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PHOSPHATIDYLCHOLINE-COATED SILICA AS A USEFUL STATIONARY PHASE FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PARTITION COEFFICIENTS BETWEEN OCTANOL AND WATER

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SUMMARY

Phospholipid-coated silica gel is a useful stationary phase to determine the partition coefficient between octanol and water, P_{oct} , by high-performance liquid chromatography (HPLC). A column of silica gel coated with dipalmitoyl phosphatidylcholine is easy to prepare, and is stable over a long period. With this column and an aqueous mobile phase containing acetonitrile, a good single correlation was observed between the log of the capacity factor, k' , and log P_{oct} for a wide variety of compounds. Thus, this chromatographic system, which overcomes the limitations of the standard shake-flask method and the HPLC method on an octadecyl silica column, is very useful for determining log P_{oct} .

INTRODUCTION

The partition coefficient between octanol and water, P_{oct} , has been used extensively as an hydrophobic index of bioactive compounds, especially in quantitative structure–activity relationship studies¹. The shake-flask method is the standard method for determination of P_{oct} , but has the serious limitations that it is laborious and requires relative large amounts of pure sample compounds and octanol, and rigorous conditions, especially for log P_{oct} values below -2 and above 4 (ref. 2).

High-performance liquid chromatography (HPLC) has recently been employed³ as an alternative method for determination of P_{oct} . It is usually carried out on an octadecyl silica (ODS) column eluted with mixtures of water and water-miscible organic modifiers, such as methanol and acetonitrile. The ODS system is very useful, but it also has limitations: the log of the capacity factor, k' , is found to increase linearity with increasing log P_{oct} , but this linear relationship is dependent on the chemical series, because of the dependence of the chromatographic behaviour on the hydrogen-bonding ability of the test compounds⁴.

Chromatographic conditions are required that give a single correlation between $\log k'$ and $\log P_{\text{oct}}$ for a wide variety of compounds in different chemical series. Octanol-coated silica is reported to give a single correlation⁵. However, in HPLC of hydrophobic compounds, a modifier must be added to the mobile phase to increase the solubility of the test compounds and to adjust the retention times to a suitable range. Such would dislodge the octanol from the coated silica gel.

The use of glyceryl-coated controlled-pore glass (gly-CPG) as a stationary phase and methanol-water (10:90) as the mobile phase was found to give a single correlation between $\log k'$ and $\log P_{\text{oct}}$ at a significant statistical level for various compounds with $\log P_{\text{oct}}$ values up to about 6 (ref. 6). However, in a preliminary study we found that this system also had the shortcoming that aromatic compounds with alkyl chains directly attached to the benzene ring showed anomalous behaviours⁷. This could be attributed to the fact that a certain steric configuration is required for the interaction of compounds with the site on the stationary phase. A similar problem was encountered with an ODS column^{8,9}.

Thus, at present, no generally applicable method is available for the determination of $\log P_{\text{oct}}$ by HPLC. An efficient chromatographic method should satisfy the following conditions: (i) the chromatographic system should give a single correlation between $\log k'$ and $\log P_{\text{oct}}$ for a wide variety of compounds, regardless of their hydrogen-bonding abilities and steric factors; (ii) the stationary phase should be stable in the presence of a mobile phase containing organic modifiers.

In this paper, we report that dipalmitoyl phosphatidylcholine (DPPC)-coated silica gel satisfies these conditions and is a very effective stationary phase for the determination of $\log P_{\text{oct}}$ for a wide variety of compounds. This column is stable over a long period, and is very easy to prepare reproducibly.

EXPERIMENTAL

The compounds used for HPLC were obtained from commercial sources, and were used without further purification. They were dissolved in acetonitrile at about 0.5 mg/ml. Dipalmitoyl phosphatidylcholine (DPPC) was a gift from Nippon Oil and Fats Co. (Amagasaki, Japan).

DPPC-coated silica gel was prepared essentially as follows: about 1.6 g of the silica gel Wakogel LC-10K (Wako Pure Chemical Industry, Osaka, Japan) and 30 ml of dichloromethane were placed in a round-bottomed flask. Then, a known amount of DPPC dissolved in 5 ml of dichloromethane was added, and the flask was placed in a rotary evaporator. The solvent dichloromethane was removed under reduced pressure at room temperature, and when the silica gel seemed to be dry the flask was allowed to stand under reduced pressure for about 1 h. In this way a thin layer of silica was formed on the inside wall of the flask.

About 1.3 g of the coated silica gel were transferred to a 30-ml glass bottle, and 10 ml of aqueous 20% acetonitrile were added. The suspension was stirred for about 20 min under reduced pressure to remove the air-bubbles formed on the surface of the DPPC-coated silica gel. The slurry was then packed into a stainless-steel tube (12.5 cm \times 4.6 mm I.D.) for use as a column.

HPLC was performed at 30°C with a Familic 300S (JASCO, Tokyo, Japan), connected with an UV detector UVIDEC 100V (JASCO). The mobile phase consisted

of 0.03 M phosphoric acid, pH 2.2, and acetonitrile in various proportions (% v/v). The flow-rate was adjusted to between 0.2 and 3.0 ml/min. A short column packed with DPPC-coated silica gel was connected between the pump and sample injector to reduce loss of DPPC from the main column.

The capacity factor, k' , is expressed by

$$k' = (t_R - t_0)/t_0 \quad (1)$$

where t_R and t_0 are the retention times of the test compound and an unretained compound, respectively. Potassium iodide was used as the unretained compound. Values of k' given in the text are averages of three to five determinations with different sample sizes.

RESULTS

Stability and properties of DPPC-coated silica

First we examined how k' changes with the amount of DPPC loaded on the silica gel. An uncoated silica gel column was employed as a reference; it was 25 cm instead of 12.5 cm long because the retention times on uncoated silica were less than those on the coated silica. The mobile phase was 20% acetonitrile containing phosphoric acid solution, adjusted at pH 2.2 to reduce the dissociation of acidic compounds.

Fig. 1 shows the change in k' for benzene, halogenobenzenes and halogenophenols with the amount of DPPC loaded on the silica gel. With smaller amounts of DPPC, k' tended to increase with increasing amount of DPPC, but above 70 mg DPPC per g silica gel the k' values for all compounds became constant. The change in k' with the amount of DPPC seemed to have two stages: (i) a first steep increase followed by a transient plateau; (ii) a second increase followed by a saturation plateau. These stages were especially clear for compounds with smaller k' values, such as chlorophenol and benzene (4 and 5, respectively, in Fig. 1).

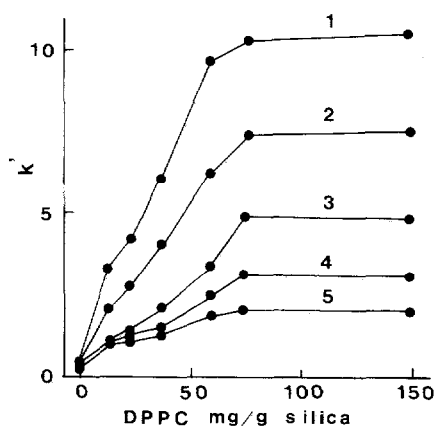


Fig. 1. Change in k' with the amount of DPPC loaded on silica gel. Mobile phase: acetonitrile-water (20:80); flow-rate, 1.0 ml/min. 1, Iodobenzene; 2, bromobenzene; 3, 4-bromophenol; 4, 4-chlorophenol; 5, benzene.

Next, the relationships between $\log k'$ and $\log P_{\text{oct}}$ with change in the amount of DPPC loaded on silica gel were examined, as shown in Fig. 2. Since the chromatographic behaviours of compounds that are capable and incapable of hydrogen-bond formation were reported to be different on an ODS column, we chose two series of compounds, halogenobenzenes as non-hydrogen-bonders (non-H-bonders) and halogenophenols as amphiprotic compounds. Fig. 2 shows that linear relationships between $\log k'$ and $\log P_{\text{oct}}$ were observed for both these series of compounds, but that they depended on the amount of DPPC loaded on the silica gel. The linear relationships can be expressed by a Collander-type equation:

$$\log P_{\text{oct}} = a + b \log k' \quad (2)$$

Values of the coefficients a and b in eqn. 2 are summarized in Table I. Fig. 2 and Table I show that below 40 mg DPPC per g silica gel, at which the first plateau in

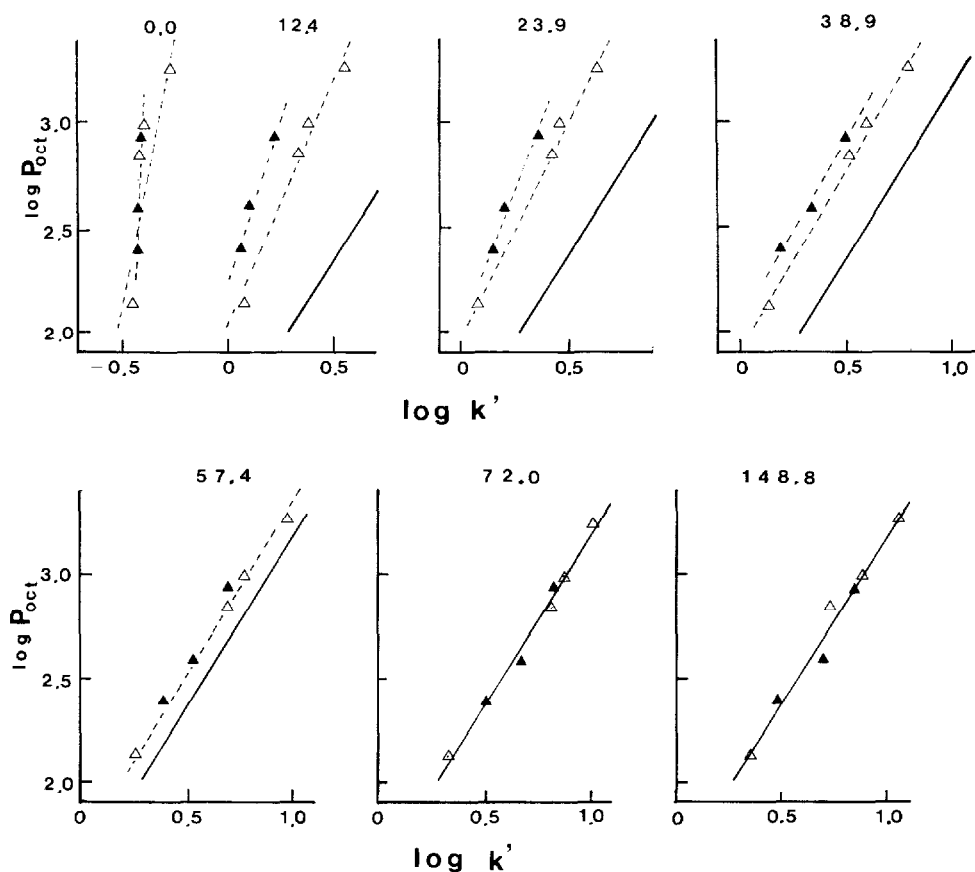


Fig. 2. Effect of DPPC coating of silica gel on the chromatographic behaviour. The relationship between $\log k'$ and $\log P_{\text{oct}}$ is shown for benzene and the halogenobenzenes chlorobenzene, bromobenzene and iodobenzene (Δ), and the halogenophenols chlorophenol, bromophenol and iodophenol (\blacktriangle). Mobile phase as in Fig. 1. The amount of DPPC loaded (mg/g silica gel) is shown at the top of each trace. Discontinuous lines are drawn based on the regression shown in Table I. Continuous lines represent the regression line for 148 mg DPPC per g silica gel.

TABLE I

EFFECTS OF THE DPPC CONTENT OF SILICA GEL ON THE COEFFICIENTS a AND b IN EQN. 2 FOR THE NON-H-BONDERS BENZENE AND HALOGENOBENZENES AND FOR AMPHIPROTIC HALOGENOPHENOLS

| DPPC (mg/g silica) | Benzene and halogenobenzenes | | Halogenophenols | |
|-----------------------|---------------------------------|------|-----------------|-------|
| | a | b | a | b |
| 0.0 | 4.81 | 4.98 | 12.40 | 22.00 |
| 12.4 | 2.06 | 2.39 | 2.31 | 3.15 |
| 23.9 | 2.00 | 2.04 | 2.10 | 2.33 |
| 38.9 | 1.93 | 1.72 | 2.06 | 1.69 |
| 57.4 | 1.69 | 1.60 | 1.65 | 1.81 |
| 72.0 | 1.61 | 1.57 | 1.58 | 1.56 |
| 148.8 | 1.60 | 1.60 | 1.66 | 1.47 |

k' seemed to be attained (*cf.*, Fig. 1), there was a difference in the straight lines for non-H-bonders and amphiprotics, but this difference became less as the DPPC content of the silica increased. In the second stage, these two series of compounds showed almost the same relationship, and the slope, b , became smaller with increasing content of DPPC. In the region of the second plateau (more than 70 mg DPPC per g silica gel) the values of a and b for the two series of compounds were the same. These changes reflect the manner of DPPC coating on the silica gel.

These results suggest that, on a column coated with enough DPPC for the second plateau, a single correlation should be obtained for a wide variety of compounds. Thus, we subsequently used a column coated with 150 mg DPPC per g silica gel, and refer it hereafter to as a DPPC-coated silica column.

Next, we examined the stability of the DPPC-coated column by determining the k' values for chlorophenol, benzene and phenol after elution of the column with various volumes of the mobile phase, 20% acetonitrile containing phosphoric acid solution (pH 2.2). Fig. 3 shows the dependence of $\log k'$ on the volume of the mobile

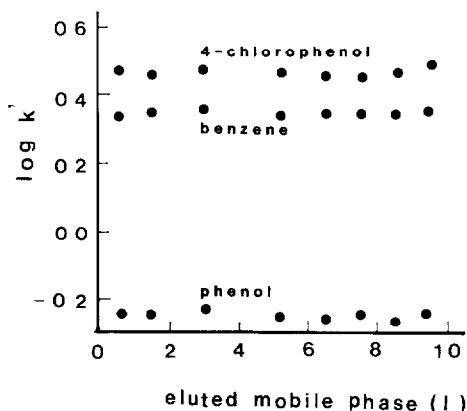


Fig. 3. Stability of the DPPC-coated silica gel column. The capacity factor $\log k'$ is plotted against the volume of the mobile phase (in liters) eluted during HPLC. Mobile phase as in Fig. 1.

phase eluted during HPLC. The values of k' for each compound did not vary with elution volumes of up to 9.5 l. The DPPC-coated silica was then removed from the column and the DPPC was extracted with dichloromethane. Thin-layer chromatography of the extract using chloroform–methanol–acetic acid (98:2:1) gave an R_F value similar to that of DPPC. These results indicate that DPPC is not dislodged significantly from silica by acetonitrile or hydrolyzed during HPLC. In practice, the DPPC-coated column is stable over a long period, *e.g.*, successive experiments at a flow-rate of 1 ml/min for at least 1 week.

Relationship between $\log k'$ and $\log P_{\text{oct}}$

We determined the $\log k'$ values of various compounds, such as benzenes, phenols, benzoic acids and benzyl alcohols, on the DPPC-coated silica gel column using various proportions of water, pH 2.2, and acetonitrile as the mobile phase. Values of $\log k'$ with mobile phase containing 10, 20 and 30% acetonitrile (the proportion of acetonitrile is shown as a subscript to k') are summarized in Table II, where the test compounds are arranged according to their hydrogen-bonding abilities: non-H-bonders, H-acceptors and amphiprotics. When the $\log P_{\text{oct}}$ values were greater than 3.6, $\log k'_{10}$ could not be determined because the t_R values were too large. Furthermore, $\log k'_{30}$ for some hydrophilic compounds could not be determined because the t_R values were too small. From the values of k' with mobile phases containing 10, 20 and 30% acetonitrile, values of k'_0 were determined by linear extrapolation of $\log k'$ to 0% acetonitrile.

Table III summarizes the linear relationships between $\log k'$ and $\log P_{\text{oct}}$ according to eqn. 2 for compounds whose k' values could be determined with all three mobile phases. It is apparent that in all cases significant linear relationships were obtained. Regression analysis showed that the hydrogen-bonding abilities of the compounds had no effect on the correlations in Table III. This indicates that the linear correlations between $\log k'$ and $\log P_{\text{oct}}$ are independent of the hydrogen-bonding abilities of the compounds contrary to the case with an ODS column⁴. Of the relationships in Table III, the correlation between $\log k'_0$ and P_{oct} is the lowest, because the change in $\log k'$ with the proportion of acetonitrile in the mobile phase was not linear, but rather curvilinear. A curvilinear change in $\log k'$ with the proportion of the organic modifier has also been reported for ODS^{4,12} and porous polymer resin¹³ columns.

Since the chromatographic behaviours on gly-CPG and ODS of alkyl-substituted benzenes were different from those of benzenes without alkyl chains, we next examined the effect on k' of an alkyl chain attached directly to the benzene ring. Table IV shows the results, with $\log k'_{20}$ as an example, for the various compounds listed in Table II. Apparently, alkyl- and non-alkyl-substituted benzenes showed the same chromatographic behaviour. The linear relationship between $\log k'_{20}$ and $\log P_{\text{oct}}$ for all the compounds is illustrated in Fig. 4.

DISCUSSION

In this study we have shown that a DPPC-coated silica gel is a very efficient stationary phase for determination of P_{oct} values for a variety of compounds. Its advantages are: (i) it can be prepared easily and reproducibly; (ii) it is very stable

TABLE II

EFFECT OF THE PROPORTION OF ACETONITRILE IN THE MOBILE PHASE ON THE VALUES OF LOG P_{oct} AND LOG k' DETERMINED WITH A DPPC-COATED COLUMN

The percentage of acetonitrile is shown as a subscript to log k' .

| Compound | | $\log P_{oct}^*$ | $\log k'_{10}$ | $\log k'_{20}$ | $\log k'_{30}$ | |
|--------------------------------------|-----------------------------------|------------------|----------------|----------------|----------------|-------|
| Class | Name | | | | | |
| Non-H-bonders | Benzene | 2.13 | 0.41 | 0.35 | -0.38 | |
| | Chlorobenzene | 2.84 | 0.93 | 0.73 | -0.07 | |
| | Bromobenzene | 2.99 | 1.07 | 0.88 | 0.02 | |
| | Iodobenzene | 3.25 | 1.31 | 1.05 | 0.16 | |
| | Toluene | 2.69 | 0.74 | 0.56 | -0.13 | |
| | Ethylbenzene | 3.15 | 1.11 | 0.88 | 0.05 | |
| | Isopropylbenzene | 3.66 | 1.48 | 1.17 | 0.37 | |
| | <i>tert.</i> -Butylbenzene | 4.11 | - | 1.39 | 0.46 | |
| | Biphenyl | 4.04 | - | 1.45 | 0.37 | |
| | Naphthalene | 3.59 | 1.59 | 1.19 | 0.17 | |
| | H-Acceptors | Cyanobenzene | 1.56 | -0.02 | -0.21 | - |
| | | Nitrobenzene | 1.85 | 0.22 | 0.02 | - |
| 4-Nitrochlorobenzene | | 2.39 | 0.58 | 0.15 | -0.24 | |
| Anisole | | 2.10 | 0.28 | 0.09 | -0.40 | |
| 4-Nitroanisole | | 2.03 | 0.45 | 0.18 | -0.24 | |
| Phenetole | | 2.51 | 0.58 | 0.34 | -0.25 | |
| Methylbenzoate | | 2.23 | 0.49 | 0.22 | -0.35 | |
| Ethyl benzoate | | 2.64 | 0.82 | 0.52 | -0.22 | |
| Phenyl benzoate | | 3.59 | 1.53 | 1.19 | 0.21 | |
| Amphiprotics | | Phenol | 1.48 | 0.02 | -0.24 | - |
| | | 2-Chlorophenol | 2.15 | 0.59 | 0.35 | -0.47 |
| | | 3-Chlorophenol | 2.50 | 0.85 | 0.55 | -0.29 |
| | 4-Chlorophenol | 2.39 | 0.62 | 0.48 | -0.25 | |
| | 2,6-Dichlorophenol | 2.93 | 0.83 | 0.66 | -0.20 | |
| | 4-Bromophenol | 2.59 | 0.78 | 0.69 | -0.26 | |
| | 4-Iodophenol | 2.93 | 1.06 | 0.84 | -0.14 | |
| | 4-Nitrophenol | 1.91 | 0.54 | 0.16 | - | |
| | 4-Methylphenol | 1.94 | 0.38 | 0.14 | - | |
| | 3,4-Dimethylphenol | 2.23 | 0.50 | 0.37 | -0.48 | |
| | 4-Ethylphenol | 2.26 | 0.51 | 0.38 | -0.39 | |
| | 4-(<i>n</i> -Propyl)phenol | 3.11** | 1.21 | 0.84 | 0.03 | |
| | 4-(<i>tert.</i> -Butyl)phenol | 3.31 | 1.27 | 0.97 | 0.06 | |
| | 2,6-Di(<i>sec.</i> -Butyl)phenol | 4.80** | - | 1.72 | 0.59 | |
| | Benzoic acid | 1.87 | 0.17 | -0.15 | - | |
| | 4-Chlorobenzoic acid | 2.65 | 0.91 | 0.58 | -0.22 | |
| | 4-Bromobenzoic acid | 2.86 | 1.09 | 0.71 | -0.13 | |
| | 4-Iodobenzoic acid | 3.02 | 1.13 | 0.84 | -0.01 | |
| | 3-Hydroxybenzoic acid | 1.50 | -0.01 | -0.31 | - | |
| | 4-Hydroxybenzoic acid | 1.58 | -0.15 | -0.29 | - | |
| | 4-Nitrobenzoic acid | 1.89 | 0.36 | 0.16 | - | |
| | 4-Methylbenzoic acid | 2.27 | 0.53 | 0.20 | -0.43 | |
| 3,4-Dimethylbenzoic acid | 2.69** | 0.91 | 0.57 | -0.19 | | |
| 4-Ethylbenzoic acid | 2.65** | 0.96 | 0.53 | -0.26 | | |
| 4-(Isopropyl)benzoic acid | 3.37** | 1.31 | 1.04 | 0.06 | | |
| 4-(<i>tert.</i> -Butyl)benzoic acid | 3.80** | - | 1.03 | 0.46 | | |
| 4-Phenylbenzoic acid | 3.62** | - | 1.31 | 0.22 | | |

(Continued on p. 54)

TABLE II (continued)

| Compound | | $\log P_{\text{oct}}^*$ | $\log k'_{10}$ | $\log k'_{20}$ | $\log k'_{30}$ |
|----------------|----------------------------|-------------------------|----------------|----------------|----------------|
| Class | Name | | | | |
| Benzyl alcohol | 4-Chlorobenzyl alcohol | 1.96 | 0.34 | 0.04 | — |
| | 3,5-Dichlorobenzyl alcohol | 2.90** | 0.97 | 0.69 | -0.16 |
| | 3-Iodobenzyl alcohol | 2.55** | 0.67 | 0.43 | -0.31 |
| | 4-Methylbenzoyl alcohol | 1.59 | 0.04 | -0.21 | — |
| | 3,4-Dimethylbenzyl alcohol | 2.10** | 0.36 | 0.11 | -0.44 |

* From ref. 10.

** Calculated by Rekker's method¹¹.

TABLE III

VALUES OF COEFFICIENTS a AND b IN EQN. 2 WITH MOBILE PHASES CONTAINING ACETONITRILEValues in parentheses are 95% confidence limits. n = Number of compounds; r = Correlation coefficient; s = standard deviation. The percentage of acetonitrile is shown as a subscript to $\log k'$.

| $\log k'$ | a | b | n | r | s |
|----------------|----------------|----------------|-----|-------|-------|
| $\log k'_0$ | 1.21 (0.17) | 1.01 (0.11) | 35 | 0.954 | 0.140 |
| $\log k'_{10}$ | 1.60 (0.09) | 1.28 (0.10) | 35 | 0.975 | 0.104 |
| $\log k'_{20}$ | 1.84 (0.08) | 1.43 (0.12) | 35 | 0.972 | 0.111 |
| $\log k'_{30}$ | 3.07 (0.05) | 2.04 (0.18) | 35 | 0.969 | 0.116 |

TABLE IV

REGRESSION COEFFICIENTS FOR $\log P_{\text{oct}} = a + b \log k'_{20}$

| Compounds | a | b | n | r | s |
|----------------------------|----------------|----------------|-----|-------|-------|
| Without alkyl substituents | 1.84 (0.05) | 1.41 (0.08) | 35 | 0.985 | 0.120 |
| With alkyl substituents | 1.78 (0.12) | 1.66 (0.15) | 17 | 0.987 | 0.139 |
| All compounds | 1.83 (0.06) | 1.51 (0.08) | 52 | 0.983 | 0.144 |

even when the mobile phase contains an organic modifier; (iii) a single correlation is obtained between $\log k'$ and $\log P_{\text{oct}}$ for a wide variety of compounds, because steric and hydrogen-bonding effects are eliminated; (iv) values of $\log P_{\text{oct}}$ of between about 1.0 and 5.0 at least can be determined rapidly and accurately with a single column.

The mode of coating of the surface of silica gel with DPPC is unknown. As shown in Fig. 2, the value of k' on the uncoated silica gel is very small, indicating that the affinity of compounds for the surface of silica gel is very low. Furthermore,

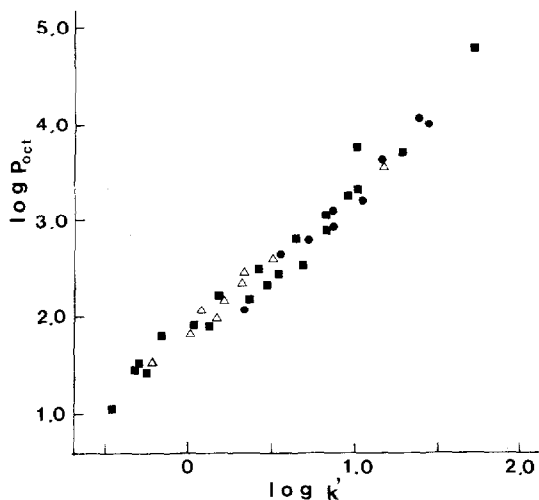


Fig. 4. Linear relationship between $\log k'_{20}$ and $\log P_{oct}$. ●, H-acceptors; △, non-H-bonders; ■, amphiprotics. Values are for the compounds listed in Table II.

from the fact that the value of k' at the second plateau is roughly twice that at the first plateau, it is possible that the k' at the first plateau corresponds to that on a monolayer of DPPC, while the k' at the second plateau corresponds to that on bi- or multi-layered DPPC. Alcohols, ethers and amides become attached to silica gel by hydrogen bonds between their electron-donor groups and the hydrogen atoms of silanols¹⁴. Therefore, DPPC in the monolayer may be adsorbed to the surface of silica gel, by bonding of polar head groups with hydrogen atoms of silanol groups, leaving the hydrophobic alkyl chains facing the mobile phase. In contrast, in the case of bi- or multi-layered DPPC, the polar head would face the mobile phase.

However, the coating cannot be uniform and homogeneous, because the DPPC content at the onset of the second phase (70 mg per g silica gel = $1 \cdot 10^{-4}$ mol per g silica gel) was too low relative to the total surface area, 400 m²/g of silica¹⁵, for formation of a complete monolayer over the whole surface; the area of a monolayer of DPPC is about 0.5 nm² per molecule (ref. 16) and thus a monolayer of 70 mg of DPPC will occupy 30 m² of silica surface, if the DPPC molecules are attached perpendicular the silica gel surface. The mode of coating of the silica gel surface with DPPC is very important for understanding the chromatographic mechanism, and will be reported elsewhere.

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