Journal of Chromatography B, 868 (2008) 20–27

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Statins (HMG-coenzyme A reductase inhibitors)–biomimetic membrane binding mechanism investigated by molecular chromatography

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ARTICLE INFO

Article history:
Received 12 November 2007
Accepted 30 March 2008
Available online 20 April 2008

Keywords:
Statins
HPLC
IAM
Binding
Thermodynamic

ABSTRACT

Many studies have demonstrated that the statin beneficial effects on cardiovascular diseases like coronary are linked to their hypocholesterolemic properties. These lipid-lowering drugs are the first-line pharmacologic therapy for hypercholesterolemia. In this paper, the interaction of a series of statin molecules STCOOH (pravastatin (prava), mevastatin (meva), simvastatin (simva) and fluvastatin (fluva)) with a phosphatidylcholine monolayer immobilized on to porous silica particles has been studied using a biochromatographic approach (molecular chromatography). The immobilized artificial membrane (IAM) provided a biophysical model system to study the binding of the statin molecules to a lipid membrane. For all the test statin molecules, linear retention plots were observed at all temperatures. An analysis of the thermodynamics (i.e., enthalpy (ΔH°), entropy ($\Delta S^{\circ *}$)) of the interaction of the statin molecules with the immobilized monolayer was also carried out. The ΔH° and $\Delta S^{\circ *}$ values were negative due to van der Waals interactions and hydrogen bonding between the statin molecules with the polar head groups of the phospholipid monolayer (polar retention effect). The statin elution order was: Prava $\lll$ Meva < Atorva $\ll$ Simva < Fluva. This result associated with IC50 data of each statin molecule confirmed that pravastatin, which exhibited the lowest association with the lipid monolayer, was taken up by a membrane transporter. In addition, the logarithm of the statin retention factor with the lipid membrane extrapolated to a total aqueous bulk solvent at pH 7.0 ($\log k'_{w-\mathrm{IAM}}$) was measured and compared to the octanol–water partition coefficient ($\log P$). The observed significant correlation showed an affinity enhanced with the increase in the molecule lipophilicity and confirmed that the hydrophobic forces played an important role in the statin molecule–biomembrane association mechanism. As well, the affinity of STCOOH to IAM is high and changes slightly with the bulk solvent pH, because the number of protons linked to binding is low. This confirmed the importance to take into account the electrostatic interaction in this association mechanism. At pHs lower than ≈7.0, the binding process is accompanied by protons release and at higher pHs protons are taken up. A change in the phospholipid monolayer phosphate group pK_a has been proposed to contribute to the positive number of protons exchanged at pHs higher than ≈7.0. This demonstrated that this phosphate residue could function as a general base/proton shuttle by facilitating the deprotonation of STCOOH, i.e., this hyper-reactive residue in the IAM surface could play a catalytic function.

1. Introduction

Cholesterol is an important part of a healthy body. However, a high level of cholesterol in the blood, i.e., hypercholesterolemia, represents a major risk for coronary heart disease. Indeed, hypercholesterolemia has been acknowledged, since mid-20th century, as a major heart disease risk factor [1,2]. Atherosclerosis, the most common cause of vascular diseases and the principal cause of coronary diseases, is due to the presence of atherome plate containing cholesterol, lipids and blood cells. This obstruction of arteries can involve cardiac vascular accidents like coronary heart disease [3]. Thus, it is very important to use drugs to reduce the cholesterol levels, more especially the LDL-Cholesterol (Low Density Lipoprotein-Cholesterol).

These 20 last years, an important number of therapeutic drug classes for treated patients with dyslipidemia, i.e., hypercholesterolemia have been developed such as statins, fibrates, niacin and others lipid-lowering drugs. Statins or 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors are the first-line pharmacologic therapy for hypercholesterolemia [4–6]. Indeed, statins block hepatic synthesis of cholesterol by inhibition of rate-limiting enzyme (HMG-CoA reductase) for hepatic

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doi:10.1016/j.jchromb.2008.03.034

cholesterol biosynthesis and upregulate LDL receptors in the liver, resulting in decreased levels of circulating cholesterol [7]. The hepatic enzyme, HMG-CoA reductase catalyzes the formation of mevalonate, the crucial and committed step in the biosynthesis of cholesterol, isoprenoids and other lipids and these statin molecules have high affinity for the enzyme active site [7]. In humans, HMG-CoA reductase is the rate-limiting step in cholesterol synthesis and represents the sole major drug target for contemporary cholesterol-lowering drugs [7,8]. Statins are a well-established class of drugs in the treatment of hypercholesterolemia, and have been shown to significantly reduce the risk of cardiovascular morbidity and mortality in the world [5,9–11]. Indeed, many studies have been demonstrated the important place on statins in the treatment of hypercholesterolemia and many diseases like heart, renal transplantations, atherosclerosis, Alzheimer [12–14]. Statins are classified according to their origin: fungal metabolites (natural statins) like mevastatin, simvastatin, pravastatin and synthetic statins like atorvastatin, fluvastatin [1].

Statins, drugs of the 21st century, were used by 20 million people and are the most prescribed medications in the world [13]. Therefore, it is very important to know pharmacologic and pharmacokinetic processes of their lipid-lowering drugs in human because the ability to cross biological membrane strongly affects the pharmacokinetic behaviour of drugs and their capacity to access the receptor site. At present, the reference parameter to predict the solute molecule passive diffusion through these biological barriers is lipophilicity, expressed as the logarithm of the solute partition coefficient between an organic solvent and an aqueous phase ($\log P$) [15]. This parameter is however only useful when polar group interactions between the solute and the phospholipids bilayers are minimal or absent [16]. It lacks structural similarities to cell membranes, reflecting only the hydrophobicity of a compound and is not suitable for highly polar and ionic compounds [17–21]. Since phospholipids are the main lipidic constituents of biomembranes, their use as partitioning phase can be expected valuable to mimic drug–biomembrane interactions.

Recently, immobilized artificial membranes (IAMs) have been introduced as HPLC column packing materials and this development unfolded new perspectives for rapid evaluation of drug partitioning into cell membranes [22,23]. IAMs consist of phosphatidylcholine residues covalently bound to silica propylamine and consequently mimic fluid phospholipid bilayer [24–27]. This technique is an acceptable method for the prediction of membrane permeability of drugs [22,23,28] particularly for ionizable compounds due to the fact that the position of the polar compounds in biomembranes is strongly affected by electrostatic and/or hydrogen bound interactions with phospholipids [29,30]. IAMs were convenient model of drug passive transport across all cells including target cells. Excellent correlations have been demonstrated between IAM chromatography indices and biological systems such as the prediction of the intestinal absorption of structurally diverse compounds [23,28,31] and of skin permeability coefficients [32].

The aim of this work was to study the association mechanism of a series of statin molecules with an immobilized artificial membrane in order to predict their ability to cross biological membrane. Moreover the thermodynamic driving forces for the statin molecules with phosphatidylcholine monolayers were analyzed.

2. Experimental and method

2.1. Solvents and samples

The five statins (STCOOH) were represented in Fig. 1. Pravastatin, mevastatin, atorvastatin, simvastatin and fluvastatin were purchased from Sigma and VWR (Paris, France). Water was obtained from an Elgastat option water purification (Odil Talant, France) fitted with a reverse osmosis cartridge. Methanol was supplied by Carlo Erba (Val de Reuil, France). Sodium dihydrogenophosphate and di-natriumhydrodenophosphate were obtained from Prolabo and Merck (Paris, France), respectively. The mobile phase consisted of 60/40 (v/v) 0.05 mol L^{-1} sodium phosphate buffer (PBS)–methanol adjusted at different pH varying between 5.7 and 7.0 (5.7, 6.0, 6.3, 6.7 and 7.0). The buffer was stocked for 1, 2 and 4 h at ambient room temperature to study the accuracy of their pH values [33]. No fluctuations were observed. The maximum relative difference of mobile phase pH value was always lower than 0.3%. To avoid the presence of significant non-linear effects, the solute amount added onto the column corresponded to the smallest sample size allowing the detection of the statin molecule in all operating conditions. Statin solutions were prepared in the mobile phase at a concentration of 1 mM and 20 μL was injected at least three times. As well, experiments were carried out over the temperature range 10–35 °C (10, 15, 20, 25, 30 and 35 °C) and at a 254 nm detection wavelength. The chromatographic system was left to equilibrate at each temperature for at least 30 min before each experiment. To study this equilibration, the retention time of fluvastatin was measured after 22, 23 and 24 h. The maximum relative difference between retention times of this solute molecule was never more than 0.6%, meaning that after 30 min the chromatographic system was sufficiently equilibrated for use [34]. Throughout the study, the flow rate was maintained constant and equal to 0.5 mL/min.

2.2. Apparatus

The HPLC system consisted of a Hitachi L 7100 pump (Merck, Nogent sur Marne, France), a Rheodyne 7125 injection valve (Cotati, CA, USA) fitted with a 20 μL sample loop, and a Shimadzu UV–vis detector. Statin retention data were obtained with a 100.0 mm × 4.6 mm IAM–PC–DD2 (immobilized artificial membrane–phosphatidylcholine–drug discovery) packing (Interchim, Montluçon, France). The column stationary phase consisted of diacyl double chain ester phosphatidylcholine (PC) ligands surface-bounded to an aminopropylsiloxane bonded silica substrate and was end capped by mixed propionic and decanoic alkylamine groups. A major advantage of this particular IAM chromatographic stationary phase (i.e., IAM–PC–DD) is that it had the shortest retention times of commercialized IAM packing [35–37]. It was also more readily and reproducibility synthesized commercially. Moreover, the IAM–PC–DD packing was stable like the ester packing leading a better modelization of the drug membrane transport [36,37].

2.3. Thermodynamic relationships

The solute retention on the IAM stationary phase can be evaluated using the retention factor k':

$$k' = \frac{t - t_0}{t_0} \quad (1)$$

where t is the solute retention time and t_0 is the column void time. To obtain the thermodynamic retention time, i.e., the accurate measure of solute retention, t was determined by calculating the first moment of the peak as previously described [38]. The void time was determined using the mobile phase peak. The retention times and column void time were corrected for the extracolumn void time. It was assessed by injections of solute onto the chromatographic system when no column was present.

At infinite dilution, i.e., under linear elution conditions, and assuming that non-specific interactions between solute and chro-

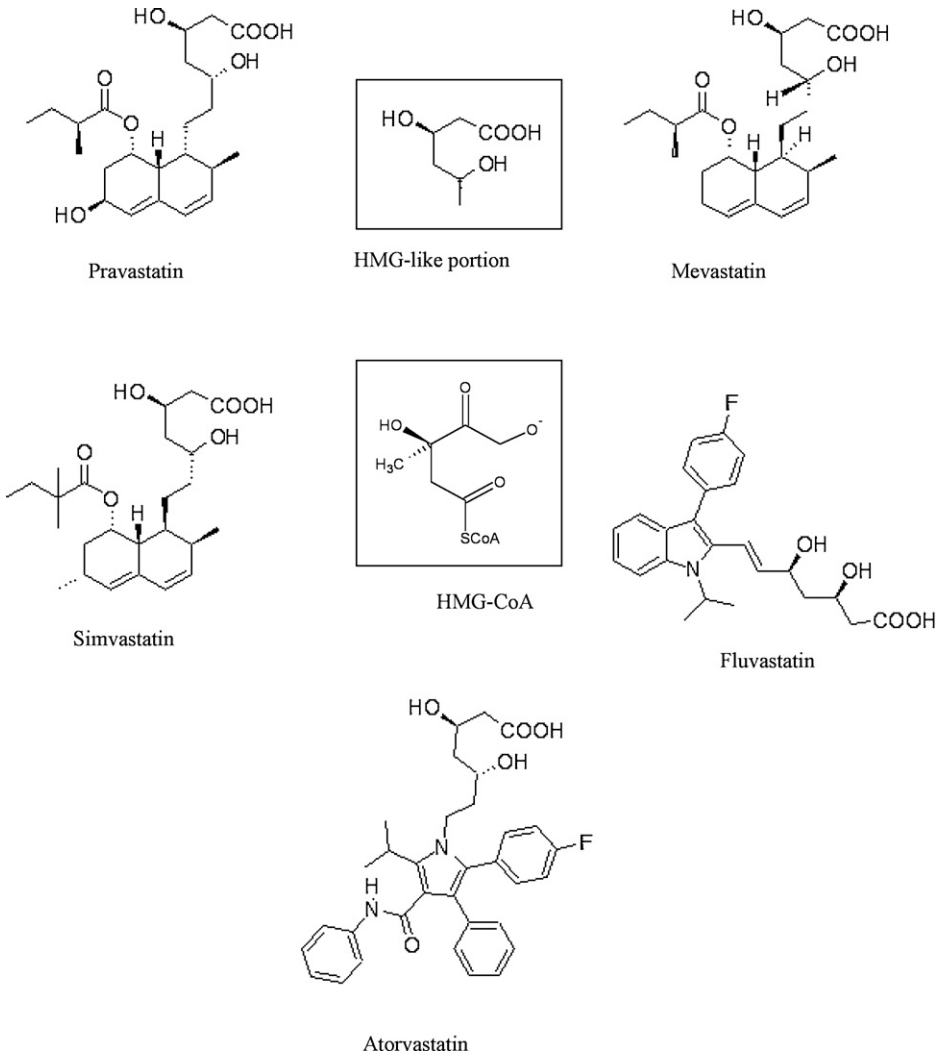

Fig. 1. Chemical structures of the statins (STCOOH), the HMG-like portion and the HMG-CoA.

matographic support was negligible, the retention factor k' can be related to the association constant K between the statin molecule and IAM as follows:

$$k' = \sigma K \tag{2}$$

where σ is equal to the ratio of the active binding site number in the column (m_L) over the void volume of the chromatographic column (V_M).

Valuable information about the processes driving the statin–IAM association mechanism can be further gained by examining the temperature dependence on statin retention [39,40]. The temperature dependence of the retention factor is given by the following relation:

$$\ln k' = \left(\frac{-\Delta H^\circ}{RT}\right) + \Delta S^{\circ *} \tag{3}$$

with

$$\Delta S^{\circ *} = \frac{\Delta S^\circ}{R} + \ln \sigma$$

where ΔH° and ΔS° are, respectively the enthalpy and entropy of transfer of the statin molecule from the bulk solvent to the IAM stationary phase, T is the absolute temperature and R is the gas constant. If the IAM stationary phase, solute molecules and solvent properties are independent of temperature and ΔH° and ΔS° are temperature invariant, a linear van't Hoff plot is obtained. From the slope and intercept ΔH° and $\Delta S^{\circ *}$ can be calculated.

2.4. Lipophilicity determination

The retention time t of each statin molecule was determined on six different organic modifier–phosphate buffer mobile phase ratios. Using Eq. (1), the retention factor k' was calculated for each methanol fraction Φ (v/v) in the mixture methanol/phosphate buffer (0.05 mol L^{-1}, pH 7.0). According to the solubility parameter concept [41], the relationship between the solute retention on the IAM surface and the methanol fraction Φ can be described by the following equation:

$$\log k' = A\Phi^2 - S\Phi + D \tag{4}$$

where A, S and D are constants. D represents the logarithm of the statin retention factor for a total aqueous mobile phase, i.e., $\Phi = 0$ and was noted $\log k'_{w-\mathrm{IAM}}$. The $\log k'_{w-\mathrm{IAM}}$ value was derived from polynomial extrapolation at $\Phi = 0$ of plots of $\log k'$ versus Φ (Eq. (4)) for each statin molecule. The use of $\log k'_{w-\mathrm{IAM}}$ as hydrophobic parameter was demonstrated by Hulshoff and Perrin [42]. Indeed, the molecular lipophilicity (i.e., the partition coefficient of a solute molecule between an aqueous phase and a lipid phase) is the reference parameter

Table 1
Experimental lipophilicity ($\log k'_{w-\mathrm{IAM}}$), computed lipophilicity ($\log P$), median inhibitory concentration (IC50, nmol L^{-1}) and pK_a values of statin molecules

Statins	$\log k'_{w-\mathrm{IAM}}$	$\log P$	IC50	pK_a
Prava	3.36	2.20	44.00	4.36
Meva	4.06	4.00	23.00	4.40
Atorva	5.35	5.70	10.00	3.95
Simva	4.90	4.70	9.00	4.45
Fluva	4.45	4.50	18.00	4.15

to predict passive diffusion through the biological membranes [15]. As well, excellent correlations have been demonstrated between IAM chromatography indices and biological systems such as the prediction of the intestinal absorption of structurally diverse compounds [23,28,31] and of skin permeability coefficients [32].

The computed lipophilicity (i.e., the octanol–water partition coefficient) $\log P$, used as well to predict drug transport across biological membranes and derived from an atomic fragment database using *XLogP* software, were taken from the PubChem database (Table 1) [43].

2.5. Bulk solvent pH effects

When the pH of the bulk solvent changed, a fuller description is essential, which explicitly maintains conservation of mass of each species and take into account binding of H^+ to the IAM, STCOOH, and the complex IAM·STCOOH:

$$\mathrm{IAM(H^+)_A + STCOOH(H^+)_B + } n_{\mathrm{H^+}}\mathrm{H^+} \rightleftarrows \mathrm{IAM \cdot STCOOH(H^+)_C} \quad (5)$$

where $n_{H^+} = C - (A + B)$ is the number of protons linked to this STCOOH binding reaction of IAM.

The association constant of this equilibrium was given by

$$K = \frac{[\mathrm{IAM \cdot STCOOH}]}{[\mathrm{IAM}][\mathrm{STCOOH}][\mathrm{H^+}]^n} \quad (6)$$

Eq. (6) can be rewritten as

$$K = \frac{K_0}{[\mathrm{H^+}]^n} \quad (7)$$

where K_0 is the K values for $[H^+] = 1$ mol L^{-1}. Taking the logarithm of Eq. (7) gives

$$\log K = \log K_0 - n_{\mathrm{H^+}} \log[\mathrm{H^+}] \quad (8)$$

After derivation of Eq. (8) we obtained:

$$\frac{\partial \log K}{\partial \mathrm{pH}} = n_{\mathrm{H^+}} \quad (9)$$

Combining Eqs. (1) and (9), the following is obtained:

$$\frac{\partial \log k'}{\partial \mathrm{pH}} = n_{\mathrm{H^+}} \quad (10)$$

3. Results and discussion

3.1. Thermodynamic parameters

The retention factor k' of each statin under study was determined with the immobilized artificial membrane in the entire range of temperature, i.e., from 10 to 35 °C. Experiments were carried out with various pH ($5.7 \leq \mathrm{pH} \leq 7.0$) of the phosphate buffer. All the experiments were repeated three times. The variation coefficients of the k' values were less than 1% indicating a high repeatability and good stability for the chromatographic system. Eq. (3) showed that with an invariant statin–biomembrane association mechanism

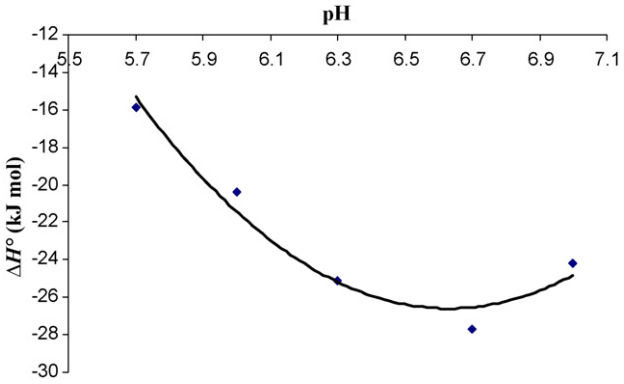

Fig. 2. ΔH° (kJ mol^{-1}) vs. pH for pravastatin.

over the temperature range being studied, the association enthalpy ΔH° remained constant and a plot of $\ln k'$ in relation to $1/T$ leads to a straight line with an enthalpic slope and entropic origin. For all statins, when the temperature increased there was a decrease in the statin–biomembrane association. Linear van't Hoff plots were obtained with correlation coefficients r higher than 0.98 for all fits. It was previously demonstrated that for some solute molecule, their binding with a phosphatidylcholine monolayer can lead to a phase transition of the IAM surface around 25 °C [44]. In this work, the observed linear relationships excluded a phase transition of the immobilized phosphatidylcholine in the stationary phase over the range of experimental temperatures as it was observed for bile salt–membrane interactions [45]. According to Eq. (3), these linear van't Hoff plots provided a conventional way of calculating the thermodynamic parameters. All the statins exhibited a similar parabolic variation ($r^2 > 0.90$) for the thermodynamic data regardless of the bulk solvent pH (Figs. 2 and 3). For example the corresponding equations for pravastatin were

$$\Delta H^\circ = 13.03\mathrm{pH}^2 - 172.72\mathrm{pH} + 545.98 \quad r^2 = 0.96 \quad (11)$$

$$\Delta S^{\circ *} = 5.55\mathrm{pH}^2 - 74.03\mathrm{pH} + 235.15 \quad r^2 = 0.97 \quad (12)$$

Additional experiments were carried out at extracellular pH, i.e., 7.4. For each statin molecule, the difference between the experimental thermodynamic parameters (i.e., using van't Hoff plots as explained above) and the values calculated with the second order equations were always <0.5% showing that the polynomial expressions (i.e., ΔH° and $\Delta S^{\circ *}$ vs. pH) can be used to model the statin retention with the IAM surface at pH 7.4. Negative enthalpies indicated that it was energetically more favourable for the statin molecule to be associated with the lipid surface. Negative entropies showed an increase in the order of the chromatographic system

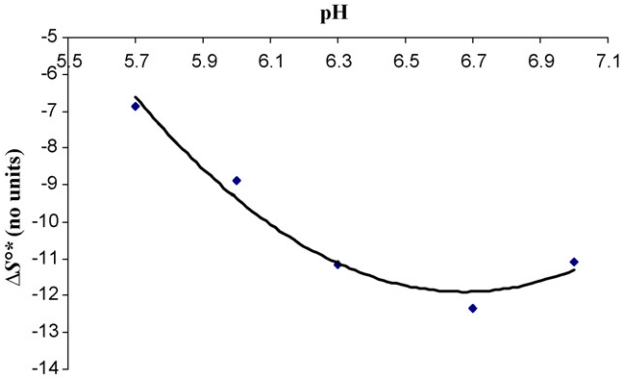

Fig. 3. $\Delta S^{\circ *}$ (no units) vs. pH for pravastatin.

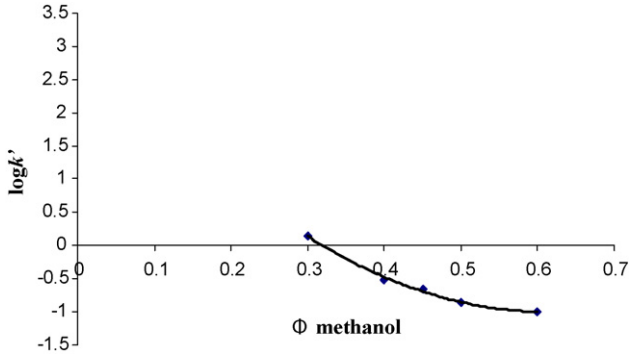

Fig. 4. Plot of log k' vs. methanol fraction Φ (v/v) for pravastatin at 25 °C.

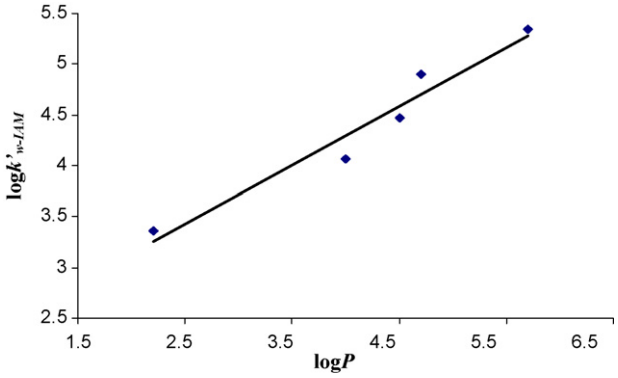

Fig. 5. Plot of log $k'_{w-\mathrm{IAM}}$ vs. log P for the five statin molecules.

when the statin molecules were transferred from the bulk solvent to the phospholipid monolayer. This transfer was enthalpically driven and can be described as the replacement of weak statin/solvent interactions by strong statin/lipid surface interactions. This indicates that enthalpic factors associated with hydrogen bonding and van der Waals interactions (characterized by negative enthalpy changes at these temperatures [46]) of the statin molecules with the lipid monolayer dominate the binding rather than entropic factors relate to the changes in the mobility of the statin molecules and the lipid monolayer fluidity. These results confirmed the great importance of the polar sites of the IAM surface on this association process (polar retention effect). On the IAM surface, the statin elution order was

$$\text{Prava} \lll \text{Meva} < \text{Atorva} \ll \text{Simva} < \text{Fluva}$$

Pravastatin exhibited the lowest association with the lipid monolayer. This can be explained by the lowest hydrophobicity of this statin molecule [9,11,13,47]. In order to gain further insight into the effect of lipophilicity in this statin–IAM association, a comparison between log $k'_{w-\mathrm{IAM}}$ and log P was carried out.

The log k' value was plotted against the methanol fraction Φ (v/v) at 25 °C for each statin molecule. For example, the plot of pravastatin was shown in Fig. 4. The slope of the plots log k' versus Φ was similar for all statins. log k' values decreased with the increase percentage of methanol (Φ) in the mobile phase. This decrease was not linear and had a polynomial shape. In our study, the polynomial correlation of log k' versus Φ displayed r values ranging from 0.99 to 1.00 for all statins. From Eq. (4) the log $k'_{w-\mathrm{IAM}}$ values were obtained for the five statin molecules (Table 1).

These log $k'_{w-\mathrm{IAM}}$ values were compared with the log P values and the following regression Eq. (13) was obtained:

$$\log k'_{w-\mathrm{IAM}} = 0.58 \log P + 1.99 \quad r^2 = 0.95 \tag{13}$$

A visual inspection of the relationship between log $k'_{w-\mathrm{IAM}}$ and log P was provided by Fig. 5. This significant correlation suggested that the retention behaviour is similar for all the statin molecules and they may mainly interact with the lipophilic part of IAM chains. The IAM stationary phase contain non-polar packing material but this column with phosphatidyl chain analogues offers polar heads as first contact site for solutes [48]. This result showed an affinity enhanced with the increase in the molecule lipophilicity and confirmed that the hydrophobic forces played an important role in the statin molecule–biomembrane association mechanism [49]. As well, a second comparison between statin passive diffusion and their potency to inhibit the HMG-CoA reductase was carried out. For this, the median inhibitory concentration of each statin molecule (i.e., IC50) was used to illustrate the statin relative potency to block the crucial enzyme (i.e., HMG-CoA reductase) in the biosynthesis of cholesterol. In all the statin molecules, the HMG-like portion (Fig. 1) is covalently linked to a rigid hydrophobic group, which ranges from very hydrophobic to partly hydrophobic. Consistent with the presence of the HMG-like moiety, all statins are competitive inhibitors of the enzyme with respect to binding of HMG-CoA (Fig. 1) [7]. However, the active site of this enzyme is located in a long carboxyl terminal domain in the cytosol [50]. Thus, to inhibit this enzyme, the statin molecules must cross the biological membrane. The IC50 values were presented in Table 1 (data from [7]). The plot log $k'_{w-\mathrm{IAM}}$ versus IC50 was illustrated in Fig. 6. This plot showed that the IC50 values have been successfully correlated ($r^2 > 0.96$) with the log $k'_{w-\mathrm{IAM}}$ values. This relationship showed that more a molecule cross the cellular membrane by passive diffusion and reached its active site more it is potent. For example pravastatin, which exhibited the lowest association with the IAM surface, presented an IC50 equal to 44.00 nmol L^{-1}. This IC50 value showed that pravastatin was less potent than other statins which were in contrary more lipophilic with IC50 values twice (mevastatin) or four times (simvastatin) lower [51]. Indeed, pravastatin is relatively hydrophilic with a log $P = 2.20$ and a log $k'_{w-\mathrm{IAM}} = 3.36$ compared with other statins (Table 1). Therefore pravastatin, which presents a greater hydrophilicity, would have lower rates of passive diffusion [51]. This result confirmed previous studies showing that pravastatin was taken by a membrane transporter [14,52,53].

Among the five statins, fluvastatin exhibited the lowest thermodynamic data. For example, at pH 7.0 $\Delta H^\circ = -36.06$ kJ mol^{-1} and $\Delta S^{\circ *} = -12.60$ (no units). This can be explained by the increase of the apparent lipophilicity of this statin molecule due to its amphiphilic character and to the intra-molecular hydrogen bonds between the carboxylate group ($-COO^-$) and the hydroxyl group

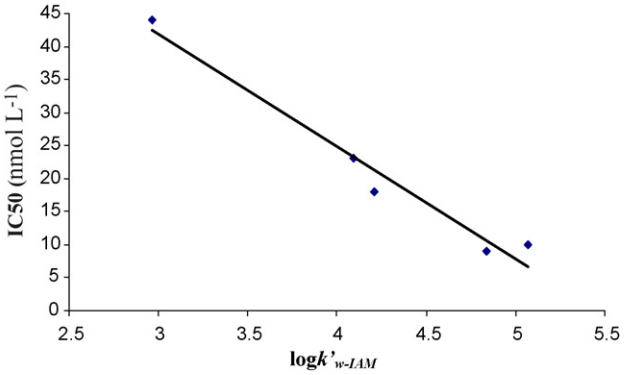

Fig. 6. Comparison between experimental lipophilicity (log $k'_{w-\mathrm{IAM}}$) at 37 °C and the median inhibitory concentration (IC50, nmol L^{-1}) values of statin molecules.

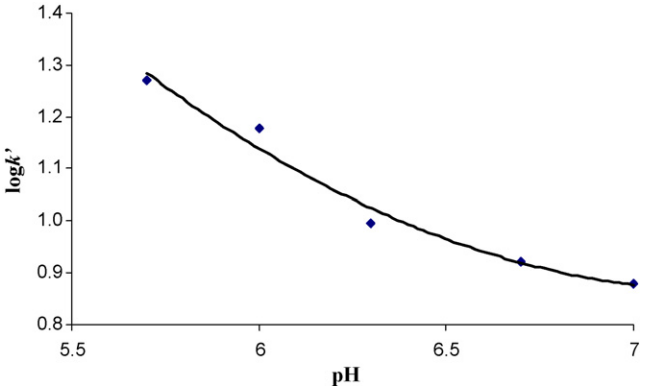

Fig. 7. $\log k'$ vs. pH for simvastatin at 25 °C.

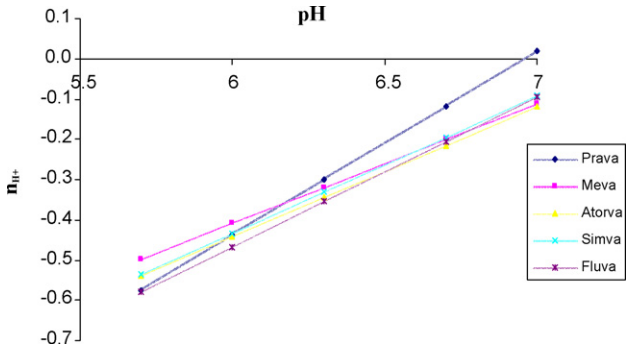

Fig. 8. n_{H^+} vs. pH for the statin molecules at 25 °C.

(−OH) ($\log P = 4.50$ and $\log k'_{w-IAM} = 4.45$) [43,46]. As well, the polar substituent on the compound (−F substituent, two −OH substituents) on the fluvastatin enhanced the lipid surface affinity by increasing hydrogen bonds between the solute molecule and the polar head groups of the IAM surface. This was associated with the lowest entropy state classically attributed to the release of the water molecules surrounding the solute molecule when the fluvastatin was transferred into the phospholipid monolayer [54].

3.2. pH effect on statin–IAM association

The logarithm of the retention factor k' was plotted against the pH at 25 °C, for each statin molecule and for a variation range of pH ($5.7 \leq pH \leq 7.0$). The slope of the plots $\log k'$ versus pH was similar for all statins. Fig. 7 reports the curve obtained for simvastatin. The line shown in Fig. 7 was obtained by fitting the experimental data to a quadratic function of the pH ($r^2 \geq 0.98$). The agreement between the experimental data and this second order relationship between $\log k'$ and pH is excellent and suitable for accurate description of the statin–lipid surface association in the whole pH range analyzed. At pH 7.4, for each statin molecule, the difference between the experimental $\log k'$ data and the values calculated using these second order relationships were always <0.6%. Thus, the second order polynomial between $\log k'$ and pH can be used at extracellular pH. As well, this second order relationship demonstrated that this plot showed a curvature at a critical pH_c value around 7.0. More precisely the theoretical critical pH_c values were, respectively equal to 6.96, 7.37, 7.36, 7.27 and 7.25 for pravastatin, mevastatin, atorvastatin, simvastatin and fluvastatin. For $pH \ll pH_c$ an initial decrease in the statin–IAM surface association was observed, followed by a weak variation in the statin molecule affinity for the binding surface at $pH > pH_c$. The non-linearity of the plot $\log k'$ versus pH showed that hydrophobic effect, van der Waals and hydrogen bonds were not the only forces which intervene in this association mechanism. For the very low pH values ($pH \ll pH_c$), the five statin molecules were in a molecular form and their carboxylate group was not ionized (Fig. 1). It is well known that the interactions between ionic species in aqueous solution are characterized by small positive enthalpy and entropy changes [33,55]. For example, ΔH° and $\Delta S^{\circ *}$ values determined for the association of Zn^{2+} on human albumin were, respectively equal to 30.21 J mol^{-1} and 19.33 (no units) [56]. Thus, for $pH < pH_c$, as ΔH° and $\Delta S^{\circ *}$ became progressively more negative when pH increased (Figs. 2 and 3), the repulsion between the phosphate groups of the IAM surface and the carboxylate group negatively charged of the statin molecule increased (according their pK_a values (Table 1), all the statin molecules were negatively charged in this pH range). This decrease of the electrostatic interactions was accompanied by a reduction of the statin–IAM surface association process. These electrostatic repulsions between the phosphate head groups of the IAM surface and the carboxylate group negatively charged of the statin molecule demonstrated that the lack of cholesterol had no influence on the statin–IAM association. Indeed, cholesterol is considered as a sterol (a combination steroid and alcohol), and had no ionized groups. When cholesterols are incorporated into a phospholipid bilayer, phospholipid head groups provide "cover" to shield the non-polar part of cholesterol from exposure to water [57]. Indeed, according to a study of Tsamaloukas and co-workers, cholesterol was intercalated between lipids without occupying much space in the head group region, so that it was screened from water by the head groups like under an umbrella [58]. From Eq. (10) the slope of the curve $\log k'$ versus pH gives the number of protons H^+ ($n_{H^+} = \partial \log k'/\partial pH$) at the statin–IAM interface implied in the binding process. The negative values of n_{H^+} reflected the exclusion of H^+ when statin bound to the phospholipid monolayer. Fig. 8 showed that the magnitude of n_{H^+} values decreased linearly with pH and was equal to zero at $pH \approx 6.96$ for pravastatin for example. At 25 °C, the pH values for which $n_{H^+} = 0$ were 7.37, 7.36, 7.27 and 7.25 for, respectively mevastatin, atorvastatin, simvastatin and fluvastatin (Fig. 8). Thus for all the solute molecules at pH lower than ≈7.0, the number of protons exchanged was negative which means that a residue of the IAM surface or of the statin molecule should decrease its pK_a value as a consequence of binding and release protons. At this point it is interesting to consider that a common property of the IAM surface is the lowering of pK_a of the carboxylic group of bound STCOOH. Potentiometric measurements have revealed that the carboxylic proton of STCOOH is released quantitatively to the buffer upon binding at pH lower than ≈7.0 [59]. This carboxylic group could be responsible for the negative number of protons exchanged. At pH higher than ≈7.0, protons are taken up upon binding, which means that a residue of the IAM surface or of the statin molecule increases its pK_a. The phosphate group of the phospholipid monolayer is related via a hydrogen bonding to the carboxylic group of STCOOH and could play a role in the deprotonation of STCOOH. An increase in the pK_a of this phosphate group in the IAM surface upon binding would lead to a positive number of protons exchanged. A proton shuttling role for the phosphate group (i.e., a catalytic function) was thus shown for the first time. There are several reasons that can explain these changes in pK_a values. For instance, a variation in the micropolarity of the environment surrounding the polar sites of the IAM surface as a result of statin binding is a possibility. As well, the enthalpy change ΔH° is the sum of a term which is independent of the buffer and a term proportional to the change of ionization of the buffer. Since the number of protons released is practically zero around 7.0, the intrinsic binding enthalpy which does not depend on the ionizations of the groups of the statin molecules or the IAM surface were thus, respectively −24.21, −26.73, −29.45, −31.97 and

Table 2
Thermodynamic parameters ΔH° (kJ mol^{-1}) and $\Delta S^{\circ *}$ of statin molecules binding to IAM at pH 7.0

Statins	ΔH° (kJ mol^{-1})	$\Delta S^{\circ *}$ (no units)
Prava	−24.21	−11.07
Meva	−26.73	−9.81
Simva	−29.45	−9.93
Atorva	−31.97	−11.65
Fluva	−36.06	−12.60

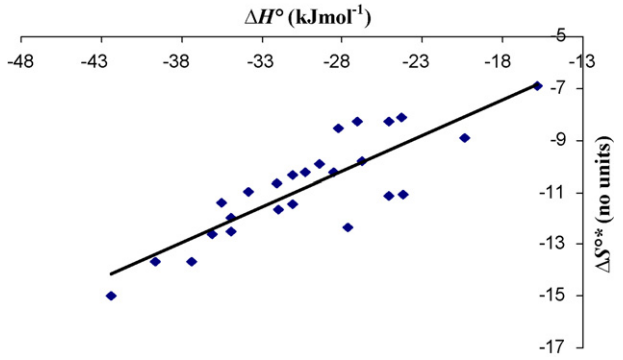

Fig. 9. ΔH° (kJ mol^{-1}) vs. $\Delta S^{\circ *}$ for all statin molecules and pH values of the bulk solvent.

−36.06 kJ mol^{-1} for pravastatin, mevastatin, atorvastatin, simvastatin and fluvastatin (Table 2).

3.3. Enthalpy–entropy compensation

Enthalpy–entropy compensation (EEC) temperature is a useful thermodynamic approach to the analysis of physico-chemical data [23]. Mathematically the entropy–enthalpy compensation can be expressed by the following equation:

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G^\circ{}_\beta \quad (14)$$

$\Delta G^\circ{}_\beta$ is the corresponding Gibbs free energy variation at the compensation temperature β. According to Eq. (14), when enthalpy–entropy compensation is observed with a group of compounds in a particular chemical interaction, all the compounds have the same free energy $\Delta G^\circ{}_\beta$ at the temperature β [60]. The plot ΔH° versus $\Delta S^{\circ *}$ at 25 °C (Eq. (14)) was drawn for the statin molecules for all the pH values (Fig. 9). A regression line was obtained ($r^2 > 0.84$). According to several authors [61,62], it can be deduced that the fraction of the free energy that arises from the enthalpy contributions is the same for all the statin molecules and the pH bulk solvent. Similarly, the fraction of the total free energy arising from the entropy contributions is the same. But, since different mechanisms could result in the same proportion of enthalpy and entropy relative to the overall free energy, it cannot be deduced rigorously that the statin molecule association mechanism on the IAM surface was independent of the statin molecule structure and the pH bulk solvent. However, all the studied molecules have similar biological activity. These two conditions (EEC and similar biological effects) seem to imply a similarity of properties of all the studied statins.

4. Conclusion

In this present work, the statin binding mechanism on the IAM surface was examined. The results demonstrated that statin retention was pH-dependent. Indeed, for pH $\ll$ pH$_c$ (pH$_c \approx$ 7.0) an initial decrease in the statin–IAM surface association was observed, followed by a weak variation in the statin molecule affinity for the binding surface at pH > pH$_c$. Thus, for pH < pH$_c$ when pH increased, the electrostatic repulsion between the phosphate groups of the IAM surface and the carboxylate group negatively charged of the statin molecule increased. This decrease of the electrostatic interactions was accompanied by a reduction of the statin–IAM surface association process. At pHs lower than ≈7.0, the binding process is accompanied by protons release and at higher pHs, protons are taken up. A change in the phosphate group pK_a of the phospholipid monolayer has been proposed to contribute to the positive number of protons exchanged at pHs higher than 7.0 and its catalytic role on the deprotonation of STCOOH was shown. Moreover, the thermodynamic data of this association process demonstrated that this statin binding mechanism on the IAM surface was principally governed by hydrogen and van der Waals bonds to which were added electrostatic and hydrophobic secondary interactions. These hydrophobic interactions were confirmed by the establishment of a significant correlation between the logarithm of the statin retention factor with the lipid membrane extrapolated to a total aqueous bulk solvent at pH 7.0 ($\log k'_{w-\mathrm{IAM}}$) and the octanol–water partition coefficient ($\log P$). Enthalpy–entropy compensation suggested that statins have an identical retention mechanism with the phospholipid monolayer. As well, it was shown that pravastatin exhibited the lowest association with the IAM lipid monolayer. This result associated with IC50 data of each statin molecule confirmed that, its active uptake into hepatocytes needed a membrane transporter. Many studies have demonstrated that this transporter was the OATP-C hepatic (a Na^+-independent organic anion transporter peptide-C). OATPs (organic anion transporting polypeptides) are a group of membrane solute carriers expressing in multiple tissues including the liver, lungs, and kidneys. Among this superfamily, OATP-C is expressed in the human liver and involved in the hepatic uptake of a number of important substrates, including therapeutic drugs. Mevastatin, atorvastatin, simvastatin and fluvastatin exhibited higher affinity with IAM surface than pravastatin. Thus, these statins could efficiently cross the cellular membrane without a transporter by passive diffusion. Further experiments are now in progress in our laboratory in order to immobilize OATPs on the IAM surface so as to get further insight into the role of these transporters on the statin molecule diffusion into the cell membrane.

Acknowledgements

This work was supported by Grants 2006 from Direction Générale de la Recherche from Programme National de Biotechnologie, Ministère de la Recherche, France.

References

[1] T. Carbonell, E. Freire, Biochemistry 44 (2005) 11741.
[2] G. Devuyst, J. Bogousslavsky, Schweiz. Med. Wochenschr. 130 (2000) 1157.
[3] S. Bellosta, F. Bernini, N. Ferri, P. Quarato, M. Canavesi, L. Arnaboldi, R. Fumagalli, R. Paoletti, A. Corsini, Atherosclerosis 137 (1998) 101.
[4] R. Bitzur, H. Cohen, D. Harats, Harefuah 145 (2006), 831.
[5] R.H. Ho, R.G. Tirona, B.F. Leake, H. Glaeser, W. Lee, C.J. Lemke, Y. Wang, R.B. Kim, Gastroenterology 130 (2006) 1793.
[6] D.J. Maron, S. Fazio, M.F. Linton, Circulation 101 (2000) 207.
[7] E. Istvan, Atheroscler. Suppl. 4 (2003) 3.
[8] S.T. Mosley, S.S. Kalinowski, B.L. Schafer, R.D. Tanaka, J. Lipid Res. 30 (1989) 1411.
[9] C. Chen, R.J. Mireles, S.D. Campbell, J. Lin, J.B. Mills, J.J. Xu, T.A. Smolarek, Drug Metab. Dispos. 33 (2005) 537.
[10] S. Bellosta, R. Paoletti, A. Corsini, Circulation 109 (2004) 50.
[11] D. Nakai, R. Nakagomi, Y. Furuta, T. Tokui, T. Abe, T. Ikeda, K. Nishimura, J. Pharmacol. Exp. Ther. 3 (2001) 861.
[12] R.A. Brain, T.S. Reitsma, L.I. Lissemore, K. Bestari, P.K. Sibley, K.R. Solomon, Environ. Sci. Technol. 40 (2006) 5116.
[13] M.H. Davidson, T.T. Toth, Prog. Cardiovasc. Dis. 47 (2004) 73.
[14] J.W. Park, R. Siekmeier, M. Merz, B. Krell, W. Marz, D. Seidel, S. Schuler, W. Gross, Int. J. Clin. Pharmacol. Ther. 40 (2002) 439.
[15] A.J. Leo, C. Hanoch, D. Elkins, Chem. Rev. 71 (1971) 525.
[16] J.A. Rogers, A. Wong, Int. J. Pharm. 6 (1980) 339.

[17] L.G. Herbette, D.W. Chester, D.G. Rhodes, Biophys. J. 49 (1986) 91.
[18] L.G. Herbette, G. Gaviraghi, T. Tulenko, R. Preston-Mason, J. Hypertens. 11 (1993) 13.
[19] R. Kaliszan, A. Nasal, A. Bucinski, Eur. J. Med. Chem. 29 (1994) 163.
[20] R.P. Mason, D.W. Chester, Biophys. J. 56 (1989) 1193.
[21] S. Ong, H. Liu, X. Qui, G. Bhat, C. Pidgeon, Anal. Chem. 67 (1995) 755.
[22] S. Ong, H. Lui, C. Pidgeon, J. Chromatogr. A 774 (1996) 113.
[23] C. Pidgeon, S. Ong, H. Lui, M. Pidgeon, A.H. Dantzig, J. Munroe, W.J. Hornback, J.S. Kasher, J.S. Glunz, T. Szczerba, J. Med. Chem. 38 (1995) 590.
[24] J. Li, J. Sun, S. Cui, Z. He, J. Chromatogr. A 1132 (2006) 174.
[25] T. Salminen, A. Pulli, J. Taskinen, J. Pharm. Biomed. Anal. 15 (1997) 469.
[26] D. Vrakas, C. Giaginis, A. Tsantili-Kakoulidou, J. Chromatogr. A 1116 (2006) 158.
[27] T.E. Yen, S. Agatonovic-Kustrin, A.M. Evans, R.L. Nation, J. Ryand, J. Pharm. Biomed. Anal. 38 (2005) 472.
[28] M. Genty, G. Gonzàlez, C. Clere, V. Desangle-Gouty, J.Y. Legendre, Eur. J. Pharm. Sci. 12 (2001) 223.
[29] A. Avdeef, K.S. Box, J.E.A. Comer, C. Hibbert, K.Y. Tom, Pharm. Res. 15 (1998) 209.
[30] F. Barbato, D.G. Martino, L. Grumetto, M.I. La Rotondo, Eur. J. Pharm. Sci. 22 (2004) 261.
[31] E. Deconinck, H. Ates, N. Callebaut, E. van Gyseghem, Y. vander Heyden, J. Chromatogr. A 1138 (2007) 190.
[32] A. Nasal, M. Sznitowska, A. Bucinski, R. Kaliszan, J. Chromatogr. A 692 (1995) 83.
[33] C. Andre, Y.C. Guillaume, Talanta 63 (2004) 503.
[34] C. Andre, L. Ismaili, J. Millet, M. Thomassin, Y.C. Guillaume, Chromatographia 57 (2003) 771.
[35] G.W. Caldwell, J.A. Masucci, M. Evangelisto, R. White, J. Chromatogr. A 800 (1998) 161.
[36] S. Ong, S.J. Cai, C. Bernal, D. Rhee, X. Qui, C. Pidgeon, Anal. Chem. 66 (1994) 782.
[37] C. Pidgeon, U.V. Venkatoron, Anal. Biochem. 176 (1989) 36.
[38] D.S. Hage, J. Chromatogr. B 3 (2002) 768.
[39] K. Miyabe, Anal. Chem. 74 (2002) 2126.
[40] T. Fornstedt, P. Sajonz, G. Guiochon, J. Am. Chem. Soc. 119 (1997) 1254.
[41] P.J. Schoenmakers, J.R. Gant, A. Hartkopf, P.H. Weiner, J. Chromatogr. 128 (1976) 65.
[42] A. Hulshoff, S.H. Perrin, J. Chromatogr. 129 (1976) 263.
[43] http://www.ncbi.nlm.nih.gov/sites/entrez?db=pccompound.
[44] H. Mozsolits, T.H. Lee, H.J. Wirth, P. Perlmutter, M.I. Aguilar, Biophys. J. 77 (1999) 1426.
[45] D.E. Cohen, M.R. Leonard, J. Lipid Res. 36 (1995) 2251.
[46] P.D. Ross, S. Subramanian, Biochemistry 20 (1981) 3096.
[47] B.A. Hamelin, J. Turgeon, Trends Pharmacol. Sci. 19 (1998) 26.
[48] F. Darrouzain, P. Dallet, J.P. Dubost, L. Ismaili, F. Pehourcq, B. Bannwarth, M. Matoga, Y.C. Guillaume, J. Pharm. Biomed. Anal. 41 (2006) 228.
[49] W. Melander, C.S. Horvath, High Performance Chromatography: Advances and Perspectives, vol. 23, Academic Press, New York, 1986.
[50] J. Roitelman, E.H. Olender, S. Bar-Nun, W.A. Dunn, R.D. Simoni, J. Cell Biol. 117 (1992) 959.
[51] F. McTaggart, Atheroscler. Suppl. 4 (2003) 9.
[52] B. Hsiang, Y. Zhu, Z. Wang, Y. Wu, V. Sasseville, W. Yang, T.G. Kirchgessner, J. Biol. Chem. 274 (1999) 37161.
[53] M. Yamazaki, H. Suzuki, M. Hanano, T. Tokui, T. Komai, Y. Sugiyama, Am. J. Physiol. 264 (1993) 36.
[54] C. Andre, C. Guyon, M. Thomassin, A. Barbier, L. Richert, Y.C. Guillaume, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 820 (2005) 9.
[55] C.S. Horvath, W. Melander, I. Molnar, J. Chromatogr. 125 (1976) 129.
[56] C. Andre, Y.C. Guillaume, J. Chromatogr. B 801 (2004) 221.
[57] J. Huang, G.W. Feigenson, Biophys. J. 76 (1999) 2142.
[58] A. Tsamaloukas, H. Szadkowska, P.J. Slotte, H. Heerklotz, Biophys. J. 89 (2005) 1109.
[59] A.M. Caccuri, G. Antonin, P.G. Board, M.W. Parker, M. Nicotra, M. Lo Bello, G. Federici, G. Ricci, Biochem. J. 344 (1999) 419.
[60] R.R. Krug, Ind. Eng. Chem. Fundam. 19 (1980) 50.
[61] J. Li, P. Carr, J. Chromatogr. 12 (1994) 105.
[62] R. Ranatunga, M.F. Vitha, P. Carr, J. Chromatogr. A 946 (2002) 47.