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Enantioseparation of drugs by means of continuous bed (monolithic) columns in nano-liquid chromatography#

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A chiral continuous bed was realized by a simple one-step synthesis and used to perform enantioseparations of drugs in nano-liquid chromatography (nano-LC). The capillary format of columns of this miniaturized technique allows to easily prepare in situ the chromatographic media. The continuous bed (also called monolithic) column was synthesized employing water-soluble comonomers and hydroxypropyl-β-cyclodextrin (HP-β-CD) was selected as a chiral selector. In order to make possible the one-step synthesis, HP-β-CD was previously activated as the allyl derivative. Several polymeric mixtures were studied to increase the enantiorecognition capability of the column. Analyses were carried out employing aqueous mobile phases, and the effect of organic modifiers and pH on enantiomeric resolution was studied. Among tested compounds, nomifensine and naproxen were baseline resolved, using a mobile phase containing respectively 20/80 methanol / water and 20/80 acetonitrile / water, both buffered with 0.1% triethylamine-acetate. A partial resolution was also achieved for praziquantel, metomidate and 5-methyl-5-phenyl-hydantoin.

Key words: nano-liquid chromatography, chiral continuous bed, hydroxypropyl-β-cyclodectrin, drug enantiomers

INTRODUCTION

Several molecules of biological, pharmaceutical or environmental interest possess one or more chiral centres. These are responsible for the existence at least of two enantiomers, which show different behaviors in a chiral environment. Drug enantiomers, e. g. can differ in their pharmacological and toxicological properties, as a consequence of different stereoselective interactions with biological matrices (i. e. receptors). These diverse interactions can result in qualitative and / or quantitative differences in the therapeutic or adverse effects of the drug [1, 2].

Conventional analytical separation techniques employed to discriminate chiral compounds are chromatographic (gas chromatography, liquid chromatography, supercritical fluid chromatography) and electrophoretic ones. With all these techniques, the enantiodiscrimination is possible selecting an appropriate chiral selector which can be either bound (by a covalent bond, absorption or entrapped) to the stationary phase contained in the column, directly to the wall of the column, or dissolved in the mobile phase as a chiral additive [3–5].

A class of chiral selectors, widely employed for enantioseparations, are native and derivatized cyclodextrins (CDs). Their high enantiorecognition capability is provided by different kinds of interactions they can establish with chiral compounds. CDs are cyclic oligosaccharides and have a

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shape of a truncated cone with a relatively hydrophobic cavity. Every glucopyranose ring in CD has five chiral centers. The external rims of the cone possess hydrophilic character, due to the presence of hydroxyl groups. CDs are able to form transient diastereomeric inclusion-complexes with enantiomers by means of the cavity. At the same time, the hydroxyl groups of glucopyranose units also play a very important role in the enantioresolution process by adsorption and / or hydrogen bonding interactions. CDs can be easily modified by derivatization of the hydroxyl groups on the external rims, leading to CD derivatives with different characteristics, such as higher solubility, different depth of cavity, different interaction sites, etc. [6–8].

Recently miniaturization has been successfully introduced in the field of analytical chemistry, and miniaturized versions of conventional liquid chromatography were developed, i. e. capillary / nano-liquid chromatography (CLC/nano-LC). Nano-LC offers several advantages, e. g. good efficiency, short analysis time, use of a small amount of reagents (i. e. mobile and stationary phases) with a consequent low environmental pollution, low sample requirement and easy coupling with MS [9, 10].

In case of chiral separations, where expensive chiral stationary phases or chiral mobile phase additives have to be employed, nano-LC results are very useful since they allow to perform analysis with a very small amount of this costly material.

In nano-LC three different types of capillary columns can be used: i) packed, ii) open tubular and iii) continuous bed (monolithic) columns.

Packed capillary columns are widely employed since they offer high selectivity and high efficiency. A large variety of stationary phases is available, since those developed and used in HPLC are also suitable for nano-LC. However, the preparation of packed capillary columns and the necessity of frits to retain the stationary phase represent challenging tasks related to their utilization. Open tubular columns are less complicated to prepare and do not need frits, but as drawbacks they show poor selectivity and low sample loading capability. Alternatively to packed and open tubular columns, continuous bed technology has been applied in nano-LC. The use of this material offers some advantages, summarized as follows. They can be easily prepared in situ due to the capillary format of the columns employed in nano-LC (internal diameters, i. d., of 10–100 μm) and no frits are needed to retain the stationary phase. Furthermore, these separation media are characterized by high permeability and consequently very low back-pressures. Huge variety of monomers available for synthesis of such materials makes it possible to obtain columns with different chromatographic properties [11, 12].

The polymer-based continuous beds can be mainly obtained in two ways: i) using polar and amphiphilic water-soluble acrylic comonomers in the presence of salt or a polymer or ii) using organic solvent soluble comonomers in the presence of co-solvent, so-called porogen.

In this work a chiral continuous bed using water-soluble comonomers was prepared by one-step synthesis, selecting hydroxypropyl-β-cyclodextrin (HP-β-CD) as a chiral selector. To make possible the one-step synthesis, HP-β-CD has been previously activated using allyl-glycidylether.

EXPERIMENTAL

Chemicals

All solvents were of the analytical reagent grade. Glacial acetic acid (99.8% pure), formic acid (99% pure), acetonitrile (ACN), acetone, iso-propanol (i-PrOH), ethanol (EtOH) and methanol (MeOH), orthophosphoric acid 85% were purchased from Carlo Erba (Milan, Italy). Sodium hydroxide, 1,4-bis(acryloyl) piperazine > 99% (PDA), triethylamine > 99% (TEA) were from Fluka Chemie AG (Buchs, Switzerland).

N-isopropylacrylamide (IPA), methacrylamide 98% (MA) and ammonium sulfate 99.999%, allyl-glycidylether (AGE) were obtained from Aldrich (Steinheim, Germany). Ammonium persulfate (APS) and N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) were from Bio-Rad Laboratories (Hercules, CA, USA).

Ultrapure water was prepared with a Milli-Q system (Millipore / Waters, Milford, MA, USA). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was from FDS Publications (Trowbridge Wilts, BA, UK). Racemic naproxen, ibuprofen, ketoprofen, suprofen, indoprofen, carprofen, nomifensine, praziquantel, metomidate, 5-methyl-5-phenyl-hydantoin, and alprenolol were from different sources and were used without further purification.

Fused silica capillary TSP100375 (100 μm i. d., 375 μm O. D.) was purchased from Composite Material Services (Block, UK).

Stock solutions of chiral compounds were set up dissolving the appropriate amount of each analyte in MeOH in order to obtain concentrations of 1 mg/mL. Before the runs, stock solutions were diluted with water at the desired concentrations (50 μg/mL). Mobile phases were daily prepared and ultrasonicated before their use.

Instrumentation

Experiments were carried out with a liquid chromatographic system composed of a conventional gradient LC pump and a UV/VIS on-column detector. Samples were introduced into the column by a nano injector valve (Sepaserve GmbH, Munster, Germany). The injection valve had a 50 μL loop that was used for both sample injection or mobile phase reservoir. Nano flows (few hundreds nL/min) were obtained using a static splitting device consisting of a stainless steel tee connected from one side to the pump with a peek tube; to the second one with a fused silica capillary (50 μm i. d. × 50 cm) to the waste, and finally to the injection valve via a stainless steel tube (500 μm i. d. × 3 cm).

Injection was done by introducing the sample mixture into the valve and injecting for 10 s. Afterwards, the loop was

filled with the mobile phase. The HPLC pump operated in the isocratic mode, delivering methanol to the split and then to the modified injection valve containing the selected mobile phase. Methanol was continuously recycled.

Allylation of HP-β-CD

The allylation of HP-β-CD was done slightly modifying a procedure previously reported [13, 14]. Briefly, about 0.391 g HP-β-CD was dissolved in 2 mL NaOH 2% w/v and the solution was heated until reaching 50 °C. Next, AGE was added in small aliquots (30 μL each 30 min, per a total volume of 180 μL), continuously stirring at 50 °C. The reaction was left to proceed for 3 h. After that, the solution was cooled at room temperature and 0.2 g $NaBH_4$ was added to reduce unreacted epoxy groups, stirring for 30 min. Finally, the solution was neutralized with phosphoric acid (85%) and kept in the refrigerator (4 °C) for further experiments.

Preparation of continuous bed columns

Continuous bed columns were prepared by in situ polymerization of the selected monomers.

For this reason, a fused silica capillary was chemically modified by the attachment of the methacryloxypropyl groups to the inner wall of the capillary. Reaction consisted of the following steps: approximately 3 m of the capillary were in turn washed with 0.1 M hydrochloric acid for 10 min, distilled water, 1 M sodium hydroxide, distilled water and acetone using a water jet vacuum pump. Afterward, a solution of 30% (v/v) methacryloxypropyl trimethoxysilane in acetone was drawn into the capillary and left overnight. The following day the capillary was washed with acetone, distilled water and cut into pieces of an appropriate length for preparing chromatographic beds.

The polymeric mixture contained PDA, bifunctional monomer, as a crosslinker, MA as a linear monomer, and IPA as a non-polar monomer, to give hydrophobic behavior to the stationary phase.

All the comonomers, ammonium sulfate and the initiator APS (10% aqueous solution), were dissolved in an appropriate volume of the aqueous buffered solution containing the allyl-activated HP-β-CD. The solution was degassed by vacuum and drawn into the capillary piece by capillary forces. Both ends of the capillary were sealed with a rubber and put into a thermostated oven at 65 °C for 3 h, exploiting thermal initiation.

Several polymeric mixtures were tested and their composition is reported in Table 1.

RESULTS AND DISCUSSIONS

One-step *in situ* polymerization of chiral continuous bed

As mentioned above, one of the main advantages of the preparation of a continuous bed is the possibility to change the composition of the mixture of comonomers in order to obtain a separation media appositely tailored for compounds under examination. This gain can be exploited for chiral separation as well, where enantiomer separations are obtained as a consequence of different interactions with the stationary phase, including stereospecific and non-stereospecific.

Stereospecific interactions are exclusive of the chiral selector, while non-stereospecific interactions can be due to the matrix supporting the chiral selector. Once the chiral selector is chosen, the polymerization mixture can be modified, for instance, changing hydrophobicity of the polymeric matrix, in order to decrease retention time, increase efficiency or selectivity among different classes of compounds [15].

Several works in literature showed HP-β-CD enantiomer recognition capability towards a large number of compounds, including acidic, basic and neutral ones, both when it was used as a chiral mobile phase additive (CMPA) or incorporated into a stationary phase.

When HP-β-CD and in general CD are used as CMPA, analyses are carried out in combination with a reverse phase column and the enantiomer resolutions achieved are a consequence of interactions with both the achiral stationary phase and the cyclodextrin [16–18].

In the previous study we have used HP-β-CD as CMPA in nano-LC for the separation of some non-stereoidal anti-inflammatory drugs [19]. The enantiomer resolutions were obtained employing a RP-C18 packed column as well as a monolithic one, working with a mobile phase containing high percentage of water. Even if the packed column showed higher selectivity, the monolithic column allowed faster analysis and to operate with a completely aqueous mobile phase, due to its high permeability and amphiphilic properties.

Considering those results, the aim of this work was synthesis of the monolithic columns containing covalently attached HP-β-CD as a chiral selector and possessing at the same time amphiphilic behavior.

The synthesis of continuous beds was realized employing acrylamide comonomers by a very simple one-step in situ polymerization, where the chiral selector (HP-β-CD) also possessed vinyl groups to take part into polymerization process.

Table 1. **Composition of the polymerization mixture**

Capillary column	MA, mg	PDA, mg	IPA, mg	$(NH_4)_2SO_4$ (500 mg/mL), μL	HP-β-CD-AGE, μL	APS (10% w/v), μL
I	10	20	30	4	196	2
II	10	20	30	2	198	3
III	20	20	20	2	198	3
IV	15	30	15	2	198	3
V	10	40	30	2	198	4
VI	10	20	30	2	198*	3

* Concentration of HP-β-CD-AGE was increased 1.5 times. See text for other explanations.

The derivatization of HP-β-CD was carried out with a simple procedure which did not need purification or isolation steps.

Allylation of HP-β-CD

As above mentioned, the synthesis of allyl-HP-β-CD was carried out following a procedure already published, with a slight modification. At first, the concentration of HP-β-CD in the solution was about 0.08 M, while the ratio between HP-β-CD and AGE was about 1 : 7 (HP-β-CD-AGE[1]). After the synthesis of I–IV columns (see Table 1), in order to increase the enantiorecognition capability of the columns, it was decided to increase HP-β-CD concentration in the solution for allylation reaction, keeping constant the ratio between the CD and AGE. In the first attempt, HP-β-CD concentration was increased twofold (HP-β-CD-AGE[2]), however, when the solution was added to the polymerization mixture, a double-phase system was created. Therefore, HP-β-CD concentration was increased 1.5 times (HP-β-CD-AGE[3]) and the solution was compatible with the polymerization mixture.

Composition of continuous bed and chromatographic enantioseparations

Working with water soluble monomers, there are some limitations concerning the use of hydrophobic monomers, just because of their reduced solubility in an aqueous solution. However, IPA, which is soluble in that media, is able to give hydrophobic interaction as well and was selected as a monomer for the polymeric mixture.

Preliminary experiments were carried out selecting a polymeric mixture composed by 50% (w/w) IPA (with relatively high hydrophobicity) and about 30% (w/w) PDA, the crosslinker, for a total comonomers concentration (%T) of about 24%. The selected amount of salt was appropriate to have a good permeability of the column. Thermal initiation was selected for the polymerization, because it gives better hydrodynamic properties of the continuous bed [12].

Based on our previous experience with HP-β-CD as CMPA and on data reported in literature [19–21], a mobile phase with high percentage of water or even totally aqueous, properly buffered, was selected to perform experiments. Table 2 reports the list of analytes which were enantioseparated employing the monolithic column above described, while the chemical structure of those compounds is reported in Fig. 1.

The first analyzed compounds were some non-steroidal anti-inflammatory drugs (NSAIDs), i. e. naproxen, ibuprofen, suprofen, flurbiprofen, ketoprofen, and carprofen. They did not elute within 1 h, with a completely aqueous mobile phase, even buffered at different pH, in the pH 2.5–5 range. For this reason, the addition of an organic modifier was studied, namely of ACN, MeOH, and i-PrOH. Among them, the presence of ACN, even at 10–20%, strongly reduced the analysis time, except for carprofen that was not eluted in 1 h. However, only naproxen was in some degree enantioresolved, obtaining the best result working with 20% ACN in a mobile phase buffered with 0.1% TEA-Ac. With percentage of an organic modifier higher than 30%, enantiomer resolution was completely lost.

A series of different chiral drugs was also examined, including nomifensine, praziquantel, 5-methyl-5-phenylhydantoin, metomidate, and alprenolol. Among them, nomifensine enantiomers only were resolved even in a completely aqueous mobile phase (MP). The addition of MeOH (10–20%) gave rise to improved enantiomer resolution and reduced analysis time. The combination of TEA and Ac resulted in a good peak shape, with these analytes as well, and it was selected as buffer for further studies.

Then, in order to increase enantiomer resolution, the composition of column I was slightly modified for the preparation of column II, halving the amount of salt. The salt strongly affects the permeability of the column by perfusive channels formation, especially when continuous beds with low polarity

Table 2. **Enantiomer recognition capability of different continuous beds tested**

Column I	k_1	k_2	α	*Rs*	MP
Naproxen	9.14	9.58	1.05	0.61	A
Nomifensine	1.60	2.01	1.26	0.64	B
Column II					
Naproxen	6.73	7.13	1.06	0.62	A
Nomifensine	2.18	2.80	1.28	1.07	B
Column IV					
Nomifensine	2.38	2.99	1.26	0.307	C
Column VI					
Naproxen	8.15	8.90	1.09	1.32	A
Nomifensine	3.91	5.13	1.31	1.79	B
Metomidate	3.48	3.78	1.09	0.69	D
Praziquantel	5.18	5.94	1.15	0.81	B
5-methyl-5-phenyl-hydantoin	2.92	2.97	1.02	0.38	D

Mobile phase (MP): A = 20/80 ACN/water (v/v) buffered with 0.1% TEA-Ac; B = 20/80 MeOH/water (v/v) buffered with 0.1% TEA-Ac; C = 0.1% TEA-Ac; D = 10/90 ACN/water (v/v) buffered with 0.1% TEA-Ac. k_1 and k_2 are the retention factors of the first and second eluted enantiomer, respectively.

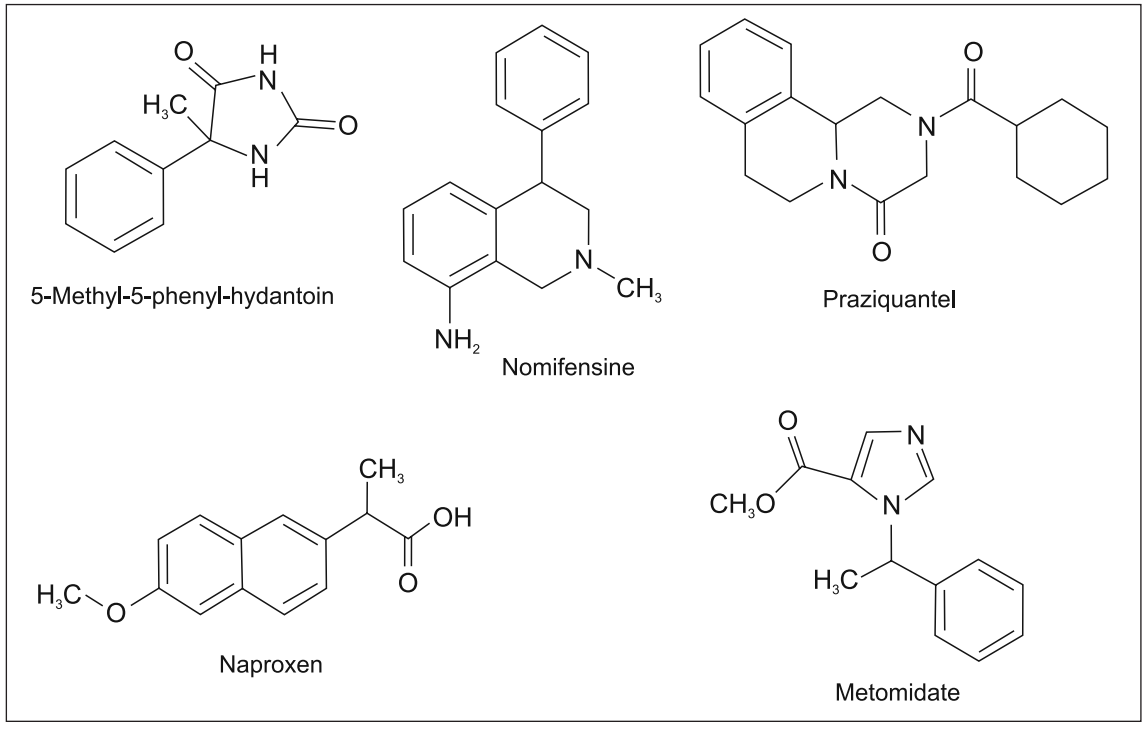

Fig. 1. Chemical structures of studied compounds

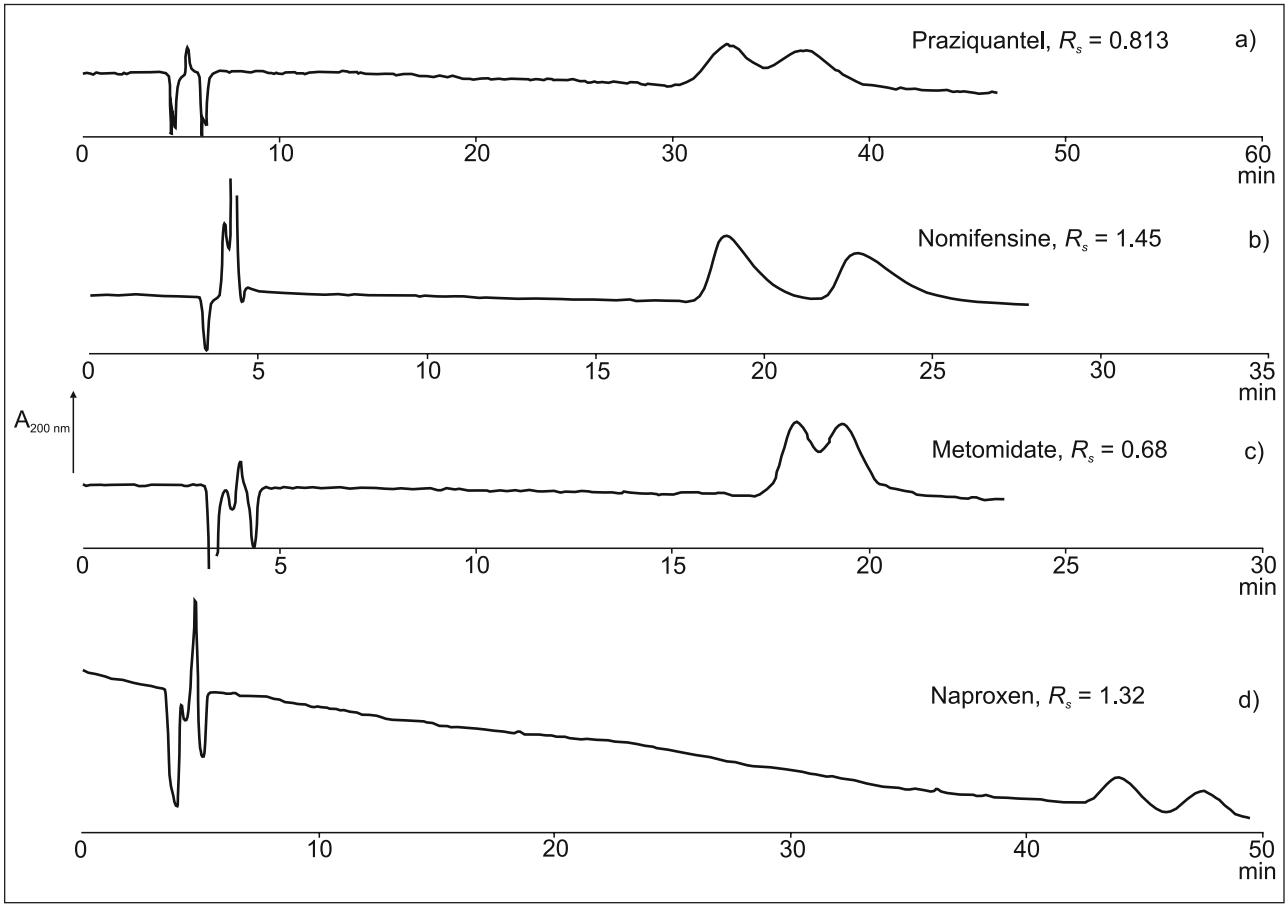

Fig. 2. Enantiomeric separation of drugs. Capillary column 100 µm i. d. × 19 cm; mobile phase: a) 20/80 MeOH/water buffered with 0.1% TEA-Ac; b) and c) 10/90 ACN/water buffered with 0.1% TEA-Ac; d) 20/80 ACN/water buffered with 0.1% TEA-Ac

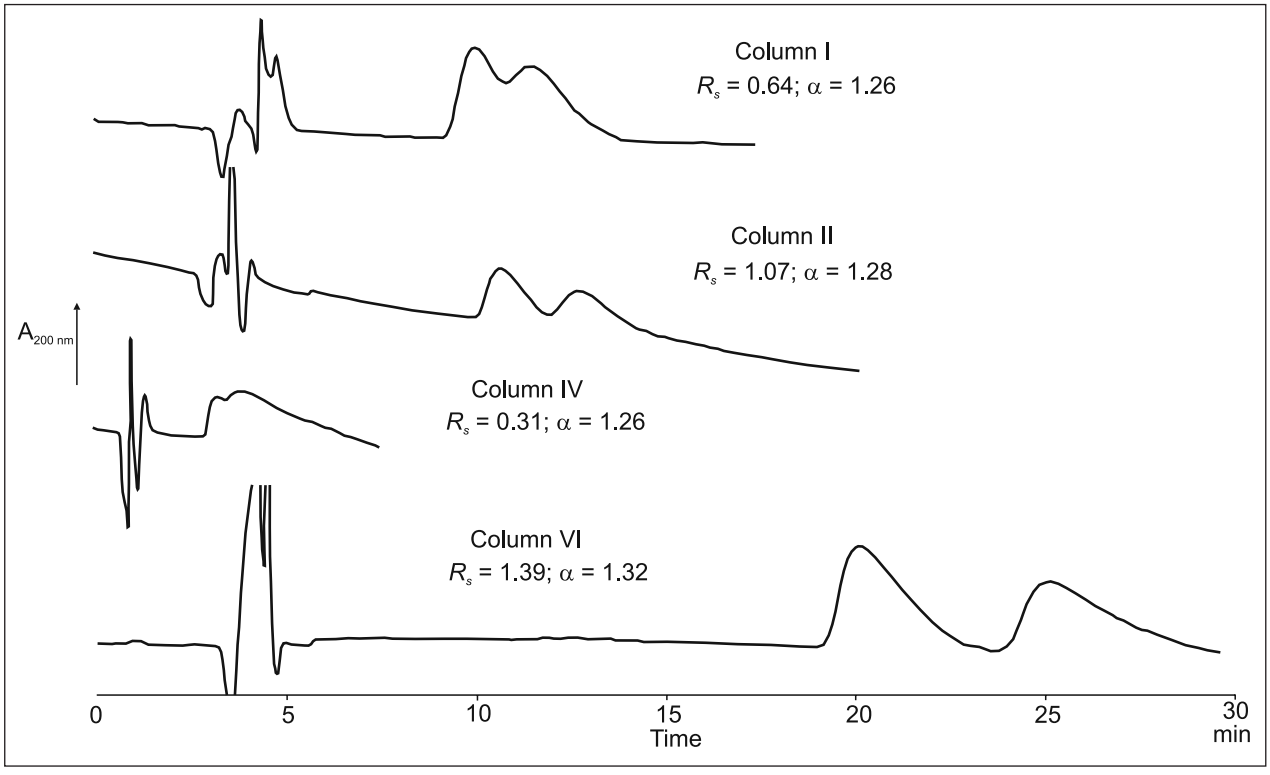

Fig. 3. Comparison of nomifensine enantiomers separations obtained with different columns. All analyses were performed using the same mobile phase (MP: 20/80 MeOH/water buffered with 0.1% TEA-Ac) and approximately at the same linear velocity, except for column IV, where linear velocity was increased to allow peak detection

have to be synthesized [12]. A little improvement was observed for the enantiomer separation of nomifensine and naproxen.

Especially to reduce the retention of NSAIDs on the continuous bed, the composition of polymerization mixture was changed, keeping constant the amount of HP-β-CD-AGE (column III–IV, Table 2).

Even if the retention times were lower, no NSAID compound was resolved and a minute resolution of nomifensine was observed with column IV. In column V, the crosslinker percentage was increased up to 50% of total comonomers, increasing also %T up to 29%. The %T influences the morphology of the continuous bed, and increasing %T the smallest perfusive channels should be expected, with, consequently, the highest efficiencies and resolution. However, in our experiments, despite of increased retention time, no enantiomer resolution was observed.

Afterward, we decided to use a more concentrated solution of HP-β-CD-AGE[3], as described above, to prepare a new monolithic column (column VI), keeping the other components of the polymerization mixture at the quantities reported for column II.

Column VI allowed to baseline resolve nomifensine and naproxen, while a partial resolution was achieved for praziquantel, metomidate and 5-methyl-5-phenyl-hydantoin. Representative chromatograms are reported in Fig. 2, while in Fig. 3 a comparison of enantiomer separations obtained with different columns is given for nomifensine.

The last attempt to improve the chiral separation of the studied compounds was done projecting a chiral gradient column (from 100% to 50% polymeric mixture used for column VI), as it was demonstrated previously for reversed phase separations using gradient stationary phases [22]. However, the results obtained were similar to the correspondent isotropic column (70% mixture of the monomers used for column VI), and in both cases enantiomer resolutions were lower than those obtained with column VI. This could be due to the insufficient focusing effect obtained for the analytes due to relatively low chiral selector concentrations at the zones of maximum polymerization mixture concentrations.

CONCLUSIONS

In this work one-step synthesis of chiral continuous beds was realized in a very simple way. The capillary columns were employed in nano-LC to perform enantiomer separations of some drugs. After optimization of the composition of the polymeric mixture, nomifensine and naproxen enantiomers were baseline separated, while for other compounds lower enantiomer resolution was observed. The achieved results are promising and further studies will be carried out in order to improve the performance of the chiral continuous bed capillary columns.

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VAISTŲ ENANTIOMERŲ ATSKYRIMAS PANAUDOJANT SKYSČIŲ NANOCHROMATOGRAFIJĄ SU IŠTISINĖMIS (MONOLITINĖMIS) NEJUDRIOMIS FAZĖMIS

S a n t r a u k a

Chiralinės ištisinės nejudrios fazės buvo susintetintos vienpakopės sintezės metu ir panaudotos vaistų enantiomerų skysčių nanochromatografijai. Kapiliarinis kolonėlių formatas šiuo miniatiūriniu metodu įgalina *in situ* lengvai susintetinti nejudrią fazę. Ištisinės (taip pat vadinamosios monolitinės) kolonėlės buvo susintetintos panaudojant vandenyje tirpius komonomerus ir hidroksipropil-β-ciklodekstriną (HP-β-CD), kuris buvo pasirinktas kaip chiralinis selektorius. Vienos pakopos sintezė buvo įgyvendinta iš anksto aktyvuojant HP-β-CD kaip alilo darinį. Buvo tirta keletas monomerų mišinių siekiant padidinti kolonėlių chiralinio atpažinimo gebą. Organinių modifikatorių ir pH efektas enantiomerų skiriamajai gebai buvo analizuotas panaudojant vandenines judrias fazes. Tarp išbandytų junginių nomifensinas ir naproksenas buvo visiškai atskirti naudojant judrias fazes, sudarytas atitinkamai iš 20/80 metanolio / vandens ir 20/80 acetonitrilo / vandens su 0,1 % trietilamino acetato priedais. Dalinis enantiomerų atskyrimas buvo pasiektas skirstant prazikantelį, metodidatą ir 5-metil-5-fenil-hidantoiną.