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Short communication

Development and validation of a HPLC-UV method for the determination in didanosine tablets

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Abstract

A simple, rapid, sensitive and specific reversed-phase high performance liquid chromatographic method involving ultraviolet detection (HPLC-UV) was developed for analysis of didanosine in drug substance and formulated products, tablets. Chromatography was carried out on a pre-packed, Lichrospher 100 Rp-8 (5.0 μm, 250 mm × 4.0 mm) column using 0.01 M sodium acetate solution:methanol (85:15, v/v) adjusted to pH 6.5 with acetic acid as mobile phase at a flow rate of 1.5 ml/min and a 248 nm detection. Hypoxantine was confirmed as the main degradation product. The assay was linear over the concentration range of 50–150 μg/ml ($R \approx 0.999$). The method was validated for accuracy and precision.

Keywords: Didanosine; Validation; HPLC-UV; tablets

1. Introduction

The didanosine, (2′,3′-dideoxyinosine; ddI) is a dideoxy analogue of the purine nucleoside inosine that potently inhibits the replication of the human immunodeficiency virus (HIV) (Fig. 1) [1,2]. Analogue to other nucleoside inhibitors, this compound also requires intracellular metabolism to the active triphosphate, 2′,3′-dideoxyadenosine-5′-triphosphate (ddATP), which acts as a competitive inhibitor of HIV reverse transcriptase or as a DNA chain terminator [1,2].

Although a few methods for the determination of the pharmacokinetic profile of didanosine is already described on literature [3–12], there is no official or analytical method for the direct analysis of didanosine bulk materials and pharmaceutical formulations. Therefore, the aim of this study is to develop a selective and sensitive HPLC-UV method for the determination of didanosine in drug substance and formulated products (tablets) suitable for routine quality control analysis and stability tests.

2. Experimental

2.1. Chemicals and reagents

Didanosine reference standard and hypoxantine were provided by Cristalia Laboratory (Brazil) while 100 mg

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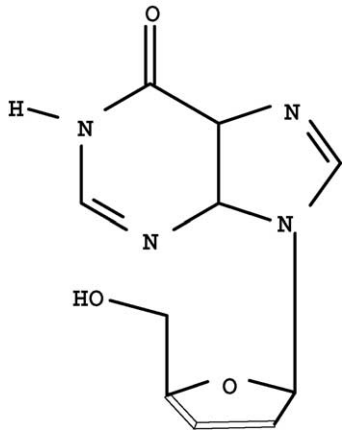

Fig. 1. Didanosine structure.

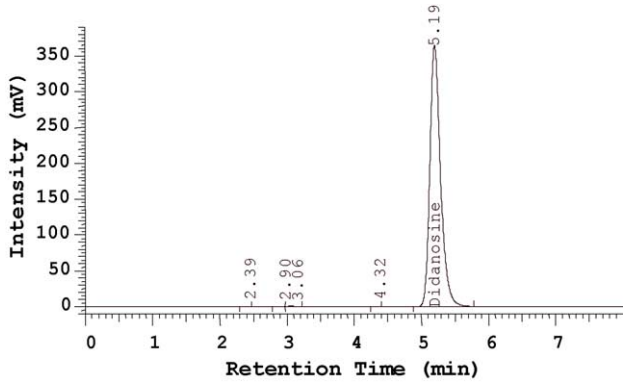

Fig. 2. Elution profile of didanosine.

didanosine tablets with the composition: didanosine 100 mg; excipients (sodium cyclamate; saccharin sodium; magnesium stearate; orange flavor; magnesium hydroxyde, calcium carbonate; explotab) qs 910 mg to tablets were obtained from Vital Brazil Institute. HPLC-grade methanol, acetonitrile and glacial acetic acid were obtained from Fisher Scientific (Springfield, NJ, USA). Deionized water was prepared by Milli-Q system (Millipore, MA, USA). All other chemicals used were of analytical grade unless otherwise indicated.

2.2. Apparatus and chromatographic conditions

The development of the method, and validation work were performed on a Merk-Hitachi HPLC system (Germany) consisted of a L-7000 pump, a Rheodine 7725/i injector and a L-7400 variable-wavelength UV detector. The method/intermediate precision studies were also performed on a Shimadzu LC-10A (Kyoto, Japan). Chromatographic separation was carried out at room temperature with Lichrospher 100 Rp-8 (5.0 μm, 250 mm × 4.0 mm) column from Merck (Germany). For the mobile phase, 0.01 M sodium acetate solution was prepared and then mixed with methanol in ratio of 85:15 (v/v). The pH was adjusted to 6.5 with glacial acetic acid prior to the dilution to 1000 ml. Finally the mobile phase was filtered through a 0.45 μm membrane filter and degassed. The injections volumes for samples and standards were 20 μl and eluted at a flow rate of 1.5 ml/min at room temperature. The eluents were monitored at 248 nm.

2.3. Standard solution

A working standard solution containing 100 μg/ml didanosine was prepared by dissolving didanosine reference standard in mobile phase. The mixture was sonicated for 30 min or until the standard dissolved completely.

2.4. Sample preparation

Twenty tablets, each containing 100 mg didanosine were accurately weighed and finely powdered. A quantity of powder equivalent to 20 mg ddI was weighted and transferred to a 200 ml volumetric flask. Only the didanosine was dissolved in the mobile phase. After that, the preparation followed the same procedure described for the standard solution preparation. Then 20 μl of these solutions was injected in to column and chromatogram was recorded and shown in Fig. 2.

2.5. Method validation

The linearity was tested at a concentration range of 50–150 μg/ml. For the determination of linearity, standard calibration curve was used. These standards were tested six times in agreement to the International Conference on Harmonization [13]. A calibration curve was constructed and the proposed method was evaluated by its correlation coefficient and intercept value, calculated in the corresponding statistic study (ANOVA) ($p < 0.05$). Characteristic parameters for regression equation ($y = a + bx$) of the HPLC method obtained by least squares treatment of the results was used to confirm the good linearity of the method developed. The homoscedasticity for the calibration curve was tested using Cochran's test (G = largest variance/sum of variances) [14].

The accuracy of the assay was measured by analyzing three spiked samples of ddI (75, 100 and 125%). According to the ICH recommendations [13], precision must be considered at two levels, repeatability and intermediate precision. Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment. On that account, a six-sample replicates were consecutively tested in the same equipment at a concentration of 100% (100 μg/ml) of the regular analytical working value. The intermediate precision expresses the within-laboratories variations, was assessed by using different equipments, analysts and days to analyze three samples six times.

3. Results and discussion

3.1. Methods development and optimization

This isocratic-mode method with UV detection was developed for the determination of the active ingredient, didanosine, at the 100% level, and its main degradation product, hypoxantine. Firstly, two reversed-phase columns were tested, a Lichrospher 100 Rp-8 Merck (5.0 μm, 250 mm × 4.0 mm) and Lichrospher 100 Rp-18 Merck (5.0 μm, 250 mm × 4.0 mm). The system suitability studies were carried out as specified in ICH [13,15]. These parameters include column efficiency (N), resolution time (R_t), tailing factor (T) and capacity factor (k'). As shown in Table 1, the number of theoretical plates for C-8 (5175) was higher than 2000, which is the minimum value to consider the method acceptable.

The mobile phase consisted of 0.01 M sodium acetate solution and methanol at various ratios (75:25, 80:20, 85:15 (v/v)) was tested as starting solvent. The variation at the mobile phase leads to considerable changes in the chromatographic parameters (Table 1). However, the proportion acetate:methanol at a ratio of 85/15 (v/v) yielded the best results (Table 1).

Our data showed that the variation of the pH (5.0, 6.0, 6.5) of the mobile phase did not have any significant effects on the HPLC-UV chromatographic resolution (Table 1). Although the retention times (5.38–5.45), capacity factor (9.77–9.89), tailing factor (1.28–1.29) and plate counts (5165–5252) of didanosine showed little change in the pH variation (5.0–6.5), it was necessary to maintain pH value of the mobile phase at 6.5, due the possible degradation of didanosine.

In order to study the effect of excipients on quantification of ddI, a placebo was prepared using sodium cyclamate; saccharin sodium; magnesium stearate; orange flavor; magnesium hydroxyde, calcium carbonate; and explotab qs 910 mg to tablets. The results revealed no interference of the excipients as shown in Fig. 3.

Literature described both 248 and 254 nm as the UV wavelengths suitable for monitoring didanosine [16,17]. In order to obtain a satisfactory and fully detection for this new method, 3D-UV–vis spectra of standard didanosine solution were obtained (not shown). Based on the highest UV absorbance for didanosine, 248 nm was chosen for detection of this new HPLC-UV method.

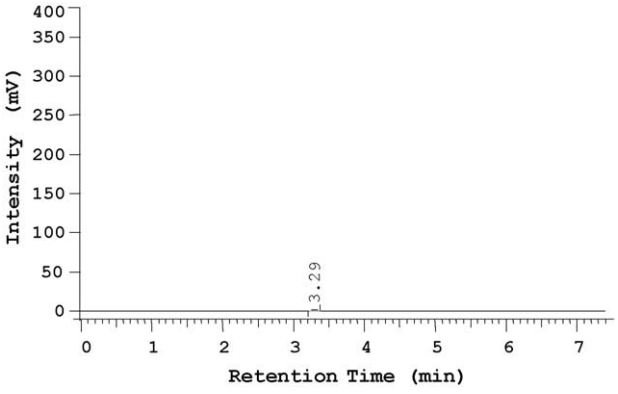

Fig. 3. Excipients elution profile using the standard parameters conditions.

3.2. Method validation

3.2.1. Stress testing

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used [18]. Related species relevant to quality control of didanosine were determined through stress studies involving heat, acid and oxidation conditions [15–18]. Fig. 4A shows the effect of the exposition of a didanosine standard solution to 100 °C during 1 week. The chromatogram revealed that, although using a high temperature, didanosine (R_t = 5.48 min) was not fully degraded and its degradation products were placed separately displaying different retention times (R_t = 2.48, 3.03, 3.18, 4.83 min). Contrastingly, in acidic condition (HCl 0.1 N), didanosine was much more instable and totally degraded to a main

Table 1
System suitability study

Experimental conditions								
Parameter	Columns[a]		Mobile phase (v/v)[b]			pH[c]		
	C8	C18	75:25	80:20	85:15	5.0	6.0	6.5
Rt	5.38	4.80	2.93	3.79	5.32	5.44	5.45	5.38
T	1.63	1.27	1.51	1.39	1.27	1.29	1.28	1.28
k'	8.93	9.76	4.87	6.58	9.65	9.88	9.89	9.77
N	5175	1965	3320	4055	5249	5208	5252	5165

The parameters monitored of the experimental conditions established were peak retention time (R_t), tailing factor (T), capacity factor (k') and theoretical plates (N).

[a] The experiment was performed using mobile phase of 0.01 M sodium acetate solution mixed with methanol at a ratio 85:15, pH 6.5.

[b] Mobile phase is consisted of 0.01 M sodium acetate solution mixed with methanol, pH 6.5, using C8 as the column of choice.

[c] The experiment was performed using mobile phase of 0.01 M sodium acetate solution mixed with methanol at a ratio 85:15 using C8 as the column of choice.

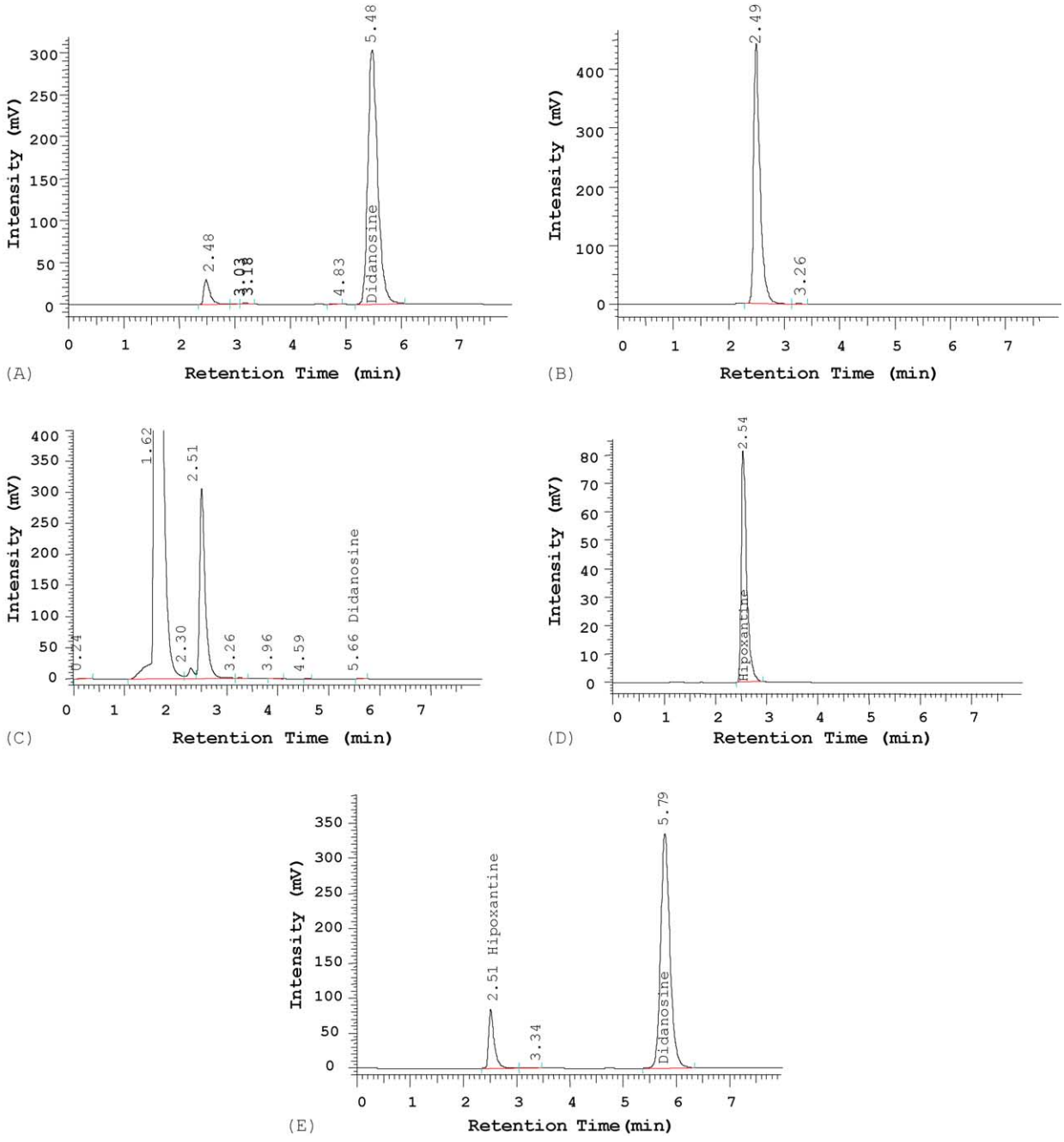

Fig. 4. Elution profile of didanosine obtained after different stress conditions: 100 °C during one week (A), acidic condition (B), oxidation process (C), hypoxantine (D) didanosine and hypoxantine mixture (E).

product, which was eluted from the C-8 column at 2.49 min (Fig. 4B) [17].

The oxidation process of the didanosine sample using a hydrogen peroxide solution 3% (R_t = 1.62 min) did not induce the degradation in a higher extent than that observed for the acidic condition. However, this stress condition formed the highest number of degradation products that neither altered the didanosine retention time nor was superposed in the same area of the antiviral in the chromatogram (Fig. 4C). Anyway, in the three stress conditions tested, a 2.45 ± 0.05 min product was always detected.

Literature describes that hypoxantine is the main product of didanosine degradation. In order to characterize the main degradation product observed on this new method, hypoxantine was analyzed separately (Fig. 4D) and mixed with didanosine (Fig. 4E), using the didanosine experimental parameters already established. The results obtained with hypoxantine (R_t = 2.51 ± 0.05) indicated that the peak observed in our HPLC-UV profiles (R_t = 2.49 ± 0.05) was probably this degradation product (Fig. 4A–D). The identification of the hypoxantine was confirmed by means of DAD detector and by comparing the UV spectra of the

Table 2
Summary data of precision and calibration curve of the ddI HPLC determination

Precision[a]								Calibration curve[a]			
Test		Mean (μg/ml)			RSD (%)			Approximate level (μg/ml)	Mean (μg/ml)	RSD (%)	Variance (μg/ml)
		S1	S2	S3	S1	S2	S3	50	49.96	0.08	0.002
Repeatability		100.48	98.08	97.48	0.50	0.51	0.27	75	75.07	0.08	0.004
								100	100.00	0.14	0.02
Intermediate precision								125	124.26	0.11	0.02
Equipment 1	Analyst 1	100.48	98.08	97.48	0.50	0.51	0.27	150	148.30	0.07	0.012
Equipment 1	Analyst 2	101.14	98.88	98.25	0.45	0.46	0.48	Slope	0.981		
Equipment 2	Analyst 1	99.70	98.60	98.65	0.52	0.51	0.50	*y*-intercept	1.152		
Equipment 2	Analyst 2	100.21	98.70	98.63	0.63	0.53	0.39	Correlation coefficient	0.999		

[a] The experiment was performed using C8 as the column of choice and 0.01 M sodium acetate solution mixed with methanol (85:15) as mobile phase.

degradation product and hypoxantine (not shown). More important this degradation product did not interfere in the detection analysis of the didanosine (Fig. 4E).

The accuracy was evaluated by the recovery of didanosine (100 μg/ml) at three different levels (75, 100, and 125%), using three preparations for each level tested three times. The mean recovery data (mean ± R.S.D.) for each level were within accepted values (100.26 ± 0.86%, 100.79 ± 1.40% and 100.03 ± 1.12% respectively).

Therefore, these results indicated a good accuracy of the method for determination of didanosine. The mean recovery will be 100 ± 2% at each concentration [15].

3.2.2. Linearity

A calibration curve was constructed and the proposed method was evaluated by its correlation coefficient (0.999) and intercept value (1.152) (Table 2).

Characteristic parameters for regression equation ($y = a + bx$) of the HPLC method obtained by least squares treatment of the results confirmed the good linearity of the method developed (Table 2).

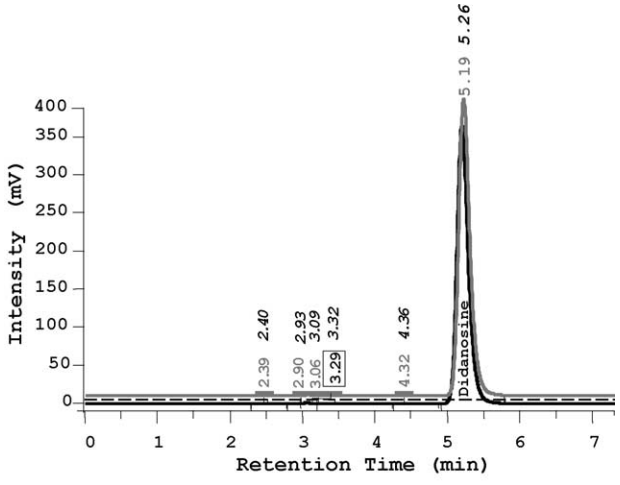

Fig. 5. Elution profile of standard didanosine (gray), didanosine tablets (black) and the tablets excipient (dotted with parameters boxed) using the HPLC-UV method parameters.

The homoscedasticity for the calibration curve was tested and *G*-value obtained in this study (0.344) was smaller than the critical value ($G = 0.684$), indicating that variances were not significantly different.

3.2.3. Solution stability

The stability of didanosine in standard and sample preparation was evaluated by storing the solutions for 24 h, at room temperature, and testing in triplicate at the intervals of 11, 16 and 24 h during the day. The results obtained (mean = 100.14, 99.77, 99.85 and R.S.D. = 0.19, 0.28, 0.23, respectively) revealed that samples retained a potency of 100 ± 1% as compared to a freshly prepared standard over a 24 h period.

3.2.4. Precision

The data of Table 2 showed that average results of repeatability were within 98 ± 2.0 and R.S.D. was 0.4, which indicated a good precision. The obtained results of intermediate precision also indicated a good method precision. The R.S.D. obtained with all the values together was 0.48 [15].

3.2.5. Selectivity of the method

In order to test the selectivity of the present developed method for the analysis of formulated products, Vital Brazil Institute didanosine tablets were analyzed and compared to the standard didanosine and excipients chromatograms (Fig. 5). The parameters results obtained with the didanosine tablets showed that the method was suitable for determination of didanosine in pharmaceutical formulations.

4. Conclusion

A rapid and reliable isocratic HPLC-UV method for determination of didanosine has been developed and validated. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including accuracy, linearity, recovery and precision data. It is a highly specific and precise analytical procedure and its chromatographic run time of 6 min allows the analysis of a large number of samples in a short period of time. Therefore, this HPLC-UV method can be used as a routine sample analysis.

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