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Investigation of the pharmacokinetics and determination of cholesteryl carbonate zidovudine in rat plasma by non-aqueous reversed-phase high performance liquid chromatography with UV detection

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Abstract

A simple reversed phase high-performance liquid chromatographic method was established for the separation and quantification of a novel prodrug of zidovudine in rat plasma. Zidovudine was one of the original drugs used to treat HIV infection. Appropriate aliquots of rat plasma were spiked with cholesteryl carbonate zidovudine (AZTC) and treated with acetonitrile to precipitate plasma proteins. The supernatant after supercentrifugation was collected and an aliquot of 20 μL was injected directly into an HPLC system consisting of a Diamonsil™ C_{18} column and a diode array detector. The mobile phase consisted of methanol (85%, v/v) and diethyl ether (15%, v/v) at a flow rate of 1.0 mL/min. The extraction recoveries of AZTC at the three concentrations examined were all higher than 80%. The HPLC assay was linear over the concentration range 0.5–80 μg/mL. A one-compartment model with apparent first-order elimination was used to describe the plasma concentration–time profile for AZTC after administration via the tail vein. The mean elimination half-life ($t_{1/2}$) was 292.4 min. This RP-HPLC method will be useful for the evaluation of the pharmacokinetics of AZTC in rats.

Keywords: Cholesteryl carbonate zidovudine; Pharmacokinetics; HPLC; Rat plasma

1. Introduction

Cholesteryl carbonate zidovudine (AZTC) (Fig. 1) is a new prodrug of zidovudine (AZT) which was firstly proposed by Bailey et al. [1]. It is known that the elimination of AZT from the body is very rapid and it has to be administered frequently in order to maintain therapeutic concentrations in the target organs and tissues, which increases its dose-dependent toxicity and side effects. In the early 1990s, some authors reported that a lower peak plasma level and a longer t_{max} of AZT were observed following the oral administration of a C18 ester prodrug [2,3]. Based on these findings, a series of prodrugs of AZT, including AZTC, were synthesized and studied in our laboratory [4,5]. Another important advantage of AZTC is its increased lipophilicity compared with AZT, which makes it more suitable for liposomal preparations.

We have established a simple reversed phase high-performance liquid chromatographic method and validated its suitability for separating and quantifying AZTC in rat plasma. In addition, we have investigated the pharmacokinetics of AZTC in rats.

2. Experimental

2.1. Instruments

The HPLC system consisted of an Elite P230 high pressure continuous flow pump (Elite, Dalian, China) with a DAD 230 detector. The detector was set to scan from 199 to 618 nm and 266 nm was the wavelength used for quantification. The HPLC system and data collection were controlled by EChrom 2000 DAD Data System (Elite, Dalian, China).

2.2. Chromatographic conditions

The chromatographic column used was a Diamonsil™ ODS C_{18} column (200 mm × 4.6 mm, 5 μm) (Dikma technologies,

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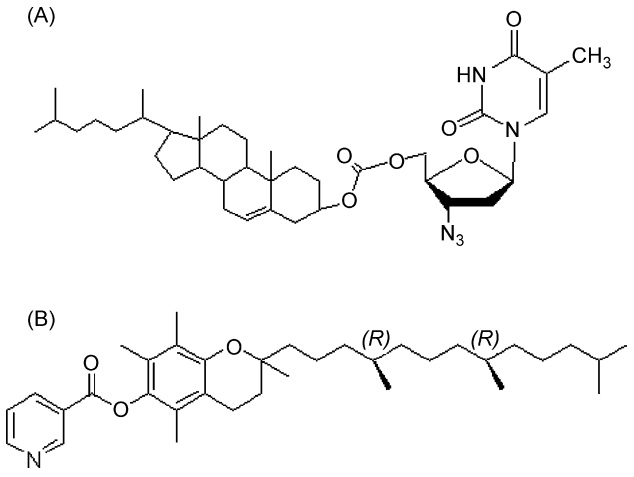

Fig. 1. The chemical structure of (A) AZTC; (B) internal standard.

USA) and the HPLC guard cartridge system was a KJO4282 type filled with the same materials (10 mm × 4.6 mm, 5 μm) (Phenomenex, Guangzhou, China). The determination was performed at room temperature. The mobile phase was composed of methanol–diethyl ether (85:15, v/v), previously filtered through a cellulosic Millipore membrane with a pore size of 0.45 μm (Xinya, Shanghai, China). The flow rate was 1.0 mL/min and the injection volume was 20 μL.

It must be noted that appropriate safety precautions should be taken because of the explosive nature of diethyl ether.

2.3. Materials and reagents

Cholesteryl carbonate zidovudine (purity > 98.5%) was derived from AZT and cholesteryl chloroformate under the action of the catalyzer, triethylamine, isolated by silica-resin chromatography and purified by recrystallization. The identity and purity of AZTC was confirmed by IR, NMR and HPLC. The internal standard, α-tocopheryl nicotinate (TN) (Fig. 1), was purchased from Saike Pharmacy Corporation (Zhejiang, China) and purified further in our laboratory until the purity was over 99.8%. Soybean lecithin was purchased from Shanghai Taiwei Pharmacy Corporation Limited (Shanghai, China). Methanol and diethyl ether were of HPLC grade and purchased from Hanbon Science and Technology Company Limited (Jiangsu, China), and other chemicals used in our research were of analytical grade. Wistar rats were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University.

The present experiments have been approved by the animal experiment administration center of Shenyang Pharmaceutical University.

2.4. Preparation of plasma standards and controls

A standard stock solution containing AZTC was prepared in methanol (1.0 mg/mL) and stored at 4 °C. A total of eight standard solutions, from 0.5 to 80 μg/mL in methanol, were made by sequential dilution. Plasma standard solutions were prepared by spiking different working standard solutions into drug-free rat plasma, which was further diluted to give final concentrations from 0.5 to 80 μg/mL AZTC. Plasma controls were prepared with different stock solutions at concentrations of 0. 5, 10 and 80 μg/mL.

2.5. Sample preparation

Rat blood (0.5 mL) was placed in heparinized 1.5 mL centrifuge tubes and immediately centrifuged at 400 × *g* for 5 min. 0.2 ml of the upper plasma layer was transferred to clean 1.5 mL centrifuge tubes and stored at −20 °C. The frozen plasma was allowed to thaw at room temperature before use. A mixture of acetonitrile containing the internal standard (40.8 μg/ml) was added to the samples and vortexed for 2 min. Then, the samples were centrifuged at 5000 × *g* for 10 min, the supernatant was transferred to a clean vial and a 20 μL aliquot of this solution was injected directly into the HPLC for analysis.

2.6. Preparation of formulation

AZTC and soybean lecithin in proper ratio were dissolved in ethanol, and then 5 mL preheated pH7.4 phosphate buffered solution was added under a rapid agitation when the mixture of AZTC and lecithin was evaporated to be viscous. After about 20 min we got the primary liposomes. The primary liposomes were sonicated and extruded through 0.45 and 0.22 μm polycarbonate membranes. The mean diameter of the final AZTC liposomes was below 100 nm.

2.7. Limits of detection and quantitation

The limit of detection (LOD) was the lowest analyte concentration that could be detected with a signal-to-noise ratio of 3. The limit of quantification (LOQ) was the lowest analyte concentration that could be measured with a signal-to-noise ratio of 10 [6].

2.8. Linearity

Plasma standard solutions were injected in triplicate and the ratios of the observed analyte peak area to internal standard were calculated. Calibration curves were constructed using the ratios versus the concentration of analyte with least square method [7]. Linear regression analysis of the data gave the slope, intercept and correlation coefficient. The analyte concentration in each sample was calculated from the regression equation.

2.9. Extraction recovery

The extraction recovery of AZTC at three different concentrations can be calculated by the following equation:

$$\text{Recovery}\,(\%) = \frac{A_1}{A_2} \times 100$$

where A_1 is the signal of quality control samples treated by the above sample preparation method; A_2 signal of untreated standard solution with according concentration.

2.10. Precision and accuracy

The accuracy was the degree of the agreement between the found analyte concentration and the actual analyte concentration. The accuracy of the method was calculated by [(found concentratin − added concentration) × 100/added concentration].

The intra-day precision of the assay was determined by assaying three quality control samples at low, moderate and high concentrations of analyte within the same day ($n = 3$). The inter-day precision of the samples was analyzed on five different days ($n = 5$). Both the intra-day and inter-day precision were evaluated as a percentage relative standard deviation (%R.S.D.).

2.11. Stability studies

The stability of the stock solution at 4 °C and room temperature and analyte in samples at −20 °C were examined on a weekly basis under the above HPLC conditions.

2.12. Pharmacokinetics

Male Wistar rats (250 g ± 10) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (China). The animals were raised in a controlled environment at constant temperature (25 °C) and 45% relative humidity. A single dose of liposomal AZTC (10 mg/kg) was administered via the tail vein to the rats ($n = 3$). One rat was used per time point. Blood samples (500 μL) were collected in heparinized tubes at 1, 5, 15, 60, 180, 360, 540, 720, 1080 min after drug administration. The pharmacokinetic model and pharmacokinetic parameters of AZTC in rats were studied based on the data obtained.

3. Results

3.1. Chromatographic behavior and specificity

The maximum UV absorption of AZTC was 266 nm. Quality control solutions were injected and analyzed under the above chromatographic conditions. The endogenous components did not interfere with the analyte peak. The retention time of AZTC under the chromatographic conditions used was about 7.6 min and the %R.S.D. was 1.7. The retention time of internal standard was about 8.9 min. The chromatograms of (A) blank plasma, (B) blank plasma spiked with AZTC and internal standard (TN), (C) an extract of plasma obtained from a rat given liposomal AZTC (10 mg/kg) at 1 min are shown in Fig. 2.

3.2. Limits of detection and quantitation

Under the above conditions, the limit of detection was 0.1 μg/ml. The limit of quantitation was 0.4 μg/ml with relative standard deviation (R.S.D.) of 4.4%.

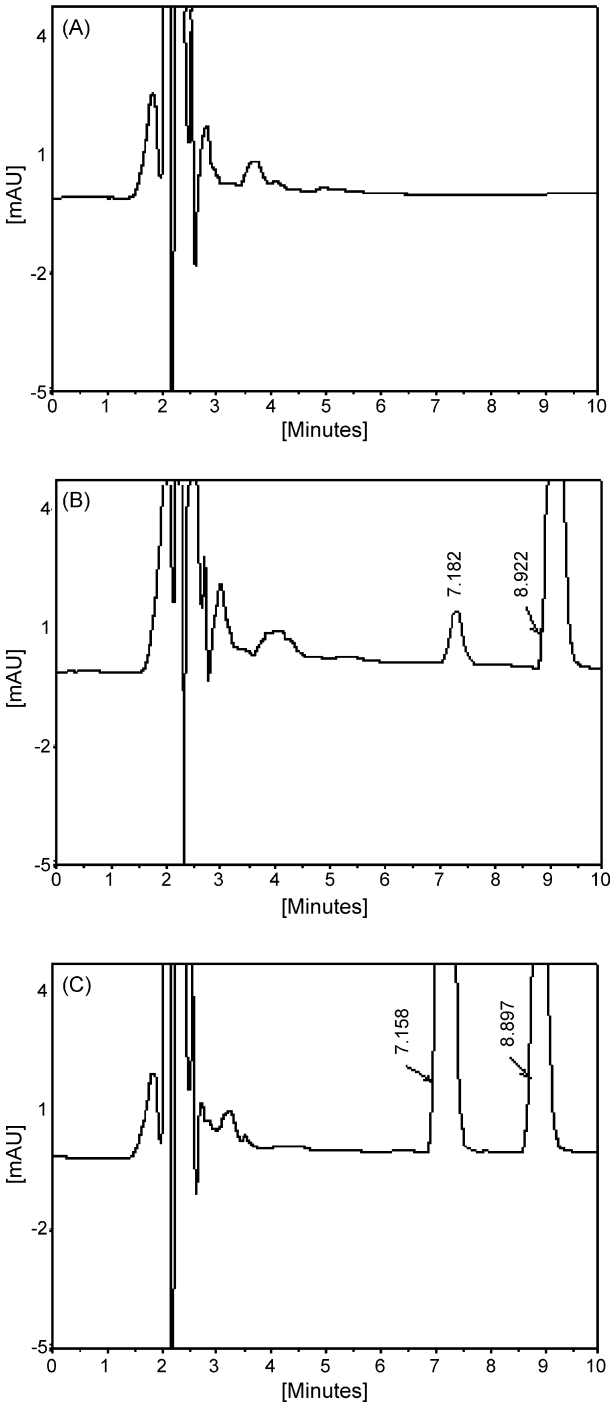

Fig. 2. Chromatograms of (A) blank plasma; (B) blank plasma spiked with AZTC (0.5 μg/ml) and internal standard; (C) an extract of plasma obtained from a rat 1 min after administered of liposomal AZTC (10 mg/kg).

3.3. Extraction recovery

The extraction recoveries of AZTC in plasma are presented in Table 1. All the recoveries were over 80.0%.

3.4. Linearity

The mean linear calibration curve was $y = 0.002254x + 3.763$, $r^2 = 0.9835$. The standard deviations of the slope and inter-

Table 1
The extraction recovery of AZTC in rat plasma ($n = 3$)

Sample	Concentration (μg/mL)	Extraction recovery (%)	R.S.D. (%)
AZTC	0.5	81.5 ± 2.55	3.13
	10	85.7 ± 2.37	2.77
	80	87.3 ± 2.61	2.99

Table 2
Intra-day precision and accuracy of the method for the determination of AZTC in rat plasma ($n = 3$)

Added concentration (μg/mL)	Found concentration (μg/mL)	R.S.D. (%)	Accuracy (%)
0.5	0.46 ± 0.04	8.70	−8.00
10	9.33 ± 0.34	3.64	−6.70
80	80.21 ± 1.80	2.24	0.26

cept of the regression line were 5.49×10^{-6} and 2.01×10^{-4}, respectively. The regression equation and determination coefficient (r^2) showed an good linearity over the concentration range 0.5–80 μg/mL.

3.5. Precision

The precision and accuracy of the method are shown in Tables 2 and 3. From the results given in these tables, precision and accuracy studies in rat plasma showed an acceptable R.S.D. values. The relative errors were lower than 10% and accuracy were high for both intra-day and inter-day studies.

3.6. Stability

In the studies, the analyte in methanol and plasma were investigated. The stock solution was stable for at least 8 week when stored at 4 °C (R.S.D. < 3.5%). The analyte in rat plasma were stable at −20 °C at least 2 weeks (R.S.D. < 6.9%).

3.7. Preliminary pharmacokinetics

The mean plasma concentration–time profile of AZTC for three rats receiving intravenous liposomal AZTC is shown in Fig. 3. AZTC elimination following a single dose of liopsomal AZTC exhibited a first-order kinetics according to the equation $C = C_0 e^{-kt}$, where C_0 is the concentration of AZTC at 0 min, C concentration of AZTC at any time point t, and k first-order rate constant expressed in units of concentration per hour. The basic pharmacokinetic parameters were calculated according to the equations for one compartment model, that is $t_{1/2} = \ln 2/k$, $V_d = X_0/C_0$, $AUC_{0\to\infty} = C_0/k$, $Cl = V_d k$.

Table 3
Inter-day precision and accuracy of the method for the determination of AZTC in rat plasma ($n = 5$)

Added concentration (μg/mL)	Found concentration (μg/mL)	R.S.D. (%)	Accuracy (%)
0.5	0.47 ± 0.03	6.38	−6.00
10	9.68 ± 0.29	3.00	−3.20
80	79.36 ± 1.54	1.94	−0.80

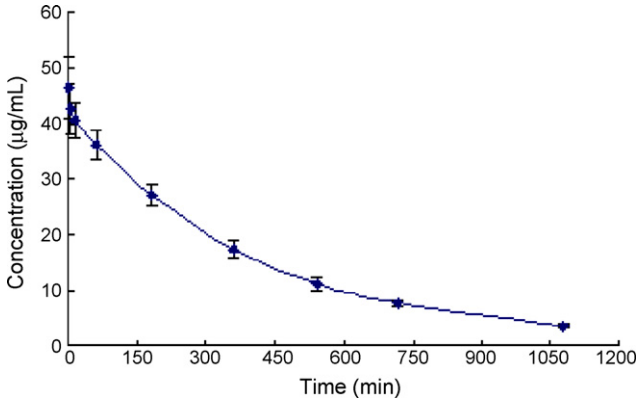

Fig. 3. Mean plasma concentration–time profile of AZTC for three rats receiving intravenous liposomal AZTC.

Table 4
Basic pharmacokinetic parameters of AZTC in rats after intravenous administration of liposomal AZTC (10 mg/kg)

Parameter	Result
$t_{1/2}$ (min)	307.5 ± 10.3
C_0 (μg/mL)	43.1 ± 1.5
k (min^{-1})	0.002254 ± 0.00072
$AUC_{0\to\infty}$ (μg min/mL)	19126 ± 1121.5
Cl (mL/min)	0.1271 ± 0.0087
V_d (mL)	56.3 ± 4.2

From the concentration–time profile (Fig. 3) and the pharmacokinetic parameters (Table 4), we found that concentration of AZTC in blood decreased dramatically after a single tail venous administration of the liposomal AZTC (10 mg/kg). The results indicate that AZTC has degraded and metabolized into its degradation product. By using another mobile phase methanol:water (65:35), we proved that one major degradation product of AZTC was AZT.

4. Discussion

In the present study, we have developed a rapid, sensitive and accurate RP-HPLC method with diode-array detection for the determination of AZTC in rats. It is a useful reference method for pharmacokinetic investigations of other prodrugs of zidovudine.

In this study, all kinds of chromatographic columns such as C_8, C_2, CN, NH_2 and Si were tried to separate AZTC to impurities and obtain an appropriate retain time of AZTC, but no satisfactory results were achieved. So we adopted non-aqueous reverse phase chromatography, that is the mobile phase consists of one organic solvent with strong polarity and another organic solvent with poor polarity [8–11]. In order to avoid exposing to diethyl ether or any potential explosion caused by it, mixtures such as methanol–acetonitrile, isopropanol–acetonitrile, acetonitrile–isopropanol–*n*-hexane, acetone–acetonitrile were used in non-aqueous RPLC, but we failed to obtain satisfactory separation and good peak shape. Finally we had to perform the

experiment on the basis of the present mobile phase methanol and diethyl ether.

It should be noted that zidovudine would be released after AZTC is administered to rats, so the determination of AZT in plasma could gave information on the metabolism and degradation of AZTC in vivo. However, according to our experiments, AZT has a very different chromatographic behavior from that of AZTC, which makes it difficult to analyze both compounds under the same chromatographic conditions. For this reason, we have tried many different kinds of chromatographic column, such as C_8, CN and NH_2 columns, but unfortunately all failed to produce a good AZT or AZTC peak because of the interference of the solvent peak with AZT peak. We also carried out a preliminary investigation of a gradient elution method, but we had a serious problem obtaining a stable baseline.

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