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A novel biochromatographic Oatp2 column to study the transmembrane transport of statins

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ABSTRACT

In a previous paper, using a biophysical model system to study the passive diffusion of the statin molecules through the cell membrane, our group demonstrated that statins could cross biological membrane by passive diffusion (Sarr et al. [40]). However, in the liver, the uptake of statins would also be mediated by organic anion transporting polypeptides (Oatps) like Oatp2 a member of this family. Thus, a novel biochromatographic approach was developed in our laboratory to study the transmembrane transport of statins and an Oatp2 inhibitor via this carrier family. For this, the Oatp2 protein was immobilized via its amino groups on a chromatographic support using an "in situ" immobilization technique. For the first time, using this novel biochromatographic concept, the effect of magnesium chloride salt ($MgCl_2$) on the pharmacomolecule–Oatp2 binding was investigated. It was shown an Mg^{2+}-dependent pharmacomolecule–protein association and a potential facilitated diffusion of these pharmacomolecules into biological membrane. This association process was due to the central positive potential pore of the Oatp2. Indeed, at pH 7.4, all the pharmacomolecules studied were ionized (i.e. negatively charged) and so interact with this positive potential pore. However, an increase of the Mg^{2+} concentration led a decrease of the pharmacomolecule–Oatp2 association attributed to ion pair formations between the Mg^{2+} cation and molecules. Moreover, the decrease of this affinity could be explained by an ion attraction between the Cl^- anion of the $MgCl_2$ salt and the positively charged pore of the protein. This novel biochromatographic column could be useful to find a specific reversible inhibitor for these transporters and so open new perspectives to be investigated.

1. Introduction

Statins or HMG-CoA-reductase inhibitors are widely used in clinics [1,2]; therefore it is very important to know their pharmacologic and pharmacokinetic processes in human because the ability to cross biological membrane (by passive or active diffusion) affects strongly the pharmacokinetic behaviour of drugs and their capacity to access the receptor site. It is well known that, after oral administration, drugs are absorbed and distributed throughout the body by passive diffusion processes. For many drugs like statins, membrane transporters limit or facilitate these processes in an active way. For the liver, the uptake of statins is mediated by organic anion transporting polypeptides [3,4]. As well, many recent publications have been demonstrated the active uptake of many statins, like pitavastatin, fluvastatin, pravastatin and rosuvastatin by the organic anion transporting polypeptides into the liver [5–7]. Oatps form a superfamily of sodium-independent transport systems that mediate the transmembrane transport of a wide range of compounds [8,9]. They play an important role in drug absorption, disposition, and excretion [4,10]. Oatps proteins are highly expressed in the physiological barriers in the body, including the liver, intestine, kidney, endothelial cells of the brain capillaries and epithelial cells in the choroid plexus [5,11,12]. These transporters are generally believed to function as electroneutral anion exchangers [13]. One of these families consists of the Oatp-related transporters and includes organic anion transporting polypeptide 2 (Oatp2; Slc21a5) which is a liver transporter that mediates the uptake of a variety of structurally diverse compounds [14–16]. A study of Li et al. demonstrated that Oatp2 is a bidirectional transporter and can mediate either net uptake or efflux of organic solutes [15]. Based on hydropathy analysis, this family of transporter peptides consists of membrane proteins with 12 putative membrane-spanning domains [17]. They function as sodium-independent exchangers or facilitators [17,18].

The uptake of some phamacomolecules by the Oatp2 transporter was abolished or decreased by some compounds such as rifamycin. Indeed, many studies have been demonstrated that this hydrophobic antibiotic exhibits differential inhibition on Oatp2 and interfere with organic anion uptake [19,20]. However, several

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questions remain unanswered such as the development of specific inhibitors for individual Oatps and the elucidation of interactions of Oatps with other proteins [11].

Magnesium plays important roles in the structure and the function of the human body. Magnesium is involved in more than 300 essential metabolic reactions [21]. Recent work showed that the beneficial effects of the statin drugs are paralleled and complemented by those of magnesium [22]. Indeed, Mg^{2+}–ATP is the controlling factor for the rate-limiting enzyme in the cholesterol biosynthesis sequence that is targeted by the statin pharmaceutical drugs [22]. Several results support the hypothesis that defective membrane function could be the primary lesion underlying the cellular disturbances that occur in Mg deficiency [23–25].

In this study, the effect of magnesium chloride salt on the statin and rifamycin/Oatp2 binding was analyzed using a novel biochromatographic approach to provide further insight into the role of ionic interactions in binding of these anionic substances to Oatp2 protein.

2. Experimental and methods

2.1. Apparatus

The HPLC system for these measurements consisted of a Hitachi L 7100 pump (Merck, Nogent sur Marne, France), a Rheodyne injection valve with a 20 μL sample loop (Cotati, CA, USA), and a Shimadzu UV–vis detector. The MODULO-CART HS UPTISPHERE 3 NH_2 (50 mm × 4.6 mm ID), where the amine support (terminal $–NH_2$ groups) was bound onto 3 μm silica particle size of 120 Å pore diameter, was purchased from Interchim (Montluçon, France).

2.2. Reagents

Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. Pravastatin (prava), mevastatin (meva), atorvastatin (atorva), fluvastatin (fluva), and rifamycin (rifa) were purchased from Sigma and VWR (Paris, France). The four statins and rifamycin were depicted in Fig. 1. Oatp2 was obtained from Interchim (Montluçon, France). *N,N′*-Disuccinylimidyl carbonate (DSC) was purchased from Sigma (Paris, France). Magnesium chloride was purchased from Merck (Paris, France). Potassium dihydrogen phosphate and dipotassium hydrogen phosphate used for the preparation of the mobile phases were of analytical grade and purchased from Prolabo and Merck (Paris, France). The preparation of the Oatp2 column via the "in situ" technique is given below.

2.3. Immobilization technique of Oatp2 on DSC-activated aminopropyl silica

The "in situ" immobilization technique was used to prepare a novel Oatp2 column in our laboratory. The immobilization of Oatp2 via the amino groups of the protein on to aminopropyl silica pre-packed column activated with DSC was carried out as follows [26,27]. Briefly, the column was first washed with acetonitrile at flow-rate 0.5 mL/min. Then the stationary phase was activated by recycling 2.25 g of DSC in 100 mL acetonitrile for 18 h at the same flow-rate followed by washing first with 60 mL acetonitrile and then with 60 mL of 1 mM phosphate buffer (pH 7.0). A solution of 500 μg (0.5 mg) Oatp2 was dissolved in 10 mL of a phosphate buffer (1 mM) and the protein solution continuously circulated through the column at a flow-rate of 0.3 mL/min. After 40 h the column was washed with 200 mL of 20 mM phosphate buffer (pH 7.4), 200 mL of a 0.5 M solution of NaCl and finally with 100 mL of a 0.2 M glycine solution to block the remaining activated groups.

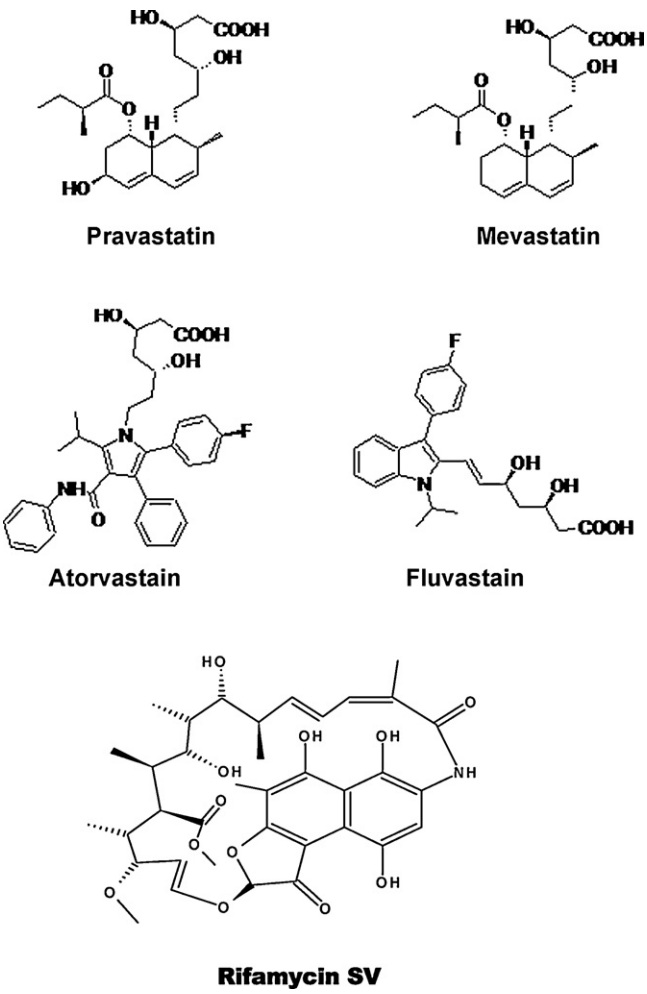

Fig. 1. Chemical structures of statins and rifamycin.

The column was rinsed with a 50 mM solution of phosphate buffer (pH 7.4). The total mass of immobilized protein (425 μg) in the column was determined by elemental analysis. For this analysis, four fractions of the stationary phase were removed from the head to the end of the column.

The maximum relative difference of the amount of immobilized protein between these different measurements was always 0.5% making a homogeneous protein distribution in the column from the ends to the core.

2.4. Chromatographic operating conditions

The mobile phase consisted of 0.05 M sodium phosphate buffer (pH 7.4; human plasma pH). The concentration range of Mg^{2+} varied, respectively, from 0 to 3 mM (including its biological concentration range 0.75–1.00 mM). 7 Mg^{2+} cation values (i.e. 0.0, 0.6, 1.0, 1.4, 1.8, 2.2 and 3.0 mM) were included in this range. Experiments were run over the temperature range 10–35 °C and at 254 nm and 300 nm detection wavelengths for, respectively, statins and rifamycin. For all the experiments, no phosphate buffer pH difference was observed. Throughout the study, the flow-rate was maintained constant and equal to 0.3 mL/min. 20 μl of the most concentrated sample was injected and the retention time was measured. For the determination of the adsorption isotherms (see below), for each statin or rifamycin studied, the equilibration of the column was carried out with 15 concentrations of solute molecule (0–7 μM) in the mobile phase to obtain a stable detection. 20 μL of

the most concentrated solute sample was injected at least 3 times and the retention time was measured.

2.5. Langmuir distribution isotherms

Using the Oatp2 stationary phase, statins and rifamycin could tightly bind to the matrix of the column. The determination of the Langmuir distribution isotherms was described in a previous study for the analysis of specific binding sites of a series of acetylcholine esterase inhibitors on an acetylcholine esterase stationary phase [28]. For this, the perturbation technique was used [29–32]. This method makes possible the determination of adsorption isotherms by measuring the retention times of small sample sizes injected onto a column equilibrated with sample solutions at different concentration levels. Briefly, the column is first equilibrated with a solution containing the compound dissolved in a non-adsorbable solvent. Then a small sample volume containing higher concentration of the compound is injected onto the column. After the injection, the equilibrium condition is disturbed and the perturbation waves reach the column outlet, a peak is registered by the detector. Therefore, if statins and rifamycin bound on two sites on the stationary phase, i.e. a specific site (site A with an adsorption constant K_A) and a column saturation capacity α_A and a second site which is non-specific (sites B with an adsorption constants K_B and a column saturation capacity α_B), then the solute retention factor (k) directly proportional to the slope of its adsorption isotherm is given by the following equation [29–32]:

$$k = \frac{t - t_0}{t_0} = \phi \frac{dC_S}{dC_m} = \phi \left(\frac{\alpha_A K_A}{(1 + K_A C_m)^2} + \frac{\alpha_B K_B}{(1 + K_B C_m)^2} \right) \quad (1)$$

Eq. (1) was fitted to the solute retention factor k by a non-linear regression and the parameters $k_A = \phi K_A \alpha_A$ and $k_B = \phi K_B \alpha_B$ corresponding to the retention contributions of the two kinds of sites under linear conditions were calculated. Valuable information about the processes driving statins (or rifamycin)–Oatp2 association mechanism can be further gained by examining the temperature dependence of statin and rifamycin retention [33,34]. Under linear conditions, the temperature dependence of the retention factor is given by the following relationship:

$$\ln\ k = \left(-\frac{\Delta H^\circ}{RT} \right) + \left(\frac{\Delta S^\circ}{R} \right) + \ln \phi \quad (2)$$

where R is the gas constant, T is the column temperature in Kelvin, ΔH° and ΔS° are, respectively, the solute enthalpy and entropy changes accompanying the transfer of the statins and rifamycin from the bulk solvent to the Oatp2 surface. If the Oatp2 stationary phase, statins and rifamycin and solvent properties are temperature invariant, a linear van't Hoff plot is obtained and from the slope ΔH° and ΔS° can be calculated.

3. Results and discussion

3.1. Langmuir distribution isotherms and column stability

As immobilization of Oatp2 on silica support could lead to non-specific interactions, the retention contributions of these two kind of sites, i.e. k_A and k_B were determined from Eq. (1) at pH 7.4 and 298 K. For each statin and the rifamycin molecule and for each solute concentration in the mobile phase, the most concentrated sample was injected into the chromatographic system and its retention factor was determined (see Section 2.5). The variation coefficients of the k values were <0.2% indicating a high reproducibility and a good stability for the chromatographic system. Using a weighted non-linear regression (WNLIN) procedure, the constants of Eq. (1) were used to estimate the retention factors. The slope of the curve representing the variation of the estimated retention factors (k) (Eq. (1)) versus the experimental values (0.999; ideal is 1.000) and r^2 (0.997) indicate that there is an excellent correlation between the predicted and experimental retention factors. The non-linear regression coefficient r^2 and the F value (from the Fisher test with the confidence level at 95%) were determined. These are shown in Table 1. The F value constitutes a more discriminating parameter than the r^2 value when assessing the significance of the model equation. From the full regression model, a Student's t-test was used to provide the basis for the decision as to whether or not the model coefficients were significant. Results of Student's t-test show that no variable can be excluded from the model. These results showed that Eq. (1) describes accurately the association behaviour of statins and rifamycin with Oatp2. As well an important conclusion can be given from these data, i.e. the interactions between statins or rifamycin with the matrix of the stationary phase were neglected (the k_A and k_B values were given in Table 1 and $k_B \ll k_A$). Therefore the non-specific binding sites of the Oatp2 column could be negligible. To evaluate the column to column reproducibility, three Oatp2 columns were prepared under identical conditions. The retention factors were obtained with rifamycin and mevastatin used as tested analytes (Table 2). The mobile phase consisted was a 0.05 M sodium phosphate buffer pH 7.4 and the column temperature was maintained equal to 25 °C. The results showed that the technique was reliable and reproducible. As well, typical reproducibility of this Oatp2 column in retention time measured as relative standard deviation was <0.6%. After half a year and more than 40 times injections, the decrease for the values of retention factor on this column was <1.7%.

Table 1
Values of the retention contribution of the two kind of sites k_A and k_B, the retention factor k ($k = k_A + k_B$) (extrapolated at $C_m = 0$) and the non-linear regression coefficients r^2 and F (Langmuir model; Lang), for the statin molecules and rifamycin at a bulk solvent pH 7.4 and $T = 298$ K, standard deviations are in parentheses.

Solute molecule	k_A	k_B	k	r^2; F-Lang
Pravastatin	0.43 (0.01)	0.01 (0.01)	0.44 (0.01)	0.9997; 7882
Mevastatin	0.79 (0.01)	0.03 (0.01)	0.82 (0.01)	0.9999; 700,895
Atorvastatin	1.53 (0.01)	0.02 (0.01)	1.55 (0.01)	0.9999; 585,875
Fluvastatin	2.10 (0.01)	0.035 (0.01)	2.13 (0.01)	0.9998; 9284
Rifamycin	1.64 (0.01)	0.02 (0.01)	1.66 (0.01)	0.9999; 460,980

3.2. Thermodynamic origins of the solute molecule binding to Oatp2

In this section, the k values of each statin and rifamycin representing their retention on the Oatp2 stationary phase were determined in the entire range of temperatures with various Mg^{2+} concentration values in the mobile phase at pH 7.4 and for a sample concentration in the mobile phase equal to zero; i.e. $C_m = 0$. The plots of $\ln k$ in relation to $1/T$ were determined with a correlation coefficient r^2 higher than 0.97. Using Eq. (2) the enthalpy values (ΔH°) were calculated. For all the pharmacomolecules, ΔH° depended on the magnesium concentration (Fig. 2).

Table 2
Evaluation of retention factor for Oatp2 column to column, reproducibility.

Column	Retention factor k	
	Mevastatin	Rifamycin
1	0.82 (0.01)	1.66 (0.01)
2	0.79 (0.02)	1.68 (0.01)
3	0.84 (0.02)	1.67 (0.02)

Mobile phase: 0.05 M sodium phosphate buffer pH 7.4, column temperature: 25 °C. Standard deviations in parentheses.

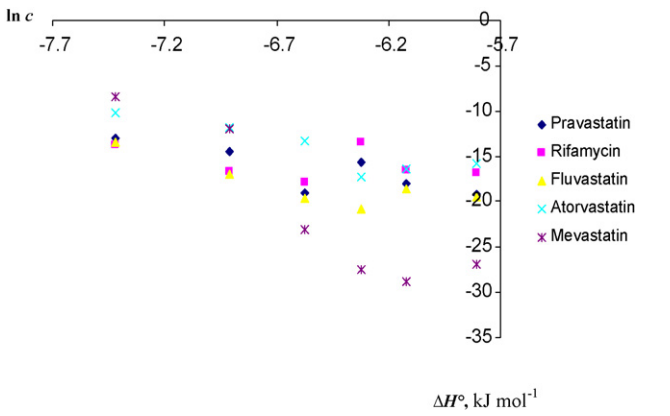

Fig. 2. Enthalpies (ΔH°, kJ mol^{-1}) of all phamacomolecules vs. ln c.

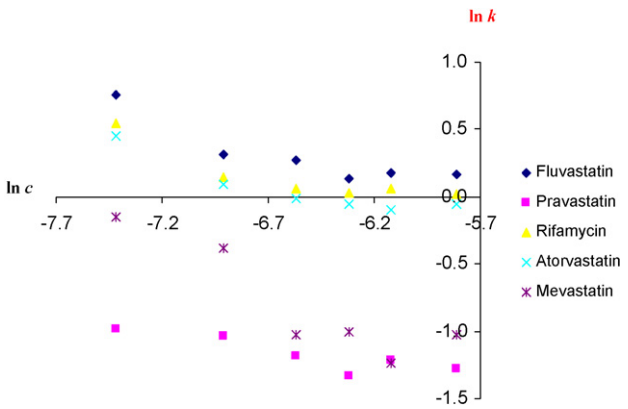

Fig. 3. Ln k of pharmacomolecules vs. ln c in the mobile phase at $T = 35\,^{\circ}$C.

The enthalpy change of association was negative indicating that the pharmacomolecule–Oatp2 formation is enthalpically governed. This means that van der Waals interactions and hydrogen bonding (both characterized by negative enthalpy changes at these temperatures) are engaged at the pharmacomolecule–Oatp2 interface confirming strong interactions between the pharmacomolecules and the Oatp2 protein.

On this new Oatp2 column, the retention order of the pharmacomolecules was prava < meva < rifa < atorva < fluva. Meier-Abt and co-workers proposed a common transport mechanism for all Oatps, whereby substrates are translocated through a central, positively charged pore in a rocker-switch type of mechanism [35]. Many recent publications have been demonstrated the active uptake of statins by organic anion transporting polypeptides into the liver [5–7]. Moreover, an inspection of the protein active site indicates that the positively charged pore of the protein interacts with the negatively charged carboxylate group of the molecules. Indeed, many studies have been demonstrated that Oatp2 was an electroneutral anion exchanger [13,15,36]. The positive electrostatic pore potential of this protein should also facilitate the binding and the transport of negatively charged compounds [14]. Meier-Abt et al. suggested equally that Arg 181 (arginine 181) was responsible for the positive potential of the pore and represented the only charged/pore that could contribute to the substrate-binding site of the Oatp family including the Oatp2 [35]. Therefore, the negatively charged carboxylate group of statins and rifamycin at pH 7.4 interact with the central positively charged pore of the Oatp2. Thus, it was a favourable electrostatic interaction of statins and rifamycin with the positive potential of the Oatp2 active site.

These data showed that pravastatin exhibited a lower retention on Oatp2 protein at the physiological pH 7.4. Indeed a study of Kobayashi et al. demonstrated that pravastatin was transported at acidic pH by OATP-B [37] which is an organic anion transporter. Therefore, it is suggested that the uptake of pravastatin may be low at the basolateral membrane of liver because the pH in these regions is not so acidic. Thus, the uptake of pravastatin was better at acidic pH than neutral pH. This result could be explained by the low retention of this pharmacomolecule on Oatp2 protein at pH 7.4.

As well, rifamycin exhibited similar results as observed for statins (Fig. 2) suggesting that rifamycin could be a competitive inhibitor because it was bound on the same protein active site than statins [19]. Indeed, many studies showed that the competitive inhibition pattern suggests the possibility that rifamycin might represent Oatp substrate [19,38]. Furthermore, this inhibition would be reversible because rifamycin was bound to Oatp2 protein through weaker non-covalent interactions such as hydrogen bonds and van der Waals forces. Indeed, irreversible inhibitors covalently modify a protein and display time-dependent inhibition [39]. The kinetic determination of irreversible inhibitor association with Oatp2 can be carried out in our future study using the parameters of protein activity inhibition.

In order to gain further insight into the mechanism of statins and rifamycin binding to Oatp2, the magnesium concentration (c) effect was studied. The plots ln k versus ln c were drawn. c varied from 0.6 to 3.0 mM. Fig. 3 reports the curves obtained for all pharmacomolecules at $T = 35\,^{\circ}$C. These plots showed that the pharmacomolecule retention decreased when salt concentration increased in the mobile phase. It can be explained by the ionic attraction with the negatively charged carboxylate group of two statins and the Mg^{2+} cation which decreased the pharmacomolecule interaction with the protein active site. Indeed, a previous study [40] had demonstrated that the Mg^{2+} cation and the carboxylate group negatively charged of two statin molecules could form an ion pair. Consequently, the ionic attraction between the positively charge side chain of Arg 181 and the negatively charged carboxylate group of the molecules [35] decreased and thus the pharmacomolecule–Oatp2 affinity was decreased. Moreover, the decrease of this affinity could be explained by an ion attraction between the Cl^{-} anion of the $MgCl_2$ salt and the positively charged pore of the protein. Thus, the pharmacomolecule association decrease with the protein was also attributed to a competition effect between the salt ions and the charged pharmacomolecules for binding with Oatp2.

It was also important to note that this unfavourable association observed when the Mg^{2+} concentration increased was accompanied by a low decrease of the thermodynamic data corresponding to this molecular association which became weakly negative (Fig. 2). In many studies, increasing Mg^{2+} concentration in the mobile phase involved a decrease of the water solubility of apolar compounds by electrostriction of the mobile phase [40,41]. This induced a change in water activity, i.e. the hydrophobic effect, due to its osmotropic character [42,43]. This hydrophobic effect was reflected by an increase of the thermodynamic terms that became less negative [40,44].

Considering $n_{Mg^{2+}}$ as the excess of magnesium cation at the pharmacomolecule–Oatp2 binding, k can be linked to the change in salt concentration, c, using the following equation [45]:

$$\frac{\partial \log k}{\partial \log Mg^{2+}} = -n_{Mg^{2+}} \quad (3)$$

where $n_{Mg^{2+}}$ is, respectively, the net number of salt ions displaced or bound in forming the pharmacomolecule–Oatp2 association.

The $n_{Mg^{2+}}$ values were determined from the slope of the curve ln k versus ln c (Fig. 3). These values were shown in Table 3. The positive values of $n_{Mg^{2+}}$ reflected the decrease of the

Table 3
The $n_{Mg^{2+}}$ values of pharmacomolecule–Oatp2 association at all the temperatures studied.

Temperature (°C)	Pravastatin	Mevastatin	Atorvastatin	Fluvastatin	Rifamycin
10	0.00	0.32	0.19	0.24	0.09
15	0.09	0.37	0.23	0.29	0.18
20	0.15	0.39	0.26	0.31	0.19
25	0.16	0.74	0.34	0.28	0.22
30	0.17	0.86	0.39	0.33	0.25
35	0.19	0.88	0.56	0.45	0.31

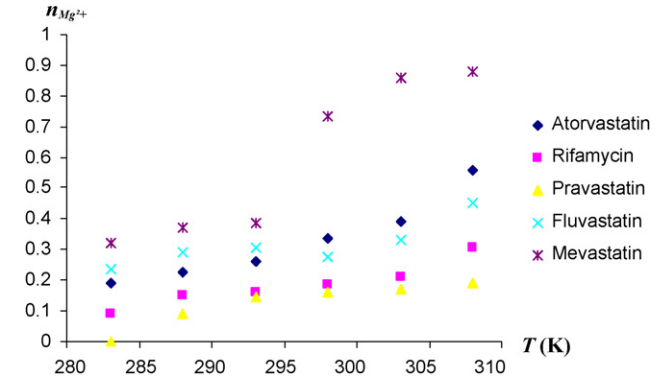

Fig. 4. Temperature dependence of the released magnesium ($n_{Mg^{2+}}$) of all pharmacomolecules.

pharmacomolecule–Oatp2 association. Indeed, the ionic association between Mg^{2+} and the carboxylate groups of two molecules could explain the positive number of Mg^{2+} exchanged and also the decrease of the pharmacomolecule–Oatp2 binding. Hinz et al. [46] established that the magnesium binding heat effect ($\Delta H_{Mg^{2+}}$) associated with a magnesium release during a binding reaction can be described by the following simple function:

$$\Delta H_{Mg^{2+}} = -2.3RT^2 \left(\frac{\partial n_{Mg^{2+}}}{\partial T} \right)_{Mg^{2+}} \quad (4)$$

For example, Fig. 4 reports the curves obtained for pravastatin, atorvastatin and rifamycin. As the temperature increased the $n_{Mg^{2+}}$ also increased and ($\partial n_{Mg^{2+}} / \partial T_{Mg^{2+}}$) had a positive value. For example, at 35 °C the corresponding $n_{Mg^{2+}}$ value of fluvastatin was 0.45. Thus, from Eq. (4), $\Delta H_{Mg^{2+}}$ has a negative value, and as the Mg^{2+} concentration increased, the binding enthalpy contributes favourably to the free energy of binding. As well, using the above relation, the $\Delta H_{Mg^{2+}}$ value was determined at 35 °C for fluvastatin and rifamycin −12.34 kJ mol^{-1} and −13.05 kJ mol^{-1}, respectively. A same order of magnitude was obtained during the Mg^{2+}–Human Serum Albumin (HSA) binding [44].

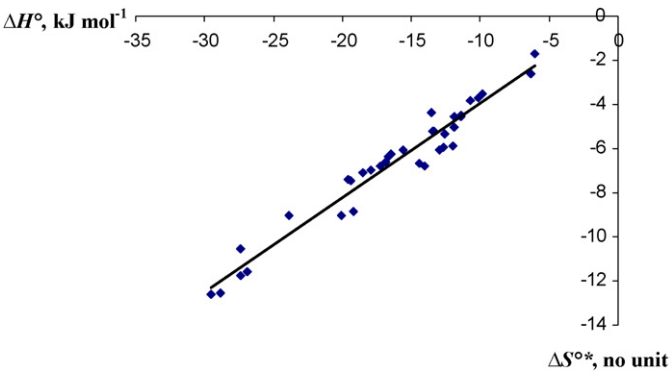

Fig. 5. Enthalpy–entropy compensation of statins and rifamycin at all the magnesium concentrations.

In order to determine the pharmacomolecule binding mechanism on Oatp2 protein, an enthalpy–entropy compensation was also investigated. The plot ΔH° versus $\Delta S^{\circ *}$ was drawn for all the pharmacomolecules and at all the Mg^{2+} concentrations in the mobile phase (Fig. 5). The coefficient correlation for the linear fit was equal to 0.98. This degree of correlation can be considered adequate to verify enthalpy–entropy compensation, indicating that statins and rifamycin bound effectively on the same positive potential pore of Oatp2 protein.

4. Conclusion

In this paper, a novel biochromatographic column was developed to study the binding affinity of statins and rifamycin to an organic anion transporting polypeptide 2 (Oatp2). For this, the Oatp2 protein was immobilized via the amino groups of the protein on a chromatographic support. For the first time, in order to elucidate the pharmacomolecule–Oatp2 binding mechanism, the role of the magnesium chloride salt was examined at pH 7.4. It was shown that the pharmacomolecule–protein association was Mg^{2+}-dependent. Indeed, an increase of the Mg^{2+} concentration led a decrease of this association due to ion pair formations between (i) the Mg^{2+} cation and pharmacomolecules and (ii) Cl^- anion and the positive potential pore of the protein. This binding affinity decrease was also accompanied with a positive number of magnesium release ($n_{Mg^{2+}}$). Our work indicated that our biochromatographic approach could soon become very attractive for studying the transmembrane transport of other substrates. As well, this novel biochromatographic column could be useful to find a specific inhibitor for these transporters and so open new perspectives to be investigated in the future.

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