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High-performance liquid chromatography of some purine and pyrimidine derivatives on silica in hexane–isopropanol–ethylene glycol mobile phases

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Abstract

An alternative approach for the separation of polar solutes in chromatographic systems consisting of unmodified silica and mobile phases containing solvents with limited mutual solubility has been developed. The investigation of ternary mobile phases consisting of hexane, isopropanol and ethylene glycol has shown that the stationary liquid phase is generated dynamically in the pores of silica, even in the mobile phase not saturated with a polar component. If the phase ratio of the column reaches 0.1 partition dominates over adsorption and such mixed partition–adsorption systems show a good column efficiency and peak symmetry of some purine and pyrimidine derivatives.

Keywords: Mobile phase composition; Partition–adsorption systems; Purines; Pyrimidines

1. Introduction

Reversed-phase chromatography has become a popular method for separation and analysis of organic substances. Nevertheless, the reversed-phase mode is not always the best or the only choice for the given analytical task. The use of normal-phase separations in combination with reversed-phase ones can offer additional possibilities in process and quality control. However, normal-phase chromatography is not widely used for separation of highly polar solutes, partly because of a poor peak shape. In order to improve it, one can apply better purified silica [1], or polar chemically bonded phases [2]. Another approach is based on using some untypical mobile phases. Solvent-generated liquid–liquid systems are a good example of such application of chemically unmodified silica. According to this method [3–10], two or three immiscible solvents are equilibrated and, after the separation of layers, the less polar layer is used as a 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. The partition mode offers two advantages: versatility and reproducibility [10]. But the extensive presaturation of the mobile phase with the stationary phase and precise thermostatting of the stock reservoir and column are the main drawbacks of the solvent-generated liquid–liquid systems [5].

Our previous investigations [11–13] showed that a stationary liquid phase is generated dynamically in the pores of silica, even in the mobile phases not saturated with the polar component. Such mixed partition–adsorption (MPA) systems are free from

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the above mentioned disadvantages of solvent-generated liquid–liquid systems [12]. The mixture of ethylene glycol (EG) in ethyl acetate (EA) was tested as a mobile phase for the first MPA system [11]. But the limited solubility of EG in EA restricts the elution strength of binary systems. It was demonstrated that the addition of methanol (MeOH) to the mobile phase increased the elution strength of the MPA system. For this reason some of the polar solutes were tested in ternary mobile phases EA–MeOH–EG [13].

However, it is still a problem how to decrease the elution strength of the MPA system, if necessary. On the other hand, earlier investigated MPA systems [11–13] have a restriction in ultraviolet transparency.

Table 1
Mobile phases consisting of hexane, isopropanol and ethylene glycol and correspondent phase ratio

No.[a]	Mobile phase composition (%)			Phase ratio (V_s/V_m)
	HEX	IPA	EG	
1	66	34	0	0
2	66	33	1	0.01
3	66	32	2	0.03
4	66	29	5	0.09
5	66	28	6	0.17
6	66	27	7	0.28
7[b]	66	24	10	0.34
8	71	24	5	0.22
9	79	18	3	0.23

[a]Points 1–9 in Fig. 1.
[b]One of the coexisting phases (saturated with EG).

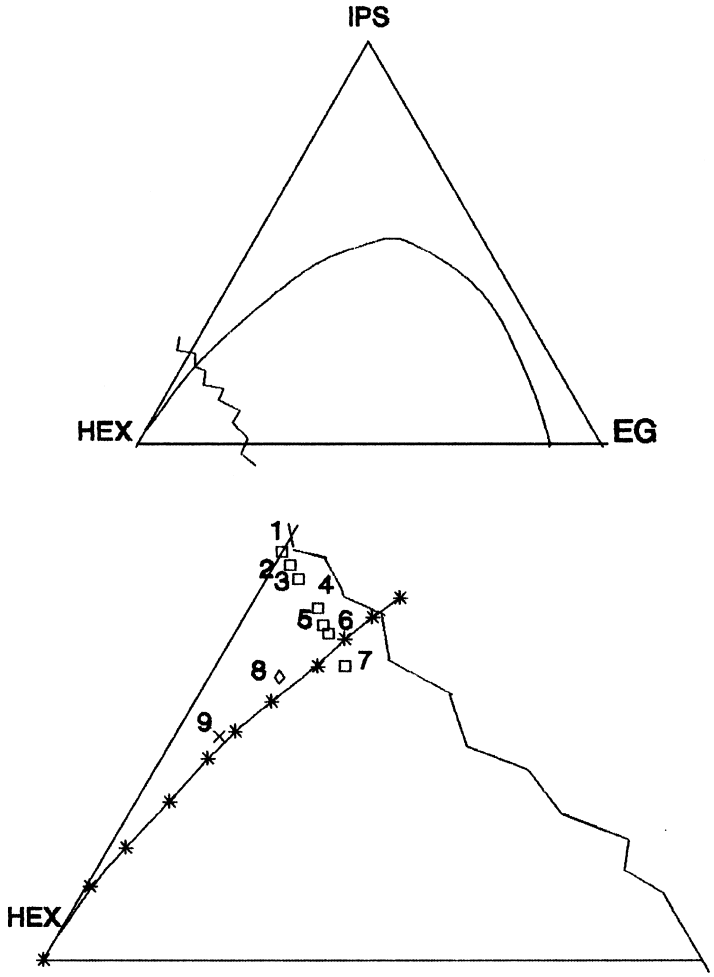

Fig. 1. Triangular phase diagram of ternary system HEX–IPA–EG at ambient temperature.

If detection at wavelengths <240 nm is required the best choice is hexane (HEX) and isopropanol (IPA). Therefore for the enlargement the number of MPA systems ternary mobile phases consisting of HEX, IPA and EG was investigated and the aim of the present work was to find the conditions when the mobile phases HEX–IPA–EG are good MPA systems.

2. Experimental

The chromatographic measurements were performed on a Gilson model 302 HPLC system, equipped with a spectrophotometer (λ=254). The 150×4.6-mm columns were packed with unmodified silica Zorbax SIL, 5–6 μm (DuPont). The mixtures of HEX with IPA and EG were studied as mobile phases. All the solvents were purchased from commercial sources and of analytical grade were and used without any pretreatment. The flow-rate was 1.0 ml/min. The samples (10–25 μl, 0.025–0.1 mg/ml) were injected via Rheodyne 7125 sampling valve.

The column conditioning was performed before each series of retention measurements. It included flushing with 50 ml of 34% IPA in HEX followed by the mobile phase under study. Usually 60 ml of the latter eluent were sufficient to obtain constant retention values.

The capacity factors of the solutes under study (k') and theoretical plate number (N) were calculated according to the usual expressions [14]. The system mobile phase volume was regarded to be equal to the benzene retention volume. The peak width for column efficiency calculations was measured at half height. The phase ratio of the column (V_s/V_m) was

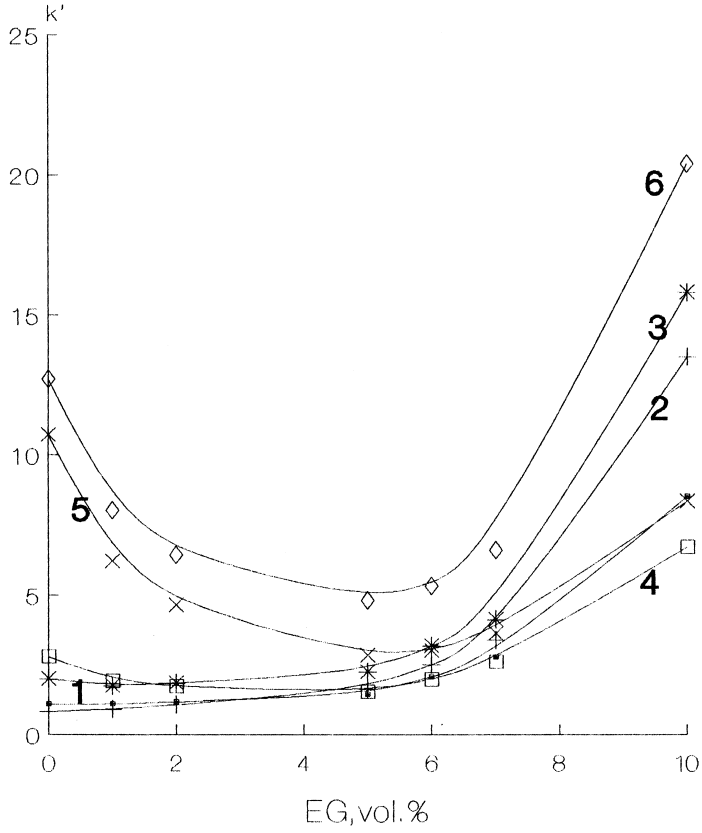

Fig. 2. Effect of ethylene glycol concentration in ternary mobile phase Nos 1–7 (Table 1) on retention of the solutes under study: ftorafur (1); 5-fluorouracil (2); uracil (3); theophylline (4); caffeine (5); theobromine (6).

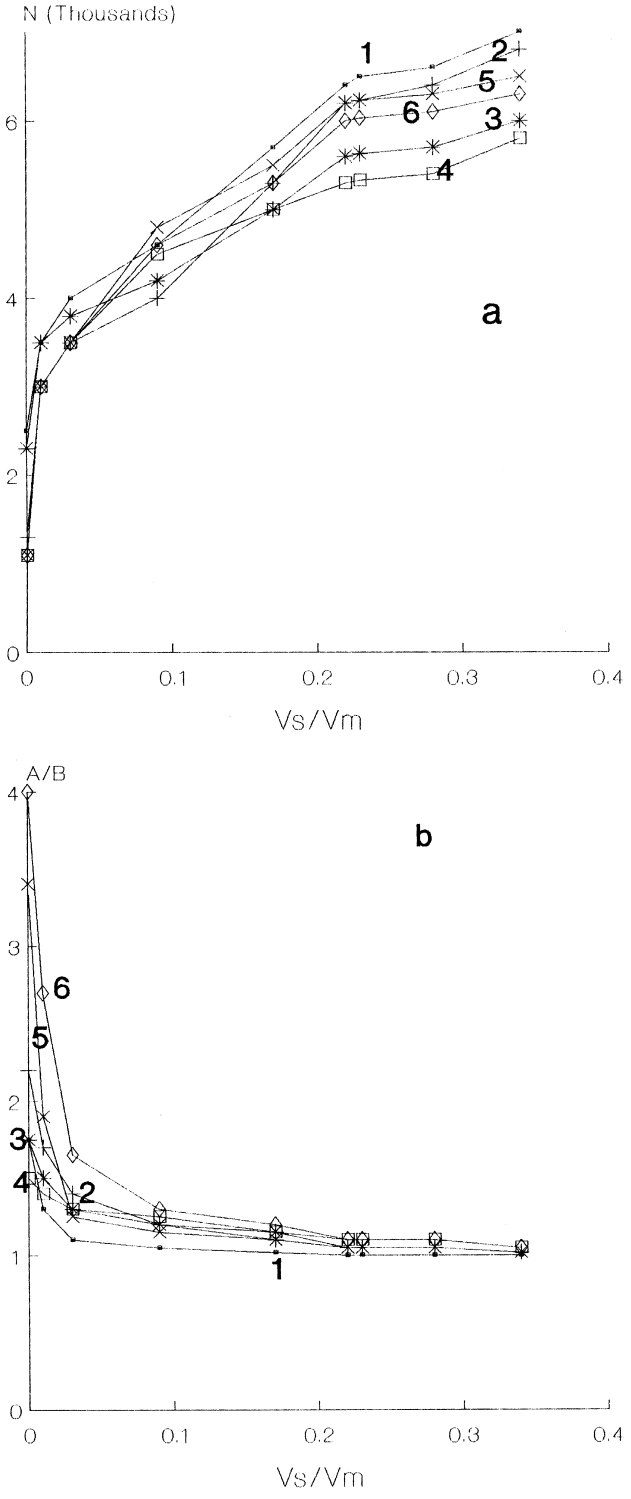

Fig. 3. Relationship between the column phase ratio (Table 1) and theoretical plate number (a) and peak asymmetry (b). Solutes 1–6, Fig. 2.

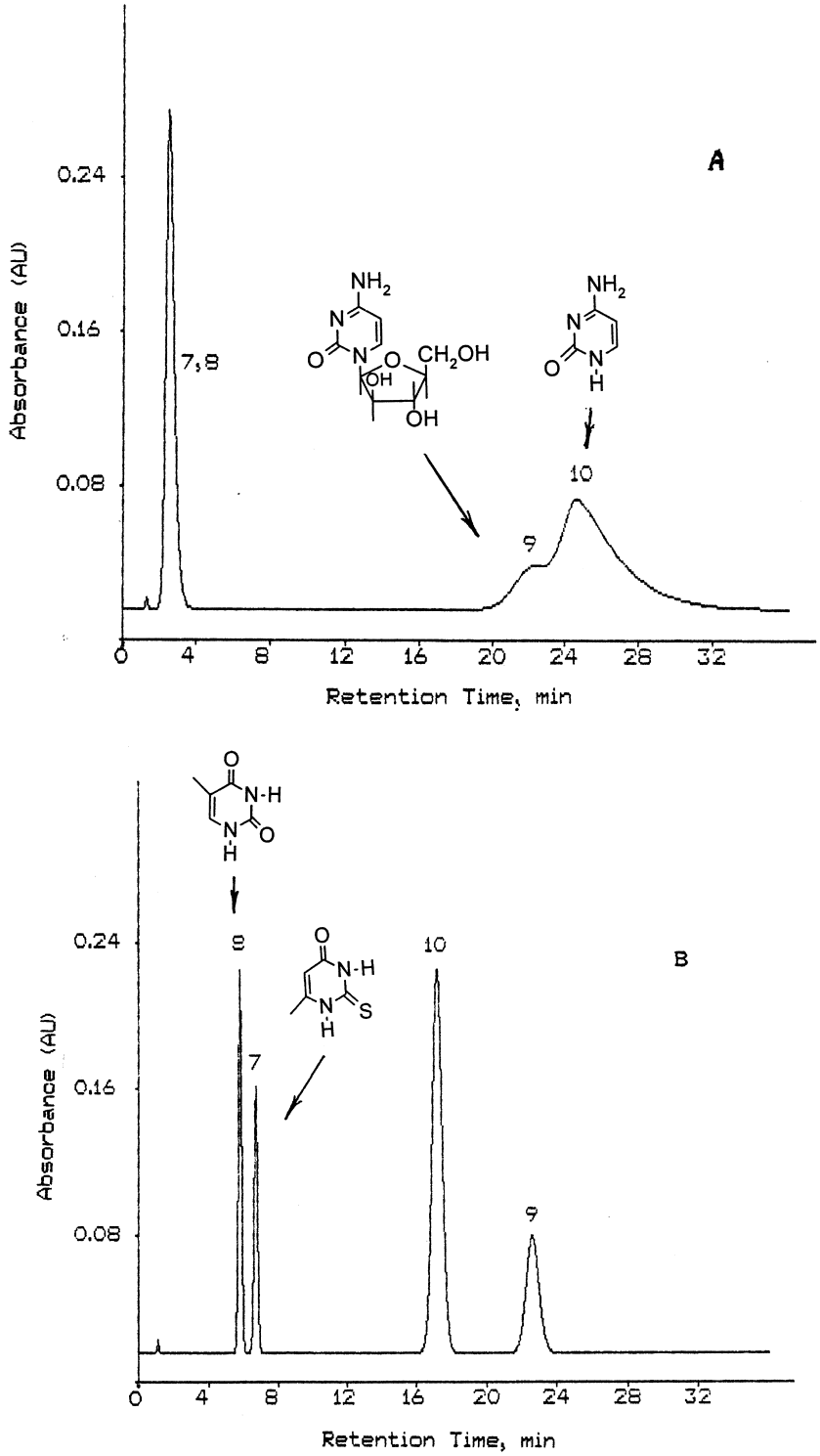

Fig. 4. Separation of the solutes studied (7–10) of the adsorption mode (A) and MPA mode (B). Mobile phase (A) No. 1; mobile phase (B) No. 8 (Table 1); column, Zorbax SIL, 150×4.6 mm; flow-rate, 1.5 ml/min; detector, UV, 254 nm; ambient temperature; 0.32 AUFS. Injection volume, 25 μl; sample concentration in mobile phase, 0.05 mg/ml (7,8), 0.2 mg/ml (9,10). Solutes, 6-methyl-2-thiouracil (7), thymine (8), cytarabine (9), cytosine (10).

Table 2
Specification of silica columns

No.	Column			Packing material		
	Trade name	Dimensions (mm)	Particle size (μm)	Pore size (Å)	Surface area (m^2/g)	Pore volume (ml/g)
1	Zorbax SIL	150×4.6	5–6	70	350	0.8
2	Zorbax SIL	150×4.6	5–6	70	350	0.8
3	Silasorb 600	250×4.0	6	60	600	1.0
4	Supelcosil LC-SI	250×4.6	5	100	170	0.6
5	Nova Pak Silica	150×3.9	4	60	120	0.3

calculated according to Ref. [12]. The peak asymmetry was calculated by determination of the A/B ratio at 10% of peak height [15].

Values of liquid–liquid equilibrium curve of the ternary system HEX–IPA–EG were determined by mutual titration (microburet was used) of a 50-ml vigorously mixed binary system IPA–HEX (5:95, 10:90, 15:85, and so on) with the EG at ambient temperature. If turbidity was observed, the solution achieved saturation.

The eluents for MPA systems were prepared by the EG slow addition to the vigorously mixed IPA–HEX to ensure complete homogenization. The eluent for the solvent-generated liquid–liquid system (Table 1, No. 7) was mixed vigorously during 6 h at constant temperature to ensure a complete demixing. The conjugated polar phase was not removed from the eluent container when pumping the apolar phase through the column.

3. Results and discussion

Chromatographic systems with silica and represented in Table 1 mobile phase consisting of HEX, IPA and EG were investigated. Since such ternary solutions contain poorly mutually soluble components, the dynamic generation of a stationary liquid phase on silica can be expected when such solutions are used as mobile phases [11–13].

Fig. 1 represents the triangular phase diagram for the ternary system HEX–IPA–EG. Point 1 in Fig. 1 corresponds to the binary mobile phase (No. 1 in Table 1) with adsorption mechanism of retention. Point 7 below the equilibrium curve corresponds to the ternary system No. 7 split into two coexisting liquid phases, one of which is presaturated with EG, the other with HEX. The first of the coexisting phases is applied as a mobile phase, and solvent-generated liquid–liquid system with partition mechanism of retention is formed [3–10]. Points 2–6 and 8–9 above the equilibrium curve correspond to the compositions which are homogeneous and differ from others by the degree of a saturation with EG. The mechanism of sorption in such systems is mixed, involving adsorption on the silica surface and partition. The contribution of each process depends on a volume of the deposited liquid phase.

Table 3
Adsorption (*A*) (mobile phase No. 1 in Table 1) and MPA mode (mobile phase No. 8 in Table 1)

Column		5-Fluorouracil						Separation factor[b], α	
No.[a]	Phase ratio (V_s/V_m)	Efficiency (N)		Peak asymmetry (A/B)		Retention (k')		*A*	MPA
		A	MPA	*A*	MPA	*A*	MPA		
1	0.22	1300	6200	2.2	1.05	0.83	4.51	3.33	0.67
2	0.22	1400	6200	2.1	1.05	0.74	4.47	3.45	0.68
3	0.25	1300	6300	2.2	1.05	0.77	4.88	4.39	0.71
4	0.19	1400	4800	2.0	1.20	0.42	3.14	3.07	0.72
5	0.13	1100	2000	2.7	1.50	0.34	2.45	2.91	0.71

[a]See Table 2.
[b]k' (theophylline)/k' (5-fluorouracil).

Fig. 2 represents the relationship between capacity factors and EG concentration in mobile phases. It is not linear due to EG-rich liquid stationary phase formation in the silica pores. Let us assume that under the condition of typical adsorption chromatography with strong enough eluent (34% IPA in HEX) benzene is not adsorbed, and in this case its retention volume corresponds to the mobile phase volume V_m or total volume V_{mo} within column, apart from silica. The formation of the dynamically generated stationary liquid phase having volume V_s leads to decrease in the mobile phase volume V_m, and the total volume Vmo in this case is $V_{mo}=V_m+V_s$. It is possible to estimate the volume of the stationary phase (V_s) as the difference between the total volume (V_{mo}) and the mobile phase volume (V_m): $V_s=V_{mo}-V_m$, where V_s is the volume of dynamically generated stationary liquid phase; V_{mo} is retention volume of benzene in typical adsorption mode; V_m is retention volume of benzene in dynamically modified system.

The values of calculated phase ratio (V_s/V_m) are represented in Table 1. It is seen (Fig. 3a,b) that if the phase ratio value is less than 0.03, adsorption dominates under these circumstance, and the column efficiency and peak symmetry for such systems are poor. If V_s/V_m is within 0.03 and 0.1, the peak symmetry and the column efficiency are better than in the adsorption mode, but worse than in the partition mode. But if the column phase ratio is higher than 0.1 the partition dominates over adsorption and such mixed partition–adsorption (MPA) systems (Table 1, Nos 5–6, 8–9) show good column efficiency and peak symmetry. Fig. 4 demonstrates the advantage of MPA mode versus adsorption mode.

The silica columns represented in Table 2 were tested as the stationary phases for comparison of

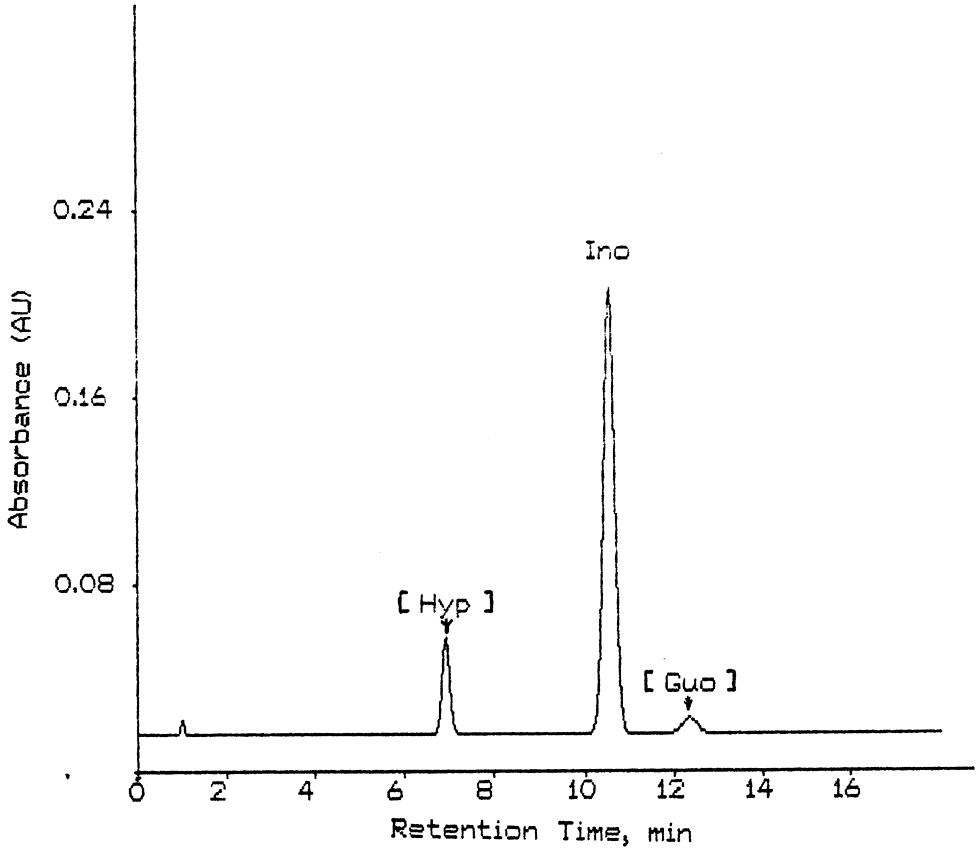

Fig. 5. Determination of impurities in crude inosine (Ino). Mobile phase No. 6 (Table 1); column, Zorbax SIL, 150×4.6 mm; flow-rate, 1.5 ml/min; detector, UV, 254 nm; ambient temperature; 0.32 AUFS. Injection volume, 50 μl; sample concentration in mobile phase, 0.2 mg/ml. Hypoxanthine (Hyp) and guanosine (Guo) were identified by co-elution with standards.

column efficiency and peak symmetry as well as retention and selectivity in adsorption and MPA mode (Table 3). It can be concluded from Tables 2 and 3 that the volume of the dynamically generated stationary phase is larger on the silicas with larger surface areas and pore volume. But in all cases MPA mode has advantage versus adsorption mode in column efficiency and peak symmetry. It is seen that retention is stronger on the silicas with larger surface area. But the selectivity obtained in the MPA mode does not differ much on different silica columns and, therefore, such MPA systems are advantageous over adsorption systems and are applicable in analytical practice. The findings (Figs. 5 and 6) have demonstrated that MPA systems are applicable in normal-phase chromatography of some purine and pyrimidine derivatives.

In order to estimate the flexibility of HEX–IPA–EG MPA systems the concentration of components in mobile phases was varied. The obtained results suggest that the selectivity does not change very markedly with alteration in polar solvents concentration and proportion, and when the concentration of hexane is the same the proportion of EG and IPA does not affect retention very much. But when the concentration of polar components in ternary mobile phases is decreased an increase in retention is observed. If considerable selectivity alterations are necessary the best approach is to use another MPA system (Fig. 7).

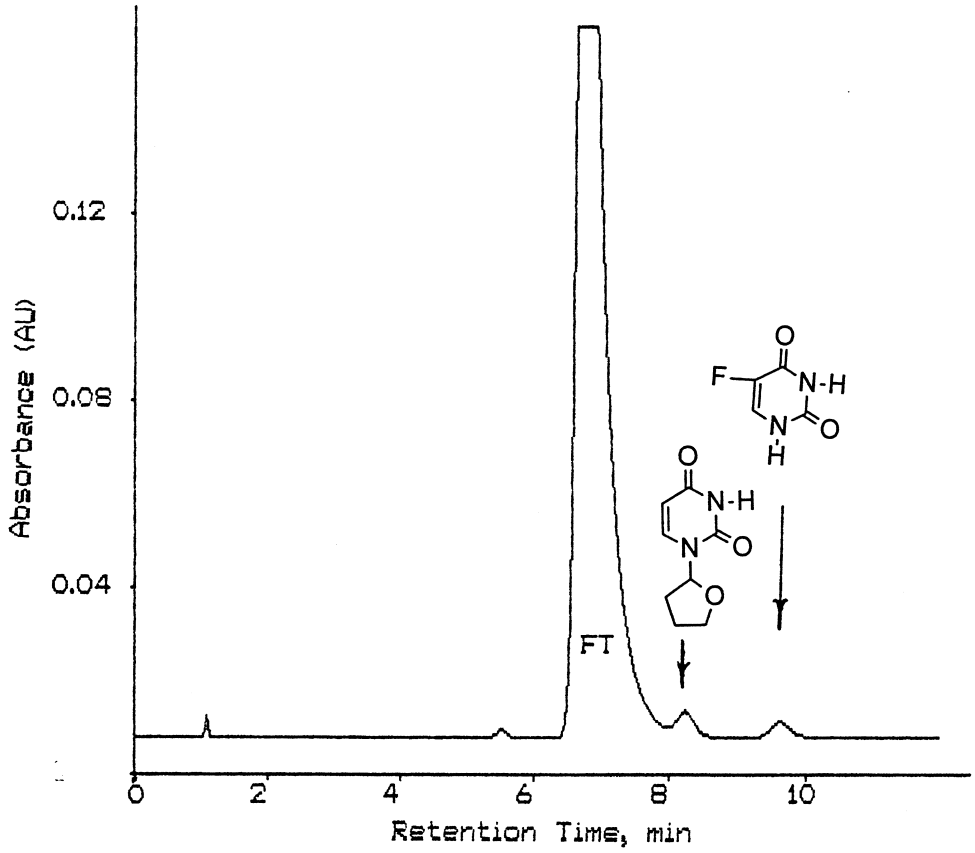

Fig. 6. Determination of impurities in ftorafur (FT) substance. Mobile phase No. 9 (see Table 1); column, Zorbax SIL, 150×4.6 mm; flow-rate, 1.5 ml/min; detector, UV, 254 nm; ambient temperature; 0.16 AUFS. Injection volume, 50 μl; sample concentration in mobile phase, 1.0 mg/ml. 1-(2-Tetrahydrofuryl)uracil and 5-fluorouracil were identified by co-elution with standards.

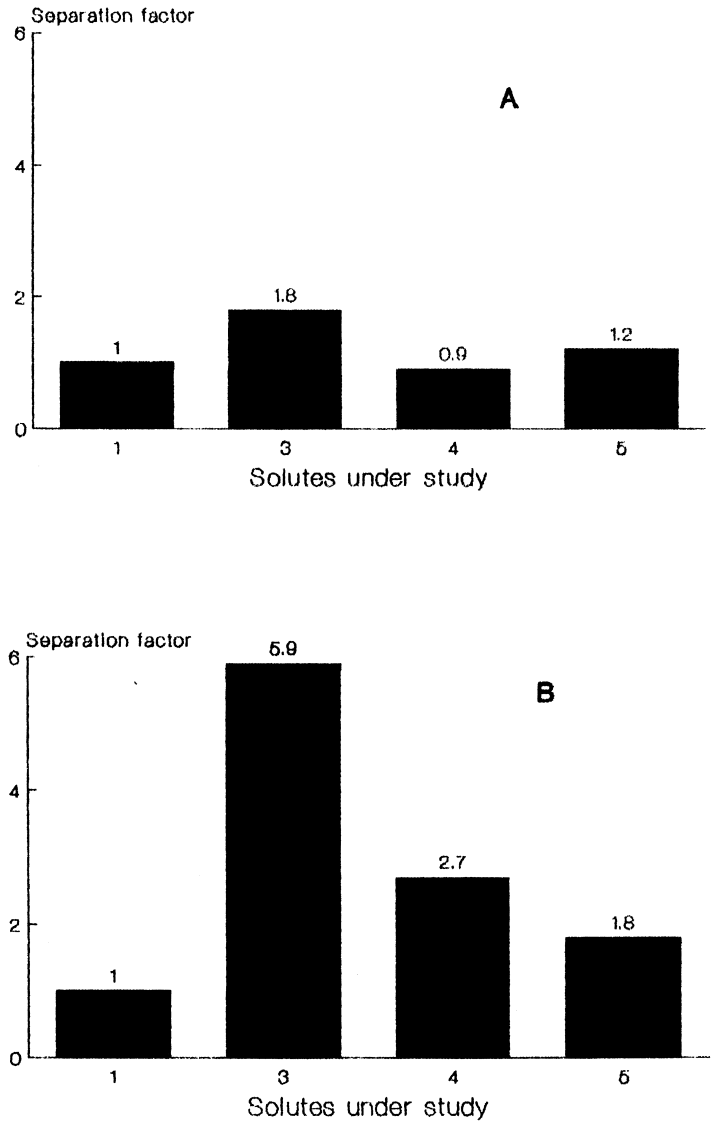

Fig. 7. Separation factor α for solutes 1,3–5 (Fig. 2) in MPA systems: (A) mobile phase No. 6 (Table 1); (B) EA–EG (96:4).

4. Conclusions

Changes in retention characteristics, column efficiency and peak symmetry are caused by a transition from adsorption to partition mechanism of sorption. A stationary liquid phase is generated dynamically, and if the phase ratio exceeds 0.1 partition dominates over adsorption. The volume of the dynamically generated stationary phase is higher on the silicas with larger surface areas and pore volume. HEX–IPA–EG MPA systems show good column efficiency and peak symmetry for some purine and pyrimidine derivatives and are applicable in analytical practice. The substitution of EA by HEX and IPA in the MPA system results in a specific selectivity and elution strength. Such mobile phases

have additional advantages of improved ultraviolet (UV) transparency.

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