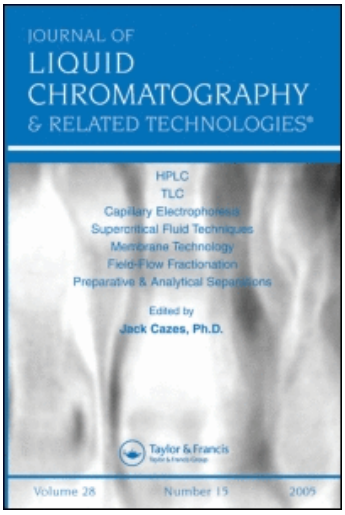

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Evaluation of Reversed Phase Columns for the Analysis of Diphenylmethane Derivatives

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To cite this Article Martinsen, A. , Hyvonen, S. and Gynther, J.(1996) 'Evaluation of Reversed Phase Columns for the Analysis of Diphenylmethane Derivatives', Journal of Liquid Chromatography & Related Technologies, 19: 16, 2579 — 2592
To link to this Article: DOI: 10.1080/10826079608014039
URL: http://dx.doi.org/10.1080/10826079608014039

J. LIQ. CHROM. & REL. TECHNOL., 19(16), 2579–2592 (1996)

EVALUATION OF REVERSED PHASE COLUMNS FOR THE ANALYSIS OF DIPHENYLMETHANE DERIVATIVES

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ABSTRACT

Four reversed phases were tested in RPLC of newly synthesized diphenylmethane derivatives. Non-deactivated Kromasil C-8 and Beckman C-18 and deactivated Supelco LC-8-DB and Supelco LC-18-DB reversed phases were evaluated. Column supplier, deactivation and the presence of amine modifier (triethylamine, TEA) were used as variables and capacity factors (k) and asymmetry factors (As) were measured. Two different mobile phase systems were used: Acetonitrile: ammonium acetate (0.1 M) and methanol: potassium dihydrogen phosphate (0.02 M). According to the results of this comparative study, non-deactivated Kromasil C-8 and Beckman C-18 were found to be the most suitable phases for our applications e.g., synthesis purity and lipophilicity studies concerning antihistamine-like compounds. For most of the compounds involved in this study, the use of these phases requires TEA as an amine modifier. Addition of the amine also made it possible to use the deactivated phases, e.g., Supelco C-8 and C-18 columns. The RPLC method was used to evaluate the lipophilicity of some DPPE derivatives, compared to known antihistaminergics.

INTRODUCTION

Recently, a para-diphenylmethane compound, N,N-diethyl-2-[4(phenylmethyl)phenoxy] ethanamine HCl (DPPE), has been proven to be a suitable ligand in binding to the anti-estrogenic binding site (AEBS), as well as to intracellular histamine (H_{ic} receptor.[1,2] DPPE has shown greater potency in inhibition of Con A-stimulated DNA synthesis than pyrilamine (an H_1-receptor antagonist), cimetidine (an H_2-receptor antagonist) and verapamil (a calcium channel blocking drug), thus being a potential drug candidate.[3] We have started synthezising DPPE and its analogues in order to develop more and more useful ligands for AEBS and H_{ic}-receptor binding studies. The aim of this study was to develop high performance liquid chromatographic (HPLC) methods for synthesis, product purity and lipophilicity assessment studies for these new compounds. Recently, the anticancer effects of DPPE itself have been studied in a clinical trial, where the compound was analyzed from clinical samples by means of HPLC using ultraviolet light (UV) detection.[4] The selection of a suitable stationary phase for RPLC of these basic and rather lipophilic compounds is crucially important. Deactivated reversed phase packings should be excellent for strongly basic analyses.[5-7] Therefore, they could be used without an amine modifier, which permits their possible application in LC-MS studies in the thermospray (TSP) mode.[8,9] However, also non-deactivated packings were included to this study, one reason being their economical price. Nonetheless, when using non-deactivated columns, it seemed likely that it would be necessary to include an amine modifier for the present compounds, because of their basic nature and amine structure.[10,11]

The retention (as measured by the capacity factors) and peak shapes (asymmetry factors) of DPPE derivatives were studied using conventional and deactivated C_8 and C_{18} reversed phase columns. The following parameters were studied:

1) effect of column supplier, stationary phase chain length and deactivation;

2) the nature of the organic solvent

3) effect of the addition of the amine modifier (triethylamine).

When the column with optimal characteristics had been determined, we also examined

4) effect of pH of the mobile phase and

5) influence of the percentage of organic solvent.

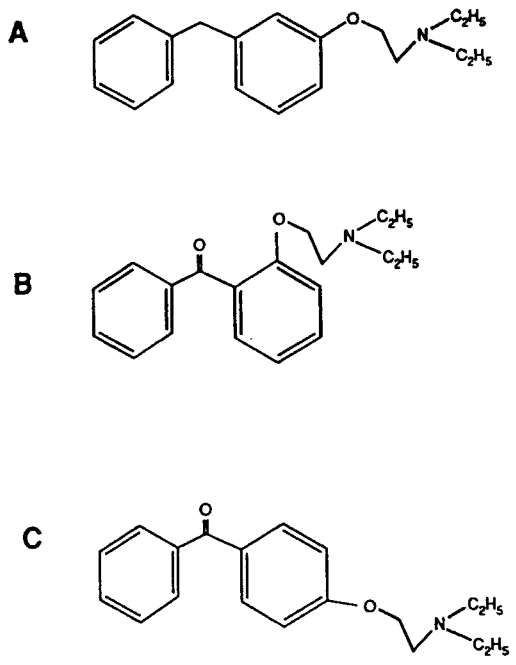

Figure 1. Structures of A) DPPE, B) 2-DBPE and C) 4-DBPE.

Only DPPE derivatives were included in these further studies. Finally, the most suitable system was applied to lipophilicity studies of DPPE derivatives and antihistamines (H_1-receptor antagonists), the latter compounds being structurally and pharmacologically closely related to DPPE.

MATERIALS AND METHODS

Chemicals

The syntheses of N,N-diethyl-2-[4-(phenylmethyl)phenoxy] ethanamine HCl (DPPE) and its derivatives (Fig. 1) have been characterized by spectral data (EI-MS, ^1H and ^{13}C NMR), but also studied by chromatography [HPLC-UV, HPLC-MS (TSP)[12]].

The following analytical grade chemicals were used: Astemizole, clemastine, carbinoxamine, cinnarizine, cyclizine, diphenhydramine,

pheniramine, phenyltoloxamine and pyrilamine (Sigma, St. Louis, MO, USA), ammonium acetate (Merck), potassium dihydrogen phosphate (Merck), concentrated acetic acid (Merck), sodium nitroprusside (Merck) and triethylamine (Fluka).

HPLC-grade solvents, methanol and acetonitrile, were purchased from Labscan. Before being used for HPLC, the mobile phases were filtered through a 0.45 μm filter, and degassed with helium.

Liquid Chromatographic System

High performance liquid chromatography was performed with a system consisting of a Beckman programmable solvent module 116, a Beckman variable wavelength UV-detector 166 (set at 240 nm), System Gold data module (Beckman Instruments, San Ramon, CA), Marathon autosampler (Sparks, The Netherlands) equipped with column thermostat and a Rheodyne 7080-080 loop (20 μl) injector (Rheodyne, Cotati, CA). The flow rate was 1.0 mL/min.

Columns

The following columns were used: Supelco LC-8-DB and LC-18-DB, 5 μm, 150x4.6 mm, from Supelco Inc (Bellefonte, PA, USA), Kromasil C-8, 5 μm, 150x4.6 mm from Eka Nobel, Surte, Sweden) and Beckman C-18, 5 μm, 250x4.6 mm from Beckman Instruments (San Ramon, CA, USA).

Mobile Phases

Two main types of mobile phases were used, under isocratic conditions:

1) acetonitrile: 0.1 M ammonium acetate buffer (65:35, v/v) with pH 6.4, and

2) methanol: 0.02 M potassium dihydrogen phosphate buffer (80:20, v/v) with pH 4.5.

Both mobile phase combinations were used, both with and without triethylamine (20 mM), when evaluating the columns. Later, when a suitable column was chosen for further studies, the percentages of organic solvent were varied for both mobile phase types. Also, the pH scale was tested between pH 3.0 and 7.0 for the most applicable mobile phase composition. For LC-MS studies, the mobile phase was acetonitrile: 0.1 M ammonium acetate buffer (65:35, v/v) with apparent pH adjusted to 6.5.

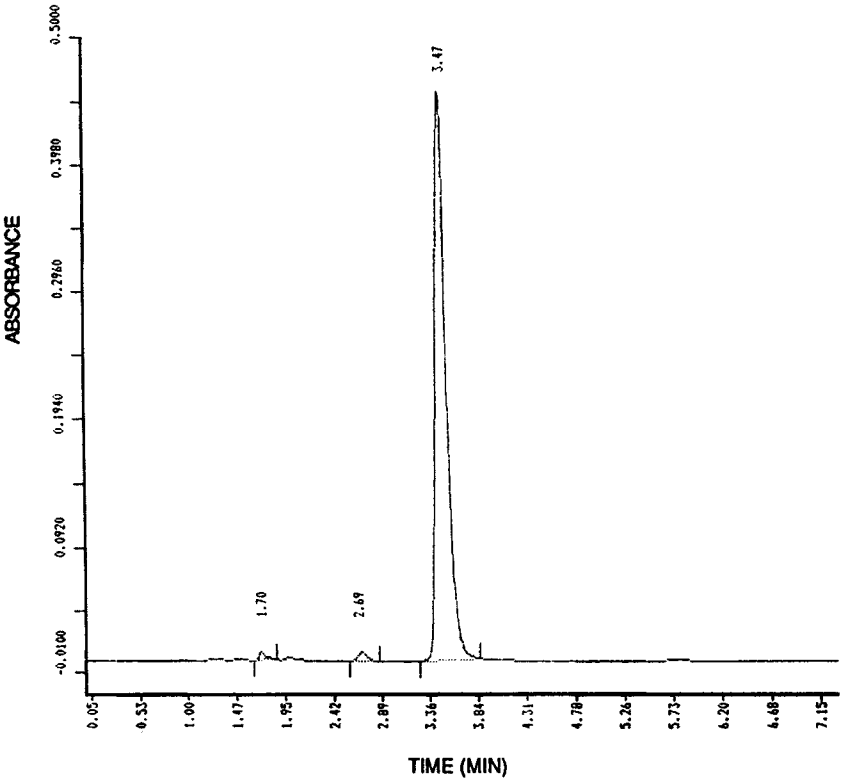

Figure 2. A chromatogram of 2-DBPE. Column: Kromasil C-8, 150x4.6 mm, 5 μm particle size, Mobile phase: Acetonitrile: 0.1 M ammonium acetate with 20 mM TEA, pH 6.5 (50:50), UV detection at 240 nm.

Solutions

Stock solutions (1 mg/mL) of both diphenylmethane derivatives and reference compounds (H_1-antihistamines) were prepared by dissolving them in methanol and filtering the solutions through a Millex-filter disc (0.22 μm, Millipore, Bedford, MA, USA).

Stock solutions were kept at -20 °C in the dark. Standard solutions (100 μg/mL) were prepared as methanolic dilutions. Aliquots of 20 μL of standard solutions were injected onto the column.

Table 1

Capacity Factors and Asymmetry Factors for Non-Deactivated Columns using Acetonitrile/Ammonium Acetate Buffer and Methanol/Phosphate Buffer

Column	DPPE				2-DBPE				4-DBPE			
	with TEA		without TEA		with TEA		without TEA		with TEA		without TEA	
	k	As	k	As	k	As	k	As	k	As	k	As
Acetonitrile : 0.1M Ammonium Acetate Buffer (65:35)												
Beckman C-18	2.34	1.8	3.88	5.8	1.35	1.0	2.11	5.8	1.33	2.0	1.91	5.5
Kromasil C-8	1.29	1.0	1.64	3.0	0.81	1.0	0.96	1.3	0.85	1.0	1.02	1.7
Methanol : 0.02M Potassium Dihydrogen Phosphate Buffer (80:20)												
Beckman C-18	1.07	2.0	3.23	4.5	0.65	2.0	1.80	9.0	0.60	2.0	1.56	6.0
Kromasil C-8	0.77	1.0	1.11	2.3	0.48	1.0	0.72	1.5	0.48	1.0	0.81	1.8

Table 2

Capacity Factors and Asymmetry Factors for Deactivated Columns using Acetonitrile/Ammonium Acetate Buffer and Methanol/Phosphate Buffer

Column	DPPE				2-DBPE				4-DBPE			
	with TEA		without TEA		with TEA		without TEA		with TEA		without TEA	
	k	As	k	As	k	As	k	As	k	As	k	As
Acetonitrile : 0.1M Ammonium Acetate Buffer (65:35)												
Supelco C-8	2.16	1.4	2.18	2.5	1.66	1.6	1.48	1.8	1.67	1.4	1.52	2.7
Supelco C-18	2.00	1.0	3.45	4.3	1.23	1.0	2.05	3.2	1.21	1.3	1.80	2.6
Methanol : 0.02M Potassium Dihydrogen Phosphate Buffer (80:20)												
Supelco C-8	0.94	2.3	2.58	2.7	0.76	2.3	3.83	2.3	0.79	2.3	3.65	3.0
Supelco C-18	1.56	2.3	3.80	6.2	0.98	1.5	2.17	3.9	0.89	1.0	2.00	3.7

Calculations

The following parameters were used for the column evaluation. The capacity factor (k) was calculated using the relationship $(t_r-t_o)/t_o$, where t_r was the retention time of the compound and t_o that of a nonretained compound, measured by using sodium nitroprusside. The peak asymmetry factor was measured at 10 % of the peak height using the ratio of the widths of the rear and front sides of the peak.[13,14]

RESULTS AND DISCUSSION

Non-Deactivated RP Columns and the Effect of the Amine Modifier

Three diphenylmethane derivatives: DPPE, 2-DBPE, 4-DBPE, were first chromatographed with conventional C_8 and C_{18} reversed phases. These compounds were studied with and without triethylamine (TEA), using two types of mobile phases: one containing acetonitrile and acetate buffer and the other mobile phase containing methanol and phosphate buffer. Experimental data showing capacity factors and asymmetry factors when using non-deactivated C_8 and C_{18} phases for RPLC of diphenylmethane derivatives are shown in Table 1.

It is evident that triethylamine is needed with every non-deactivated reversed phase. The situation is the same with both types of mobile phases. Optimal chromatographic conditions were obtained with the Beckman C_{18} column, apparently due to its length (250 mm) and capacity (octadecyl chains). It seems that this column is most suitable for RPLC-UV studies of synthetic products of new diphenylmethane derivatives and related compounds. For lipophilicity studies a C_8 phase with a 15 cm column (Kromasil) should be optimum, as can be seen in the chromatogram of 2-DPPE (Fig. 2), and the portion of organic solvent could be reduced to obtain suitable retention behavior as was found later.

Deactivated RP Phases and the Effect of Amine Modifier

The significance of column deactivation was one of the most crucial points in this investigation. Recently, this parameter has been studied in the RPLC of α-tocopherol analogues.[15] In our studies, the results indicate that column deactivation is a significant factor in RPLC of diphenylmethane derivatives. However, the addition of an amine modifier, in this case triethylamine, has greater effects on capacity factors and peak symmetry of diphenylmethanes than column deactivation (Table 2). When Supelco LC-8-DB with acetonitrile:0.1 M acetate buffer (65:35) was used for DPPE, one obtained k and As values of 2.18 and 2.5 without TEA.

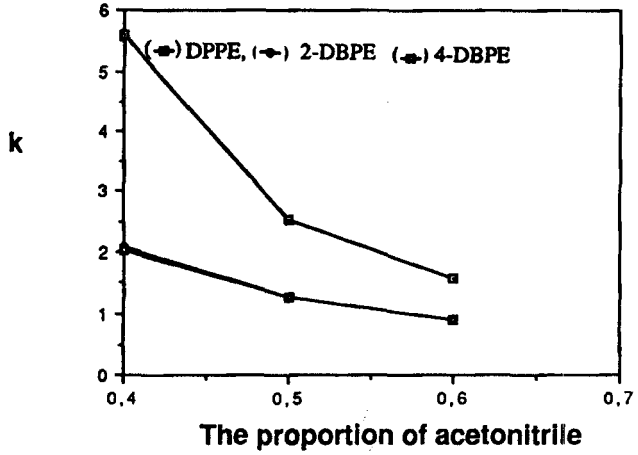

Figure 3. Effect of the proportion of acetonitrile on the capacity factors of DPPE derivatives. Conditions as in Fig. 2 with the exception that pH was 6.0.

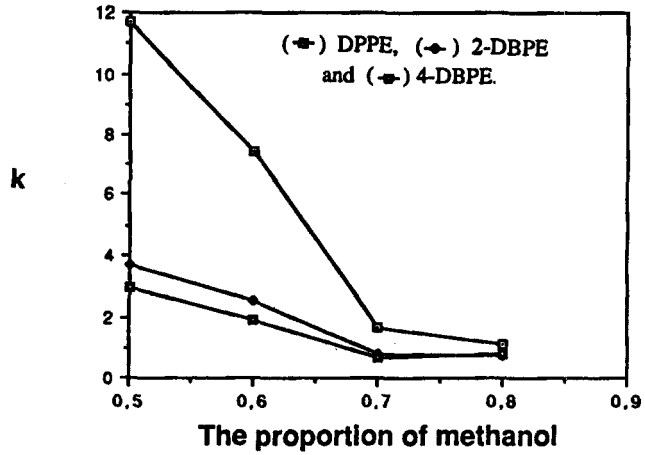

Figure 4. Effect of the proportion of methanol on the capacity factors of DPPE derivatives. Conditions as in Fig. 2. with the exception that mobile phase was MeOH: KH_2PO_4 (0.02M), pH4.5.

When 20 mM TEA had been added to the buffer of the mobile phase, the corresponding values were 2.16 and 1.4, respectively. TEA affects both retention times and peak shapes. When DPPE is chromatographed without TEA, first with a nondeactivated column, Kromasil C-8 (Table 1) and subsequently with a deactivated column, Supelco LC-8-DB (Table 2), the differences in capacity factors and asymmetry values are not so dramatic as is seen with the addition of TEA. Thus, for RPLC of diphenylmethanes, the use of an amine modifier is preferable also in the case of deactivated columns.

For LC-MS studies performed in the TSP mode, a deactivated Supelco LC-8-DB was chosen. This was due to the fact that TEA is not suitable for the solvent system used in TSP, and therefore the Supelco column was the best choice. The non-deactivated RP columns tested were not able separate the sample components sufficiently well to obtain TSP spectra.

Effect of the Organic Solvent

As described before, two main types of mobile phases were used initially, one based on acetonitrile and ammonium acetate buffer (65:35), one consisting of methanol and phosphate buffer (80:20). These combinations were tested with almost all the columns involved, with and without triethylamine.

For further method development with Kromasil C-8, both acetonitrile/acetate buffer and methanol/phosphate buffer combinations were examined in more detail: The percentage of organic solvent was varied. The proportions of acetonitrile (Fig. 3) and methanol (Fig 4) were varied when DPPE, 2-DBPE and 4-DBPE were chromatographed in the presence of 20 mM TEA at pH 6.5 (Acn:NH_4Ac 0.1 M) or pH 4.5 (MeOH:KH_2PO_4 0.02 M). The aim was to use the methanolic mobile phases principally for lipophilicity investigations, but it was found that column back pressures were too high (2.9 kpsi) for routine analyses. This was the case with every suitable proportion of methanol. Thus, the only reasonable alternative for this column and purpose was to use acetonitrile as the organic solvent, even though methanol is generally preferred for log k determinations by RPLC.[10,16]

Effect of the pH of the Mobile Phase and the Column Temperature

After the selection of the mobile phase composition, e.g., Acn: NH_4Ac 0.1 M with 20 mM TEA (50:50), the pH scale was tested with this solution running through the Kromasil C-8 column. The effect of the pH was studied also for the mobile phase consisting of MeOH: KH_2PO_4 0.1 M with 20 mM TEA (60:40), although this composition was not suitable for permanent use because of its pressure inducing effects as described above.

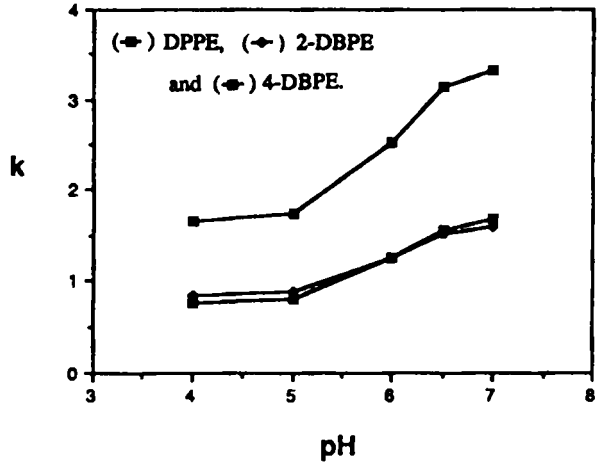

Figure 5. Effect of pH of the mobile phase on capacity factors of DPPE derivatives. Conditions as in Fig. 2.

It is worth noting that, should other types column be used, i.e., which do not exhibit such pressure rises as seen here (for instance a shorter or not so tightly packed column, e.g. C-18 phase), the methanolic mobile phase may be very useful.

The capacity factors of DPPE derivatives clearly depend on the pH of the mobile phase of the acetonitrile/acetate buffer (Fig. 5). pH is one of the most important factors for modification if one is trying optimize the mobile phase of RPLC for lipophilicity studies of new molecules in drug design.[16] The effect of temperature was not remarkable. The change of ambient temperature to 40 °C had little effect on the retention parameters of these compounds (Table 3). Therefore, we continued to use an ambient temperature (25 °C), because, when working at this relatively high pH (6.5), it should prolong column lifetime compared with working at elevated temperatures.[17]

Applications

Purity studies for synthetic DPPE compounds were performed with HPLC with both variable wavelength detection and with diode array detection (unpublished results). Identification and purity studies with HPLC-MS (thermospray) method for DPPE and its derivatives have been described elsewhere.[12]

Table 3

The Effect of Temperature on the Capacity Factors and Asymmetry Factors of DPPE Derivatives*

Compound	40 °C	25 °C
DPPE	k = 2.62 As = 1.7	k = 2.53 As = 2.3
2-DBPE	k = 1.30 As = 1.3	k = 1.26 As = 1.0
4-DBPE	k = 1.33 As = 2.0	k = 1.25 As = 1.0

* Conditions as in Figure 2.

Table 4

Log k Values Obtained by RPLC and Log P Values Obtained from the Literature* for Nine Antihistamines**

Compound	Log k	Log P
Astemizole	0.488	4.10
Clemastine	0.750	5.05
Cinnarizine	1.437	6.14
Cyclizine	0.481	3.97
Diphenhydramine	0.136	3.36
Pheniramine	-0.102	2.02
Phenyltoloxamine	0.519	3.90
Carbinoxamine	0.046	2.17
Pyrilamine	0.136	2.77

* Reference 18. ** Chromatographic conditions as in Figure 2.

The special application described here is the evaluation of lipophilicity of DPPE and related compounds. One of the main areas of interest for our project is to produce lipophilicity data to characterize the physicochemical properties of new compounds and also to use this data in QSAR and CoMFA studies with respect to drug design.

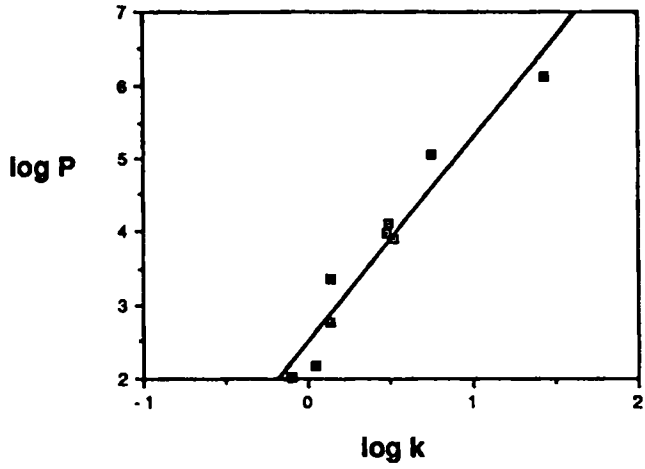

Figure 6. Correlation of log k and log P values of antihistamines. Log k values were obtained at the same liquid chromatographic conditions as in Fig. 2.

Table 5

Log P Values Calculated According to Equation (1) and Log P Values Obtained by Shake-Flask Method for DPPE and its Derivatives

Compound	Log k	Log P (calc.)	Log P (obs.)
DPPE	0.503	4.07	4.55
2-DBPE	0.195	2.97	3.51
4-DBPE	0.211	3.02	3.71

HPLC is especially suitable for this kind of work since it is much more compatible and faster for lipophilicity determinations than conventional methods (e.g. shake flask method), and also provides more data about the molecular properties of the drug than can be obtained with simple lipophilicity determination.[10] We selected the Kromasil C-8, 5 μm, (150x4.6 mm) to be the RPLC column for our lipophilicity studies of the new diphenylmethane derivatives and also for further method development in this field. It is a tightly packed, and a well end-capped column, though it is not actually deactivated. It is not expensive, and its lifetime seems to be quite long. Initially, we used Acn: 0.1 M NH_4Ac buffer with 20 mM TEA (50:50), pH 6.5 as the mobile phase selected in this study. The peaks produced by DPPE compounds are not very sharp in these conditions, but they are symmetric and, importantly, their

retention is optimal for generating their log k values as well as log k values of a set of structurally related antihistamines (Table 4). When subjecting the log k values obtained here and log P values from the literature[18] to linear regression (Fig. 6), an equation:

$$y=2,4602 + 2,8338x \ (R=0.97) \qquad (1)$$

was derived. According to this equation, log P values for DPPE and its derivatives can be calculated. These values are in accordance with those determined with the shake-flask method (Table 5).

According to this investigation, non-deactivated columns may be worthy of evaluation; they may be the columns of choice. For instance, non-deactivated columns, Beckman C-18, 250 mm of length and Kromasil C-8, 150 mm of length were suitable for purity studies and lipophilicity investigations, respectively. They were better than deactivated columns, especially when one considers also the economics of their use, as in the case of Kromasil C-8.

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Received March 18, 1996
Accepted April 8, 1996
Manuscript 4120