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Short communication

Determination of fludarabine in a pharmaceutical formulation by LC

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1. Introduction

Chronic lymphocitic leukemia (CLL) is a malignant disease characterized by a monoclonal proliferation of mature-appearing lymphocytes [1]. It has an overall incidence of 2.4 per 100 000 inhabitants and its clinical course is highly variable, although the identification of prognostic determinants has led to the definition of disease stages and to the development of prognostic systems [2–5].

High response rates have most recently been reported for the fluorinated adenine analogue fludarabine phosphate (Fig. 1), which proved to be highly active in patients with advanced CLL even after failure of conventional regimens. Fludarabine (9-β-D-arabynofuranosyl-2-fluoroadenine monophosphate) is relatively resistant to deamination by adenosine deaminase; it is remarkably well tolerated [6,7].

Because of its relative insolubility, F-ara-A, is administered as its monophosphate form (Fludara®). It was used for many experimental investigations and all clinical trials.

For estimation of fludarabine in biological fluids, several LC methods have been used [8–10]. One of these [9] determined fludarabine concentration in plasma by HPLC with fluorescence detection. Another work [10] reported the determination of fludarabine in plasma from rabbits by HPLC, TLC and NMR. For the pharmacological analysis of fludarabine phosphate in swine an HPLC method with UV detection was used [8]. In all methods internal standards were not used.

This paper describes a validated, rapid and reproducible reversed phase high pressure liquid chromatographic (RP-LC) method for the deter-

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mination of fludarabine phosphate in a pharmaceutical formulation. The method uses a C_{18} reversed phase column, with detection in the UV region and an internal standard technique. The procedure proposed is simple and thus may be of advantage when the main task is to investigate the fludarabine stability and pharmacokinetics in order to provide the most reliable management of patients undergoing anticancer therapy.

2. Experimental

2.1. Apparatus

The HPLC apparatus was a Perkin-Elmer chromatographic system (Series 410 Liquid Chromatograph) equipped with a septumless injector (Rheodyne 7125-075) and a column heater (Perkin-Elmer TC 931). A variable wavelength diode array detector (Perkin-Elmer LC 235) was used. Peak area integrations were performed using a chromatographic data system (Perkin-Elmer LCI 100 Laboratory Computing Integrator). A Vydac reversed-phase C_{18} column (25 cm × 4.6 mm i.d., particle size 10 μm), thermostated at 25°C, was used as stationary phase.

2.2. Reagents

Lichrosolv® methanol was purchased from Merck (Darmstadt, Germany). Water used in the mobile phase was deionized, distilled and filtered through a 0.22-μm Millipore filter (Bedford, USA) before use. Sodium phosphate bibasic (Rudipont, Milan, Italy) and citric acid (Carlo Erba, Milan, Italy) were all analytical grade.

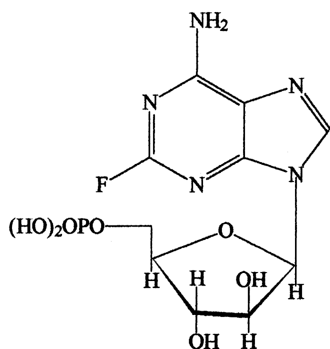

Fig. 1. Fludarabine phosphate.

The dosage of Fludarabine phosphate in commercial formulation was carried out with Fludara® vials (Schering, Milan, Italy). The composition of one vial was: 50 mg fludarabine phosphate, 50 mg mannitole and 10 mg sodium hydroxide.

Fludarabine standard was supplied by Sigma-Aldrich (Milan, Italy).

2.3. Standard preparation

Two stock solutions were prepared by weighing 7 mg of fludarabine phosphate and 20 mg of internal standard fludarabine into two 20-ml volumetric flasks. The substances were dissolved and diluted to volume with methanol.

2.4. Sample preparation

The content of a vial was weighed (110 mg) and a portion (11 mg), equal to 5 mg fludarabine phosphate, 5 mg mannitol and 1 mg sodium hydroxide, was withdrawn and dissolved in 10 ml of methanol. From the methanolic solution, 7 ml were withdrawn and diluted to the mark with methanol in a 10-ml volumetric flask (conc. 0.35 mg/ml). The solution obtained was repeatedly analyzed by HPLC in order to dose the active principle.

2.5. Chromatographic conditions

The mobile phase was a mixture (pH 4.1) of methanol and phosphate buffer 0.1 M (20/80, v/v). The buffer was prepared by mixing an aqueous solution of sodium phosphate bibasic (71.6 g/100 ml) (A) and citric acid (21 g/100 ml) (B). According to F.U. IX ed., the withdrawn volumes were 40 ml of solution (A) and 60 ml of solution (B). The flow rate was 0.8 ml/min. The UV detector wavelength was set at 265 nm and an attenuation of 0.05 a.u.f.s. was used.

Table 1
Calibration curve

Concentration of fludarabine phosphate (mg/ml)	$A/A_{I.S.}$	Slope	R.S.D. slope	Intercept	R.S.D. intercept	r^2
0.0350	0.1267 ± 0.0097	3.52998 ± 0.0113365	0.0021010	0.005668 ± 0.0664624	0.0104420	0.999973
0.1050	0.3785 ± 0.0259					
0.1750	0.6229 ± 0.0302					
0.1925	0.6874 ± 0.0210					
0.3500	1.2398 ± 0.0116					

2.6. Calibration curve

Aliquots of the standard stock solution of fludarabine phosphate were pipetted into five different 10-ml volumetric flasks. The internal standard stock solution (1 ml) was put into each flask and the solutions were diluted to the mark with methanol. The final concentrations of fludarabine phosphate were respectively 0.0350, 0.1050, 0.1750, 0.1925, 0.3500 mg/ml. Five determinations were carried out for each solution. Peak areas were recorded for all the solutions. The correlation graph was constructed by plotting the peak areas obtained at the optimum wavelength of detection versus the injected amounts.

Linearity of the standard was determined by chromatographing five standard solutions in a range of 0.035–0.35 mg/ml. Linear regression analysis of the peak area response versus the concentration gave a linear response (Table 1).

The system precision was determined by chromatographing six injections of the standard solution and calculating the relative standard deviation (R.S.D.) of the peak area responses with the proposed chromatographic method. The R.S.D.% for standard was 0.5163.

The chromatographic procedure was applied to determine fludarabine phosphate content in a pharmaceutical formulation. The assay values are presented in Table 2.

3. Results and discussion

Methanol and phosphate buffer was chosen as the best mobile phase for the separation, since by using other solvents, as the organic modifiers, it was not possible to achieve a resolution. The symmetry of the peaks was satisfactory and with the separation obtained the peaks were just resolved down to baseline and areas could be measured accurately. Capacity factors were reproducible under the experimental conditions used, since the coefficient of variation (C.V.) ranges from 1.0 to 1.4 for within-day and from 2.2 to 3.1% for between-day studies. The proposed chromatographic method was assessed for linearity, precision, ruggedness, robustness and stability.

Table 2
Fludarabine analysis in pharmaceutical dosage form

Sample	Found (mg)	Recovery (%)
1	0.3450	98.57
2	0.3485	99.57
3	0.3494	99.82
4	0.3503	100.08
5	0.3493	99.80
6	0.3499	99.97
7	0.3495	99.85
8	0.3494	99.82
9	0.3508	100.22
10	0.3504	100.11
11	0.3496	99.88
Mean	0.3492	
Mean recovery (%)		99.79
R.S.D. (%)		0.4429

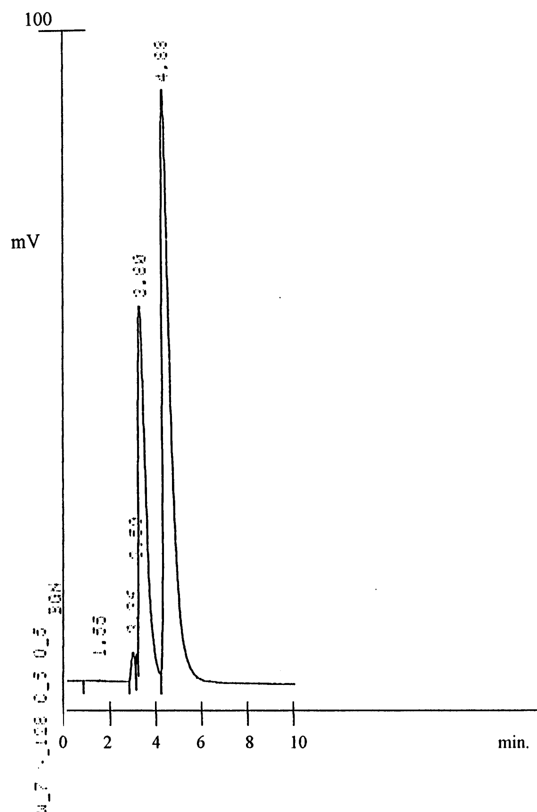

Fig. 2. Chromatogram of a solution containing fludarabine phosphate ($t_r = 3.80$) and fludarabine ($t_r = 4.88$) at concentrations of, respectively, 0.175 and 0.04 mg/ml, on a Vydac C_{18}. The mobile phase was a mixture of methanol and phosphate buffer solution 0.1 M (20/80, v/v). Detection was at 265 nm.

In order to establish the validity of the proposed HPLC method, three samples from different vials were assayed in triplicate.

A robustness test was carried out to investigate which experimental parameters might influence the quantitative results. The variables taken into account were column temperature, mobile phase pH and percentage of organic modifier. The proposed method was found to be robust with regard to the column temperature. Retention time showed no significant change by varying the temperature from 25 to 65°C. When changing the pH values from 4.1 to 7.0 and the organic modifier percentage in the mobile phase, only a slight shift in the retention times was obtained.

The proposed HPLC method was used by analysts employing two different instruments to analyze the same sample. The results showed no statistical differences between operators and instruments.

The stability of the sample solution at 20°C, 24 h and 1 month after preparation, was verified by re-assaying. Any decomposition of fludarabine phosphate was noticed in the sample. Moreover a sample solution was heated gradually up to 65°C for over 24 h and only a degradation equal to 4% was observed.

4. Conclusions

The chromatographic conditions described permitted the separation of fludarabine phosphate and the internal standard in about 5 min. The relative chromatogram is shown in Fig. 2. The identification of chromatographic peaks was carried out by comparison of retention time and subsequent enrichment of the sample.

The proposed HPLC method was found to be reproducible, linear, precise, robust, rugged, and may be useful for pharmacokinetic investigations or routine control of fludarabine in pharmaceutical forms.

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