Journal of Chromatography, 626 (1992) 181–186
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 510

High-performance liquid chromatography of some purine and pyrimidine derivatives on silica in formamide–ethyl acetate mobile phases

H. A. Kažoka and V. D. Shatz

Institute of Organic Synthesis, 21 Aizkraukles Street, 226006 Riga (Latvia)

(First received June 3rd, 1992; revised manuscript received July 15th, 1992)

ABSTRACT

The chromatographic system with a silica sorbent and formamide–ethyl acetate as the mobile phase, amongst others, has been studied. It was established that the liquid stationary phase is deposited in the pores of silica during its equilibration with the eluent. The dynamically generated liquid–liquid system showed very good column efficiency and peak symmetry for the polar solutes under study. The mechanism of sorption is mixed, involving adsorption on the silica surface and partition. The contribution of each process depends on the volume of deposited liquid phase. In favourable cases the phase ratio of the system can reach 0.3. The sensitivity of the retention values to the temperature is usually not greater than that in traditional chromatographic modes on silica, although column thermostatting sometimes may be desirable. The disadvantage of such systems is a high consumption of solvents for reequilibration after work with chemically different eluents. In routine analysis, when this disadvantage is not critical, such systems can be useful because of their unique selectivity.

INTRODUCTION

Unmodified silica is traditionally used in normal-phase chromatography with mobile phase of low or medium polarity. Under these conditions, adsorption is usually the dominating retention mechanism, therefore the peak shapes of highly polar solutes are usually unsatisfactory and such substances are recommended to be analysed in the reversed-phase mode.

We believe that the potential of unmodified silica for separation of such highly polar solutes has been underestimated. The chromatograms on silica can be improved significantly if some untypical mobile phases are used.

Solvent-generated liquid–liquid systems are a good example of such application of chemically unmodified silica. According to this method [1–5], two or three immiscible solvents are equilibrated and, after the separation of layers, the less polar layer is used as the mobile phase. The polar component of this layer wets the silica surface better than the non-polar one, and covers it with a film of liquid that acts as a stationary phase in liquid–liquid partition chromatography.

One of the main advantages of partition systems is the linearity of the sorption isotherms and, consequently, the symmetrical shape of the chromatographic peaks. It was noted in the literature [1,4] that the properties of solvent-generated liquid–liquid systems are highly dependent on the temperature. Therefore such systems were not considered to be important in practice.

It has also been shown that the bulk liquid stationary phase can be formed not only by saturated solutions of polar solvents in less polar ones. The system consisting of silica and mixtures of ethyl acetate and ethylene glycol (EG) has been studied [6], and it was shown that the contribution of the

---

Correspondence to: Dr. H. A. Kažoka, Institute of Organic Synthesis, 21 Aizkraukles Street, 226006 Riga, Latvia.

0021-9673/92/$05.00

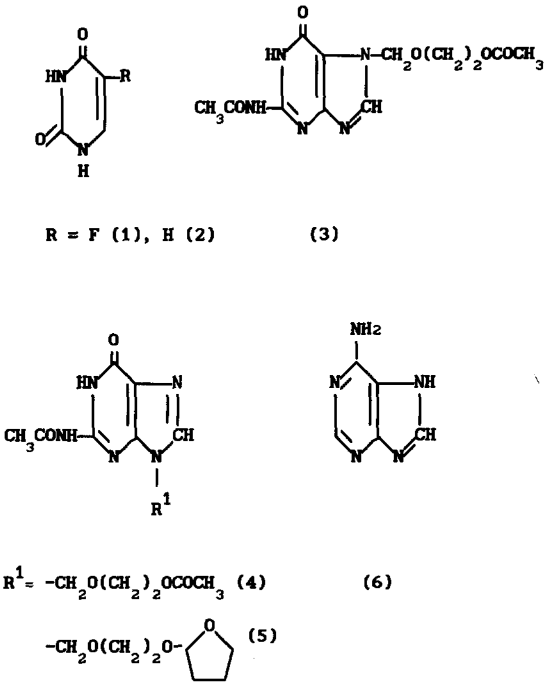

Fig. 1. Molecular structures of the solutes under study.

partition process to the retention is significant even when the concentration of EG is *ca.* 2%, *i.e.*, one-third of the saturation concentration.

Other solvent mixtures can be used in such chromatographic systems, and each of them is expected to have a different selectivity. For example, it was shown [4,5,7–9] that formamide-containing mobile phases can be used for the separation of phenols, steroids. alkaloids, and barbiturates. The goal of the present work was to investigate mixtures of ethyl acetate and formamide, and to estimate the system sensitivity to temperature changes.

EXPERIMENTAL

The solutes under study are shown in Fig. 1.

The chromatographic measurements were performed on DuPont Model 8800 HPLC system, equipped with a spectrophotometer ($\lambda = 254$ or 270 nm) and a refractive index detector. The columns (250 × 4.6 mm I.D.) were packed with unmodified silica Zorbax SIL (DuPont). Mixtures of ethyl acetate (EA) and acetonitrile (MeCN) with methanol (MeOH), ethylene glycol (EG) and formamide (FA) were studied as the mobile phases. The flow-rate was 2.0 ml/min. The samples (10–25 μl, 0.025–0.1 mg/ml) were injected with a Rheodyne 7125 sampling valve.

The column and mobile phase reservoir were kept at 23°C (typically) with the aid of a water-bath.

The columns were conditioned before each series of retention measurements by flushing with 50 ml of MeOH and 50 ml of EA or MeCN, followed by the mobile phase under study. Usually 350 ml of eluent were sufficient to obtain constant retention values.

The capacity factors of the solutes under study were calculated according to usual formula.

The system mobile phase volume was taken as equal to the benzene retention volume. The total volume within the column, apart from the silica, V_{mo}, was measured with mobile phases that did not modify the packing, *e.g.*, pure EA or MeCN. The deposition of liquid stationary phase in the pores of silica leads to a decrease of the mobile phase volume, V_m. This allows the volume of the stationary phase, V_s, to be calculated:

$$V_s = V_{mo} - V_m \tag{1}$$

The phase ratio, ϕ, of the column was calculated according to the formula

$$\phi = V_s/V_m \tag{2}$$

The peak width for column efficiency calculations was measured at half height, and the asymmetry factor S was calculated according to ref. 10.

The temperature coefficient, V, of the capacity factor was calculated according to the formula

$$V = \frac{k'_1/k'_2 - 1}{T_2 - T_1} \tag{3}$$

where k'_1 and k'_2 are the capacity factors at temperature T_1 and T_2, respectively ($T_1 < T_2$).

RESULTS AND DISCUSSION

In our previous work [6] the relationship between the molar concentration (C) of the polar component in the mobile phase and the retention was studied for eluents consisting of ethyl acetate, methanol and ethylene glycol. It was shown that, in the case of MeOH–EA mobile phases, log k' was linear with log C. The slope of the straight lines was in 0.5–

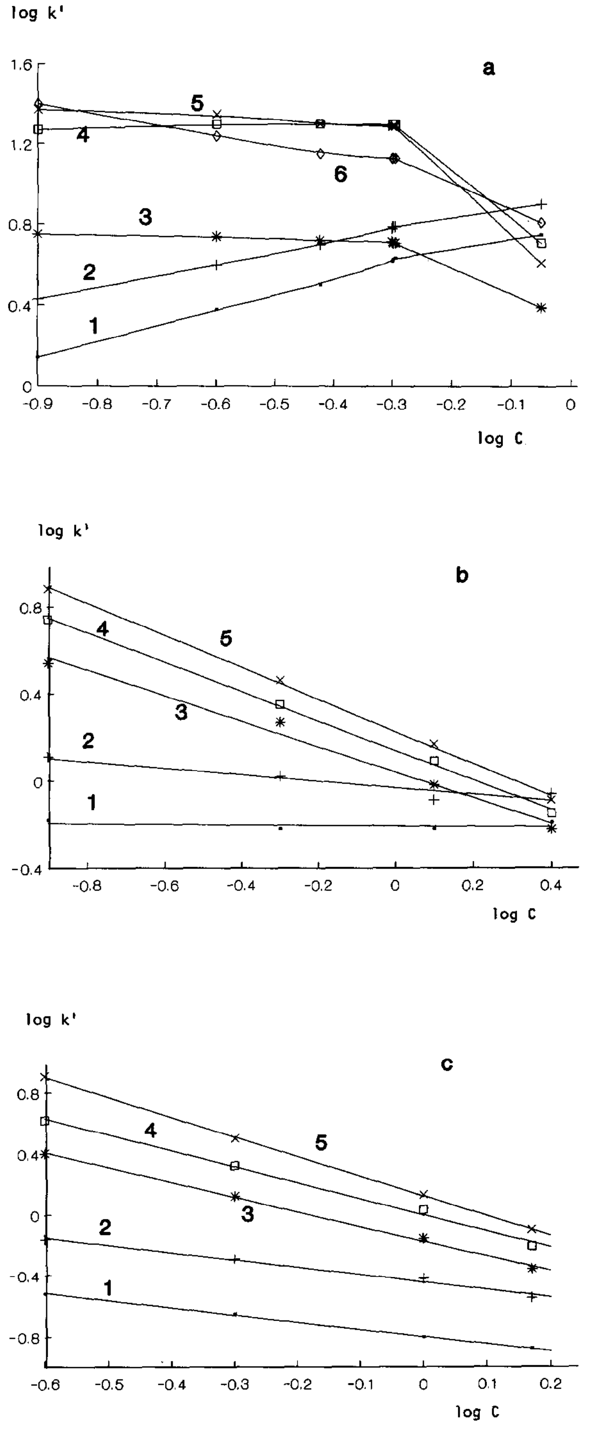

Fig. 2. Relationship between the concentration of polar solvent and capacity factors. Mobile phases: (a) FA–EA; (b) FA–MeCN; (c) MeOH–MeCN.

1.3 range, which is typical for normal phase chromatography. Linearity was not observed for EG–EA mobile phases. This was evidence of demixing of the mobile phase and formation of a liquid stationary phase in the pores of silica.

The relationship between the molar concentration of FA in the mobile phase and the capacity factors of the compounds under study is presented in Fig. 2a. The plots are very unusual and different from those observed for EG–EA eluents. The capacity factors of pyrimidines increased in the entire range of concentrations studied. The retention of purines decreased very slightly in range 0.5–2% (v/v) of FA. A further increase in the formamide concentration up to the miscibility limit caused a sharp drop of retention.

Such behaviour was observed only when the non-polar component of the mobile phase was EA, which is partly miscible with FA. The data for FA–MeCN and MeOH–MeCN eluents (Fig. 2b and c) show the usual linear behaviour. The slopes (except those for pyrimidines in FA–MeCN eluent) are in the 0.5–1.3 range, which is typical for adsorption chromatography.

The column dead-volume measured in different mobile phases can serve as a direct indicator of bulk liquid phase formation. It can be presented as a function of the volume of solvent pumped through the column, or as a function of the solvent composition. Fig. 3 shows the dependence of the phase ratio on the concentration of polar solvents in MeCN and

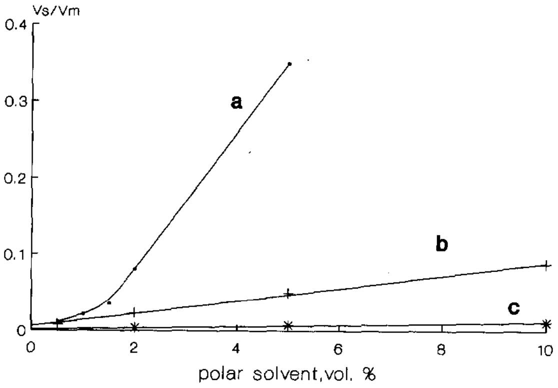

Fig. 3. Dependences of the column phase ratio on the concentration of polar solvent. Mobile phases: (a) FA–EA; (b) FA–MeCN; (c) MeOH–MeCN.

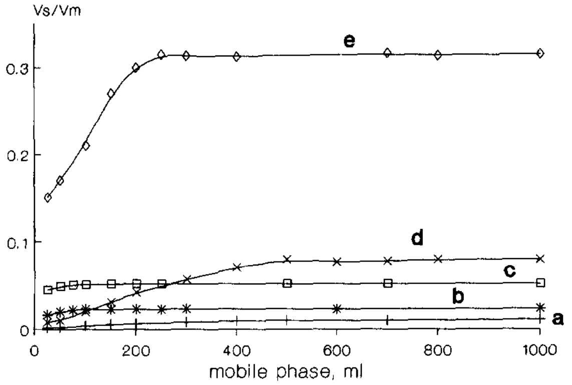

Fig. 4. Equilibration of the column with the mobile phases: (a) 0.5% FA in EA; (b) 2% FA in MeCN; (c) 5% FA in MeCN; (d) 2% FA in EA; (e) FA–saturated EA.

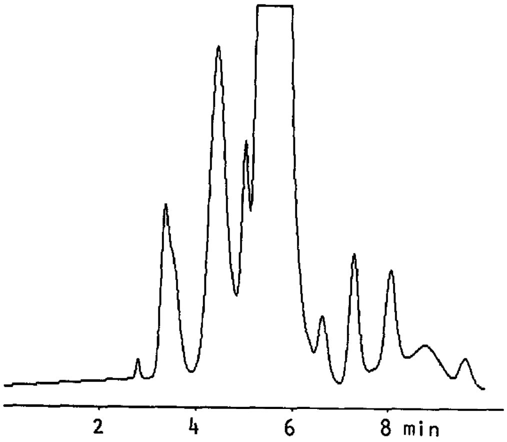

Fig. 5. Chromatogram of 3 mg of solute **5** in FA–saturated EA mobile phase. Injection volume, 100 μl.

EA. It can be seen that some amount of the stationary phase is generated in all three cases. The volume of the stationary phase formed depends on the difference between the polarities of the mobile phase components. In the case of MeOH–MeCN, the volume of the stationary phase is negligible: it is much larger in FA–MeCN. Both MeOH–MeCN and FA–MeCN systems are completely miscible and, therefore, the volume of the stationary phase increases linearly with increase of the polar solvent concentration. This was the reason why no anomalous log k'–log C relationships were observed for these mobile phases. In FA–EA, where the solubility is limited, we observed a much larger volume of the stationary phase formed and the relationship between C and ϕ is not linear.

Fig. 4 shows the stabilization of ϕ values during equilibration of the column with binary mobile phases. It can be seen that, when significant deposition of the stationary phase takes place, a relatively large volume of the eluent is needed to reach the constant properties of the system. The phase ratio of the system with saturated FA–EA solvent reaches 0.3. This high value makes such systems promising in preparative separations (Fig. 5).

The generation of a liquid stationary phase is accompanied by an improvement of the column efficiency and peak symmetry (Table I). This effect is especially significant for strongly retained solutes. In the usual adsorption chromatography systems ($\phi = 0$), the column efficiency and peak symmetry of substances 3–5 are much lower than those of weakly retained solutes, and also lower than is stated in the quality certificate of the given column. Table I shows that, for the systems with a significant degree of modification by the mobile phase ($\phi \geqslant 0.02$), and in the systems with the dominatant partition mechanism ($\phi > 0.3$), the peak symmetry and column efficiency are better than in adsorption systems and are almost independent of retention values.

The temperature coefficients, V, of the capacity factors for different compounds and systems are compared in Table I. The temperature range was 18–28°C, which is typical for most laboratories. It can be seen that the sensitivity of the retention to column temperature depends on the type of eluent. If the eluent is not saturated with the polar component, this sensitivity is no higher than in usual adsorption chromatography. If it is saturated, the sensitivity is dependent on the nature of the polar solvent. For FA–EA systems it is similar to that usually observed, whereas EG–EA systems show rather high V values. This means that, in many cases, temperature effects will not prevent the practical application of the discussed approach, although some kind of solvent and column thermostatting is desirable.

Systems with solvent-generated stationary phases seem to promise the possibility of changing the selectivity of separation, and even mechanisms, without changing the column installed in the instrument. The following two series of experiments were

TABLE I

RETENTION, PEAK SHAPE PARAMETERS AND TEMPERATURE COEFFICIENTS OF RETENTION IN DIFFERENT MOBILE PHASES

Mobile phase		Parameters[a]	Solutes under study					
Composition	ϕ		1	2	3	4	5	6
MeOH–EA (2:98)	0.00	k'	0.63	1.88	5.96	10.54	16.86	–
		N	2600	3000	500	350	350	–
		S	1.67	2.00	3.33	3.33	5.00	–
		V	0.010	0.010	0.010	0.010	0.015	–
MeOH–MeCN (2:98)	0.00	k'	0.39	0.88	2.11	2.85	4.22	–
		N	4800	5500	4700	5300	4800	–
		S	1.25	1.54	2.00	2.22	2.50	–
		V	0.010	0.013	0.015	0.015	0.020	–
FA–MeCN (2:98)	0.02	k'	0.59	1.05	1.80	2.28	3.02	14.5
		N	5000	6000	7000	7600	7400	2000
		S	1.11	1.18	1.18	1.18	1.18	2.50
		V	0.010	0.010	0.012	0.012	0.014	0.017
EG–MeCN (2:98)	0.02	k'	0.43	0.88	1.68	1.86	2.78	–
		N	6000	6300	4600	4600	4000	–
		S	1.05	1.05	1.25	1.25	1.25	–
		V	0.017	0.014	0.015	0.015	0.016	–
FA–EA (1.5:98.5)	0.04	k'	3.17	5.00	5.30	20.10	20.10	14.1
		N	7600	7600	6000	6200	6200	4400
		S	1.02	1.02	1.02	1.05	1.05	1.33
		V	0.012	0.014	0.011	0.012	0.012	0.010
FA–EA (2:98)	0.08	k'	4.30	6.10	5.15	20.00	19.60	13.6
		N	9300	9300	7600	8400	8400	6000
		S	1.02	1.02	1.02	1.02	1.02	1.25
		V	0.016	0.020	0.010	0.010	0.010	0.014
EG–EA (2:98)	0.02	k'	1.29	2.60	2.83	7.00	7.90	16.8
		N	7500	8200	8200	7400	7000	2300
		S	1.02	1.02	1.02	1.05	1.05	2.00
		V	0.016	0.017	0.010	0.010	0.010	0.016
EG–EA (4:96)	0.06	k'	1.08	2.04	1.53	2.87	3.19	10.1
		N	7800	8200	8000	7700	7000	2300
		S	1.02	1.02	1.02	1.02	1.02	1.67
		V	0.032	0.032	0.012	0.013	0.015	0.020
FA–saturated EA	0.35	k'	5.57	7.92	2.48	5.14	4.21	6.5
		N	8700	8700	7000	7000	7000	7600
		S	1.02	1.02	1.02	1.02	1.02	1.05
		V	0.010	0.012	0.012	0.010	0.011	0.014
EG–saturated EA	0.33	k'	4.37	9.29	1.70	5.23	4.82	22.7
		N	7600	7900	6000	6300	6300	3700
		S	1.02	1.02	1.02	1.02	1.02	1.43
		V	0.13	0.14	0.040	0.080	0.070	0.11

[a] N = Number of theoretical plates; S = peak symmetry.

TABLE II

REPRODUCIBILITY OF RETENTION TIMES

Solutes under study	Retention time, t_R ± S.D. (min) ($n = 6$)			
	FA–EA (2:98)		FA–saturated EA	
	Set I	Set II	Set I	Set II
1	3.95 ± 0.08	5.32 ± 2.20	6.65 ± 0.08	6.52 ± 1.61
2	5.85 ± 0.10	7.52 ± 2.66	9.29 ± 0.08	8.98 ± 2.00
3	7.46 ± 0.06	8.33 ± 1.00	4.63 ± 0.07	4.11 ± 0.35
4	19.93 ± 0.70	24.56 ± 5.90	8.04 ± 0.08	7.17 ± 0.65
5	21.23 ± 0.55	22.55 ± 1.30	7.04 ± 0.10	6.22 ± 0.40
6	19.35 ± 0.50	21.47 ± 1.75	9.14 ± 0.18	8.31 ± 1.15

performed to assess the degree of reversibility of column modification by the mobile phases studied. In one series of experiments (set 1 in Table II), the column was washed as described in Experimental, then equilibrated with the same mobile phase (EA saturated with or containing 2% of FA). Such washing–equilibration cycles were repeated six times. The standard deviation of the obtained retention times was used as a measure of reproducibility. After each equilibration, the whole set of retention times was measured. In the second set of experiments (set 2 in Table II), equilibration of the column and measurement of the retention with FA–EA mobile phase were followed by several separations performed with other solvent compositions (*e.g.*, EG–EA, FA–MeCN, MeOH–EA). The system was washed again and equilibrated with the initial FA–EA mobile phase. The results (Table II) show that the reproducibility of capacity factors is much better in the first series of experiments. This means that modification of the sorbent is not completely reversible. This observation means that, when working with solvent-generated stationary phases, frequent changes of the mobile phase composition cannot be recommended.

ACKNOWLEDGEMENT

This investigation was performed within Grant No. 424 of the Latvian Republic.

REFERENCES

1 J. F. K. Huber, M. Pawlowska and P. Markl, *Chromatographia*, 17 (1983) 653.
2 J. F. K. Huber, M. Pawlowska and P. Markl, *Chromatographia*, 19 (1984) 19.
3 J. P. Crombeen, S. Heemstra and J. C. Kraak, *Chromatographia*, 19 (1984) 219.
4 J. P. Crombeen, S. Heemstra and J. C. Kraak, *J. Chromatogr.*, 282 (1983) 95.
5 J. P. Crombeen, S. Heemstra and J. C. Kraak, *J. Chromatogr.*, 286 (1984) 119.
6 V. D. Shatz and H. A. Kažoka, *J. Chromatogr.*, 552 (1991) 23.
7 H. Poetter, M. Hulm and S. Schumann, *J. Chromatogr.*, 391 (1985) 440.
8 H. Engelhardt and N. Weigand, *Anal. Chem.*, 45 (1973) 1149.
9 H. Engelhardt, J. Asshauer, U. Neue and N. Weigand, *Anal. Chem.*, 46 (1974) 336.
10 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 1979, p. 222.