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Mechanism of separation on cholesterol–silica stationary phase for high-performance liquid chromatography as revealed by analysis of quantitative structure–retention relationships

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Abstract

The retention characteristics of a newly synthesized stationary phase were determined for reversed-phase high-performance liquid chromatography obtained by chemical immobilization of cholesterol on spherical silica gel. For a designed series of analytes the retention factors, $\log k$, were determined at several compositions of the methanol-water mobile phase. Logarithms of retention factor corresponding to a hypothetical pure water eluent, $\log k_w$, were calculated by extrapolation of the linear relationships of individual $\log k$ data versus volume percent of methanol. The series of 24 test analytes were characterized structurally by means of the logarithms of *n*-octanol–water partition coefficients, $\log P$, by a set of the linear solvation energy relationship (LSER)-based descriptors of the polarity and bulkiness of the analytes and by structural descriptors of analyte size and polarity acquired by molecular modelling. Quantitative structure–retention relationships (QSRR) were derived by multiple regression analysis using the three groups of structural descriptors of analytes and the $\log k_w$ data determined on the new stationary phase. For the sake of comparison the corresponding QSRR equations were also derived for retention parameters determined on a standard octadecylsilica and on the so-called immobilized artificial membrane (IAM) stationary phase. The QSRR analysis clearly proved distinctive retention properties of the new cholesterol–silica stationary phase. It has been concluded that the new phase may possess valuable analytical specificity. Its application for modelling penetration of xenobiotics through biological membranes appears rather unlikely. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Reversed-phase high-performance liquid chromatography (RP HPLC); Cholesterol–silica stationary phase; Quantitative structure–retention relationships (QSRR); Linear solvation-energy relationships (LSER); Molecular modelling; Chemometrics

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1. Introduction

Modelling of permeation of drugs and drug candidates through biological membranes is typically based on the standard measure of hydrophobicity (lipophilicity), namely the logarithm of *n*-octanol–water partition coefficient, log *P* [1]. The log *P* scale is a valuable reference scale of hydrophobicity. It has been chosen rather arbitrary, however, and there are evidences that other systems than the *n*-octanol–water partition system may better model individual biological (pharmacological) properties of analytes [2,3].

Diversified partition systems are most conveniently provided by the reversed-phase high-performance liquid chromatography (RP HPLC) [4–6]. Advantages of RP HPLC retention parameters determined on specific stationary phases over log *P* in modelling individual pharmacological properties of drug analytes have been reported by several authors [3,7–9]. Special attention with that respect called the phospholipid–silica stationary phases introduced by Pidgeon and co-workers [10,11], i.e. the so-called immobilized artificial membrane (IAM) phases. Chromatographic hydrophobicity parameters determined on the IAM columns appeared especially suitable for modelling pharmacokinetics of drugs [12–14].

Cholesterol is an important component of biological membranes. Therefore, it seemed interesting to prepare a stationary phase for RP HPLC with cholesterol chemically immobilized on silica and to investigate its hydrophobic properties. The cholesterol-based stationary phases for HPLC also call analytical interest [15–17]. This is because of the expected specific separation properties of the presumed liquid crystal structure of the immobilized cholesterol layer.

The best means to characterize objectively and in a quantitative manner new stationary phase materials for all the modes of chromatography appears to be the analysis of quantitative structure–retention relationships (QSRR) [2,3,5–7]. There are several recent publications employing QSRR to identify those stationary phases for RP HPLC which possess distinctive separation properties. QSRR provide insight into molecular mechanism of separation operating in individual chromatographic systems and hence help to rationally design phases of required properties [3,7,18–27]. In this paper QSRR equations will be derived and discussed for retention data determined on a cholesterol-bound silica stationary phase for RP HPLC in relation to reference stationary phases.

2. Experimental

2.1. Column

The cholesterol-bound silica stationary phase was synthesized [28] using the highest quality spherical silica gel, Kromasil-100A (Eke Nobel AB, Bohus, Sweden). As a chemical modifier of silica surface γ-aminopropylotriethoxysilane (Wacker GmbH, München, Germany) was employed. Cholesterol used for synthesis was purchased from Sigma-Aldrich (Gillingham, UK). The cholesterol stationary phase obtained was thoroughly characterized physicochemically by ^{13}C-NMR in solid state, FT-IR and elemental analysis [29]. For the stationary phase used to slurry-pack the column which was employed in this work, the density of the silica surface coverage with the cholesterol ligands was 2.64 μmol m^{-2} and the number of ligands on the area unit was 1.58 nm^{-2}.

The chemical structure of the new stationary phase under study is illustrated in Fig. 1.

2.2. Analytes and their structural descriptors

A series of structurally diverse analytes designed for QSRR studies was used [18,26,30]. The compounds were selected in way that their structural descriptors were not intercorrelated mutually and they spanned a relatively wide range of values [31].

Three kinds of structural descriptors of test analytes were considered in QSRR analysis. The first was the logarithm of *n*-octanol–water partition coefficient, log *P*, taken from a compilation by Hansch et al. [32]. The second kind of analyte structural descriptors were the linear solvation energy relationships (LSER) [35] parameters according to Abraham [26,33,34]. The third group

of descriptors were the parameters determined by calculation chemistry (HyperChem package with the extension ChemPlus, HyperCube, Waterloo, Canada): highest atomic electron excess charge in the molecule of the analyte, δ_{min} (in electrons), total dipole moment, μ (in Debyes), and water-accessible molecular surface area, SAS (in Å^2).

Structural descriptors of test analytes are assembled in Table 1.

2.3. Chromatographic parameters

Test analyses were chromatographed using a Merck-Hitachi (Wien, Austria) apparatus equipped with an integrator and a variable-length UV detector.

Retention coefficients, k, were calculated for three to eight compositions of the methanol–water eluent assuming a signal of sodium nitrite as a dead time marker. Linear relationships were determined between $\log k$ and volume percent of methanol in eluent. The slopes, S, and intercepts, $\log k_w$ (Chol), of the relationships are given in Table 2, along with the correlation coefficients, R, and the numbers of data points, n, used in regressions.

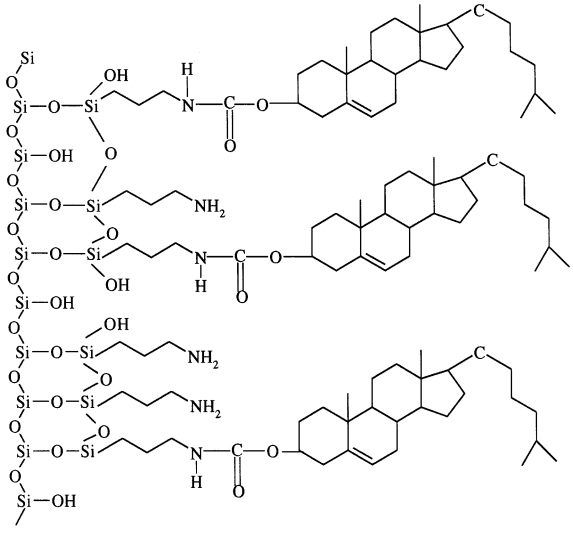

Fig. 1. Chemical structure of the cholesterol-bound silica stationary phase.

In Table 2 are also collected retention parameters previously determined on two other stationary phase materials. The $\log k$ (IAM) values were determined on the so-called immobilized artificial membrane material (IAM. PC. MG column, Regis, Morton Grove, IL; acetonitrile: buffer pH 7.0 10:90% v/v as the mobile phase) [26]. The $\log k_w$ (Poly) values were determined by extrapolation of $\log k$ data from several methanol–water eluent systems to a hypothetical pure water solvent employing a commercial octadecylsilica column Polygosil-60-5-C18 (Macherey-Nagel GmbH, Duren, Germany) [36].

2.4. Statistical analysis

Retention parameters from Table 2 were subjected to a multiple regression analysis (MRA) in terms of structural descriptors. Observing the requirements of statistically significant and physically meaningful MRA [31] the appropriate descriptors were selected and collected in Table 1.

All the calculations were run on a personal computer employing the Statgraphics package (Manugistics, Rockville, MD).

3. Results and discussion

At first the intercorrelations were checked between $\log k_w$ (Chol) determined on the new phase and the retention parameters for test solutes determined on the two reference columns: $\log k$ (IAM) and $\log k_w$ (Poly). The following equations resulted:

$$\log k_w \text{ (Chol)} = 1.7930(\pm 0.1376) + 1.3202(\pm 0.1144) \log k \text{ (IAM)} \quad (1)$$

$$n = 24;\ R = 0.9264;\ s = 0.5631;\ F = 133;\ p \leq 10^{-4}$$

$$\log k_w \text{ (Chol)} = 0.3407(\pm 0.1641) + 1.0056(\pm 0.0610) \log k_w \text{ (Poly)} \quad (2)$$

$$n = 24;\ R = 0.9618;\ s = 0.4094;\ F = 271;\ p \leq 10^{-4}$$

Table 1
Structural descriptors of test series of analytes used in analysis of quantitative structure-retention relationships (QSRR)[a]

No.	Analyte	Log P [b]	R_2 [c]	π_2^H [c]	α_2^H [c]	β_2^H [c]	V_x [c]	δ_{min}	μ^2	SAS
1	*N*-Hexylbenzene	5.52	0.591	0.5	0.00	0.15	1.562	−0.2104	0.03880	415.40
2	1,3,5-Triisopropylbenzene		0.627	0.4	0.00	0.22	1.985	−0.2057	0.00624	478.27
3	1,4-Dinitrobenzene	1.47	1.13	1.63	0.00	0.41	1.065	−0.3418	0.00012	312.07
4	3-Trifluoromethylphenol	2.95	0.425	0.87	0.72	0.09	0.969	−0.2454	4.39321	302.54
5	3,5-Dichlorophenol	3.62	1.02	1.1	0.83	0.00	1.020	−0.2434	1.98246	306.77
6	4-Cyanophenol	1.60	0.94	1.63	0.79	0.29	0.930	−0.2440	10.9693	290.61
7	4-Iodophenol	2.91	1.38	1.22	0.68	0.20	1.033	−0.3021	2.51856	301.47
8	Methylphenylether	2.11	0.708	0.75	0.00	0.29	0.916	−0.2116	1.56000	288.13
9	Benzamide	0.64	0.99	1.5	0.49	0.67	0.973	−0.4334	12.8450	293.30
10	Benzene	2.13	0.61	0.52	0.00	0.14	0.716	−0.1301	0.00000	244.95
11	Chlorobenzene	2.89	0.718	0.65	0.00	0.07	0.839	−0.1295	1.70824	269.49
12	Cyclohexanone	0.81	0.403	0.86	0.00	0.56	0.861	−0.2944	8.83278	269.31
13	Dibenzothiophene	4.38	1.959	1.31	0.00	0.18	1.379	−0.2709	0.27457	364.54
14	Phenol	1.47	0.805	0.89	0.60	0.30	0.775	−0.2526	1.52028	256.72
15	Hexachlorobutadiene	4.78	1.019	0.85	0.00	0.00	1.321	−0.0750	0.06708	352.14
16	Indazole	1.77	1.18	1.25	0.54	0.34	0.905	−0.2034	2.39011	285.46
17	Caffeine	−0.07	1.5	1.6	0.00	1.35	1.363	−0.3620	13.3298	367.02
18	4-Nitrobenzoic acid	1.89	0.99	1.07	0.62	0.54	1.106	−0.3495	11.7786	321.77
19	*N*-Methyl-2-pyrrolidinone	−0.54	0.491	1.5	0.00	0.95	0.820	−0.3532	12.9168	270.53
20	Naphtalene	3.30	1.34	0.92	0.00	0.20	1.085	−0.1277	0.00000	313.25
21	4-Chloronphenol	2.39	0.915	1.08	0.67	0.20	0.898	−0.2482	2.18448	280.38
22	Toluene	2.73	0.601	0.52	0.00	0.14	0.716	−0.1792	0.06916	274.50
23	Benzonitrile	1.56	0.742	1.11	0.00	0.33	0.871	−0.1349	11.1222	277.91
24	Benzoic acid	1.87	0.73	0.9	0.59	0.40	0.932	−0.3651	5.85156	288.00

[a] Log P, logarithm of *n*-octanol–water partition coefficient; R_2, the excess molar refraction; V_x, McGowan's characteristic volume; π_2^H dipolarity/polarizability; α_2^H, hydrogen-bond acidity; β_2^H, hydrogen-bond basicity; δ_{min}, highest atomic excess charge in the molecule; μ^2, square of total dipole moment; SAS, solvent (water)-accessible molecular surface area.
[b] According to Ref. [32].
[c] According to Refs. [26,33,34].

where n is the number of data points used to the derive regression equation, R is correlation coefficient, s is standard error of estimate, F is value of the F-test of significance and p is significance level; numbers in parenthesis are standard deviations of individual regression coefficients.

Correlation described by Eq. (2) is evidently higher than that in Eq. (1). This confirms the expected similarity of the hydrocarbonaceous phases: octadecyl–silica and cholesterol–silica. Certainly, 1-myristoyl-2-[(13-carboxyl)-tridecoyl]-*sn*-3-glycero-phosphocholine ligand of the IAM-type phase provides specific inputs to retention due to its polar fragments.

Correlation in Eq. (2) is relatively high but not absolute. Hence, the hydrophobicity parameter determined on the new cholesterol–silica phase is not identical with that normally assessed using the common octadecylsilica phases. This observation is confirmed by the correlations between the three chromatographic parameters considered and log P. These are as follows:

$$\log k_w \text{ (Chol)} = 0.6202(\pm 0.1767) + 0.8363(\pm 0.0660) \log P \quad (3)$$

$$n = 23;\ R = 0.9405;\ s = 0.4506;\ F = 161;\ p \leq 10^{-4}$$

$$\log k \text{ (IAM)} = -0.7685(\pm 0.1784) + 0.5965(\pm 0.0666) \log P \quad (4)$$

$$n = 23;\ R = 0.8902;\ s = 0.4550;\ F = 80;\ p \leq 10^{-4}$$

$$\log k_w \text{ (Poly)} = 0.3524(\pm 0.0931) + 0.7928(\pm 0.0348) \log P \quad (5)$$

$n = 23;\ R = 0.9804;\ s = 0.2375;\ F = 520;\ p \leq 10^{-4}$

As regards correlation with standard reference hydrophobicity parameter, $\log P$, the new cholesterol–silica reversed-phase material is localized between the modern, deactivated octadecylsilica material and the biological membrane mimicking material having polar outermost moieties.

Multiple regression analysis relating retention parameters (Table 2) to the LSER-based parameters of Abraham (Table 1) gave the following equations:

$$\log k_w \text{ (Chol)} = 0.4550(\pm 0.4041) - 0.7481(\pm 0.2935)\alpha_2^H - 3.1141(\pm 0.3095)\beta_2^H + 3.312(\pm 0.3313)V_x \quad (6)$$

$n = 24;\ R = 0.9575;\ s = 0.4523;\ F = 73;\ p \leq 10^{-4}$

$$\log k_w \text{ (IAM)} = -1.0941(\pm 0.3011) + 0.6889(\pm 0.2066)R_2 - 2.4247(\pm 0.2408)\beta_2^H + 1.8592(\pm 0.2603)V_x \quad (7)$$

$n = 24;\ R = 0.9470;\ s = 0.3535;\ F = 58;\ p \leq 10^{-4}$

$$\log k_w \text{ (Poly)} = 0.4194(\pm 0.2322) - 0.6906(\pm 0.1675)\pi_2^H - 0.4051(\pm 0.1581)\alpha_2^H - 2.3655(\pm 0.1968)\beta_2^H + 3\ 3574(\pm 0.1616)V_x \quad (8)$$

Table 2
Slope, S, and intercept, $\log k_w$ (Chol), of linear relationship between logarithms of retention factors determined on cholesterol–silica column and volume percent of methanol in methanol–water eluent[a]

No.	Analyte	Slope S	Intercept $\log k_w$ (Chol)	Correlation coefficient r	Sample size (n)	Log k (IAM)[b]	Log k_w (Poly)[c]
1	*N*-Hexylbenzene	−0.0592	5.5269	0.9999	4	2.056	5.186
2	1,3,5-Triisopropylbenzene	−0.0656	6.0963	0.9999	5	2.428	6.037
3	1,4-Dinitrobenzene	−0.0300	2.0735	0.9948	7	0.157	1.620
4	3-Trifluoromethylphenol	−0.0444	3.2194	0.9992	6	1.234	2.59
5	3,5-Dichlorophenol	−0.0435	3.7398	0.9995	5	1.895	2.946
6	4-Cyanophenol	−0.0331	1.8862	0.9923	8	0.771	1.288
7	4-Iodophenol	−0.0385	3.0596	0.9993	6	1.593	2.424
8	Methylphenylether	−0.0332	2.4282	0.9991	6	0.31	2.099
9	Benzamide	−0.0293	1.2629	0.9980	7	−0.099	0.777
10	Benzene	0.0326	2.3664	0.9984	6	0.093	2.022
11	Chlorobenzene	−0.0384	3.0489	0.9997	6	0.655	2.572
12	Cyclohexanone	−0.0244	1.2470	0.9995	4	−0.607	1.027
13	Dibenzothiophene	−0.0468	4.4631	0.9998	4	2.132	3.788
14	Phenol	−0.0294	1.6489	0.9881	8	0.366	1.197
15	Hexachlorobutadiene	−0.0534	4.8077	0.9989	4	1.998	4.406
16	Indazole	−0.0341	2.1380	0.9978	7	0.71	1.608
17	Caffeine	−0.0284	1.4102	0.9957	8	−0.396	0.847
18	4-Nitrobenzoic acid	−0.0132	1.0929	0.9975	3	−0.228	1.903
19	*N*-Methyl-2-pyrrolidinone	−0.0232	0.5202	0.9949	6	−1.497	0.195
20	Naphtalene	−0.0421	3.5423	0.9997	5	1.33	3.032
21	4-Chlorophenol	−0.0366	2.6810	0.9990	6	1.124	1.984
22	Toluene	−0.0364	2.8695	0.9996	6	0.436	2.613
23	Benzonitrile	−0.0337	2.0456	0.9934	7	0.154	1.679
24	Benzoic acid	−0.0139	0.8227	0.9605	3	−0.736	1.667

[a] r is the correlation coefficient of the relationship and n is the number of retention data points considered. In two last columns are retention data parameters considered for the sake of comparison and determined independently on the immobilized artificial membrane column, $\log k$ (IAM) [26], and on the Polygosil column, $\log k_w$ (Poly) [36].

[b] Taken from Ref. [26].

[c] After Kaliszan et al. [36].

$n = 24;\ R = 0.9897;\ s = 0.2200;\ F = 228;\ p \leq 10^{-4}$

The LSER-based structural descriptors of significance in Eqs. (6)–(8) are: R_2—the excess molar refraction; V_x—the so-called McGowans characteristic volume which can be calculated simply from molecular structure and which reflects the size (bulkiness) of the analyte; π_2^H—the measure of dipolarity/polarizability of the analyte which can be determined through gas-chromatographic and other measurements; α_2^H—the effective or summation hydrogen-bond acidity of the analyte; β_2^H—the effective or summation hydrogen-bond basicity of the analyte [33–35]. The regression coefficients at individual descriptors in Eqs. (6)–(8) are assumed to account for the complementary property of the chromatographic system (i.e. net property of the mobile/stationary phase system).

There is a negative term $-0.6906(\pm 0.1675)\ \pi_2^H$ present in Eq. (8), whereas the π_2^H term is insignificant in Eq. (6). This can be interpreted that in the Chol system the dipolarity/polarizability attractive interactions are roughly of the same magnitude between an analyte and the stationary phase, on the one hand, and the same analyte and polar eluents, on the other. In the case of Poly systems the dipolarity/polarizability attractions by the stationary phase are weak and analogous attractions by polar eluent predominate. This would indicate a higher polarity of the Chol column. It may be explained assuming that the aminocarboxy moieties of the Chol phase (Fig. 1) are partially accessible to analytes.

The LSER-based Abraham parameters predict best the retention on the octadecylsilica Poly column (Fig. 2) as it was the case also with log P. This could be expected because Abraham's parameters were actually designed to predict log P. The differences between the Chol and the Poly columns are reflected by the presence or absence of individual descriptors in Eqs. (6) and (8) and by the magnitude of respective regression coefficient. Considering the coefficient at α_2^H in Eqs. (6) and (8) one can conclude that the complementary hydrogen-bond acceptor properties of the stationary phase are weaker in case of cholesterol–silica than in case of Polygosil. The same is true regarding the hydrogen-bond donor properties of the two stationary phases which appear to be stronger in the case of Poly phase as reflected by smaller absolute value of the regression coefficient β_2^H (we assume that the same eluent system used with both phases provides the same negative inputs to retention). Probably the hydrogen-bond donating and accepting silanols are more accessible in case of Polygosil than cholesterol–silica. In the latter case they are screened by aminopropylsilica moieties.

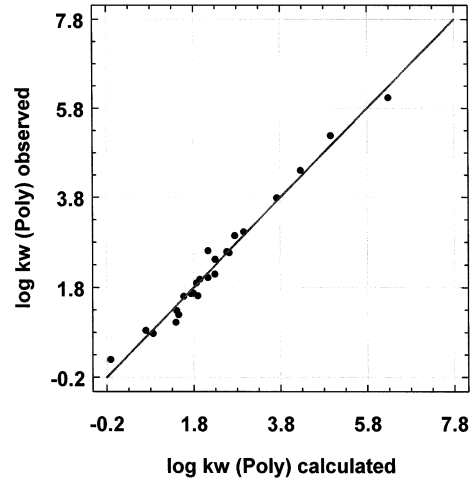

Fig. 2. Relationship between logarithms of retention parameter determined on a Polygosil-60-5-C18 column and corresponding to pure water eluent, $\log k_w$ (Poly), observed experimentally and calculated by Eq. (8).

The form of Eq. (7) describing retention on an IAM-type column is distinctive from that of both Eqs. (6) and (8) thus clearly indicating the specificity of this column.

The third set of structural descriptors of analyses presented in Table 1 are molecular modelling parameters, δ_{min}, μ and SAS. The following QSRR equations were derived employing these parameters:

$$\log k_w \text{ (Chol)} = -0.9856(\pm 0.6817) + 5.3280(\pm 1.4095)\delta_{min} - 0.1079(\pm 0.0262)\mu^2 + 0.0176(\pm 0.0020)\ \text{SAS} \quad (9)$$

$n = 24;\ R = 0.9477;\ s = 0.5003;\ F = 59;\ p \leq 10^{-4}$

$$\log k_w \text{ (IAM)} = -1.7215(\pm 0.8136) - 0.1157(\pm 0.0264)\mu^2 + 0.0094(\pm 0.0025)\ \text{SAS} \quad (10)$$

$n = 24;\ R = 0.8127;\ s = 0.6257;\ F = 20;\ p \leq 10^{-4}$

$$\log k_w \text{ (Poly)} = -1.8069(\pm 0.6076) + 4.5478(\pm 1.2563)\delta_{min} - 0.0965(\pm 0.0233)\mu^2 + 0.0183(\pm 0.0018)\ \text{SAS} \quad (11)$$

$n = 24;\ R = 0.9548;\ s = 0.4459;\ F = 69;\ p \leq 10^{-4}$

Again there is a close similarity of regression models for Chol and Poly phases and a dissimilarity with the IAM phase. Eqs. (9) and (11) have quite a good retention prediction potency (see Fig. 3 for illustration). This is especially worth to notice because the descriptors used can easily be calculated for any structural formula of an analyte. The parameters are also readily interpretable in physical sense. They show that retention increases with increasing *SAS*, i.e. the area of contact with molecules forming chromatographic systems. The *SAS* parameter reflects the ability of an analyte molecule to participate in nonspecific, dispersive (London-type) intermolecular interactions. These attractive interactions are stronger between a given analyte molecule and the bulky ligands of a stationary phases than between the same analyse molecule and the small molecules of eluent (water, methanol, acetonitrile). Hence, the net effect of dispersive interactions will increase retention. We assume that dipole moment reflects the ability of an analyte to take part in attractive dipole–dipole and/or dipole-induced dipole interactions. Such interactions will be stronger between a given molecule of analyte and the polar molecules of eluent, on the one hand, than between the same molecule and the basically nonpolar ligands of stationary phase, on the other hand. Hence, the net inputs to retention provided by μ^2 are negative in Eqs. (9)–(11). The same holds true for an another analyte polarity parameter, δ_{min} (it is a negative quantity!). A larger value of the coefficient at δ_{min} in Eq. (9) than in Eq. (11) confirms the previous conclusion that the Chol phase is more polar than Poly.

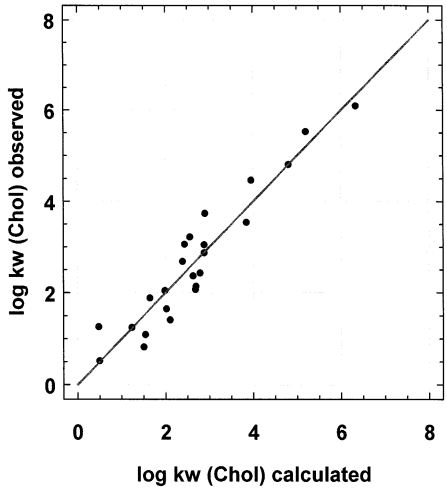

Fig. 3. Relationship between logarithms of retention parameter determined on the cholesterol-bound silica column and corresponding to pure water eluent, $\log k_w$ (Chol), observed experimentally and calculated by Eq. (9).

In conclusion it should be stressed that the new cholesterol–silica stationary phase possesses different retention properties than the typical hydrocarbonaceous silica phases for reversed-phase HPLC. It had also been demonstrated to be different from the IAM phases, as theoretically expected. Unique retention characteristics of the Chol phase may find specific analytical applications. The phase is less likely to find an application in modelling of the penetration of xenobiotics through biological membranes. For that application the inputs to retention on Chol due to an analyte-accessible aminocarboxy moiety of the ligand appears to be an obstacle. Probably the spacer arm binding cholesterol to silica matrix should be a longer hydrocarbon chain. Also, the cholesterol moiety should be bound that way that the hydroxyl group was free and hence able to interact with analytes as is the case with the cholesterol molecules dispersed in biological membranes.

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