Use of Accelerator Mass Spectrometry to Measure the Pharmacokinetics and Peripheral Blood Mononuclear Cell Concentrations of Zidovudine

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ABSTRACT: The remarkable sensitivity of accelerator mass spectrometry (AMS) is finding many new applications in pharmacology. In this study AMS was used to measure [^{14}C]-Zidovudine (ZDV) concentrations at the drug's site of action (peripheral blood mononuclear cells, PBMCs) following a dose of 520 ng (less than one-millionth of the standard daily dose) to a healthy volunteer. In addition, the pharmacokinetics of this microdose were determined and compared to previously published parameters for therapeutic doses. Microdose ZDV pharmacokinetic parameters fell within reported 95% confidence intervals or standard deviations of most previously published values for therapeutic doses. Blood, urine, stool, saliva, and isolated PBMCs were collected periodically through 96 h postdose and analyzed for ZDV and metabolite concentrations. The results showed that ZDV is rapidly absorbed and eliminated, has one major metabolite, and is sequestered in PBMCs. ^{14}C mass balance assessments indicated a significant portion of ZDV remained after 96 h with a much prolonged elimination half-life. Results of this study demonstrate the usefulness of microdosing and AMS as a tool for studying the pharmacokinetic characteristics, including PBMC concentrations, of ZDV and underscore the value of AMS as a tool with which to perform pharmacokinetic and mass balance studies using trace amounts of radiolabeled compound. J Pharm Sci

Keywords: AZT; accelerator mass spectrometry; AMS; isotope; human; ZDV; microdosing; microdose; absorption; kinetics; pharmacokinetics; leukocytes; cell loading

INTRODUCTION

Accelerator mass spectrometry (AMS) was developed 30 years ago in nuclear accelerator laboratories to directly count individual ions of rare isotopes at natural levels of abundance. In AMS, atomic ions are accelerated at very high energy (often millions of electron volts) and resolved at unit resolution using simple dipole mass spectrometry. Acceleration to million electron volt energies provided an analysis so sensitive that it was only appropriate for isotopes having concentrations of parts per billion to

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parts per quadrillion in an isolated elemental sample.[1]

Sensitive quantification of one isotope in particular, carbon-14 (^{14}C), had an immediate impact on the field of carbon dating. Prior to AMS, carbon dating was exclusively done by radioactive decay counting.[2] Decay counting of long-lived isotopes such as ^{14}C (5730 years), however, is an inefficient means of detection; as a result, large sample masses (10's of grams) and extended count times (weeks) were needed. As an example, to obtain 10000 counts (a count being a radioactive decay event) in a one disintegration per minute sample requires 6.94 days of uninterrupted counting (even though a 1 dpm sample contains >4.35 billion ^{14}C atoms or 7.2 fmol ^{14}C). In contrast, AMS measures 10000 counts (here a count being the collision of the accelerated atom with the detector) through direct ion counting in as little as 30 s. Thus AMS quantifies ^{14}C as stable mass isotope, providing subattomole detection sensitivity from milligram-sized samples.[3]

AMS changed radiocarbon dating through enabling rapid quantification of trace radioactivity in small samples and the same change is taking place in biosciences and pharmaceutical development. Using a radioactive isotope such as ^{14}C is not new to drug development: clinical Absorption, Distribution, Metabolism, Elimination (ADME) studies frequently use a ^{14}C-labeled tracer with liquid scintillation detection as a means of quantifying uptake and route of elimination of a drug without specific knowledge of its molecular structure. However, AMS enables these same evaluations with at least a 1000-fold reduction in radioactive dose applied to the human test volunteers. Indeed, AMS can virtually eliminate radiation exposure issues associated with the administration of radioactivity to humans as typical radioactive doses are a few pCi/g (typical <100 nCi for a 70 kg person) for long duration tracing (weeks or months);[4,5] radiation risks are much lower than commonly accepted risks such as air flight.[1] This opens up sensitive subpopulations to ethical tracer studies (i.e., infants and even pregnant women).

The high sensitivity of AMS towards the ^{14}C radiolabel also empowers a new clinical paradigm known as microdosing. Microdosing involves the administration of doses that are deemed too small to elicit harm to healthy test volunteers. The Food and Drug Administration (FDA) has defined it in a January 2006 guidance document addressing exploratory Investigational New Drug Applications (exIND). Under this guidance [http://www.fda.gov/cder/guidance/7086fnl.htm], human pharmacokinetic parameters of new drug candidates can be studied using doses of either <100 μg or $<1/100$ of the expected pharmacologically active dose. The exIND provides a mechanism to obtain key human absorption and pharmacokinetic data on chemical entities with a very abbreviated (and low cost) animal toxicology package prior to introduction in man. It is a human screening tool expected to complement the battery of animal, *in vitro*, and *in silico* tools now applied in lead optimization and clinical candidate nomination. This concept has created a new clinical category, dubbed Phase 0, which precedes the traditional, IND driven, Phase 1 testing. In the majority of cases, AMS is the only analytical detector capable of quantifying microdoses *in vivo*.

Zidovudine (ZDV), generically known as azidothymidine (AZT), is a reverse transcriptase inhibitor with antiviral activity against human immunodeficiency virus (HIV-1 and HIV-2) and other retroviruses.[6,7] The US FDA approved ZDV in 1987 for the treatment of patients with HIV infection, and it remains a component of many recommended initial treatment regimens.

The ordinary adult ZDV dosage for patients with HIV ca. 600 mg per day, which is commonly administered on a long-term basis as an oral 300 mg b.i.d. dose. ZDV is rapidly and well absorbed after oral administration, with approximately 54–75% bioavailability. ZDV is widely distributed throughout the body with 20–38% protein binding. The plasma half-life ($T_{1/2}$) of ZDV is approximately 0.8–1.5 h and concentrations in saliva are about the same as blood levels.[8] ZDV undergoes hepatic metabolism to its 5′-*O*-glucuronide metabolite (GZDV, also called GAZT), which lacks anti-HIV activity (see Fig. 1). Both ZDV and GZDV are eliminated through the kidneys, with urine recovery of ZDV and GZDV accounting for 14% and 74%, respectively, of the dose following oral administration.[8]

ZDV diffuses into peripheral blood mononuclear cells (PBMCs) passively, where it is triphosphorylated by intracellular kinases.[9] It is the intracellular anabolite that competes with endogenous nucleotides for incorporation into the replicating HIV DNA, resulting in termination of viral replication.[10] Phosphorylation decreases the membrane permeability of ZDV, thereby sequestering it inside the cell, and is a rate-limiting step in the anti-HIV effectiveness of ZDV.[10] Uptake and intracellular phosphorylation of ZDV by

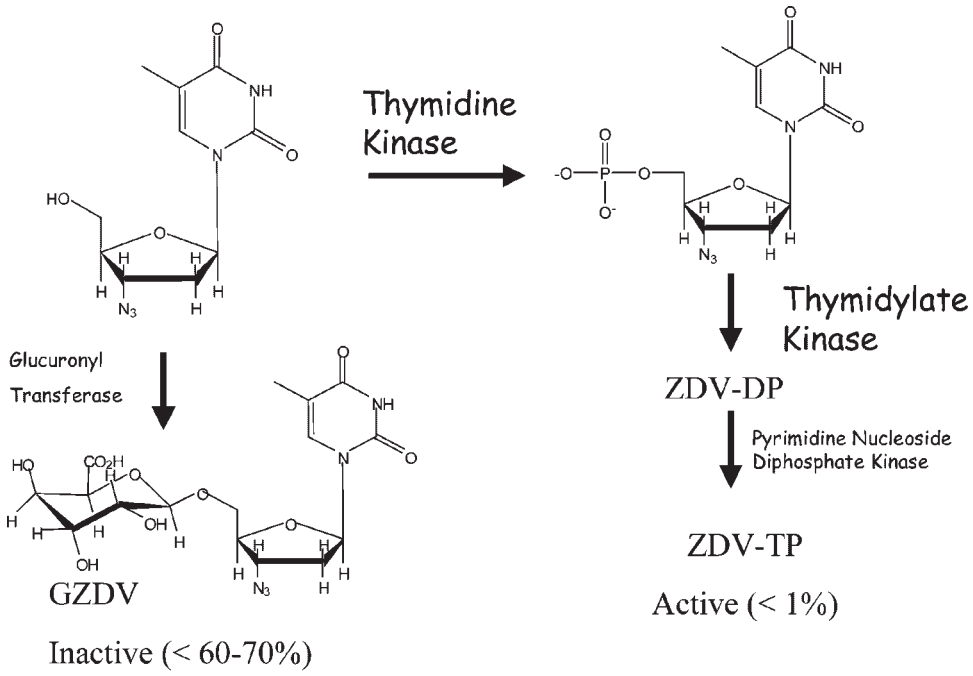

Figure 1. The chemical structure of ZDV and its primary pathways of metabolism. A majority of ZDV in the circulation is processed by glucuronyl transferase in the liver, resulting in the inactive compound GZDV that is eliminated through the kidneys. A minor portion of ZDV is taken up by PBMCs and phosphorylated by thymidine kinase and thymidylate kinase to the active antiviral compound ZDV-triphosphate.

PBMCs are highly variable and are not correlative with plasma and extracellular ZDV concentrations, ZDV dose, or exposure. As a result, intracellular concentrations of ZDV, particularly its triphosphate form are viewed as a superior marker of ZDV efficacy (as well as toxicity).[11] Because less than 1% of the total administered ZDV dose is found in PBMCs, the clinical study of the control of ZDV uptake and phosphorylation requires highly sensitive analytical methodology.

The present study was carried out to demonstrate the utility of AMS in the study of human pharmacokinetics and mass balance of a microdose (520 ng) of ZDV six orders of magnitude smaller than conventional therapeutic doses. We hypothesized that the plasma and PBMC pharmacokinetic parameters of this microdose would be linear with those previously determined for therapeutic doses. Using the microdose approach with one male subject, the metabolic and PBMC disposition of ZDV was determined after oral ingestion of a single microdose (520 ng, 100 nCi). Analysis of total excreta, plasma kinetics and rates of elimination were determined through direct measurement of ^{14}C levels. Specific parent and metabolite information were generated through fractionation of plasma specimens using liquid chromatographic (LC) techniques prior to ^{14}C-AMS analysis.

MATERIALS AND METHODS

Chemicals

^{14}C-labeled ZDV ([2-^{14}C]-3′-azido-3′-deoxythymidine; 53 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA) in an ethanol suspension with radiochemical purity of >99%. The product was reduced to dryness under streaming nitrogen and brought up in sterile water to a concentration where 5 mL of formulation would deliver 520 ng of ZDV, or 3.784 kBq (102 nCi) of radioactivity. This dose aliquot was transferred to a polypropylene cryogenic tube and stored frozen at −60°C until shipment to the clinical facility. The material was shipped on dry ice and stored at −60°C until the morning of dosing. ^{14}C content of all chemicals used in preparation of the drug were determined by AMS prior to use.

Unlabeled ZDV and ZDV glucuronide (3′-azido-3′-deoxythymidine β-D-glucuronide sodium salt;

GZDV) were purchased from Toronto Research Chemical, Inc. (North York Ontario, Canada). Tributyrin (glycerol tributyrate) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and dissolved in methanol at a concentration of 50 mg/mL for use as low ^{14}C carbon diluent (referred to as tributyrin diluent hereafter). All solvents and other chemicals unless otherwise noted were obtained from VWR (Brisbane, CA).

Study Conduct and Subject Selection

This study was conducted in accordance with the ethical standards that have their origin in the Declaration of Helsinki and the ICH-GCP Guidelines of April 1, 2001, and the US 21 Code of Federal Regulations addressing clinical studies. The study protocol and informed consent form were approved prior to commencement by an independent investigational review board (Aspire Independent Review Board, San Diego, CA). Informed consent was obtained from the study subject prior to commencement of any study procedures.

The subject was a healthy nonsmoking male, 34 years old, with a body mass index of 24.5 kg/m^2. He reported no medical history of prior participation in a clinical trial with a ^{14}C-labeled compound or other excessive exposure to radiation, no history of gastrointestinal, hepatic, or renal abnormalities, no participation in any other clinical trial within 30 days prior to enrollment in this study, and no use of any prescription or over the counter medications within 72 h prior to administration of the study drug. Clinical laboratory tests for chemistry, complete blood count, and urinalysis were all within normal ranges, and tests for hepatitis A and C, HIV, and urine screen for illicit drugs were all negative. The prestudy physical examination, including vital signs and ECG, were also normal. The study subject fasted from 8 h prior to study drug administration until 4 h postdose. Caffeine was restricted to less than the equivalent of two cups of coffee from 24 h prior study drug administration until completion of the last study procedure. No concomitant medications were administered during the study.

Dose Administration

Dose administration and sample collections were carried out at the Covance Clinical Research Center, Honolulu, HI. The dose aliquot was thawed on the morning of administration. The participant consumed the entire volume of the dose aliquot as well as two 5 mL rinses of the container with purified water, followed by another 240 mL of water from a clean cup.

Sample Collections

Blood was collected predose, at 15, 30, 45 min and at 1, 1.5, 2, 3, 4, 6, 9, 12, 16, 24, 48, 72, and 96 h postdose (17 draws, 340 mL total blood volume). Four 5 mL aliquots were collected into EDTA Vacutainer® tubes at each time point. Plasma from two 5 mL aliquots of blood were committed to total ^{14}C; ZDV parent and metabolite analysis while PBMCs were isolated from the remaining tubes as described below.

Single urine and fecal specimens were obtained predose for determination of "background" levels of ^{14}C. Cumulative urine voids were collected and pooled by intervals of 0–3, 3–6, 6–12, 12–24, and 24–48 h postdose while the subject was confined to the research unit. The subject was given instruction on urine collection techniques and provided with containers to collect all urine from 48–72 and 72–96 h postdose. Cumulative fecal voids were collected and pooled in 24-h intervals from 0 to 96 h postdose, with use of appropriate outpatient containers from 48 to 96 h. All urine and fecal specimens were weighed in tared containers and stored frozen after collection ($-20^\circ C$). Urine and stool samples were refrigerated during collection intervals while the subject was confined to the clinic, but no special precautions were taken to keep the specimens chilled during the outpatient collection periods.

Saliva samples were obtained at 0, 1, 2, 3, 4, and 6 h postdose by voluntary expectoration. A 15-min water restriction was imposed prior to each saliva collection. The subject was asked to expectorate into clean 50-mL polypropylene conical tubes. Each sample was collected within 3 min of the nominal time, with a minimum of 0.5–1.0 mL of saliva collected at each time point.

Sample Processing

Plasma for ZDV analysis was separated by centrifugation of the blood samples for 10 min at 1500*g* and transferred to cryogenic vials for storage at $-60^\circ C$ within 60 min of collection from the subject.

Complete blood counts with differential were obtained at baseline and 96 h following dose using a Beckman Coulter LH 750B counter (Clinical Laboratories of Hawaii, Honolulu, HI).

PBMCs were harvested using commercially available Accuspin lymphocyte prep tubes (Sigma, St. Louis, MO). Briefly, whole blood (10 mL in EDTA vacutainers) was poured into the upper chamber of the Accuspin System-Histopaque tubes and centrifuged at 100*g* at 20°C for 10 min. Following discard of the supernatant and three centrifugation/wash cycles of the mononuclear cell pellet with phosphate buffered saline, the pelleted cells were then resuspended in 500 μL of 2-propanol and water (50:50) and dispersed by vortex action. A 125-μL aliquot (1/4 of the volume) of the suspension was transferred to a quartz vial containing 125 μL of tributyrin diluent. Samples were processed for AMS as described below (^{14}C Quantification by AMS). The remaining material was capped and placed in storage at −60°C.

Total fecal samples (24-h collections) were diluted 1 part stool to 5 parts (w:v) homogenization solution (2-propanol:1 N KOH in water; 50:50) in a sealed container and shaken for 20 min at high speed (10 cycles per second) to disperse the stool. A 200 μL aliquot was added to 1.6 mL of tributyrin diluent with mixing. Urine samples were prepared by addition of 200 μL of urine to1.6 mL of tributyrin diluent with mixing. Aliquots (30-μL) of diluted stool or urine samples were transferred to quartz vials for AMS processing. Aliquots of neat plasma (30 μL) and saliva (60 μL) were similarly transferred to quartz vials and processed without admixture.

Ultra-Performance Liquid Chromatography and Analysis

Plasma samples were prepared for LC separation as follows. Plasma (100 μL) was added to acetonitrile (200 μL) in a 1.5 mL polypropylene microfuge tube. The microfuge tube was capped, mixed by vortex action for 30 s and centrifuged (3000*g* for 10 min). The supernatant (150 μL) was quantitatively transferred to a clean microfuge tube and concentrated to dryness under reduced pressure. The residue was reconstituted in 100 μL of Mobile Phase A to which was added 10 μL of an equimass standard solution of unlabeled ZDV and GZDV (2.5 mg/mL each in Mobile Phase A) for cochromatography. The sample was syringe filtered (0.45 μm PVDF membrane, Acrodisc®) and placed in capped sample vials for LC analysis.

The LC apparatus (Waters Acquity™ Ultra Performance Liquid Chromatograph; UPLC) was fitted with an Acquity UPLC C18 column (2.1 mm × 50 mm, 1.7 μm particle size). Gradient separation was achieved using a binary solvent system: Mobile Phase A consisted of 0.1% trifluoracetic acid in water/acetonitrile (95:5); Mobile Phase B consisted of 0.1% trifluoracetic acid in acetonitrile. The flow composition was held at 95:5 A/B for 2 min followed by a linear gradient to 50:50 over 1 min. The flow rate was held at 0.5 mL/min for the duration of the analysis. Column effluent was monitored at 254 nm.

Eluent fractions from the UPLC effluent were collected at regular intervals (12 s) to establish full radiochromatograms at the 45-min and 1-h time points. Full radiochromatograms were not collected at the other time points, yet the specific eluent fractions coinciding with ZDV and GZDV based upon UV monitoring of the column effluent were collected. To ensure quantitative recovery, the fraction window was 3 s wider on the front and back end of the UV trace peak being monitored with the software. All eluent fractions were collected in quartz sample vials. The fractions were supplemented with 440 μL of tributyrin diluent in methanol, and taken to dryness under vacuum.

^{14}C Quantification by Accelerator Mass Spectrometry

The specimen-containing vials were dried under reduced pressure and processed to graphite using the procedure described by Ognibene et al.[12] Background samples containing only tributyrin were prepared in triplicate for baseline ^{14}C determination. Processing of samples for AMS determinations of ^{14}C was carried out by Vitalea Science, Inc. (Woodland, CA). Dried specimens (UPLC fraction, plasma, urine, stool, mononuclear cells, DNA) were reduced to filamentous graphite according to published procedures.[12] The resulting graphite/cobalt mix was pressed into aluminum cathodes and analyzed for ^{14}C/C using either a 1 MV NEC compact Pelletron (housed at Lawrence Livermore National Lab, Livermore, CA) or a 200 kV Bio-MICADAS (Paul Scherrer Institut, Zurich, Switzerland). Both machines delivered similar results based upon repeat measurements of duplicated specimens.

Typical AMS measurement times were <3 min/sample with a counting precision of ≤2%. The assay precision was determined from the amount of scatter in the results obtained from the analysis of individually prepared replicates ($N \geq 3$) of plasma, urine, and stool and averaged about 4% for neat specimens and <7% for processed specimens (PBMCs, LC fractions). The $^{14}C/^{13}C$ ratios of unknowns were normalized to measurement of identically prepared standards of known isotope concentration (Oxalic acid, NIST 4990C). A limit of quantification (LOQ) for neat analyses was defined as five times the standard deviation of the assay imprecision associated with predose samples.

Data Analysis

Excess ^{14}C concentrations over natural abundance (or background) were converted to ^{14}C-ZDV equivalents using the specific activity of the ZDV and carbon content of the fractions. The amounts of ^{14}C excreted in urine or feces were expressed as a percentage of the administered dose of ^{14}C. Pharmacokinetic parameter analysis was performed by noncompartmental analysis using WinNonlin (v 4.1; Pharsight Corp., Mountain View, CA). Area under the (concentration-time) curve (AUC) values for the ZDV and GZDV were determined using the trapezoidal rule (linear interpolation) from time zero to infinity. Plasma peak concentrations (C_{max}) and times were determined directly from the data points. Rate constants (λ_z) for the elimination phase were estimated by linear regression analysis of semi-log plots of the data with the slope of the line equal to λ_z. Half-lives ($T_{1/2}$) were calculated as $\ln 2/\lambda_z$.

RESULTS

Subject Enrollment

A single male volunteer in good health was chosen for this experiment and a summary of his characteristic and biochemical data are provided in Table 1. The subject reported no adverse events during the course of the study and no clinically significant abnormal clinical laboratory results were observed.

UPLC Separation of ZDV and GZDV in Plasma

Plasma samples collected through the 3 h time point were fractionated *via* UPLC. GZDV eluted from the column in the 1.2-min fraction while ZDV was recovered in the 1.6-min fraction. Representative ^{14}C measurements in fractionated plasma from the 0.75 and 1.5 h time points are shown in Figure 2.

Table 1. Biochemical and Subject Characteristics

	Baseline	96 h
Age	34	
BMI (kg/m^2)	24.5	
Height (m)	1.78	
Weight (kg)	77.4	
WBC (10^9/L)	7.2	6.2
% Lymphocytes	34	42
Absolute count (10^9/L)	2.5	2.9

Plasma Pharmacokinetics

Plasma concentrations of ZDV and GZDV over the 3 h following dose administration are shown in Figure 3. Concentrations were obtained by AMS measurement of ^{14}C in UPLC fractions previously determined to contain either ZDV or GZDV. Peak ZDV concentrations were measured to be 12.6 fM (3.25 pg/mL) at 0.5 h, while peak GZDV concentrations were measured to be 28.1 pM (12.44 pg/mL) at 0.75 h. Concentrations at 3 h following dose administration were 5% and 12% of peak values

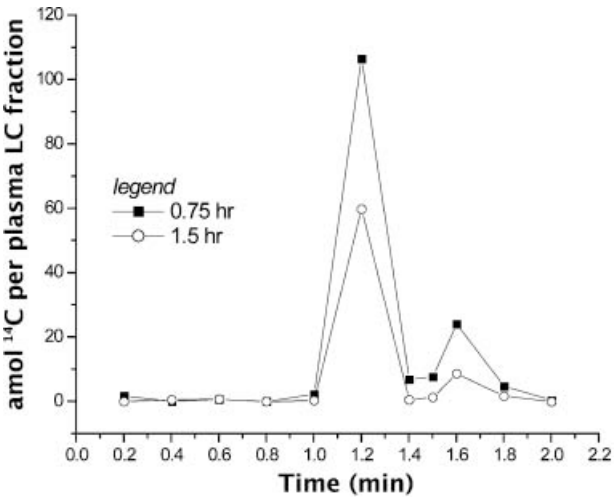

Figure 2. Full radiochromatogram illustrating total ^{14}C recovered from plasma precipitates of samples collected at the 0.75 h (filled squares) and 1.5 h (open circles) time points. GZDV and ZDV were eluted from the column at 1.2 and 1.6 min, respectively. Peak ^{14}C concentrations contained approximately 0.014 disintegrations per minute of activity.

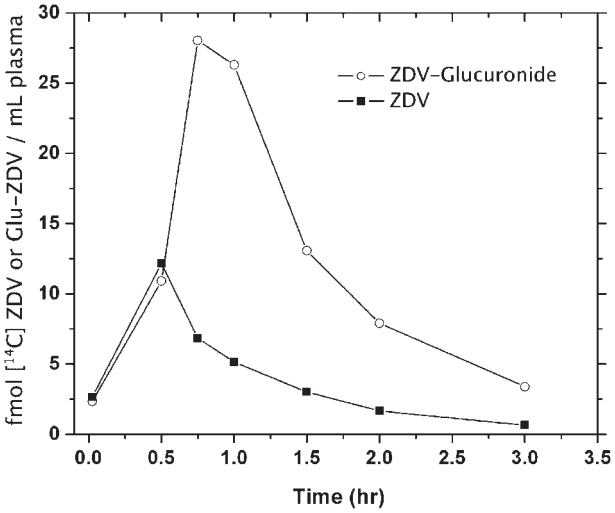

Figure 3. Time-concentration profile of [^{14}C]-ZDV (closed squares) or [^{14}C]-GZDV (ZDV = Glucuronide, open circles) measured from plasma precipitates of samples collected during the first 3 h following [^{14}C]-ZDV administration.

for ZDV and GZDV, respectively. Pharmacokinetic parameters of the current microdose of ZDV and GZDV, as well as those of commonly cited previous studies at therapeutic doses for comparison are shown in Table 2. Values for some parameters are also dose-normalized in order to assess their linearity across the wide range of doses. In general, ZDV and GZDV dose-normalized pharmacokinetic parameters for the subject enrolled in the present study fell within the reported 95% confidence interval or standard deviation of previously published values. Elimination half-life data were slightly less for both ZDV (0.68 h compared to previous reports ranging from 0.8 to 1.5 h) and GZDV (0.79 h compared to previous reports ranging from 1.0 to 1.4 h) in this volunteer, but may be within the variability of this parameter.

Peripheral Blood Mononuclear Cell and Saliva Concentrations

Concentrations of ^{14}C in isolated PBMC preparations up to 16 h postdose are shown in Figure 4. Peak concentration in plasma was achieved in parallel with ZDV (T_{max} of 0.5 h) but a peak concentration of 87.8 fg/mL blood that indicated that ZDV was sequestered at the target cell. The first order elimination half-life in PBMCs (1.69 h) was slightly longer than in plasma and there was a flat portion of the concentration-time curve that remained relatively constant between 6 and 16 h, suggesting a portion of ZDV is retained in the PBMCs with a longer elimination half life, likely representative of the phosphorylated and DNA-incorporated fractions that do not exchange with the plasma.

Saliva concentrations of ^{14}C at baseline, 1, 2, 3, 4, and 6 h following dose administration are shown in Figure 5. Peak concentrations were measured in the 1 h sample and appeared to disappear with an initial elimination half-life of ~0.6 h, followed by a slower rate approaching 1 h at the 6 h time point.

Table 2. Kinetic Parameters Comparison of Pharmacokinetic Parameters of ZDV and GZDV Administered as an Oral Microdose Compared with Previously Published Parameters for Oral Therapeutic Doses

	520 ng microdose	Wang et al.[22]	Drew et al.[23]	Blum et al.[9]
Dose (mg)	0.00052	300	200	134–670
Formulation	Oral solution	100 mg capsules	Oral syrup	Oral solution
ZDV T_{max} (h)	0.5	0.52	0.46	0.42–0.70
GZDV T_{max} (h)	0.75	0.71	n/a	n/a
ZDV C_{max}/dose (1/mL)	6.25 E−06	5.47 E−06	6.15–7.75 E−06	3.78–5.67 E−06
GZDV C_{max}/dose (1/mL)	2.39 E−05	3.14 E−05	n/a	n/a
ZDV AUC_{inf}/dose (h/mL)	5.69 E−06	6.70 E−06	8.25–9.05 E−06	5.67–6.36 E−06
GZDV AUC_{inf}/dose (h/mL)	2.39 E−05	4.07 E−05	n/a	1.12–1.91 E−05
% ZDV in urine	~15%	n/a	n/a	14.0%
% GZDV in urine	~74%	n/a	n/a	75.0%
ZDV CL (L/h)	175.7	149.3	105.3–121.2	157.2–176.4
GZDV CL (L/h)	41.8	31.8	n/a	52.4–89.3
ZDV $T_{1/2}$ (h)	0.68	1.43	1.03	1.21
GZDV $T_{1/2}$ (h)	0.79	1.4		1.00

ZDV molecular weight = 267.24, GZDV molecular weight = 447.

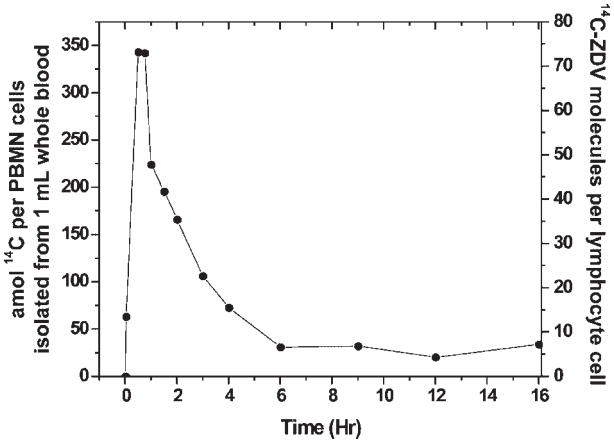

Figure 4. Time-concentration profile illustrating total ^{14}C content of PBMN cells isolated from whole blood following [^{14}C]-ZDV administration. The right-hand y-axis depicts the estimated number of ZDV molecules per lymphocyte cell based on cell counts and assumed 1:1 ratio of ^{14}C to ZDV molecules.

^{14}C Mass Balance Assessment

Cumulative collection of ^{14}C in urine and feces samples over the 96 h following dose administration are shown in Figure 6A, while the plasma concentrations of total ^{14}C are shown in Figure 6B. ^{14}C was recovered predominantly in the urine, reaching nearly 85% of the administered dose within 24 h. At 96 h, 89% of the administered ^{14}C had been recovered in the urine, ~74% as GZDV, while less than 5% was recovered in feces. Urine recovery paralleled plasma concentration data, as plasma concentrations fell to less than 5% of peak values within the first 24 h. In addition, both urine/feces recovery data and plasma concentration data suggest a small portion of ZDV remains after 96 h with a much prolonged elimination half-life.

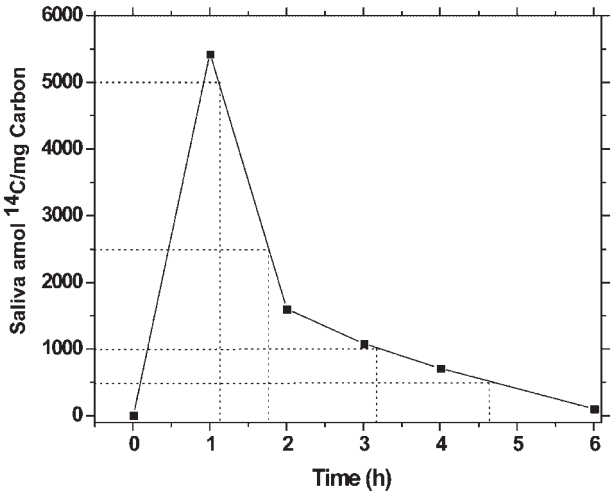

Figure 5. Time profile of ^{14}C as a portion of overall carbon content of saliva samples collected during the first 6 h following [^{14}C]-ZDV administration. ^{14}C concentrations were highest 1 h following the dose, and were still above the limit of detection at the 6 h time point.

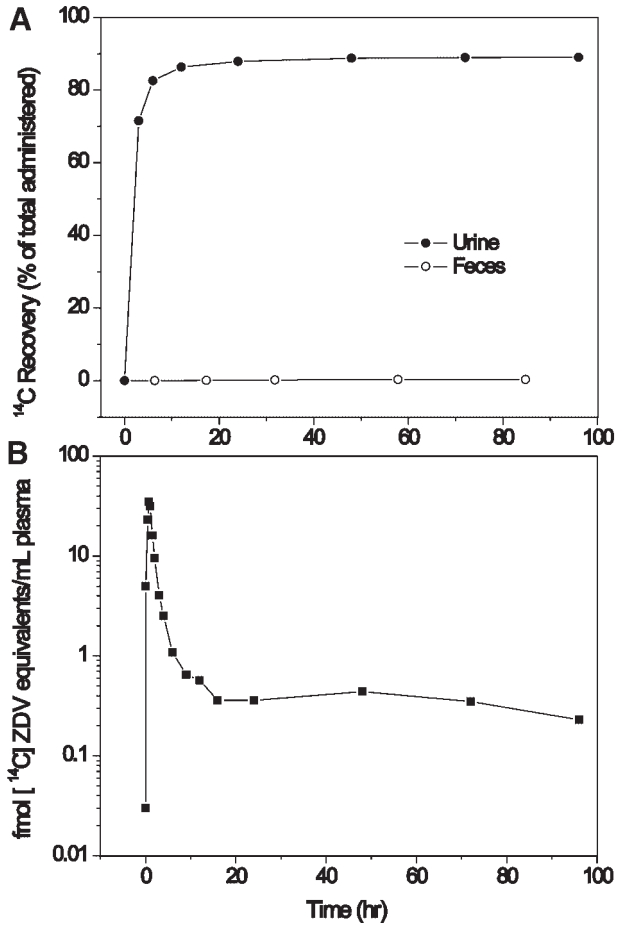

Figure 6. (A) Time profile of overall ^{14}C recovery from urine and feces as a percent of total ^{14}C administered. Data demonstrate nearly complete absorption and renal excretion of ZDV and its metabolites. (B) Time profile of $\log_{10}$ ^{14}C concentrations in plasma, demonstrating a prolonged elimination half-life for a portion of the ^{14}C administered.

DISCUSSION

The objective of this study was to quantify *via* AMS the pharmacokinetic parameters of a drug that undergoes metabolism following microdose level administration. We also chose to evaluate whether AMS could provide sufficient sensitivity to quantify tissue specific uptake of the label, in

our case PBMCs, following a single exposure to the drug. This information is important to establishing the benefits and limitations of microdosing.

According to a recently released FDA guidance for exploratory IND studies, a microdose is defined as "less than 1/100th of the dose of a test substance calculated (based on animal data) to yield a pharmacologic effect of the test substance with a maximum dose of $\leq$100 μg (for imaging agents, the latter criterion applies)." Although by definition microdose studies do not contribute to pharmacodynamic or toxicologic knowledge about a compound, the sensitivity of AMS as an analytical method enables the characterization of pharmacokinetic properties of a compound. The predictive value of pharmacokinetic parameters determined from microdoses rests upon the assumption that the measured microdose parameters bear a linear relationship to those of therapeutic doses. To date there are few published examples to support this assumption in either animals or humans. In rats, linearity of plasma C_{max} and AUC has been demonstrated for fluconazole and tolbutamide over dose ranges of more than 1000-fold.[13] In dogs, the pharmacokinetic properties of a nucleoside analog demonstrated linearity across a 50-fold dose range.[14] The most comprehensive examination dose linearity between microdoses and therapeutic doses to date are the "Consortium for Resourcing and Evaluating AMS Microdosing (CREAM)" studies.[15] These studies were designed to demonstrate the utility of AMS as a decision-making tool in early drug development. Five drugs (warfarin, ZK253—a test drug from Schering, diazepam, midozalam, and erythromycin) were selected based on various difficult pharmacokinetic challenges they presented in early clinical development. Of the four drugs that completed analysis, microdose PK data reflected pharmacological dose PK for three of the compounds (ZK253, diazepam, and midozalam) and provided important metabolism data for the fourth (warfarin). The fifth, erythromycin, was not detectable in plasma due to acid lability of the formulation. Table 2 compares the PK parameters of ZDV following the 520 ng dose of the current study with previously published data. This comparison suggests dose proportional PK properties across a dose range greater than 100000-fold and similar first order plasma elimination phase kinetics.

The current study represents the second report using AMS technology to study the pharmacokinetics of an HIV drug. Sarapa et al.[16] have previously employed AMS to study the effect of multiple therapeutic oral doses of nelfinavir on the systemic metabolism of both nelfinavir and its active metabolite. Using these methods, they were successful in determining upregulated drug metabolism to be the cause of decreased bioavailability following multiple oral dosing. The present study further establishes the usefulness of AMS in determining the pharmacokinetic parameters of a compound and its metabolites following administration of doses orders of magnitude lower than those needed for therapy or conventional ^{14}C mass balance studies using liquid scintillation.

Microdosing techniques are especially useful for pharmacokinetic evaluation of compounds with extensive tissue retention, such as the DNA incorporation of nucleoside analogues. In our study, specific analysis of the forms of the label within the cell were not quantified, but are not beyond the reach of AMS sensitivity using defensible blood volumes (each measurement was made from PBMC harvested from 10 mL of whole blood). AMS was first used in biomedical research to determine levels of genotoxin binding to rodent DNA,[17] where sensitivities of one ^{14}C per 10^{11} bases (1 per 30 cells) were demonstrated, a level that is twice the natural ^{14}C content of the DNA. Thus, it is possible to quantify incorporation of ^{14}C-ZDV-TP into lymphocyte DNA at one incorporation per cell or better if the DNA is cleanly separated from all unbound labeled metabolites, which has been shown possible using modern isolation columns for genetic DNA isolation from labeled small molecule toxins.[18] A 1 mL blood sample contains on the order of 10^6 PBMCs, whose DNA contain about 30000 natural ^{14}C atoms, whereas the incorporation of one ^{14}C-ZDV-TP per cell incorporates 10^6 ^{14}C atoms, or 2 amol ^{14}C which is detectable by AMS.

Because AMS requires ^{14}C-labeled study compounds, a mass-balance evaluation of the analytical data was performed. We found recovery of 89% of the administered ^{14}C within the urine with 70% of that appearing in the 0–3 h collection. Less than 5% of the label was recovered in the feces. Because quantitation is based only on ^{14}C concentrations, pharmacologic mass balance studies are possible when the radiolabel is diluted with unlabeled material. This later phase application offers the dual benefit of reduction in consumption of radiolabel with the fortuitous decrease in radiation exposure to study subjects.

Previous comparisons between AMS and LSC in balance type studies have shown the AMS method to deliver results indistinguishable from LSC.[19,20] AMS quantification of separated biomolecular species provides high sensitivity and specificity for determination of the kinetics and dynamics of drug candidates at very low chemical or radioactive exposures, opening the way to studies of; (i) extremely potent receptor-binding therapeutics, (ii) nontoxic doses of candidate compounds for early first-in-human testing, and (iii) human metabolism and protein binding using unprecedented small sample volumes. In the present study, we demonstrate the ability of AMS to quantify the general pharmacokinetic parameters of ZDV and its metabolites, as well as the uptake of ZDV into PBMCs using a relatively small quantity of whole blood. These findings underscore how the advanced sensitivity of AMS offers potential to increase the margin of safety and information gained from the study of compounds with complex metabolism. This tool will prove to be of particular value in studies involving patient populations in which studies are severely limited by safety and technical issues such as pregnant women, pediatrics, and the infirmed elderly.

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