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The use of phospholipid modified column for the determination of lipophilic properties in high performance liquid chromatography

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ABSTRACT

A new chromatographic stationary phase obtained by coating a reversed phase amide column with phosphatidylcholine based liposomes solution to yield a phospholipid modified column (PLM). The modification is achieved by the dynamic coating method which recycles the coating solution through the column in a closed loop for a period of 24 h. The chromatographic properties of the new column have changed significantly as compared to the original amide column due to the phospholipid coating. A good correlation was observed between n-octanol/water log *P* values and the logarithm of the retention factor obtained on the PLM column for a large number of solutes. In addition the PLM column was characterized using the linear solvation energy relationship (LSER). The values of the LSER system constants for the PLM column were calculated and were found to be very close to those of the n-octanol/water extraction system thus suggesting that the PLM column can be used for the estimation of n-octanol/water partition coefficient and serve as a possible alternative to the shake-flask method for lipophilicity determination. In addition, the results suggest that the PLM column can provide an alternative to other phospholipid-based column such as the IAM and the DPC columns.

1. Introduction

Lipophilicity is an important parameter in the process of designing new drugs. This physicochemical parameter is measured as the ratio of the concentration of a drug candidate between two immiscible phases, most often n-octanol/water. By the accepted definition, lipophilicity is the logarithm of the thermodynamic partition coefficient, log *P* [1,2] of the solute between the two immiscible solvents. The partition coefficient is traditionally determined by the shake flask method [3]. However this method is relatively slow, it requires large amount of a pure compound and tends to be complicated due to emulsion problems. In addition, different experimental method produced different values of log *P* for the same solute. Therefore, there was an increasing demand for developing an alternative analytical method for indirect determination of log *P*.

High performance liquid chromatography, in reversed phase separation mode, has been recognized as a potential method for lipophilicity determination [4–7]. The chromatographic method requires small amount of material, not necessarily pure, it is relatively fast and easily automated. The chromatographic approach is based on a correlation model between known log *P* values and chromatographic retention data (log *k*′), using standard set of solutes.

$$\log P = a \log k' + b \quad (1)$$

where *a* and *b* are regression constants for the slope and intercept accordingly.

The correlation model above is for neutral solutes. For ionized solutes the correlation model is between log *D* and log *k*′ where *D* is the distribution coefficient of the ionized solute in a n-octanol/water extraction system.

Various stationary phases have been examined in order to evaluate their lipophilicity prediction ability [8–15]. Some of these phases showed good correlation with log *P*. Criticism concerning the ability of conventional stationary phases to simulate the phospholipid bilayer of biological membranes, mainly due to the differences in the structures of the phases [16], led to the development of immobilized membrane stationary phases. The first immobilized membrane stationary phase is known as immobilized artificial membrane (IAM). IAM column is prepared by the covalent binding of phospholipid to propyl amino molecule on the surface of silica particles [17–20]. IAM may contain a single or a mixture of phospholipids, and among the phospholipids used are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglcerol (PG), phosphatidic acid (PA) and phosphatidylserine (PS) ligands [18]. These bonded phospholipid molecules are assumed to resemble biological membrane and, therefore, to emulate interactions of solutes or drugs with the

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phospholipids bilayers in the membranes. Vrakas et al. used an IAM PC DD2 column to determine the retention behavior at pH 7.4 for 43 neutral and basic drugs and correlated it to n-octanol/water partition coefficient log P (r^2 = 0.88) [21].

Liposomes are spherical vesicles form by aggregation of amphiphilic phospholipid molecules. Structurally, the liposomes resemble cell membrane and are extensively used as models mimicking the structure and function of membranes [22,23].

Immobilized liposome chromatography (ILC) is an additional tool for utilization of membrane lipids as components of chromatographic stationary phases [24]. ILC uses stationary phases where liposomes are immobilized into the support particles by either steric [25,26], covalent [27], hydrophobic [24] or electrostatic means. Lundahl et al. prepared ILC column by sterically entrapping liposomes in the pores of gel beads using freeze–thaw fusion. The specific retention factors of several drugs on that column correlate reasonably well with drug permeability in Caco-2 cell media [28]. Beigi et al. compared the retention of a set of compounds on an IAM-PC column and on the PC liposome column finding a moderate correlation (r^2 = 0.83) between the two columns [29]. Osterberg et al. studied the chromatographic retention of drug molecules on immobilized liposomes columns prepared from egg phospholipids and from chemically pure phospholipids. The correlation of the partition of drugs (log K_s) versus their log P_{oct} values showed three separate rectilinear relationships depending on the charge of the compound [30].

An alternative method for the application of liposomes and phospholipids as stationary phase is through dynamic coating. The stationary phase is prepared by dynamically coating phospholipids on a reversed phase column. Krause et al. described the preparation and properties of a noncovalent immobilized artificial membrane stationary phase. The column was prepared by pumping a phospholipid suspension through a reversed phase – RP-18 column [31]. Different phospholipids (PC, sphingomyeline, PE and PS) were examined with the use of RP-8, or RP-18 [32–35]. A modification of a reversed phase column (RP-8) by dynamically coating it with an aqueous solution of PC based liposomes (SUV) was previously reported by Tsirkin and Grushka [36]. The chromatographic behavior of the modified reversed phase column was changed and variations in retention factors and selectivity were obtained.

In principle, dynamic coating should provide a simple and economic method to prepare phospholipid-based stationary phases for the prediction of partition coefficient, log P. The present study describes the preparation and application of a lipid-based biomembrane stationary phase. The phase was prepared by dynamically coating egg-phosphatidylcholine-based liposomes on a commercial column – phospholipid modified (PLM) column. The selected column to coat was an Ascentis RP-amide which is a polar embedded column with an amide group attached to the hydrophobic chain. The polar group of the stationary phase is predicted to better mimic the structure of natural membrane. Once coated with the phospholipids, we measured retention data for a set of solutes and drugs and compared the changes in the column selectivity before and after coating. In addition, the suitability of the phospholipid-coated column to predict the n-octanol/water partition coefficient (log P) was assessed.

The PLM column was characterized using the linear solvation energy relationship (LSER) model of Abraham [37]. The solvation parameter model determines the contributions of the intermolecular interactions responsible for the retention in a given column. The model can be expressed as (Eq. (2)):

$$\log k' = c + eE + sS + aA + bB + vV \quad (2)$$

The model consists of a sum of products. Each product represents a different intermolecular interaction. The capital letters, E, S, A, B and V are the descriptors representing the solute properties. The corresponding system constants are indicated by the lower case letters. Each product expresses a contribution to the retention: eE takes into account the contribution due to electron lone pair interactions, sS represents dipole interaction, aA and bB take into account hydrogen-bond interaction and vV measures hydrophobic interaction via the differences in cavity formation ability in the two phases [38–40]. The system constants were calculated by multiple linear regression analysis for a set of log k' values of neutral solutes with known solutes descriptors.

In this study we will use the LSER analysis in order to characterize the PLM column to better understand the interactions between the new stationary phase and the solutes. Also, we will compare the system constants of the PLM column with those of the n-octanol/water partition system.

2. Experimental

2.1. Materials

Egg yolk phosphatidylcholine, Lipoid E PC S, which has fatty acid contents of mainly 33% palmitic acid, 14% stearic acid 27% oleic acid and 17% linolenic acid was obtained from Lipoid GMBH (Ludwigshafen, Germany).

The following standards compounds and drugs were purchased from Sigma Aldrich (Rehovot, Israel): acetone, 2-butanone, 3-pentanone, 2-hexanone, 2-heptanone, 2-octanone, acetophenone, propiophenone, butyrophenone, valerophenone, catechol, m-aminophenol, o-aminophenol, o-cresol, ethylbenzene, nitrobenzene, caffeine, antipyrine, aniline, 3-cholroaniline, acetaminophen, quinoline, 3-bromoquinoline, cortisone-21-acetate, corticosterone, cortisone, hydrocortisone, prednisolone, prednisone, hydrocortisone-21-acetate, naproxen, flurbiprofen, indoprofen, fenbufen, fenoprofen Ca^{2+} salt hydrate, ibuprofen, alprenolol hydrochloride, acebutolol hydrochloride, atenolol, nadolol, pindolol, propranolol hydrochloride, sotalol hydrochloride, metoprolol tartrate, lidocaine, procaine hydrochloride, prilocaine hydrochloride, mepivacaine hydrochloride, tetracaine hydrochloride. Hydroquinone, benzonitrile, chlorobenzene and m-toluidine were purchased from Merck (Germany). Phenol was obtained from J.T. Baker. P-cresol, m-cresol, toluene, anisole and p-nitroaniline were purchased from BDH Chemicals Ltd. (Poole, England). Solute solutions were prepared by dissolving the compounds in the mobile phase. The solutions were filtered through a 0.22 μm filter before injection.

The water used throughout was purified and deionized with Seradest SD 2000 system (Germany). HPLC grade methanol, used as the organic modifier, was purchased from J.T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA). Chloroform HPLC grade, was purchased from Carlo Erba Reagenti (Rodano MI, Italy).

The buffer part of the mobile phase consisted of 0.02 M disodium hydrogenphosphate (Sigma Aldrich, St. Louis, MO, USA) and was adjusted to pH 7.4 with phosphoric acid (J.T. Baker, Mallinckrodt Baker, Deventer, Netherlands). The mobile phase was prepared by mixing the appropriate buffer at a desired pH with methanol (20:80%, v/v, methanol/sodium phosphate buffer). The column void volume was estimated from the retention of sodium nitrate dissolved in the mobile phase measured at 230 nm. All measurements were performed in triplicate and the average is reported.

2.2. Instrumentation

Ascentis® RP-amide HPLC column, 50 mm × 4.6 mm, 5 μm (Supelco Analytical) was used as the column for the dynamic coating.

Table 1
LSER solute descriptors.

Solute	Descriptors					Reference
	V	*E*	*S*	*A*	*B*	
Acetone	0.547	0.179	0.7	0.04	0.51	[46]
2-Butanone	0.6879	0.166	0.7	0	0.51	[47]
3-Pentanone	0.829	0.143	0.68	0	0.51	[48]
2-Hexanone	0.968	0.136	0.68	0	0.51	[48]
2-Heptanone	1.111	0.055	0.663	0	0.51	[47]
2-Octanone	1.252	0.108	0.68	0	0.51	[48]
Acetophenone	1.0139	0.767	1.06	0	0.48	[47]
Propiophenone	1.155	0.8	0.85	0	0.51	[47]
Butyrophenone	1.2957	0.8	0.95	0	0.51	[47]
Phenol	0.7751	0.722	0.736	0.744	0.3	[47]
Hydroquinone	0.8338	1.063	1.27	1.06	0.57	[49]
Resorcinol	0.8338	0.98	1.11	1.09	0.52	[49]
Catechol	0.8338	0.97	1.1	0.88	0.47	[50]
m-Aminophenol	0.8747	1.13	1.15	0.65	0.79	[38]
o-Aminophenol	0.8747	1.11	1.1	0.6	0.66	[38]
m-Nitrophenol	0.9493	1.05	1.57	0.79	0.23	[38]
p-Cresol	0.916	0.82	0.87	0.57	0.32	[47]
m-Cresol	0.916	0.822	0.88	0.57	0.34	[50]
o-Cresol	0.916	0.84	0.86	0.52	0.31	[46]
Toluene	0.8573	0.564	0.516	0	0.14	[47]
Nitrobenzene	0.8906	0.871	1.11	0	0.28	[50]
Benzonitrile	0.8711	0.779	1.123	0	0.33	[47]
Anisole	0.916	0.71	0.75	0	0.29	[47]
Caffeine	1.3632	1.5	1.6	0	1.33	[47]
Antipyrine	1.5502	1.32	1.5	0	1.48	[50]
4-Chlorophenol	0.8975	0.895	0.745	0.949	0.2	[47]
Aniline	0.8162	0.996	0.985	0.254	0.5	[47]
3-Chloroaniline	0.939	1.05	1.1	0.3	0.36	[50]
Acetaminophen	1.1724	1.06	1.63	1.04	0.86	[12]
m-Toluidine	0.957	0.946	0.95	0.23	0.55	[47]
o-Toluidine	0.9571	0.966	0.92	0.23	0.59	[47]
o-Nitroaniline	0.9904	1.18	1.37	0.3	0.36	[47]
p-Nitroaniline	0.9904	1.22	1.83	0.45	0.38	[47]
m-Nitroaniline	0.9904	1.2	1.71	0.4	0.35	[50]
Quinoline	1.044	1.268	0.97	0	0.51	[47]
3-Bromoquinoline	1.2193	1.64	1.23	0	0.42	[12]

All chromatographic measurements were performed on a Waters 2690 separation module equipped with a Waters 996 photodiode array detector. All measurements were monitored at wavelength of 230 or 254 nm, mobile phase flow rate was 1.0/2.0 mL/min, and temperature was controlled at 30 °C.

2.3. Dynamic coating of the stationary phase

The column was prepared by immobilizing the phospholipid on the HPLC column. 0.38 g of egg-PC were dissolved in chloroform and evaporated by nitrogen. The lipid film was redissolved in a solution of methanol:water (1:1) and passed through a mini extruder (Avanti Polar Lipids, Alabaster, AL) equipped with a 100 nm pore size filter. This process produced large unilamellar vesicle (liposomes). The solution was diluted in the mobile phase to give a 1 mM liposomes solution. The lipid loading was achieved by recycling the solution through the column by the HPLC pump in a close loop for a period of 24 h. The loading was performed at 30 °C and at a flow rate of 1 mL/min. The coated phase was stored in water. When needed, the lipid phase of the column can be washed with 100% methanol.

Quantification of the amount of phosphatidylcholine loaded on the column was done by monitoring the coating solution before and after the coating process using a UV-VIS spectrophotometer (Shimadzu's UV 1601; Agentek, Israel). A second method for quantification used inductively coupled plasma (ICP) on the actual column packing measuring the phosphorus concentration in the stationary phase. The analysis was done by the analytical laboratory at the Geological Survey institute of Israel.

2.4. Lipophilicity and structural parameters

Log *P*, the logarithm of the partition coefficient, values were obtained from Syracuse Research Corporation's Physprop database and from experimental shake-flask data of Lombardo et al. [12]. Log *D* values for monoprotic acids and bases were calculated from the following equations [41] (Eqs. (3) and (4)) respectively:

$$\log D_{\text{acids}} = \log P + \log \frac{1}{1 + 10^{\text{pH}-\text{p}K_a}} \quad (3)$$

$$\log D_{\text{bases}} = \log P + \log \frac{1}{1 + 10^{\text{p}K_a-\text{pH}}} \quad (4)$$

The pK_a and pK_b data were obtained from SciFinder© (American Chemical Society).

The solute descriptors for the linear solvation energy relationship were obtained from the literature; see Table 1.

3. Results and discussion

3.1. The dynamic coating of egg phosphatidylcholine liposome based stationary phase

3.1.1. Initial quantification of the egg PC coating

Quantification of the amount of phosphatidylcholine loaded on the column was done by measuring the UV absorbance of the coating solution before and after the coating process. Based on the absorbance difference it was calculated that about 23 mg of the phospholipid were adsorbed by the column. This value agrees well with results published previously [32,35].

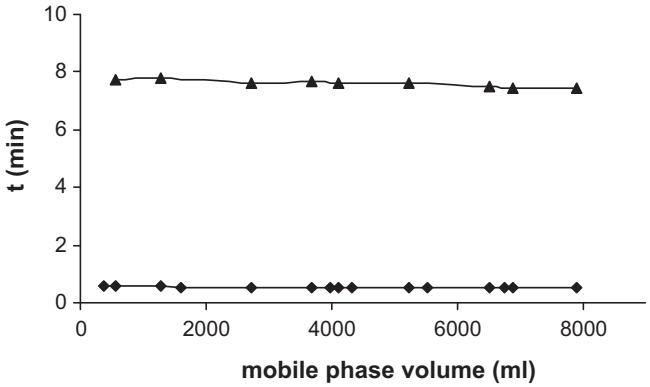

Fig. 1. Stability of the PLM coated column. Changes in the retention time of the void volume marker and the retention factor of resorcinol: (♦) retention time sodium nitrate; (▲) retention factor resorcinol.

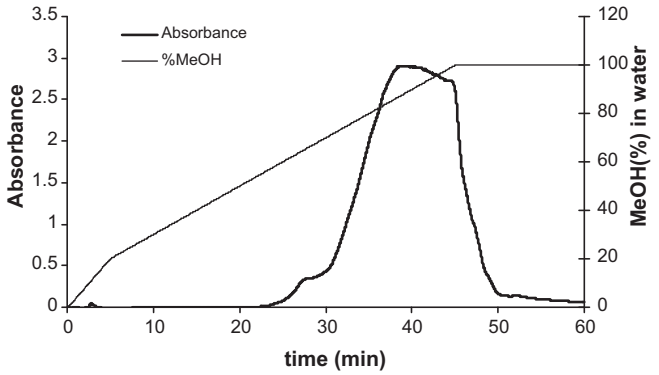

Fig. 2. Absorbance of the released egg PC coated in a linear gradient washing (increasing concentration of methanol in water 0–100% in 60 min, 1 mL/min, UV detection, 240 nm.

Second method for quantification of the phospholipid loaded on the column was done using inductively coupled plasma (ICP) to determine the amount of phosphorus in the stationary phase. The packing of the PLM column and of the RP-Amide were removed from the respective columns, analyzed by ICP and compared in order to evaluate the amount of phosphorus added by the coating process. The PLM column was actually divided into two parts one containing the front half of the packing and the second containing the back half one of the column in order to examine the homogeneity of the phospholipid coating.

The results from the ICP showed an increase from 7 ppm phosphorus in the uncoated column to an average of 4000 ppm phosphorus in the coated column. A 10% difference in the P content was observed between the two halves of the column, with the front half being richer in phosphorous. These results confirmed that phospholipids were coated on the column packing and that the coating was relatively uniform throughout the column length. The amount of phospholipid adsorbed on the column, calculated from the phosphorus concentration was 63 mg.

Although the results of the two methods were different, the calculations of the amount of lipid in the column were indirect and therefore not precise. The fact that both results were of the same order of magnitude was sufficient for proving that coating with phospholipids was accomplished and for estimating the amount of the adsorbed phospholipid on the column.

3.1.2. Reproducibility and stability of egg-phosphatidylcholine coating

The reproducibly of the coating procedure of the PLM column was about 8%. Retention measurements for each analyte, including the sodium nitrate, were performed in triplicate and the average reported, with the relative standard deviation (RSD) of the measurements were less than 1%.

In order to assess the stability of the phospholipid modified column, multiple retention experiments were done. The retention time of void volume marker and the retention factor of a chosen solute (resorcinol) were measured over two weeks of constant column use (Fig. 1) with a mobile phase of phosphate buffer with 20% methanol. The results indicate that the coating of the stationary phase was stable for a reasonable period of time; less than 1.5% RSD in the retention time.

The phospholipid stationary phase was washed out in a linear gradient elution of methanol in order to test the stability of coating to varying amounts of the organic modifier. The mobile phase gradient (from 0% to 100% methanol within 60 min) was monitored by UV detection at 240 nm (Fig. 2). The UV trace indicates that at methanol contents below 55% there was no measurable desorption of the coated stationary phase. Appreciable desorption of the phospholipids began to occur at 75% methanol and above. Thus, at the methanol levels used in this study, the coated column was stable for the durations of the experiments.

3.2. Chromatographic properties of neutral solutes and drugs

The chromatographic behavior of the coated column was examined by comparing retention time changes of neutral solutes. The retention factors, k', of 36 neutral solutes were measured on the RP-amide column before and after coating with the phospholipids; see Table 2. Each value of k' is an average of three determinations. The average standard deviation for all the measurements was around 0.01 with no large deviations observed. The retention behavior of the solutes on the modified column has clearly changed as compared to the uncoated column. The influence of the coating was different for each solute. In general, for solutes that can participate in hydrogen bonding, for example phenol derivatives or nitro anilines derivatives, the retention factors increased in the presence of the phospholipids. For all other solutes the retention decreased on the modified column.

The changes in the retention of the solutes due to the phospholipid coating resulted in the selectivity changes. Table 3 shows some examples of the changes in the selectivity between the RP-amide column and the PLM column for different pairs of solutes. Selectivity factor α for two solutes is defined by the ratio of their retention factors. Selectivity values less than 1 ($\alpha < 1$) on the PLM column indicate reversal in the retention order of the two solutes as compared to the retention order on the original column. The results in Table 3 demonstrate that the presence of the phospholipids significantly changed the column selectivity. For some pairs of solutes, the selectivity increased; for example in the case of acetophenone and 3-chloroaniline α increased from 1.32 to 3.39. For other pairs of solutes, the selectivity decreased or retention reversal occurred, as in the case of m-aminophenol and 2-butanone where α changed from 1.25 to 0.28. Note that in the case of these two solutes the selectivity actually increased substantially on the PLM column.

Different groups of drugs were examined on the coated column. The retention factors of the drugs before and after the coating are detailed in Table 2. The drugs included steroid hormones (neutral drugs at pH 7.4), local anesthetics and β-blockers (basic drugs that are ionized at pH 7.4) and NSAID's (acidic drugs; ionized at pH 7.4). As can be observed from Table 2, the presence of the phospholipid in the stationary phase reduced the retention times for all drugs except two (pindolol and propranolol). The most significant change in the retention time was observed for the steroid hormones where the retention times decreased by an order of magnitude.

Table 2
Lipophilicity values, k' and $\log k'$ determined for solutes in their neutral form and for drugs on the RP-amide column and on the PLM column.

No.	Solute	$\log P$	k'		$\log k'$	
			Ascentis RP-amide	PLM	Ascentis RP-amide	PLM
1	Acetone	−0.24	0.582	0.178	−0.235	−0.749
2	2-Butanone	0.29	1.600	0.580	0.204	−0.236
3	3-Pentanone	0.99	4.468	1.797	0.650	0.255
4	2-Hexanone	1.38	14.411	5.065	1.159	0.705
5	2-Heptanone	1.98	41.286	16.632	1.616	1.221
6	2-Octanone	2.37	148.487	58.336	2.172	1.766
7	Acetophenone	1.58	16.726	10.605	1.223	1.026
8	Propiophenone	2.19	48.678	32.433	1.687	1.511
9	Butyrophenone	2.77	139.913	87.806	2.146	1.944
10	Phenol	1.46	11.069	17.092	1.044	1.233
11	Hydroquinone	0.59	1.777	2.799	0.250	0.447
12	Resorcinol	0.8	3.131	7.735	0.496	0.888
13	Catechol	0.88	4.045	9.786	0.607	0.991
14	m-Aminophenol	0.21	1.284	2.075	0.109	0.317
15	o-Aminophenol	0.62	2.471	3.895	0.393	0.590
16	m-Nitrophenol	2	37.439	65.864	1.573	1.819
17	p-Cresol	1.94	28.344	40.873	1.452	1.611
18	m-Cresol	1.9	25.705	40.155	1.410	1.604
19	o-Cresol	1.95	28.334	44.905	1.452	1.652
20	Toluene	2.73	82.034	105.245	1.914	2.022
21	Nitrobenzene	1.85	22.084	24.544	1.344	1.390
22	Benzonitrile	1.56	13.462	10.699	1.129	1.029
23	Anisole	2.11	32.658	36.191	1.514	1.559
24	Caffeine	−0.07	3.712	0.346	0.570	−0.461
25	Antipyrine	0.38	4.818	0.529	0.683	−0.277
26	4-Chlorophenol	2.39	70.985	138.316	1.851	2.141
27	Aniline	0.9	3.446	3.991	0.537	0.601
28	3-Chloroaniline	1.88	22.035	35.958	1.343	1.556
29	Acetaminophen	0.51	2.358	1.366	0.373	0.135
30	m-Toluidine	1.4	9.368	9.668	0.972	0.985
31	o-Toluidine	1.32	8.515	8.548	0.930	0.932
32	o-Nitroaniline	1.85	25.301	36.408	1.403	1.561
33	p-Nitroaniline	1.39	13.635	21.011	1.135	1.322
34	m-Nitroaniline	1.37	11.412	18.130	1.057	1.258
35	Quinoline	2.03	24.045	13.005	1.381	1.114
36	3-Bromoquinoline	3.03	151.001	122.512	2.179	2.088
Steroid hormones						
37	Cortisone-21-acetate[a]	2.1		80.888		1.908
38	Corticosterone	1.94	342.087	44.254	2.534	1.646
39	Cortisone	1.47	140.710	16.012	2.148	1.204
40	Hydrocortisone	1.61	162.351	21.153	2.210	1.325
41	Prednisolone	1.62	164.511	23.873	2.216	1.378
42	Prednisone	1.46	121.168	14.193	2.083	1.152
43	Hydrocortisone-21-acetate[a]	2.19		58.527		1.767
NSAIDs[b]						
44	Naproxen	0.18	25.201	5.454	1.401	0.737
45	Flurbiprofen	0.90	105.593	18.935	2.024	1.277
46	Indoprofen	−0.24	28.515	2.307	1.455	0.363
47	Fenbufen	0.35	75.862	10.304	1.880	1.013
48	Fenoprofen	0.70	62.581	8.707	1.796	0.940
49	Ibuprofen	0.98	84.167	9.483	1.925	0.977
β-Blockers[b]						
50	Alprenolol	1.33	100.371	78.985	2.002	1.898
51	Acebutolol	0	39.870	8.687	1.601	0.939
52	Atenolol	−1.61	2.169	0.901	0.336	−0.045
53	Nadolol	−0.97	9.878	4.756	0.995	0.677
54	Pindolol	−0.06	14.372	20.972	1.158	1.322
55	Propranolol	1.73	112.419	247.140	2.051	2.393
56	Sotalol	−1.55	2.293	1.891	0.360	0.277
57	Metoprolol	−0.09	23.014	9.518	1.362	0.979
Local anestheics[b]						
58	Lidocaine	1.28	153.803	24.069	2.187	1.381
59	Procaine	0.29	13.476	10.061	1.130	1.003
60	Prilocaine	1.45	47.312	15.209	1.675	1.182
61	Mepivacaine	1.18	48.245	9.099	1.683	0.959
62	Tetracaine[a]	2.61		111.064		2.046

[a] Solutes with long retention time, did not elute from the column.
[b] For the ionized drugs $\log D^{7.4}$ parameters were presented and were calculated by Eqs. (3) and (4).

Table 3
Changes in the selectivity between different pairs of solutes. A change from $\alpha > 1$ to $\alpha < 1$ means reversal in the retention order.

No.	Solutes	Selectivity, α	
		Ascentis RP-amide	PLM
1	m-Aminophenol/2-butanone	1.25	0.28
2	Hydroquinone/acetaminophen	1.33	0.49
3	Resorcinol/aniline	1.10	0.52
4	3-Pentanone/antipyrine	1.08	0.29
5	m-Nitroaniline/benzonitrile	1.18	0.59
6	p-Nitroaniline/2-hexanone	1.06	0.24
7	Acetophenone/3-chloroaniline	1.32	3.39
8	Nitrobenzene/quinoline	1.09	0.53
9	p-Cresol/anisole	1.15	0.89
10	m-Nitrophenol/2-heptanone	1.10	0.25
11	4-Chlorophenol/toluene	1.16	0.76
12	Butyrophenone/2-octanone	1.06	0.66

3.3. Relationship between the retention factor and partition coefficient – log P or log $D^{7.4}$ on the phospholipid modified column

To examine the ability of the coated column to emulate the *n*-octanol/water partition system, the correlations between log *P* values and log *k*′ for the set of neutral solutes and drugs were examined. The correlated data are shown in Fig. 3. The regression data for the correlation, at 95% confidence level, are as follow:

(a) For the set of neutral solutes:

$$\log P = 1.07(\pm 0.06) \log k' + 0.35(\pm 0.08) \quad n = 38;$$
$$r^2 = 0.90; \quad s = 0.28; \quad F = 324 \tag{5}$$

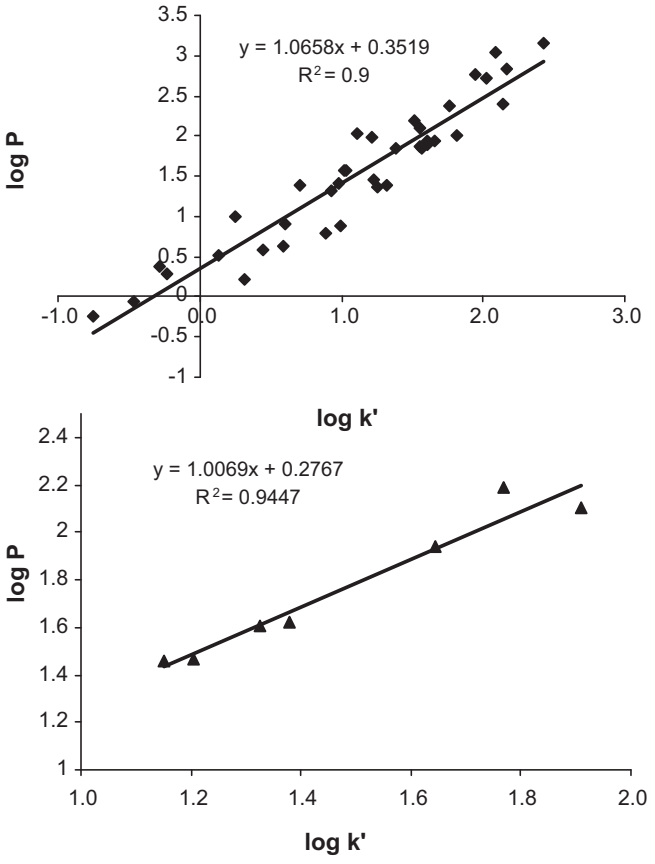

Fig. 3. (a) Relationship between log *P* and log *k*′ for a set of neutral test solutes. (b) Relationship between log *P* and log *k*′ for a set of neutral drugs – steroid hormones.

(b) For the set of neutral drugs (steroid hormones):

$$\log P = 1.01(\pm 0.11) \log k' + 0.28(\pm 0.16) \quad n = 7;$$
$$r^2 = 0.94; \quad s = 0.08; \quad F = 85 \tag{6}$$

The values in the parentheses represent the standard deviation, *n* is the number of solutes, r^2 is the squared correlation coefficient, *s* is the standard error and *F* is the Fischer's test value.

For the neutral solutes and drugs, good correlations between log *P* and log *k*′ were observed. For both correlations the slope is close to unity and the intercept value is small, which implies that the two processes, the retention on the coated column and partition mechanism in the *n*-octanol/water extraction system, are homoenergetic; that is, the changes in the free energies due to the transfer from one phase to another are equivalent in both systems.

To gain better understanding of the interactions that govern the retention mechanism, the neutral solutes were grouped according to their ability to participate in hydrogen bonding. The correlation between log *k*′ and log *P* values for solutes that are non hydrogen bond donors is described by Eq. (7) while Eq. (8) shows the correlation for solutes that are hydrogen bond donors

$$\log P = 1.08(\pm 0.03) \log k' + 0.57(\pm 0.05) \quad n = 19;$$
$$r^2 = 0.99; \quad s = 0.13; \quad F = 1122 \tag{7}$$

$$\log P = 1.08(\pm 0.07) \log k' + 0.10(\pm 0.09) \quad n = 19;$$
$$r^2 = 0.93; \quad s = 0.17; \quad F = 228 \tag{8}$$

Eqs. (7) and (8) show an improvement in the correlation between log *P* and log *k*′ for both solute sets. The significant improvement in Eq. (7) indicates that solutes without hydrogen bond donating capability behave in a very similar way in the n-octanol/water partition system and in the PLM column. Eq. (8) shows the correlation between log *P* and log *k*′ for the solutes capable of forming hydrogen bonds with the phospholipid phase as donors. Similar to Eq. (7), there is an improvement in the correlation, albeit to a lesser extant. The presence of the phospholipids in the stationary phase augmented the hydrogen bonding interactions thus, producing a different retention mechanism and resulting in longer retentions.

For the ionized drugs, the correlation between the logarithm of the distribution coefficient (log $D^{7.4}$) and log *k*′ was examined. The drugs comprised of basic β-blockers and local anesthetics and acidic NSAID's. All measurements were performed with phosphate buffer at pH 7.4 as detailed in the experimental part, on Ascentis RP-amide column before and after coating with the Egg phosphatidylcholine solution. Table 4 summarizes the correlation data for the ionized drugs before and after the coating. The data show that the correlation between log $D^{7.4}$ and log *k*′ improved for all drugs by the phospholipid coating. The improvement indicates that the phospholipid environment imitates better the n-octanol/water partition system. In particular, very good correlation was obtained for the β-blockers. The moderate correlations for the local anesthetics and for the NSAID's are due, most likely, to two factors. In the first place, the log $D^{7.4}$ values were calculated from estimated log *P* and pK_a values and deviations from the experimental values are observed frequently, especially when intermolecular H-bonding is possible. Secondly, at pH 7.4 residual silanols in the column are ionized and negatively charged and can give rise to ion exchange interactions [11]. Such interactions make it more difficult to obtain a good correlation between log *D* and log *k*′.

Table 4
Correlation between $\log D^{7.4}$ and $\log k'$ for ionized drugs on the RP-amide column (before the coating) and on the phospholipid modified (PLM) column according to Eq. (1): $\log D^{7.4} = a \log k'^{7.4} + b$.

Drugs	*n*	*a* (slope)	*b* (intercept)	r^2	*s*	*F*
β Blockers – RP-amide column	8	1.80 (±0.20)	−2.37 (±0.27)	0.93	0.35	81
β Blockers – PLM column	8	1.49 (±0.13)	−1.72 (±0.17)	0.96	0.27	135
Local Anesthetics – RP-amide column	4	0.95 (±0.52)	−0.54 (±0.88)	0.63	0.39	3
Local Anesthetics – PLM column	5	1.66 (±0.51)	−0.82 (±0.79)	0.78	0.45	10
NSAID's – RP-amide column	6	1.54 (±0.48)	−2.21 (±0.85)	0.72	0.28	10
NSAID's – PLM column	6	1.32 (±0.38)	−0.69 (±0.35)	0.75	0.26	12

3.4. Comparison of retention characteristics using the linear solvation energy relationship (LSER) model

LSER is used as a tool to understand the intermolecular processes in the new phospholipid-modified column. The LSER system constants of the RP-amide column and of the PLM column are compared to the system constants of the n-octanol/water extraction system. Similarity in the system constants indicates similarity in the intermolecular interactions of the various partitioning systems. The model was applied to the logarithm of the measured retention factors from which the system constants were calculated by means of the multiple linear regression analysis. The system constants represent the difference in solvation properties between the two phases of each separation system. The sign and magnitude of each constant indicate its significance to the retention mechanism in chromatography and to the partitioning in the extraction system. In general, a positive sign of the coefficients means a preference for interaction with the stationary phase in the chromatographic system or with the n-octanol phase in the extraction system, thus contributing to longer retention times or to higher $\log P$ values. A negative sign indicates stronger interactions with the mobile phase in chromatography and as a result shorter retention times or preference for the aqueous phase resulting in lower $\log P$ values.

The system constants of the Ascentis RP-amide and the PLM column are summarized in Table 5. For sake of comparison, Table 5 includes the system constants for the n-octanol/water extraction system based on shake-flask $\log P$ data [42] and the constant for two other phospholipid-based columns [43,44]. Table 5 shows that the presence of the phospholipid in PLM column changed the system constants and as a result the PLM column is more similar to the n-octanol/water partitioning system.

From the system constants of the PLM column, we can conclude that dipolarity *s* and hydrogen bond acidity *b* contribute negatively to $\log k'$, while the excess molar refraction *e*, the hydrogen bond basicity *a* and the cohesion and dispersion interactions *v*, lead to an increase in $\log k'$. Thus, the stationary phase of the phospholipid is less dipolar and less hydrogen bond acidic than the mobile phase but more polarizable, more hydrogen bond basic and more hydrophobic than the mobile phase. The large positive value of the hydrogen bond basicity in the modified column is noteworthy. In a standard reversed phase system, the *a* constant is usually negative or close to zero, meaning that the aqueous mobile phase is slightly stronger basic hydrogen bond former than the stationary phase. In the modified column, the situation is reversed. This reversal in the sign emphasizes the change in the column properties brought about by the presence of the phospholipids in the stationary phase.

The magnitude of the *b* and *v* constants shows the importance of these two factors in determining the retention and the partition of the solutes. The strong acidic hydrogen bond capabilities of the mobile phase in the chromatographic system or the aqueous phase in the extraction system, as compared to the stationary phase or the n-octanol phase, are responsible for the more negative value of *b*. From Table 5 it can be seen that the *b* value for the n-octanol/water system is the most negative. Modifying the column with the phospholipids decreases the *b* value (more negative), making the modified column more like the extraction system. We assume that the above behavior is due to the blockage of the residual silanols groups by the phospholipids. The presence of phospholipids screens the silanols groups, which are responsible for some of the hydrogen bond interactions of the stationary phase. In the RP-amide column there is no screening and therefore the value of *b* is less negative.

The *v* constant represents the differences in cavity formation and dispersion interactions associated with solute transfer between the two phases. In all systems (Table 5) the *v* value is positive representing the preference of the cavity formation in the stationary phase and therefore more hydrophobic interactions with the stationary phase. In the PLM column there is a small decrease in the *v* value. The reason for that decrease is probably due to a decrease in the hydrophobicity of the stationary phase resulting from the presence of the phosphate group at the end of each phospholipid chain. The polarity of the phosphate group reduces the difference between the two phases thus decreasing the *v* value.

The constant *s*, describing dipole type interactions, increases with the phospholipid coating and it is closer in value to the n-octanol/water extraction system. As for constant *e*, representing the lone pair electron interactions, its value is identical for the n-octanol/water extraction system and the PLM column, which means identical lone pair electron interactions between the solutes and the two separation techniques.

Based on the analysis of the system LSER constants, as detailed in Table 5, we can state that the PLM column and the n-octanol/water extraction system are very similar in terms of intermolecular interactions.

A comparison of the PLM column system constants with those of an immobilized artificial membrane (IAM) column with mobile

Table 5
LSER system constants for the RP-phase chromatographic systems, for the n-octanol/water system and for two other phospholipid-based columns.

Separation system	System constants						Statistics			
	c	*v*	*e*	*s*	*a*	*b*	r^2	*SE*	*F*	*n*
Ascentis RP-amide column – 20% MeOH	−0.57	3.38	0.21	−0.57	−0.08	−2.32	0.98	0.10	259	36
Phospholipid modified column (PLM) – 20% MeOH	−0.32	3.17	0.56	−0.70	0.34	−3.16	0.97	0.14	230	38
n-Octanol/water $\log P$ [42]	0.09	3.81	0.56	−1.05	0.03	−3.46	0.995	0.12	23162	613
IAM – 10% MeCN [43]	−1.04	1.87	0.81	−0.42	0.69	−1.99	0.993	0.124	287	27
DPC – 20% MeCN [43]	−1.08	2.24	0.57	−0.50	−0.01	−2.59	0.98	0.096	203	46
IAM – 20% [44]	−1.20	2.57	0.65	−0.31	0.22	−2.50	0.997	0.056	965	34

phase containing 10% MeCN, dipalmitoyl phosphatidylcholine coated silica phase (DPC) column of Miyake et al. [45] and system constants for IAM column calculated for 20% methanol through the use of solvent strength gradient [44] shows significant differences between the constants. While the difference in the mobile phases contributes to the difference in the system constants, Table 5 shows, nonetheless, that the PLM system is closer to the ocatnol/water system than the other three systems.

4. Conclusions

The phosphatidylcholine modified column, prepared by dynamic coating, was characterized and examined as a model for lipophilicity measurement. The column was found to be stable and reproducible. The modification of the column resulted in changes of the chromatographic behavior and in different column selectivity. The correlation between $\log P$ values and $\log k'$ of different neutral solutes was examined and good correlations were observed.

The modified column was characterized using the linear solvation energy relationship (LSER) to determine the contributions of intermolecular interactions responsible for the retention on RP-HPLC phase. The values of the LSER system constants for the PLM column were calculated and were found to be very close to those of the n-octanol/water extraction system. These results confirmed that the phospholipid modified column can be used to estimate the n-octanol/water partition coefficient and, thus, to serve as an alternative to the shake-flask method for lipophilicity determination. In addition, the PLM column can provide an alternative to other phospholipid-based column such as the IAM and the DPC columns.

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