Separation of enantiomers of chiral weak acids with polysaccharide-based chiral columns and aqueous-organic mobile phases in high-performance liquid chromatography: Typical reversed-phase behavior?

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16 **Abstract**

17 When polysaccharide-based chiral columns are used in combination with aqueous-
18 organic mobile phases for the separation of enantiomers in high-performance liquid
19 chromatography the separation mode is commonly called “reversed-phase” in analogy to
20 achiral separations. In several earlier and recent studies on neutral and basic chiral
21 analytes it was shown by our and other groups that due to multiple type of interactions
22 involved in selector-selectand binding and enantioselective recognition with
23 polysaccharide derivatives, the above mentioned separation system may not always
24 behave like a reversed-phase system. In the present study additional examples of non-
25 reversed-phase behavior are described for the first time for weak acidic chiral analytes. In
26 addition, the reversal of enantiomer elution order was observed again for the first time for
27 several analytes based on water-content in the mobile phase.

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29 *Keywords:*

30 Enantioseparations, chiral HPLC, polysaccharide-based chiral stationary phases, reversal
31 of enantiomer elution order

32

1. Introduction

Although the mobile phase used in the early stage of development of polysaccharide-based chiral stationary phases (CSP) for liquid-phase separation of enantiomers was pure alcohol [1] later Okamoto et al. recommended alcohol-hydrocarbon mobile phases (called “normal phase”) for the successful separation of enantiomers with polysaccharide phenylcarbamate-based CSPs [2]. Applicability of aqueous-alcohol mixtures as mobile phase was also mentioned in this early paper but was not recommended. The reason for discouraging the use of water:alcohol mixtures as mobile phase was based on the belief that hydrogen bonding and/or dipole-dipole interactions between chiral analytes and polysaccharide-based chiral selectors (CS) are essential to chiral discrimination and these interactions may be significantly diminished in aqueous media (water can compete for hydrogen interaction sites with both analytes and the CS).

In fact, since aromatic polysaccharide-phenylcarbamates contain rather large hydrophobic regions (groves or ravines) theoretically hydrogen bond formation between these materials and chiral analytes seems possible also in aqueous media [3]. Independent of the fact whether hydrogen bonding interactions are essential to chiral discrimination the reality is that polysaccharide-based CSPs are very useful for the separation of enantiomers also in combination with pure polar organic, as well aqueous-organic mobile phases [3-9]. The last mode is especially preferred for bioanalytical studies, as well as for HPLC–MS coupling due to better compatibility with bioanalytical samples (a direct sample injection is possible after simple protein precipitation). In addition, sample components become ionized easier in this mode for MS detection and the mobile phase is safer for the MS detection.

In achiral reversed-phase separations, analyte retention increases with increasing water content in the mobile phase. The opposite behavior has been extensively studied in the chromatographic literature within the technique called hydrophilic interaction liquid chromatography (HILIC). Similar behavior, contrary to RP retention has been reported lately for chiral separations by our [3, 10, 11] and other [12, 13] groups. It was proposed that the balance between hydrophilic interactions (residing with carbamate or ester moieties) and hydrophobic interaction (associated with phenyl moieties) may result in behavior contrary to reversed-phase HPLC. Thus, based on analyte characteristics and separation conditions, polysaccharide-based CSPs may behave either as RP-like or HILIC-like stationary phases.

In order to better understand the reasons of this dual behavior of polysaccharide-based CSPs and the role of hydrogen-bonding and of hydrophobic interactions in analyte retention and chiral recognition with these materials, the gradual transition from polar-organic to aqueous-organic mobile phases was investigated in this study with chiral weak acids as test compounds. Special attention was paid to changes in enantiomer elution order that suggest significant changes in the balance of various interactions taking place between analytes and polysaccharide-based CSs.

2. Experimental

2.1 Materials

The chiral test compounds acenocoumarol, carprofen, 2-(3-chlorophenoxy)-propionic acid, coumachlor, coumatetralyl, difenacoum, fenoprofen, flurbiprofen, hexobarbital, ibuprofen, indoprofen, ketoprofen, ketorolac, mandelic acid, mandelic acid ethylester, S-

naproxen, 2-phenoxypropionic acid, pyranocoumarin, proglumide, **suprofen**, sulindac, warfarin, were commercially available from Sigma-Aldrich (St. Louis, MI, USA). Racemic naproxen was provided by Dr. Salvatore Fanali, Institute of Chemical Methodologies, Rome, Italy. Racemic zaltoprofen and its enantiomers were provided by Prof. Jong Seong Kang from College of Pharmacy, Chungnam National University, Daejeon, Korea. Benzobamyl halonal and nembital were provided from the Chair of Organic Chemistry, Tomsk Polytechnic University, Tomsk, Russian Federation. The structures of studied analytes are shown in Fig. 1. HPLC-grade methanol, acetonitrile and water, as well as chemical grade formic acid were supplied by Karl Roth (Karlsruhe, Germany). Chiral columns Lux-Amylose-1, Lux Amylose-2, Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3 and Lux Cellulose-4 were provided by Phenomenex Inc. (Torrance, CA, USA). Chiral column prepared with cellulose tris(3,5-dichlorophenylcarbamate) covalently immobilized on silica was provided by Enantioseparation GmbH (Münster, Germany). The structures of chiral selectors are shown in Fig. 2. All columns were of 250x4.6 mm dimensions packed with silica particles of 5 micrometer nominal particle size.

2.2. Instruments

All HPLC experiments were performed with an Agilent 1200 HPLC instrument (Agilent Technologies, Waldbronn, Germany) equipped with a G1367C HiP ALS-SL autosampler, G1316B TCC-SL temperature controller, G1311A quaternary pump and G1314D VWD variable wavelength detector. The Chemstation software (version B.03.02-SR2) was used for instrument control, data acquisition and data processing.

HPLC separations were performed at 20 °C at 1 ml/min mobile phase flow rate if not mentioned otherwise. All mobile phases contained 0.1% formic acid (v/v). UV detection was performed at 240 nm. The absolute configuration or optical rotation sign of enantiomers were assigned based on spiking experiments with enantiomerically pure standards or correlation with earlier publications in which the authors have used optical rotation and circular dichroism detectors sequentially connected to a UV-detector.

3. Results and Discussions

In this work no statistical summary on the applicability of each chiral column or mobile phase for separation of enantiomers of studied set of weak chiral acids is provided. For part of the analytes included in this study such statistics for normal phase and polar-organic mode separations has been previously published in refs. [14] and [15], respectively.

3.1. Transition from methanol to aqueous methanol

Methanol as a protic solvent may engage in hydrogen-bonding interactions with chiral selector, as well as with chiral analyte. Therefore, it is expected that hydrogen-bonding interactions between analyte and chiral selector play a lesser role in chiral recognition and separation of enantiomers in pure methanol as a mobile phase. Thus, in most cases when water is added to methanol used as a mobile phase, no drastic decrease of hydrogen-bonding type and other polar interactions may be expected. However, hydrophobic interactions may increase with increasing content of water leading to increased retention of analytes. Whether or not increased retention results in better

enantioresolution, together with contribution of hydrophobic interactions in enantioselective recognition, will depend on the counteracting effects of plate numbers (commonly decrease) and separation factor (commonly increase).

For all 182 combinations of chiral analytes and chiral columns (26 chiral analytes and 7 chiral columns) the retention factors increased after addition of water to the mobile phase even at the lowest concentration studied (5% v/v). This typical behavior is shown for coumachlor on Lux Cellulose-3 column in Fig. 3. The splitting of the curve commonly taking place at higher content of water in the mobile phase indicates that the separation of enantiomers can be observed above a particular water content in the mobile phase. Only in few cases the enantioseparation achieved in pure methanol, as a mobile phase, was lost upon addition of water and could not be achieved again at higher concentrations of water in the mobile phase. There were few cases where enantioseparation was observed in pure methanol, which disappeared at the intermediate content of water in the mobile phase and reappeared again with the same elution order at the higher content of water in the mobile phase. It is clear that with increasing content of water in the mobile phase the contribution of hydrophobic interactions to analyte retention and enantioseparation increases. However, the preferential binding of one over the other enantiomer by the chiral selector may not necessarily revert when the contributions of various forces part of selector-selectand interactions and enantioselective recognition change with changing mobile phase composition (in particular its water content). The chiral recognition pattern (binding affinity of enantiomers to chiral selector) based on hydrogen bonding and other polar interactions at no or lower content of water in the mobile phase may be the same as the binding pattern

based on hydrophobic interactions that are intensified at higher content of water in the mobile phase. In such a case no reversal in enantiomer elution order based on the content of water in the mobile phase is to be expected. Only in the case when enantiomer affinity pattern based on the above mentioned two different groups of interactions (polar and hydrophobic) are opposite to each other, **enantiomer elution order (EEO)** reversal brought about by the increasing water content in the mobile phase may occur. Interesting example of **EEO** reversal with increasing content of water in the mobile phase was observed for pyranocoumarin on Lux Cellulose-4 column (data not shown) and for coumatetralyl (Fig. 4) and hexobarbital on Lux Cellulose-1 column (data not shown). The effect of content of water in the alcohol-hydrocarbon mobile phases on the EEO of some basic analytes on polysaccharide-based chiral columns was mentioned in few earlier studies [16-18].

For the chiral agrochemical difenacoum all four stereoisomers were baseline resolved in a short time on Lux Cellulose-1 column with methanol as a mobile phase (Fig. 5). Noteworthy, the addition of water to methanol although prolonged the retention but led to peak overlapping and widening and thus decrease of resolution.

3.2. Transition from acetonitrile to aqueous acetonitrile

Acetonitrile as aprotic solvent should not interfere with hydrogen-bonding type interactions taking place between chiral selectors and chiral analytes. Therefore, water as a strongly competing species for the hydrogen bonding sites should displace the analyte from any hydrogen-bonding between chiral selector and selectand. The net outcome of this role water could play in **acetonitrile**-based mobile phase is a significant reduction of analyte retention with increasing content of water in the system. This exact effect was

observed for all 182 combinations of chiral analytes and chiral selectors included in this study at a water content in acetonitrile of up to around 20%. Typical dependence of analyte retention on the content of water in acetonitrile is shown on the example of ketoprofen enantiomers on Lux Amylose-2 column in Fig. 6. Under such conditions (water content up to around 20% in acetonitrile) the polysaccharide phenylcarbamates and ester-type chiral selector exhibit rather “HILIC-like” than reversed-phase behavior. Such behavior is associated with a decrease in retention with the gradual addition of water to the mobile phase made primarily of ACN followed by a reversal in this trend: a gradual increase in retention at high water content. Such behavior is not observed in mobile phase made of methanol, in which case only increase in retention was observed for all 182 analyte-chiral column combinations.

This marked difference in the role water plays in analyte retention when added to ACN and to methanol deserves careful consideration. In both mobile phases systems ACN:water and methanol:water analyte retention is a function of the balance between hydrophilic (hydrogen-bonding) and hydrophobic types of interactions taking place between selector and selectand. In both cases, hydrophobic interactions play a primary role in retention as soon as the mobile phase conditions allow for such interactions and also as soon as hydrogen bonding is minimized. In methanol **hydrogen bonding interactions between analyte and chiral selector** play only minimal role given the strong hydrogen-bond ability of methanol. The addition of water can only diminish the strong eluting strength of methanol (a common mobile phase modifier in RP) bringing about an increase in analyte retention. In contrast, the addition of water to acetonitrile will first

drastically reduce hydrogen-bond induced retention and at higher water levels reduce the eluting strength of ACN (as in RP separations).

Thus, hydrogen bonding-type interactions are prevalent at low water content in acetonitrile (typically below 20%) while hydrophobic interactions take over commonly above 20% water content. This behavior can be visualized with the separation of coumatetralyl enantiomers on Lux Amylose-1 column shown in Fig. 7. It is interesting to note that the affinity pattern of coumatetralyl enantiomers caused by hydrogen-bonding (polar) type and hydrophobic type interactions towards amylose tris(3,5-dimethylphenylcarbamate) chiral selector is the same. Therefore, no reversal of EEO based on the content of water in acetonitrile was observed in this case. From the retention point of view very similar trend was observed on cellulose tris(3,5-dimethylphenylcarbamate)-based chiral column Lux Cellulose-1 (Fig. 8). Thus, enantioselectivity was observed in 100% acetonitrile, it was lost at 5% (v/v) water and was achieved again at 20% of water in acetonitrile and above. However, from the viewpoint of chiral recognition pattern a significant difference was observed between these two separation systems. Firstly, in the mobile phase where hydrogen bonding-type interactions were not disrupted (100% acetonitrile) the affinity pattern of coumatetralyl enantiomers toward the two chiral selectors was opposite. However, in the mobile phase in which hydrophobic interactions were favored (>10% water (v/v) in acetonitrile) the affinity pattern of coumatetralyl enantiomers towards both chiral selectors was the same. Based on this discussion it is clear that a reversal in EEO was observed for coumatetralyl enantiomers on Lux Cellulose-1 column based on the water content in acetonitrile (Fig. 8). Similar to this observation, based on the results shown in Figs. 9a-d, it becomes

evident that hydrogen-bonding-based and hydrophobic-interaction-based recognition affinity patterns are opposite for ibuprofen and warfarin enantiomers on Lux Cellulose-3 (Figs. 9a and 9b, respectively), for hexobarbital enantiomers on Lux Cellulose-1 (data not shown) and cellulose (3,5-dichlorophenylcarbamate) (Fig. 9c) and for ketoprofen enantiomers on Lux Amylose-2 column (Fig. 9d). As shown in Fig. 10 not only EEO may be altered based on the content of water in the mobile phase but also the elution order of stereoisomers not related to each other as enantiomers. Although this is not a major topic of this study it is interesting to mention that the elution order of difenacoum stereoisomers is quite different on different chiral columns (see Figs. 5 and 10).

3.3 The effect of n-hexane addition to methanol or acetonitrile

In order to get a better idea about the specificity of water modifier and hydrogen bonding interactions in chiral recognition with polysaccharide-based CSs, the effect of non-hydrogen bonding modifier, n-hexane to methanol and acetonitrile on the retention and separation of coumatetralyl enantiomers was studied on two chiral columns, Lux Cellulose-1 and Lux Amylose-1. Since the CSs in these two columns have the same pendant groups but different polymer backbone (cellulose and amylose, respectively), it was also interesting to test what is the difference between their response to non-hydrogen-bonding modifier (n-hexane). In the case of methanol the addition of n-hexane to the mobile phase up to 20% led to the decrease of retention by 7% and 8% for the first and second eluting enantiomers, respectively on Lux Cellulose-1 column and by 2% on Lux Amylose-1 column (no separation of enantiomers was observed at any composition of the mobile phase on this column). Comparison of these results with the scenario shown

in Fig. 4 for Lux Cellulose-1 column indicates that the column responds very differently to the **addition of water and n-hexane to methanol**. In particular, in the case of water addition in the same percentage (up to 20%, v/v) the drastic increase (instead of slight decrease in the case of n-hexane) of the retention and even the reversal of the enantiomer affinity pattern towards the chiral selector took place. In the case of Lux Amylose-1 column no separation of enantiomers was observed but the effects of n-hexane and water **addition** on the retention of co-eluted peaks were again opposite to each other. The slight decrease of retention due to the **addition** of n-hexane up to 20% (v/v) and significant increase of retention in the case of water **addition** in the same percentage were observed (Data not shown).

The effect of n-hexane **addition** to acetonitrile on the retention and enantioselectivity on both, Lux Cellulose-1 and Lux Amylose-1 columns is shown in Figs. 11a and 11b, respectively. On the Lux Cellulose-1 column the retention decreased with increasing n-hexane content what looks apparently similar to the effect of water **addition** shown in Fig. 8. However, in the case of water **addition** the enantiomer affinity pattern toward chiral selector reverted while the selectivity of separation did not change even quantitatively in the case of n-hexane **addition**. This is of course a drastic difference between the effects of water and n-hexane **addition** to acetonitrile on the behavior of the separation system. Very interesting effect of n-hexane **addition** was observed on separation of coumatetralyl enantiomers on Lux Amylose-1 column. In particular, with increasing content of n-hexane in the mobile phase the retention of the less retained enantiomer decreased while the retention of more retained enantiomer increased. This enantioselective effect of n-hexane **addition** to acetonitrile led to the increase of

separation selectivity with increasing n-hexane content in the mobile phase (Fig. 11b). Appearance or increase of separation selectivity due to introduction of non-polar modifier in the mobile phase is quite common with polysaccharide-based CSPs [19]. Due to non-polar modifier commonly the retention of both enantiomers increases and the longer residence time of the analyte on the chiral selector, but not necessarily a redistribution of interactive forces between the chiral analyte and chiral selector affects the separation factor. In the example described in Fig. 11a the separation improves not as a result of increased residence time but most likely due to increased contribution of enantioselective forces into overall energy of selector-selectand interactions. Similar examples of additives on separation **selectivity** has been described in our earlier study [20].

4. Conclusions

The results represented in this paper allow for drawing the following conclusions:

- 1) polysaccharide-based chiral columns when used with aqueous-organic mobile phase may not always behave like typical reversed-phase chromatographic systems; this is especially true at low content of water (<20% v/v) in acetonitrile.
- 2) When the hydrogen-bonding interactions-based affinity **pattern** of enantiomers towards a chiral selector is opposite to that based on hydrophobic-type interactions, a reversal of enantiomer elution order may be observed based on the content of water in the mobile phase; this phenomenon seems to be more common in aprotic solvent-water systems than in protic solvent-water systems. Detailed studies on the effect of water addition to organic mobile phase may provide useful information for better understanding of chiral recognition mechanisms with polysaccharide-based chiral selectors.

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360 **Legends:**

361 Fig. 1 Structure of studied analytes.

362 Fig. 2 Structure of chiral selectors in the chiral columns under this study.

363 Fig. 3 Dependence of retention on the content of water in methanol for coumachlor

364 enantiomers on Lux Cellulose-3 column. For experimental conditions see sections

365 2.1 and 2.2.

366 Fig. 4 Separation of enantiomers of coumatetralyl on Lux Cellulose-1 with methanol (a),

367 methanol-water (95-5, v/v) (b), methanol-water (80-20, v/v) (c) and methanol-water

368 (70-30,v/v) (d) as mobile phases. In all cases mobile phase contained 0.1% formic

369 acid. For other experimental conditions see sections 2.1 and 2.2.

370 Fig. 5 Separation of stereoisomers of difenacoum on Lux Cellulose-1 in methanol-formic

371 acid (100/0.1, v/v) as the mobile phase. For other experimental conditions see

372 sections 2.1 and 2.2.

373 Fig. 6 Dependence of retention of ketoprofen enantiomers on the content of water in

374 acetonitrile on Lux Amylose-2 column. For experimental conditions see sections

375 2.1 and 2.2.

376 Fig. 7 Separation of enantiomers of coumatetralyl on Lux Amylose-1 with acetonitrile (a),

377 acetonitrile-water (95-5, v/v) (b), acetonitrile-water (90-10, v/v) (c) as mobile

378 phases. In all cases mobile phase contained 0.1% formic acid. For other

379 experimental conditions see sections 2.1 and 2.2.

Fig. 8 Separation of enantiomers of coumatetralyl on Lux Cellulose-1 with acetonitrile (a), acetonitrile-water (95-5, v/v) (b), acetonitrile-water (80-20, v/v) (c) as mobile phases. In all cases mobile phase contained 0.1% formic acid. For other experimental conditions see sections 2.1 and 2.2.

Fig. 9 Separation of enantiomers of (a) ibuprofen on Lux Cellulose-3 with acetonitrile-water (50-50, v/v) and acetonitrile-water (20-80, v/v) as mobile phases, (b) enantiomers of warfarin on Lux Cellulose-3 with acetonitrile and acetonitrile-water (40-60, v/v) as mobile phases (Separation temperature was 5°C), enantiomers of hexobarbital on Cellulose (3,5-dichlorophenylcarbamate) with acetonitrile and acetonitrile-water (50-50, v/v) as mobile phases (Separation temperature was 5°C), enantiomers of ketoprofen on Lux Amylose-2 with acetonitrile, acetonitrile-water (60-40, v/v) and acetonitrile-water (20-80, v/v) as mobile phases, (Separation temperature was 5°C). For other experimental conditions see sections 2.1 and 2.2.

Fig. 10 Separation of stereoisomers of difenacoum on Lux Cellulose-1 in acetonitrile (a), acetonitrile-water (90/10, v/v) (b), acetonitrile-water (70/30, v/v) (c) and acetonitrile-water (50/50, v/v) (d) as mobile phases. In all cases mobile phase contained 0.1% formic acid. For other experimental conditions see sections 2.1 and 2.2.

Fig. 11 The effect of n-hexane addition to acetonitrile on separation of coumatetralyl enantiomers on (a) Lux Cellulose-1 and (b) Lux Amylose-1 columns. For other experimental conditions see sections 2.1 and 2.2.

Fig. 1

Cellulose Derivatives, R=		Amylose Derivatives, R=	
Lux-Cellulose-1		Lux Amylose-1
Lux-Cellulose-2		Lux Amylose-2
Lux-Cellulose-3
Lux-Cellulose-4
Cellulose-(3,5-dichlorophenylcarbamate)

Fig. 2

Fig. 3

Fig. 4

Fig. 5

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Fig. 6

Fig. 7

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Fig. 9

Fig. 10

Fig. 11