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Liquid chromatographic separation of zalcitabine and its stereoisomers

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Abstract: A liquid chromatographic method capable of separating and quantitating the stereoisomers of zalcitabine has been developed and validated. The separation was achieved with an Astec Cyclobond I — RSP column and a mobile phase of 0.25% triethylamine in water adjusted to a pH of 6.5 with glacial acetic acid. All enantiomers were found to exhibit a linear response in the range of 0.1–10% in the presence of 100% zalcitabine. Precision of analysis was found to be less than 1.5% at a level of 1% relative to zalcitabine. The limit of detection for two of the three enantiomeric impurities was determined to be 0.05% relative to zalcitabine. The detection limit for the third was found to be 0.1%. This method was successfully applied to the analysis of reference standards and several production scale batches. All of these materials were found to be stereochemically pure to a level of 99.8% or better.

Keywords: *Zalcitabine (ddc); chiral LC; Stereoisomer separation; modified cyclodextrin column.*

Introduction

Traditionally, drug development has not concerned itself with the resolution of mixtures of chiral compounds into individual optical isomers, as clinical efficacy was usually demonstrated using a racemic mixture. Hence, the optical purity of such materials was typically not monitored. However, recently released FDA guidelines require sponsors of new medicinal products either to market active ingredients which possess chirality either as single enantiomers or, alternatively, to tightly control the composition of the racemic mixture [1–3]. The latter approach requires rigorous justification. In these new guidelines, stereoisomers other than that designated as the active substance are to be treated as impurities which are by-products of the synthetic procedure and hence must be routinely monitored. Alternatively, sufficient evidence must be shown that the stereoisomeric purity of the drug substance remains constant from batch to batch, to justify not testing for enantiomeric purity in a quality control regimen. The development of chiral liquid chromatographic (LC) columns capable of resolving racemic mixtures into individual enantiomers on an analytical scale has also prompted the FDA to require evidence of lack of contamination from enantiomeric impurities. One of the most popular types of columns are those based on cyclodextrins. Cyclodextrins (CyDs) are macrocyclic polymers of glucose composed of glycosidic residues connected together to form a doughnut shaped toroidal molecule with a hydrophobic interior. CyDs composed of six, seven and eight residues are denoted as α-CyD, β-CyD and γ-CyD, respectively [4–5]. CyDs are capable of including molecules which satisfy certain size and shape criteria. Due to their inherent chirality, CyDs also possess the capability of discriminating between optical isomers, which has resulted in their utility as LC stationary phases [6–7].

Zalcitabine (4-amino-1-β-D-2′,3′-dideoxy-ribofuranosyl-2-(1,H)-pyrimidinone, 2′,3′-dideoxycytidine, ddC) has been approved by the FDA for combination therapy with Retrovir (azidothymidine, AZT) for the treatment of individuals infected with the human immunodeficiency virus (HIV) or those who have developed symptoms of acquired immune deficiency syndrome (AIDS). Zalcitabine is a nucleoside analogue member of the AIDS drug class known as reverse transcriptase inhibitors [8–11], which stall advancement of the virus by causing premature termination of the HIV–

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DNA synthetic process. The zalcitabine molecule possesses two stereogenic centres and, therefore, four potential optical isomers, as is shown by the structures in Fig. 1. The active substance is the 2R-*cis* isomer. Since the synthesis of zalcitabine from cytidine protects the chiral centres and hence results in a single enantiomeric form [12] and because it was decided to avoid the rigorous justification needed to market this compound as a racemate, our laboratory sought to develop and validate a method capable of resolving zalcitabine from its stereoisomers. This method had to have the ability of separating and quantitating low levels of the enantiomeric impurities in the presence of a large excess of zalcitabine. The method would then be used to analyse batches of zalcitabine representative of production scale for the presence of the enantiomeric impurities and show that they were indeed pure with respect to enantiomeric contamination.

Experimental

Instrumentation

The liquid chromatograph used in this study was a modular system composed of a high-pressure pump (model 600E; Waters Associates, Milford, MA, USA), autosampler/injector (model 715; Waters Associates) and an absorbance detector (model 783; Applied Biosystems, Foster City, CA, USA). The column used was a Cyclobond I RSP (25 cm × 4.6 mm, 5 μm i.d.; Advanced Separation Technologies, Whippany, NJ, USA). A silica precolumn was used to saturate the mobile phase and extend column life. All chromatographic analyses were performed at ambient laboratory temperature (approximately 22°C). A PeakPro laboratory automation system (Beckman Instruments, Allendale, NJ, USA) was used to collect and analyse the signal from the detector. All weighing operations were performed on an electronic balance (model AT201; Mettler Instruments, Hightstown, NJ, USA).

Chemicals and reagents

Zalcitabine and its related stereoisomers were obtained from the Synthesis Services Department at Hoffmann-La Roche Inc. (Nutley, NJ, USA). Triethylamine (Kodak Chemical, Rochester, NY, USA) and glacial acetic acid (Fisher Scientific, Springfield, NJ, USA) were obtained from commercial sources

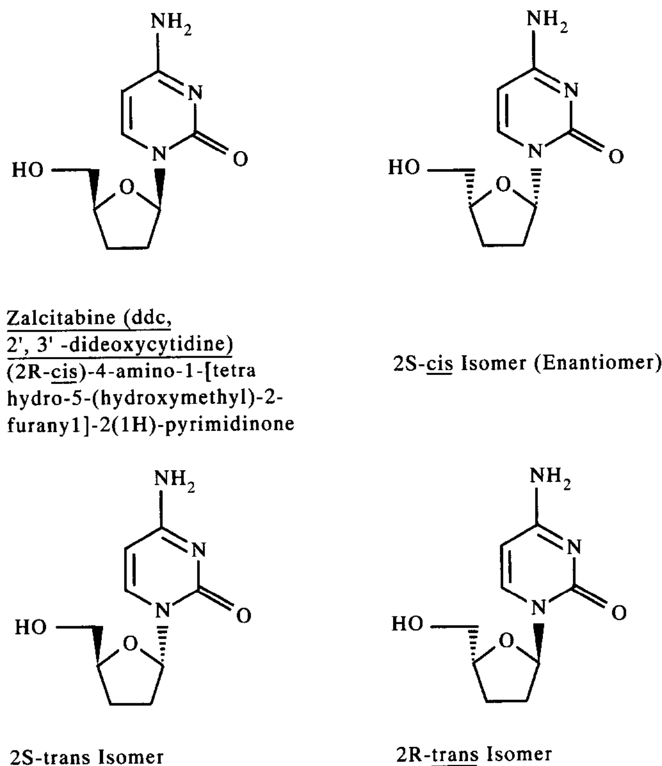

Figure 1
Structures of zalcitabine and related stereoisomers.

and used without further purification. Distilled, deionized water was generated by a NanoPure water system (Barnstead, Dubuque, IA, USA).

Preparation of mobile phase

The mobile phase was prepared by dissolving 2.5 ml of triethylamine in 1 l of water. The pH of this 0.25% solution was adjusted to 6.5 with glacial acetic acid. The pH was measured with a digital pH/ion meter (model 901; Orion, Boston, MA, USA).

Chromatographic conditions

The mobile phase was filtered and degassed by vacuum filtration prior to use. The flow rate was kept constant at 0.25 ml min^{-1}. The output wavelength of the detector was set at 270 nm. The response time was 20 ms. A 5 μl aliquot of each sample was injected.

Preparation of standards

Approximately 10 mg each of zalcitabine, 2S-*cis*, 2R-*trans* and 2S-*trans* stereoisomers were weighed into four separate 100-ml volumetric flasks and diluted to volume with water. Five, 10, 20, 50 and 100 μl aliquots from each of the three impurity stock solutions were added to a 10-ml volumetric flask and diluted to volume with zalcitabine reference solution to yield 0.05, 0.1, 0.2, 0.5 and 1.0% solutions of each isomer relative to zalcitabine.

Preparation of samples

Approximately 5 mg of zalcitabine drug substance was accurately weighed into a 50 ml volumetric flask and diluted to volume with water to give a final concentration of 0.1 mg ml^{-1}.

System suitability

A 20 μl aliquot of a four component reference solution containing 5 μl each of the four 10 mg ml^{-1} standard solutions was injected onto the LC. The capacity factor, number of theoretical plates, tailing factor (at 5% of the peak height) and resolution were determined as described in USP XXII [13]. The LC system was deemed to be suitable for use when the resolution between zalcitabine and both the 2S-*cis* and 2S-*trans* isomers was greater than or equal to 1.7.

Method validation

To determine the linearity, solutions of the 2S-*cis*, 2R-*trans* and 2S-*trans* isomers ranging in concentration from 0.05 μg ml^{-1} to 10 μg ml^{-1} each in 100 μg ml^{-1} of zalcitabine were prepared. These solutions, corresponding to 0.05–10% impurity levels relative to zalcitabine, were than injected onto the LC system. The limit of detection (LOD), defined as the concentration of analyte producing a peak height signal twice that of the baseline noise, was determined for the three enantiomeric impurities.

System precision of the method was determined at 0.2, 1.0 and 100% of impurity level relative to 100 μg ml^{-1} of zalcitabine. Each of these solutions were injected onto the LC system five times. The mean response, standard deviation and percentage relative standard deviation were calculated for each component.

Results and Discussion

The ability of CyDs to discriminate between optical isomers of guest molecules forms the basis for their use as chiral stationary phases for LC. One would predict that the zalcitabine molecule, by virtue of its structure, would fit snugly in a cyclodextrin cavity and be effectively separated. It has been demonstrated that other nucleotides and nucleosides can be separated by chiral LC by using CyDs as mobile phase additives [14]. Recent work has shown that another nucleoside analog, 2′,3′-dideoxyinosine (ddI), is capable of being complexed with β-CyD, which concurrently increases its hydrolytic stability [15]. Initial work in our laboratory with the stereoisomeric separation of zalcitabine involved the use of an "unmodified" β-cyclodextrin column and a mobile phase of 0.01 M potassium phosphate buffered to pH 7.0 at a flow rate of 0.3 ml min^{-1}. This system resulted in resolution values of 1.2 and 3.2 between zalcitabine and the 2S-*cis* and 2S-*trans* isomers, respectively. It was felt that these method conditions did not result in adequate specificity for the 2S-*cis* enantiomer, and thus modifications to this method were sought. The addition of an organic modifier (methanol) and the use of a lower pH buffer did little to improve the separation, so that alternative separation schemes were investigated. Resolution was not even improved by the use of an α-1-acid glycoprotein column. A close examination of the mechanism on a molecular scale was

performed in an attempt to improve the separation.

In order for a complete separation of stereoisomers to occur on a CyD column, two prerequisites for chiral recognition must be met [16]. The first requires the analyte to have a proper structural fit inside the CyD cavity. In the case of β-CyD, analytes possessing a six-membered aromatic ring can be accommodated easily in the CyD interior. The second requires the chiral analyte to possess some substituent group at or near the stereogenic centre to interact in an attractive or repulsive fashion with the secondary hydroxyl groups at the mouth of the CyD cavity. Although the pyrimidinone ring on the zalcitabine molecule satisfies the first prerequisite and is most probably the major site of inclusion, it appears from the separation obtained with the unmodified β-CyD column that the second criterion is not being satisfied. In order to increase the potential for interaction between the host molecule and the CyD cavity, the use of a derivatized CyD column was considered. The β-CyD RSP column consists of β-CyDs in which seven of the secondary hydroxyl groups have been converted to hydroxypropylether moieties. The advantages of the use of this phase for chiral separations compared to the normal β-CyD include increased flexibility, whereby the hydroxypropyl group can rotate to achieve an orientation which provides the maximum degree of interaction with the guest molecule [17]. This increased interaction leads to a higher degree of hydrogen bonding between the CyD and the analyte which serves to immobilize the latter molecule causing an increased stability of the host–guest complex [18]. Finally, the larger substituent groups on the CyD torus increase steric interactions near the centre of chiralty, especially for enantiomers which have bulky substituents located at or near the stereogenic center. The β-CyD RSP column has been successfully used to separate the stereoisomers of two structurally similar nucleoside analogues, 2′-deoxy-3′-thiacytidine [19] and 2′-deoxy-3′-oxacytidine [20].

The use of the CyD-RSP column improved the degree of interaction between the zalcitabine and the CyD cavity, which resulted in baseline separation of all four stereoisomers, as is shown by the chromatogram in Fig. 2. The chromatographic parameters for all four peaks are summarized in Table 1. Note that all peaks are well resolved from each other within a

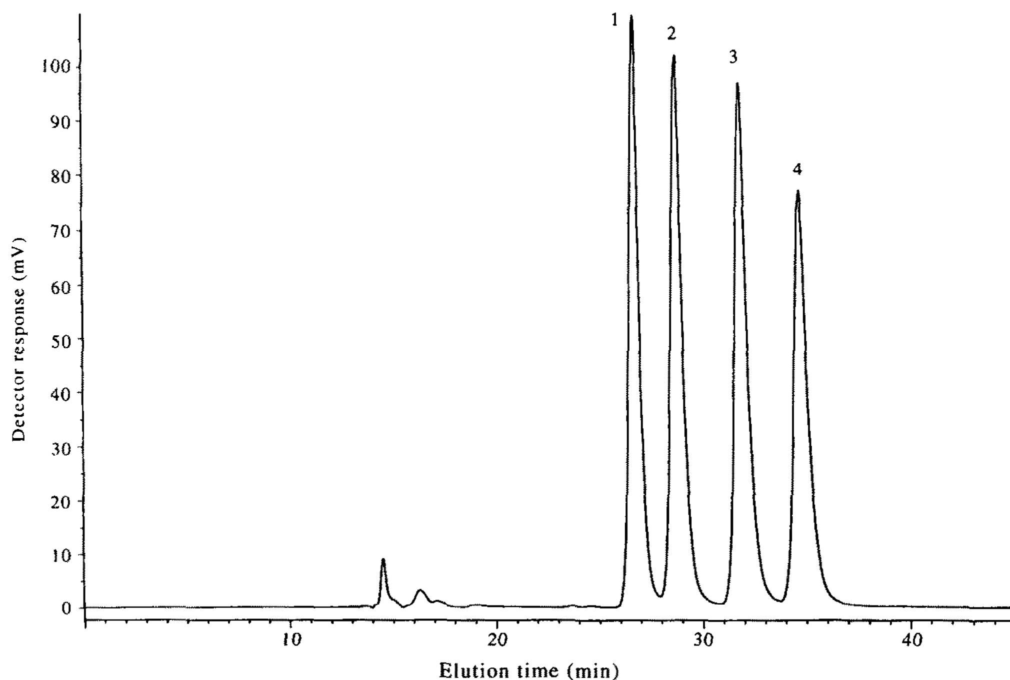

Figure 2
Representative chromatogram showing the separation of zalcitabine and related stereoisomers. Conditions as noted in the text. 1 = 2S-*cis* isomer; 2 = zalcitabine; 3 = 2S-*trans* isomer; 4 = 2R-*trans* isomer.

Table 1
Chromatographic parameters

Component	Elution time (min)	Peak width (s)	Efficiency ($N \times 10^{-4}$)	Capacity factor (k')	T_f	Resolution ratio
2S-*cis*	24.9	56	1.13	2.12	1.38	
						2.20
Zalcitabine	27.1	62	1.12	2.39	1.27	
						2.12
2S-*trans*	29.4	67	1.10	2.68	1.41	
						2.30
2R-*trans*	32.2	75	1.07	3.03	1.47	

Note: All chromatographic parameters calculated as per USP XXII [13].

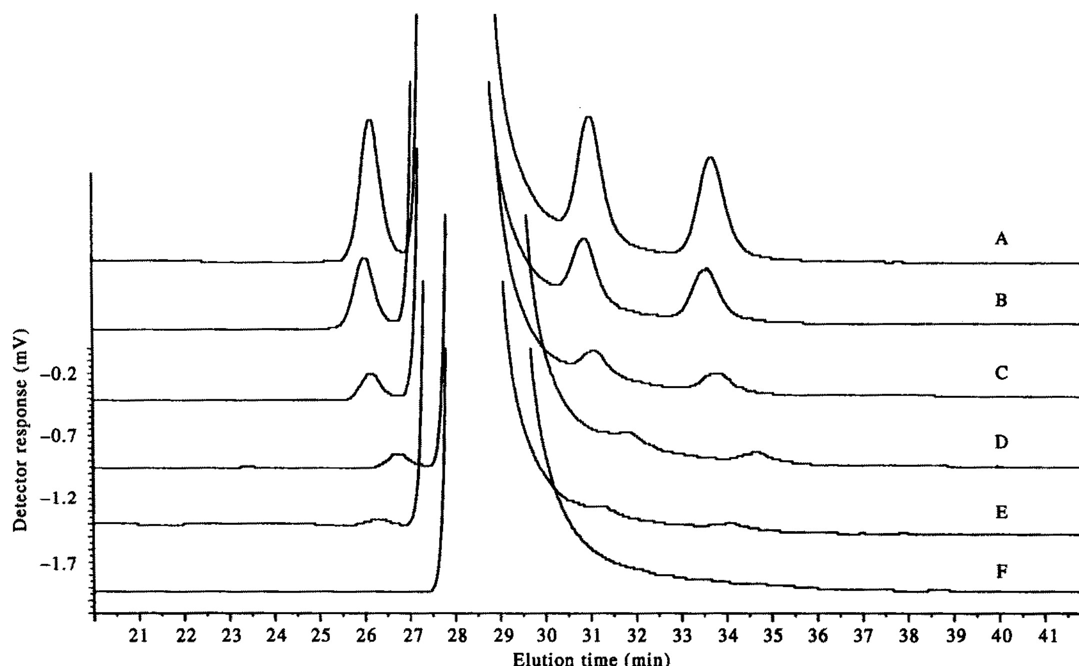

Figure 3
Representative chromatograms of stereoisomeric impurities in the presence of 100% zalcitabine. A = 1%, B = 0.5%, C = 0.2%, D = 0.1%, E = 0.5%, F = Typical production lot.

40 min run time. The method was found to be quite precise at high levels with system precision values of 0.15, 0.31 and 0.31% being obtained for six replicate injections of standards of 2S-*cis*, 2S-*trans* and 2R-*trans* isomers, respectively. The true test of the method came at levels in which the aforementioned three isomers were treated as impurities in the zalcitabine drug substance. At levels of 0.2% in the presence of 100% zalcitabine, the 2S-*cis*, 2S-*trans* and 2R-*trans* isomers gave system precision values of 3.6, 15.5 and 5.1%, respectively. At 1.0% these values improved to 1.3, 1.1 and 1.4%, respectively. The detector response for all three isomers was found to be linear, as is shown in Fig. 3. The correlation coefficient for the three isomers were determined to be 0.99998 for the 2S-*cis*, 0.99984 for the 2S-*trans* and 0.99990 for the 2R-*trans* isomers. The limit of detection (LOD) for two of the three isomers was determined to be approximately 0.05% in the presence of an excess of zalcitabine. The third component, the 2S-*trans* isomer, gave an LOD value of 0.1%. Since this component elutes just after zalcitabine, its LOD was somewhat higher. It should be noted that these LOD values were obtained in solutions which contained a great excess of zalcitabine to approximate the conditions in which the method would be used. It was felt that all LOD values were acceptable. Therefore, the method was pronounced suitable for use as a limit test for the various stereoisomers where these components would be present at orders of magnitude less than zalcitabine.

Although the system precision values at low levels of the stereoisomers are higher, they were found to be adequate to apply this

method to the analysis of real production scale batches of material. The purpose of this analysis was to ascertain the stereoisomeric purity of material manufactured in a real production environment. A total of 18 lots of material were tested according to this method as well as two lots of zalcitabine purified further to produce reference standard grade material. All lots were found to be pure to a level of 99.8% or better. In only three cases was a trace found of any other than the desired stereoisomeric form. To further demonstrate the utility of this method, solid state samples of zalcitabine stored at 100°C for 4 weeks were analysed for stereoisomeric purity. This was done to investigate whether thermal stress had any effect on the proportions of the isomers. After 4 weeks, no trace of any of the stereoisomers could be found, which shows that any thermal decomposition of zalcitabine does not involve the stereogenic centres of the molecule.

Conclusions

We have shown the validity of a method for the separation of zalcitabine and its related stereoisomers. The aforementioned method is simple, rapid and is sufficiently sensitive to detect small amounts of stereoisomeric contamination in production scale batches of zalcitabine drug substance. Results of the analysis of several commercial batches indicated that only the pure enantiomeric form is produced. Furthermore, thermally stressed zalcitabine samples did not show any increase in the level of the stereoisomers, indicating that any degradation pathways of the molecule do not involve the chiral centres.

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