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Enantioseparation of racecadotril using polysaccharide-type chiral stationary phases in polar organic mode

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Abstract

Enantioseparation of the antidiarrheal drug, racecadotril, was investigated by liquid chromatography using polysaccharide-type chiral stationary phases in polar organic mode. The enantiodiscrimininating properties of 4 different chiral columns (Chiralpak AD, Chiralcel OD, Chiralpak AS, Chiralcel OJ) with 5 different solvents (methanol, ethanol, 1-propanol, 2-propanol, and acetonitrile) at 5 different temperatures (5–40 °C) were investigated. Apart from Chiralpak AS column the other 3 columns showed significant enantioseparation capabilities. Among the tested mobile phases, alcohol type solvents were superior over acetonitrile, and significant differences in enantioselective performance of the selector were observed depending on the type of alcohol employed. Van't Hoff analysis was used for calculation of thermodynamic parameters which revealed that enantioseparation is mainly enthalpy controlled; however, enthropic control was also observed. Enantiopure standard was used to determine the enantiomer elution order, revealing chiral selector—and mobile-phase dependent reversal of enantiomer elution order. Using the optimized method (Chiralcel OJ stationary phase, thermostated at 10 °C, 100% methanol, flow rate: 0.6 mL/min) baseline separation of racecadotril enantiomers (resolution $= 3.00 \pm 0.02$) was achieved, with the R-enantiomer eluting first. The method was validated according to the ICH guidelines, and its application was tested on capsule and granules containing the racemic mixture of the drug.

KEYWORDS
chiral separation, elution order, HPLC, sinorphan, van't Hoff analysis

1 | INTRODUCTION

Racecadotril (RAC) ((*RS*)-Benzyl *N*-[3-(acetylthio)-2-benzylpropanoyl]glycinate) (Figure 1) is a lipophilic prodrug of thiorphan, which acts as a neutral endopeptidase inhibitor, preventing the natural breakdown of the enzyme substrates, including enkephalins.[1] Due to the antisecretory action of the substrate, the drug is currently in use as an effective antidiarrheal agent, displaying comparable efficacy to loperamide, but a superior tolerability and side effect profile.[2,3] The enantiomers of the active metabolite, thiorphan, have a strong and equipotent in vitro affinity on the enkephalinase enzyme[4]; therefore, RAC is marketed as a racemic mixture. However, the individual enantiomers display different potencies in a wide variety of other pharmacological effects.[4,5] The *R*-enantiomer (retorphan or dexecadotril) entered clinical trials as an intestinal antisecretory agent,

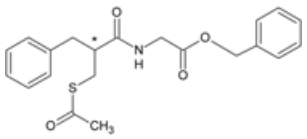

FIGURE 1 Chemical structure of RAC. The asterisk denotes the asymmetric carbon atom

while the *S*-enantiomer (sinorphan or ecadotril) was believed to have beneficial cardiovascular effects.[6,7] The development of ecadotril was halted, due to the lack of efficacy of the drug for the treatment of heart failure.[8] Based on the different pharmacological properties of the optical antipodes, suitable chiral method development is necessary for the analysis of the enantiomers. Chiral separation based on HPLC resolution of enantiomers using chiral stationary phases (CSPs) is the golden standard in this field. Polysaccharide-type CSPs based on phenylcarbamate or ester-derivatives have long been established as one of the most versatile chiral selectors and are extensively used for the chiral separation of various structurally different molecules.[9,10] Most of currently available chiral polysaccharide-based columns can be used in normal-phase, reversed-phase, and polar organic mobile phase modes, making these CSPs universally applicable.[9] Nowadays, polar organic mode has been well established for analytical and preparative-scale enantioseparation due to their advantages, such as short analysis time, high efficiency, better signal/noise ratio, and commonly higher solubility of the analytes in the mobile phase.[11-13]

To the best our knowledge, validated chiral methods are not available for RAC, despite the growing interest in analytical characterization of the drug.[14-17] Because of the different pharmacological effects of RAC enantiomers and possibilities of future chiral switches, development of a sensitive, precise, and reliable enantioselective method is required.

The major aim of the present study was to develop a novel, validated chiral liquid chromatographic method for the analysis of RAC enantiomers using polysaccharide-type CSPs in polar organic mode. The in-depth analysis of different chromatographic parameters was also intended in order to evaluate their influence on enantioseparation.

2 | MATERIALS AND METHODS

2.1 | Materials

Polysaccharide-type chiral columns with identical dimensions (250 × 4.6 mm, 10-μm particle size) Chiralpak AD (based on amylose tris(3,5-dimethylphenylcarbamate)), Chiralcel OD (based on cellulose tris(3,5 dimethylphenylcarbamate)), Chiralcel OJ (based on cellulose tris(4-methylbenzoate)), and Chiralpak AS (based on amylose tris[(*S*)-α-methylbenzyl carbamate]) were products of Daicel Corporation (Tokyo, Japan). Gradient grade methanol (MeOH), ethanol (EtOH), 1-propanol (PrOH), 2-propanol (IPA), acetonitrile (ACN), acetic acid, and acetone were purchased from Merck (Darmstadt, Germany). RAC and triethylamine (TEA) were ordered from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), while sinorphan (*S*-RAC) was purchased from Toronto Research Chemicals (Toronto, Canada). Ultrapure, deionized water was prepared by a Milli-Q Direct 8 Millipore system (Milford, MA, USA). Hidrasec® 100-mg capsules and Hidrasec® Baby 10-mg granules (Bioprojet Europe, Paris, France) were obtained from a local pharmacy in Budapest, Hungary.

2.2 | LC-UV analysis

LC-UV analysis was carried out on an Agilent 1260 Infinity HPLC system (G1312B binary gradient pump, G1367E autosampler, G1315C diode array detector) (Agilent Technologies, Waldbronn, Germany). Agilent Masshunter B.04.00 software was used for data analysis. UV detection was performed at 210 nm. RAC stock solution was prepared at 1 mg/mL in MeOH, and further dilutions were made with the same solvent to reach concentrations in the range of 2 to 50 μg/mL. An injection volume of 10 μL was used, and 3 parallel measurements were performed in each case.

For the determination of hold-up time, acetone was used. Performance of the separation system was monitored in terms of resolution (R_s) and selectivity factor (α), according to the usual formulae:

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \quad (1)$$

$$\alpha = \frac{k_2}{k_1} \quad (2)$$

where t_1 and t_2 are the retention times, w_1 and w_2 are the extrapolated peak widths at the baseline, and k_1 and k_2 are the retention factors for the first and second eluting enantiomers, respectively.

Other chromatographic conditions including validation processes are given in the "Results and Discussion" section.

2.3 | Sample preparation

Sample preparation from capsules and granules was performed in a similar manner to that described by Prabu et al.[14] Briefly, the content of 10 capsules or granules were weighed and afterwards ground and mixed in a mortar. Then, 100-mL MeOH was added to an accurately weighted portion of this powder equivalent to 10 mg of RAC. The sample was sonicated for 30 minutes at 25 °C and centrifugated for 10 minutes applying 4000 rpm (Sartorius 2-16P benchtop centrifuge, Goettingen, Germany). The clear supernatant was filtered through a 0.22-μm pore size syringe filter. An appropriate dilution was made with MeOH to obtain the final concentration of 10 μg/mL.

3 | RESULTS AND DISCUSSION

3.1 | CSP and mobile phase screening

The 4 chiral selectors used in this work differ from each other in terms of polysaccharide backbone (cellulose or amylose), type of pendant group (benzoate or carbamate), and/or nature of the substituent (3,5-dimethylphenyl, 4-methylbenzyl, (*S*)-α-methylbenzyl). Chiralpak AD and Chiralcel OD columns differ only in the polysaccharide backbone while Chiralpak AD and Chiralpak AS as well as Chiralcel OD and Chiralcel OJ differ only in the nature of the substituents (Figure 2). These differences in the molecular structure of the monomers have a great impact on the tridimensional structures of the chiral polymers. The substituents are thought to delimitate nanocavities or so-called "chiral grooves" of different sizes and different molecular environments in the structure of the helical polymers. Differences in chiral recognition mechanisms on polysaccharide-type selectors are considered to be strongly related to the differences in molecular environment of the chiral cavities in these selectors.

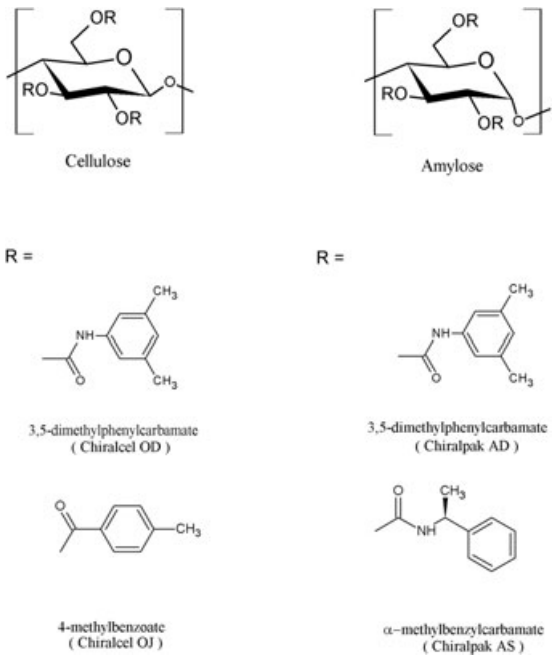

FIGURE 2 Structures of the CSPs and corresponding commercial names of the columns employed in this study

Indeed, noticeable differences in the main chromatographic parameters (k_1, α, R_s) can be observed from the application of 100 different chromatographic conditions (5 mobile phases on 4 chiral columns at 5 different temperatures, Table 1).

The obtained data indicate that 3 out of the 4 polysaccharide columns showed chiral discrimination towards RAC enantiomers. Using the Chiralpak AS column, based on amylose tris[(*S*)-α-methylbenzylcarbamate], no chiral separation was observed, regardless of the mobile phase applied. Moreover, the calculated retention factors were also the lowest in this case, indicating only weak interactions between the enantiomers and the chiral selector. Given its unsuccessful application for the chiral separation of the enantiomers, regardless of the experimental factors applied, this column was eliminated from further evaluations.

Baseline separations were observed on Chiralpak AD column with MeOH and PrOH, on Chiralcel OD column with MeOH and IPA and on Chiralcel OJ column with MeOH and PrOH as mobile phase. Using ACN, only a partial resolution was observed. It is common for all columns that the retention factors were the lowest using pure ACN as the mobile phase, and generally, this was accompanied with the lowest enantioselectivities in almost all cases.

While the protic alcohols employed are stronger H-bond competitors than ACN, the later could be disadvantageous for π-π interactions. Given the poor retention factors and almost no enantioselectivity observed with the use of ACN, regardless of the CSP employed, it can be assumed that hydrophobic interactions between the phenyl group of a CSP and aromatic groups of the solute may also greatly modulate retention and enantioselective interactions on the employed CSPs. In several earlier studies, apart from H-bonds, the importance of π-π interactions between the selector and selectand were highlighted based on chromatographic data obtained for structurally similar analytes,[18-20] while later, further insights were provided using complementary techniques on HPLC data, IR data, and molecular simulations.[21-23] Moreover, in their recent reports, Cirilli et al highlighted the importance of uncommon solvophobic interactions in explaining the exceptionally high enantioselectivity values obtained for 3-(phenyl-4-oxy)-5-phenyl-4,5-dihydro-(1H)-pyrazole,

TABLE 1 Chromatographic data, retention factor of the first eluting enantiomer (k_1), selectivity factor (α), resolution (R_s), and elution sequence on the polysaccharide-type columns in polar organic mode. Flow rate: 0.5 mL/min

Column	Eluent	Parameter	Temperature (°C)					Elution sequence
			5	10	20	30	40	
Chiralpak AD	MeOH	k_1	2.23	1.96	1.46	1.09	0.78	S < R
		α	1.24	1.23	1.20	1.18	1.16	
		R_s	1.9	2.6	3.1	2.8	2.1	
	EtOH	k_1	2.39	2.04	1.55	1.14	0.81	S < R
		α	1.16	1.16	1.15	1.15	1.14	
		R_s	1.5	1.6	1.7	1.7	1.7	
	PrOH	k_1	1.03	0.93	0.72	0.58	0.44	R < S
		α	1.51	1.47	1.38	1.31	1.27	
		R_s	2.4	2.4	2.0	1.7	1.5	
	IPA	k_1	0.98	0.67	0.47	0.33	0.23	R < S
		α	1.01	1.01	1.04	1.07	1.10	
		R_s	0.1	0.1	0.2	0.4	0.4	
	ACN	k_1	0.39	0.32	0.25	0.20	0.16	S < R
		α	1.08	1.11	1.16	1.15	1.14	
		R_s	0.2	0.4	0.5	0.6	0.6	
Chiralcel OD	MeOH	k_1	0.49	0.45	0.37	0.31	0.26	R < S
		α	1.24	1.23	1.20	1.18	1.16	
		R_s	1.7	1.6	1.4	1.1	0.8	
	EtOH	k_1	0.32	0.30	0.26	0.22	0.19	
		α	1.31	1.30	1.27	1.26	1.24	
		R_s	1.3	1.3	1.1	1.0	0.8	
	PrOH	k_1	0.47	0.41	0.35	0.30	0.25	
		α	1.24	1.23	1.21	1.19	1.17	
		R_s	1.1	1.0	0.9	0.8	0.7	
	IPA	k_1	0.81	0.70	0.51	0.35	0.27	
		α	1.56	1.53	1.48	1.45	1.40	
		R_s	2.2	2.1	2.0	1.8	1.5	
	ACN	k_1	0.23	0.21	0.17	0.14	0.13	
		α	1.23	1.23	1.19	1.17	1.15	
		R_s	1.1	1.0	0.8	0.3	0.2	
Chiralpak AS	MeOH	k_1	0.20	0.18	0.15	0.12	0.10	-
		α	1.00					
		R_s	-					
	EtOH	k_1	0.18	0.16	0.11	0.09	0.07	
		α	1.00					
		R_s	-					
	PrOH	k_1	0.14	0.12	0.07	0.03	0.01	
		α	1.00					
		R_s	-					
	IPA	k_1	0.63	0.59	0.51	0.45	0.38	
		α	1.00					
		R_s	-					
	ACN	k_1	0.02	0.01	0	0	0	
		α	1.00					
		R_s	-					
Chiralcel OJ	MeOH	k_1	0.81	0.73	0.59	0.48	0.38	S < R
		α	1.31	1.30	1.27	1.25	1.22	
		R_s	3.1	3.1	2.8	2.4	1.9	
	EtOH	k_1	0.98	0.87	0.68	0.49	0.40	-
		α	1.00					
		R_s	-					
	PrOH	k_1	1.03	0.92	0.72	0.61	0.50	R < S

(Continues)

TABLE 1 (Continued)

Column	Eluent	Parameter	Temperature (°C) 5	10	20	30	40	Elution sequence
		α	1.71	1.63	1.54	1.46	1.40	
		R_s	2.3	2.2	2.0	1.8	1.6	
	IPA	k_1	1.16	0.90	0.68	0.50	0.41	R < S
		α	1.17	1.16	1.14	1.11	1.09	
		R_s	0.4	0.6	0.8	1.1	1.1	
	ACN	k_1	0.05	0.04	0.04	0.04	0.03	-
		α	1.00					
		R_s	-					

further underlining the importance of interactions between apolar portions of the selector and selectand amplified under polar organic conditions.[24,25]

Also, on most of the employed CSPs, there is no linear correlation between the polarity of the mobile phase and retention times of the analytes. As the polarity of the linear alcohols decreases in the order MeOH > EtOH > PrOH, an increase in analyte retention would be expected with the increase in alcohol chain length. However, this behaviour was only observed on Chiralcel OJ column; in all of the other cases, MeOH produced a stronger retention than the other 2 linear alcohols, which can also further indicate the involvement of apolar interactions between the CSP and the enantiomers. Similar observations were also recently described by Sardella et al.[26] and also Cirilli et al.[24]

However, it is hard to set up a general trend from the experimental data obtained. It has already been shown that alcohols of different size and bulkiness can be incorporated in the CSPs structure and can also induce conformational changes in the helical structure of the polysaccharide selectors, which result in different stereo environments.[27,28]

During investigation of the elution sequence of the enantiomers, chiral selector—and mobile-phase dependent reversal of enantiomer elution order were observed. On Chiralcel OD column the elution order was *R*-RAC, followed by *S*-RAC, and it was independent of the mobile phase employed, while on the Chiralpak AD column MeOH, EtOH, and ACN, the *S*-enantiomer, while using PrOH or IPA the *R*-enantiomer eluted first. On cellulose-type Chiralcel OJ column using pure MeOH as mobile phase the elution order was *S*-RAC followed by *R*-RAC enantiomer, while using PrOH or IPA a reversal of elution order was observed (Table 1, Figure 3).

The chiral selector-dependent reversal of elution order observed between Chiralpak AD and Chiralcel OD column is frequently explained by the conformational difference between the 2 CSPs.[29-31] While both polymers have the same side chain (3,5-dimethylphenylcarbamate) and the same monomeric building blocks (1➔4-linked-D-glucopyranose), cellulose presents β, while amylose presents α linkage types.[32] This difference in linkage-type results larger chiral cavities and weaker intrapolymer H-bond in the cellulose derivative, when compared with the amylose-based polymer.[33] The structural differences lead to different molecular environments of the chiral cavities affecting the affinity pattern of the CSPs towards the enantiomers.

On both Chiralpak AD and Chiralcel OJ columns, PrOH and IPA produced a reversal of elution order, when compared with the other solvents. Application of the bulkier IPA as mobile phase resulted in lower enantioselectivities and generally lower retention on both columns, when compared with its unbranched isomer, PrOH. The obtained results illustrate the complementarity of different separation systems based on polysaccharide CSPs. It has been shown once again that even fine changes in chiral selector structure and/or mobile phase have a great impact on both retention and enantioselectivity of the chiral columns.

During method development, equally high enantioresolution (R_s = 3.1) was observed on Chiralpak AD and Chiralcel OJ column using MeOH as mobile phase; however, retention times were lower, and the peak shapes were better using Chiralcel OJ column. Further method optimizations were thus performed using this column with pure MeOH as mobile phase.

The effects of mobile phase additives such as TEA (0.01–0.1 v/v%), acetic acid (0.01–0.1 v/v%) and their mixtures were also investigated. These modifiers did not influence the quality of the enantioseparation significantly.

For further optimization, the effect of flow rate on enantiodiscrimination and retention factor was also investigated. Varying flow rate between 0.3 and 0.9 mL/min resulted in decreased retention times and resolution values. To achieve a shorter analysis time with appropriate resolution, 0.6 mL/min flow rate was

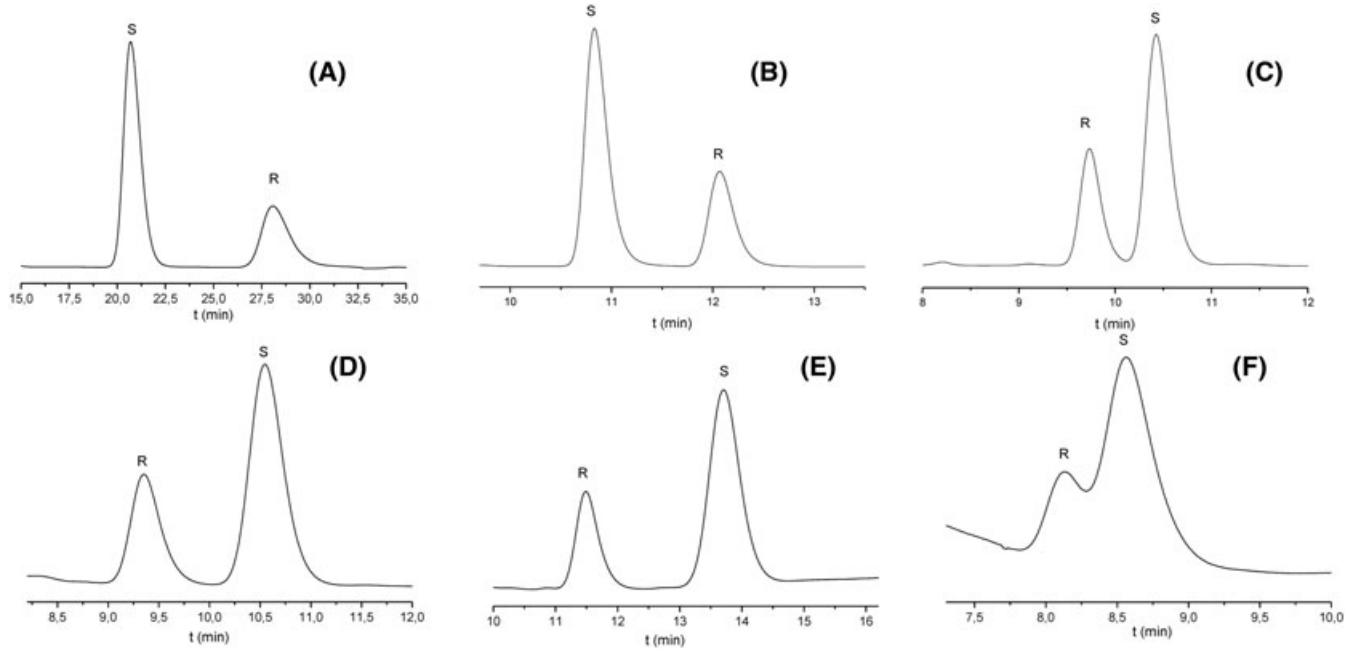

FIGURE 3 Chiral separation and elution order of RAC enantiomers on different CSPs with different mobile phases. Chromatographic conditions: (A) Chiralpak AD with MeOH, (B) Chiralcel OJ with MeOH, (C) Chiralcel OD with MeOH, (D) Chiralpak AD with PrOH, (E) Chiralcel OJ with PrOH, (F) Chiralcel OD with PrOH (250 × 4.6 mm, 10-μm particle size, flow rate: 0.5 mL/min, temperature: 10 °C)

chosen as optimum. The effect of the temperature between 5 and 40 °C range was also investigated, and 10 °C has been found as optimum. Further details regarding temperature effect can be found in the following section.

3.2 | Effect of column temperature on chiral separation of RAC. Determination of thermodynamic parameters

During chiral separations, temperature changes affect both analyte retention and enantioselective interactions.[34-36] In order to obtain information about the mechanistic aspects of chiral discrimination process, the differences in the change of standard enthalpy Δ(ΔH°) and standard enthropy Δ(ΔS°) for the 2 enantiomers moving from the mobile to the stationary phase were calculated according to the modified van't Hoff equation:

$$\ln \alpha = -\frac{\Delta\Delta \mathrm{H}^{\circ}}{\mathrm{RT}} + \frac{\Delta\Delta \mathrm{S}^{\circ}}{\mathrm{R}} \quad (3)$$

where R is the universal gas constant, T is the temperature, expressed in Kelvin, while α is the selectivity factor. In the present study, the classical van't Hoff method was undertaken, which assumes that analyte retention is only due to enantioselective interactions with the stationary phase. However, for a more realistic approach, contributions of both enantioselective and nonenantioselective interactions need to be considered.[37-40]

In order to evaluate the effect of temperature on retention and selectivity of RAC enantiomers and calculate thermodynamic parameters, temperature was varied between 5 and 40 °C (278–313 K) with 3 replicates, results being presented as averages. From the thermodynamic data, the isoenantioselective temperatures (T_{iso}) were also calculated as the ratio between Δ(ΔH°) and Δ(ΔS°). At this temperature, enthalpy and entropy compensations cancel each other, the 2 enantiomers co-elute and no separation occurs. The temperature dependence of retention factor and selectivity are summarized in Table 1, while the calculated thermodynamic data are presented in Table 2.

Comparison of the retention factors on all investigated columns reveals that all of the recorded k_1 values decreased with increasing temperature. The same trend was observed regarding α values almost in all cases, except on Chiralpak AD column using IPA and ACN as mobile phase. In the case of IPA, α increased when temperature increased, while in the case of ACN, the lnα vs 1/T plot can be divided into 2 regions, which could mean that the linear van't Hoff plots reflect different overall binding situations in the investigated temperature ranges (Table 2 and Figure 4). Similar observation were observed on Chiralcel OJ[41] and Chiralpak AD columns[42] and more recently also on an isopropyl carbamate-CF6-based CSP.[43]

TABLE 2 Thermodynamic parameters, Δ(ΔH°), Δ(ΔS°), TxΔ(ΔS°), Δ(ΔG°), van't Hoff equation, correlation coefficients, and Q values on different polysaccharide stationary phases

Column	Mobile phase	van't Hoff equation	R^2	$-\Delta(\Delta H°)$ (kJ mol^{-1})	$-\Delta(\Delta S°)$ (J mol^{-1} K^{-1})	$-T\times\Delta(\Delta S°)_{298K}$ (kJ mol^{-1})	$-\Delta(\Delta G°)_{298K}$ (kJ mol^{-1})	$T_{(iso)}$ (°C)	Q*
Chiralpak AD	MeOH	$\ln k_1 = 2598.4x - 8.517$	0.9957	4.5	12.9	3.8	0.7	77	1.2
		$\ln k_2 = 3139.6x - 10.062$	0.9964						
		$\ln\alpha = 541.2x - 1.546$	0.9986						
	EtOH	$\ln k_1 = 2639.8x - 8.606$	0.9955	0.4	0.2	0.06	0.3	1830	6.7
		$\ln k_2 = 2688.4x - 8.629$	0.9958						
		$\ln\alpha = 48.6x - 0.023$	0.9908						
	PrOH	$\ln k_1 = 2098.7x - 7.499$	0.9985	3.7	10.0	3.0	0.8	100	1.3
		$\ln k_2 = 2547.1 - 8.699$	0.9963						
		$\ln\alpha = 448.4 - 1.200$	0.9924						
	IPA	$\ln k_1 = 3387.5x - 12.294$	0.9935	−1.8	−6.6	−1.9	0.1	5	0.9
		$\ln k_2 = 3167.4 - 11.500$	0.9915						
		$\ln\alpha = -220.1x + 0.793$	0.9905						
	ACN(5–20 °C)	$\ln k_1 = 2231.1 - 8.994$	0.9903	−3.3	−12.6	−3.8	0.4	−9	0.9
		$\ln k_2 = 1829.3 - 7.472$	0.9910						
		$\ln\alpha = -401.8x + 1.522$	0.9919						
	ACN(20–40 °C)	$\ln k_1 = 2053.4x - 8.378$	0.9995	0.7	1.1	0.3	0.4	340	2
		$\ln k_2 = 2137.4x - 8.515$	0.9997						
		$\ln\alpha = 84.0x - 0.137$	0.9984						
Chiralcel OD	MeOH	$\ln k_1 = 1572.9 - 6.365$	0.9998	1.4	3.2	1.0	0.4	161	1.5
		$\ln k_2 = 1739.7 - 6.749$	0.9997						
		$\ln\alpha = 166.8x - 0.384$	0.9940						
	EtOH	$\ln k_1 = 1368.4 - 6.040$	0.9990	1.2	2.1	0.6	0.6	303	1.9
		$\ln k_2 = 1513.5 - 6.292$	0.9992						
		$\ln\alpha = 145.1x - 0.252$	0.9924						
	PrOH	$\ln k_1 = 1533.7x - 6.295$	0.9960	1.2	2.5	0.8	0.4	201	1.6
		$\ln k_2 = 1677.8x - 6.600$	0.9956						
		$\ln\alpha = 144.1x - 0.3042$	0.9934						
	IPA	$\ln k_1 = 2804.1x - 10.275$	0.9965	2.1	4.0	1.2	0.9	263	1.8
		$\ln k_2 = 3059.2x - 10.751$	0.9975						
		$\ln\alpha = 255.1x - 0.476$	0.9973						
	ACN	$\ln k_1 = 1491.7x - 6.847$	0.9990	1.5	3.8	1.1	0.4	134	1.4
		$\ln k_2 = 1677.1x - 7.302$	0.9994						
		$\ln\alpha = 185.4x - 0.455$	0.9918						
Chiralcel OJ	MeOH	$\ln k_1 = 1871.9 - 6.932$	0.9987	1.4	2.9	0.9	0.6	224	1.7
		$\ln k_2 = 2043.7 - 7.278$	0.9988						
		$\ln\alpha = 171.8x - 0.346$	0.9991						
	PrOH	$\ln k_1 = 1792.0 - 6.421$	0.9982	4.1	10.5	3.1	1.0	122	1.3
		$\ln k_2 = 2288.5 - 7.678$	0.9981						
		$\ln\alpha = 496.5x - 1.257$	0.9942						
	IPA	$\ln k_1 = 2570.1 - 9.142$	0.9990	1.6	4.6	1.4	0.3	87	1.2
		$\ln k_2 = 2769.6 - 9.696$	0.9991						
		$\ln\alpha = 199.5x - 0.554$	0.9974						

*$Q = \Delta(\Delta H°)/T\times\Delta(\Delta S°)_{298K}$.

The Δ(ΔH°) value provides information on the relative ease of transfer of analytes from the mobile to the stationary phase.[37] These values ranged from −4.5 to 3.3 kJ/mol. Positive Δ(ΔH°) values were observed only on Chiralpak AD column using pure IPA or ACN as mobile phase, indicating that in these cases, from an enthalpic point of view the transfer of the analytes from the mobile phase to the stationary phase is unfavourable.[44] Interestingly the highest negative and also the highest positive Δ(ΔH°) value was observed on Chiralpak AD column: in the case of ACN the highest positive Δ(ΔH°) value while in the case of MeOH the lowest negative Δ(ΔH°) value were calculated. These observations further underline the role of the mobile phase in the enantiomer recognition. The analysis of Δ(ΔS°) shows similar trends: negative Δ(ΔH°) values are accompanied

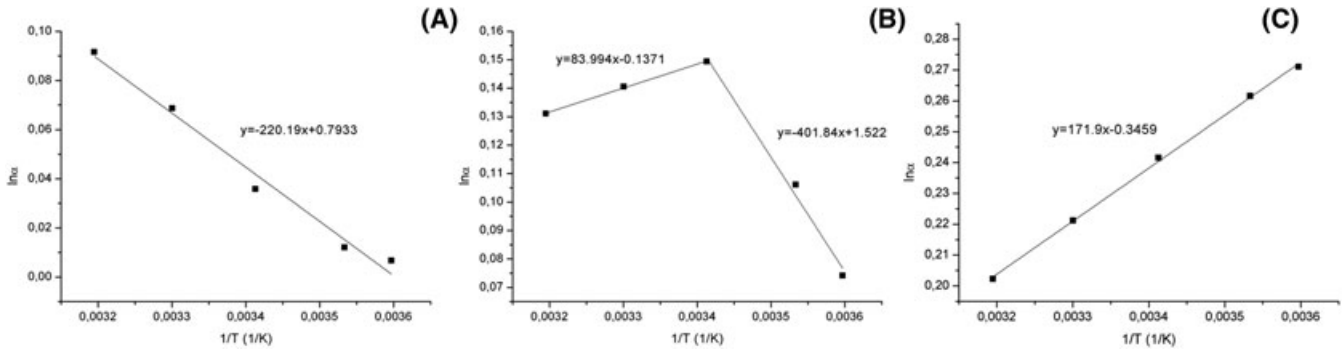

FIGURE 4 van't Hoff plots of ln α vs 1/T for RAC enantiomers on (A) and (B) Chiralpak AD and (C) Chiralcel OJ CSPs. Chromatographic conditions: 250 × 4.6 mm, 5-μm particle size columns, mobile phase (A) IPA, (B) ACN, (C) MeOH, flow rate: 0.5 mL/min, temperature: (5–40 °C)

by a negative Δ(ΔS°) values; moreover, the largest Δ(ΔH°) value accompanied the largest Δ(ΔS°) value.

The thermodynamic parameter Δ(ΔG°) provides information on the strength of binding between selector and selectand, more negative values indicating a more efficient binding. The highest Δ(ΔG°) values were obtained on Chiralcel OJ using PrOH as mobile phase. Comparing the values obtained, once again, no linear trend can be observed between chain length of unbranched alcohols and Δ(ΔG°) values.

The relative contribution of enthalpic and entropic terms to the free energy of adsorption can be visualized through the enthalpy/entropy ratio Q.[45,46] Comparison of Q values revealed that the enantioseparation was mainly enthalpically controlled in most cases as reflected by Q larger than 1 values. Interestingly, enthropy controlled enantioseparations (Q < 1) were observed using Chiralpak AD as stationary phase and IPA or ACN as mobile phase. However, it should be noted that the Q value is only 0.9 in each cases.

3.3 | Method validation

Method validation was performed according to ICH Q2 (R1) guidelines by studying linearity, sensitivity (LOD, LOQ), accuracy, and precision.[47]

Linearity was assessed over concentration levels ranging from 2 to 50 μg/mL of racemic RAC, at 6 concentration levels, each being analyzed in triplicate. Calibration plots were constructed by plotting peak areas against corresponding concentration of enantiomers. Slope, intercept, and correlation coefficients were determined by least squares polynomial regression analysis. Regression analysis pointed towards a linear relationship, with the following equations: $y = 11.627x - 2.579$ ($R^2 = 0.9995$) and $y = 11.726x - 2.471$ ($R^2 = 0.9994$) for the *S*-enantiomer and *R*-enantiomer, respectively. Zero was included in 95% confidence interval of the intercept for both enantiomers, a residual sum of squares plot was also performed, and the residuals were randomly scattered around the zero line.

Parameters for LOD and LOQ were determined at 3/1 and 10/1 signal to noise ratios, respectively. The LOD and LOQ values for both enantiomers were found to be 0.28 and 0.95 μg/mL, respectively.

Intra-day and inter-day precision was assessed by injecting racemic RAC standards at 3 concentration levels (2, 10, and 50 μg/mL) in 3 parallel runs on the same day and on 3 successive days, respectively. Precision was determined from the RSD% of backcalculated concentrations, results being under 5% for both enantiomers in all cases.

Repeatability was further checked for retention times and R_s with 6 parallel injections at 10 μg/mL concentration. The RSD% of retention times and R_s were under 0.6% and 2%, respectively, for each enantiomer.

The accuracy of the method was verified through the recovery test: an appropriate amount of racemic RAC tablet powder was weighted dissolved in methanol, and the solution was spiked with racemic standard at 3

TABLE 3 Results obtained during quantification of RAC enantiomers from 2 commercially available pharmaceutical products ($n = 3$ replicates for each product)

	Declared enantiomer quantity, mg		Found enantiomer quantity, mg (n = 3)	
Pharmaceutical product	***S*-RAC**	***R*-RAC**	***S*-RAC**	***R*-RAC**
Hidrasec® capsule (100 mg)	50 mg	50 mg	50.15 mg ± 0.11	49.83 mg ± 0.18
Hidrasec® baby granules (10 mg)	5 mg	5 mg	5.03 mg ± 0.03	4.95 mg ± 0.02

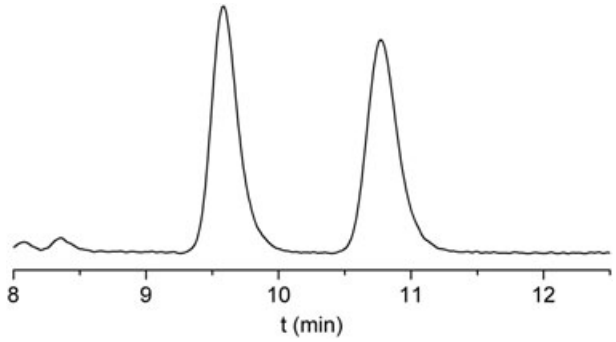

FIGURE 5 Representative chromatogram for the chiral separation of RAC enantiomers from commercially available dosage forms. Chromatographic conditions: Chiralcel OJ column, 250 × 4.6 mm, 10-μm particle size columns, mobile phase 100% MeOH, flow rate: 0.6 mL/min, temperature: 10 °C

different concentrations (2, 10, 50 μg/ml), each solution being analyzed in triplicate. The percentage recovery values of the enantiomers varied from 98.9% to 100.1%.

In light of these results, it can be stated that the developed method is suitable for determination of RAC enantiomers and proved to be reliable, linear, precise, and accurate.

3.4 | Analysis of pharmaceutical preparations

The validated method was applied for the determination of RAC enantiomers in commercially available capsules and granules (Hidrasec®). Good agreements were obtained between the value claimed by manufacturer and that determined by the HPLC method (Table 3). Moreover, no interferences were observed from the drug formulation excipients on the chromatograms. Figure 5 shows a representative chromatogram obtained during method applicability study.

4 | CONCLUSION

Chiral separation of RAC enantiomers was evaluated on 4 different polysaccharide-based CSPs, using 5 different solvents in polar organic mode. Evaluation of chromatographic data revealed that even fine changes in the chiral selector and/or mobile phase structure and properties can lead to large differences in retention and enantioselectivity of the applied systems. CSP and mobile phase-dependent reversal of enantiomer elution order and entropy-controlled enantioseparations were also observed, showing once again the great potential of these systems in enantiomeric quality control.

Based on the observations of the preliminary HPLC runs, a simple and rapid direct chiral HPLC method was developed and optimized for the simultaneous estimation of the RAC enantiomers on Chiralcel OJ column. The stationary phase, thermostated at 10 °C using 100% methanol with 0.6 mL/min succeeded in the baseline separation of RAC enantiomers ($R_s = 3.00 \pm 0.02$), was achieved within 12 minutes. The method was validated and proved to be linear, accurate and precise. Applicability of the method was checked by analyzing commercial pharmaceutical preparations. The developed method further underlines the ease of use of polar organic mode in chiral separations.

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