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SEPARATION OF PROPRANOLOL ENANTIOMERS USING CHIRAL HPLC

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ABSTRACT. Enantiomers of pharmaceuticals may exhibit different effects because the organism serves as a chiral selector. Enantiomers can now be identified and separated using analytical techniques such as gas chromatography and high-performance liquid chromatography. The nonselective β-adrenergic blocker propranolol is used as a racemate in therapy, but only the S(-) isomer has been shown to be active. The aim of this study was to determine the mobile phase composition that would achieve optimal retention and satisfactory resolution between enantiomers and to confirm the order of elution of the enantiomers. The enantioseparation was tested in the n-heptane/ethanol/ammonia, n-heptane/ethanol/diethylamine, n-heptane/isopropanol/ ammonia and n-heptane/isopropanol-/diethylamine systems. Optimal separation of enantiomers (t_1=4,708; t_2=5.264; R_s=1.75) was achieved with the mobile phase n-heptane/ethanol/diethylamine 80/20/0.1. The elution order was based on the retention time of the pure S(-)isomer. It was confirmed that the R(+) isomer shows higher retention.

Keywords: propranolol; enantioseparation; chiral stationary phases; HPLC.

INTRODUCTION

Enantiomers (from the Greek words *ἐνάντιος* – opposite, and *μέρος* – part) are stereoisomers that relate to each other like an object and its mirror image and cannot overlap. Enantiomers have the same physical properties but differ into the direction of deflection of the plane from linearly polarized light. The enantiomer that rotates the plane of polarized light clockwise is dextrorotatory and is designated by (+) or d-enantiomer, while the enantiomer

that rotates the plane of polarized light counterclockwise is levorotatory and is designated by (-) or l-enantiomer (PÁLOVICS *et al.*, 2018).

Enantiomers are also chemically identical when their behavior toward optically inactive compounds are observed, while the two enantiomers react with optically active molecules at different rates, yielding as products two diastereoisomers with different physicochemical properties (PÁLOVICS *et al.*, 2018; FOGASSY *et al.*, 2006; FAIGL *et al.*, 2010). An equimolar mixture of the two enantiomers is a racemate (±) and shows no optical activity. A compound with two or more stereocenters that can overlap with their mirror image and contain a plane of symmetry is a *meso* compound (from Greek *meso* - middle). Few optically active drugs are used in therapy as pure enantiomers; most are administered as racemates. Enantiomers of drugs can have different activities because the organism act as a chiral selector. The enantiomer that has the desired pharmacological activity is called the eutomer (from the Greek word *eú* – good). The enantiomer that does not exhibit the desired activity, has a weaker pharmacological activity or may even be toxic is called distomer (from the Greek word *dys* – bad). Whether the racemate or the pure enantiomer is used in pharmacotherapy depends on the side effects of the pharmacologically inactive enantiomer, the differences in pharmacodynamics, pharmacokinetics, toxicity of the two enantiomers, and economic reasons, since the synthesis of pure enantiomers is a complex and expensive procedure (PÁLOVICS *et al.*, 2018; FOGASSY *et al.*, 2006).

For this reason, analytical methods have been developed to identify and separate of the active enantiomer from the less active or inactive enantiomer. The most used methods are gas chromatography (GC) (SCHURIG, 2001; TARAFDER and MILLER, 2021) and high-performance liquid chromatography (high pressure chromatography, chiral HPLC) (CHANKVETADZE, 2012; TARAFDER and MILLER, 2021). High pressure liquid chromatography is the most common separation method for the analysis of enantiomers. Chiral HPLC can be a direct or indirect method (WITTE, 1993). The indirect method involves a chiral separation, based on the derivatization of a mixture of enantiomers with a pure chiral reagent and the formation of diastereoisomers that can be separated by the classical HPLC method (WITTE, 1993; THOMPSON, 2005). The direct method requires the use of chiral mobile phases or the use of chiral stationary phases. Separation of enantiomers on chiral stationary phases is the most widely used method in enantiomer analysis today (THOMPSON, 2005). The stationary phase consists of an optically pure substance that can be chemically bound or physically applied to the carrier. The carrier for the stationary phase is usually silica gel or amino-propyl derivatized silica gel.. The basis of chiral separation is the so-called chiral recognition, i.e., the ability of a chiral stationary phase to react to different degrees with two enantiomers, leading to their separation. The nature of chiral recognition is determined by various interactions that lead to the formation of short-lived diastereoisomeric complexes with the stationary phase; and the most important interactions are: π-π interactions, hydrogen bonds, dipole-dipole interactions, formation of inclusion complexes and sterically determined interactions. To separate enantiomers, at least three of the above interactions must occur simultaneously, at least one of which must be stereoselective (ARMSTRONG *et al.*, 1986; FISCHER *et al.*, 1991; ZHANG *et al.*, 2003; HROBONOVÁ *et al.*, 2005; THOMPSON, 2005; SINGH *et al.*, 2015).

Propranolol (1-((1-methylethyl)amino)-3-(1-naphthalenyloxy)-2-propanol) (Figure 1), belongs to the group of nonselective β adrenergic blockers, used mainly to threat cardiovascular diseases (hypertension, angina pectoris, arrhythmias). The chemical structure of propranolol is aryloxypropanolamine, an optically active compound. The racemate is used in therapy, but only the S(-) isomer has been shown to be active (SINGH *et al.*, 2015; https://www.nhs.uk/medicines/propranolol/).

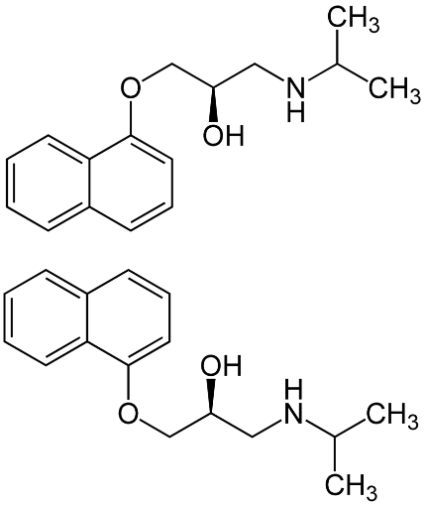

Figure 1. Propranolol enantiomers.

In view of all this, the aim of this study was the following: to define the composition of the mobile phase that achieves the most optimal retention and satisfactory resolution between enantiomers and to confirm the elution order of enantiomers, which may be important in pharmaceutical-technological, pharmacological, and toxicological studies.

MATERIALS AND METHODS

Equipment and reagents

Liquid chromatographs: Agilent Technologies HP1200, analytical balance: AdventurerTM - Pro (Ohaus Corporation, Pine Brook, USA).

Standard substances: propranolol hydrochloride racemate – working standard, propranolol hydrochloride S(-) isomer - working standard.

Reagents and solutions: n-heptane, Sigma Aldrich; ethanol, Sigma Aldrich; isopropanol, Sigma Aldrich; ammonia, 25%, Sigma Aldrich; diethylamine, Sigma Aldrich.

Chromatographic conditions

Chromatographic column: ChiralPak® IA, 250-4.6 5µm (silica gel coated with amylose tris-(3,5-dimethylphenylcarbamate).

Mobile phase: a mixture of n-heptane and ethanol, n-heptane and isopropanol in various volume ratios, with and without addition of an alkaline modifier (ammonia or diethylamine) (Tab. 1).

Table 1. Volume ratios of solvents in the mobile phase.

ChiralPak IA	**n-heptane / ethanol / NH_3** *(v/v/v)*		
	80:20:0.1	70:30:0.1	60:40:0.1
	n-heptane / ethanol / diethylamine *(v/v/v)*		
	80:20:0.1	70:30:0.1	60:40:0.1
ChiralPak IA	**n-heptane / isopropanol / NH_3** *(v/v/v)*		
	80:20:0.1	70:30:0.1	60:40:0.1
	n-heptane / isopropanol / diethylamine *(v/v/v)*		
	80:20:0.1	70:30:0.1	60:40:0.1

Flow rate: 1ml/min
Injection volume: 20μL
Column temperature: 25°C
UV detection: 220 nm

Preparation of solutions

-Preparation of the working standard of the racemate
Transfer 5 mg of propranolol hydrochloride into a 10 ml volumetric flask, dissolve in 5 ml of methanol and fill the volumetric flask with methanol up to the mark. The concentration of the prepared solution is 0.5 mg/ml.

-Preparation of the working standard of S(-) isomer
Transfer 5 mg of propranolol hydrochloride S(-) isomer into a 10 ml volumetric flask, dissolve in 5 ml of methanol and fill the volumetric flask with methanol up to the mark. The concentration of the prepared solution is 0.5 mg/ml.

- Preparation of mobile phases with modifiers:
-0.1% ammonia in ethanol
Transfer 0.1 ml of 25% ammonia into a 100 ml volumetric flask and fill the volumetric flask with ethanol up to the mark.

-0.1% ammonia in isopropanol
Transfer 0.1 ml of 25% ammonia into a 100 ml volumetric flask and fill the volumetric flask with isopropanol up to the mark.

-0.1% diethylamine in ethanol
Transfer 0.1 ml of diethylamine into a 100 ml volumetric flask and fill the volumetric flask with ethanol up to the mark.

-0.1% diethylamine in isopropanol
Transfer 0.1 ml of diethylamine into a 100 ml volumetric flask and fill the volumetric flask with isopropanol up to the mark.

Carrying out the chromatographic procedure

Under the described chromatographic conditions, the column is conditioned with the selected mobile phase (n-heptane/ethanol/NH3 60/40/0.1 (v/v/v), 70:30:0.1 (v/v/v) and 80:20:0.1 (v/v/v) as well as with the mobile phase n-heptane/ethanol/diethylamine 60/40/0.1 (v/v/v), 70:30:0.1 (v/v/v) and 80:20:0.1 (v/v/v)) until a stable base line is reached. The working standard of the racemate propranolol-hydrochloride is injected into the conditioned chromatographic system and the chromatographic behavior of the racemic mixture is observed. If, under the given chromatographic conditions, separation of enantiomers occurs, in order to determine the order of elution, a solution of the S(-) isomer is injected into the chromatographic system. The order of elution of the isomers is confirmed by reading the retention time of the pure S(-) isomer.

Of the chromatographic parameters, the retention time (retention parameter) and the resolution factor as a separation parameter were monitored.

RESULTS AND DISCUSSION

Enantioseparation was tested in the system n-heptane/ethanol/ammonia, n-heptane /ethanol/diethylamine, n-heptane/isopropanol/ammonia and n-heptane/isopropanol/diethylamine in the following volume ratios: 80/20/0.1, 70/30/0.1 and 60/40/0.1 (v/v/v). The following parameters were monitored as system responses: retention time (t_r), resolution factor (R_s).

By using a solution of the pure S(-) isomer under the same chromatographic conditions under which the racemate separation was performed, the order of elution from the column was determined based on the retention time of the pure S(-) isomer. The test results are shown in Tables 2–5 .

Table 2. Values of tested parameters with mobile phase n-heptane/ethanol/ammonia on ChiralPak IA column.

		n-heptane / ethanol / NH_3 (v/v/v) 60/40/0.1	
		$t_{r(min)}$	***R_s***
I	**Propranolol**	4.129	
II		4.508	1.22
		n-heptane / ethanol / NH_3 (v/v/v) 70/30/0.1	
		$t_{r(min)}$	***R_s***
I	**Propranolol**	4.585	
II		5.064	1.35
		n-heptane / ethanol / NH_3 (v/v/v) 80/20/0.1	
		$t_{r(min)}$	***R_s***
I	**Propranolol**	5.087	
II		5.869	1.27

Table 3. Values of tested parameters with mobile phase n-heptane/ethanol/diethylamine on ChiralPak IA column.

		n-heptane / ethanol / diethylamine (v/v/v) 60/40/0.1	
		$t_{r(min)}$	***R_s***
I	**Propranolol**	3.926	
II		4.254	1.27
		n-heptane / ethanol / diethylamine (v/v/v) 70/30/0.1	
		$t_{r(min)}$	***R_s***
I	**Propranolol**	4.207	
II		4.635	1.55
		n-heptane / ethanol / diethylamine (v/v/v) 80/20/0.1	
		$t_{r(min)}$	***R_s***
I	**Propranolol**	4.708	
II		5.264	1.75

Table 4. Values of tested parameters with mobile phase n-heptane/isopropanol/ammonia on ChiralPak IA column.

		n-heptane / isopropanol / NH_3 (v/v/v) 60/40/0.1	
		$t_{r(min)}$	R_s
I	**Propranolol**	-	
II		4.787	-
		n-heptane / isopropanol / NH_3 (v/v/v) 70/30/0.1	
		$t_{r(min)}$	R_s
I	**Propranolol**	-	
II		5.243	-
		n-heptane / isopropanol / NH_3 (v/v/v) 80/20/0.1	
		$t_{r(min)}$	R_s
I	**Propranolol**	-	
II		6.297	-

Table 5. Values of the tested parameters with the mobile phasen-heptane/isopropanol/diethylamine on the ChiralPak IA column.

		n-heptane / isopropanol / diethylamine (v/v/v) 60/40/0.1	
		$t_{r(min)}$	R_s
I	**Propranolol**	-	
II		4.027	-
		n-heptane / isopropanol / diethylamine (v/v/v) 70/30/0.1	
		$t_{r(min)}$	R_s
I	**Propranolol**	-	
II		4.465	-
		n-heptane / isopropanol / diethylamine (v/v/v) 80/20/0.1	
		$t_{r(min)}$	R_s
I	**Propranolol**	-	
II		5.177	-

The results shown in the Tables 2–5 indicate that the ChiralPak® IA column showed better enantioselectivity when the mixture of n-heptane/ethanol/diethylamine in the ratio 80/20/0.1 (v/v/v) was present in the mobile phase.

Figure 2 shows the chromatograms: top-S(-) isomer and bottom-racemic mixture of propranolol.

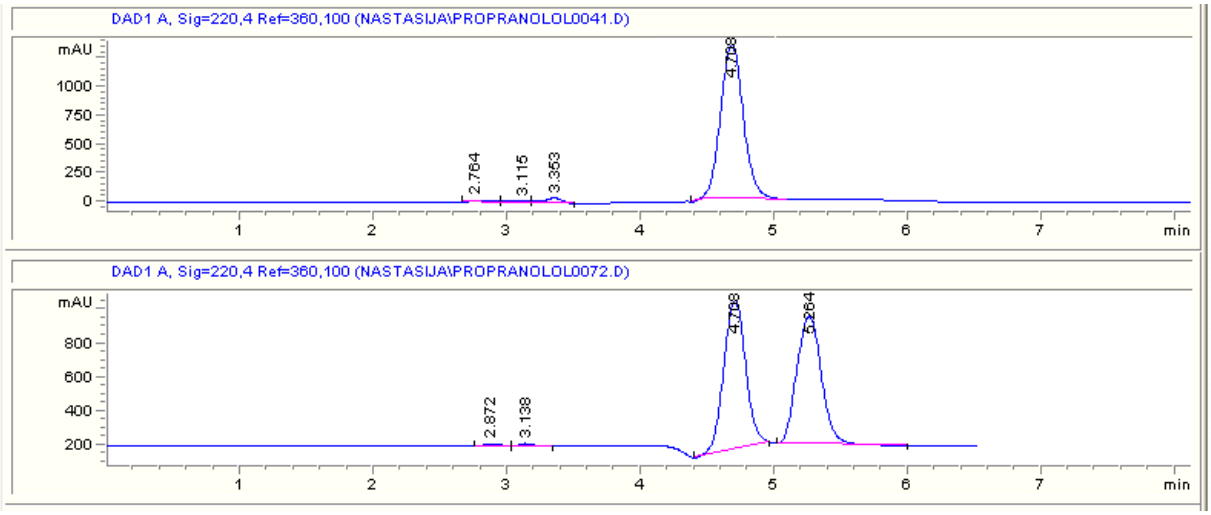

Figure 2. Chromatograms of propranolol (top - pure S(-) isomer;
bottom - racemic mixture of propranolol).

This work demonstrate the direct enantioseparation of propranolol enantiomers on modified amylose as a chiral stationary phase (ChiralPak®IA, 250-4.6.5µm (silica-gel coated with amylose tris-(3,5-dimethylphenylcarbamate)). Data on the stability and compatibility of the stationary phase were used in the choice of solvent for the mobile phase. It is known that mixtures of alkanes, alkyl ethers with the addition of a small amount of isopropanol or ethanol are the most commonly used mobile phase when working with derivatized stationary phases on amylose, and this mobile phase composition served as the initial mobile phase in enantioseparation (WITTE, 1993).

A mixture of n-heptane and isopropanol was chosen, for the initial mobile phase, with 20-40% isopropanol of. In the chromatographic system thus defined, separation of the enantiomers (R_s=0) was not achieved, and the peaks were stretched, so that ethanol, an alcohol with stronger eluting power, was added as a polar modifier in the mobile phase instead of isopropanol in the same percentage. By varying the percentage of ethanol in the mobile phase, it was shown that satisfactory retention of the molecules on the stationary phase was achieved, at a percentage of 20%-40%, but no separation was achieved.

For enantioseparation, the type and number of interactions between the compound and the stationary phase, as well as the number of stereoselective interactions, are of great importance. In general, stereoselective interactions are achieved by polar amino or hydroxyl groups forming proton donor-acceptor bonds with the stationary phase. Propranolol (Figure 1) is a secondary amine and exists in the form of the hydrochloride salt. Protonation of the polar amino group reduces the possibility of forming proton acceptor bonds between the molecule and the stationary phase (molecule-chiral selector), which reduces the enantioselectivity of the stationary phase. This problem can be solved by adding an alkaline modifier (ammonia, diethylamine). According to the manufacturer's data, the optimal amount of alkaline modifier for the mentioned phases is in the range of 0-10 mM/l of mobile phase, and ammonia and diethylamine were added in amounts of 0.1% and 0.2%, respectively. The presence of alkaline modifier allows the compounds to be in molecular form during the analysis, i.e., the secondary amino group is available for interaction with the stationary phase.

The addition of an alkaline modifier (ammonia/diethylamine) to the mobile phase did not have a major effect on the retention of the compound but had a positive effect on the enantioseparation. Since it was shown at the beginning that there was no significant difference in enantioseparation when a 0.1% or 0.2% was used, a 0.1% of alkaline modifier was used in further work.

A mixture of n-hexane/ethanol/ammonia, n-hexane/ethanol/diethylamine, n-hexane/ isopropanol/ammonia and n-hexane/isopropanol/diethylamine in the volume ratios of 80/20/0.1, 70/30/0.1 and 60/40/0.1 (v/v/v) was also used as the mobile phase, but no enantioseparation was achieved with these mobile phases.

Analyzing the retention time (t_r) and the resolution factor (R_s) values, we can say that the optimal conditions for enantioseparation were achieved with the mobile phase n-heptane/ ethanol/diethylamine 80/20/0.1 (v/v/v). The enantiomer that forms a less stable diastereoisomeric complex with the stationary phase and elutes first rotates the plane of polarized light to the left (S(-) isomer), while the enantiomer that forms a more stable diastereoisomeric complex and elutes second rotates the plane of polarized light to the right (R(+) isomer). It is known that the absolute configuration of the (+) isomer of propranolol is R and that of the (-) isomer is S.

CONCLUSION

Analysis of the resolution factor (Rs) values, shows that the optimal conditions for enantioseparation were achieved with the mobile phase n-heptane/ethanol/diethylamine 80/20/0.1 (v/v/v) (t_1=4.708, t_2=5.264, R_s=1.75).

Based on the results obtained with a solution of the pure S(-) isomer, it was found that when the racemates were separatedof, the S(-) isomer eluted first from the column, and that the R(+) isomer had a greater affinity for the stationary phase.

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