Department of Analytical Chemistry[1], Faculty of Pharmacy, Ankara University, Department of Pharmaceutical Technology[2], Gülhane Military Medical Academy, Etlik, Ankara, Turkey

Validated RP-HPLC method for the assay of zalcitabine in drug substance, formulated products and human serum

B. Uslu[1], A. Savaşer[2], S. A. Özkan[1], Y. Özkan[2]

Received October 16, 2003, accepted November 18, 2003

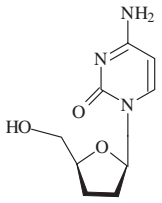
Assoc Prof. Dr. Yalç ın Özkan, Gülhane Military Medical Academy, Department of Pharmaceutical Technology, TR-06018 Etlik, Ankara, Turkey
yozkan@gata.edu.tr

Pharmazie 59: 604–607 (2004)

A HPLC method for zalcitabine determination in bulk form, pharmaceutical dosage forms and human serum has been developed and validated. The proposed method was conducted using a reverse phase technique, and UV monitoring at 265 nm. The mobile phase consisted of methanol: 0.01 M NaH_2PO_4 (85 : 15; v/v) adjusted to pH 4.62 with 1 M NaOH. The detector response was linear in the range of 0.015–50 μg mL^{-1}. The limit of detection and the limit of quantification of the procedure were 0.0066 μg mL^{-1} and 0.022 μg mL^{-1}, respectively. The retention time was 2.5 min for zalcitabine and 3.5 min for the internal standard. No interferences from tablet additives were observed and analysing tablets containing zalcitabine proved the applicability of the method. This method was also applied for the determination of zalcitabine in spiked human serum samples.

1. Introduction

Zalcitabine is a nucleoside reverse transcriptase inhibitor derived from cytidine with activity againts retroviruses including HIV. It is used in the treatment of HIV infection, usually in combination with other anti-retroviral drugs. Zalcitabine is absorbed from the gastrointestinal tract with a bioavailability of greater than 80% (PDR 2003; Sweetman 2002). Binding to plasma proteins is negligible.



A survey of the literature has not revealed any determination method from the pharmaceutical dosage forms of zalcitabine, however few HPLC methods (Fan 2001; Kalin 1988; Magnani 1989; Morris 1994; Harker 1994; Simon 2001) have been reported for the determination of this compound in biological fluids. These published techniques include complicated sample preparation, extraction steps and column switching procedure for determination of zalcitabine in human serum samples (Fan 2001; Kalin 1988; Magnani 1989; Morris 1994; Harker 1994; Simon 2001). To the best of our knowledge, no assay has been published for the determination of zalcitabine in pharmaceutical dosage forms.
Owing to the widespread use of HPLC in routine analysis, it is important that good HPLC methods are developed and that these are thoroughly validated (Uslu 2002; Özkan 2002, 2003; Savaşer 2003).

The main objective of this work has been to develop a simple, sensitive, reliable, time and money saving fully validated HPLC method with UV detection for the determination of zalcitabine in human serum and pharmaceuticals.

2. Investigations, results and discussion

The preliminary trails using different compositions of mobile phases consisting of methanol, acetonitrile and water on reversed-phase stationary phases did not give good peak shape and resolution. With a mobile phase consisting of methanol and 0.01 M NaH_2PO_4 in the ratio 85 : 15 at pH 4.62, the resolution between solvent front, compound and IS were found to be suitable. The best flow rate was obtained as 0.7 mL min^{-1}. Under these conditions, good results were obtained in terms of shape of the peak, sensitivity and retention time for raw material, pharmaceutical dosage form and serum samples. The retention times of zalcitabine were varied between 2.48 and 2.52 min.
Deflazacort was chosen as the internal standard (IS) because it not only gave the best peak shape but also gave the better resolution and shorter retention time compared to other potential internal standards.
The USP defines parameters that can be used to determine system suitability prior to analysis (USP 24, 2000). These parameters include tailing factor, capacity factor, resolution, selectivity factor, and RSD % of retention times and RSD % of peak area or height for repetitive injections. At least two of these criteria are required to demonstrate system suitability for any method (Snyder 1997). System suitability tests were carried out on freshly prepared standard stock solutions of zalcitabine. Tailing and capacity factors were 1.17 and 2.37 for zalcitabine and 1.00 and 3.68 for

Table 1: Analytical parameters for the determination of zalcitabine

Linearity range (μg mL^{-1})	0.015–50
Slope of calibration graph	0.172
Intercept	0.0193
Correlation coefficient (r)	0.999
RSD% of slope	0.001
RSD% of intercept	0.017
Detection limit (μg mL^{-1})	0.0066
Quantification limit (μg mL^{-1})	0.022

IS, respectively. The retention times in mobile phase were used as hold-up time for the capacity factor calculations. The present chromatographic conditions ensure adequate retention of both compounds, since capacity factor values obtained satisfied the conditions (between 2 and 10). Resolution and selectivity factors for this system were 1.65 and 1.55, respectively. The method has enabled good resolution of analytes, since values of resolution factors of adjacent peaks were greater than 1.0. The retention times of zalcitabine raw material, tablets and serum samples were 2.52, 2.52 and 2.49 min, giving RSD % of 0.13, 0.14 and 0.20, respectively. All the values for different system suitability parameters checked were found to be in agreement with the USP requirements.

Peak area ratios (A_{sample}/A_{IS}) were plotted against corresponding concentrations in the range of 0.015 – 50 μg mL^{-1}. Table 1 shows the calibration characteristics and related validation, parameters of zalcitabine. The correlation coefficient was found greater than 0.999 and RSD % of slope and intercept of the calibration equation were very low. These parameters established the precision of the proposed method. Necessary statistical data of the regression equations, such as LOD, LOQ values were also shown in Table 1. The LOD and LOQ values were based on the standard deviation of the response (shown as s) and the slope of the corresponding calibration curve (shown as m) using the following equations:

$$LOD = 3.3\ s/m\,; \qquad LOQ = 10\ s/m$$

(Swartz 1997; Riley 1996).

Intra-day precision and accuracy of the method were evaluated by assaying freshly prepared solutions five times at three different concentrations of zalcitabine (Table 2). Inter-day precision and accuracy of the method calculated from the individual recovery data were evaluated by assaying freshly prepared solutions, five times for five different days over a 2 weeks period. The RSD ranged from 0.42 to 0.83% for intra-day and 0.51 to 0.93% for inter-day results.

The stability of the reference substance was checked by analyzing a prepared standard solution of zalcitabine in mobile phase aged at + 4 °C in the dark against a freshly prepared sample. The obtained results showed that the working reference solutions were stable for up to 7 days. The peak area ratio for the assay reference solutions over 7 days did not change significantly.

Table 2: Within-day and between-day precision of zalcitabine standards

Theoretical concentration (μg · mL^{-1})	Within-day measured concentration (μg · mL^{-1})[a]			Between-day measured concentration (μg · mL^{-1})[b]		
	Mean	RSD %	Bias %	Mean	RSD %	Bias %
2.5	2.48	0.83	0.8	2.49	0.93	0.4
5	4.98	0.42	0.4	4.92	0.51	1.6
10	9.97	0.43	0.3	9.89	0.80	1.1

[*] Mean values represent five different zalcitabine standards for each concentration
[**] Between-day reproducibility was determined from five different runs over a 2 weeks period

Table 3: Results of the determination and the recovery analysis of zalcitabine in tablet dosage forms

Labeled claim (mg per tablets)	0.375
Mean of amount found (mg)*	0.371
RSD % of amount found	0.71
Added (mg)	0.10
Recovered (mg)**	0.101
Recovery %	100.51
RSD % of recovery	0.34

*, ** Each value is the mean of five experiments

The proposed method was applied to the determination of zalcitabine in its pharmaceutical dosage form, the results in Table 3 indicate satisfactory accuracy and precision of the

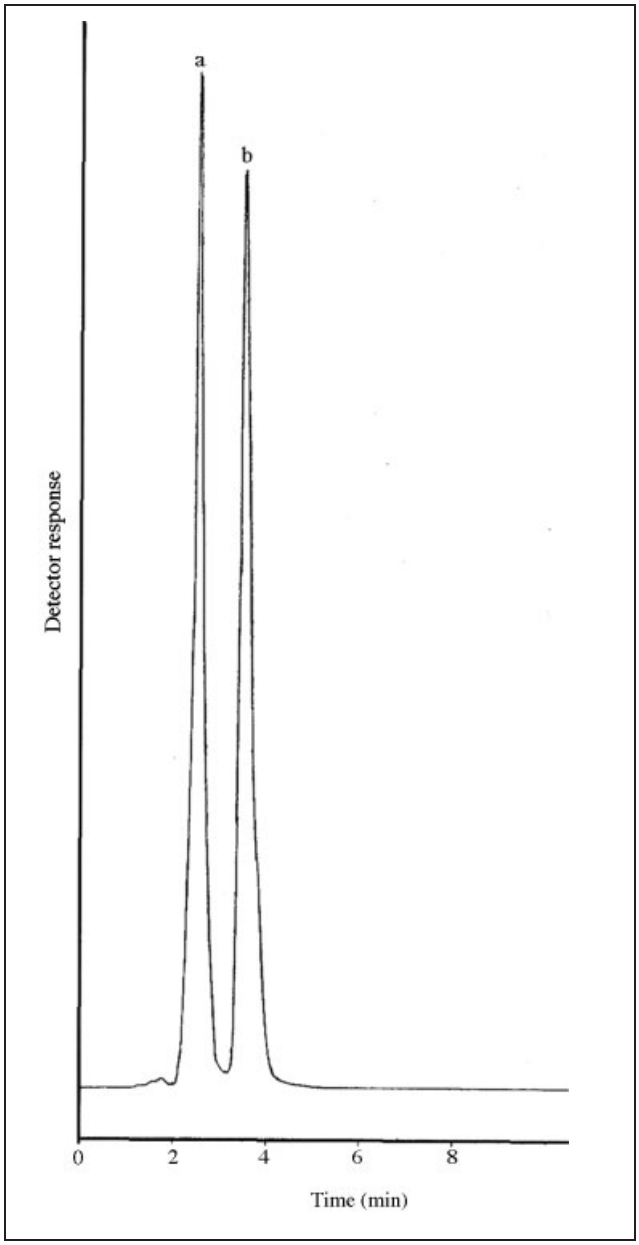


Fig. 1 Chromatogram obtained from a tablet dosage form containing 5 μg mL^{-1} zalcitabine (a) and 10 μg mL^{-1} deflazacort (b) (IS)

method. Common tablet excipients, such as starch, lactose, talc, magnesium stearate, Avicel, etc. did not interfere with the assay. The proposed method could be used for the determination of zalcitabine without prior seperation of the excipients. The results showed the drug content of this product to be in accordance with the labeled claim. The mobile phase resolved the two compounds very efficiently into two distinct sharp peaks of zalcitabine and IS obtained from the tablets as shown in Fig 1. According to Fig 1, zalcitabine was eluted, forming well-shaped, symmetrical single peaks, well separated from the solvent front. No interfering peaks were found in the chromatogram due to the tablet excipients. In order to check the interferences from the excipients of the tablet dosage forms, recovery studies were carried out using by the standard addition technique. The accuracy of the results was further tested by recovery experiments by adding a known amount of pure compound to pre-analyzed samples of the formulations. Recovery experiments using the developed assay procedure further indicated the absence of interference from commonly encountered pharmaceutical excipients used in the selected formulation. The recovery results are summarized in Table 3.

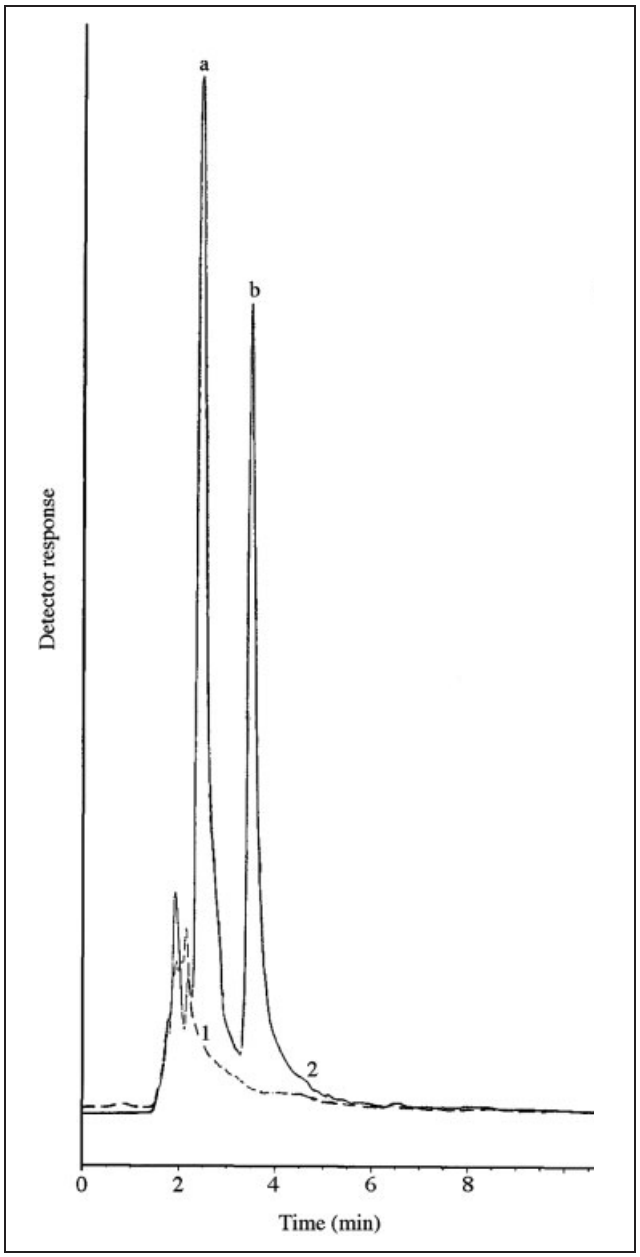


Fig. 2: Chromatogram of blank serum (1) and serum spiked (2) with 7.5 μg mL^{-1} zalcitabine (a) and 10 μg mL^{-1} deflazacort (b) (IS)

Table 4: Results obtained for zalcitabine analysis from human serum

Zalcitabine added (μg · mL^{-1})	2.5	5.0	7.5
n	5	5	5
Zalcitabine found (μg · mL^{-1})	2.47	4.96	7.48
RSD %	0.54	0.75	0.33
Average recovery %	98.64	99.20	99.65
RSD % of recovery	0.54	0.76	0.35

Applying the proposed method to human serum, the recovery studies were done in human serum samples. Fig. 2a shows a typical chromatogram of an extract of fresh blank serum and Fig. 2b shows a chromatogram obtained when the method was applied to spiked serum containing 7.5 μg mL^{-1} zalcitabine and 10 μg mL^{-1} IS. Analysis of drugs from biological samples generally requires extensive time-consuming sample preparation, use of expensive organic solvents and other chemicals. In our proposed method, the serum proteins and other potential endogenous substances are precipitated by the addition of acetonitrile and the mixture is vortexed and centrifuged at 5000 rpm. The supernatant is taken carefully and diluted with mobile phase, directly injected to the column and analyzed. Good recovery results of zalcitabine were obtained from the serum samples.

The proposed method gives a good resolution between zalcitabine and IS within a short analysis time (< 5 min). The proposed method is very simple, rapid, sensitive and reproducible and does not involve complex instrumentation or complicated sample treatment. The high percentage of recovery shows that the method is free from interferences with excipients used in the formulation. The obtained results from the spiked human serum samples demonstrate a high level of precision and enough sensitivity for the determination of zalcitabine in serum. Thus this method can be easily adopted for the routine quality control analysis of zalcitabine in pharmaceutical dosage forms and human serum samples and may be regarded for routine analysis of zalcitabine in quality control laboratories.

3. Experimental

3.1. Instrumentation

HPLC was performed using a HP Chromatographic System (Hewlett Packard, Avondale, USA). It consisted of a isocratic Quat pump Model G 1311 A (HP, Avondale, USA) connected with a HP G 1314 A UV-VIS detector (HP, Avondale, USA) operating at 265 nm, a G 1328 A (Cotati, CA) injection valve, with a 20 μL loop. The chromatographic data was collected and analysed using HP Chemstation for LC and LC/MS system (Hewlett Packard, Avondale, USA). The separation was carried out at ambient temperature, on a reversed-phase Waters spherisorb column (250 × 4.6 mm; 5 μm particle size). The chromatographic separation was performed using an isocratic mode. The mobile phase employed was methanol: 0.01 M NaH_2PO_4 (85 : 15 v/v) adjusted to pH 4.62 with 1 M NaOH. The flow rate was 0.7 mL min^{-1}. Deflazacort was used as internal standard (IS).

3.2. Chemicals and reagents

Zalcitabine was obtained as a gift sample from Roche Pharm. Ind. (Istanbul, Turkey). Deflazacort (IS) was kindly supplied from Aventis-Pharma Ind. (Istanbul, Turkey). HPLC grade methanol and sodium dihyrogen phosphate were purchased from Merck (Darmstadt, Germany). All other chemicals were analytical reagent grade quality. Doubly distilled water was used for preparing mobile phase.

3.3. Preparation of the standard calibration graph

Stock solutions of zalcitabine and IS (1 mg · mL^{-1} of each) in mobile phase were prepared separately. These stock solutions were diluted with

mobile phase. Standards for the calibration curve were prepared from 0.015 to 50 $\mu g \cdot mL^{-1}$ in mobile phase. The stability of the solution of zalcitabine during analysis was determined by repeated analysis of samples during the course of the experiment on the same day and on different days after storing in the refrigerator. System suitability test parameters were also obtained.

The concentration of IS was maintained at a constant level of 10 $\mu g \cdot mL^{-1}$.

3.4. Precision, accuracy and ruggedness

The within day and between day precision and accuracy and the ruggedness of the proposed HPLC method were estimated by assaying five replicate samples at three different concentrations. The RSD % was calculated to check the ruggedness and precision of the method. The accuracy was expressed as % bias (Swartz 1997; Riley 1996).

3.5. Estimation of zalcitabine from tablets

Not less than ten tablets were weighed, crushed and combined. Five accurately weighed quantities of this powder equivalent to 1.0 mg zalcitabine were taken in different 10 mL volumetric flasks and diluted with mobile phase, sonicated for 10 min, and then completed to the volume with the mobile phase. The above solution was filtered through 0.45 μm Whatman filter paper. After filtration, appropriate solutions were prepared by taking suitable aliquots of clear filtrate and addition of the appropriate internal standard, diluting them with mobile phase in order to obtain the final solution. 20 μl of this solution was injected and chromatograms were recorded.

3.6. Recovery from tablets

Known amounts of pure drug were added to the different pre-analysed formulations of zalcitabine including a constant level of internal standard, and the mixtures were analyzed by the proposed method. The percent recovery calculated by comparing the concentration obtained from spiked samples with the concentration actually added. Thus, the effect of common tablet formulation excipients on chromatograms (e.g. tail, broading etc.) was investigated. After five repeated experiments, the recoveries were calculated.

3.7. Recovery studies in spiked human serum samples

Drug free serum samples, obtained from healthy individuals (after obtaining their written consent), were stored frozen until assay. After gentle thawing, 2 mL aliquots of serum were spiked with an appropriate amount of zalcitabine (dissolved in mobile phase), 1000 μl acetonitrile (for denaturation and precipitation of serum proteins). The tubes were vortexed for 5 min and then centrifuged for 10 min at 5000 × g. The supernatant was taken carefully. Serum samples including various concentrations of zalcitabine and a constant amount of internal standard were injected into the HPLC column.

References

Fan B, Steward JT (2001) Determination of zidovudine/zalcitabine/nevirapine in human plasma by ion-pair HPLC. J Liq Chromatogr Rel Tech 24: 3017–3026.

Harker AJ, Evans GL, Hawley AE, Morris DM (1994) High-performance liquid chromatographic assay for 2′-deoxy-3′-thiacytidine in human serum. J Chromatogr B Biomed Appl 657: 227–232.

Kalin JR, Hill DL (1988) Determination of 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine in biological samples. J Chromatogr 431: 184–191.

Magnani M, Rossi L, Bianchi M, Cucchiarini L, Stocchi V (1989) Reversed-phase liquid chromatographic determination of 2′,3′-dideoxycytidine in human blood samples. J Chromatogr 491: 215–220.

Morris DM, Selinger K (1994) Determination of 2′-deoxy-3′-thiacytidine (3TC) in human urine by liquid chromatography: direct injection with column switching. J Pharm Biomed Anal 12: 255–264.

Özkan SA, Uslu B (2002) A rapid HPLC assay for the determination of lamivudine in pharmaceuticals and human serum. J Liq Chromatogr Rel Techn 25: 1447–1456.

Özkan Y, Savaşer A, Taş Ç, Uslu B, Özkan SA (2003) Drug dissolution studies and determination of deflazacort in pharmaceutical formulation and human serum samples by RP-HPLC. J Liq Chromatogr Rel Techn 26: 2141–2156.

Physicians Desk Reference (PDR) (2003), 57th Ed. Published by Medical Economics Company Inc., Montvale, N.J, p. 2898.

Riley CM, Rosanske TW (1996) Development and validation of analytical methods, Elsevier Science Ltd, New York.

Savaşer A, Özkan C K, Özkan Y, Uslu B, Özkan SA (2003) Development and validation of a RP-HPLC method for the determination of valacyclovir in tablets and human serum and its application to the drug dissolution studies. J Liq Chromatogr Rel Techn 26: 1755–1767.

Simon VA, Thiam MD, Lipford LC (2001) Determination of serum levels of thirteen human immunodeficiency virus-suppressing drugs by high-performance liquid chromatography. J Chromatogr A 913: 447–453.

Snyder LR, Kirkland JJ, Glajch JL (1997) Practical HPLC method development. 2nd Ed. John Wiley and Sons Inc., New York.

Swartz ME, Krull I S (1997) Analytical method development and validation, Marcel Dekker, New York.

Sweetman SC (Ed.), (2002) Martindale, The Complete Drug Reference, 33th Ed., Pharmaceutical Press, London, p. 664.

The United States Pharmacopoeia (2000), The USP 24rd Ed.; [CD-ROM], Easton, Rand Mc Nally, Tounton, M.A.

Uslu B, Özkan SA (2002) Determination of lamivudine and zidovudine in binary mixture using first derivative spectrophotometric, first derivative of the ratio spectra and HPLC-UV methods. Anal Chim Acta 466: 175–185.