Journal of Chromatography, 605 (1992) 1–17
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 189

Adsorption isotherms of cholesterol and related compounds in non-aqueous reversed-phase chromatographic systems

Pavel Jandera* and Georges Guiochon

Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1501 and Division of Analytical Chemistry, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120 (USA)

(First received January 14th, 1992; revised manuscript received March 17th, 1992)

ABSTRACT

The adsorption isotherms of cholesterol, cholesteryl acetate, cholesteryl formate and cholestanone were measured on two different chemically bonded C_{18} silica columns, using acetonitrile–dichloromethane or acetonitrile–*n*-hexane as mobile phases. In all instances the experimental isotherms are nearly but not extactly linear, exhibit significant deviations from Langmuir isotherm behavior and are fairly well described by a three-parameter quadratic equation. However, the best representation of the experimental data is obtained with a four-parameter quadratic isotherm. A model was worked out to account for these isotherms and to attempt an explanation of the physical meaning of the isotherm parameters on the basis of the limited solubility of the compounds studied in the bulk liquid phase, of two-layer adsorption and of an association between the sorbed molecules. A detailed study of the dependence of the isotherm coefficients on various experimental parameters (*e.g.*, type and specific surface area of the C_{18} phase, type and concentration of the solvents in the mobile phase) gave results in qualitative agreement with the model.

INTRODUCTION

The experimental determination of the equilibrium isotherms of the components of a mixture in chromatographic systems, the modeling of these isotherms and the study of their relationships to band profiles are important problems in preparative chromatography. Accurate predictive calculations of the individual band profiles by computer simulations require the exact description of these equilibrium isotherms [1]. From such profiles, precise optimum values of the experimental conditions for maximum production rate can be derived.

Because high solute concentrations are required to achieve high throughputs in preparative chromatography, the adsorption isotherms are rarely linear in the concentration range of interest. The simple Langmuir isotherm [2] describes properly the sorption equilibrium behavior in a few cases only, or in a low concentration range [3]. It usually fails because it assumes an ideal behavior of both the solution and the adsorbed layer [2], and it does not take into account the secondary effects which are important at high concentrations, such as the interactions between adsorbed molecules, the solvation effects or solubility limitations.

To account for these deviations from Langmuir adsorption behavior, a number of more sophisticated models have been suggested. The best known isotherm equations are the bilangmuir [4], the Fowler [5], the Volmer [6], the quadratic [7,8], the Toth [9] and the Unilan isotherms [10]. The formalism of the more rigorous models, based on

Correspondence to: Professor G. Guiochon, Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1501, USA.

* On leave from the Institute of Chemical Technology, Pardubice, Czechoslovakia.

0021-9673/92/$05.00

statistical thermodynamics [6–11], takes into account several phenomena ignored by the Langmuir model. In so doing, however, these models include more parameters, which explains, at least in part, their greater success in describing experimental data: they are more flexible. Conversely, it is often difficult to correlate these coefficients to the physical nature of the phenomena taking place in a given liquid–solid system and to give them a physical meaning.

Models of isotherms can be considered from two entirely different viewpoints. On the empirical front, they are fitting models, or equations used to fit to the experimental data for the purpose of representing simply these data in further calculations. From a theoretical point of view, they are the translation in mathematical language of our ideas of what is or should be the behavior of molecules in phase equilibria. A confusion arises easily because poor theoretical models can be good fitting models (*e.g.*, the Langmuir model). A good fit alone is never a proof of the theoretical value of a model.

Most studies on liquid–solid adsorption have been carried out using relatively simple compounds. Because of the development of the applications of preparative chromatography, there is a need for experimental data and for modeling of these data in the case of compounds more representative of those to which the method is routinely applied. The availability of series of results on similar compounds could help separation chemists in finding the most suitable equations to fit to their data.

In a recent study dealing with the simulation of the elution bands of a poorly soluble compound [12], we observed significant deviations from the Langmuir adsorption behavior for cholesterol in non-aqueous reversed-phase (NARP) systems. An empirical combination of a linear and a Langmuir isotherm was successfully fitted to the experimental data sets. This behavior appeared to be related to the limited solubility of cholesterol in most solvents commonly used in NARP and to a likely association of the flat hydrophobic molecules of steroid in the adsorbed layer.

The aim of this work was a deeper investigation of this adsorption behavior, a broadening of the earlier study to closely related steroids and a comparison of the results obtained when fitting the adsorption data to several isotherm equations.

THEORETICAL

Langmuir model

Although the Langmuir adsorption isotherm can easily be derived from statistical thermodynamics [6–11], its original derivation is based on a kinetic argument [2]. The equation is obtained by equating the adsorption and desorption rates:

$$\frac{dq}{dt} = k_1 C(q_s - q) \tag{1a}$$

$$-\frac{dq}{dt} = k_2 q \tag{1b}$$

where C and q are concentrations of the compound in the mobile and stationary phase, respectively, k_1 and k_2 are adsorption and desorption rate constants, respectively, and q_s is the adsorbent specific saturation capacity (*i.e.*, saturation capacity per unit mass). The Langmuir isotherm is

$$q = \frac{\frac{k_1}{k_2} \cdot q_s C}{1 + \frac{k_1}{k_2} \cdot C} = \frac{bq_s C}{1 + bC} = \frac{aC}{1 + bC} \tag{2}$$

where a ($= bq_s$) and b ($= k_1/k_2$) are auxiliary constants. The model assumes localized adsorption in a monolayer, no molecular interactions in either phase, an ideal solution and an ideal adsorbed layer.

Bilayer adsorption isotherm

The original Langmuir isotherm can be improved by introducing additional phenomena in the adsorption model in an attempt to make it more realistic. For example, we can consider that adsorption proceeds in two adsorption layers instead of a monolayer, with some competition between the first and the second layers. Then, the adsorption isotherm in the first layer is given by eqn. 1 (with $q = q_1$), and the following equations give the rates of adsorption and desorption from the second layer:

$$\frac{dq_2}{dt} = k_1^* C(q_1 - q_2) \tag{3a}$$

$$-\frac{dq_2}{dt} = k_2^* q_2 \tag{3b}$$

where q_1 and q_2 are the adsorbate concentrations in the first and the second layers, respectively, and k_1^*

and k_2^* are the adsorption and desorption rate constants for the competitive adsorption in the second layer, respectively. Combining eqns. 1 and 3 gives the following isotherm equation for the total concentration q in the stationary phase, assuming two-layer adsorption (bilayer model):

$$q = q_1 + q_2 = \frac{A_1C + A_2C^2}{1 + B_1C + B_2C^2} \quad (4)$$

Eqn. 4 is a special case of a quadratic isotherm with the coefficients A_1, A_2, B_1 and B_2 given as

$$A_1 = \frac{k_1}{k_2} \cdot q_s \quad (5a)$$

$$A_2 = 2 \cdot \frac{k_1}{k_2} \cdot \frac{k_1^*}{k_2^*} \cdot q_s \quad (5b)$$

$$B_1 = \frac{k_1}{k_2} + \frac{k_1^*}{k_2^*} \quad (5c)$$

$$B_2 = \frac{k_1}{k_2} \cdot \frac{k_1^*}{k_2^*} \quad (5d)$$

Clearly, the isotherm given in eqn. 5 depends only on three parameters, the two rate constant ratios (k_1/k_2, k_1^*/k_2^*) and the total specific saturation capacity of the adsorbent (q_s). In spite of this, the isotherm in eqn. 4 is different from the general quadratic isotherm derived by statistical thermodynamics, also a three-parameter equation. Statistical thermodynamics gives as general equation for the isotherm the ratio $C\,P'(C)/P(C)$, where $P(C)$ is a polynomial of degree n an $P'(C)$ its first differential [5–7]. The quadratic isotherm is written as

$$q = \frac{q_sC(b_1 + 2\,b_2C)}{1 + b_1C + b_2C^2} \quad (6)$$

Although the correct relationship (eqn. 5) holds between the coefficients A_2 and B_2, it does not hold between A_1 and B_1, and eqns. 4 and 6 are not equivalent.

Previous workers have used the three-parameter eqn. 6 in the calculation of chromatographic band profiles [13]. An isotherm equation similar to eqn. 4 was derived by Svoboda [8], following an approach similar to ours. However, the rationale for introducing a "blocking coefficient" in the denominator of eqn. 4 and not in the numerator (which makes the isotherm a four-parameter equation) is unclear.

If the approach followed for the derivation of eqn. 4 is applied to model the adsorption isotherm on a surface covered with two different types of independent adsorption sites, we obtain the bilangmuir isotherm. This isotherm is formally a special case of the quadratic isotherm where the parameters B_1 and B_2 are those given in the eqn. 4, but where the parameters A_1 and A_2 are different from the case of a bilayer adsorption and are

$$A_1 = q_{s1} \cdot \frac{k_1}{k_2} + q_{s2} \cdot \frac{k_1^*}{k_2^*} \quad (7a)$$

$$A_2 = (q_{s1} + q_{s2}) \frac{k_1}{k_2} \cdot \frac{k_1^*}{k_2^*} \quad (7b)$$

where q_{s1} and q_{s2} are the specific saturation capacities for the two types of adsorption sites of the adsorbent. If we compare eqns. 5a, 5b, 7a and 7b, we see that, formally, the bilayer adsorption model corresponds to a two-types-of-sites model with $q_{s2} = 0$ and $q = 2q_{s1}$.

Associative–bilayer adsorption isotherm

In the bilayer model of adsorption, the solute molecules of the second layer compete for adsorption on the molecules adsorbed in the first layer. This is a form of loose association. We can consider another model of association of the adsorbed molecules. As a first approximation, if the association is due primarily to dispersive (or hydrophobic) interactions, the adsorption of a molecule on the primary adsorbed layer does not change much the number of the adsorption sites available, as the area this molecule exposes to the mobile phase is approximately equal to the area it occupies when sorbed in the first layer. In such a case, the rate of adsorption on the second layer is not limited by the number of molecules adsorbed on the first layer as it is in the formulation of eqn. 3a and the equation for the adsorption rate now reads

$$\frac{dq_2}{dt} = k_1^*Cq_1 \quad (8)$$

The same procedure used above yields the following adsorption isotherm equation:

$$q = \frac{A_1C + A_2C^2}{1 + B_1C} = \frac{a_1C}{1 + bC} + a_2C \quad (9)$$

and the parameters A_1, A_2 and B_1 in eqn. 9 are given by

$$A_1 = \frac{k_1}{k_2} \cdot q_s \tag{10a}$$

$$A_2 = \frac{k_1}{k_2} \cdot \frac{k_1^*}{k_2^*} q_s \tag{10b}$$

$$B = \frac{k_1}{k_2} \tag{10c}$$

with the relationships

$$a_1 = A_1 - \frac{A_2}{B}; \quad a_2 = \frac{A_2}{B}; \quad b = B \tag{11}$$

Eqn. 9 is another particular case of the quadratic isotherm equation, different from eqns. 4 and 6. It can be rewritten as the combination of a linear and a Langmuir isotherm. If the association rate constant $k_1^* = 0$, eqn. 9 becomes the equation of the classical Langmuir isotherm.

The actual association process can be intermediate between a free association and the competition for the adsorption sites on the primary layer. In this case, the number of adsorption sites in the second layer would be equal to the number of molecules adsorbed in the first layer minus the number of molecules adsorbed in the second layer multiplied by a coefficient p, between 0 (bilayer–association model) and 1 (bilayer model). Eqn. 3a, for the adsorption rate on the second layer, would read

$$\frac{dq}{dt} = k_1^*(q_1 - pq_2) \tag{12}$$

and the adsorption isotherm becomes

$$q = \frac{\frac{k_1}{k_2} \cdot q_s C + \frac{k_1}{k_2} \cdot \frac{k_1^*}{k_2^*} \cdot q_s(1+p)C^2}{1 + \left(\frac{k_1}{k_2} + \frac{k_1^*}{k_2^*} p\right)C + \frac{k_1}{k_2} \cdot \frac{k_1^*}{k_2^*} \cdot pC^2} \tag{13}$$

Eqn. 13 is the general equation of a quadratic isotherm, with four parameters, k_1/k_2, k_1^*/k_2^*, q_s and p. Eqn. 13 becomes eqn. 9 for $p = 0$ and eqn. 4 for $p = 1$. The actual value of p should obviously depend on the solute and the phase system.

Limited solubility isotherm

Another complicating factor in the sorption process may be the limited solubility of the studied compound in the mobile phase. A provision for this effect can be included in the adsorption model. We can set a limiting condition on the number of available "sites" in the bulk liquid phase in the equation giving the desorption rate. This is done by analogy to the limitation of the adsorption rate by the saturation capacity, q_s (eqn. 1a):

$$-\frac{dq_1}{dt} = k_2 q_1(C_x - C) \tag{14a}$$

$$-\frac{dq_2}{dt} = k_2^* q_2(C_x - C) \tag{14b}$$

where C_x is the limiting solubility concentration of the solute in the bulk liquid phase.

With this solubility limitation, the same derivation procedure as above leads to the following equation for the monolayer (Langmuir) adsorption isotherm:

$$q = \frac{\frac{k_1}{k_2} \cdot q_s \cdot \frac{C}{C_x}}{1 + \left(\frac{k_1}{k_2} - 1\right)\frac{C}{C_x}} = \frac{aC}{1 + bC} \tag{15}$$

Eqn. 15 is formally the same as the classical Langmuir isotherm equation, but it shows that in the case of a limited solubility the sorption capacity q_s can no longer be calculated as the ratio of the two isotherm coefficients a and b. The maximum stationary phase concentration which can be reached in such a system is obtained by setting $C = C_x$ in eqn. 15 (then $q = q_s$). Eqn. 15 cannot be used for the solutes which have an unlimited solubility in the bulk liquid phase.

A limited solubility of the studied component in the bulk liquid is a sign of a lack of compatibility between this solute and the solvent, *i.e.*, of a moderate energy of interaction between their molecules. When the absolute value of the energy of the solute–solvent molecular interactions is smaller than the energy of the solute–solute molecular interactions, a poor solubility is likely to be observed, and to be accompanied by association between the sorbed molecules and by multi-layer adsorption.

If we take into account eqn. 12 for the adsorption rate on the second layer, together with a limited solubility in the bulk liquid, we can modify the isotherm eqn. 14 to read

$$q = \frac{\frac{k_1}{k_2} \cdot q_s \cdot \frac{C}{C_x} + \frac{k_1}{k_2} \cdot q_s \left[\frac{k_1^*}{k_2^*}(1+p) - 1\right]\frac{C^2}{C_x^2}}{1 + \left(\frac{k_1}{k_2} + \frac{k_1^*}{k_2^*} \cdot p - 2\right)\frac{C}{C_x} + \left(\frac{k_1}{k_2} - 1\right)\left(\frac{k_1^*}{k_2^*} \cdot p - 1\right)\frac{C^2}{C_x^2}} \quad (16)$$

Eqn. 16 describes another particular case of the quadratic isotherm, with the coefficients A_1, B_1, A_2 and B_2 defined otherwise than the coefficients in eqns. 4, 6 and 9. Eqn. 16 depends on five parameters, C_x, k_1/k_2, k_1^*/k_2^*, p and q_s.

It results from eqn. 16 that, at equilibrium with a saturated solution ($C = C_x$), the solute concentration in the stationary phase is given by $q = q_s(1 + p)/p$. Hence q should be twice the monolayer capacity when only competitive adsorption takes place in the second layer. On the other hand, q should increase indefinitely with decreasing $C - C_x$ if pure association with already adsorbed solute molecules is expected. The latter case would appear unrealistic, as it assumes that the number of adsorbed layers is unlimited. Experimental data confirm that, when the liquid-phase concentration becomes close to the saturation limit, there is no equivalent in liquid–solid adsorption to the pore condensation observed in gas–solid adsorption when the partial pressure approaches the vapor pressure.

In conclusion, if molecular association does take place, the proportionality constant p should be positive and values of q larger than double the monolayer saturation capacity q_s could be expected.

Fowler isotherm

The Fowler isotherm [5] is another three-parameter isotherm which takes into account the adsorbate–adsorbate molecular interactions through the use of an empirical interaction energy parameter χ. Except for these interactions, the same assumptions are made in the derivation of this model as for the Langmuir model. The Fowler isotherm equation is written as

$$bC = \frac{q}{q_s - q} \cdot e^{\chi \cdot \frac{q}{q_s}} \quad (17a)$$

or

$$q = \frac{bq_sC\, e^{-\chi \cdot \frac{q}{q_s}}}{1 + bC\, e^{-\chi \cdot \frac{q}{q_s}}} \quad (17b)$$

The Fowler isotherm equation was originally derived by statistical thermodynamics [5].

Data handling

The coefficients of the Langmuir isotherms were derived by linear regression of the plots of C/q *versus* C, derived from the experimental data [3]. The computations were performed on an IBM (Boca Raton, FL, USA) Model 50 Z personal computer. The VAX 8650 computer (Digital Equipment, Marlboro, MA, USA) of the Computer Center of the University of Tennessee and the SAS software were used for fitting all the other isotherm equations to the experimental data, using non-linear regression.

EXPERIMENTAL

Instrumentation

An HP 1090M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA), equipped with a DR solvent delivery system, an automatic sample injector with a 250-μl sample loop, a temperature-controlled column compartment, a diode-array UV detector and a data workstation was used to acquire the data necessary for the determination of the equilibrium isotherms.

Stainless-steel columns (250 mm × 4.6 mm I.D.) were packed in the laboratory with the following adsorbents, using a high-pressure slurry technique: Impaq RG2010-C_{18} (PQ Corp.), 10-μm C_{18}-bonded silica, silica average pore size 200 Å, surface area 246 m^2/g, amount of bonded material 16.85% C, bonding density 3.65 μmol/m^2, surface area after derivatization 139 m^2/g, dead volume V_M = 3.12 ml, phase ratio ϕ = 0.331, packing density 0.5 kg/l; and Nucleosil 500-C_{18} (Macherey–Nagel, Düren, Germany), 7-μm C_{18}-bonded silica, pore size 500 Å, surface area 35 m^2/g, V_M = 3.17 ml, ϕ = 0.308.

Solutes

Cholesterol, cholestanone, cholesteryl acetate and cholesteryl formate (all 99+% grade, Sigma, St. Louis, MO, USA) were dissolved in the mobile phases used for the determination of the isotherms, at concentrations approximately 10% below the solubility limits to avoid possible precipitation in the instrument lines.

Mobile phases

Spectroscopic-grade acetonitrile, dichloromethane, *n*-propanol and *n*-hexane (Burdick & Jackson, Muskegon, MI, USA) were used to prepare the mobile phases by mixing in the appropriate ratios. *n*-Hexane was dried and stored over molecular sieves 3A. All the solvents were filtered through a 0.45-μm filter (Millipore, Milford, MA, USA) before the preparation of the mobile phase. The mobile phase and the sample solution used for the determination of the adsorption isotherm were degassed continuously in the liquid chromatograph by stripping with helium. The mobile phase flow-rate was set at 1–3 ml/min, depending on the solute retention.

Determination of equilibrium isotherms

The equilibrium isotherms were measured using the frontal analysis method as described previously [14,15]. The mobile phase was stored in one of the solvent flasks of the solvent-delivery system, the solution of the solute under study, in a solvent of same composition as the mobile phase, in another flask. The gradient-delivery system was used to pump and mix the solutions needed for the frontal analysis experiments.

The ratio of the flow-rates of the two solutions controls the concentration of the solute delivered continuously to the column. It was adjusted from 0 to 100% in successive 10% steps. Time was allowed for the stabilization of the detector signal after each concentration change. The flow-rate and the column temperature (35°C) were kept constant during all the experiments.

In each experiment the solute concentration in the stationary phase was determined from the integral mass balance equation, using the experimental retention volume (inflection point of the breakthrough curve), corrected for the volume of the tubing between the mixing point of the liquids pumped in each channel and the column top, as described previously in more detail [15]. All the experiments were repeated at least twice.

The experimental results are presented in table form. All concentrations are in g/l.

RESULTS AND DISCUSSION

The experimental isotherms of the four compounds studied on the Impaq C_{18} column, with pure acetonitrile, and of cholesterol on the Impaq and Nucleosil C_{18} columns, with a series of acetonitrile–dichloromethane and acetonitrile–*n*-hexane mixtures as mobile phases, are shown as symbols in Figs. 1–4. Some of these experimental isotherms appear to be close to straight lines, especially in pure acetonitrile (Fig. 1), but there is a significant curvature at low solute concentrations and the data cannot be fitted accurately to straight lines. This is obvious in Figs. 2 and 3.

We successively fitted the four isotherm equations discussed in the previous section (eqns. 2, 4, 9 and 16) to the experimental results, in an attempt to find the equation having the smallest number of parameters that would fit these data. We discuss the sets of parameter values obtained with each model to show the extent of their agreement and/or their inconsistencies with these models.

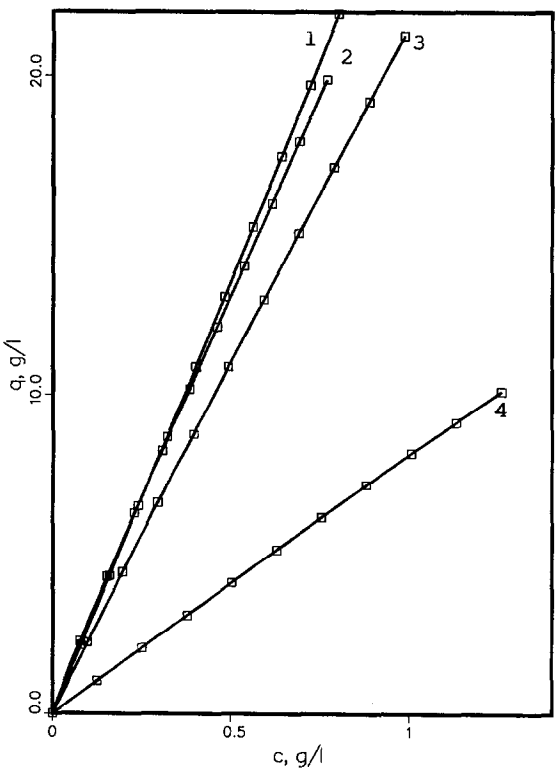

Fig. 1. Isotherms of (1) cholesteryl formate, (2) cholesterol, (3) cholestanone and (4) cholesteryl acetate on an Impaq RG2010-C_{18} column in pure acetonitrile at 35°C. Symbols, experimental data measured by frontal analysis; solid lines, best fits to the three-parameter quadratic isotherm (eqn. 9). c, q = Concentrations of the solutes in the mobile and stationary phase, respectively.

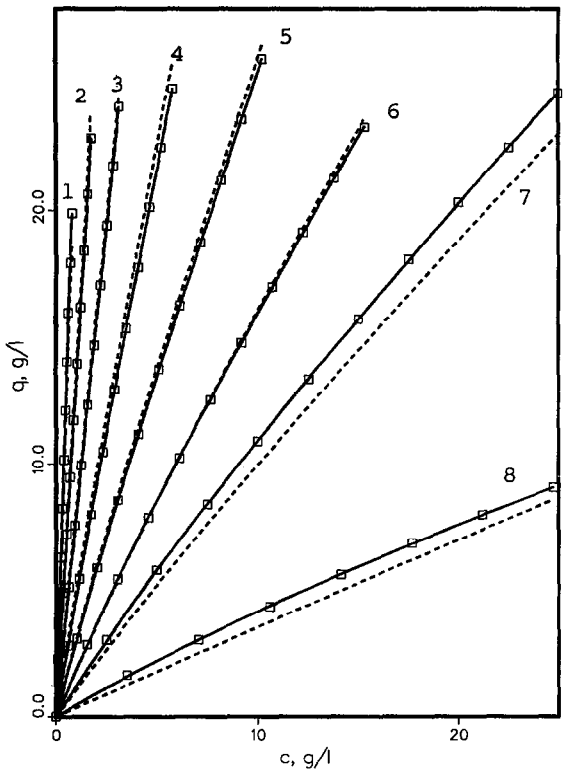

Fig. 2. Isotherms of cholesterol on an Impaq RG2010-C_{18} column in solutions containing (1) 0%, (2) 10%, (3) 20%, (4) 30%, (5) 40%, (6) 50%, (7) 60% and (8) 80% dichloromethane in acetonitrile at 35°C. Symbols, experimental data measured by frontal analysis; solid lines, best fits to the three-parameter quadratic isotherm (eqn. 9); dashed lines, isotherms calculated using the relationships derived in this work between the parameters A_1, A_2 and B of this isotherm and the concentration of dichloromethane in the mobile phase (from Table VII).

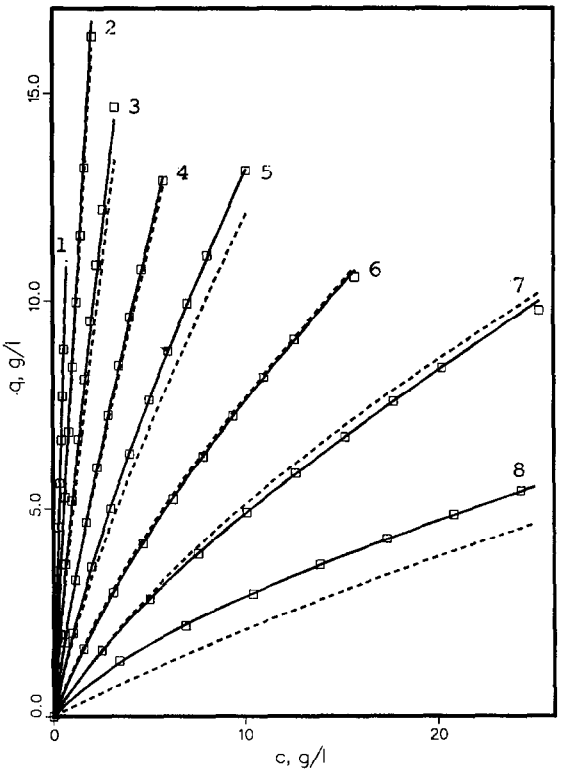

Fig. 3. Isotherms of cholesterol on a Nucleosil 500-C_{18} column in solutions containing (1) 0%, (2) 10%, (3) 20%, (4) 30%, (5) 40%, (6) 50%, (7) 60% and (8) 80% dichloromethane in acetonitrile at 35°C. Symbols, experimental data measured by frontal analysis; solid lines, best fits to the three-parameter quadratic isotherm (eqn. 9); dashed lines, isotherms calculated using the relationships derived in this work between the parameters A_1, A_2 and B of this isotherm and the concentration of dichloromethane in the mobile phase (from Table VII).

Langmuir isotherm

Although this isotherm equation broadly describes the main features of the experimental results, the shape of the Langmuir isotherm (eqn. 2) deviates significantly from the profile of the experimental isotherms, making an accurate fit impossible. The correlation coefficients of the fitted Langmuir equations were between 0.82 and 0.98 and the mean relative error of the values of q predicted from the best-fit Langmuir isotherm was between 1 and 3%. The best values of the parameters a and b of the Langmuir isotherms fitted to the experimental data on the two C_{18} columns with acetonitrile–dichloromethane as mobile phases are given in the Tables I (Impaq RG2010-C_{18}) and II (Nucleosil 500-C_{18}).

The parameter b of the Langmuir isotherm for cholesteryl formate in pure acetonitrile is negative, which does not make any physical sense within the framework of the Langmuir model. The reason for this behavior is that, differing from all other isotherms, the experimental isotherm of this compound is convex downwards, which explains the negative value for b and is inconsistent with the Langmuir model.

The data in Tables I and II show that the parameters a and b of the Langmuir isotherm of cholesterol decrease with increasing concentration of dichloromethane in acetonitrile. This is in agreement with the reversed-phase retention mechanism. Increasing the concentration of a less polar solvent

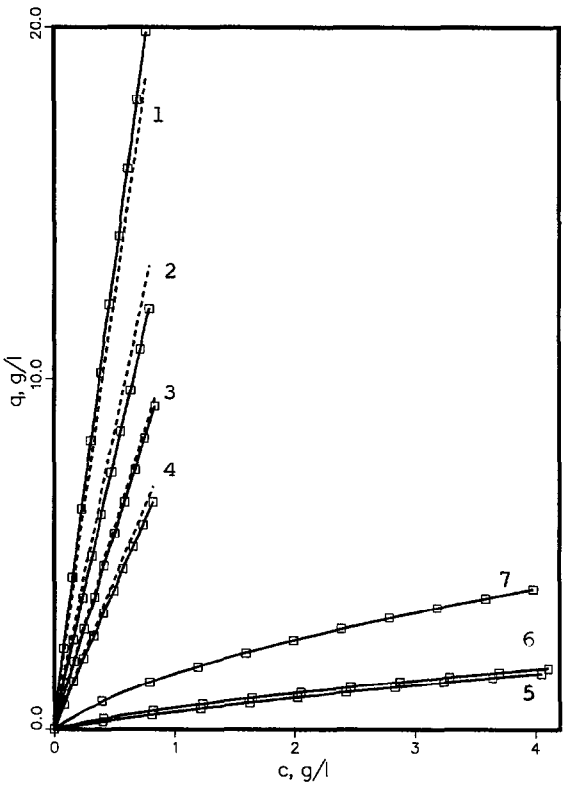

Fig. 4. Isotherms of cholesterol on an Impaq RG2010-C_{18} column in solutions containing (1) 0%, (2) 5%, (3) 10%, (4) 13.6%, (5) 98%, (6) 99% and (7) 100% *n*-hexane in acetonitrile at 35°C. Symbols, experimental data measured by frontal analysis; solid lines, best fits to the three-parameter quadratic isotherm (eqn. 9); dashed lines, isotherms calculated using the relationships derived in this work between the parameters A_1, A_2 and B of this isotherm and the concentration of dichloromethane in the mobile phase (from Table VII).

in a more polar solvent increases the solubility and decreases the retention of hydrophobic molecules such as the steroids studied here, and hence decreases the ratio of the adsorption and desorption rate constants, k_1/k_2.

A comparison of the dependence of the isotherm parameters on the composition of the mobile phase for the two C_{18}-bonded silica columns shows that the values of the parameter a of the Langmuir isotherm are always higher for the Impac C_{18} than for the Nucleosil C_{18}, whereas the opposite holds true for the parameter b (Tables I and II). The larger retention volume and the higher specific saturation capacity for Impaq C_{18} than for Nucleosil C_{18} are in agreement with the higher specific surface area of the former stationary phase.

We expect the specific surface area to have little effect on the rate constants of adsorption and desorption, whereas the saturation capacity should be proportional to this area. The large variation of the apparent specific saturation capacity ($q_s = a/b_L$) with increasing dichloromethane concentration in Tables I and II does not seem compatible with the

TABLE I

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE LANGMUIR ISOTHERM

Stationary phase, Impac RG2010-C_{18}. n.a. = Not applicable.

Solute[a]	φ[b]	a	b	q_s (mg/ml)
1	0	22.78	0.064	356
2	0	8.66	0.078	111
3	0	25.45	−0.031	n.a.
4	0	28.79	0.179	161
4	10	14.29	0.063	227
4	20	8.25	0.028	295
4	30	4.8	0.023	209
4	40	2.97	0.018	165
4	50	1.85	0.015	123
4	60	1.21	0.010	121
4	80	0.46	0.010	46

[a] Solutes: 1 = cholestanone; 2 = cholesteryl acetate; 3 = cholesteryl formate; 4 = cholesterol.
[b] Solvent composition (% dichloromethane in acetonitrile).

TABLE II

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE LANGMUIR ISOTHERM

Stationary phase, Nucleosil 500-C_{18}.

φ[a]	a	b	q_s (mg/ml)
0	17.26	0.264	65
5	11.67	0.168	69
10	9.16	0.114	80
30	3.21	0.083	39
40	1.96	0.055	36
50	1.05	0.038	28
60	0.63	0.027	23

[a] Solvent composition (% dichloromethane in acetonitrile).

Langmuir model assumptions. The surface area occupied by one adsorbed molecule on the surface should not depend to that extent on the solvent composition, even if the values of the saturation capacity at high dichloromethane concentrations are not considered, because of their relative lack of accuracy (too small a retention).

One can calculate that a saturation capacity of 100 mg/ml for cholesterol (MW = 386.64) corresponds to 0.26 mol/l. With a packing density of 0.5 kg/l and a surface area of 139 m^2/g, this would give a surface area of 45 $Å^2$ per cholesterol molecule, a small value for this flat molecules, but still a likely order of magnitude. An estimate of the maximum saturation capacity with a Langmuir isotherm can be derived from the surface area, assuming spherical molecules with a density of 1.5 (thus, packing to a fluid of 0.90 density). The diameter of such a molecule would be 9.35 Å. A dense monolayer (hexagonal distribution) of such spheres on the silica surface (area 69.5 m^2/ml) would include $9.1 \cdot 10^{19}$ molecules for a mass of 58 mg. Because a dense packing is impossible, some pores are not accessible to cholesterol and this molecule is flat and may be solvated, a saturation capacity of the order of 10–30 mg/ml would be more reasonable. The values in Tables I and II are unrealistically high, confirming the failure of the Langmuir model.

Three-parameter eqn. 9

The results of a fit of the three-parameter quadratic isotherm (eqn. 9) to all the experiment data sets is shown by the solid lines in Figs. 1–4. The agreement is much better than with the Langmuir model, with an average relative difference (model error) between the experimental values of q and the values calculated from the best-fit three-parameter quadratic isotherm between 0.05 and 0.8%. The best values obtained for the parameters a_1, a_2 and b of this isotherm equation (eqn. 9) when fitted to our experimental data are given in the Tables III (Impaq RG2010-C_{18}) and IV (Nucleosil 500-C_{18}). Also included in these tables are the values of A_1, A_2, the adsorption capacity, q_s, and the ratio of the second-layer association and desorption rate constants, k_1^*/k_2^*, derived from the values of a_1, a_2 and b (eqns. 10a–c and 11).

The values of the parameter b of the three-parameter quadratic isotherm equation are all positive, but they differ significantly between the individual compounds. For all the solutes, except cholesteryl formate, the ratio of the first layer adsorption and desorption rate constants ($b = k_1/k_2$) is greater than the corresponding rate constant ratio for the association in the second layer (k_1^*/k_2^*). The opposite behavior of cholesteryl formate results in a negative value for the parameter a_1 (but positive values for

TABLE III

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE THREE-PARAMETER QUADRATIC ISOTHERM

Stationary phase, Impaq RG2010-C_{18}. See eqn. 10, with $q_s = A_1/B = (a_1 + a_2)/b$, $k_1/k_2 = B$ and $k_1^*/k_2^* = A_2/A_1 = a_2b/(a_1 + a_2)$.

Solute[a]	φ[b]	a_1	a_2	b	q_s (mg/ml)	A_1	A_2	k_1^*/k_2^*
1	0	2.93	20.12	1.11	21	23.05	22.3	0.97
2	0	1.51	7.67	3.13	2.9	9.2	24.0	2.62
3	0	−11.3	36.77	0.076	335	25.5	2.79	0.11
4	0	12.89	25.11	22.50	1.70	38.0	565.0	14.9
4	10	3.19	12.64	3.82	4.1	15.8	48.3	3.05
4	20	1.34	7.42	1.48	5.9	8.8	11.0	1.25
4	30	1.05	3.97	0.40	12.6	5.0	1.60	0.32
4	40	0.86	2.28	0.22	14.3	3.14	0.50	0.16
4	50	0.66	1.27	0.10	19.3	1.93	0.13	0.07
4	60	0.47	0.78	0.05	25	1.25	0.04	0.03
4	80	0.23	0.25	0.04	12	0.48	0.01	0.02

[a] Solutes: 1 = cholestanone; 2 = cholesteryl acetate; 3 = cholesteryl formate; 4 = cholesterol.
[b] Solvent composition (% dichloromethane in acetonitrile).

TABLE IV

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE THREE-PARAMETER QUADRATIC ISOTHERM

Stationary phase, Nucleosil 500-C_{18}. See eqn. 10, with $q_s = A_1/B = (a_1 + a_2)/b$, $k_1/k_2 = B$ and $k_1^*/k_2^* = A_2/A_1 = a_2b/(a_1 + a_2)$.

φ^a	a_1	a_2	b	q_s (mg/ml)	A_1	A_2	k_1^*/k_2^*
0	6.96	13.92	11.64	1.8	20.9	162.0	7.76
5	5.17	9.92	7.45	2.0	15.1	73.9	4.90
10	4.26	7.56	4.47	2.6	11.8	33.8	2.86
30	1.62	1.76	0.43	7.85	3.4	0.75	0.22
40	1.06	0.96	0.25	8.1	2.02	0.24	0.12
50	0.69	0.45	0.13	8.8	1.14	0.06	0.05
60	0.57	0.24	0.16	5.1	0.81	0.04	0.04

[a] Solvent composition (% dichloromethane in acetonitrile).

A_1, A_2 and B) and in an isotherm which is slightly concave upward, whereas the isotherms of all the other compounds tested are slightly convex upward, as normally expected.

The data in Tables III and IV show that the parameters a_1, a_2 and b_T of the three-parameter quadratic isotherm of cholesterol decrease with increasing concentration of dichloromethane in acetonitrile, and so does the ratio k_1^*/k_2^*. As explained above in the case of the Langmuir isotherm, this is in agreement with the reversed-phase retention mechanism. Increasing the concentration of a less polar solvent in a more polar solvent not only increases the solubility and decreases the retention of this hydrophobic molecule by decreasing the ratio of the first-layer adsorption and desorption rate constants, k_1/k_2. At the same time as the solubility of cholesterol in the mobile phase increases, we expect that the ratio of the second-layer association rate constants, k_1^*/k_2^*, should decrease with increasing compound solubility in the bulk liquid phase. This is what we observe in the Tables III and IV.

However, we expect the model to give comparable values of the specific saturation capacities for the four steroids whose molecules have comparable sizes, and a nearly constant value for the specific saturation capacity of cholesterol when the dichloromethane concentration is increased. The experimental results are certainly not in agreement with this prediction. The order of magnitude is reasonable except at very low dichloromethane concentrations where the isotherm is nearly linear and the coefficients inaccurate. The increase in the saturation capacity with increasing dichloromethane concentration could be explained by a decreasing degree of solvation of the cholesterol molecule and an increasing solubility of the bonded alkyl chains in the mobile phase. More investigations are needed in this area.

Comparing Tables III and IV, we see that the parameters a_1 and a_2 are higher on Impaq C_{18} than on Nucleosil C_{18}, whereas the values obtained for the parameters b and k_1^*/k_2^* on the two phases are close, with the exception of the data measured with pure acetonitrile as the mobile phase. We expect the specific surface area to have little effect on the ratios of the rates of adsorption and desorption in the first and second adsorption layers but to affect to a greater extent the adsorption capacity, which is the result observed. The saturation capacity for Nucleosil 500-C_{18} is between two and three times smaller than for Impac C_{18}, in agreement with the four times smaller specific surface area and the 2.5 times larger average pore size, making the adsorbent surface more accessible.

We attempted to fit the Fowler isotherm equation (eqn. 17) to the experimental data sets for the C_{18} columns. These attempts were unsuccessful, probably because, at a given adsorption capacity and initial isotherm slope, the initial curvature of the isotherm increases with increasing value of χ, while the comparison between the results obtained with the Langmuir and the three-parameter isotherm shows that experimental results are better repre-

sented by an equation which has a smaller initial curvature than the Langmuir isotherm.

Four-parameter isotherms

Not surprisingly, a fit of the four-parameter quadratic isotherm equation (eqn. 4) to the experimental data sets, using non-linear regression, gave better results than those obtained with the three-parameter equation (eqn. 9). The values of the parameters A_1, A_2, B_1 and B_2 of this isotherm for the four solutes and the different stationary and mobile phases investigated are reported in the Tables V and VI, together with the parameters A_1, A_2 and B of the three-parameter isotherm (eqn. 9) calculated from the values of the parameters a_1, a_2 and b in Tables III and IV, using eqn. 10a–c.

The values calculated for the parameters A_1 and A_2 of the three- and four-parameter isotherm equations are generally close. In contrast, the values obtained for the parameters B_1 of the four-parameter isotherm equation differ significantly from the value of the parameter b in the Tables III and IV. For this reason, we used the parameter B_1 of the four-parameter isotherm instead of the parameter b in eqns. 10a–11 for the calculation of the coefficient A_2 of the three-parameter isotherm in Tables V and VI. A comparison between these tables and the Tables III and IV shows the extent of the differences.

Variation of isotherm parameters with mobile phase composition

Like the parameters a_1, a_2 and b, the parameters A_1, A_2 and B_1 of either the three- or the four-parameter isotherms decrease with increasing dichloromethane concentration in the mobile phase. By analogy with the well known dependence of the retention factors on the mobile phase composition in reversed-phase systems [16], we can expect the following empirical relationships to describe properly the ratios k_1/k_2 and k_1^*/k_2^* as a function of the concentration of dichloromethane in the mobile phase, φ:

$$\frac{k_1}{k_2} = k_0 e^{-\kappa\varphi} \tag{18a}$$

$$\frac{k_1^*}{k_2^*} = k'_0 e^{-\kappa'\varphi} \tag{18b}$$

where κ is a numerical coefficient.

If these empirical equations apply, we can expect an exponential decrease in the parameters A_1, A_2 and B of the three-parameter isotherm (eqn. 9) with increasing mobile phase concentration of dichloromethane. For the four-parameter isotherms (eqns. 13 and 16), a similar dependence is expected for A_1 and A_2. The results of the linear regressions of the logarithms of the isotherm parameters of cholesterol

TABLE V

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE THREE-PARAMETER (EQN. 9) AND THE FOUR-PARAMETER QUADRATIC (EQNS. 4 AND 16) ISOTHERMS

Stationary phase, Impaq RG2010-C_{18}; mobile phase, acetonitrile–dichloromethane. Solutes and φ as in Tablé I; A_1 (T) and A_2 (T) were calculated from eqn. 10a–c using the parameters a_1 and a_2 of the isotherm (eqn. 9) from Table III and the values of B_1 for b.

Solute	φ	A_1	A_2	B_1 (l/g)	B_2 [(l/g)^2]	A_1(T)	A_2(T)
1	0	23.11	33.61	1.62	0.019	23.11	32.56
2	0	9.12	19.60	2.59	−0.014	9.17	19.86
3	0	25.48	2.35	0.07	−0.007	25.43	2.44
4	0	34.49	292.2	11.94	−0.254	37.99	299.9
4	10	15.72	43.15	3.43	−0.006	15.83	43.38
4	20	8.75	10.28	1.39	−0.001	8.77	10.31
4	30	5.14	3.36	0.80	0.004	5.02	3.16
4	40	3.30	1.56	0.61	0.003	3.14	1.40
4	50	2.11	0.90	0.56	0.004	1.93	0.71
4	60	1.42	0.55	0.50	0.003	1.25	0.39
4	80	0.56	0.14	0.35	0.002	0.48	0.09

TABLE VI

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE THREE-PARAMETER (EQN. 9) AND THE FOUR-PARAMETER QUADRATIC (EQNS. 4 AND 16) ISOTHERMS

Stationary phase, Nucleosil 500-C_{18}; mobile phase, acetonitrile–dichloromethane. Solutes and φ as in Table II; isotherm parameters as in Table V.

φ	A_1	A_2	B_1 (l/g)	B_2 [(l/g)^2]	A_1(T)	A_2(T)
0	20.43	134.77	9.73	−0.216	20.88	135.48
5	14.88	64.30	6.37	−0.110	15.09	63.16
10	11.30	39.36	5.30	−0.064	11.82	40.03
30	3.96	3.15	1.32	0.039	3.38	2.33
40	2.58	1.54	1.05	0.023	2.02	1.01
50	1.27	0.91	0.98	0.023	1.41	0.44
60	0.95	0.40	0.81	0.012	0.81	0.19

on the concentration of dichloromethane in the mobile phase are reported in Table VII. These results are in general agreement with our other conclusions. The correlation coefficients are best for A_1, acceptable for A_2 and poor for B_1. No such dependence is observed for B_2.

The dashed lines in Figs. 2 and 3 show the three-parameter quadratic isotherms (eqn. 9) calcu-

TABLE VII

DEPENDENCE OF THE ISOTHERM PARAMETERS ON THE MOBILE PHASE COMPOSITION

Slopes (s) and intercepts (i) of the regression equations relating the mobile phase composition (φ) and the natural logarithms of the parameters A_1, A_2, B_1 and B_2 of the four-parameter quadratic isotherm (eqns. 4 and 16) and of the parameters A_1 and A_2 of the three-parameter quadratic isotherm (eqn. 9) of cholesterol. Isotherm parameters as in Table III; R^2 = correlation coefficient. Columns: I = Nucleosil 500-C_{18}; II = Impaq RG2010-C_{18}. Solvents: ACN = acetonitrile; DCM = dichloromethane; HEX = n-hexane; %HEX = 0–13.6%; %ACN = 0–2%.

Column	Mobile phase	φ	Parameter	Intercept	Slope	R^2
I	ACN–DCM	%DCM	A_1	2.968	−0.0519	0.9961
I	ACN–DCM	%DCM	A_2	4.618	−0.0975	0.9814
I	ACN–DCM	%DCM	B_1	2.039	−0.0430	0.9285
I	ACN–DCM	%DCM	A_1 (T)	2.990	−0.0557	0.9958
I	ACN–DCM	%DCM	A_2 (T)	4.693	−0.1114	0.9884
II	ACN–DCM	%DCM	A_1	3.282	−0.0498	0.9893
II	ACN–DCM	%DCM	A_1	4.623	−0.0905	0.9402
II	ACN–DCM	%DCM	B_1	1.570	−0.0399	0.8021
II	ACN–DCM	%DCM	A_1 (T)	3.347	−0.0527	0.9887
II	ACN–DCM	%DCM	A_2 (T)	4.703	−0.0966	0.9525
II	ACN–HEX	%HEX	A_1	3.542	−0.1009	0.9999
II	ACN–HEX	%HEX	A_2	5.774	−0.1498	0.9118
II	ACN–HEX	%HEX	B_1	2.571	−0.0639	0.6830
II	ACN–HEX	%HEX	A_1 (T)	3.641	−0.1061	0.9998
II	ACN–HEX	%HEX	A_2 (T)	5.798	−0.1500	0.9154
II	ACN–HEX	%ACN	A_1	0.967	−0.7364	0.9274
II	ACN–HEX	%ACN	A_2	0.763	−0.6171	0.8923
II	ACN–HEX	%ACN	B_1	0.804	−0.1535	0.9413
II	ACN–HEX	%ACN	A_1 (T)	0.854	−0.7048	0.9705
II	ACN–HEX	%ACN	A_2 (T)	0.141	−0.2787	0.7887

lated using the values of the parameters A_1, A_2 and B derived from the regression equations in Table VII, *i.e.*, using a single equation to account for the equilibrium isotherm in the whole range of mobile phase composition investigated. The agreement between this general isotherm equation and the experimental data is very good over a broad range of mobile phase compositions, from 0 to 50% dichloromethane. At higher dichloromethane concentrations, the differences between experimental and calculated data are more significant, but are still reasonably small below 80%.

The intercepts of the linear regressions of the logarithm of the parameter A_1 on the dichloromethane concentration in the mobile phase are higher on the Impaq C_{18} column than on the Nucleosil C_{18} column (Table VII). This was expected, as these intercepts depend on the adsorption capacity, which should be higher for Impaq C_{18} which has the larger specific surface area. On the other hand, the intercepts of the regressions for B_1 and the slopes for all three parameters of eqn. 9 do not depend on the saturation capacity and, according to eqns. 10a–c and 18a and b, should be far less affected by the type of C_{18}-bonded silica used. In agreement with these considerations, the values found for the two columns tested are close: -0.052 and -0.050 for the slopes of the A_1 regression, -0.098 and -0.091 for the slopes of the A_2 regression and -0.043 and -0.040 for the slopes of the B_1 regression.

Comparison between the models

The results discussed so far indicate that the four-parameter quadratic isotherm (eqn. 4) gives a very accurate representation of the experimental data. To a first approximation, however, these data are also in good agreement with a model assuming association between sorbed molecules in the second layer, but without competition (three-parameter quadratic isotherm, eqn. 9).

The small difference between the quality of the representation afforded by these two models is explained by the small values of the parameter B_2 in the four-parameter model, generally close to zero. The values of B_2 are lower for Impaq C_{18} than for Nucleosil C_{18}, except for cholesterol in pure acetonitrile. On both columns, the values of B_2 for cholesterol are negative in mobile phases containing 0–20% of dichloromethane in acetonitrile. They increase with increasing concentration of dichloromethane until small positive values are achieved at high concentrations. Because of the systematic character of this variation, it is unlikely that the negative values of B_2 at low concentrations could result from experimental errors or from errors made in fitting the data.

Isotherm model with limited solubility (eqn. 16)

Both four-term quadratic isotherms, whether taking into account full (eqn. 4) or partial (eqn. 13) competition for adsorption in the second layer, predict positive values for all the isotherm parameters and cannot explain negative values of B_2 for cholesterol and its esters in pure acetonitrile or in acetonitrile-rich mobile phases. However, the parameters A_2, B_1 and/or B_2 can be either positive or negative for the four-parameter quadratic isotherm model which takes into account a limited solubility of the solute in the mobile phase (eqn. 16).

The two adsorption–desorption rate constant ratios k_1/k_2 and k_1^*/k_2^* in eqn. 16 cannot be compared directly with the corresponding ratios estimated on the basis of the three-term isotherm (eqn. 9) because in the latter isotherm the ratios do not take into account the influence of the solubility of the solutes in the mobile phase. Considering the experimental solubilities of cholesterol determined recently and published elsewhere [12], we can calculate the values of these two rate constant ratios and of the proportionality constant p, knowing the constants of the four-parameter quadratic isotherm and assuming the validity of eqn. 16. The values obtained are reported in the Table VIII for the Impaq C_{18} column. On the basis of the parameters in this table, the dependence of the constant B_1 on the concentration of dichloromethane in the mobile phase can be explained as follows.

The proportionality constant p in eqn. 16 represents the fraction of molecules sorbed in the second layer which are not available for direct association with other solute molecules in the bulk liquid phase; p can be expected to increase with increasing solubility in the mobile phase, while the two rate constant ratios k_1/k_2 and k_1^*/k_2^* decrease. In mobile phases with low dichloromethane concentrations, the two rate constant ratios of cholesteryl esters and cholesterol are probably significantly higher than unity

TABLE VIII

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE FOUR-PARAMETER QUADRATIC ISOTHERM (EQN. 16) WHEN SOLUBILITY IS LIMITED

Stationary phase: Impaq RG2010-C_{18}. φ = % dichloromethane in acetonitrile. The values of the parameters k_1/k_2, k_1^*/k_1^*, p and q_s of the quadratic isotherm (eqn. 16) are calculated from the best values of the parameters A_1, A_2, B_1 and B_2 of the quadratic isotherm (eqn. 4), using the experimental solubilities C_x of cholesterol in mixtures of acetonitrile and dichloromethane on the Impaq RG2010-C_{18} column.

φ	C_x (g/l)	k_1/k_2	k_1^*/k_2^*	p	pk_1^*/k_2^*	q_s
0	0.872	11.43	7.40	0.133	0.981	2.631
10	2.241	8.69	6.15	0.162	0.996	4.053
20	3.932	6.46	4.62	0.216	0.998	5.322
30	6.794	6.39	4.42	0.234	1.033	5.465

and the coefficients A_2 and B_1 are positive. If p is low, however, the product pk_1^*/k_2^* can be lower than unity and B_2 is negative. When the concentration of dichloromethane increases, p increases more rapidly than the ratio k_1^*/k_2^* decreases, the product pk_1^*/k_2^* becomes higher than unity and the term B_2 is positive. However, because the solubility C_x also increases, B_2 is close to zero.

Most probably, the proportionality constant p increases with decreasing polarity of the molecule. Since the order of group polarity is keto < ester < hydroxyl, p should be larger for cholestanone than for cholesteryl esters, and larger for these esters than for cholesterol. This could explain why B_2 is positive for cholestanone in pure acetonitrile.

Isotherms with acetonitrile–n-hexane solutions

The results obtained with solutions of either *n*-hexane or dichloromethane in acetonitrile as mobile phases were very similar. The quality of the fit of the different isotherm equations used here to the experimental data measured on the Impaq C_{18} column with mobile phases containing moderate concentrations (below 14%) of *n*-hexane in acetonitrile is similar to that observed with acetonitrile–dichloromethane mobile phases. With these latter solutions, the average errors on the calculated values of q are 0.6–1% for the Langmuir isotherm, 0.06–0.2% for the three-parameter isotherm (eqn. 9) and 0.02–0.08% for the four-parameter quadratic isotherm (eqn. 4).

The average errors with mobile phases containing small concentrations of acetonitrile (below 3%) in *n*-hexane were 1–2.7% for the Langmuir isotherm, 0.3–0.7% for the three-parameter isotherm and 0.05–0.24% for the four-parameter isotherm. It was not possible to measure isotherms in mobile phases containing between 3 and 85% of acetonitrile in *n*-hexane, because these solvents are not miscible in this range of concentrations. In Fig. 4, we show the best three-parameter equation (solid lines) fitted to the experimental data (symbols). Attempts to fit the Fowler isotherm to the experimental data failed as they did with isotherm data obtained with mixtures of acetonitrile and dichloromethane.

In Table IX, we give the values of the parameters a and b_L of the Langmuir isotherm and of the parameters a_1, a_2 and b_T of the three-parameter isotherm (eqn. 9) of cholesterol on Impaq C_{18} with acetonitrile–*n*-hexane mobile phases, together with the ratios of the second layer association–desorption rates k_1^*/k_2^* calculated from eqns. 10 and 11. In mobile phases having a high acetonitrile content, the parameters of both the Langmuir and the three-parameter quadratic isotherms decrease with increasing *n*-hexane concentration, in agreement with the assumptions of the reversed-phase mechanism. On the other hand, in *n*-hexane-rich mobile phases, the isotherm parameters decrease with increasing acetonitrile concentration, which is expected for normal-phase systems. This behavior can probably be attributed to the existence of residual, unshielded silanol groups at the surface of the stationary phase.

TABLE IX

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE LANGMUIR AND THE THREE-PARAMETER ISOTHERMS

Stationary phase, Impac RG2010-C_{18}; mobile phase, acetonitrile–*n*-hexane. φ = % *n*-hexane in acetonitrile; parameters of the isotherms as in Table I.

φ	a	b_L (l/g)	a_1	a_2	b_T (l/g)	k_1^*/k_2^*
0	28.79	0.179	12.89	25.11	22.50	14.9
10	11.60	0.080	2.52	10.83	18.78	15.2
13.6	8.32	0.082	1.21	7.71	8.22	7.1
98	0.54	0.097	0.35	0.27	0.45	0.19
99	0.88	0.167	0.64	0.37	0.66	0.24
100	2.16	0.304	1.95	0.57	0.87	0.20

In Fig. 4, we show that the isotherms of cholesterol are more strongly curved in mobile phases having a high *n*-hexane concentration (conditions under which the normal-phase mechanism tends to control the retention) than in mobile phases having a high acetonitrile concentration, for which the reversed-phase mechanism applies. The ratio k_1^*/k_2^* should decrease with increasing solubility of cholesterol, *i.e.*, with increasing concentration of *n*-hexane in the mobile phase. This is the result observed.

The comparison of the values of the parameters A_1, A_2 and B calculated from a_1, a_2 and b of the three-parameter isotherm (eqn. 15) with the values of the corresponding parameters determined by fitting the four-parameter quadratic isotherm (eqn. 4) in Table X shows good agreement for A_1, fair agreement for A_2 in acetonitrile-rich mobile phases and poor agreement for A_2 in mobile phases with low concentrations of acetonitrile and for B_1. The parameters of the linear relationship between the logarithm of the isotherm parameters and the mobile phase concentration of *n*-hexane (at high acetonitrile concentrations) or of acetonitrile (at high *n*-hexane concentrations), φ, were in general agreement with eqn. 18. However, the quality of the correlation decreases in the order $A_1 > A_2 > B_1$ (see Table VII). These results are similar to those obtained with acetonitrile–dichloromethane solutions. The slopes of these relationships are steeper for *n*-hexane than for dichloromethane, in agreement with the lower polarity and higher solvent strength of *n*-hexane in non-aqueous reversed-phase systems (Table VII).

The dashed lines in Fig. 4 compare the data and the three-parameter isotherms (eqn. 9) calculated with the values of the parameters A_1, A_2 and B derived from the regression equations in Table VII. The degree of agreement achieved between the experimental data at high acetonitrile concentrations and the predictions of this general isotherm whose parameters are obtained as a function of φ is similar to the agreement seen in Figs. 2 and 3 for acetonitrile–dichloromethane mixtures as mobile phases. As with these solutions, the three-parameter quadratic equation is more suitable than the Langmuir isotherm to describe the experimental isotherm of cholesterol on a C_{18} column in acetonitrile–*n*-hexane solutions, but for precise calculations and for the interpretation of the isotherms, the four-parameter quadratic equation (eqn. 4), with a term B_2 different from zero, should probably be preferred.

The values of the parameters B_2 of the four-parameter quadratic isotherm of cholesterol are negative in mobile phases having a high acetonitrile concentration and positive in mobile phases having a high *n*-hexane concentration. This behavior can be explained by considering the isotherm eqn. 16, derived for the case of a limited sample solubility in the mobile phase, such as happens with the acetonitrile–dichloromethane solutions. In solutions having a low *n*-hexane concentration, the ratio of the second-layer adsorption and desorption rate constants is likely to decrease with increasing cholesterol solubility, *i.e.*, with increasing *n*-hexane concentration in the mobile phase, and the proportionality constant p is also expected to be small. Consequently, the product pk_1^*/k_2^* is lower than unity and, because the ratio of the first-layer adsorption and desorption rate constants, k_1/k_2, is obviously larger than unity, the parameter B_2 is negative, as it is with the solutions having a low dichloromethane concentration in acetonitrile (see above).

In solutions having a high *n*-hexane concentration, the cholesterol solubility is higher than in the solutions concentrated in acetonitrile. Consequently, p is higher and k_1^*/k_2^* is lower with these solutions. It remains possible, however, that the effect of the proportionality constant p predominates and that the product pk_1^*/k_2^* is larger than unity, as in the

TABLE X

REPRESENTATION OF THE EXPERIMENTAL DATA WITH THE THREE-PARAMETER (EQN. 9) AND THE FOUR-PARAMETER QUADRATIC (EQNS. 4 AND 16) ISOTHERMS

Stationary phase, Impaq RG2010-C_{18}; mobile phase, acetonitrile–*n*-hexane. φ = % of *n*-hexane in acetonitrile; isotherm parameters as in Table V.

φ	A_1	A_2	B_1 (l/g)	B_2 [(l/g)^2]	A_1(T)	A_2(T)
0	34.50	292.20	11.944	−0.254	37.99	299.9
10	12.67	103.75	9.72	−0.104	13.35	105.2
13.6	8.72	32.08	4.27	−0.078	8.92	32.9
98	0.68	0.71	1.61	0.077	0.62	0.72
99	0.99	0.90	2.00	0.110	1.01	0.74
100	2.96	2.43	2.19	0.180	2.52	1.25

TABLE XI

ABILITY OF THE MODELS TO ACCOUNT FOR THE EXPERIMENTAL DATA

Solutes as in Table I. Mobile phases: AC = acetonitrile; DCM = dichloromethane; HEX = *n*-hexane. Mean relative errors (%) of the concentrations q of (1) cholestanone, (2) cholesteryl acetate, (3) cholesteryl formate and (4) cholesterol calculated at equilibrium on the Impaq RG2010-C_{18} (1) or Nucleosil 500-C_{18} (2) column when using the Langmuir isotherm (L, eqn. 2), the three-parameter quadratic isotherm (T, eqn. 9) and the four-parameter quadratic isotherm (Q, eqns. 4 and 16).

Column	Mobile phase	φ	Solute	Mean relative error (%)		
				L	T	Q
1	DCM–AC	0% DCM	2	0.7	0.03	0.02
1	DCM–AC	0% DCM	3	0.23	0.06	0.02
1	DCM–AC	0% DCM	4	1.4	0.09	0.03
1	DCM–AC	10% DCM	4	1.1	0.04	0.03
1	DCM–AC	20% DCM	4	0.72	0.02	0.02
1	DCM–AC	30% DCM	4	0.8	0.11	0.04
1	DCM–AC	40% DCM	4	1.1	0.17	0.03
1	DCM–AC	50% DCM	4	1.1	0.28	0.07
1	DCM–AC	60% DCM	4	1.0	0.34	0.09
1	DCM–AC	80% DCM	4	1.3	0.45	0.12
2	DCM–AC	0% DCM	4	1.7	0.08	0.09
2	DCM–AC	5% DCM	4	1.8	0.14	0.07
2	DCM–AC	10% DCM	4	1.7	0.42	0.20
2	DCM–AC	30% DCM	4	1.5	0.41	0.07
2	DCM–AC	40% DCM	4	2.1	0.54	0.17
2	DCM–AC	50% DCM	4	1.7	0.78	0.40
2	DCM–AC	60% DCM	4	2.1	0.51	0.23
1	HEX–AC	10% HEX	4	0.8	0.06	0.01
1	HEX–AC	14% HEX	4	0.6	0.06	0.01
1	AC–HEX	1% AC	4	2.7	0.53	0.24
1	AC–HEX	2% AC	4	1.0	0.31	0.21

solutions of dichloromethane and acetonitrile which are concentrated in dichloromethane.

CONCLUSIONS

Cholesterol and many similar compounds have a limited solubility in most of the mobile phases used in non-aqueous reversed-phase chromatography. The Langmuir isotherm cannot describe successfully the adsorption behavior of these solutes in such chromatographic systems.

As demonstrated by the results obtained by fitting different isotherm equations to the experimental data (Table XI), a three-parameter quadratic isotherm (eqn. 9) accounts fairly well, as a first approximation, for the adsorption behavior of cholesterol and the related compounds studied on C_{18}-bonded phases in non-aqueous reversed-phase systems. This model accounts especially well for the moderate curvature of the experimental isotherms in the low concentration range. The values of the parameters of this isotherm and their variations with the composition of the solution are in qualitative agreement with the model of non-competitive adsorption or association of the solute molecules on the first adsorbed layer.

The four-parameter quadratic equation based on an isotherm model taking into account the limited solubility of the solute in the mobile phase, together with a non-stoichiometric competition of the adsorbate molecules in the second layer for access to the adsorption sites on the first layer, provides a good fit to the experimental data. The variation of the parameters of this isotherm for Impaq C_{18} with the mobile phase composition is in semi-quantitative agreement with the prediction of the model. With other systems, it is possible to explain qualitatively the observed dependencies of the isotherm param-

eters on the composition of the solution.

The experimental results described demonstrate the usefulness of the isotherm model presented here. This model can explain, at least qualitatively, the single-component experimental isotherms of cholesterol and of some related compounds. This model is also most convenient to fit the experimental data. However, the acquisition of a larger amount of such data would be necessary to prove the physical validity of the model and explain the physical meaning of its parameters.

ACKNOWLEDGEMENTS

We acknowledge the generous gift of the Impaq RG2010-C_{18} sample and detailed information regarding this product by Neil T. Miller (PQ Corp., Conshohoken, PA, USA). This work was supported in part by grant CHE-8901382 from the National Science Foundation and by the cooperative agreement between the University of Tennessee and the Oak Ridge National Laboratory. We acknowledge support of our computational effort by the University of Tennessee Computing Center.

REFERENCES

1 A. M. Katti and G. Guiochon, *Adv. Chromatogr.*, 31 (1991) 1.
2 I. Langmuir, *J. Am. Chem. Soc.*, 38 (1916) 2221.
3 J. D. Andrade, in J. D. Andrade (Editor), *Surface and Interfacial Aspects of Biomedical Polymers*, Vol. 2, Plenum Press, New York, 1985, p. 35.
4 R. J. Laub, *ACS Symp. Ser.*, 297 (1986) 1.
5 R. H. Fowler and E. A. Guggenheim, *Statistical Thermodynamics*, Cambridge University Press, Cambridge, 1939.
6 D. M. Ruthven, *Principles of Adsorption and Adsorption Processes*, Wiley, New York, 1984.
7 T. L. Hill, *Introduction to Statistical Thermodynamics*, Addison-Wesley, Reading, MA, 1960.
8 V. Svoboda, *J. Chromatogr.*, 518 (1990) 77.
9 J. Toth, *Acta Chim. Acad. Sci. Hung.*, 69 (1971) 311.
10 J. M. Honig and L. H. Reyerson, *J. Phys. Chem.*, 56 (1952) 140.
11 M. D. Levan and T. Vermeulen, *J. Phys. Chem.*, 85 (1981) 3247.
12 P. Jandera and G. Guiochon, *J. Chromatogr.*, 588 (1991) 1.
13 G. Guiochon, S. Golshan-Shirazi and A. Jaulmes, *Anal. Chem.*, 60 (1988) 1856.
14 E. Cremer and J. F. K. Huber, *Angew. Chem.*, 73 (1961) 461.
15 S. Golshan-Shirazi, S. Ghodbane and G. Guiochon, *Anal. Chem.*, 60 (1988) 2630.
16 P. Jandera and J. Churáček, *Gradient Elution in Column Liquid Chromatography*, Elsevier, Amsterdam, 1985.