Separation and elution order of the enantiomers of some β -agonists using polysaccharide-based chiral columns and normal phase eluents by high-performance liquid chromatography

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Abstract

In this study separation of enantiomers of 8 chiral β -agonists were studied on 6 polysaccharide-based chiral columns in polar-organic and alcohol-hydrocarbon mobile phases. No separation of enantiomers was observed on any column with polar-organic mobile phase eluents such as pure methanol, ethanol or acetonitrile. Most of the chiral analytes were resolved into enantiomers when alcohol-hydrocarbon type mobile phases were used. The most successful column was Lux Cellulose-2 on which all 8 chiral analytes were baseline resolved into enantiomers at least with one mobile phase used. The reversal of enantiomer elution order was observed dependent on the chemistry of the chiral selector and the composition of the mobile phase.

Keywords: Beta agonist, enantiomer elution order, enantioseparations, HPLC, mobile-phase additives, polysaccharide-based chiral columns.

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1. Introduction

The β -agonists are phenethanolamine derivatives used in human medicine for long time as uterine relaxants, cardiostimulants and for asthma treatment [1]. The majority of currently used β -agonists represent racemic mixtures. The pharmacological activity of the β -agonist usually resides in (R)-enantiomer [2]. (S)-Enantiomers are believed to have no bronchodilator effect and induce unwanted effects [2]. For instance, earlier studies showed that S,S-formoterol inhibits the beneficial effects of R,R-formoterol on anti-inflammatory cellular surface marker expression and cytokine secretion [3, 4].

Although separation of enantiomers is widely used for analytical and preparative purposes in academia and industry, there is no theory that would allow predicting a separation result based on analyte structure, nature of chiral selector and mobile phase composition. The primary question in this field is to understand the nature of forces causing the preferential binding of one enantiomer to the chiral selector over the other [5]. From a practical point of view, the enantiomer elution order (EEO) reversal can have an impact on the determination of the enantiomeric purity [6, 7]. The impurity can be quantified with better accuracy and precision while it elutes before the major peak [6].

Composition of the mobile phase may cause a reversal in the chiral recognition pattern. This makes mobile phase composition of great interest from a practical point of view (in some cases allowing the adjustment of the EEO), as well as for a better understanding of enantiomer recognition mechanisms. Reversal of EEO in high-performance liquid chromatography (HPLC) with polysaccharide-based chiral columns

have been reported based on the percentage of the major component of the mobile phase [7-9], and the minor acidic and basic additives in the mobile phase [10-13].

In literature separation of enantiomers of some chiral β -agonists are described using HPLC [14-25], supercritical fluid chromatography (SFC) [26], capillary electrophoresis (CE) [17, 23, 27-29], capillary electrochromatography (CEC) [30] and micellar electrokinetic chromatography (MEKC) [31]. Some studies were devoted to investigation of enantiomer migration order and chiral recognition mechanisms in CE [17]. Until now, almost no dedicated studies have been published on the EEO of these chiral compounds in HPLC.

In the present work, HPLC separation of eight chiral β -agonists derivatives was studied on six different columns having a polysaccharide backbone (amylose and cellulose) with polar organic and alcohol-hydrocarbon mobile phases. Along with the successful separation of analyte enantiomers, the emphasis of this study was on differences in EEO depending on the chemistry of a chiral selector, mobile phases and mobile-phase additives.

2. Experimental

2.1. Chemicals and Reagents

The commercially available chiral β -agonists, butoxamine (BUT), cimbuterol (CIM), clenbuterol (CLN), clenpenterol (CLP), isoxsuprine (ISO), ritodrine (RIT), terbutaline (TER) and tulobuterol (TUL) (Fig. 1), as well as analytical grade formic acid (FA), diethylamine (DEA) and mobile phase components, such as chromatographic grade methanol, ethanol, 2-propanol, acetonitrile and n-hexane were supplied from Sigma

Aldrich (Steinheim, Germany). Lux series of polysaccharide based chiral columns (Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, Lux Cellulose-4 and Lux Amylose-2) with the dimensions 4.6 x 250 mm, 3 μ m and the Lux Amylose-1 with 5 μ m particle size were provided by Phenomenex Inc. (Torrance, CA, USA). The schematic structures of their chiral selectors are shown in ref. [9].

2.2. Instruments and Chromatographic Conditions

The Agilent 1100 series HPLC system (Santa Clara, CA, USA) was used for the method development studies, equipped with a G1379A degasser, G1311A quaternary pump, G1313 auto injector and G1315B diode array detector. The detection was performed at 225 nm. The chromatographic separations were performed at 25 °C with the flow rate of 1 mL min⁻¹. For the data acquisition and data handling the Chemstation[®] software was used. Injection volume was fixed as 5 μ L for all experiments. An isocratic mobile phase consisting of a varied mixture of n-hexane, 2-propanol containing FA and/or DEA % 0.1 (v/v) were used for the separation of enantiomers.

2.3. Fraction Collection

The enantiomers of the chiral compounds in this study were collected in house by micropreparative chromatography on suitable analytical columns with the flow rates of 1 to 2 mL min⁻¹. The collected effluents were evaporated at 25 °C and the purified enantiomers were used for spiking of the racemic samples in order to obtain non-racemic mixtures of enantiomers (enantiomeric ratio approximately 1:2).

3. Results and Discussions

3.1. General overview on separations

As mentioned in experimental section, six analytical columns with various chiral selectors were used with the hydrocarbon-alcohol (different compositions of n-hexane and 2-propanol) and polar organic mobile phases, such as methanol, ethanol, acetonitrile, 2-propanol. These mobile phases were used with various combination of acidic and/or basic additives. The enantiomeric separations of β -agonist derivatives were initially performed in the polar organic solvents that are indicated above.

Four out of six studied chiral columns contained cellulose derivatives as chiral selector (Lux Cellulose-1 to 4), while two columns contained amylose derivatives (Lux Amylose-1 and 2) as chiral selector. Only the Lux Cellulose-3 column had benzoate based chiral selector while the others had a polysaccharide carbamate-based selector. Within the carbamate derivatives, two of them contained only methyl group as a substituent. Furthermore, three of them included both methyl and chlorine substituents on the phenyl moieties.

Polar organic mobile phases were not successful for the separation of enantiomers of chiral β -agonists on 6 studied polysaccharide-based chiral columns with acidic and/or basic additives. Successful separations of enantiomers of all 8 studied analytes were obtained by using hydrocarbon-alcohol eluents at least on one chiral column. Under the optimized conditions the most successful column was Lux Cellulose-2 that separated the enantiomers of all 8 chiral compounds. The success rates of other chiral columns (considering the baseline separation with $R_s \geq 1.5$) ranked in the following order: Lux Cellulose-2 > Lux Cellulose-4 = Lux Amylose-1 > Lux Amylose-2 > Lux Cellulose-3 >

Lux Cellulose-1. Many complimentary separations were observed with various column and mobile phase combinations. These results are summarized in Table 1 and discussed below from the viewpoint of EEO.

3.2. The effect of polysaccharide backbone

As mentioned above, all columns have the same D-glucopyranose units making up the natural polymers used for their preparation, but the difference between amylose and cellulose is that the glucopyranose units in amylose are linked through α -1,4-linkage while in cellulose through β -1,4-linkages. The effect of linkage type on chiral resolving ability of polysaccharide-based chiral columns is well known [7, 32, 33-35]. In the present study with n-hexane/2-propanol mixture as the mobile phase the separation of enantiomers of ISO, CLP and RIT (data not shown) were obtained on Lux Amylose-1 column while no separations for the same pairs of enantiomers were observed on Lux Cellulose-1 column (Fig. 2).

3.3. Carbamate- vs. benzoate-type chiral selectors

The reversal of EEO for CIM was observed between cellulose-carbamate (Lux Cellulose -1, -2, -4) and cellulose-benzoate (Lux Cellulose-3) based chiral columns (Fig. 3). Based on this experiment it cannot be proved that the observed EEO reversal is caused by the linkage-type between the polysaccharide backbone and aromatic pendant groups since the substituents on the latter are also not the same.

3.4. The effect of the nature of substituents on the phenyl moiety on retention and separation of enantiomers

The nature of the substituents on the phenyl moieties of polysaccharide phenylcarbamates have an effect on separation and elution order of enantiomers. The appearance, lost, decrease or increase of separation, as well as a reversal of EEO, can be observed depending on the nature of substituents [7, 10, 13, 32]. The EEO reversal is the most interesting effect from the viewpoint of better understanding of chiral recognition mechanisms.

The reversal of elution order of TUL, BUT, ISO and CIM enantiomers were observed on two amylose based columns, Lux Amylose-1 and Lux Amylose-2 with studied hydrocarbon-alcohol type mobile phases (Fig. 4). Although the polysaccharide backbone is the same in the chiral selectors in these two columns, their pendant groups differ significantly in terms of the nature and location of substituents.

3.5. The effect of the additives to mobile phase on retention and separation

The absence or presence of acidic and/or basic additives in the mobile phase can play an important role in the separation of enantiomers of chiral compounds [7, 10, 32-36]. For this reason, acidic (formic acid, FA) and/or basic (diethylamine, DEA) compounds were added to the mobile phase in the present study.

3.5.1. No additive vs. basic additive

The basic additives in the mobile phase can interact with acidic sites on the CSP surface and bring about decreased nonselective retention and improve resolution of

enantiomers [32]. Additionally, basic additives may also suppress the ionization of basic chiral analytes and thus intensify their interaction with hydrophobic moieties part of polysaccharide-based CSPs. In the present study, addition of DEA to the mobile phase commonly caused the decrease of retention, improved peak shape and sometimes decrease of separation selectivity. As illustrated in Fig. 5 for separation of BUT enantiomers on Lux Cellulose-1 column, the analysis time decreased approximately 2.5 times and peak shape improved significantly when DEA was added to the mobile phase. As shown for CLP on Lux Cellulose-4 column the resolution almost disappeared due to a drastic decrease of retention when DEA was added to the mobile phase (Fig. 6). In contrast to the above mentioned, on Lux Cellulose-3 column the enantiomers of IZO were stronger retained and better separated due to DEA addition to the mobile phase (Fig. 7). As shown in Fig. 8 there was almost no effect of DEA additive to the mobile phase for IZO on Lux Amylose-1 column. Thus, due to multiple interactions the basic additive may enter with carrier of a chiral selector, chiral selector itself or affecting ionization degree of the analyte, the overall effect of basic additive on the retention and separation may be multivariate and dependent on the nature of chiral analyte, chiral stationary phase (CSP) and a mobile phase.

3.5.2. *No additive vs. acidic additive*

The acidic additive of the mobile phase interacts with some basic groups which may be present (but not necessarily) on functionalized silica, as well as with basic sites of the chiral selector. In addition, acidic additives may form salts with basic analytes. Therefore, multiple effects of such additives may be observed on retention and separation

of enantiomers. The addition of acidic additives to the mobile phase when analyzing basic analytes is still considered somehow counterintuitive, although quite many interesting results are already described in the literature [9, 10, 32-34].

In the present study the addition of FA to the mobile phase commonly improved the enantioresolution. For instance, separation of the enantiomers of BUT (Fig. 5) and CLP (Fig. 6) improved significantly due to the addition of 0.1% FA (v/v) to the mobile phase. Furthermore, the enantiomers of IZO were almost not resolved on Lux Cellulose-3 column when n-hexane/2-propanol (80/20, v/v) was used as a mobile phase. Enantioresolution was observed due to the addition of FA (Fig. 7). Conversely, the separation of the enantiomers of the same compound on Lux Amylose-1 column was negatively affected by the FA additive to the mobile phase and the resolution as high as 8.6 dropped to 2.0 with this additive (Fig. 8).

3.5.3. *No additive vs. both additives*

In most cases, resolution between enantiomers were increased due to the addition of both additives, FA and DEA, to the mobile phase (Figs. 5, 6 and 7). However, in some cases, the perfect separation without any additive turned to almost no separation when both additives were simultaneously present in the mobile phase (Fig. 8).

3.5.4. *Acidic additive vs. basic additive*

As already mentioned above, for improvement of enantioseparation of basic analytes in HPLC commonly basic additives are recommended to be added to the mobile phase. However, as shown in few earlier studies, acidic additives can also be considered

as alternative, or as co-additives for the improvement of enantioseparation of basic analytes [9, 10, 32-34]. In the present study after the addition of FA instead of DEA to the mobile phase, significant increase in analyte retention and improvement of enantiomer separation were observed for TUL on Lux Cellulose-1, for BUT, CLN, CIM, RIT and CLP on Lux Cellulose-2, for BUT on Lux Cellulose-3, for ISO, BUT, CIM, TER, TUL, RIT and CLP on Lux Cellulose-4, for CLN, CLP, CIM, TUL on Lux-Amylose-1 and for CIM on Lux Amylose-2. Some examples are shown in Fig. 9.

3.6. Reversal of EEO due to the additives

The presence of additives in the mobile phase may not only affect the retention and enantioselectivity, but they can also cause a reversal of EEO [9, 10, 32-34]. Several examples were observed in this study when the additive caused a reversal of the enantiomer affinity pattern towards the chiral selector. Thus, the enantiomers of CIM were not resolved on Lux Cellulose-2 with n-hexane/2-propanol/DEA (80/20/0.1, v/v/v) as a mobile phase (Fig. 10a). However, in n-hexane/2-propanol/FA (80/20/0.1, v/v/v) the separation appeared (Fig. 10b). In the mobile phase containing both additives in the same volume percentage the EEO was opposite to that with only FA (Fig. 10c).

In case of separation of ISO on Lux Amylose-1 good separation of enantiomers was observed in n-Hex/2-propanol mobile phase without any additive (Fig. 8a). The separation improved slightly due to the additive of DEA but the elution order was the same (Fig. 8b), while the alternative additive of FA shortened the retention and the EEO reverted (Fig. 8c). In the case of equipercent amount of both additives almost no separation was observed (Fig. 8d).

An interesting example of EEO reversal based on the concentration of FA acid additive was observed in the separation system mentioned in the previous paragraph (Fig. 11). Only few examples of this kind have been described in the literature earlier [32].

Although no detailed insight can be gained into chiral recognition mechanisms at this stage, these observations seem quite promising from the mechanistic point of view.

4. Conclusion

The present study illustrates that the affinity of the enantiomers of β -agonist towards polysaccharide-based chiral stationary phases depends on multiple variables such as polysaccharide backbone, nature and the positions of substituents on chiral selector and mobile phase composition. Especially, the role of acidic and/or basic additives is complex and may result in increase, decrease, appearance and loss of enantioseparation and reversal of EEO. The practical significance of these results is that additives may not only serve as a useful tool for the adjustment of the enantiomer elution order but may also serve to better understand chiral recognition mechanisms in enantioselective intermolecular interactions. Further studies of these phenomena may provide useful information for better understanding chiral recognition mechanisms with polysaccharide-based chiral stationary phases and the optimization of separations with these materials.

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Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Success rate of the chiral columns

Chiral Column	n-hexane:IPA FA	n-hexane:IPA DEA	n-hexane:IPA FA/DEA	Polar Organic ^a	The cumulative number of separated compounds
Lux Cellulose-1	2	1	2	-	3
Lux Cellulose-2	7	2	6	-	8
Lux Cellulose-3	2	4	1	-	4
Lux Cellulose-4	7	4	6	-	7
Lux Amylose-1	6	2	6	-	7
Lux Amylose-2	1	3	2	-	4

^a Polar organic refers to methanol, acetonitrile, ethanol, isopropanol

Figure Legends

Figure 1. Chemical structures of studied compounds.

Figure 2. Separation of isoxsuprine (a, b) and clenpenterol (c, d) enantiomers on Lux Amylose-1 (a, c) and Lux Cellulose-1 (b, d). Mobile phase: n-hexane:2-propanol (80:20) for isoxsuprine and n-hexane:2-propanol:FA (80:20:0.1) for clenpenterol.

Figure 3. Separation of enantiomers of cimaterol on Lux Cellulose-1 (a), Lux Cellulose-2 (b), Lux Cellulose-3 (c) and Lux Cellulose-4 (d). Mobile phase: n-hexane:2-propanol:FA (80:20:0.1).

Figure 4. Separation of enantiomers of butoxamine (a, b), cimaterol (c, d), isoxsuprine (e, f) and tulobuterol (g, h) on Lux Amylose-1 (a, c, e, g) and Lux Amylose-2 (b, d, f, h).

Figure 5. Separation of enantiomers of butoxamine on Lux Cellulose-1 with n-hexane:2-propanol (80:20) (a), n-hexane:2-propanol:DEA (80:20:0.1) (b), n-hexane:2-propanol:FA (80:20:0.1) (c), and n-hexane:2-propanol:FA:DEA (80:20:0.1:0.1) (d).

Figure 6. Separation of enantiomers of clenpenterol on Lux Cellulose-4 with mobile phase: n-hexane:2-propanol (80:20) (a), n-hexane:2-propanol:DEA (80:20:0.1) (b), n-hexane:2-propanol:FA (80:20:0.1) (c) and n-hexane:2-propanol:FA:DEA (80:20:0.1:0.1) (d).

Figure 7. Separation of enantiomers of isoxsuprine on Lux Cellulose-3 mobile phases: n-hexane:2-propanol (80:20) (a), n-hexane:2-propanol:DEA (80:20:0.1) (b), n-hexane:2-propanol:FA (80:20:0.1) (c) and n-hexane:2-propanol:FA:DEA (80:20:0.1:0.1) (d).

Figure 8. Separation of enantiomers of isoxsuprine on Lux Amylose-1 with mobile phases: n-hexane:2-propanol (80:20) (a), n-hexane:2-propanol:DEA (80:20:0.1) (b), n-hexane:2-propanol:FA (80:20:0.1) (c) and n-hexane:2-propanol:FA:DEA (80:20:0.1:0.1) (d).

Figure 9. Separation of enantiomers of ritodrine on Lux Cellulose-4 with the mobile phases (n-hexane:2-propanol:DEA (70:30:0.1) (a), (n-hexane:2-propanol:FA (70:30:0.1) (b) and enantiomers of tulobuterol on Lux Amylose-1 with the eluents (n-hexane:2-propanol:DEA (80:20:0.1) (c), (n-hexane:2-propanol:FA (80:20:0.1) (d).

Figure 10. Separation of enantiomers of cimbuterol on Lux Cellulose-2 with mobile phases: n-hexane:2-propanol:DEA (80:20:0.1) (a); n-hexane:2-propanol:FA (80:20:0.1) (b), n-hexane:2-propanol:FA:DEA (80:20:0.1:0.1) (c).

Figure 11. Separation of enantiomers of isoxsuprine on Lux Amylose-1 with mobile phases: n-hexane:2-propanol (80:20) (a), n-hexane:2-propanol:FA (80:20:0.01) (b), n-hexane:2-propanol:FA (80:20:0.03) (c), n-hexane:2-propanol:FA (80:20:0.05) (d), n-hexane:2-propanol:FA (80:20:0.07) (e) and n-hexane:2-propanol:FA (80:20:0.1) (f)

Figure

Butoxamine

Cimbuterol

Clenbuterol

Clenpenterol

Isoxsuprine

Ritodrine

Terbutaline

Tulobuterol

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11