase (CSP), namely cellulose tris(4-chloro-3-methylphenylcarbamate) coated on silica (Sepapak-4), which showed a good enantioresolution for local anesthetics like bupivacaine and mepivacaine, was used [11, 12]. Moreover, the developed method was fully validated according to the strategy proposed by a Commission of the Société Française des Sciences et Techniques Pharmaceutiques [13]. This strategy is based on the use of accuracy profiles which take into account the total error, i.e. estimation of systematic and random errors of measurement results.

VI.2. Experimental

VI.2.1. Chemicals and reagents

Ropivacaine hydrochloride monohydrate and ropivacaine impurity G were supplied by EDQM (Strasbourg, France). Ropivacaine chemical structure is presented in fig.1. Naropin® (10mg/mL) was supplied by NV Astra Zeneca SA (Brussel, Belgium). Acetonitrile (ACN) of HPLC grade and glacial acetic acid (AcA) pro analysis were provided by Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA), diethylamine (DEA), and formic acid (FA) pro analysis were obtained from Acros Organics (Geel, Belgium). *n*-Hexane of HPLC grade was from BDH Hypersol (Poole, UK).

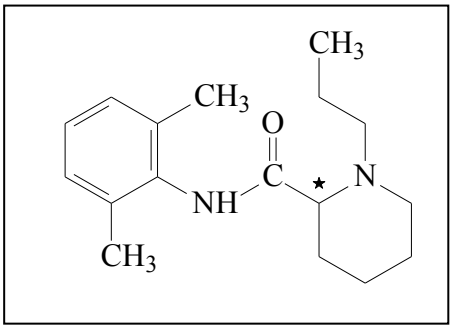

Figure 1. Chemical structure of ropivacaine.

VI.2.2. Instrumentation

The chromatographic system from Agilent Technologies (Waldbronn, Germany) consisted in a binary pump, a thermostated column compartment, a diode array detector and an automatic injector, all of 1200 series. The Chemstation software was used for system control and data acquisition. The chiral column Sepapak-4 (250 mm x 4.6 mm I.D.) was kindly provided by Sepaserve (Münster, Germany). The chiral selector adsorbed on aminopropylsilanized silica was cellulose tris(4-chloro-3-methylphenylcarbamate).

The statistical calculations for validation were performed by means of e.noval version 3.0 software (Arlenda, Liège, Belgium).

VI.2.3. Standards solutions

VI.2.3.1. Sample solutions used for method development

Different stock solutions of R- and S-ropivacaine were prepared by dissolving an accurately weighed amount of approximately 1 mg of each compound in 10 ml of ACN. The stock racemate solution was prepared by dissolving a weighed amount of approximately 1 mg for R ropivacaine and 1.1 mg for S-ropivacaine in 10 mL of ACN. This stock solution was then diluted 5-fold to obtained a final solution of 20 µg/mL.

VI.2.3.2. Sample solutions used for validation

A stock solution of R-ropivacaine was prepared by dissolving an accurately weighed amount of approximately 5 mg of this compound in 100 mL of the LC mobile phase. Then subsequent dilutions were achieved in order to obtain five calibration standards ranging from 1.0 to 15 µg/mL (m=5) (cf. Table 1). Two replicates (n=2) were prepared per concentration level. Each solution is injected one time. The number of concentration levels was sufficient to generate different regression models.

A stock solution of S-ropivacaine was prepared by diluting ten-fold the aqueous solution of Naropin (10 mg/mL) in the LC mobile phase. The final concentration of S-

ropivacaine was 1 mg/mL. Then three independent series of validation standards were prepared by spiking the correct volume of the R-ropivacaine stock solution in a sufficient volume of S-ropivacaine stock solution in order to reach final concentrations of 1, 2, 5 and 15 µg/mL (m=4) of the enantiomeric impurity (cf. Table 1). Three replicates (n=3) were prepared per concentration level and each one was injected one time.

VI.2.4. Chromatographic conditions

The mobile phases consisted in a mixture of acetonitrile, organic modifier (hexane), acidic additive and basic additive (v/v) and were pumped at a constant flow-rate of 1.0 mL.min-1. In all experiments, the basic additive percentage in the mobile phase was settled at 0.1 %. The optimal mobile phases was made up of ACN/0.1% DEA/0.2% FA/ 5% hexane. The injection volume was 10 µL. The analytes were detected photometrically at 240 nm.

Table 1. Preparation of standard solutions related to R-ropivacaine for validation.

Concentration level (% relative to 1.0 mg/mL of S-ropivacaine)	Concentration of R-ropivacaine (µg/mL)	
	Calibration standards	Validation standards
0.1	1.0	1.0
0.2	2.0	2.0
0.5	5.0	5.0
1.0	10.0	-
1.5	15.0	15.0
total	10 samples/day	12 samples/day

VI.3. Results and discussion

VI.3.1. Method development

In a previous work, an experimental design was applied for the enantioresolution of ten basic drugs, including bupivacaine and mepivacaine [12]. The optimal mobile phases consisted of ACN/0.1% DEA/0.1% FA/ 5% hexane for mepivacaine and ACN/0.1% DEA/0.2% FA for bupivacaine. For both methods, the temperature was set at 25°C. Moreover, this study highlighted the fact that the enantiomeric resolution of chiral pharmaceuticals on Sepapak-4 was influenced by the nature and the percentage of the acidic additive and hexane in the mobile phase [11, 12]. Finally, a strategy for rapid method development using a mobile phase made up of 0.2% FA, 5% hexane with ACN and 0.1% DEA at 25°C was proposed. Therefore, these experimental conditions were applied for the enantiomeric resolution of ropivacaine. As can be seen in Fig.2, under these conditions, ropivacaine enantiomers were completely resolved (Rs value: 3.0) in a relatively short analysis time (14 min).

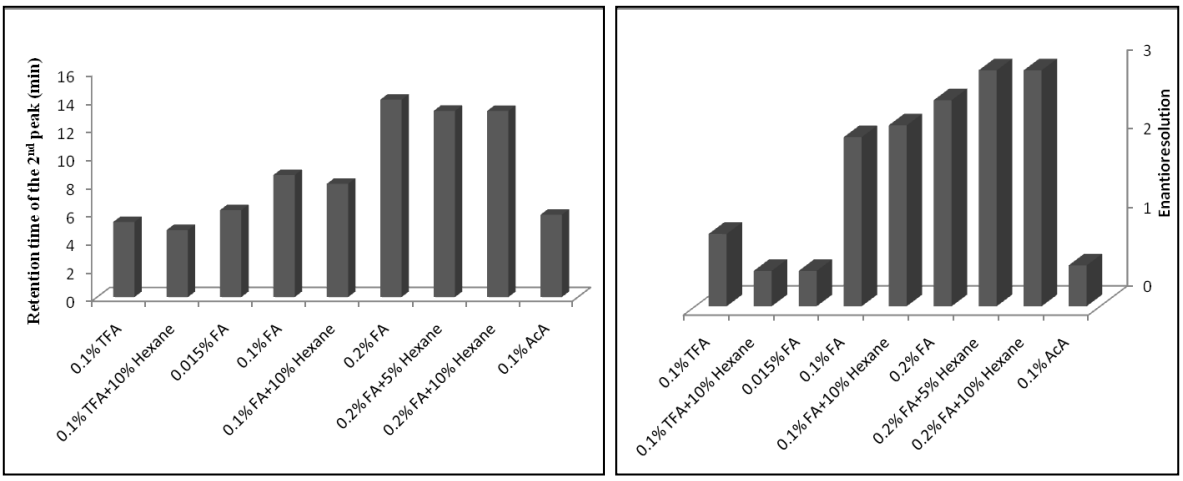

Figure 2. Influence of the nature and the percentage of the acidic additive on the retention time of the second peak and on enantioresolution.

In order to verify that the generic conditions were the best for ropivacaine enantiomers with respect to retention time and enantioresolution, experiments were performed using other acidic additives (TFA and AcA), different FA and *n*-hexane percentages (cf. Figs. 2A and B). As expected, the nature of the acidic additive has a strong influence on the enantioresolution of ropivacaine. Indeed, only FA (at a percentage of at least 0.1%) leads to a complete separation of ropivacaine enantiomers. An increase of FA concentration up to 0.2% has a positive effect on the enantioresolution and retention of the enantiomers. Moreover, the presence of 5% - 10% of n-hexane in the mobile phase gives rise to a small decrease of the analysis time and also slightly improves enantioresolution.

It is noteworthy that the elution order of ropivacaine enantiomers depends on the nature of the acidic additive. Indeed, as can be seen in Figs. 3A and B, when TFA was used as acidic additive, S-ropivacaine was the first eluting peak whereas an opposite elution order was observed with FA under these conditions. Therefore, FA was kept as acidic additive since the impurity eluted before the main compound. Indeed, to avoid possible interference in the enantiomeric purity determination caused by the tailing of the main enantiomer present in high concentration, the elution order is of utmost importance, especially when the enantioseparation is minimal [14, 15]. Here, it is clearly an advantage of these LC systems to enable a reversal of the elution order by changing the nature of the acidic additive.

As can be seen in Figs. 3C and D, the same elution order was observed using Sepapak-2 (chiral selector: cellulose tris(3-chloro-4-methylphenylcarbamate)) with similar changes in the mobile phase. Interestingly, this reversal of elution order only occurred using these two CSPs which have both electron-withdrawing (chlorine) and electron-donating (methyl) groups on the phenylcarbamate moiety. Indeed, no reversal of ropivacaine enantiomers elution order was observed using Chiralcel ODH or Sepapak-5 with either two electron-donating or two electron-withdrawing groups in positions 3 and 5 of the phenylcarbamate moiety (data not shown). Therefore, the key role of the acidic additive in the reversal of ropivacaine enantiomers elution order could be related to the simultaneous presence of methyl and chlorine groups on the phenylcarbamate moiety of Sepapak-2 and -4.

Finally, taking into account the desired elution order, a short analysis time without jeopardizing enantioresolution, a mobile phase made up of ACN/0.1% DEA/0.2% FA/5% hexane was selected for this application. Fig. 4A illustrates the chromatogram of ropivacaine enantiomers using this mobile phase at 25°C.

Nevertheless, when analyzing a validation sample, a peak system related to the presence of water in the sample appeared between ropivacaine enantiomers, which disturbed the quantification of the targeted impurity. To overcome this problem, the temperature was set at 35°C and, as can be seen in Figs. 4B and C, the peak system was then located after S-ropivacaine peak and therefore did no longer interfere in the determination of the enantiomeric impurity. However, as already observed earlier, this change of temperature had no significant effect on enantioresolution [11, 12].

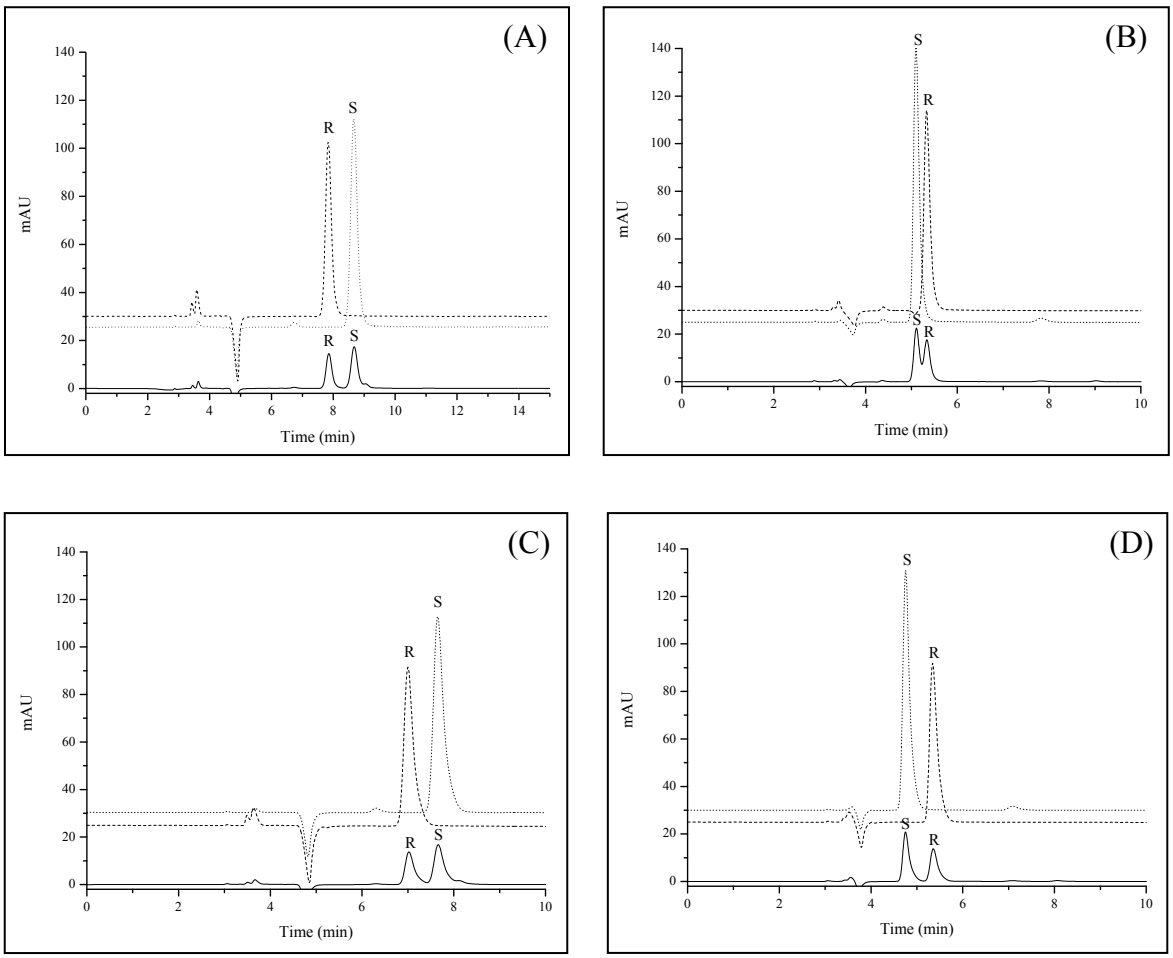

Figure 3. Chromatograms showing the reversal of ropivacaine enantiomers elution order according to the nature of the acidic additive.

Mobile phase: ACN/0.1% DEA/0.1% FA (A, C), ACN/0.1% DEA/0.1% TFA (B,D); Flow rate: 1 mL/min; Temperature: 25°C; UV detection: 240 nm; CSP: Sepapak-4 (A,B), Sepapak-2 (C,D).

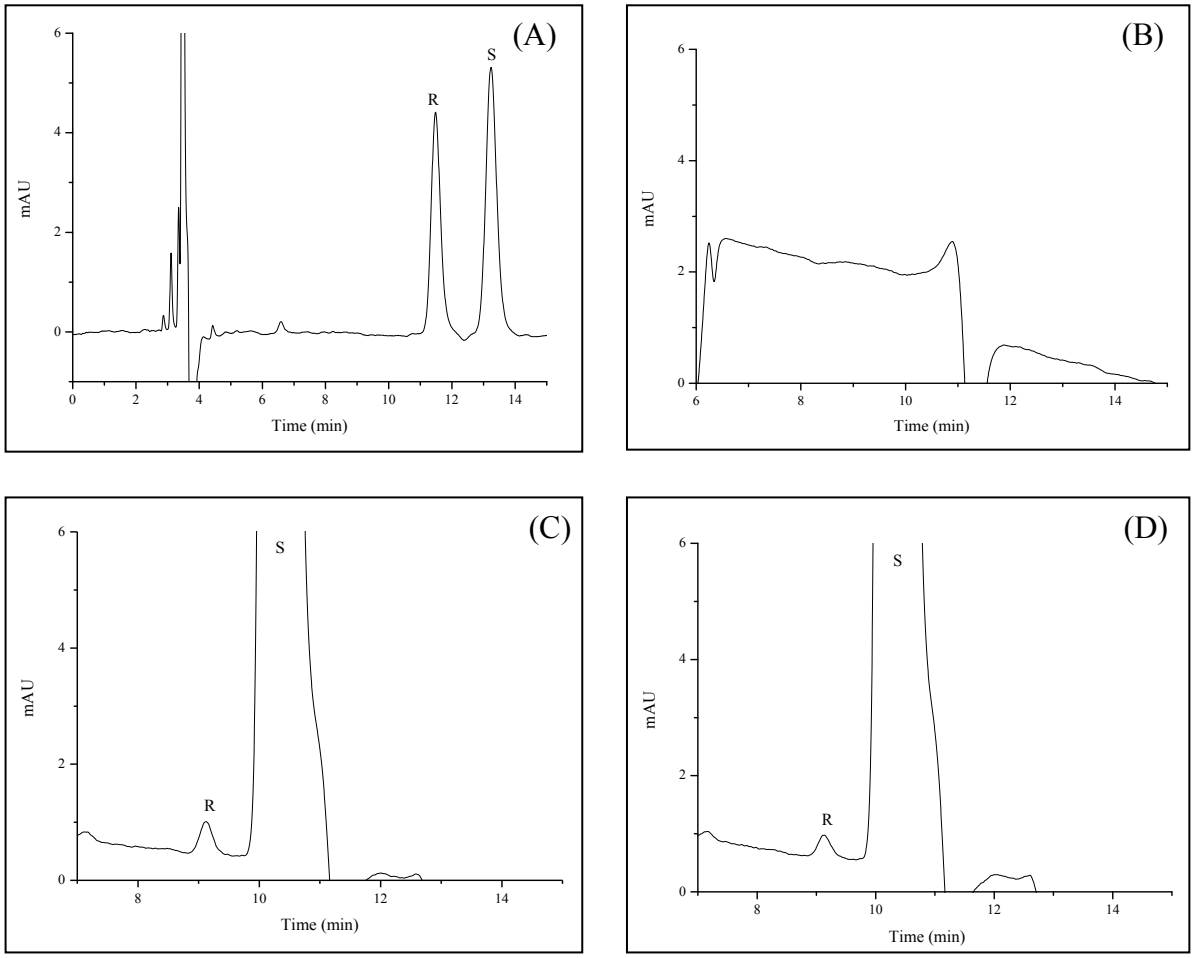

Figure 4. Typical chromatograms of a) a solution of racemic ropivacaine (20 µg/mL); b) water diluted in the mobile phase; c) a solution of S-ropivacaine (1 mg/mL of diluted Naropin®) containing R-ropivacaine (1 µg/mL); d) a solution of S-ropivacaine (1 mg/mL of diluted Naropin®).

Mobile phase: ACN/0.1% DEA/0.2% FA/5% Hexane, Flow-rate: 1 mL/min; Temperature: 25°C (A) and 35°C (B,C,D), UV: 240 nm; CSP: Sepapak-4. Other conditions see Section 2.

VI.3.2. Method validation

The validation approach, based on total measurement error and accuracy profiles as decision tool, was applied to demonstrate that the developed method is suited for its intended purpose [13, 16, 17]. This strategy was elaborated by a SFSTP (Société Française des Sciences et Techniques Pharmaceutiques) Commission. The concept of accuracy profile was also used to select the most appropriate calibration model, to determine the lower limit of quantification and the range over which the method can be considered as valid.

VI.3.2.1. Selectivity

To evaluate method selectivity, the chromatograms obtained by analyzing water diluted in the mobile phase and a sample of S- and R-ropivacaine, were compared in order to check the absence of compounds likely to interfere in the quantification of R-ropivacaine. As can be seen in Figs. 4A and B, no interference was observed at the retention time of the peak corresponding to R-ropivacaine. It is worth noting that even though the peak corresponding of water interferes slightly with S-ropivacaine peak, it does not compromise the quantification of the enantiomeric impurity.

Moreover, a solution of S-ropivacaine (1 mg/mL), obtained by diluting ten-fold Naropin®, was analysed (cf. Fig. 4D). As can be seen from this figure, a small peak, identified as R-ropivacaine by comparison of the retention times, was observed. Therefore, three independent injections of this solution was carried out daily and, at each validation day, the mean peak area of R-ropivacaine was subtracted from the responses of the validation standards.

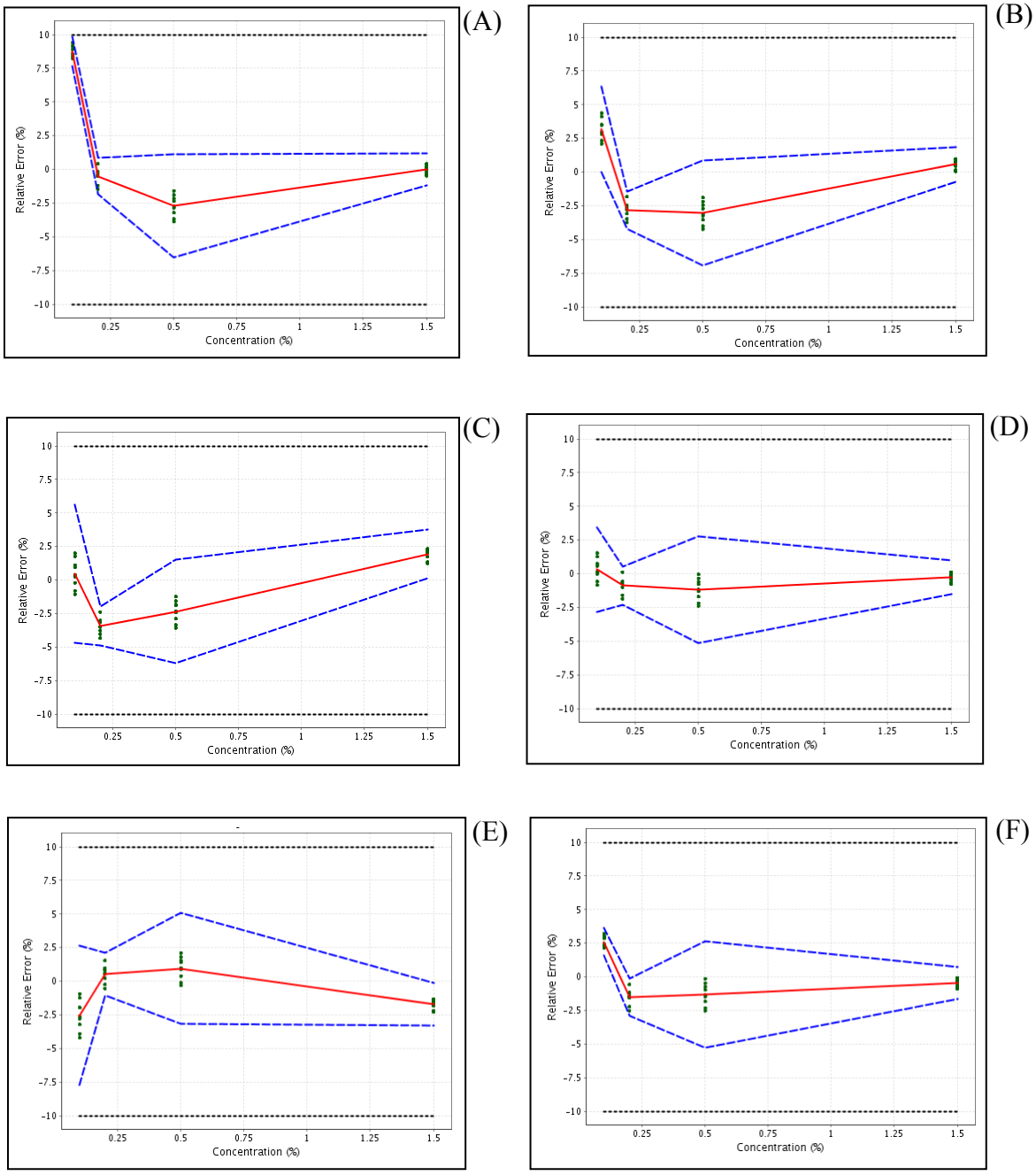

Figure 5. Accuracy profiles obtained using a) a linear regression model. b) a weighted linear regression model with a weight equal to 1/X. c) a weighted linear regression model with a weight equal to $1/X^2$. d) a linear regression model after square root transformation. e) a linear regression model after logarithm transformation. f) a quadratic regression model. The plain line is the relative error (%), the dashed lines correspond to the accuracy profile i.e. to the β-expectation tolerance limits expressed in relative error, and the dotted curves represent the acceptance limits (±10%).

VI.3.2.2. Selection of the calibration model

Several regression models were fitted to the calibration standards. From each regression line obtained, the concentrations of the validation standards were back-calculated in order to determine, at each concentration level, the mean relative bias, the relative standard deviation for intermediate precision as well as the upper and lower β-expectation tolerance limits at 95% [13].

Taking into account the validation data, several accuracy profiles were plotted to select the most suitable regression model for the intended use of the analytical method, as illustrated in Fig. 5. Six response functions, namely the simple linear regression, the weighted (1/X) and ($1/X^2$) linear regression models, the linear regression models after square root and logarithm transformation as well as the quadratic regression model, were tested. The acceptance limits were set at ±10% according to the regulatory requirements [18-20].

All these response functions allowed to demonstrate the capability of the method over the concentration range considered, since the tolerance intervals were totally included inside the acceptance limits as shown in Fig. 5. However, the linear regression model after square root transformation seems to be the best. Indeed, the relative error is the lowest especially at the two first concentration levels. The variability of the results is also weak. Table 2 presents the validation results obtained by applying this linear regression model.

VI.3.2.3.Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value and a mean experimental one. It gives information on systematic error and is expressed in term of relative bias (%). As can be seen in Table 2, the relatives biases assessed from the validation standards at four concentration levels were found quite acceptable according to the regulatory requirements, since their values are largely below the maximum of 10%, irrespective of the concentration level.

VI.3.2.4. Precision

Precision is the closeness of agreement among measurements from multiple sampling of homogeneous sample under the recommended conditions. The precision of the analytical method was estimated by calculating repeatability and time-dependent intermediate precision at each concentration level. The relative standard deviation (RSD) values were calculated from the estimated concentrations. As can be seen from Table 2, the RSD values were found to be very low (between 0.2 and 0.9%), illustrating the excellent precision of the proposed method.

VI.3.2.5. Accuracy

Accuracy refers to the closeness of agreement between the test result and the accepted value, namely the conventionally true value. The accuracy of the analytical method takes into account the total error, i.e. both systematic and random errors, related to the test result. The upper and lower β-expectation tolerance limits expressed in relative bias (%) as a function of the introduced concentrations are presented in Table 2. The different tolerance limits of the mean relative bias did not exceed the acceptance limits for each concentration level. Therefore, the developed method can be considered as accurate over the whole concentration range investigated.

VI.3.2.6. Linearity

The linearity of an analytical method is the ability within a definite range, to obtain results directly proportional to the concentration (quantity) of the analyte in the sample. For all series, a regression line was fitted on the back-calculated concentrations of validation standards as a function of the introduced concentrations by applying the linear regression model based on least squares method.

The results attesting the method linearity, namely the regression equation corresponding to that relationship with its coefficient of determination, are presented in Table 2. Moreover, in order to demonstrate the method linearity, the approach based on absolute β-expectation tolerance limits was applied. As can be seen in Fig. 6, the linearity of the present

method was demonstrated since the absolute β-expectation tolerance limits were within the absolute acceptance limits.

VI.3.2.7. Detection and quantification limits

The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. In the present study, the LOD was estimated to be the response corresponding to three times the signal to noise ratio. By applying this method, the LOD of the developed method was found to be equal to 0.02%. The limit of quantification (LOQ) of an analytical method is the lowest amount of the targeted substance which can be quantitatively determined under the experimental conditions prescribed with a well-defined accuracy, i.e., taking into account the systematic and random errors [13, 16]. As the accuracy profile was included inside the acceptance limits over the whole concentration range investigated, the first concentration level (0.1%) was considered as the LOQ, according to the selected regression model. Indeed, precision and trueness were demonstrated at this concentration level.

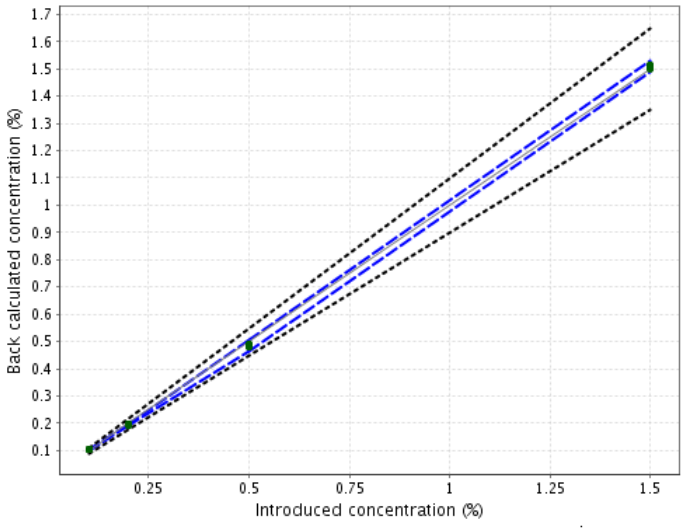

Figure 6. Linearity profile of the developed method. The plain line is the identity line: Y=X, the dashed lines correspond to the accuracy profile i.e. to the β-expectation tolerance limits expressed in absolute values. The dotted curves represent the acceptance limits (±10%) expressed in the concentration unit.

Table 2. Validation results for R-ropivacaine using the linear regression model after square root transformation.

Validation criteria	R-Ropivacaine		
Response function	Slope	Intercept	r^2
(k=3; m=5; n=2) (0.1-1.5%)			
Day 1	6.47	-0.341	0.9999
Day 2	6.50	-0.356	0.9999
Day 3	6.48	-0.337	0.9999
Trueness (k=3; n=3)			Relative bias (%)
0.1%			0.3
0.2%			-0.85
0.5%			-1.2
1.5%			-0.25
Precision (k=3; n=3)	RSD (%)		
	Repeatability		Intermediate precision
0.1%	0.5		0.9
0.2%	0.6		0.6
0.5%	0.3		0.9
1.5%	0.2		0.3
Accuracy (k=3; n=3)			Relative β-Expectation tolerance limits (%)
0.1%			[-2.79 , 3.46]
0.2%			[-2.27 , 0.57]
0.5%			[-5.12 , 2.79]
1.5%			[-1.50 , 0.99]
Linearity (k=3; m=4; n=3) (0.1-1.5%)			
Slope		0.9979	
intercept		-0.0015	
r^2		1	
LOD (%)		0.02	
LOQ (%)		0.1	

VI.4. Conclusion

In this paper, a chiral LC method with Sepapak-4 as CSP in polar organic solvent chromatography mode, was developed to determine the enantiomeric purity of S-ropivacaine in an aqueous formulation. The method development shows the reversal of ropivacaine enantiomers elution order according to the acidic additive (TFA or FA).

The method was then fully validated according to the strategy based on the accuracy profiles. Good performance with respect to selectivity, trueness, precision and accuracy, were obtained. The limits of quantification (0.1%), detection (0.02%) and the analysis time make the method suitable for rapid quality control of the enantiomeric purity of ropivacaine hydrochloride in an aqueous pharmaceutical formulation in comparison with the existing methods.

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CHAPTER VII.

LC METHOD FOR THE ENANTIOMERIC PURITY DETERMINATION OF S-AMLODIPINE WITH SPECIAL EMPHASIS ON REVERSAL OF ENANTIOMER ELUTION ORDER USING CHLORINATED CELLULOSE-BASED CHIRAL STATIONARY PHASES AND POLAR NON-AQUEOUS MOBILE PHASES

Summary

A LC method was developed and prevalidated for the enantiomeric purity determination of S-amlodipine in polar organic solvent chromatography (POSC) using chlorine containing cellulose-based chiral stationary phases (CSPs). The concentration of formic acid (FA) (0.01-0.2%) in the mobile phase containing acetonitrile as the main solvent, was found to influence the elution order of amlodipine enantiomers as well as the enantioresolution. A reversal of the enantiomer elution order of amlodipine was only observed with CSPs with both electron-withdrawing (chloro groups) and electron-donating (methyl group) on the phenyl moieties of the chiral selector, namely Sepapak-2 and Sepapak-4. The highest enantioresolution (*Rs*: 4.1) value was obtained at the lowest FA concentration (0.01 %) with Sepapak-4 as CSP and the enantiomeric impurity, R-amlodipine, eluted first under these conditions. Therefore the mobile phase selected for the prevalidation of the method consisted of ACN/0.1%DEA/0.01%FA and the temperature was set at 25°C.

The prevalidation of the method by means of the strategy based on total measurement error and the accuracy profile led to the selection of the weighted ($1/X^2$) linear regression as response function of the calibration curve within the matrix. The method was found to be selective and the limit of quantification was found to be about 0.5 µg/mL (0.05%) for R-amlodipine while the limit of detection was close to 0.2 µg/mL (0.02%). Moreover no significant matrix effect was observed.

VII.1. Introduction

Advances in chiral technology have led to the development of the pure enantiomers of new chiral drugs as well as previously marketed drugs as racemates ("*chiral switch")* [1, 2]. The need to enhance the efficacy and the safety profile of modern drugs as well as economic reasons have resulted in an active promotion of the chiral switch in the pharmaceutical companies. Indeed, the potential benefits of this strategy include improvement of the therapeutic index, simplification of the dose–response relationship and more selective pharmacodynamic profile [1, 2, 3]. Moreover, the pharmaceutical companies can save their market share from erosion due to generic competitors by developing the single-enantiomer versions of their racemic drugs in order to get new patents and monopolize the market.

Amlodipine, a long-acting L-type calcium-channel antagonist, is commercially available as a racemic mixture of (*R*)- and (*S*)-isomers. The comparison of the *R*(+)- and *S*(–)-isomers activities has shown that the *S*(–)-isomer is 1000 times more potent than the *R*(+)-isomer [4,5]. Moreover, clinical studies revealed that lower extremity edema was observed with S-amlodipine compared to the racemic mixture [6, 7, 8]. Thus, the chiral switch from racemic amlodipine to its S-enantiomer becomes evident since better tolerability and antihypertensive effects are expected.

Different methods dedicated to the determination of amlodipine enantiomers in biological fluids were reported. The enantioseparation of amlodipine was successfully carried out in liquid chromatography using α1-acid-glycoprotein column [9,10] and in capillary electrophoresis with α-CD as chiral selector in the running buffer [11]. To the best of our knowledge, there is no published methods for the enantiomeric purity determination of amlodipine.

The first aim of our work was to develop a LC method for the determination of R-amlodipine in a pharmaceutical formulation of S-amlodipine using chlorine containing cellulose-based CSP. Indeed, these CSPs have been shown to develop high chiral discrimination abilities for chiral basic drugs in polar organic solvent chromatography [12-14] The nature and concentration of the acidic additive were found to be important parameters to optimize retention and enantioresolution of the tested drugs. Then the optimized LC method was prevalidated according to the strategy proposed by a Commission of the Société

Française des Sciences et Techniques Pharmaceutiques [15]. This strategy is based on the use of accuracy profiles which take into account the total error, i.e. estimation of systematic and random errors of measurement.

VII.2. Experimental

VII.2.1. Chemicals and reagents

(R) and (S)-amlodipine base were generously provided by Prof. B. Chankvetadze. Amlodipine chemical structure is presented in Figure 1. Acetonitrile (ACN) of HPLC grade. Diethylamine (DEA), and formic acid (FA) pro analysis were obtained from Acros Organics (Geel, Belgium). Microcrystalline cellulose NF (Avicel®) was supplied by FMC Biopolymer (Brussels Belgium) and Magnesium stearate by RPL (Leuven, Belgium). Pregelatinized starch NF (Starch 1500®) and Colloidal silicon dioxide NF (Acrosil 200®) were supplied respectively by Colorcon (Indianapolis, USA) and Degussa AG (Franckfurt, Germany).

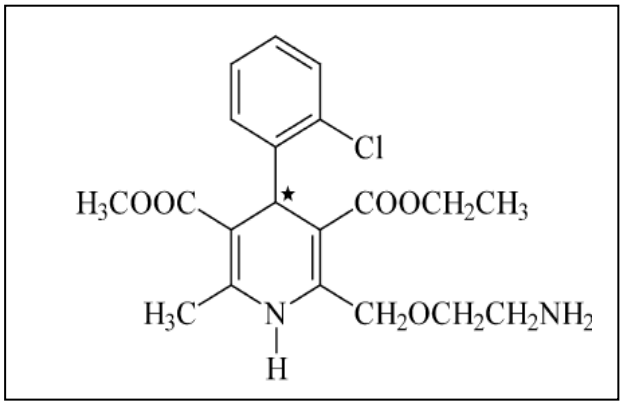

Figure 1. Chemical structure of amlodipine.

VII.2.2. Instrumentation

The chromatographic system from Agilent Technologies (Waldbronn, Germany) consisted in a binary pump, a thermostated column compartment, a diode array detector and an automatic injector, all of 1200 series. The Chemstation software was used for system control and data acquisition.

The chiral column Sepapak-2, Sepapak-4 and Sepapak-5 (250 mm x 4.6mm I.D.) was kindly provided by Sepaserve (Münster, Germany). The chiral selector adsorbed on aminopropylsilanized silica was cellulose tris (3-chloro-4-methylphenylcarbamate), cellulose tris (4-chloro-3 methylphenylcarbamate) and cellulose tris (3,5-dichlorophenylcarbamate) for Sepapak-2, Sepapak-4 and Sepapak-5 respectively, in the amount of 25% (w/w).

The statistical calculations for validation were performed by means of e.noval version 3.0 software (Arlenda, Liege, Belgium).

VII.2.3. Standards solutions

VII.2.3.1. Sample solutions used for method development

Different stock solutions of R- and S-amlodipine were prepared by dissolving an accurately weighed amount of approximately 1 mg of each compound in 10 ml of ACN. The stock racemate solution was prepared by dissolving a weighed amount of approximately 1 mg for R amlodipine and 1.1 mg for S-amlodipine in 10 mL of ACN.

VII.2.3.2. Sample solutions used for prevalidation

A stock solution of R-amlodipine was prepared by dissolving an accurately weighed amount of approximately 5 mg of this compound in 100 mL of the LC mobile phase. Then subsequent dilutions in the mobile phase were achieved in order to obtain five calibration standards ranging from 0.5 to 15 µg/mL (m=5) (cf. Table 1).

Stock matrix solution was prepared by dissolving 14.4 mg of Starch 1500® formulation [16] of amlodipine besylate without its active ingredient in the LC mobile phase. This mixture was then centrifuged at 4500 g for 5 minutes and the supernatant was used for preparation of calibration standards. Five solutions were prepared by diluting the stock solution of R- amlodipine with the matrix solution to achieve calibration concentrations ranging from 0.5 to 15 µg/ml. Two replicates (n=2) were prepared per concentration level. Each solution was injected one time. The number of concentration levels was sufficient to generate different regression models with and without matrix.

Table 1. Preparation of standard solutions related to R-amlodipine for prevalidation

Concentration level (% relative to 1.0 mg/mL of S-amlodipine)	Concentration of R-amlodipine (µg/mL)	
	Calibration standards wihout the matrix	Calibration standards within the matrix
0.05	0.5	0.5
0.1	1.0	1.0
0.2	2.0	2.0
0.5	5.0	5.0
1.5	15.0	15.0
total	10 samples/day	10 samples/day

VII.2.4. Chromatographic conditions

The mobile phases consisted of a mixture of acetonitrile, acidic additive and basic additive (*v/v*) and were pumped at a constant flow-rate of 1.0 mL.min-1. In all experiments, the basic additive percentage in the mobile phase was settled at 0.1 %. The injection volume was 20 µL. The analytes were detected photometrically at 240 nm and the temperature was settled at 25°C in all experiments.

VII.3. Results and discussion

VII.3.1. Method development

The screening of the chromatographic conditions for the enantioseparation of amlodipine was performed using two different mobile phases, namely ACN/0.1%DEA/0.1%TFA and ACN/0.1%DEA/0.2%FA (*v/v/v*), with a temperature settled at 25°C. These conditions were selected in previous work using three chlorine containing cellulose based CSPs, namely Sepapak-2, Sepapak-4 and Sepapak-5 [12,13].

The mobile phase with formic acid (FA) as acidic additive showed good results with respect to enantioresolution, even if the retention times were higher with this additive, irrespective of the nature of the CSP (data not shown). On the contrary, the use of the mobile phase containing TFA as acidic additive did not give rise to the enantioseparation of amlodipine.

In order to optimize the analysis time and the enantioresolution for amlodipine, the concentration of FA in the mobile phase was tested from 0.01 and 0.2%. The results showed a significant effect of the FA concentration in the mobile phase on the retention and resolution of amlodipine enantiomers.

VII.3.1.1. Effect of FA concentration on retention: reversal of elution order

Figs. 2 A, B and C present the retention times of the enantiomers of amlodipine on the three CSPs, using different concentrations of FA as acidic additive in the mobile phase. As can be seen in these figures, the retention of amlodipine enantiomers depends on the nature of the CSP. The lowest concentration of FA gave rise to the lowest retention of amlodipine enantiomers (<15 min) on all these CSPs. At higher FA concentrations, the retention times increased and passed through a maximum at 0.1% FA for Sepapak-2 and Sepapak-4 and at 0.05% FA for Sepapak-5. Moreover, the highest retention times obtained for amlodipine enantiomers using Sepapak-5 were lower compared to those found on the other CSPs.

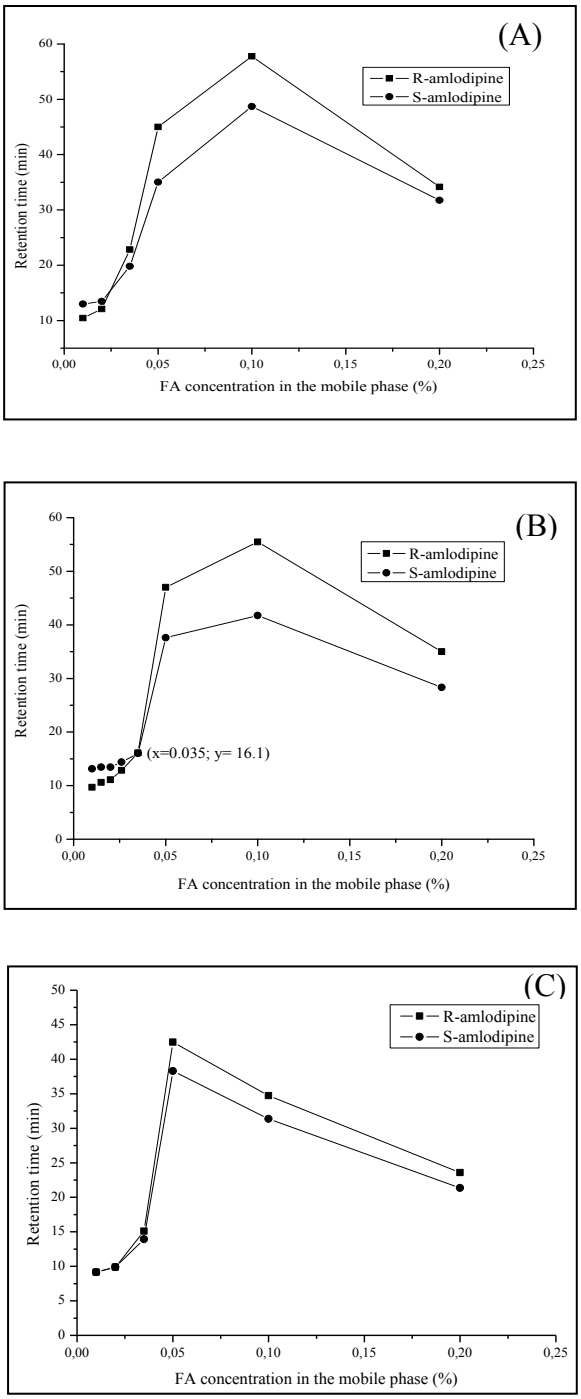

Figure 2. Influence of the percentage of formic acid (FA) on the retention and elution order of amlodipine enantiomers.

Mobile phase: ACN/0.1% DEA/(0.01- 0.2)% FA; Flow rate: 1 mL/min; CSP: Sepapak-2 (A), Sepapak-4 (B), Sepapak-5 (C). Other conditions see Section 2.

It is particularly important to note that a reversal of the enantiomer elution order was observed on both Sepapak-2 and Sepapak-4. In both cases, R-amlodipine eluted first at lower FA concentrations, less than 0.02 % with Sepapak-2 and less than 0.035% with Sepapak-4 in the mobile phase, while S-amlodipine eluted first at higher FA concentrations (cf. chromatograms obtained with Sepapak-4 in Figs. 3 A and B). No reversal of the enantiomer elution order was obtained on Sepapak-5.

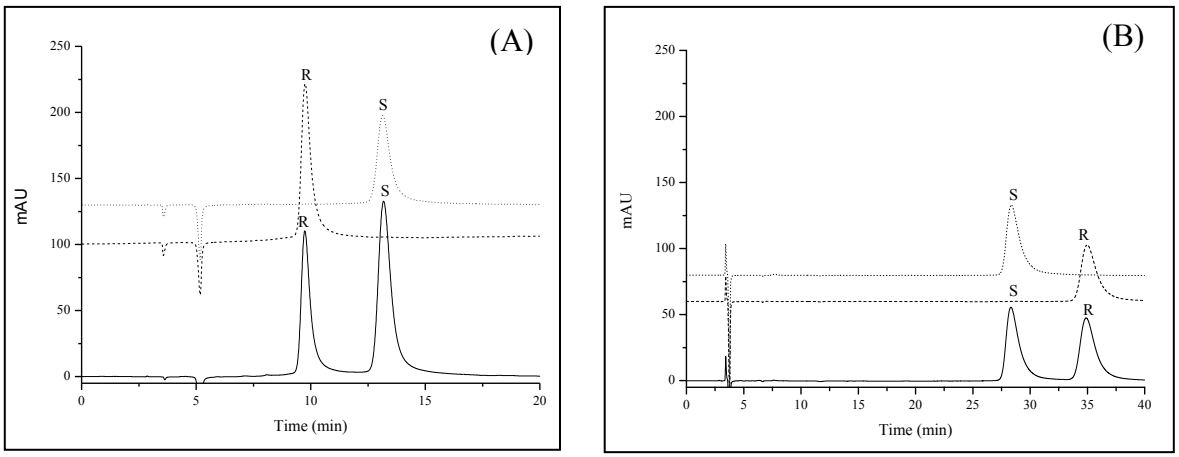

Figure 3. Chromatograms showing the reversal of amlodipine enantiomers elution order according to the concentration of the acidic additive (FA).

Mobile phase: ACN/0.1% DEA/0.01% FA (A), ACN/ 0.1% DEA/0.2% FA (B); Flow rate: 1 mL/min; CSP: Sepapak-4. Peaks: (R) R-amlodipine and (S) S-amlodipine: Others conditions see Section 2.

This unusual phenomenon is a key issue in LC determination of the enantiomeric purity of chiral drugs [17,18]. Indeed, it is usually desirable to detect the minor component in front of the major one in this kind of analysis because several studies have shown that the limits of detection and quantification were lower and that the reproducibility of the determination was higher for the minor enantiomer when it eluted first [19-21].

Many reversals of elution order were described in literature. In 1988, a reversal of the elution order was observed by Hermansson and Schill for the enantioseparation of pseudoephedrine using an α-acid glycoprotein-based (AGP) stationary phase. The authors have related this phenomenon to the addition of octanoic acid to the mobile phase [22]. Two years later, Haginaka et al. reported a reversal of the elution order of propranolol enantiomers and its ester derivatives depending on the nature of the organic modifier added to the mobile phase using ovomucoid-bonded silica as CSP [23]. A temperature-dependent elution order reversal was first reported by Pirkle and Murray using a π-basic proline-derived CSP in 1993 [24]. Okamoto et al. reported in 1991 the first reversal of the enantiomer elution order on a cellulose-based CSP (Chiralcel OJ) [25]. They found that the reversal of elution order of pyriprofen enantiomers (an insect growth regulator) using this CSP was associated with the change in the mobile phase of the nature of the organic modifier (alcohols). Balmér et al. [26] observed also a temperature-dependent reversal of the enantiomer elution order when a derivative of metoprolol, H170/40, was enantioseparated with Chiralcel OD as CSP. In almost all these reversals of the enantiomeric elution order, the authors suggested that solvation and/or conformation of both the solute enantiomers and the CSP can be affected by the change of the nature of the polar organic modifier. Likewise, changes in temperature can induce conformational changes of a polymeric selector. Recently, we reported the reversal of the elution order of ropivacaine enantiomers induced by the change of the nature of the acidic additive (TFA or FA) in the mobile phase using the same chlorine containing cellulose-based CSPs [14].

The reversal of elution order of amlodipine enantiomers observed on Sepapak-2 and Sepapak-4 could be related to the presence of both electro-withdrawing (chlorine) and electron-donating (methyl) substituents on the phenyl moieties of the chiral selector since no reversal was obtained on Sepapak-5, which possesses only chloro substituants on its phenyl moieties. Indeed, these mixed substituents may create a regular arrangement of grooves along the carbohydrate (cellulose) chains which can serve as enantioselective binding pockets, as suggested by Lämmerhofer [27]. A change of the FA concentration in of the mobile phase could have induced a modification of the conformation of the polymeric selector that leads to the reversal of the elution order of amlodipine enantiomers.

VII.3.1.2. Effect of FA concentration on the enantioresolution

As can be seen in Figure 4, the enantioresolution of amlodipine was found to strongly depend on the FA concentration in the mobile phase. As expected, enantioresolution values passed through zero, corresponding to a FA concentration of 0.035 % when Sepapak-4 was used as CSP, due to the reversal of the elution order of amlodipine enantiomers at this FA concentration. A similar trend can be observed with Sepapak-2 and the concentration corresponding to the coelution of both enantiomers of amlodipine is situated in this case between 0.02 and 0.03% FA (cf. Fig. 2 A). Even though enantioresolution values started to increase at FA concentrations higher than 0.02% when Sepapak-5 was used as CSP, the se values remained lower than those obtained with the two other CSPs.

It is noteworthy that the highest enantioresolution value (*Rs*: 4.1) was obtained using Sepapak-4 with a FA concentration of 0.01% in the mobile phase. Therefore, the mobile phase selected for the prevalidation of the method for the enantiomeric purity determination of S-amlodipine was made of ACN/0.1% DEA/ 0.01% FA since the enantiomeric impurity (i.e. R-amlodipine) is then the first eluting peak.

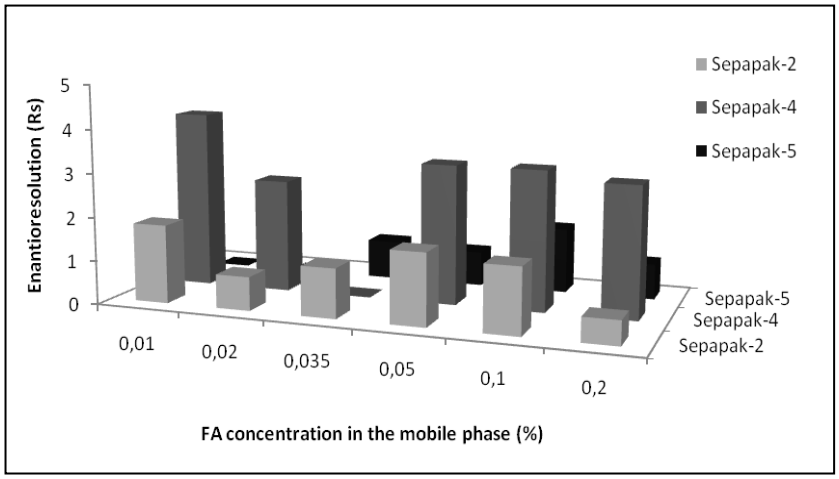

Figure 4. Influence of FA concentration in the mobile phase on the enantioresolution of amlodipine using the three chlorine containing CSPs.
Mobile phase: ACN/0.1% DEA/(0.01- 0.2)% FA; Flow rate: 1 mL/min;
Other conditions see Section 2.

VII.3.2. Prevalidation

On the basis of the prevalidation protocol proposed by the "Société Française des Sciences et Techniques Pharmaceutiques (SFSTP)" Commission, the experiments achieved during the prevalidation step deal with the analysis of the response function and the selection of the most appropriate model for the calibration curve in the validation step. For that purpose, the SFSTP approach based on two-sided 95% β-expectation tolerance intervals for total measurement error including systematic and random errors and accuracy profiles obtained with and without the matrix was used [15, 28-30]. The concept of accuracy profile was also applied to estimate the limit of quantification (LOQ) and the range over which the method could be validated. Moreover, the method selectivity was evaluated and the limit of detection (LOD) was estimated.

VII.3.2.1. Selectivity

Typical chromatograms obtained after analysis of matrix solution containing or not R-amlodipine as well as a reference solution of the compound of interest were compared in order to check the absence of components likely to interfere in the quantification of R-amlodipine. As can be seen in Figs. 5A, B and C, no interference was observed at the retention time of the peak corresponding to R-amlodipine. Moreover, matrix solution containing S-amlodipine (1 mg/mL) and spiked with 1 µg/mL (Fig. D) or 5 µg/mL (Fig. E) of R-amlodipine was analysed (cf. Fig. 5D and E). As can be seen in these figures, the peak of R-amlodipine can be identified and quantified.

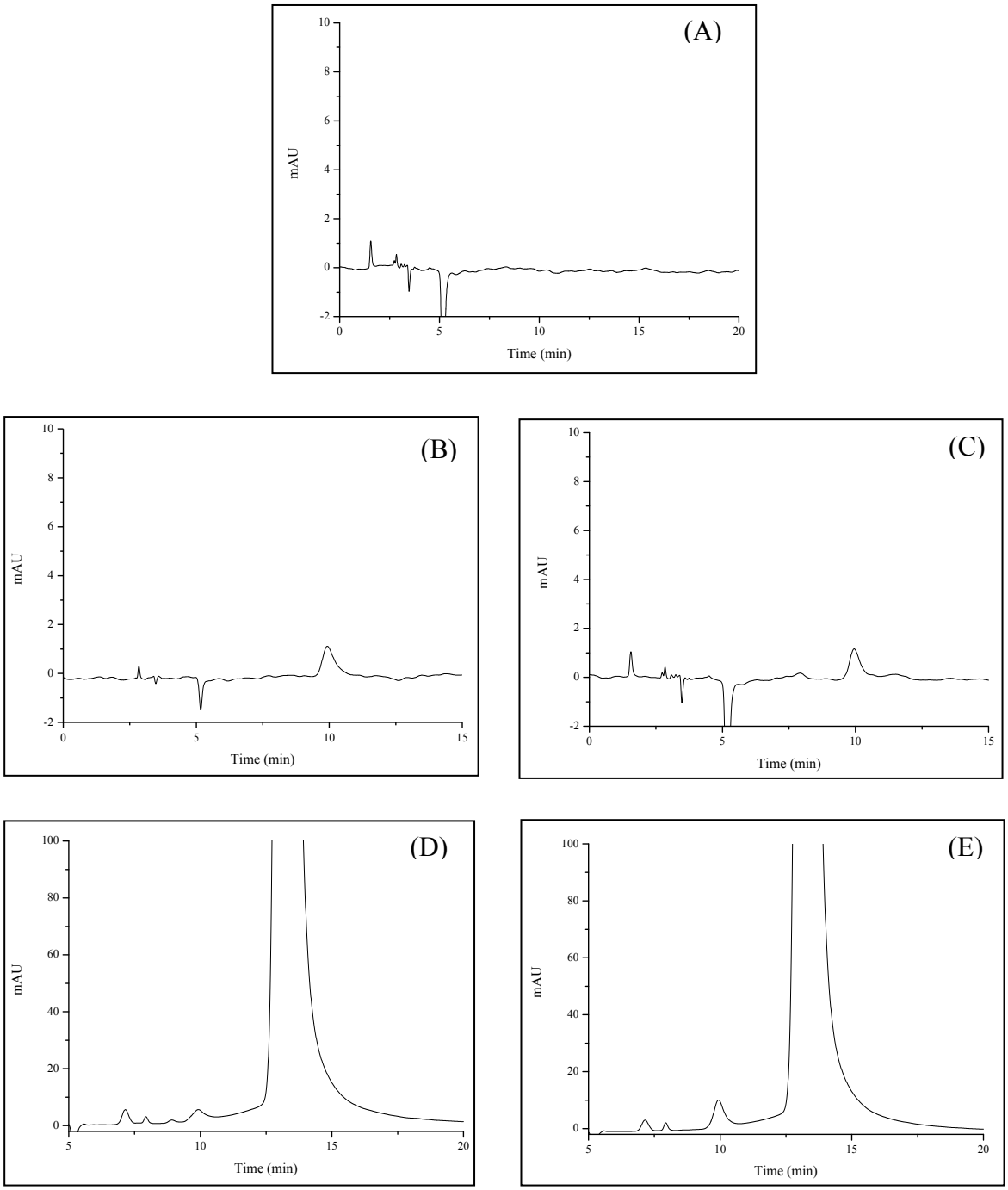

Figure 5. Typical chromatograms of a) a matrix solution; b) R-amlodipine diluted in the mobile phase (1 µg/mL); c) R-amlodipine spiked in the matrix solution (1 µg/mL); d) a solution of S-amlodipine (1 mg/mL) spiked with R-amlodipine (1 µg/mL); e) a solution of S-amlodipine (1 mg/mL) spiked with R-amlodipine (5 µg/mL).
Mobile phase: ACN/0.1% DEA/0.01% FA, Flow-rate: 1 mL/min; CSP: Sepapak-4. Other conditions see Section 2.

VII.3.2.2. Selection of the calibration model

Two independent calibration solutions with and without the matrix were prepared each day of prevalidation.

Several regression models were fitted to the calibration standards prepared with and without matrix. From each regression model obtained, the concentrations of the calibration standards were back-calculated in order to determine, at each concentration level, the mean relative bias, the relative standard deviation for intermediate precision as well as the upper and lower β-expectation tolerance limits at 95% [15].

Taking into account the prevalidation data, several accuracy profiles were plotted to select the most suitable regression model for the intended use of the analytical method, as illustrated in Figure 6. Four response functions (with and without the matrix), namely the simple linear regression, the weighted ($1/X$ and $1/X^2$) linear regression models and the linear regression model obtained after logarithm transformation were tested. The acceptance limits were set at ±5% according to the regulatory requirements [15, 28-30].

All these response functions, except the simple linear model, allowed testing the capability of the method over the concentration range considered, since the tolerance intervals were totally included inside the acceptance limits as shown in Fig. 6. However, the weighted ($1/X^2$) linear regression model observed with the matrix seems to be the best. Indeed, the relative error is the lowest especially at the two first concentration levels (cf. Fig. 6F). The variability of the results is also weak. However, the matrix effect seem to be limited. Table 2 presents the prevalidation results obtained by applying this weighted linear regression model.

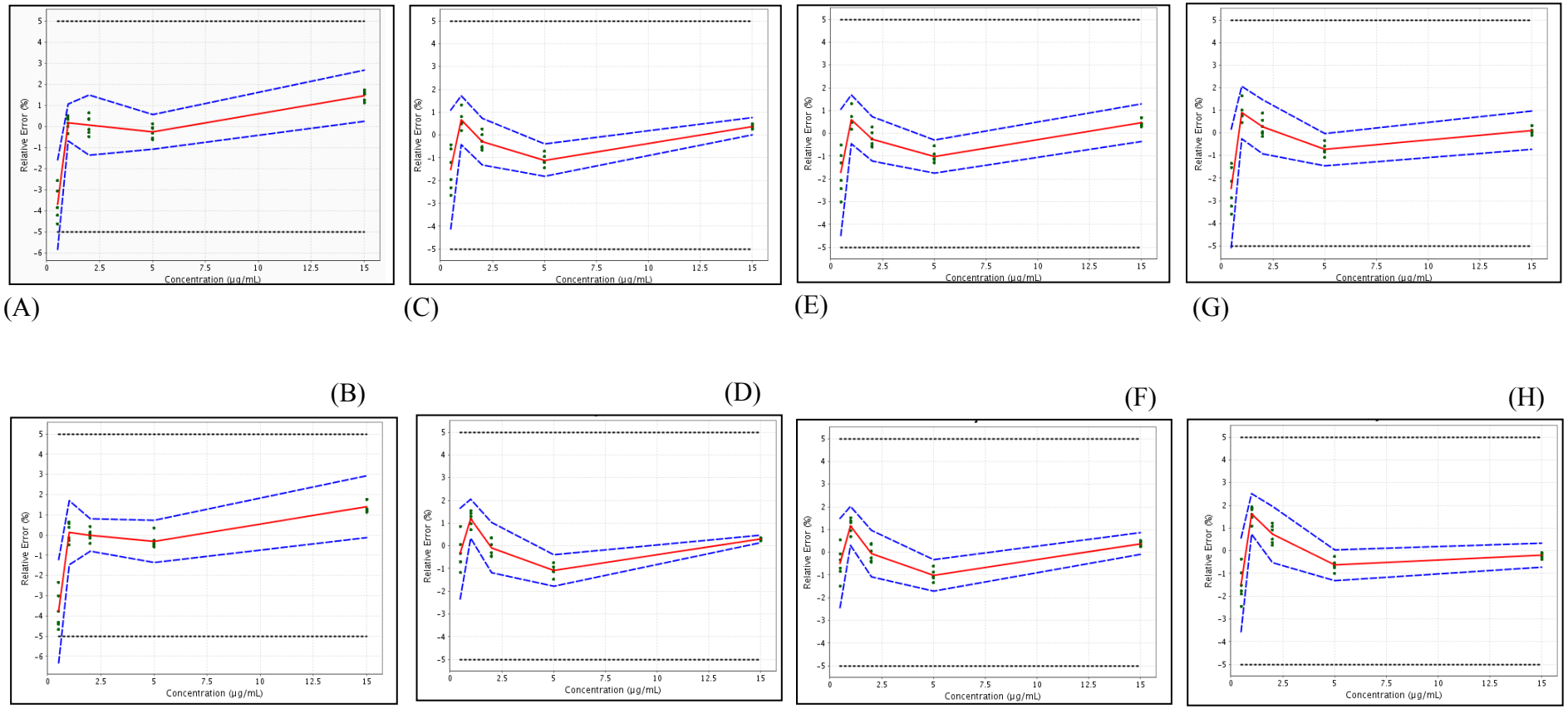

Figure 6. Accuracy profiles with (down) and without (upper) the matrix obtained using a;b) a linear regression model. c;d) a weighted linear regression model with a weight equal to 1/X. e;f) a weighted linear regression model with a weight equal to $1/X^2$. g;h) a linear regression model after logarithm transformation. The plain line is the relative error (%), the dashed lines correspond to the accuracy profile i.e. to the β-expectation tolerance limits expressed in relative error, and the dotted curves represent the acceptance limits (±5%).

VII.3.2.3.Trueness

Trueness refers to the closeness of agreement between a conventionally accepted value or reference value and a mean experimental one. It is expressed in terms of relative bias (%). As can be seen in Table 2, the relative biases assessed from the calibration standards within the matrix at five concentration levels were found quite acceptable, since their values are largely below 5%, irrespective of the concentration level.

VII.3.2.4. Precision

Precision was estimated by calculating repeatability and time-dependent intermediate precision at five concentration levels. The relative standard deviation (RSD) values were calculated from the estimated concentrations. As can be seen in Table 2, the RSD values were found to be very low (between 0.06 and 0.7%), illustrating the very low variability of the proposed method.

VII.3.2.5. Accuracy

Accuracy refers to the closeness of agreement between the test result and the accepted value, namely the conventionally true value. The accuracy of the analytical method takes into account the total error related to the test result. The upper and lower β-expectation tolerance limits expressed in relative bias (%) as a function of the introduced concentrations in the matrix are presented in Table 2. The different tolerance limits of the mean relative bias did not exceed the acceptance limits for each concentration level. Therefore, the developed method could be considered as accurate over the whole concentration range investigated. However, it should be more interesting to perform the validation within a restrictive concentration range, namely 1 to 15 µg/mL corresponding to 0.1 and 1.5 % of R-amlodipine in S-amlodipine pharmaceutical formulation.

Table 2. Prevalidation results for R-amlodipine using the weighted ($1/X^2$) linear regression model within the matrix

Prevalidation criteria	R-amlodipine		
Response function	Slope	Intercept	r^2
(k=3; m=5; n=2) (0.5-15µg/mL)			
Day 1	36.73	-0.9147	0.9999
Day 2	36.64	-0.7149	0.9999
Day 3	36.68	-0.7773	0.9999
Trueness (k=3; n=2)			Relative bias (%)
0.5 µg/mL			0.48
1.0 µg/mL			1.16
2.0 µg/mL			-0.05
5.0 µg/mL			-1.02
15 µg/mL			0.39
Precision (k=3; n=2)	RSD (%)		
	Repeatability		Intermediate precision
0.5 µg/mL	0.7		0.7
1.0 µg/mL	0.3		0.3
2.0 µg/mL	0.4		0.4
5.0 µg/mL	0.3		0.3
15 µg/mL	0.06		0.12
Accuracy (k=3; n=2)		Relative β-Expectation tolerance limits (%)	
0.5 µg/mL			[-2.44 , 1.48]
1.0 µg/mL			[0.31 , 2.01]
2.0 µg/mL			[-1.07 , 0.97]
5.0 µg/mL			[-1.73 , -0.31]
15 µg/mL			[-0.08 , 0.85]
Linearity (k=3; m=5; n=2) (0.5-15µg/mL)			
Slope		1.004	
intercept		-0.0148	
r^2		1	
LOD (µg/mL)		0.15	
LOQ (µg/mL)		0.5	

VII.3.2.6. Linearity

The linearity of an analytical method is the ability within a definite range, to obtain results directly proportional to the concentration (quantity) of the analyte in the sample. For all series, a regression line was fitted on the back-calculated concentrations of calibration standards as a function of the introduced concentrations by applying the linear regression model based on least squares method.

The slope and intercept of the equation corresponding to that relationship as well as the coefficient of determination are presented in Table 2.

VII.3.2.7. Detection and quantification limits

The limit of detection (LOD) is the smallest quantity of the targeted substance that can be detected, but not accurately quantified in the sample. In the present study, the LOD was estimated using the mean intercept of the calibration model and the residual variance of the regression. By applying this computation method, the LOD of the developed method was estimated to be equal to 0.15µg/mL.

The limit of quantification (LOQ) of an analytical method is the lowest amount of the targeted substance which can be quantitatively determined under the experimental conditions prescribed with a well-defined accuracy [15, 28]. As the accuracy profile was included inside the acceptance limits over the concentration range investigated, the first concentration level (0.05%) could be considered as the LOQ, according to the selected regression model.

VII.4. Conclusion

The determination of the enantiomeric purity of S-amlodipine in a pharmaceutical formulation was developed in POSC using chlorine containing cellulose based CSPs. The results showed a reversal of the elution order of amlodipine enantiomers, related to the concentration of FA in the mobile phase. This reversal of the enantiomer elution order occurred only on cellulose-based CSPs with both electron-withdrawing (chlorine atoms) and electron-donating (methyl group) on the phenyl moieties of the chiral selector.

Furthermore, the method was prevalidated according to the strategy based on the accuracy profile. The method was found to be selective, precise and accurate and no matrix effect could be observed. The response function appropriate for this method was found to be the weighted ($1/X^2$) linear regression model. The limit of quantification was 0.5µg/mL (0.05%) of R-amlodipine. However, for the method validation it would be better to settle the lowest concentration at 1µg/mL (0.1%) of R-amlodipine.

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CHAPTER VIII.

GENERAL CONCLUSIONS AND PERSPECTIVES

The recognition abilities of three new chiral stationary phases, Sepapak-2, and Sepapak-4 and Sepapak-5, towards the enantiomers of thirteen (13) chiral basic pharmaceuticals with widely different structures and polarities, have been studied in polar organic solvent chromatography (POSC) with acetonitrile as the main solvent.

In the first part of this thesis, we studied the influence of temperature, organic modifiers, nature and concentration of acidic additves on retention, selectivity and enantioresolution of these chiral drugs using Sepapak-4. An univariate approach was used for that purpose. The temperature (15-35°C) was found to have a rather limited effect on enantioresolution. However, for celiprolol, a significant efficiency enhancement led to enantioresolution improvement. Polar (methanol) and non-polar (n-hexane) organic modifiers had opposite effects on retention and enantioresolution indicating a normal-phase behaviour.The addition of n-hexane (10%) in the mobile phase increased the retention and enantioresolution of most of the studied compounds, unlike methanol. The importance of the nature and concentration of the acidic additive (TFA, FA and AcA) in the mobile phase on retention, selectivity and enantioresolution was also demontrated. FA was found to induce the highest retention while the lowest was obtained using TFA. Moreover the influence of the acidic additive (FA and AcA) concentration in the mobile phase on retention and enantioresolution was found to be rather molecule dependent even though FA generally led to better enantioresolution values compared to AcA. Therefore, two groups could be distinguished according to the acidic additive. The first group consisted of all studied beta blockers (except oxprenolol, propranolol and sotalol) and prilocaine for which enantioresolution was better with TFA as acidic additive. The second group for which a better enantioseparation was obtained with FA, was constituted by the rest of the studied chiral compounds. Although the mechanisms of chiral recognition could not be completely explained, the acidic additive effects seems be related to the presence of both electron-withdrawing (chlorine) groups and electron-donating (methyl) groups on the phenyl moieties of the chiral selector of Sepapak-4.

In the second part of this thesis, experimental designs were used to perform the screening and optimization of the factors previously studied. The screening step performed using a fractional factorial design confirmed that the effects of the acidic additive and n-hexane on enantioresolution were significant while temperature and the nature of the basic additive were found to have a limited effect. The optimization step carried out with a face

centered central composite design led to optimal enantioresolution values and the deduced conditions for rapid screening could be applied to other chiral compounds.

In the third part of this thesis, three rapid screening conditions, namely ACN/0.1% DEA/0.1%TFA, ACN/0.1% DEA/0.2% FA and ACN/0.1% DEA/0.2% AcA, were applied to the three studied CSPs (Sepapak-2, Sepapak-4 and Sepapak-5). They revealed the complementarity of the chiral discrimination abilities of these CSPs in polar organic solvent chromatography. Obviously, a rapid screening could be carried out with only four experiments involving Sepapak-4 with ACN/0.1% DEA/0.1%TFA and ACN/0.1% DEA/0.2% FA as mobile phases and Sepapak-5 with ACN/0.1% DEA/0.2% FA and ACN/0.1% DEA/0.2% AcA as mobile phases.

In the application part of this thesis, enantioseparation methods using these CSPs were developed to evaluate the enantiomeric purity of S-ropivacaine and S-amlodipine, respectively, in pharmaceutical formulations. With both compounds, a phenomenon of reversal of the enantiomer elution order could be observed. The reversal of the enantiomer elution order of ropivacaine caused by the change of the nature of acidic additive (TFA or FA) in the mobile phase occurred only on the two CSPs with both electron-withdrawing (chlorine) and electron donating (methyl) groups on the phenyl moieties of the chiral selector, Sepapak-2 and Sepapak-4. The reversal of the enantiomer elution order of amlodipine caused by the change in the formic acid concentration in the mobile phase occurred on the same CSPs as for ropivacaine. The change of the nature or the concentration of the acidic additive could have induced a modification of the conformation of the chiral selector, leading to the reversal of the enantiomer elution order.

The optimised method for the enantiomeric purity evaluation of S-ropivacaine in a pharmaceutical formulation (Naropin, 10 mg/mL aqueous solution) was validated by means of the strategy based on the use of the accuracy profiles. The method truness, precision and accuracy were determined and found to be satisfactory over the working concentration range. A limit of quantification (LOQ) of 0.1% was reached while the limit of detection (LOD) was 0.02% for R-ropivacaine.

Regarding amlodipine, the developed method was prevalidated according to the same approach as for ropivacaine. The selectivity, linearity and precision of the method were

demonstrated. Futhermore, no matrix effect was observed and the concentration range for method validation was found to be 1-15µg/mL.

As a general conclusion, this thesis work demonstrates the essential role that the mobile phase acidic additive plays in the optimization of enantioseparations using these chlorine containing cellulose-based CSPs together with polar organic mobile phases. Indeed, the retention, selectivity and enantioresolution of chiral basic compounds and even the enantiomer elution order for some molecules, such as ropivacaine and amlodipine, can be modulated by the nature and concentration of the acidic additive in the mobile phase.

Among the three tested CSPs, it is no doubt Sepapak-4 that must be considered as the first choice in this POSC mode, since this CSP was found to give the highest resolution power towards the enantiomers of basic drugs in most cases. Sepapak-5, with two chloro groups on its phenyl moieties, has shown significant differences in chiral recognition properties compared to Sepapak-4 and can therefore be seen as an interesting alternative.

At the end of this thesis, we are perfectly conscious of the fact that there are still several other aspects that it would have been worth studying but we have not been able to tackle them by lack of time.

For example, it would be interesting to study more in detail the behavior of these CSPs towards acidic and neutral compounds in order to extend the proposed rapid screening strategy to these classes of compounds.

Several polar organic solvents were tested as main components of the mobile phase but only acetonitrile, which was found to be the most suitable solvent in preliminary experiments, was used in this thesis. It would certainly be worth testing POSC systems with other solvents, such as methanol or ethanol, either pure or in mixtures, since they might lead to different chiral recognition mechanisms on these CSPs.

Finally, it would be also of interest to investigate the use of these CSPs in other column formats, in particular in columns with small diameters, either in micro-LC or in CEC, and to try to couple these columns with a mass spectrometric detection in order to develop highly sensitive methods for the stereoselective determination of small amounts of drug enantiomers in biological fluids.

APPENDIX I

List of publications

1."**Enantioseparation of basic pharmaceuticals using cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral stationary phase and polar organic mobile phases**"

K.S.S. Dossou, P. Chiap, B. Chankvetadze, A.-C. Servais, M. Fillet, J. Crommen, Journal of Chromatography A 1216 (2009) 7450-7455. DOI: 10.1016/j.chroma.2009.05.081.

2."**Optimization of the LC enantioseparation of chiral pharmaceuticals using cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral selector and polar non-aqueous mobile phases**"

K.S.S. Dossou, P. Chiap, B. Chankvetadze, A.-C. Servais, M. Fillet, J. Crommen, Journal of Separation Science 33 (2010) 1699-1707. DOI: 10.1002/jssc.201000049.

3."**Development and validation of LC method for the enantiomeric purity determination of S-ropivacaine in a pharmaceutical formulation using a recently commercialized cellulose-based chiral stationary phase and polar non-aqueous mobile phase**"

K.S.S Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen, Journal of Pharmaceutical and Biomedical Analysis 54 (2010) 687-693 DOI: 10.1016/j.jpba.2010.10.020.

4."**Evaluation of chlorine containing cellulose-based chiral stationary phases for the LC enantioseparation of basic pharmaceuticals using polar non-aqueous mobile phases**"

K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen, Journal of Separation Science, in press.

5."**LC method for the enantiomeric purity determination of S-amlodipine with special emphasis on reversal of enantiomer elution order using chlorinated cellulose-based chiral stationary phases and polar non-aqueous mobile phases**"

K.S.S. Dossou, P.A. Edorh, P. Chiap, B. Chankvetadze, A.-C. Servais, M. Fillet, J. Crommen,

(submitted for publication in Journal of Pharmaceutical and Biomedical Analysis).

APPENDIX II

List of oral communications, posters and contributions to oral communications throughout the Ph.D.

"**Development and validation of LC method for determination of enantiomeric purity of S-ropivacaine in a pharmaceutical formulation using a recently commercialized cellulose based chiral stationary phase and non-aqueaous mobile phases.**"

K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen.

Poster presentation at Drug Analysis 2010 (Antwerp, Belgium), September 21-24, 2010.

"**Evaluation of recently developed cellulose-based chiral stationary phases for the LC enantioseparation of basic pharmaceuticals using polar non-aqueous mobile phases.**"
K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen.

Poster presentation at 34th International Symposium on High Performance Liquid Phase Separations and related techniques, "HPLC 2009" (Dresden, Germany), June 2009.

"**A screening study of chiral pharmaceutical using tris(4-chloro-3-methylphenylcarbamate) of cellulose-based stationary phase and polar organic phases.**"

K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen.

Poster Presentation at 33rd International Symposium on High Performance Liquid Phase Separations and related techniques, "HPLC 2008" (Kyoto, Japan), December, 2008.

"**Comparison of different cellulose based chiral stationary phases for the LC enantioresolution of basic drugs using polar organic mobile phases.**"

K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen.

Contribution to oral communication at 19th International symposium on Pharmaceutical & Biomedical Analysis, "PBA 2008" (Gdansk, Poland), June 2008.

"**Enantioséparation de substances médicamenteuses basiques par chromatographie liquide à l'aide d'une nouvelle phase stationnaire chirale et de phases mobiles polaires non aqueuses.**"

K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen.

Oral communication at Forum «des Jeunes Chercheurs du CIRM» (Liege, Belgium), February 02 2008.

"**HPLC enantioseparation of basic drugs using recently developed polysacharide-based CSPs with polar organic mobile phases.**"

K.S.S. Dossou, P. Chiap, A.-C. Servais, M. Fillet, J. Crommen.

Contribution to oral communication at 12th International Meeting on Recent Development in Pharmaceutical Analysis, "RDPA 2007" (Elbe Island, Italy), September 2007.

"**HPLC enantioseparation of basic pharmaceuticals using a new polysaccharide-based chiral stationary and non-aqueous polar mobile phases.**"

K.S.S. Dossou, S. Ghiani, P. Chiap, A.-C. Servais, M. Fillet, J.Crommen.

Poster presentation at 31st International Symposium on High Performance Liquid Phase Separations and Related Techniques, "HPLC 2007" (Gand, Belgium), June 21-24, 2007.