CHROMSYMP. 222

HIGH-PERFORMANCE LIQUID–LIQUID CHROMATOGRAPHY

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SUMMARY

The applicability of liquid–liquid systems as phase systems in high-performance liquid chromatography (HPLC) was investigated. The more polar phase of the liquid–liquid system is generated *in situ* on pre-packed silica columns by pumping through the saturated mobile phase. The influence of the type of silica support on the amount of generated stationary liquid, the phase ratio, the retention and the selectivity was measured. The column efficiency and capacity of liquid–liquid systems generated *in situ* were compared with normal adsorption systems. The results obtained so far indicate that liquid–liquid systems can be a worthwhile way to increase the number of phase systems in HPLC. Their practical utility is demonstrated with a number of separations of different classes of compounds.

INTRODUCTION

Although high-performance liquid chromatography (HPLC) has been developed into a worthwhile analytical technique for the analysis of non-volatile compounds, its full separation potential is still poorly exploited. This is mainly due to the limited number of stationary phases presently available. Moreover, the reproducibility of columns leaves much to be desired as this differs from supplier to supplier and often from batch to batch.

Extension of the number of stationary phases has been achieved by modification of the existing stationary phases by means of strongly adsorbed compounds. Reversed-phase ion-pair[1,2], enantioselective phases[3] and metal-bonded phases[4] are some examples of these efforts. The application of liquid–liquid systems is another way to enlarge the number of phase systems in LC. However, the interest in these types of phase systems has drastically decreased since their applications in the late 60's when HPLC started to be developed[5]. The reason for this can be attributed to the mutual solubility of two liquid phases which necessitates extensive precautions such as presaturation and thermostatting in order to obtain stable phase systems. Moreover, the use of such liquid–liquid systems usually precludes the injection of sample solutions which differ somewhat in composition from the mobile phase and excludes gradient elution. Despite these drawbacks the number of potential liquid–liquid systems is very large and their application has definite advantages compared with the adsorption systems with respect to reproducibility and selectivity.

For that reason we have investigated the applicability of a number of dynamically generated liquid–liquid systems in HPLC.

EXPERIMENTAL

Apparatus

The liquid chromatograph comprised a reciprocating membrane pump (Orlita, Giessen, F.R.G.), a flow-through manometer, a high-pressure injection valve Model 70-10 (Rheodyne, Berkeley, CA, U.S.A.) fitted with a 20-μl sample loop and a UV-detector with variable wavelength (Model PM2 DLC1; Zeiss, Karlsruhe, F.R.G.). All columns were made of 316 stainless steel of dimensions 150 × 4.6 mm I.D. and were fitted with home-made thermostatting jackets. The double-walled stock reservoir and the column-jackets were connected with separate thermostats Type F 4391 (Haake, Berlin, F.R.G.).

Materials

All solvents were obtained from commercial sources and of analytical grade used without any pretreatment. The following silica supports were used: Sorb Sil 60-5 (Macherey, Nagel & Co., Düren, F.R.G.); Hypersil (Shandon, Runcorn, U.K.); Zorbax BP-Sil (Du Pont, Wilmington, DE, U.S.A.); LiChrosorb Si 100 and LiChrospher Si 100, 500 and 1000 (Merck, Darmstadt, F.R.G.). All supports had a declared mean particle size of 5 μm, except Zorbax and LiChrospher with mean particle sizes of 7 and 10 μm respectively.

Procedures

The columns were packed with a pressurized slurry technique using 1-propanol as suspension liquid and methanol as driving liquid up to 500 atm. LiChrospher Si 500 and 1000 were packed at the lower pressure of 200 atm. The performances of all columns were tested in the adsorption mode using n-hexane + 2 vol.% ethyl acetate as mobile phase and butylbenzene, methyl benzoate, acetophenone and p-nitroacetophenone as solutes.

Liquid–liquid systems were prepared by weighing out the component solvents, then mixing vigorously during 12 h at constant temperature. The following systems were used:

Code	Components	Composition (v/v)
A	Water–ethyl acetate	–
B	Water–methanol–chloroform 0.05 M Formic acid and 0.05 M ammonium formate	40:250:710
C	Ethylene glycol–ethyl acetate	–
D	Formamide–n-hexane–chloroform	1:9:90
E	Water–ethanol–isooctane	49:766:1185
F	Dimethyl sulphoxide–cyclohexane	–

The total accessible volume, V_0, which is the sum of the pore volume, V_p, and the interstitial volume, V_i, was determined from the elution volume of an unretained solute in the adsorption mode[6].

Corrections were made for the volume contribution of tubings, sample loop, etc. The volume of the stationary phase, V_s, was calculated from the difference of the

total accessible (V_0) and the actual retention volume, V'_0, of an unretained solute in the partition mode.

RESULTS AND DISCUSSION

The investigated liquid–liquid systems were used in the so-called normal phase mode in which the more polar phase acts as stationary phase and is generated *in situ* on silica gel by pumping through the less polar phase which is presaturated with the polar phase. If liquid–liquid distribution is the only distribution process there will be a linear relationship between k' and the phase ratio. However, solutes can also interact with the silica matrix and thus in practice the retention is governed by at least two distribution processes, adsorption and partition

$$k' = K_{ads} \cdot \frac{A_s}{V_m} + K_p \cdot \frac{V_s}{V_m} = K_{ads} \cdot \frac{A_s}{V_0} + \frac{V_s}{V_m} \left(K_{ads} \cdot \frac{A_s}{V_0} + K_p \right) \qquad (1)$$

where k' = capacity factor, K_{ads} = adsorption coefficient, K_p = liquid–liquid partition coefficient, A_s = surface area, V_m = volume of the mobile phase, V_s = volume of the stationary phase and V_0 = void volume of the column.

The magnitude of adsorption and partition differs with the type of solute, type of phase system, the stationary liquid loading and the type of silica gel. When applying the *in situ* technique to load the column with the stationary phase, one important parameter, namely the phase ratio, cannot be used as a variable to adjust the retention as the pores are always completely filled. However, in a previous paper[6] we showed that the phase ratio can be adjusted over a wide range by adjusting the temperature difference between the stock reservoir and column without significantly influencing the selectivity.

In this study we investigated the influence of the type of silica gel on the phase ratio, retention and selectivity, and paid attention to the efficiency and capacity of liquid–liquid columns generated *in situ* and to the influence of the mobile phase velocity on the retention.

In situ generation of liquid–liquid systems on various types of silica gels

As the amount of stationary phase and thus the phase ratio depends on the type of silica (*e.g.*, surface area, pore volume, pore size), the phase ratio was determined with a number of liquid–liquid systems (see Experimental) on various types of commercially available silica gels. The results of these measurements are compiled in Table I. It appears that there is no clear relationship between the maximum phase ratio and the various properties of the silica gels. Moreover, the maximum phase ratio seems to vary with the type of liquid–liquid systems. For instance, phase system B comprising a chloroform–methanol–aqueous formate buffer gives larger pore fillings than system A comprised of ethyl acetate–water. This indicates that the polarity difference, *e.g.*, the mutual solubility, between the two liquid phases plays an important rôle in the pore filling. This is also found when comparing the speed of stationary phase generation. With systems B and F the maximum stationary phase loading is reached after about 75 column volumes of mobile phase, whereas for systems A, C

TABLE I

MAXIMUM PHASE RATIO AS OBTAINED ON VARIOUS COMMERCIAL SILICA GELS

V_p = Intra-pore volume per cm^3 column volume; A = surface area of the silica gel per cm^3 column volume. ●, Spherical particles; ▲, irregular particles.

Support	Shape	A $(cm^{-1} \cdot 10^{-4})$	V_p	Liquid–liquid system*					
				A	B	C	D	E	F
Hypersil	●	90	0.35	0.43	0.66	0.58	–	–	0.53
LiChrosorb Si 100	▲	120	0.48	0.72	0.85	–	0.80	–	–
LiChrospher Si 100	●	90	0.44	–	1.16	–	1.12	0.25**	–
LiChrospher Si 500	●	18	0.30	–	0.27	–	–	–	–
LiChrospher Si 1000	●	11	0.45	–	0.08	–	–	–	–
Sorb-Sil 60***	▲	235	0.95	–	0.60	–	–	–	–
Zorbax BP-Sil	●	180	0.73	–	0.90	–	–	–	0.72

     * See *Procedures*.
    ** Stock reservoir and column at 25°C.
   *** It has been assumed that the surface area and the pore volume of Sorb-Sil 60 and LiChrosorb Si 60 are the same.

and E at least 500 column volumes of mobile phase are required. System D comprising formamide–*n*-hexane–chloroform shows a quite different behaviour. On LiChrosorb Si 100 a phase ratio of 0.2 is reached after pumping through 75 column volumes of mobile phase. However, the maximum phase ratio of 0.77 is just reached
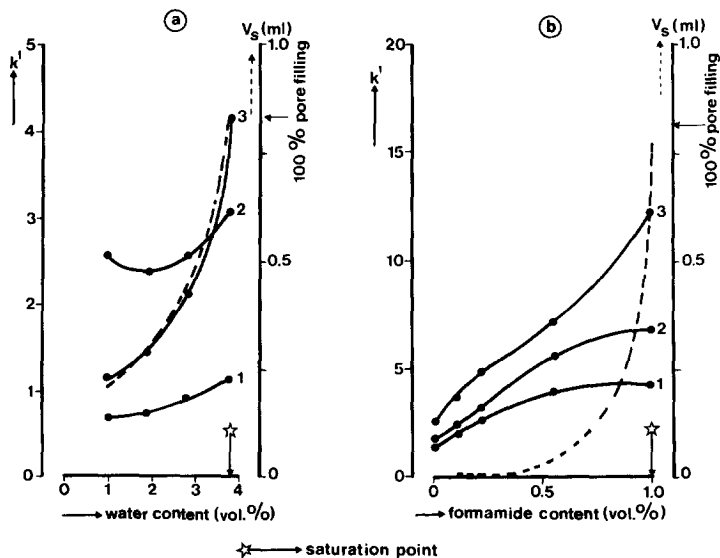

Fig. 1. Relationship between the capacity ratio, k', and the amount of generated stationary liquid on silica gel with unsaturated and saturated mobile phases, systems B and D. a, Mobile phase: system B with varying water content (1–3.8%, v/v). Silica support: Zorbax BP-Sil. Solutes: 1 = 3-hydroxycinnamic acid; 2 = hippuric acid; 3 = 3,4-diaminobenzoic acid. b, Mobile phase: system D with varying formamide content (0–1%, v/v). Silica support: LiChrosorb Si 100. Solutes: 1 = butalbital; 2 = aprobarbital; 3 = phenobarbital.

after pumping through more than 6000 column volumes of saturated mobile phase.

It is known from previous studies[7] that in adsorption chromatography the polar components of the mobile phase are preferentially adsorbed and can give rise to the formation of a significant layer of stationary liquid. In order to investigate whether this occurs with the mobile phases used in this study, we investigated the retention behaviour of some test solutes with unsaturated mobile phases of systems B and D. The results of these measurements are given in Fig. 1. With system B a stationary liquid is generated even at low water contents of the mobile phase. The amount of stationary phase increases gradually with increasing water content and then steeply rises when using a saturated mobile phase (complete pore filling). The change of k' with the water content of the mobile phase coincides to a large extent with the amount of stationary liquid generated. One compound, which shows a significant retention via adsorption, first shows a decrease of k' with increasing water content and then an increase at larger water contents. This behaviour can be explained by Snyder's competition model[8] and illustrates the transition from retention via adsorption to partition. A completely different behaviour is found with system D when measuring k' at various formamide concentrations. Up to about half the saturation concentration, k' of the test solutes increases significantly, despite almost no pore filling. This behaviour is in contrast to Snyder's competition model and indicates that the surface is modified by small amounts of adsorbed formamide which enhance significantly the retention of the selected test solutes. The synergistic effect of adsorbed compounds on surfaces on the retention of solutes has previously been demonstrated in reversed-phase[9] and reversed-phase ion-pair chromatography[10]. A
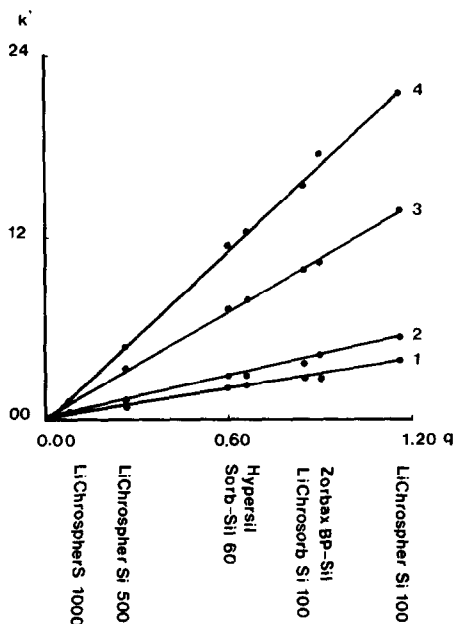

Fig. 2. Capacity ratio, k', *versus* the phase ratio as determined on various commercially available silica gels. Phase system: B. Solutes: 1 = 2,6-dihydroxybenzoic acid; 2 = nicotinic acid; 3 = 2-aminobenzenesulphonic acid; 4 = p-aminohippuric acid.

clear relationship between the amount of adsorbed formamide and k', as found with the water content of system B, cannot be found. However, the results obtained with formamide offer possibilities to modify *in situ* silica gel with specially synthesized compounds and thus enlarged the number of available normal stationary phases.

Influence of the type of silica on retention and selectivity

Eqn. 1 shows that when assuming a constant contribution of adsorption to the overall retention there will be a linear relationship between k' and the phase ratio V_s/V_m. This assumption is not so unrealistic: when increasing the stationary liquid loading the accessible surface area decreases but also V_m, the volume of the mobile phase, and thus the ratio of A_s/V_m might change only little. In an earlier paper[6] we gave some evidence that the adsorption contribution can be considered as more or less constant when changing the phase ratio by adjustment of the temperature difference between the stock reservoir and column. Although a temperature difference is an attractive way to adjust the phase ratio since only one column is needed, columns filled with different types of silica gel can be also applied according to the data given in Table I. In order to investigate this and to determine the influence of the silica matrix on the selectivity, the k' values of some test solutes were measured in phase system B on the various types of silica gel, keeping the stock reservoir and column at the same temperature. The results of these measurements are given in Table II and Fig. 2. As can be seen from Table II, the magnitude of the retention differs from silica to silica because of the difference in stationary phase loading, but the variation in selectivity is surprisingly small. This indicates that the influence of the silica matrix, *e.g.*, the adsorption, is small or at least a constant fraction of the total retention. This is supported by Fig. 2 which shows the correlation of k' for some solutes with the phase ratios as measured on the various types of silica gels. A

TABLE II

REPRODUCIBILITY OF RETENTION AND SELECTIVITY OF PHASE SYSTEM B ON VARIOUS COMMERCIALLY AVAILABLE SILICA GELS

$k_i' = \dfrac{t_{Ri} - t_{R0}}{t_{R0}}$ = capacity ratio of compound i calculated from the retention time, t_{Ri}, and the residence time, t_{R0}, of the unretained butylbenzene; $r_{ji} = k_j'/k_i'$ = selectivity factors with $k_j' > k_i'$.

Compounds (acids)	Sorb-Sil 60		Hypersil		LiChrosorb Si 100		Zorbax BP-Sil		LiChrospher Si 100	
	k_i'	r_{ji}	k_i'	r_{ji}	k_i'	r_{ji}	k_i'	r_{ji}	k_i'	r_{ji}
m-Hydroxycinnamic-	0.95	–	0.97	–	1.22	–	1.29	–	1.91	–
2,6-Dihydroxybenzoic-	2.13	2.24	2.28	2.35	2.84	2.33	2.72	2.11	3.95	2.07
Hippuric-	2.44	1.15	2.54	1.11	3.19	1.12	3.59	1.32	4.71	1.19
3-Pyridinecarboxylic-	2.83	1.16	2.92	1.15	3.64	1.14	4.44	1.24	5.46	1.16
3,4-Diaminobenzoic-	3.43	1.21	3.70	1.27	4.68	1.29	4.83	1.09	6.58	1.21
2-Aminobenzenesulphonic-	7.3	2.13	8.0	2.16	10.1	2.15	10.4	2.15	13.7	2.09
3,5-Diaminobenzoic-	9.3	1.27	10.1	1.27	12.8	1.27	13.9	1.33	18.1	1.32
p-Aminohippuric-	11.5	1.23	12.3	1.22	15.5	1.21	17.5	1.26	22.5	1.24
3-Aminobenzenesulphonic-	15.8	1.37	17.6	1.43	22.3	1.45	23.1	1.32	29.9	1.33
4-Aminobenzenesulphonic-	20.7	1.31	23.0	1.31	29.4	1.31	30.0	1.30	39.3	1.31

completely linear relationship is found which can only occur if the adsorption contribution is small or a constant fraction of the total retention. Regression analysis[11] applied to the relation between k' and V_s/V_m for ten test solutes gives a standard estimate of error, expressed as a percentage of the calculated k' at $V_s/V_m = 1.16$, of 0.8% for the highest k' to 5.3% for the lowest k'. Further insight into the rôle of adsorption can be obtained by correlating batch wise measured partition coefficients with retention data and phase ratios. Work in this direction is now in progress in our laboratory.

Performance of liquid–liquid systems generated in situ

The performance of the applied liquid–liquid systems with respect to column efficiency, capacity and influence of the mobile phase velocity on k' was investigated with system B and Hypersil and Zorbax BP-Sil as support. The possible dependence of k' on the mobile phase velocity is caused by heat production in the column due to frictional forces and/or mechanical stripping of the stationary phase by sheer forces. The two effects are difficult to distinguish experimentally. It has been demonstrated[12] that, due to heat production, a longitudinal and radial temperature gradient
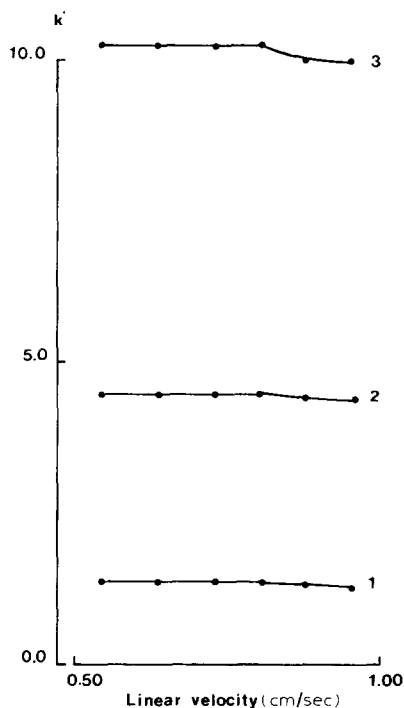

Fig. 3. Influence of the linear velocity, $\langle v \rangle$, on the capacity ratio, k', of some test solutes with phase system B. Silica support: Zorbax PB-Sil. Solutes: 1 = 3-hydroxycinnamic acid; 2 = nicotinic acid; 3 = 2-aminobenzenesulphonic acid.
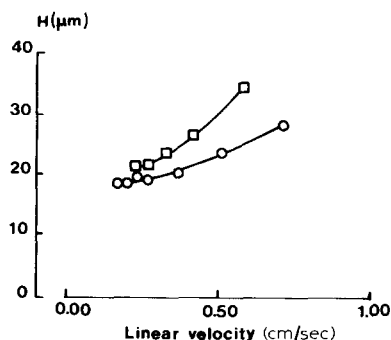
Fig. 4. $H/\langle v \rangle$ curve for some test solutes in the adsorption (O) and liquid–liquid modes (□) as measured on the same column. Silica support: Hypersil. Mobile phase: adsorption mode, n-hexane + 2% (v/v) ethyl acetate; liquid–liquid mode, phase system B. Solutes: 1,4-naphthoquinone ($k' = 3.05$) (O); nicotinic acid ($k' = 3.62$) (□).

is developed in columns which might give rise at larger mobile phase velocities to a few degrees difference in temperature between the two ends of the column and a somewhat smaller temperature difference between the core and wall of the column. As the mutual solubility of the liquid phases is temperature dependent this means that the phase ratio and thus k' will change with the mobile phase velocity. Fig. 3 shows the k' values of some solutes as a function of the mobile phase velocity. It is seen that k' decreases somewhat at larger mobile phase velocities but the change is not dramatic (about 2.5% at the highest velocity). Further, it was found that on returning from the highest velocity to the lowest the original k' values are obtained. This indicates a rapid restoration of the stationary liquid phase. It should be noted that the influence of the heat production in columns might be more significant for phase systems where the mutal solubility is strongly temperature dependent, or can lead to an increase in k' if the mutual solubility just decreases with increasing temperature.

The column efficiency of system B generated *in situ* was compared with the efficiency obtained when using the same column in a pure adsorption mode with *n*-hexane + 2 vol.% ethyl acetate as mobile phase. In order to exclude the effects of external peak broadening on the plate height measurements, solutes with $k' > 2$ were selected. The results of the measurements are shown in Fig. 4. At relatively low velocities the theoretical plate height of the liquid–liquid system is 4 d_p which is about 20% larger than in the pure adsorption mode. This is quite acceptable taking into account the larger viscosity of the mobile and stationary phases. At higher velocities
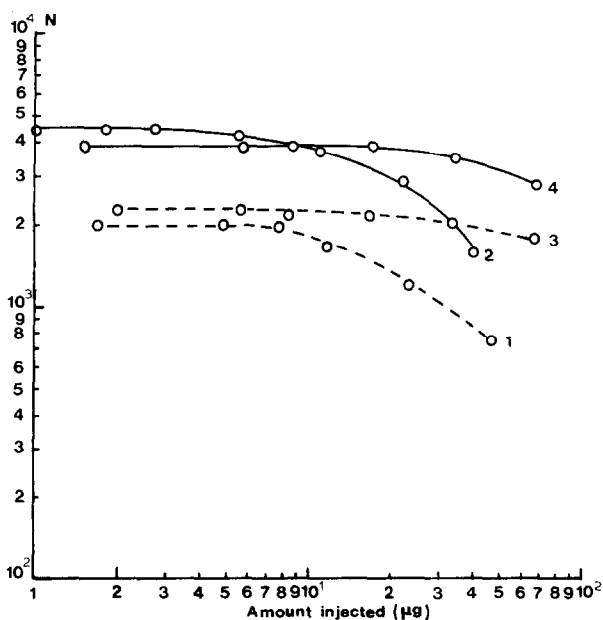

Fig. 5. Influence of the injected amount of some test solutes on the column efficiency as measured with an unsaturated (dashed line) and a saturated mobile phase, on the same column. Silica support: Zorbax BP-Sil. Mobile phase: unsaturated, system B, except 1% (v/v) of water; saturated, system B. Solutes: 1 = *p*-aminohippuric acid ($k' = 5.38$); 2 = nicotinic acid ($k' = 5.79$); 3 = hippuric acid ($k' = 2.40$); 4 = 2,6-dihydroxybenzoic acid ($k' = 2.46$).

however the plate height of the liquid–liquid system increases much more rapidly than in the adsorption mode. This indicates a slower mass transfer in the liquid–liquid system which again can be attributed partly to the larger viscosities but also to the effect of the radial thermal gradient in the column[12]. As a result of the latter effect, the retention in the core of the column is less than in the periphery (lower temperature) which causes an extra broadening of the solute band. Despite the rather steep $H/\langle v \rangle$ curve the column performance of liquid–liquid systems is quite acceptable at the commonly used linear velocities of 1–2 mm/sec. In order to improve the column efficiency, application of smaller particles (3 μm) and microbore columns, in order to minimize thermal effects, might be worthwhile with the described liquid–liquid systems.

The phase ratio in liquid–liquid systems is significantly larger than in adsorption systems[13] which should be favourable with respect to the capacity of such columns. This was investigated with a saturated and unsaturated mobile phase of system B and solutes with k' of about 2 and 5. Fig. 5 shows the dependence of the theoretical number of plates on the injected amount of solutes. For unknown reasons, the column efficiency obtained with the unsaturated mobile phase, i.e., adsorption mode, under non-overload conditions, was significantly worse than that, obtained with the saturated mobile phase, i.e. liquid–liquid mode. Although the difference in column efficiency makes comparison of the capacity of both systems less easy, no dramatic difference in capacity can be seen from Fig. 5. However, a final judgement of the capacity of liquid–liquid versus adsorption systems cannot yet be made on these preliminary results. Therefore it is necessary to carry out similar experiments with other liquid–liquid and adsorption systems.
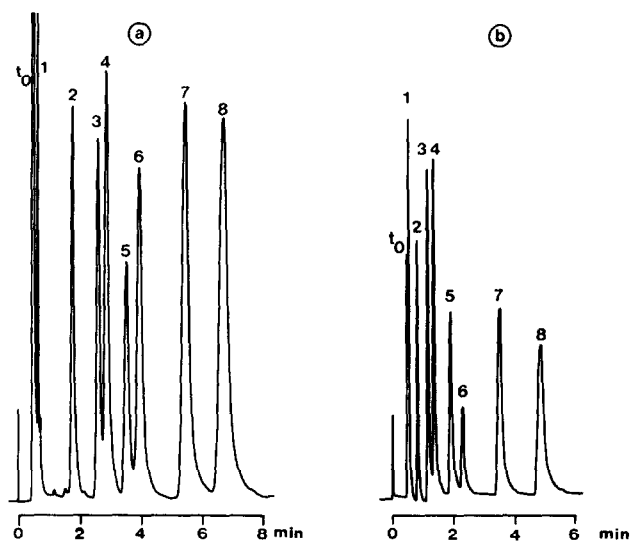

Fig. 6. Separation of some test mixtures of barbiturates (a) and phenol derivatives (b) on phase system D. Silica support: LiChrosorb Si 100. Solutes: a, 1 = methylphenobarbital; 2 = amobarbital; 3 = butalbital; 4 = cyclobarbital; 5 = unknown; 6 = aprobarbital; 7 = allobarbital; 8 = phenobarbital. b, 1 = eugenol; 2 = 2,6-dichlorophenol; 3 = o-chlorophenol; 4 = m-cresol; 5 = p-chlorophenol; 6 = phenol; 7 = m-nitrophenol; p-nitrophenol.
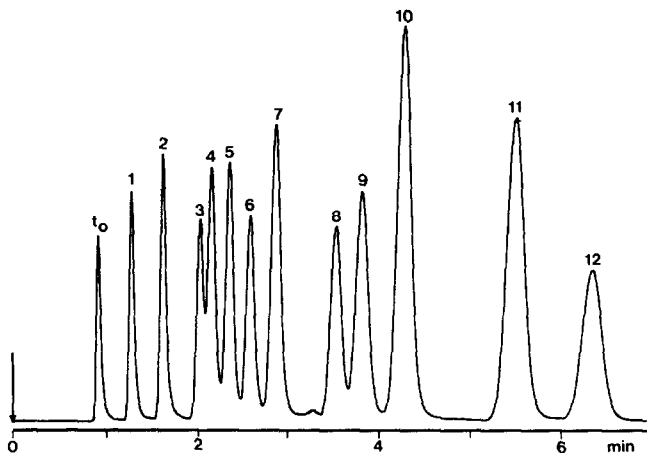
Fig. 7. Rapid separation of twelve polycyclic aromatic hydrocarbons on phase system F. Silica support: Zorbax BP-Sil. Solutes: 1 = acenaphthene; 2 = fluorene; 3 = acenaphthylene; 4 = anthracene; 5 = phenanthrene; 6 = pyrene; 7 = fluoranthene; 8 = benz[a]anthracene; 9 = chrysene; 10 = benzo[a]pyrene; 11 = benzo[g,h,i]perylene; 12 = dibenz[a,h]anthracene.

Practical utility

The practical utility of the investigated liquid–liquid systems had already been shown in a previous paper[6]. Extension of the applicability of the liquid–liquid systems is demonstrated in Figs. 6 and 7. The chloroform–hexane–formamide system seems to be very suitable for the separation of barbiturates (Fig. 6a) and phenol derivatives (Fig. 6b). A very promising liquid–liquid system is dimethyl sulphoxide–cyclohexane which was found to be particularly useful for extremely fast and selective separations of polycyclic aromatic hydrocarbons as demonstrated in Fig. 7.

CONCLUSIONS

The more polar phase of several liquid–liquid systems can be very well and reproducibly generated "in situ" on prepacked bare silica columns by pumping through the saturated mobile phase. The volume of the generated stationary phase and thus the phase ratio varies slightly with the type of liquid–liquid system, but considerably with the type of silica gel. On the various commercially available silica gels investigated in this study, phase ratios between 0.08 and 1.16 were obtained without significantly influencing the selectivity. The reproducibility of the selectivity, independent of the type of silica, is an important advantage of these liquid–liquid systems compared with adsorption systems in which the selectivity usually differs from supplier to supplier and sometimes even from batch to batch.

At commonly used mobile phase velocities (1–2 mm/sec), the column efficiency with liquid–liquid systems usually drops by about 20% compared with the efficiency obtained with the columns operated in the pure adsorption mode. However, at higher mobile phase velocities the efficiency drop is significantly larger. This can be partly attributed to stripping of the stationary phase by frictional heat production and sheer forces as k' decreases somewhat at such velocities. Preliminary experiments on the

capacity of the liquid–liquid systems show no significant differences from other liquid chromatographic systems, but a more extensive investigation is needed to confirm this. The results obtained so far indicate that, apart from extensive and precise thermostatting of the stock reservoir and column, liquid–liquid systems are well suited to HPLC and deserve more attention.

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