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An exploratory study of enantioselective behavior of Sol-Gel encapsulated human serum albumin using frontal analysis

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Abstract: Adsorption behavior of pure enantiomers and racemic mixtures of nonsteroidal anti-inflammatory drugs (ibuprofen and naproxen) on human serum albumin (HSA) was evaluated. The HSA was immobilized by Sol-Gel technique and this biomaterial was used in a chromatographic system where frontal analysis experiments were performed at pH 7.4 and temperatures of 25°C and 37°C. The association constants for enantiomers of the drugs were determined by linear adjustment for data corrected just for dead volume. In uncorrected data for non-specific retention, an inverse ratio between the number of sites and the value of the association constant was found. The participation of non-specific retention was estimated by non-linear regression and the values of association constants (Kass), which were determined considering this information, are comparable to some values reported by other methods at 37°C: 1.4 $x10^5$ and 5.7 $x10^4$ for Ibuprofen (IBU) R and S, respectively, and 2.3 $x10^5$ and $1.8x10^5$ for naproxen (NX) R and S, respectively.

Keywords: Association constant, frontal analysis, Sol-Gel, enantioselectivity, Chiral stationary phase.

INTRODUCTION

The HSA binds a broad variety of endogenous ligands. Many, mainly acidic, drugs also bind to HSA at one of two primary sites, either at warfarin-azapropazone (site 1) or at benzodiazepine (site 2) (Ghuman *et al*., 2005; Peyrin *et al*., 1999).

The HSA enantioselectivity has been observed for enantiomers of different compounds and, therefore, it is not surprising that it has been broadly used as chiral stationary phase (CSP) in liquid chromatography (Zhivkova and Russeva, 1998; Haginaka, 2001; Haginaka, 2008; Buchholz *et al*., 2002; Oravcava *et al*., 1996; Mallik and Hage, 2006; Loun and Hage, 1994; Li and Hage, 2017).

There are different techniques to study enantioselective behavior of HSA. Classical methods are equilibrium dialysis and ultrafiltration. Recent researches have utilized circular dichroism and high pressure affinity chromatography, however, the results obtained by diverse methods exhibit technique-related differences as well as those relative to experimental conditions (Zhivkova and Russeva, 1998; Hage *et al*., 1995; Cheruvallath *et al*., 1997, Itoh *et al*., 1997). For instance, in the case of warfarin some authors report a low enantioselectivity of HSA for this drug (Ghuman *et al*., 2005; Petitpas *et al*., 2001), but in other papers, sufficient HSA enantioselectivity has been found as to consider the HSA a useful chiral stationary phase for separating R and S forms of warfarin (Kim and Wainer, 2008). Cheruvallath determined values for association constant (K_{ass}) of ibuprofen and naproxen enantiomers using microcalorimetry and circular dichroism; in both cases, HSA exhibited enantioselectivity but the values obtained for K_{ass} of enantiomers by either one or the other method show some differences (Cheruvallath *et al*., 1997; Cheruvallath *et al*., 1996; Vuignier *et al*., 2013; Lammers *et al*., 2013).

In his review of chiral recognition, Lämmerhofer explains that experimental conditions, such as pH, buffer type, ionic strength, presence of organic modifiers, additives and temperature, are variables that regulate retention and enantioselectivity (Lämmerhofer, 2010); notwithstanding this, the basis for enantioselectivity has its origin in an association constant that is different for each enantiomer, whereas the chemical environment, solute molecules and adsorbent surface may assist the chiral recognition. It is thereby possible that the variation in values reported by Kass is due to variability of experimental conditions utilized in different research investigations (Haginaka, 2001; Loun *et al*., 1994; Vuignier *et al*., 2013; Lammers *et al*., 2013; Fornstedt *et al*., 1997; Yao *et al*., 2017).

Several techniques for protein immobilization have been reported as chiral selectors; the covalent bonding to the support often allows obtaining columns with active and stable proteins (Yang *et al*., 1996; Hage *et al*., 2012; Hage, 2006). Recently, Sol-Gel encapsulation technique for biomaterial development has attracted the attention because of some advantages. This process takes place

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under very soft conditions at room temperature reducing damage to the biomolecule; in addition, this latter is not bound to the support surface and, therefore, there are no orientation problems and the active site of the protein to act on the target molecule is not hidden (Vazquez-Lira *et al* ., 2003; Jin and Brennan, 2002).

Notwithstanding the foregoing, the silica-based supports appear to have some problems since surface silanol groups present non-specific retention and the trapped biomolecule loses certain percentage of its native conformation. Furthermore, these biomaterials do not resist high pressure of a normal chromatographic system at a relatively normal flow. However, the Sol-Gel method has been used in the molecules where covalent bonding is not possible (Calleri *et al*., 2011).

In this study, bioaffinity columns with immobilized HSA were developed by traditional Sol-Gel method; also, the usefulness and characteristics of this biomaterial in its capacity to differentiate values of association between enantiomers and racemic mixtures of naproxen and ibuprofen were explored by frontal analysis using a normal chromatographic system at moderate flow and pressure conditions.

Theory

Frontal analysis can be applied for determining the association constant between immobilized HSA and the analyte. The breakage curve displacement takes place at shorter times as the analyte concentration increases. The simplest analysis considers the interaction of just one type of binding site and takes into account two variables: analyte concentration passing through the biomaterial and mean position of the breakage curve (stoichiometric point). The affinity column capacity and association constant are calculated using Lineweaver-Burk equation (double reciprocal; equation 1) (Yang and Hage, 1996; Hage, 2002). However, it is common for affinity columns to present heterogeneity caused by the presence of two types of binding sites: selective high-affinity sites (due to immobilized ligand) and non-selective low-affinity sites associated with the support (Samuelsson *et al*., 2009). The bi-Langmuir model has been successfully used to describe this process. At low analyte concentrations, a simplified version if this model is equation (2). The term ($1+K_2[F]$) tends towards 1 given that K_2 is small and [F] is very low, resulting in the simplification of bi-Langmuir equation to equation 2 (Mallik *et al*., 2008).

$$\frac{1}{\mathrm{m_{app}}} = \frac{1}{(\mathrm{K_{ass} m_{sat}[F]})} + \frac{1}{\mathrm{m_{sat}}} \qquad 1$$

m_{app} are drug moles retained by equilibrium biogel
K_{ass} is drug/protein association constant
$[F]$ is molar concentration of drug in percolated solution m_{sat} represents moles of total active sites

$$m_{app} = \frac{\mathrm{m_{L1}}\ K_{a1}[F]}{1+K_{a1}[F]} + m_{l2}K_2[F] \qquad 2$$

m_{L1} represents moles of enantioselective active sites
K_{a1} is selective association constant
m_{12} are moles of non-specific binding sites
K_2 is non-specific binding constant

MATERIALS AND METHODS

Reagents

The Sigma-Aldrich fatty acid free HSA, Tetraethoxisilane (TEOS, 99% FLUKA), was used as precursor for Sol-Gel synthesis. Sigma-Aldrich racemic mixture of Ibuprofen and Naproxen and isomers (S) and Toronto-Research Chemical Inc. isomers (R) were utilized as well. The other reagents were J.T. Baker analytical grade reagents. All reagents were used without additional purification. The water was obtained from Barnstead Thermolyne 04747 model deionizer.

Sol-Gel Encapsulation

The encapsulation procedure employed herein was previously reported (Vera-Avila *et al*., 2008; Sakai-Kato *et al*., 2009), but in our case the amount of immobilized protein in the biomaterial was substantially modified from 0.7mg to 15mg in order to achieve a significant displacement of the position of analyte fronts. The first stage, precursor hydrolysis (stage in which Sol is formed), is carried out with 2.5mL TEOS, 0.1mL 0.1 M HCl and 0.4mL water. The mixture is stirred and sonicated in an ice bath for 30 minutes. Afterwards, it is left under continuous stirring by mechanical stirrer at room temperature for 4 hours.

In the second stage, a change in pH and ionic strength is provoked in order to initiate the gelation. In order to achieve HSA encapsulation, immediately prior to gelation, 2mL phosphate buffer (0.1 M) pH 7.0 with 0.9% NaCl (PBS) are added under constant stirring, followed by the addition of 1mL HSA solution at a concentration of 15mg/mL; the gelation takes place in 2-3 minutes. The hydrogel formed in this way is left to rest 10 minutes in PBS and then is cut into pieces, filtered and washed.

The maturing stage is a process of drying and aging carried out at 4°C, where hydrogel is left to lose up to 50% of weight under these conditions. The obtained xerogel is pulverized in a mortar and re-suspended in a phosphate buffer solution (0.04M), pH 7.4, and packed into 50x4.6 mm stainless steel columns.

Frontal analysis

A binary Knauer chromatograph provided with Smartline pump 1000, UV-Diode Array 2600 Detector and Eurochrom data acquisition software was used.

Table 1: (overall) kass calculation of biomaterial enantioselectivity data using equation 1

Analyte	Temperature	Kass ± ee (n=3)	Number of binding sites ± ee (n = 3)	r	α
RS-IBU	25°C	$8.7 \pm 0.92 \times 10^{4}$	$3.3 \pm 0.18 \times 10^{-8}$	0.993	
RS-IBU	37°C	$8.8 \pm 0.8 \times 10^{4}$	$3.3 \pm 0.24 \times 10^{-8}$	0.996	
R-IBU	25°C	$1.3 \pm 0.13 \times 10^{5}$	$2.9 \pm 0.21 \times 10^{-8}$	0.992	1.46
S-IBU	25°C	$7.4 \pm 1.3 \times 10^{4}$	$3.5 \pm 0.5 \times 10^{-8}$	0.993	
R-IBU	37°C	$9.5 \pm 0.23 \times 10^{4}$	$2.9 \pm 0.07 \times 10^{-8}$	0.992	1.34
S-IBU	37°C	$2.05 \pm 0.2 \times 10^{4}$	$1.0 \pm 0.06 \times 10^{-7}$	0.994	
RS-NX	25°C	$1.17 \pm 0.02 \times 10^{5}$	$3.4 \pm 0.08 \times 10^{-8}$	0.999	
RS-NX	37°C	$3.4 \pm 0.3 \times 10^{5}$	$1.13 \pm 0.07 \times 10^{-8}$	0.995	
R-NX	25°C	$2.7 \pm 0.2 \times 10^{5}$	$1.7 \pm 0.03 \times 10^{-8}$	0.984	1.33
S-NX	25°C	$1.5 \pm 0.2 \times 10^{5}$	$2.3 \pm 0.23 \times 10^{-8}$	0.994	
R-NX	37°C	$2.15 \pm 0.2 \times 10^{5}$	$1.73 \pm 0.03 \times 10^{-8}$	0.997	1.13
S-NX	37°C	$1.5 \pm 0.08 \times 10^{5}$	$2.2 \pm 0.1 \times 10^{-8}$	0.992	

Table 2: Association constants estimated using equation 2 and considering heterogeneous system and α selectivity of HSA

Analyte	Temperature	Kass ± ee (n = 3)	α	SS
RS-IBU	25°C	$1.38 \pm 0.07 \times 10^{5}$		1.23×10^{-17}
RS-IBU	37°C	$1.39 \pm 0.06 \times 10^{5}$		1.16×10^{-17}
R-IBU	25°C	$1.88 \pm 0.04 \times 10^{5}$	1.64	1.62×10^{-17}
S-IBU	25°C	$1.15 \pm 0.05 \times 10^{5}$		5.4×10^{-18}
R-IBU	37°C	$1.42 \pm 0.04 \times 10^{5}$	2.5	5.9×10^{-18}
S-IBU	37°C	$5.67 \pm 0.2 \times 10^{4}$		5.3×10^{-18}
RS-NX	25°C	$2.4 \pm 0.03 \times 10^{5}$		6.3×10^{-18}
RS-NX	37°C	$3.6 \pm 0.08 \times 10^{5}$		1.98×10^{-17}
R-NX	25°C	$2.93 \pm 0.14 \times 10^{5}$	1.67	1.11×10^{-17}
S-NX	25°C	$1.75 \pm 0.06 \times 10^{5}$		8.85×10^{-18}
R-NX	37°C	$2.32 \pm 0.04 \times 10^{5}$	1.3	1.62×10^{-18}
S-NX	37°C	$1.78 \pm 0.06 \times 10^{5}$		9.9×10^{-18}

Table 3: Comparison with reported data for association constants of racemic and pure enantiomers

	R-IBU	S-IBU	RS-IBU	R-NX	S-NX	RS-NX
Kass (25°C)	2.9×10^{5}	1.1×10^{5}	1.4×10^{5}	1.6×10^{5}	1.5×10^{5}	5.5×10^{5}
Method	MC*	HPAC***	CD**	CD**	MC*	CD**
Reference	Cheruvallatha *et al.*, 1996	Hage *et al.*, 1995	Cheruvallath *et al.*, 1997	Cheruvallath *et al.*, 1997	Cheruvallath *et al.*, 1996	Cheruvallath *et al.*, 1997
Kass (37°C)			$1.15\text{-}2.7 \times 1.0^{3}$			
Method			*In vivo*			
Reference			Lockwood *et al.*, 1985			
Kass in this work	1.9×10^{5} (25°C)	1.15×10^{5} (25°C)	1.38×10^{5} (37°C)	2.9×10^{5} (25°C)	1.75×10^{5} (25°C)	2.4×10^{5} (25°C)

*Microcalorimetry
**Circular Dichroism
***High performance affinity chromatography

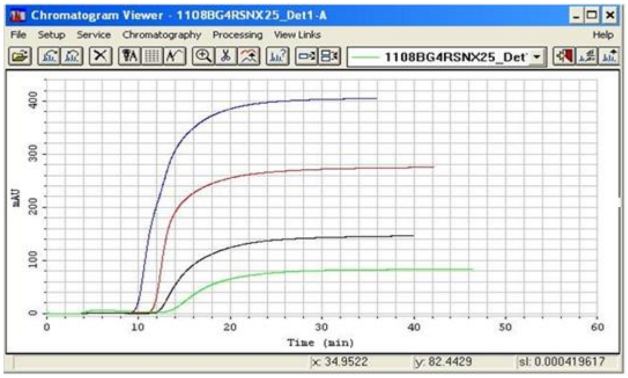

Fig. 1: Typical frontal analysis curves obtained for RS-Naproxen at 25°C on immobilized HSA column. Sodium phosphate buffer 0.04M pH 7.4, flow rate 0.3 mL/min.

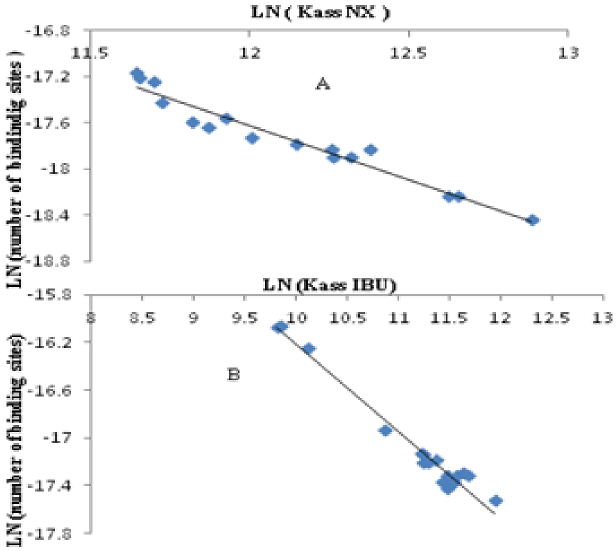

Fig. 2: Plot of LN Kap vs LN sites (A Naproxen) equation: LN Sites=-6.39-0.936 (LN Kass), (B Ibuprofen) equation: LN site= -8.89-0.733 (LN Kass)

Frontal analysis was performed by continuous application of 1.4 to 9 µM solutions of each of ibuprofen or naproxen isomers or racemic mixture to the columns with immobilized HSA at a flow rate of 0.3 mL/minute in a 0.04 M phosphate buffer solution with 0.9% NaCl, pH 7.4. The amount of (R) and (S) isomers or of racemic mixture necessary to saturate the column at each level of concentration was determined from the mean point of the obtained breakage curve. The data was corrected for dead volume determined by a lithium nitrate solution breakage curve.

The experiments were run in triplicate and the temperature was controlled by a phenomenex EHO-7057 controller at 25°C and 37°C. Eluent detection was at 220 nanometers for ibuprofen and at 230 nm for naproxen.

Calculations

Initially, data were fitted to equation 1, double reciprocal, by linear regression. Afterwards, they were analyzed considering heterogeneity of the column according to equation 2 (Matsuda *et al.*, 2015; Hage, 2017), using Statgraphics Centurion XVI program for non-linear adjustment. Adjustment evaluation was performed by calculating the residual sum of squares using equation 2 to make the estimates.

RESULTS

In this study, frontal analysis was used to evaluate the behavior of enantiomers and racemic mixtures of non-steroidal anti-inflammatory drugs (ibuprofen and naproxen) in combination with synthesized biomaterial by calculating the association constant and the number of active sites. fig. 1 shows the case of racemic mixture of naproxen at 25°C. Frontal analysis turned out to be similar for all enantiomers and racemic mixtures at both temperatures tested; the only differences were observed in the positions of breakage curves.

Data obtained on the mean position of breakage curves corrected for dead volume were analyzed in accordance with equation 1. Each plot obtained in that way presented a linear adjustment in the interval of tested concentrations with correlation coefficients ranging from 0.9842 to 0.999 [table 1]. The estimation of selectivity (α) such as the ratio of association constants of the most retained over the least retained enantiomer, corrected for the number of sites obtained from the intercept (Loun and Hage, 1994), presented in table 1, is 1.4 and 1.34 for ibuprofen at 25°C and 37°C, respectively and 1.33 and 1.13 for naproxen at 25°C and 37°C, respectively, showing a higher retention for R-isomer in both cases.

Theoretical basis for application of double reciprocal equation (eq. 1) suggests independence in the determination of association constants and the number of sites (2). In the obtained results, the behavior of active sites, determined from intercepts of linear adjustments for ibuprofen and naproxen enantiomers and racemic mixtures seems to exhibit one tendency: as the value of association constant decreases, the number of binding sites is seen to increase. fig. 2 shows an adjustment between the natural logarithm of association constant and the natural logarithm of the number of sites, which contradicts the supposed independence between parameters. (A) Naproxen, (B) Ibuprofen; correlation coefficients of these adjustments were 0.978 and 0.985 respectively.

In the application of simplified bi-Langmuir equation, eq. 2, for low solute concentrations: sites (m_{l2}) are considered unsaturated, K_2 has a very small value under conditions of very low [F] (Mallik *et al*., 2008). Data were adjusted to equation 2 by non-linear regression. For simplicity and adjustment purposes, some parameters were considered equal between enantiomers, such as K_2m_{l2} (assuming that

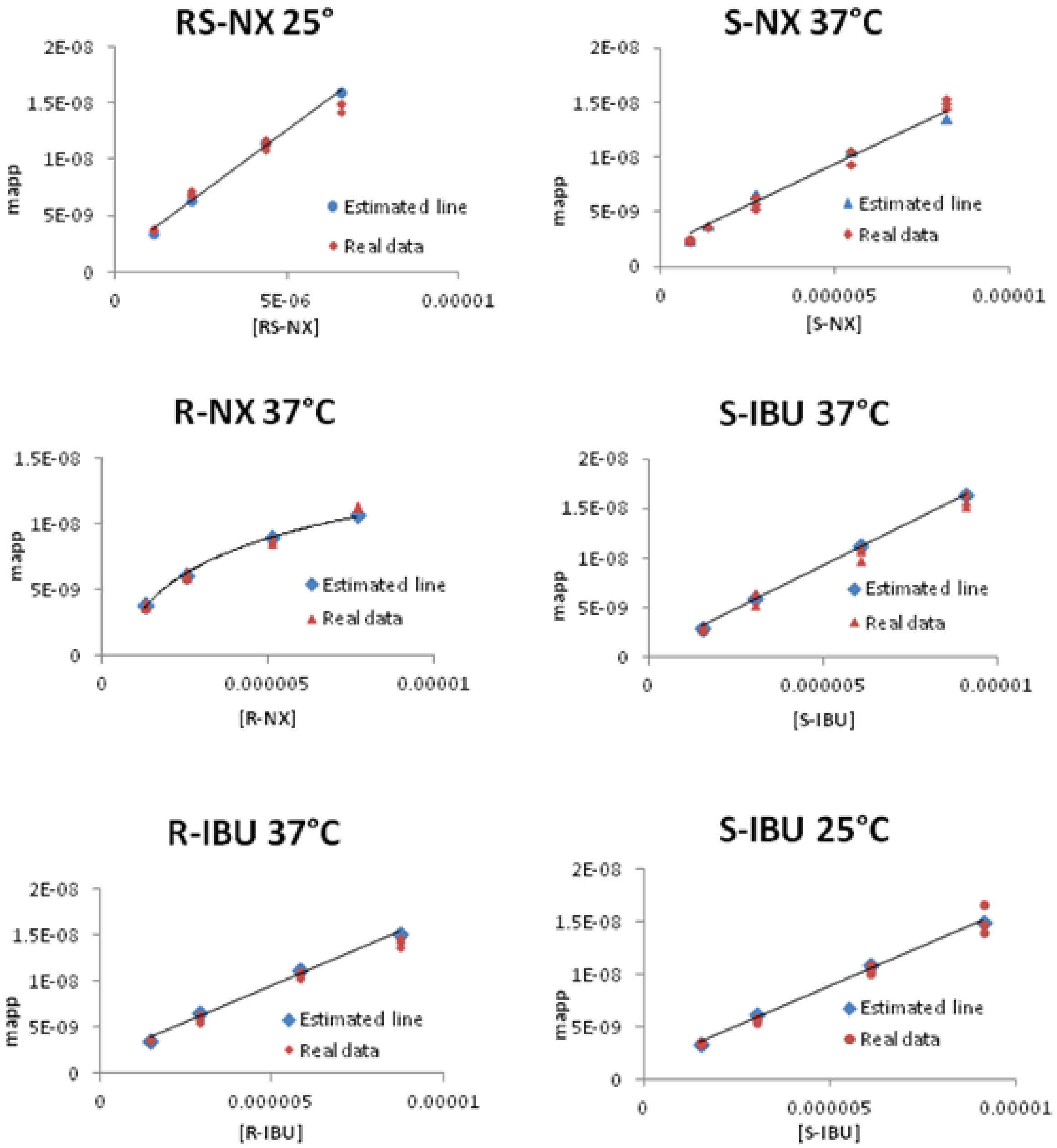

Fig. 3: Line plots estimated using simplified bi-Langmuir equation (2) and experimental points on the estimate.

these are non-enantioselective sites, they should be equal for both enantiomers). The parameters calculated using equation 2 were employed to make estimates for "m_{app}" term according to equation 2 and comparisons with experimentally obtained data; the residual sum of squares is given in table 2 and some plots that exemplify adjustment are given in fig. 3.

DISCUSSION

The exploration of data adjustments to equation 1 allows noting that different values for association between enantiomers are obtained in this biomaterial, which means selective capacity of the biomaterial under experimental conditions employed. Given the values of (α) shown in table 1, under such conditions a moderate separation of enantiomers would be achieved.

The reason for the tendency observed between the overall association constant value and the number of binding sites calculated with equation 1 (fig. 2) is not clear. Since the column preparation method was exactly the same in all cases, some random experimental variability is to be expected regarding an average number of sites, however a ratio between the value determined for association and the number of sites should have another explanation that could be related with column heterogeneity (Samuelsson *et al*., 2009; Mallik *et al*., 2008; Tweed *et al*., 1997; Tong *et al*., 2011) and the fact that this analysis only allows obtaining values of overall association constants where a combination of specific and non-specific sites is present. The information reported by other researchers (Tweed *et al*., 1997; Tong *et al*., 2011) shows that biomaterial retention involves non-specific interaction that can be significant, so the analysis should consider two types of adsorption sites: one of low energy between the analyte and non-chiral parts of protein and support and the second, of highly selective high-energy interactions responsible for enantiometric separation. The proportion of selective sites is smaller than that of non-selective sites; therefore, their saturation capacity is much lower, but their energy is much higher. Non-selective sites do not get saturated and their binding energy is very small but since they are numerous, their retention is significant.

In the adjustments carried out considering the biomaterial, an HSA-drug Kass comparable to the reported values could be expected, since attempts have been made to separate the adjustment of non-specific interferences and its proximity to reported values (table 3) indicates successful adjustment. However, more experiments defining better non-specific participation are recommendable in order to attain more precise data. When making this comparison, it is important to take into account that the association constant value, reported by different researchers, is also influenced by the techniques and experimental conditions employed; (Cheruvallath *et al*., 1997; Itoh et L., 1997; Petitpas *et al*., 2001; Yao *et al*., 2017).

Finally, upon comparing the results of tables 1 and 2 it is inferred that one way to significantly increase biomaterial enantioselectivity is to reduce non-specific retention without affecting specific retention; the factor (α) considered as the ratio of association constants shows values between 1.13 and 1.46 in table 1, whereas in table 2 these range from 1.3 to 2.5. A greater selectivity for both compounds at room temperature can be observed in table 1. However, table 2 shows that in the case of ibuprofen, specific association of S-isomer diminishes considerably as compared to R-isomer due to the increase in temperature, which gives rise to a greater biomaterial selectivity at 37°C. As the contribution of non-specific retention is so significant, biomaterial selectivity is not properly detected at 37°C in the overall Kass.

CONCLUSION

The evaluated biomaterial proved to be capable of providing different overall association constants for ibuprofen and naproxen enantiomers. However, under conditions of non-specific interaction, the separation would be poor. The association constant determined by double reciprocal equation (without considering non-specific interaction) presents an inverse ratio with moles of binding sites. This dependence between parameters seems to be related to the importance of the non-specific interaction participation in overall analyte retention.

The correct estimation of the association constant between the drug and HSA in affinity chromatography using Sol-Gel method should include an estimation of the participation of non-specific retention, because of its significant role in overall analyte retention.

Frontal analysis employed with this biomaterial prepared by Sol Gel method allowed obtaining results comparable to those achieved by using high-pressure affinity chromatography (HPAC) and other techniques with the advantages characteristic of Sol Gel.

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