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Short communication

Determination of uracil in 5-fluorouracil substance by high-performance liquid chromatography

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Abstract

Separation of 5-fluorouracil and uracil in chromatographic systems consisting of a silica column as a stationary phase and ethyl acetate or ethyl acetate partially saturated with water as a mobile phase has been studied. The results indicated that when saturation of ethyl acetate with water was chosen to reach more than 60%, such mobile phases [e.g., 2.4% (v/v) water in ethyl acetate] are useful in determining uracil in 5-fluorouracil substances.

Keywords: Mobile phase composition; Uracil; Fluorouracil; Pyrimidines; Butylbenzene

1. Introduction

5-Fluorouracil (FU) is applied in clinical practice to the treatment of various cancer forms. It is important to obtain FU substance with a high purity. Reversed-phase liquid chromatography (RPLC) on octadecyl silica columns has become a popular method for the separation of pyrimidine derivatives [1,2]. However, RPLC was not the best choice for the given analytical task. Separation of FU and uracil (Ura), a probable FU substance impurity, was difficult with octadecyl silica columns (separation factor, α, <1.2) [3,4]. For better separation of an FU/Ura pair under RPLC conditions the column, the packing of which was prepared by copolymerization of heptadecafluorodecyl acrylate with divinyl monomers as a cross-linking agent [5], or the columns packed with an Ag(I)-loaded thiol stationary phase [6] or with poly(styrene–divinylbenzene) [7] were used.

A good selectivity for an FU/Ura pair was observed under adsorption normal-phase liquid chromatography (NPLC) conditions but, at the same time, the peak shapes of the solutes under study, especially for Ura, were unsatisfactory [8].

It is known that the use of organic eluents partially saturated with water is a good choice for reducing peak tailing on silica [9–11]. Here this technique is used to separate uracil and 5-fluorouracil.

2. Experimental

2.1. RP mode

Chromatographic measurements were performed

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on a Varian ProStar high-performance liquid chromatography (HPLC) system equipped with a photodiode array detector ProStar 330 (λ=254 nm). A Zorbax SB-C_{18} column was used as a stationary phase at a temperature of 40 °C. Water was studied as a mobile phase with a flow-rate 1.0 ml/min. The samples (injection volume 50 μl, sample concentration in mobile phase 0.5 mg/ml) were injected via the ProStar Model 410 autosampler.

2.2. NP mode

Chromatographic measurements were performed on a Gilson Model 302 HPLC system, equipped with a Gilson 115 variable-wavelength detector (λ=254 nm). The samples (injection volume 5–50 μl, sample concentration in mobile phase 0.01–0.5 mg/ml) were injected via a Rheodyne 7125 sampling valve.

Ethyl acetate (dry EA), mixtures of dry EA with water-saturated EA, and 2.4% (v/v) water in EA were studied as mobile phases with a flow-rate 1.5 ml/min. Dry EA (of analytical grade in addition purified by distillation) first was divided into two volumes. One volume was saturated with water, to give 100% saturated solvent, vigorously shaken for 4 h EA and water (50:5) for complete equilibration. Saturated EA separated from the residual water. For the final mobile phases (EA with different water saturation percentages) a certain volume of saturated EA with another volume of dry EA were combined.

2.3. 2.4% (v/v) Water in dry EA preparation

EA (e.g., 203 ml) was added to a vessel equipped with a magnetic stirrer and gently stirred. Water (e.g., 5 ml) was added to EA and the mixture stirred some more to ensure a complete homogenization.

A Zorbax-SIL column was used as a stationary phase at ambient temperature. The column was conditioned before each series of retention measurements. Conditioning included flushing with 150 ml of dry EA followed by the mobile phase under study. Usually 40 ml of the latter eluent (partially water-saturated EA) was sufficient to obtain constant retention values of Ura.

The peak asymmetry (A_s) was calculated by determining the A/B ratio[1] at 10% of peak height [12]. The capacity factors of the solutes under study (k'), theoretical plate number (N) and separation factor (α) were calculated according to the usual expressions [13]. The stationary liquid phase volume (V_s) and phase ratio (ϕ) were calculated in accordance with Ref. [14].

3. Results and discussion

Chromatograms of 5-fluorouracil substance tested under RPLC and NPLC conditions are presented in Fig. 1. It can be seen that separation of an FU/Ura pair under reversed-phase conditions is not satisfactory. A good selectivity, but low efficiency was observed under common NPLC conditions (silica as a stationary phase and ethyl acetate as a mobile phase).

Separation of FU and Ura in a chromatographic system consisting of a Zorbax-SIL column as a stationary phase and ethyl acetate partially saturated with water as a mobile phase has been studied. When ethyl acetate saturation with water is more than 30% the asymmetry factor, A_s, for Ura is less than 1.5. Nevertheless, substantial improvement of the peak shape is observed when saturation reaches 60% (A_s<1.2). The decrease in the separation factor, α, from 3.8 (dry ethyl acetate as a mobile phase) to 2.5 (100% water saturation of ethyl acetate) was observed. If water saturation of ethyl acetate is 60–80%, selectivity changes slowly (α from 2.9 to 2.7).

It is postulated that under conditions of typical adsorption mode (silica as a stationary phase and dry ethyl acetate as a mobile phase) *tert*.-butylbenzene is not adsorbed. Its retention volume corresponds to the mobile phase (V_m) or total volume (V_t) within the column (apart from the silica) and it can be assumed that in the adsorption mode V_t equals V_m. The

[1] The asymmetry factor is calculated from the chromatographic peak by dropping a perpendicular at the peak apex and a horizontal line at 10% of the peak height; at the interaction, the distance to the tail of the peak along the horizontal line (distance A) divided by the distance along the horizontal line to the front of the peak (distance B) produces a ratio called the peak asymmetry factor.

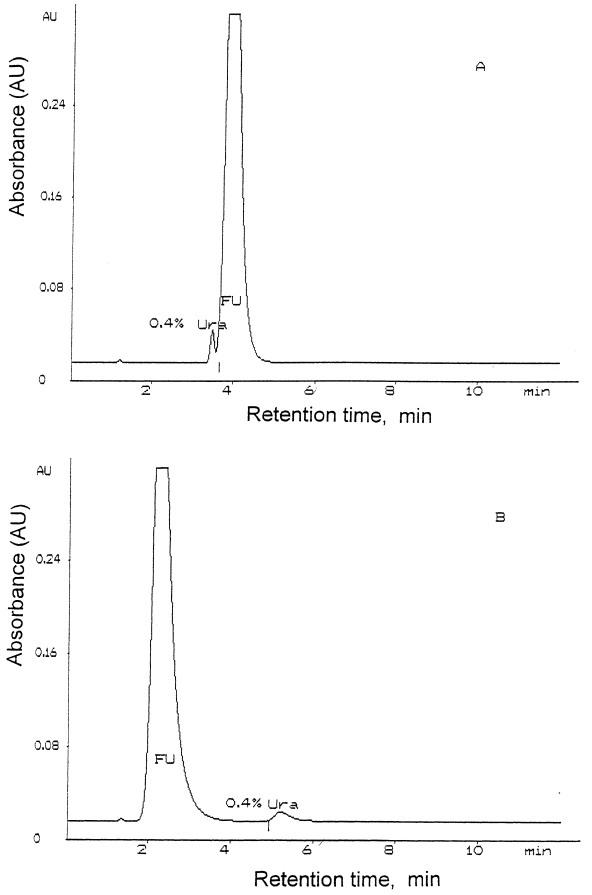

Fig. 1. Chromatograms of a test 5-fluorouracil (FU) substance under RPLC (A) and NPLC (B) conditions. Mobile phases: water (A), ethyl acetate (B); columns and other chromatographic conditions see Experimental; 0.32 AUFS. Injection volume, 50 μl; sample concentration in mobile phase, 0.5 mg/ml. Uracil (Ura) was identified by co-elution with standard.

formation of a dynamically generated liquid stationary phase in the mixed mode (water-saturated ethyl acetate as a mobile phase) leads to a decrease in *tert*.-butylbenzene retention volume (V_m), and this allows one to calculate the volume of the liquid stationary phase (V_s) according to the formula:

$$V_s = V_t - V_m$$

where V_t is the retention volume of *tert*.-butylbenzene in the adsorption mode (dry ethyl acetate as a mobile phase); and V_m is the retention volume of *tert*.-butylbenzene in the mixed mode (water-saturated ethyl acetate as a mobile phase).

Some amount of the liquid stationary phase is generated in all cases when a water–ethyl acetate mobile phase is used. If 100% saturation is achieved, the volume of the liquid stationary phase increased to 0.105 ml. The liquid stationary phase is mechanically held as a bulk film onto a solid support, and the retention mechanism in such a system is partition [15]. It can be assumed that when partially water-saturated compositions are applied, the stationary liquid phase is generated dynamically in the pores of the silica and the mechanism of sorption is mixed, involving adsorption on the silica surface and partition. The contribution of each process depends on the volume of the deposited liquid phase. At higher water contents (60–80% water saturation), the amount of the liquid stationary phase ranges from 0.04 to 0.06 ml, and it can be assumed that in such chromatographic systems partition dominates over adsorption.

Fig. 2 represents a chromatogram of FU substance tested under mixed partition–adsorption conditions with 2.4% (v/v) water in ethyl acetate as a mobile phase. The volume of the liquid stationary phase, V_s, in such a system is 0.05 ml. Good selectivity (α, 2.77) and peak shape (Ura A_s, 1.1) are observed

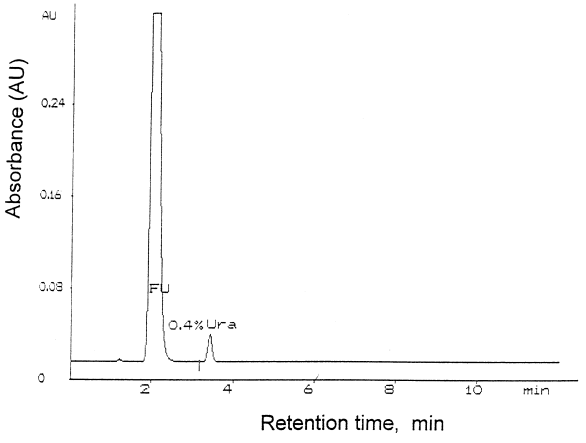

Fig. 2. Chromatogram of a test 5-fluorouracil (FU) substance under mixed partition–adsorption conditions. Mobile phase: 2.4% (v/v) water in ethyl acetate; column Zorbax-SIL; other chromatographic conditions see Fig. 1.

Table 1
Specification of silica columns

Column			Packing material			
No.	Trade name	Dimensions (mm)	Particle size (μm)	Pore size (Å)	Surface area (m^2/g)	Particle shape
1	LiChrospher Si-60	125×4.0	5	60	700	Spherical
2	Silasorb 600	250×4.0	6	60	600	Spherical
3	Kromasil 60-5 Si	150×4.6	5	60	550	Spherical
4	LiChrosorb Si-60	250×4.0	5	60	500	Irregular
5	Zorbax-SIL	150×4.6 (a–c) 250×4.6 (d)	5–6	70	350	Spherical
6	μPorasil	300×3.9	10	125	300	Irregular
7	Zorbax Rx-SIL	150×4.6	5	80	180	Spherical
8	Supelcosil LC-SI	250×4.6	5	100	170	Spherical
9	Nova-Pak silica	150×3.9	4	60	120	Spherical

when 2.4% (v/v) water in EA solution is used as a mobile phase.

The silica columns represented in Table 1 were tested as stationary phases to compare retention and peak symmetry of uracil as well as selectivity for a Ura/FU pair in the adsorption (dry ethyl acetate as a mobile phase) and mixed partition–adsorption modes [2.4% (v/v) of water in ethyl acetate]. Table 2 shows the advantage of water-saturated ethyl acetate versus dry ethyl acetate.

As can be seen from Tables 1 and 2, the retention of Ura depends on the packing material. Usually it is stronger on the silicas with larger phase ratios ($\phi = V_s/V_m$). Also, the surface activity is important, which is obviously seen from the comparison of the data on a packing material Zorbax-SIL (Nos. 5a–d). These columns are from various batches and have a different history of use (were applied in the laboratory for routine analysis for at least 1 year with traditional organic mobile phases, including acids and bases). Nevertheless, the selectivity obtained in the chromatographic system with 2.4% (v/v) water in ethyl acetate does not differ much and, therefore, such a system is applicable in analytical practice.

Table 2
Partially water-saturated ethyl acetate versus dry ethyl acetate as a mobile phase in HPLC separation of 5-fluorouracil (FU) and uracil (Ura) on silica

Column No.*	Mobile phase								
	Dry ethyl acetate				2.4% (v/v) Water in ethyl acetate				
	Retention, k'_{Ura}	Peak asymmetry, $A_{s(Ura)}$	Theoretical plates, $N_{(Ura)}$	Separation factor, $\alpha_{(Ura/FU)}$	Phase ratio, ϕ	Retention, k'_{Ura}	Peak asymmetry, $A_{s(Ura)}$	Theoretical plates, $N_{(Ura)}$	Separation coefficient, $\alpha_{(Ura/FU)}$
1	16.17	2.0	1000	4.74	0.12	5.12	1.10	5500	2.80
2	6.00	2.5	700	4.55	0.05	2.34	1.10	6400	2.78
3	4.89	2.0	2000	4.08	0.07	2.50	1.10	6400	2.78
4	8.45	2.0	850	3.00	0.08	3.00	1.15	4400	2.66
5a	2.86	2.0	1510	3.81	0.03	1.65	1.10	6500	2.77
5b	1.63	2.0	1200	3.33	0.02	0.90	1.10	7000	2.67
5c	6.00	2.5	600	4.30	0.05	2.38	1.10	6000	2.77
5d	4.25	2.0	1000	4.00	0.05	2.10	1.10	8000	2.79
6	3.84	2.5	500	2.00	0.03	1.25	1.20	3900	2.77
7	3.18	1.6	2000	3.61	0.03	1.76	1.10	7600	2.76
8	1.92	2.0	1800	1.68	0.02	0.71	1.10	7000	2.71
9	2.41	2.0	1500	4.09	0.02	1.19	1.15	4300	2.76

* See Table 1.

4. Conclusions

Although RPLC has superseded NPLC in many application areas, untypical NPLC systems consisting of a silica column and a partially water-saturated ethyl acetate as a mobile phase can play a useful role in the separation of 5-fluorouracil and uracil, and are applicable in analytical practice.

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