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Isocratic, simultaneous normal-phase LC separation of four impurities for a stavudine synthesis control

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Abstract

The isocratic separation of impurities for a stavudine synthesis control has been studied by liquid chromatography (LC). The investigations have shown that isocratic reversed-phase LC conditions were unable to achieve a good separation. It was established that isocratic normal-phase LC system with common silica column as a stationary phase and the mixture of ethyl acetate with 4% (v/v) of ethylene glycol as a mobile phase is useful in simultaneous determination of four impurities for a stavudine synthesis control.

Keywords: Stavudine; Liquid chromatography; Silica; Mobile-phase composition

1. Introduction

1-(2′,3′-Dideoxy-β-D-glycero-pent-2′-enofuranosyl)thymine or stavudine is a highly potent and selective anti-HIV agent. Among the several methods offered for the preparation of stavudine, one of the most widely used is the procedure developed by Mansuri et al. [1]. In this case, stavudine is synthesized from 2′-deoxythymidine via its 3′,5′-bis(methylsulfonyl)- and 3′,5′-anhydroderivatives. However, the successful application of this synthetic pathway required an efficient analytical method for the end-product quality control, i.e. for the detection of possible intermediates and by-products of the process in stavudine. Reversed-phase liquid chromatography (RPLC) on octadecyl silica columns has become a popular method for the separation of pyrimidine derivatives, including stavudine [1–3]. However, RPLC is not always the best or the only choice for the given analytical task. Normal-phase liquid chromatography (NPLC) can be an alternative approach to optimize LC separation [4]. A common problem for separations by NPLC is that polar compounds often show broad tailing peaks, especially for basic analytes. Peak asymmetry causes a reduction in column efficiency as well as a decrease in resolution and detection limits.

Good and stable separation conditions for some pyrimidines could be obtained by using chromatographic systems with silica as a stationary phase and a mixture of two or three solvents with a limited mutual solubility as a mobile phase [5]. Such a chromatographic system was called the mixed partition-adsorption (MPA) normal-phase mode. MPA systems allow one to separate pyrimidine derivatives on silica with a good selectivity, high column efficiency and peak symmetry [5]. The goal of this work was to find optimal conditions for isocratic, simultaneous LC separation of four impurities {thymine (1), 2′-deoxythymidine (2), 1-(3′,5′-anhydro-2′-deoxy-β-D-threo-pentofuranosyl)thymine (3) and 1-(2′-deoxy-3′,5-bis(methylsulfonyl)-β-D-erythro-pentofuranosyl)thymine (4)} for a stavudine (ST) synthesis control.

2. Experimental

2.1. Chemicals and apparatus

Thymine, 2′-deoxythymidine and all organic solvents for mobile phases were purchased from Acros Organics

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(New Jersey, USA). Stavudine, 1-(3′,5′-anhydro-2′-deoxy-β-D-threo-pentofuranosyl)thymine and 1-(2′-deoxy-3′,5′-bis-(methylsulfonyl)-β-D-erythropento-furanosyl)thymine were synthesized according to the literature procedure [1].

RPLC-mode: chromatographic measurements were performed on a Varian ProStar LC system equipped with a photodiode array detector ProStar 330 (λ = 265 nm). A Zorbax SB-C18 column was used as a stationary phase at a temperature of 40 °C. Mixtures of 0.1% (v/v) phosphoric acid with 15% (v/v) of acetonitrile or 20% (v/v) of methanol were studied as mobile phases with a flow-rate 1.5 ml/min. The samples (injection volume 10 μl, sample concentration in mobile phase 0.05 mg/ml) were injected via the ProStar 410 autosampler.

NPLC-mode: chromatographic measurements were performed on a Gilson Model 302 LC system, equipped with a Gilson 115 variable-wavelength detector (λ = 265 nm). A Zorbax Rx-SlL column was used as a stationary phase at ambient temperature. Ethyl acetate or binary mixtures {30% (v/v) of isopropanol in hexane; 4% (v/v) of ethylene glycol in ethyl acetate} were studied as mobile phases with a flow-rate 1.5 ml/min. The column was conditioned before each series of retention measurements. Conditioning included flushing with 50 ml of isopropanol-hexane (30:70) followed by the mobile phase under study. The samples (injection volume 10 μl, sample concentration in mobile phase 0.05 mg/ml) were injected via a Rheodyne 7125 sampling valve.

2.2. Data calculation

The retention factor of the solutes under study (k), separation factor (α) and theoretical plate number (N) were calculated according to the usual expressions [6].

The asymmetry factor (As = A/B) was calculated from the chromatographic peak by dropping a perpendicular at the peak apex and a horizontal line at 10% of the peak height. The distance to the tail of the peak along the horizontal line (distance A) was divided by the distance along the horizontal line to the front of the peak (distance B) [7].

The phase ratio (ϕ) of the column was calculated according to the equation [8]:

$$\phi = \frac{V_s}{V_m}$$

It is postulated that under conditions of typical adsorption mode (silica as a stationary phase and 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 formation of dynamically generated liquid stationary phase in the mixed mode (ethyl acetate partially saturated with ethylene glycol 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 equation:

$$V_s = V_t - V_m$$

where V_t is the retention volume of *tert*-butylbenzene in the adsorption mode (ethyl acetate as a mobile phase); and V_m is the retention volume of *tert*-butylbenzene in the mixed mode {4% (v/v) of ethylene glycol in ethyl acetate as a mobile phase}.

3. Results

Chromatograms of the test solutes (Fig. 1) studied under RPLC conditions are presented in Fig. 2. It can be seen that isocratic reversed-phase mode was not the best choice for a stavudine (ST) synthesis control. Separation factor, α was too small for the 1/2 pair (Fig. 2A, acetonitrile as an organic mobile phase modifier) or for the 3/ST pair (Fig. 2B, methanol as an organic mobile phase modifier). Moreover, separation factor, α was too large for the 4/3 pair, and a gradient elution was preferable in this case.

Chromatograms of a test mixture under NPLC conditions are presented in Fig. 3. A Zorbax Rx-SIL column, which is known as less adsorptive and highly purified type B silica [9], was used as a stationary phase. Low efficiency for stavudine (theoretical plate number, N, less then 3500)

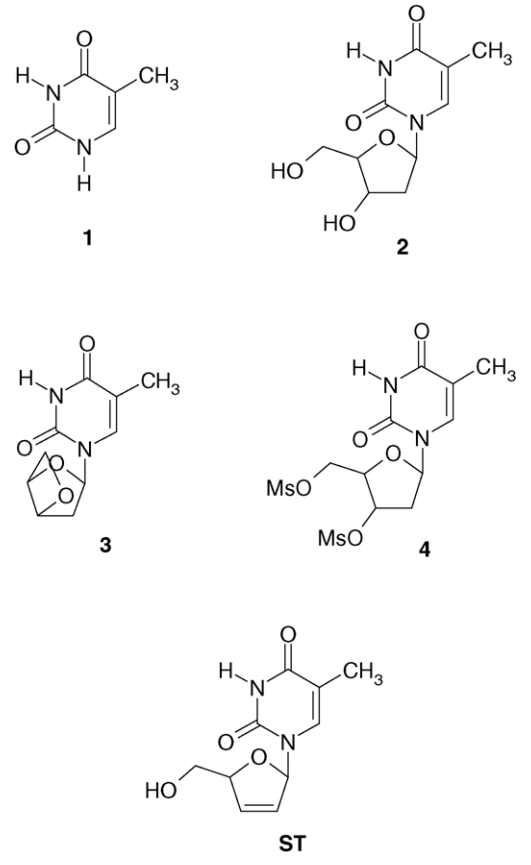

Fig. 1. Molecular structures of the solutes under study.

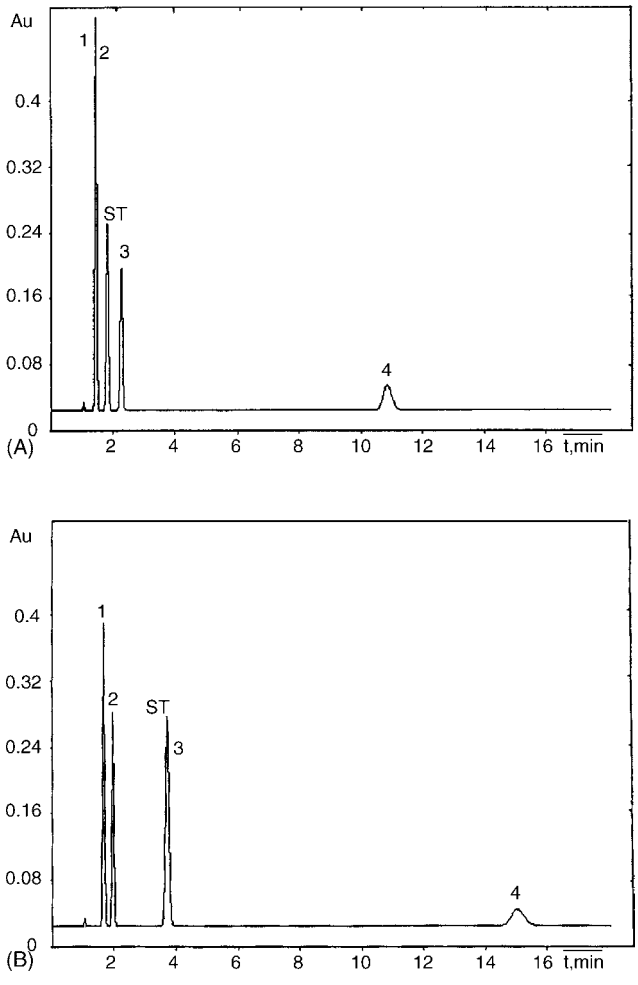

Fig. 2. Chromatograms of a test mixture (Fig. 1) under RPLC conditions. Mobile phases: 15% of acetonitrile in 0.1% phosphoric acid (A), 20% of methanol in 0.1% phosphoric acid (B); column and other chromatographic conditions see Section 2.

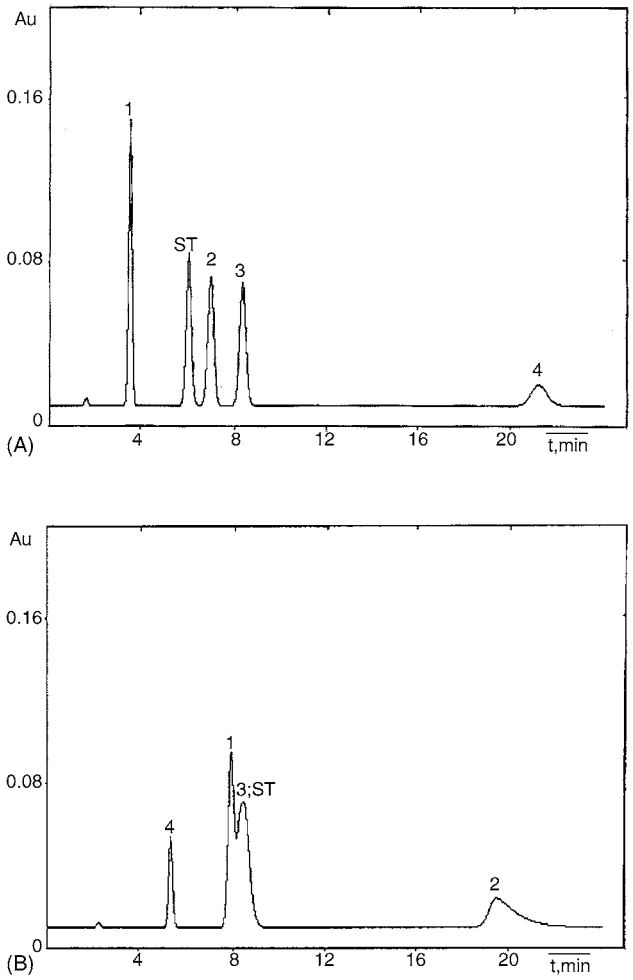

Fig. 3. Chromatograms of a test mixture (Fig. 1) under NPLC conditions. Mobile phases: 30% (v/v) of isopropanol in hexane (A), ethyl acetate (B); column and other chromatographic conditions see Section 2.

is observed under traditional NPLC conditions. It is seen (Fig. 3A) that if the isocratic NPLC system with a mixture of isopropanol–hexane as a mobile phase was used the separation is not good (large separation factor, α for the 4/3 pair). A substitution of the mobile phase isopropanol–hexane by ethyl acetate (Fig. 3B) leads to the considerable selectivity alterations (the inversion of the elution order for solutes 2 and 4). However, separation factor, α for the 1/3 pair is too small, whereas for the 3/ST pair no separation is observed, and a peak shape of 2′-deoxythymidine (solute 2) is unsatisfactory (asymmetry factor, As >1.5).

As one can see from Fig. 4, the use of 4% (v/v) of ethylene glycol in ethyl acetate as a mobile phase can improve the separation of a test mixture on a Zorbax Rx-SIL column. Such MPA system shows good peak symmetry and improves the column efficiency {N (for stavudine) 4500 theoretical plates}.

The main difference of the mobile phase under study (Fig. 4) from the traditionally used in NPLC mobile phases

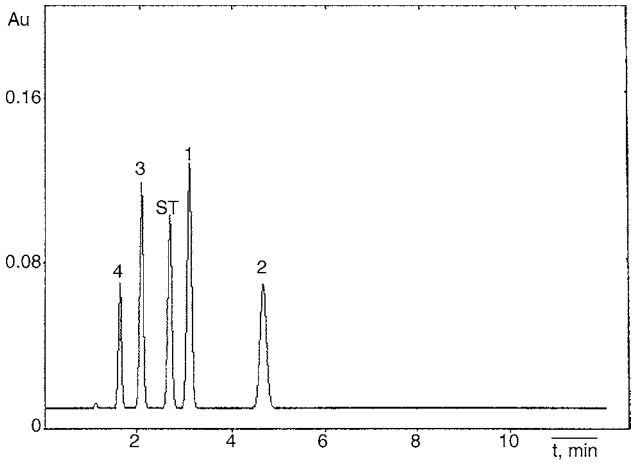

Fig. 4. Chromatogram of a test mixture (Fig. 1) under mixed partition-adsorption (MPA) normal-phase conditions. Mobile phase: 4% (v/v) of ethylene glycol in ethyl acetate; column and other chromatographic conditions see Section 2.

Table 1
Specification of silica columns

No.	Column		Packing material			
	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	Kromasil 60-5 Si	150 × 4.5	5	60	550	Spherical
3	Silasorb 600	250 × 4.0	6	60	600	Spherical
4	LiChrosorb Si-60	250 × 4.0	5	60	500	Irregular
5	Zorbax SIL	150 × 4.6	5–6	70	350	Spherical
6	Zorbax Rx-SIL	150 × 4.6	5	80	180	Spherical
7	Supelcosil LC-SI	250 × 4.6	5	100	170	Spherical

Table 2
Separation of a test mixture (Fig. 1) on silica columns (Table 1) as a stationary phase and 4% (v/v) of ethylene glycol in ethyl acetate as a mobile phase

No.	Column			2-Deoxythymidine (2)		Separatio factor (α)			
	V_t (ml)	V_m (ml)	Phase ratio	Retention factor ($k2$)	Asymmetry factor (As)	$k2/k1$	kST/$k1$	$k3$/kST	$k3/k4$
1	1.057	0.911	0.16	10.9	1.05	2.00	1.30	1.90	2.10
2	1.845	1.692	0.09	6.1	1.05	1.86	1.26	1.70	1.88
3	2.453	2.271	0.08	5.3	1.10	1.92	1.30	1.73	1.88
4	2.303	2.132	0.08	5.2	1.05	1.83	1.29	1.73	1.82
5	1.773	1.688	0.05	3.5	1.05	1.80	1.28	1.63	2.02
6	1.721	1.655	0.04	3.2	1.05	1.79	1.26	1.63	1.90
7	3.131	3.039	0.03	2.2	1.05	1.78	1.25	1.62	1.80

(Fig. 3) is a limited mutual solubility of the mobile phase components (ethylene glycol and ethyl acetate). It can be assumed that when partially ethylene glycol–saturated ethyl acetate is applied, the stationary liquid phase is generated dynamically in the pores of 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. Some amount of the liquid stationary phase is generated in all cases when ethylene glycol–ethyl acetate mobile phase is used but more than 40% saturation is desirable for a good peak symmetry of pyrimidines [5]. The composition of the mobile phase represented in Fig. 4 was chosen to reach approximately 60% saturation of ethyl acetate with the polar component {up to 6.5% (v/v) of ethylene glycol can be dissolved in ethyl acetate at room temperature [5]}.

The silica columns represented in Table 1 were tested as stationary phases under mixed partition-adsorption mode {4% (v/v) of ethylene glycol in ethyl acetate as a mobile phase} for the separation of test solutes (Fig. 1).

According to the data in Tables 1 and 2, the column phase ratio, ϕ, depends on the silica type. On the various silica gels investigated in this study, phase ratios between 0.03 and 0.16 were obtained. It can be assumed that the volume of the dynamically generated liquid stationary phase is larger on silicas with a larger surface area. The retention also depends on the packing material (retention factors, $k2$, between 2.2 and 10.9). The results (Tables 1 and 2) show that retention is stronger on the silicas with larger surface area. It can be concluded that if the phase ratio is larger, the retention is stronger. Nevertheless, it has been observed (Table 2) that the reproducibility of the selectivity is independent on the silica type (separation factors, α obtained in the MPA mode do not differ much) and, therefore, such a system is applicable in analytical practice.

4. Conclusion

The isocratic LC separation of stavudine and its four impurities was achieved with a silica column (e.g., Zorbax RX-SIL) as a stationary phase and a partially ethylene glycol-saturated ethyl acetate {4% (v/v) of ethylene glycol in ethyl acetate} as a mobile phase. A fast isocratic normal-phase LC method for simultaneous determination of four impurities can play a useful role in a stavudine synthesis control. Financial support from the Latvian Council of Science (grants Nos. 01.0177 and 01.0183) and from the Organisation for the Prohibition of Chemical Weapons (grants L/ICA/ICB/75632/03) is gratefully acknowledged.

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