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Enantiomeric separation of β-blockers and tryptophan using heparin as stationary and pseudostationary phases in capillary electrophoresis

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Abstract

The separation methods of the enantiomers of two β-blockers and tryptophan were studied using capillary electrochromatography with heparin covalently as well as non-covalently, bonded onto the capillary inner wall as stationary phase and electrokinetic chromatography with heparin as pseudostationary phase. In the case of heparin, used as a stationary phase, the method was unable to resolve enantiomers in both cases β-blockers and tryptophan. On the other hand, when heparin was used as a pseudostationary phase, the resolution of the enantiomers was obtained only with 3-aminopropyltriethoxysilane which were immobilised onto the inner phase of the capillary. The results of this study let us infer that the electrostatic, hydrophobic, and steric interactions were involved in the separation mechanisms. The separation was achieved in less than 10 minutes under the optimized conditions: 30 mM phosphate buffer (pH 2.5) with the adding of 15 mg/mL of heparin at 15°C and 10 kV. The usefulness of heparin as a chiral selector both in electrokinetic chromatography using 3-aminopropyltriethoxysilane attached to the capillary was demonstrated for the first time. The developed method was powerful, sensitive, and fast, and it could be considered an important alternative to conventional methods used for chiral separation.

KEYWORDS

APTES, capillary electrochromatography, chiral separation, electrokinetic chromatography, heparin

1 | INTRODUCTION

Many pharmaceutical compounds have one or more chiral centers that are responsible for their optical activity. The importance of chirality in the activity of these compounds has long been recognized due to the fact that those enantiomers exhibit different activities in human and animal bodies.[1] Being able to define the amount of each in a mixture has become of utmost importance for the pharmaceutical industry. Consequently, developments of methods for enantiomer separation are extremely valuable.

Currently, in the industry, the separation of chiral drugs is dominated by high performance liquid chromatography.[2-6] Gas chromatography and supercritical fluid chromatography are other techniques that have a key application in the separation of chiral drugs.[7] However, during the last decade, capillary electrophoresis (CE) has received a great attention within this field due to its high resolution, power, and selectivity.[8,9]

An important advantage of using CE to separate chiral drugs is the flexibility that this technique presents in adding, during the separation process, a variety of

compounds that could act as some chiral selectors like cyclodextrins and its derivatives,[10-12] macrolides,[13,14] and many other small molecules.[15,16] In general, chiral separations using CE can be achieved following two strategies: the first one includes adding chiral additives in the running buffer while the second one is based on the modification of the capillaries inner wall with a chiral selector.[17-19]

Many researches have focused their attention in macromolecules with chiral selection capacity such as proteins[20-22] and polysaccharides.[23-26] Among them, polysaccharides are one of the most promising chiral selectors due to the fact that they have a great versatility in the CE enantiomer separation. Many neutral polysaccharides such as dextran, dextrin,[27,28] laminaran, pullulan,[29] etc. as well as ionic polysaccharides such as heparin,[30,31] chondroitin sulphate,[32] dextran sulphate,[33] pentosan polysulphate,[34] have been successfully employed as chiral selectors in CE. The most important characteristic of polysaccharides is the low absorbance they have in UV spectra range which allows these substances to be used in CE with UV detection. Thus, this is being considered as an important advantage to perform high detection sensitivity. Another advantage of the charged polysaccharides is that they can be used in high concentration in the background electrolyte (BGE) due to their high solubility in aqueous solutions. Moreover, because of the importance of polysaccharides in biological systems,[35] the studies of their stereo-selectivity capacity have great potential applications not only to develop new methods of separation but also for future studies of selectivity interaction mechanisms between polysaccharides and different biomolecules or pharmaceutical products.

Heparin's chiral selective capacity in CE was reported for the first time by Stalcup and Agyei in 1994.[30] Heparin is a glycosaminoglycan with a molecular mass between 6000 and 30,000. It is a heterogeneous mixture of variably sulphated polysaccharide chains composed of α-(1,4)-linked D-glucosamine and also L-iduronic or D-glucuronic acids. These groups are responsible for the strong anionic character, good solubility, and electrophoretic mobility. The unit structure of heparin is shown in Figure 1.

Among the compounds selected for these experiments, β-blockers were found, which is a group of drugs used in various medical conditions especially in the treatment of cardiac arrhythmias and cardio protection after myocardial infarction.[36] Furthermore, tryptophan was used, an essential amino acid which has chiral characteristics. The two β-blockers used in this work, pindolol and propranolol, are not selective agents, which means that they have the same effect on both adrenergic receptors β-1 and β-2. The amino acid is deeply involved in the synthesis of serotonin, a hormone strongly related with stress.

The goal of this work is to transfer the acquired knowledge regarding the behaviour of heparin as a chiral selector when it was used as a stationary and pseudo stationary phase. Moreover, in this work, we have tried to explain the role of 3-aminopropyltriethoxysilane (APTES) as a stationary phase when heparin is used as an additive in the BGE.

2 | MATERIALS AND METHODS

2.1 | Materials

Heparin (H4784), APTES, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, E1769) as well as propranolol and pindolol were purchased from Sigma-Aldrich. D, L-tryptophan was brought from the British Drug Houses (UK). Dimethyl sulphoxide (DMSO) was purchased from LabScan, Dublin, Ireland. Phosphoric acid, sodium acetate, sodium hydroxide (1.0 M), and hydrochloric acid (1.0 M) were obtained from Oy FF-Chemical Ab (Yli Ii, Finland); acetone was from Mallinckrodt Baker (Deventer, The Netherlands). The pH solutions (4, 7, and 10), used for calibrating the pH meter, were purchased in Merck (Darmstadt, Germany).

2.2 | Instrumentation

The capillary electrophoretic experiments were carried out with a Hewlett-Packard ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a diode array detector (detection at 200, 214, and 254 nm) and an air-cooling

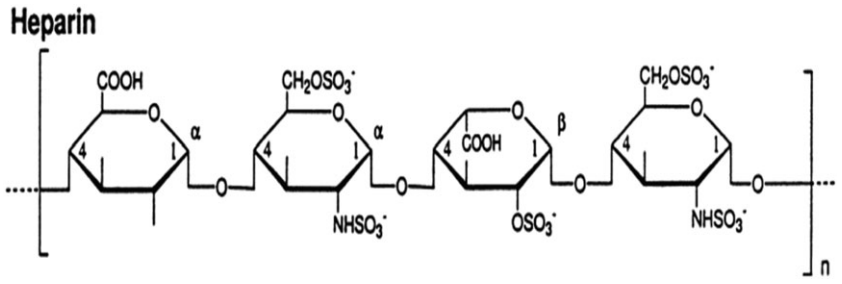

FIGURE 1 Unit structure of heparin employed for CE enantiomer separations

device for the capillary cassette. Bare fused-silica capillaries of 50 μm (I.D.) × 375 μm (O.D.) were purchased from Optronis GmbH, Kehl, Germany. The length from the capillary to the detector was of 30.0 cm with a total length of 38.5 cm for all the experiments. A Lauda Ecoline Re-104 water bath (Lauda-Königshofen, Germany) was used to control the temperature of the autosampler.

A Jenway 3030 pH meter (Jenway, Felsted, UK) and a MeterLab PHM 220 pH meter (Radiometer, Copenhagen, Denmark) were used in the pH measurements. Distilled water was further purified with a Millipore water purification system (Millipore S.A., Molsheim, France). Millipore filters (Bedford, MA, USA) were used for filtering BGEs.

2.3 | Methods

2.3.1 | Sample and buffer preparation

Stock solutions of each racemic sample were prepared in methanol (pindolol) or water (propranolol and tryptophan) at concentrations of 1.0 mg/mL. The sample solutions for enantio-separation were prepared by mixing stock solutions and diluting it with water at concentrations of 100 μg/mL. The stock solution of DMSO (1% v/v) used as EOF marker was prepared in water.

The BGEs used were phosphate buffer, ionic strength(IS) 20 to 40 mM, pH 2.5 and 3.5; also acetate buffer IS 20 to 40 mM, pH 4.5 and 5.5. Both buffers were prepared with their acids and sodium hydroxide. The pH was adjusted to 1.0 M sodium hydroxide, 1.0 M hydrochloric acid, or acetic acid, respectively.

The solutions containing chiral selector were freshly prepared by dissolving heparin in the BGEs. Before use, the BGEs were passed through 0.2-μm Millipore filters using a Millipore vacuum system. All the solutions were stored at +4°C.

2.3.2 | Coating procedures

Three types of immobilized capillaries were used: APTES-coated capillary, APTES-heparin covalently, and non-covalent coated capillary.

APTES-coated capillary

Before coating, a new capillary was pre-treated using the following procedures: 15-minute flush with 1 M NaOH, 15-minute flush with deionized water, and 15-minute flush with acetone at a pressure of 940 mbar. After preconditioning, APTES coating was attached to the inner surface of the capillary as follows: the capillary was flushed with 4% (v/v) APTES solution in anhydrous acetone at 50 mbar for 2 hours. Then, the capillary was flushed with acetone for 10 minutes, water for 5 minutes, and phosphate buffer (pH 5.5 and IS20mM) for 60 minutes in order to eliminate the excess of unbounded APTES.

APTES-heparin covalently coated capillary

After preconditioning and coating the capillary with APTES, the amino-silanized capillary was treated with heparin. Briefly, a heparin solution (3 mg/mL) dissolved in 2-mL phosphate buffer (pH 7.4 and IS = 20 mM) containing 0.25 mg of EDC was passed through the capillary for 1 hour. Finally, the capillary was rinsed with phosphate buffer (pH 7.4 and IS = 20 mM) to remove the not immobilized heparin.

APTES-heparin non-covalently coated capillary

After being coated with APTES, the heparin non-covalently coated capillary was flushed with heparin solution at 50 mbar for 40 minutes and left standing in the coating solution for 60 minutes. Finally, the capillary was rinsed with phosphate buffer at 15 minutes.

2.3.3 | Calculation

The resolution between the studied analytes was determined by Equations (1) and (2).

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) \quad (1)$$

where, R_s is resolution based on peak-width; t_1, t_2 are migration times of enantiomers; w_1, w_2 are baseline peak widths of enantiomers; and

$$R_h = (H_i - H_m)/H_i \quad (2)$$

where R_h is resolution based on peak-height. If the baseline separation of two peaks cannot be achieved, R_h is applied. H_i is peak height of the lower peak of the two peaks, and H_m is the height of the valley between the two peaks.

3 | RESULTS AND DISCUSSION

3.1 | Results

In a previous study, we developed a procedure for the covalent and non-covalent coating of heparin onto the inner phase of the capillary. At that time, the coating had the aim of studying the interaction between heparin and lipoproteins.[37] The presence of carboxylic groups all along the heparin molecule allowed us to perform the covalent interaction between the carboxylic group and amino group from APTES, which is previously attached

to the inner phase of the capillary. In covalent coating, heparin was attached to APTES using EDC as a linker. In this reaction, the terminal amine group of APTES molecule reacted with the carboxylic group of heparin. As a consequence, a stable amide bond was formed. On the contrary, non-covalent immobilization of heparin on APTES-coated capillary was based on ionic interaction between the positively charges of anime groups of APTES and the negatively charges of sulphate and/or carboxylic groups of heparin. In the current work, heparin was used as a chiral selector, and its function was evaluated as a stationary and pseudostationary phase, to study the enantio-separations of two basic chiral β-blockers, propranolol and pindolol, and one basic aminoacid, tryptophan (Figure 2).

3.2 | Capillary electrophoretic measurements in APTES coating

With the purpose of studying the stability of the APTES coating, the EOF movement was monitored using DMSO marker by six successive runs at pHs 2.5, 3.5, 4.5, and 5.5 (Figure 3A) and at 15, 25, and 37°C (Figure 3B). Above pH 5.5, the coating turns unstable, and the measurement of the EOF was imprecise. The conditions selected to perform the following experiment were pH 2.5 and 15°C.

3.3 | Chiral separation in APTES-heparin coated capillary

Despite the stability of the covalent and non-covalent heparin, the coating of the capillary exceeded our expectation. The separation of the chiral β-blockers and tryptophan would have been impossible without the addition of heparin in the BGE, even when heparin was present attached to the capillary (Figure 4).

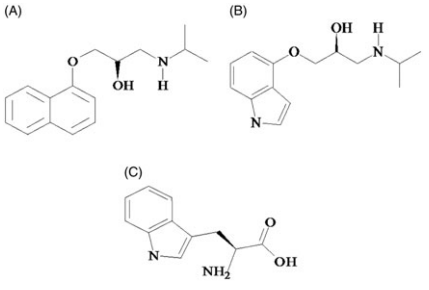

FIGURE 2 Chemical structures of drugs investigated. A, Propranolol; B, pindolol; C, tryptophan

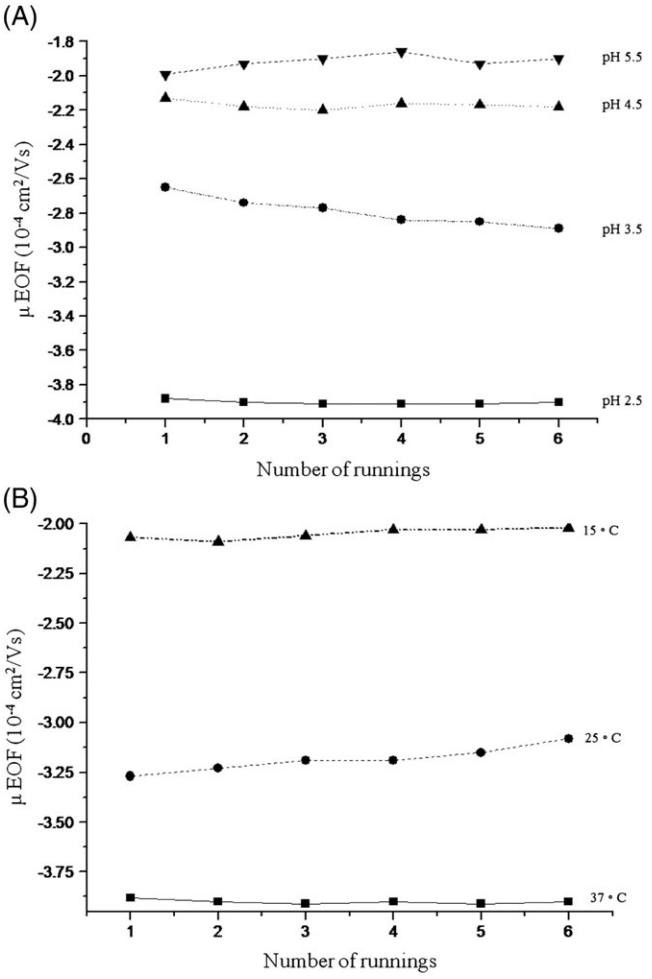

FIGURE 3 A, Behaviour of the μEOF related to different pHs. Running conditions: 30 mM phosphate buffer; applied voltage, 10 kV; temperature, 15°C; injection mode injection 0.5 psi during 10 seconds, detection at 214 nm; B, Behaviour of the μEOF related to different temperatures. Running conditions: 30 mM phosphate buffer, pH 2.5; applied voltage, 10 kV; injection mode injection 0.5 psi during 10 seconds, detection at 214 nm

3.4 | Chiral separation in APTES-coated capillary with heparin as a pseudo stationary phase

As it was mentioned earlier, this study includes the chiral separation with the aggregation of heparin as a pseudo stationary phase in a bared capillary and in an APTES coated capillary. The concentration of the heparin added to the BGE is essential to optimize the separation process. Four concentrations were evaluated (5, 10, 15, 20 mg/mL). The separation was not successful with the bared capillary. A better separation between the two enantiomers (propranolol and pindolol) and tryptophan was observed with an APTES-coated capillary and the addition of heparin to the BGE. In this sense, the amount of heparin added to the BGE was evaluated in terms of

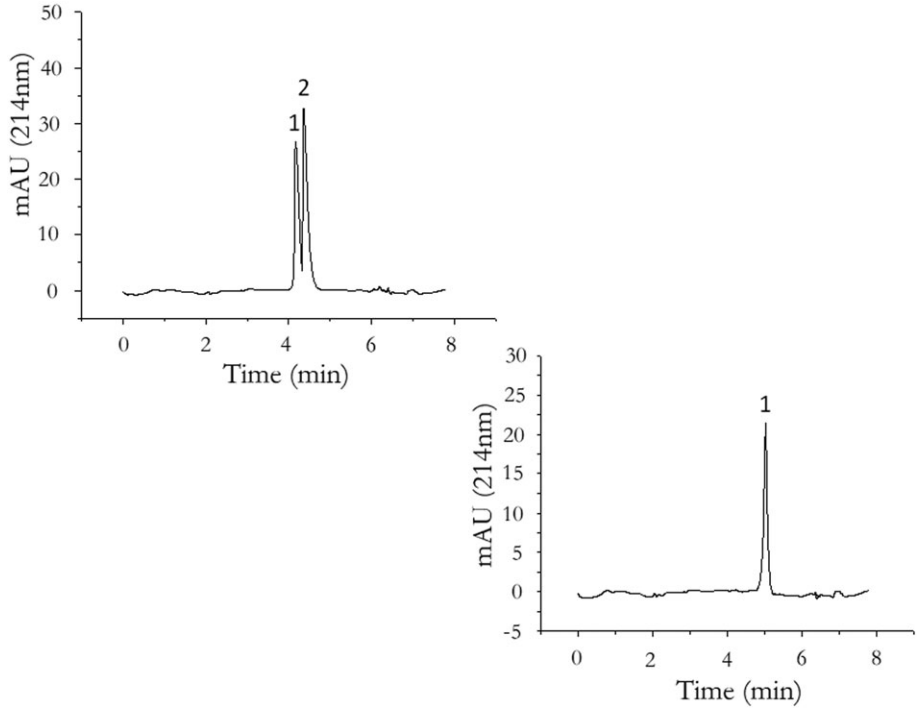

FIGURE 4 Electropherograms of the unsuccessful chiral separation of A, propanolol (peak 1) and pindolol (peak 2) and B, tryptophan using heparin as a stationary phase. Running conditions: 30 mM phosphate buffer, pH 2.5; applied voltage, 10 kV; temperature, 15 °C; injection mode: hydrodynamic, injection 0.5 psi during 10 seconds, detection at 214 nm

resolution (Table 1). As it was a convenient choice, the separations were performed with the addition of 15 mg/mL of heparin in the BGE. The presence of the heparin affects the electroosmotic flow by altering the ionic strength as well as the conductivity and viscosity of the buffer. Although the increase in ionic strength and viscosity from the addition from heparin tends to decrease the electroosmotic flow, this may be partly compensated by the increased conductivity, hence, mobility of the analytes. Also, further increases in heparin concentration were limited by concomitant increases in current.[38]

Table 2 shows the results, in terms of resolution, of the chiral separation under different conditions. The best separation and the working conditions were phosphate buffer 30 mM, pH 2.5, temperature 15°C, and voltage 10 kV with the addition of 15 mg/mL of heparin. The results of enantiomeric separations by CE with 15 mg/mL of heparin as a pseudo stationary phase are shown in Figures 5 and 6.

TABLE 1 Enantiomeric resolution with different conditions of heparin added to the BGE.

Heparin concentration (mg/mL)	R_s		
	Pindolol	Propranolol	Tryptophan
5	0.26	0.44	0.52
10	0.64	0.66	0.73
15	1.15	1.21	1.38
20	0.97	1.21	1.13

3.5 | Evaluation of the separation process

In order to consider the reliability of the results obtained during the chiral separation, we evaluated the repeatability ($n = 6$ in the same capillary) and the reproducibility (day-to-day) of the separation process. The results showed that the average retention time ($n = 6$) were 6.04 ± 0.02 minutes (peak 1) and 6.09 ± 0.02 minutes (peak 2) for propanolol and 6.24 ± 0.03 minutes (peak 3) and 6.29 ± 0.03 minutes (peak 4) for pindolol with an RSD lower than 0.4%. The reproducibility of the separation was measured during three successive days ($n = 6$), and the RSD was no more than 1.2%, confirming that the results of the separation remained stable at least for three days.

In aqueous solutions, heparin forms a helical structure in the same manner as in α-(1,4)-linked dextrins.[31] It has been postulated that the presence of the sulphate groups[39] as well as the heparin helical superstructure[40] both contribute to the enantiomeric separations. Bearing in mind the quaternary structure adopted by heparin, we could speculate that when heparin is added to BGE, the sulphate groups are exposed in the solution and, thus, are accessible to interact with chiral compounds. When heparin is attached to the inner phase of the capillary,

TABLE 2 Enantiomeric resolution of propranolol and pindolol with different running conditions using heparin as a pseudo stationary phase. Running conditions: a-pH 2.5, applied voltage 10 kV, temperature 15 °C, Injection mode injection 0.5 psi during 10 s, detection at 214 nm. b- Running conditions: 30 mM phosphate buffer, applied voltage 10 kV, temperature 15 °C, Injection mode injection 0.5 psi during 10 s, detection at 214 nm. c- Running conditions: 30 mM phosphate buffer, pH 2.5, temperature 15 °C, Injection mode injection 0.5 psi during 10 s, detection at 214 nm.

	[a]Buffer concentration (mmol/L)				[b]Buffer pH				[c]Voltage (kV)		
R_s	20	30	40	50	5.5	4.5	3.5	2.5	20	15	10
Propranolol	0.13	1.15	0.50	0.44	-	-	0.43	1.15	0.24	0.77	1.15
Pindolol	0.15	1.21	0.70	0.67	-	-	0.64	1.21	0.55	0.75	1.21

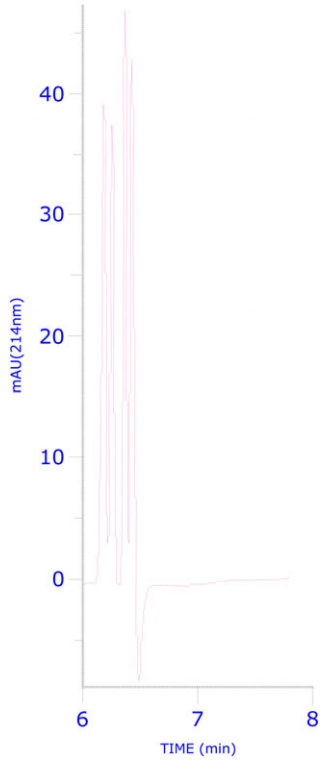

FIGURE 5 Electropherogram of the chiral separation of propranolol and pindolol using heparin as a pseudo stationary phase in an APTES-coated capillary. Running conditions: 30 mM phosphate buffer, pH 2.5; applied voltage, 10 kV; temperature, 15°C; injection mode: hydrodynamic, injection 0.5 psi during 10 seconds, detection at 214 nm

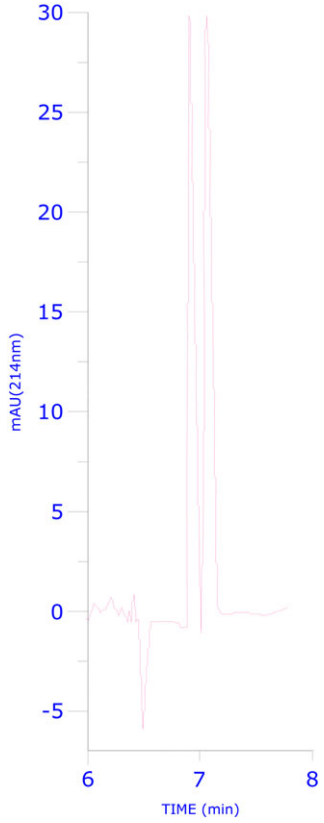

FIGURE 6 Electropherogram of the chiral separation of tryptophan using heparin as a pseudo stationary phase in an APTES-coated capillary. Running conditions: 30 mM phosphate buffer, pH 2.5; applied voltage, 10 kV; temperature, 15°C; injection mode: hydrodynamic, injection 0.5 psi during 10 seconds, detection at 214 nm

the sulphate groups could stay hidden in the structure although the carboxylic groups are the ones which take part of covalent bonding with silanols of the capillary. In this way, the access for the chiral compounds to the sulphate groups became more delicate. Moreover, the negative results in electrostatic interaction between heparin and APTES could be explained due to the fact that both carboxylic and sulphate group take part in the interaction resulting in a few negatively charged groups available to interact with the chiral compounds. As a final point, we can postulate that chiral selection capacity of heparin could be based on the interaction of the chiral compound, not only with the sulphate groups, but also with the carboxylic groups. The moment when the carboxylic groups take part of the covalent interaction, heparin loses its capacity as chiral selector.

Having in mind the explanation given above, it is logical to suggest that the affinity of heparin is higher for the tryptophan than the β-blockers due to the exposition of

the amino groups in the amino acid structure, while paying attention to the ionic interaction between the hydroxyls groups and amino groups of APTES.

4 | CONCLUSION

The present work presents a separation procedure in which heparin are added to the BGE as a pseudo stationary phase. Although the use of heparin as a pseudo stationary phase has been previously described by other colleagues, in this work the separation procedure is improved thanks to the presence of APTES attached to the inner phase of the capillary. In general, stronger interactions, as evidenced by higher resolution, were observed between heparin and tryptophan. The method described in this study are very promising in terms of resolution and reproducibility. Further work including expanding the number and types of solutes to assist in elucidating solute structural features required for successful enantioselective interactions, examining the role of the molecular weight, and the degree of sulfation for heparin must be done.

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