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Short communication

Simultaneous determination of 3′-azido-2′,3′-dideoxyuridine and novel prodrugs in rat plasma by liquid chromatography

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Abstract

3′-Azido-2′,3′-dideoxyuridine (AZDU) is a nucleoside analog structurally similar to zidovudine (AZT) with proven activity against human immunodeficiency virus (HIV). The purpose of this study was to develop and validate a high-performance liquid chromatographic (HPLC) method to quantitatively determine AZDU and its novel prodrugs in rat plasma simultaneously. A reversed-phase gradient elution HPLC method was developed to quantitate AZDU and its prodrugs, N_3-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (I), 5′-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (II), 5′-*O*-valinyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (III) and 5′-*O*-phenylalanyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (IV), in rat plasma. AZDU and its prodrugs were analyzed using an octadecyl silane column with a mobile phase consisting of 0.04 μM sodium acetate buffer, pH 5.0, and acetonitrile, running in a segmented gradient manner at a flow rate of 2 ml/min. Acetonitrile was increased from 10 to 50% during the first 8 min by 5% per min, followed by 10% per min until it reached 90% acetonitrile. 3′-Azido-2′,3′-dideoxy-5-ethyluridine (CS-85) was used as an internal standard (25 μg/ml). Compounds were detected by UV absorption at 261 nm. Extraction recoveries for all compounds were greater than 80%. Retention times of AZDU, CS-85, prodrugs I, II, III and IV were 3.3, 5.2, 9.1, 8.8, 6.3 and 7.3 min, respectively. Calibration plots were linear over the range of 0.25–100 μg/ml for AZDU and prodrugs II, III, and IV and 0.5–100 μg/ml for prodrug I. The limit of quantitation was 0.25 μg/ml for prodrugs II, III and IV and 0.5 μg/ml for prodrug I. The intra- and inter-day variations were less than 10% and accuracies were greater than 90%. This method is rapid, sensitive and reproducible for the determination of AZDU and prodrugs in rat plasma.

Keyword: 3′-Azido-2′,3′-dideoxyuridine

1. Introduction

Acquired immunodeficiency syndrome (AIDS) first diagnosed in 1981 [1–3], has become a global epidemic. While major advances have been made in the understanding of the disease and clinical treatments, there is currently no cure for AIDS. Therefore, the search for highly effective antiviral agents with low toxicity continues.

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3′-Azido-2′,3′-dideoxyuridine (AZDU, CS-87) is a synthetic nucleoside analog structurally similar to zidovudine (AZT) with proven activity against human immunodeficiency virus (HIV) and less bone marrow toxicity compared to AZT [4,5]. However, the clinical application of AZDU is limited by its relatively short half-life, extensive glucuronidation in patients and insufficient delivery to the brain and lymphatic system. Thus, research has focused on the discovery of prodrugs of AZDU with improved pharmacokinetic properties such as increased oral bioavailability, longer half-life and enhanced brain and lymphatic delivery. Previous studies have reported that prodrugs of AZDU through esterification of the 5′-OH function with various aliphatic acids showed improved pharmacokinetic distribution such as enhanced brain and lymphatic delivery and extended half-lives compared to that of parent drug AZDU [6]. Further, it has been demonstrated that the 5′-L-valyl ester of acyclovir (L-Val-ACV) enhanced the uptake of acyclovir (ACV) 10-fold as compared to the parent drug [7]. L-Val-ACV was shown to be a substrate for the oligopeptide transporter in Caco-2 cells, which may explain its improved absorption characteristics [8]. Based on these results, four compounds, N_3-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (I), 5′-*O*-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (II), 5′-*O*-valinyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (III) and 5′-*O*-phenylalanyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (IV), were synthesized as potential prodrugs of AZDU (unpublished results). The chemical structures of the compounds are illustrated in Fig. 1.

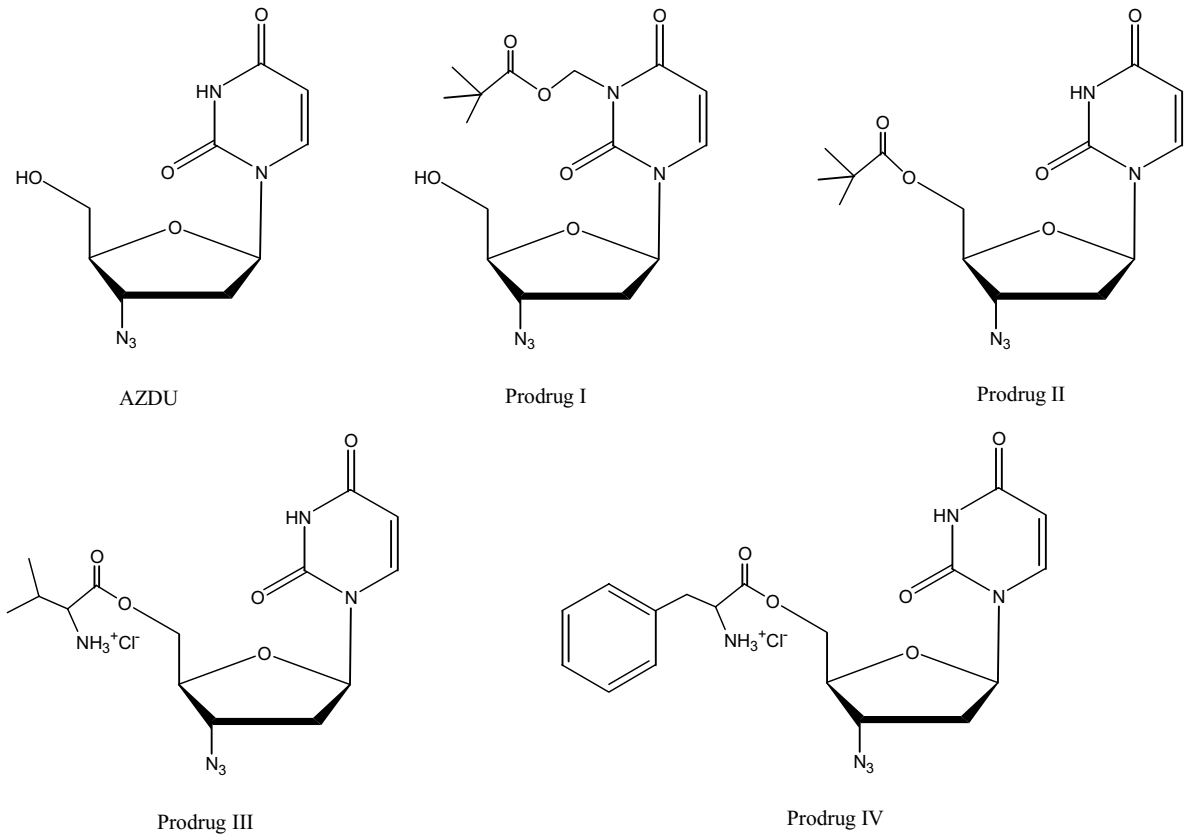

Fig. 1. Chemical structures of AZDU and prodrugs N_3-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (I), 5′-*O*-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (II), 5′-*O*-valinyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (III) and 5′-*O*-phenylalanyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (IV).

To investigate the preclinical distribution and metabolism of these prodrugs, a method for the simultaneous quantitation of the prodrugs and parent compound, AZDU, is required. High-performance liquid chromatographic (HPLC) techniques have been reported for the determination of AZDU and other prodrugs [9–13]. The objective of the present study was to develop a simple, accurate and sensitive HPLC method for the quantitation of AZDU and its potential prodrugs, N_3-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (I), 5′-*O*-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (II), 5′-*O*-valinyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (III) and 5′-*O*-phenylalanyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (IV), suitable for preclinical pharmacokinetic studies in rats.

2. Experimental section

2.1. Chemicals

3′-Azido-2′,3′-dideoxyuridine (AZDU), internal standard, 3′-azido-2′,3′-dideoxy-5-ethyluridine (CS-85), and prodrugs I, II, III and IV (Fig. 1) were synthesized in our laboratories (unpublished results). The chemical purity of each compound, as assessed by HPLC and spectral analysis, was greater than 98%. Acetonitrile, HPLC grade, and other chemicals, analytical grade, were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Preparation of standards

Standard solutions of 1.0, 10, and 100 μg/ml of the compounds were prepared in distilled water. Standard solutions were added to rat plasma to obtain calibration concentrations of 0.25, 0.5, 1, 5, 10, 20, 50, 80, 100 μg/ml. Standard solutions of all compounds were stable for at least 8 h in water and at least 2 h in rat plasma.

2.3. Extraction procedure

Plasma standards and unknown samples were added to 1.5 ml polypropylene centrifuge tubes, followed by 50 μl of internal standard, CS-85 (25 μg/ml). Ice-cold acetonitrile (0.9 ml) was added to precipitate plasma proteins. The tubes were vigorously mixed for 1 min and centrifuged for 10 min at 9000 × *g*. The supernatant was transferred to a polypropylene centrifuge tube, and excess crystalline magnesium sulfate was added. After mixing for 3 min, the tubes were centrifuged for 10 min at 9000 × *g*. The supernatant was transferred to a clean polypropylene tube and evaporated to dryness under a stream of nitrogen gas at ambient temperature. The residual film was reconstituted in 200 μl of water and transferred to a disposable 300 μl polypropylene injection tube. Fifty microliters were injected onto HPLC.

2.4. Chromatography

The HPLC system consisted of a Shimadzu LC-10A solvent delivery system, a Shimadzu SCL-10A system controller, a Shimadzu SPD-10A UV-Vis detector, SIL-10A auto injector and CR-501 chromatopac integrator. Concentrations of AZDU and its prodrugs in plasma were determined by reversed-phase high-performance liquid chromatography using an octyldecyl silane column (4.6 mm i.d. × 15 cm, 5 μm particle size; Alltech Associates, Deerfield, IL) protected by a Nova-Pak C18 guard column with particle size 4 μm at room temperature. The mobile phase consisted of solvent A, 0.04 M sodium acetate buffer, pH 5.0, and solvent B, acetonitrile. A segmented gradient elution was used to separate compounds at a flow rate of 2 ml/min. During the first 8 min, the concentration of acetonitrile increased from 10 to 50% by 5% per min and then by 10% per min until it reached 10% buffer (A) and 90% acetonitrile (B). After each analysis, the column was equilibrated for 10 min to initial conditions. Compounds were detected by UV absorption at 261 nm.

2.5. Quantitation

Concentrations of AZDU and prodrugs in unknown samples were determined from the slope of calibration plots of the peak-area ratio of AZDU or prodrug/internal standard versus the calibration standard AZDU or prodrug concentrations. Slopes were determined using linear regression analysis with a weighting factor of $1/x^2$.

2.6. Assay specifications

The extraction recoveries of AZDU and prodrugs I, II, III and IV were assessed at plasma concentrations of 0.5, 5.0 and 50 μg/ml. The recovery of internal standard was assessed at 25 μg/ml at which it was used for the assay. Three plasma samples (100 μl) containing drug and internal standard were extracted and injected. Three injections of the same amount of compound in water were directly injected. The peak areas of the compounds were measured and the percentage recovery was calculated from $100 \times (\text{peak area}_{\text{extraction}}/\text{mean peak area}_{\text{direct injection}})$.

The intra- and inter-day precision and accuracy of the analytical method were determined by analysis of six plasma samples containing 0.5, 5.0, 50 μg/ml concentrations for five compounds. Assay precision was determined by calculating relative standard deviations (R.S.D.) for each drug concentration. Accuracy was calculated by comparing measured concentrations to the known values. The limit of quantitation was calculated as the lowest compound concentration producing a signal-to-noise response ratio of at least 3 with an accuracy of at least 90% and precision at least 10%.

3. Results and discussion

The purpose of this study was to develop an HPLC analytical method for the simultaneous determination of AZDU and its prodrugs I, II, III, IV, in rat plasma. The method employed a protein precipitation

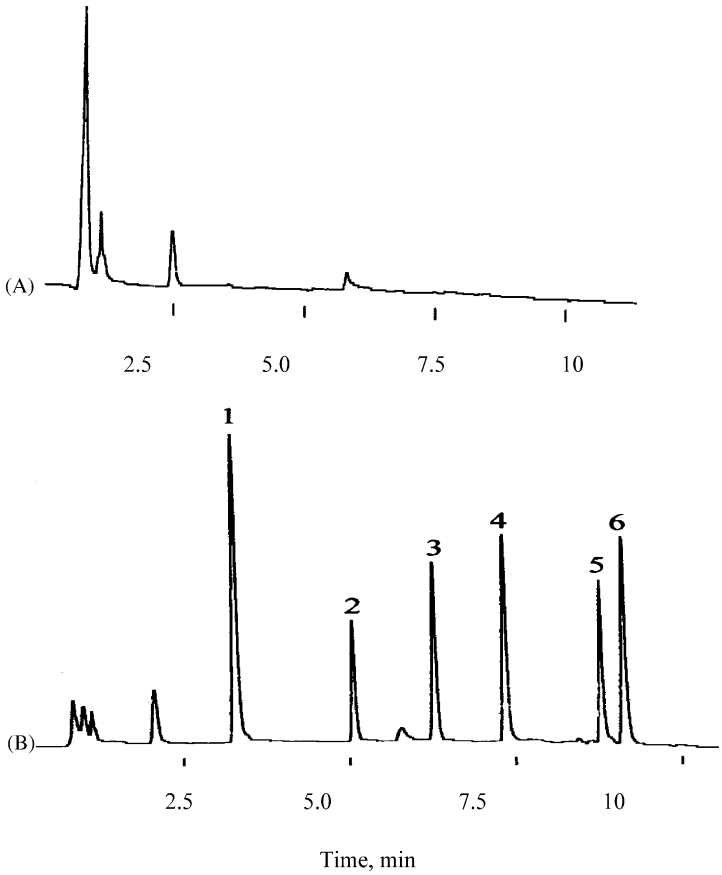

Fig. 2. Representative chromatograms for (A) blank plasma and (B) rat plasma samples with (1) AZDU, (2) internal standard (CS-85), (3) prodrug III, (4) prodrug IV, (5) prodrug II, and (6) prodrug I.

extraction procedure with slight modifications [10,13] for AZDU and its prodrugs as well as the internal standard, CS-85. AZDU and its prodrugs have a wide retention range. Under isocratic conditions, AZDU tends to be an early elute and prodrugs, especially I and II, are late elutes. Thus, to measure the parent drug, AZDU, and its prodrugs simultaneously, a gradient elution method was developed. In this method, a binary-solvent mobile phase consisted of 40 mM sodium acetate buffer (pH 5.0) and acetonitrile was used throughout the separation, with the concentration of acetonitrile (B%) increasing from 10 to 90% in a segmented fashion during each run. This method provided appropriate retention times for both AZDU and its prodrugs, while good separation was achieved.

Fig. 2 illustrates chromatograms corresponding to the extracts of blank rat plasma and rat plasma with AZDU, prodrugs I–IV and internal standard added at a concentration of 50 μg/ml. Each compound eluted with a sharp peak and distinct separation at baseline. The retention times of AZDU, CS-85, prodrugs I, II, III and IV were 3.3, 5.2, 9.1, 8.8, 6.3 and 7.3 min, respectively. Blank plasma was free of interferences at the retention times corresponding to the compounds of interest.

The limit of quantitation was 0.25 μg/ml for prodrugs II, III and IV and 0.5 μg/ml for prodrug I. Calibration plots were linear over the range of 0.25–100 μg/ml for compounds AZDU, II, III, IV and CS-85, and 0.5–100 μg/ml for prodrug I. The assay specifications including extraction recovery, intra- and inter-day precision and accuracy for AZDU and its prodrugs at 0.5, 5.0 and 50 μg/ml concentrations are presented in Table 1. The extraction recoveries for AZDU, all prodrugs and internal standard were greater than 80%. The precision of the assay was acceptable with relative standard deviations less than 10%. The accuracy of the method was greater than 90% for all the compounds at low, medium, and high concentrations.

In summary, the determination of AZDU and its prodrugs I, II, III and IV in rat plasma by the HPLC method described is simple, rapid, sensitive and reproducible. The limit of quantitation of this method is sufficiently sensitive to characterize the preclinical pharmacokinetics of the prodrugs and their biotransformation to AZDU in rats.

Table 1
Assay specifications for the simultaneous determination of AZDU and its prodrugs N_3-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (I), 5′-pivaloyloxymethyl-3′-azido-2′,3′-dideoxyuridine (II), 5′-*O*-valinyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (III) and 5′-*O*-phenylalanyl-3′-azido-2′,3′-dideoxyuridine hydrochloride (IV)

Compound	Concentration (μg/ml)	Recovery (%)	Precision (%)		Accuracy (%)	
			Intra-day	Inter-day	Intra-day	Inter-day
AZDU	0.5	95.2 ± 1.5	2.22	1.10	92.10	95.98
	5.0	94.6 ± 2.8	1.02	7.34	97.55	100.00
	50.0	90.4 ± 1.0	5.65	1.20	92.33	98.77
I	0.5	93.1 ± 5.2	7.98	9.49	96.59	90.03
	5.0	94.4 ± 3.7	9.19	8.23	95.56	92.48
	50.0	94.8 ± 5.7	6.75	8.07	99.30	97.79
II	0.5	82.2 ± 8.9	1.98	7.11	97.69	94.85
	5.0	91.1 ± 5.2	0.48	6.72	94.70	93.93
	50.0	97.1 ± 1.5	3.01	6.38	99.00	93.11
III	0.5	81.2 ± 6.8	7.31	3.37	95.43	92.78
	5.0	92.2 ± 1.1	0.68	2.85	98.90	97.87
	50.0	92.5 ± 4.2	3.51	6.93	99.52	92.81
IV	0.5	84.7 ± 6.3	3.70	7.05	92.96	93.73
	5.0	91.1 ± 2.9	2.63	7.21	97.66	96.10
	50.0	92.4 ± 1.8	1.13	3.12	92.61	96.21

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