A Method for the Quantification of Intracellular Zidovudine Nucleotides

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An assay to quantify the phosphorylation products of zidovudine (AZT) in peripheral blood mononuclear cells (PBMC) was developed. Extracts of PBMC were separated by high-performance liquid chromatography. Eluted AZT mono- (MP), di- (DP), and triphosphate (TP) were collected in separate portions. Treatment with alkaline phosphatase yielded equimolar amounts of AZT, which after solid-phase enrichment were assayed by radioimmunoassay. Detection limit was 0.1 pmol/10^6 PBMC for each nucleotide. Recoveries of 102%–118% were observed. AZT nucleotides were measured in samples from three patients receiving 250 mg of AZT every 12 h. Intracellular concentrations of AZT-MP after 1–2 h ranged from 0.9 to 1.4 pmol/10^6 PBMC and then declined to 0.3–1.1 pmol/10^6 PBMC after 4 h. AZT-DP and AZT-TP reached concentrations of 0.3–0.5 pmol/10^6 PBMC after 1–2 h and could not be detected after 4 h in any of the three patients. Duplicate determinations deviated by <20%.

Zidovudine (3′-azido-3′-deoxythymidine; AZT) has been shown to improve survival in patients with AIDS and advanced AIDS-related complex [1]. The compound is a thymidine analog that is efficiently converted intracellularly to AZT-5′-monophosphate (AZT-MP) by thymidine kinase. AZT-MP is then further phosphorylated by thymidylate kinase to the 5′-diphosphate (DP) form and finally to AZT-5′-triphosphate (AZT-TP) [2]. AZT-TP is assumed to be the active form of the drug as it competitively inhibits viral reverse transcriptase [2–4] and further causes proviral DNA chain termination [5].

Serum pharmacokinetics of AZT have been studied extensively [6]; however, no data about the extent and kinetics of in vivo phosphorylation are available. To date the intracellular anabolism of AZT and of other dideoxynucleosides has been examined only in vitro using radiolabeled compounds [2, 7–9]. A detailed knowledge about the in vivo phosphorylation is important for several reasons. First, there is a documented variability of AZT phosphorylation in various cell systems [8], and data from in vitro experiments cannot necessarily be extrapolated to the in vivo situation. Second, interindividual differences in drug metabolism are well known in clinical medicine for a variety of compounds, and it is conceivable that differences in phosphorylation may at least partly explain the observed differences in the toxicity profile and the clinical and virologic response. Finally, a better understanding of the in vivo pools and pharmacokinetics of intracellular AZT-TP might lead to improved drug schedules for individual patients. Thus we developed a method to measure the intracellular anabolites of AZT in whole blood from patients treated with this drug.

Materials and Methods

Preparation of cell extracts. From patients receiving 250 mg of AZT every 12 h or from healthy human immunodeficiency virus (HIV)–seronegative controls, peripheral blood mononuclear cells (PBMC) from 20 ml of heparinized venous blood were separated by ficoll-hypaque density gradient centrifugation. Aliquots of plasma were stored frozen at −20°C for subsequent determination of AZT concentrations by RIA. Cells were washed twice with cold PBS and counted by trypan blue dye exclusion. Nucleotides were extracted twice with 250 μl of 0.33 mol/l perchloric acid.

After neutralization of combined extracts with trioctylamine-freon [10], the ribonucleotides were degraded by adding 20 μl of 0.2 mol/l sodium periodate, followed after 2 min by 30 μl of 3 mol/l glycine. After mixing, extracts were incubated at 40°C for 1 h; 2 μl of 1 mol/l rhamnose was then added, and the samples were put on ice or stored frozen at −20°C.

Separation of AZT nucleotides. Aliquots of extracts (200 μl, corresponding to 6.8–16.5 × 10^6 PBMC) were separated by ion exchange high-performance liquid chromatography (HPLC) on a 5-μm column (250 × 4.6 mm; Supelcosil LC-SAX; Supelco, Bellefonte, PA). A linear gradient from 12 to 120 mmol/l potassium dihydrogen phosphate (KH_2PO_4) was applied within 10 min at a flow rate of 2 ml/min. Three fractions of the eluate (4 ml each) containing the AZT-MP, AZT-DP, or AZT-TP, respectively, were collected.

Collection periods were established by chromatographing a mixture of all three AZT nucleotides using UV detection at 267 nm. Beginning and end times were extended by at least 0.2 and 0.5 min on the front and back side of the peaks. This standard

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Informed consent was obtained from all study subjects; guidelines of the University Hospital Ethical Committee were followed.

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solution was freshly prepared by adding 0.07 units of alkaline phosphatase (bovine type I-S; Sigma, St. Louis) to 100 μl of 4.25 μmol/l AZT-TP (Burroughs Wellcome, Research Triangle Park, NC). After each series of separations the column was flushed with 400 mmol/l phosphoric acid for 10 min to avoid peak broadening due to column aging.

Hydrolysis of AZT nucleotides. After addition of 1 ml of 1 mol/l TRIS, pH 8.8, and 17.5 units of alkaline phosphatase, fractions were incubated at 37°C for 24 h.

Solid-phase extraction of AZT. Hydrolyzed samples were loaded onto a SepPak C18 cartridge (Waters, Milford, MA) preconditioned with 5 ml of methanol followed by 10 ml of distilled water. After washing with 10 ml of distilled water, AZT was eluted with 2 ml of methanol.

Quantification of AZT. Methanol was removed by vacuum centrifugation (Speed Vac; Savant, Farmingdale, NY). The residue was reconstituted in 200 μl of RIA buffer. Amounts of AZT were determined by RIA (ZDV-Trac RIA; Incstar, Stillwater, MN) according to the manufacturer's instructions. Standard samples containing 1.1, 2.3, 4.6, 10, 20, 76, and 272 pmol of AZT-TP in 200 μl of distilled water were assayed in duplicate as described for neutralized extracts. Calibration curves were constructed by plotting RIA counts against logarithms of standard amounts. Results of unknown samples were normalized to 10^6 PBMC based on cell counts and volumes of extracts.

Determination of analytical recovery. Blood samples from an untreated control were processed as described above, except that known amounts of AZT-TP were added to the cell pellet before perchloric acid extraction.

Cell cultures. PBMC (20×10^6) from a healthy HIV-seronegative blood donor were cultured for 24 h in 20 ml of RPMI 1640 medium (GIBCO BRL, Basel, Switzerland) supplemented with 20% fetal bovine serum (Inotech, Wohlen, Switzerland) and 10% interleukin-2 (200 units/ml; Boehringer Mannheim, Mannheim, Germany) in the presence of 2 μmol/l [^{3}H]AZT (500 dpm/pmol; cultures I and II) or 2 μmol/l cold AZT (culture III). Antibiotics were omitted. Cells were collected from the same blood donor for all three batches. Thereafter, cells were harvested by centrifugation at 600 *g* for 10 min (cultures I and III) or by ficoll separation (culture II). AZT nucleotides in cell extracts were quantified in duplicate by liquid scintillation counting of HPLC eluates (cultures I and II) [11] or by the method described above (culture III).

Results

Log-logit transformed calibration curves obtained with AZT-TP were linear from 1.1 to 272 pmol ($r < -.990$). Lower amounts were not detectable. Analytical recoveries from blank PBMC spiked with known amounts of AZT-TP are shown in table 1. Duplicate determinations deviated by <15%.

In PBMC cultured in the presence of 2 μmol/l [^{3}H]AZT for 24 h, concentrations of AZT-MP, AZT-DP, and AZT-TP were 193, 1.3, and 2.0 pmol/10^6 PBMC, respectively, after ficoll-hypaque density-gradient centrifugation. If cells were harvested by simple centrifugation, concentrations of 215, 1.7, and 1.6 pmol/10^6 PBMC were found. PBMC of the same donor treated under identical conditions but with unlabeled AZT yielded concentrations of 198 pmol/10^6 PBMC for AZT-MP, 1.8 pmol/10^6 PBMC for AZT-DP, and 2.4 pmol/10^6 PBMC for AZT-TP by RIA. These results rule out excessive loss of AZT nucleotides during sample preparation.

Table 1. Recovery from cell extracts spiked with zidovudine triphosphate.

Zidovudine (pmol/10^6 peripheral blood mononuclear cells)		
Spiked	Found	% recovery
0.20	0.23	115
0.50	0.54	108
1.4	1.5	107
2.0	2.4	118
5.0	5.1	102

NOTE. Data are means of duplicate determinations.

AZT-MP, -DP, and -TP were well separated by HPLC. Mean retention times of 5.38, 7.87, and 9.49 min, respectively, were observed, with only minor run-to-run variability within the same day (SD < 0.1 min).

Blood samples were obtained from three patients on long-term oral therapy with 250 mg of AZT every 12 h. AZT nucleotides were determined before and 1, 2, and 4 h after administration of the drug. No phosphorylation products were found before administration.

Intracellular concentrations of AZT-MP after 1–2 h were 0.9–1.4 pmol/10^6 PBMC and then declined to 0.3–1.1 pmol/10^6 PBMC after 4 h. AZT-DP and AZT-TP reached concentrations of 0.3–0.5 pmol/10^6 PBMC after 1–2 h and could not be detected after 4 h in any of the three patients (table 2).

Discussion

Quantification of intracellular AZT nucleotides has so far been done only in cell cultures treated with [^{3}H]AZT [2, 7–9]. Since radiolabeling is not feasible in human pharmacokinetic studies, a method was developed that uses the selectivity of HPLC to separate mono-, di-, and triphosphate and the sensitivity of a commercially available RIA to quantify them in PBMC obtained from patients treated with AZT.

Cell extracts contain large amounts of natural ribonucleotides, which in part coelute with AZT nucleotides in HPLC. They were destroyed by periodate oxidation [12] to limit potentially cross-reacting contaminations of RIA samples. Alkaline phosphatase activity is inhibited by inorganic phosphates present in the elution buffer. Large amounts of the

Table 2. Phosphorylation of zidovudine (AZT) in three patients receiving 250 mg every 12 h.

Patient, h	Plasma AZT (μmol/l)	AZT-MP	AZT-DP	AZT-TP
1, 0	<0.03	<0.1	<0.1	<0.1
1	2.22	1.2	0.3	0.2
2	0.72	1.4	0.3	0.3
4	0.25	1.1	<0.1	<0.1
2, 0	<0.03	ND	ND	ND
1	2.98	0.9	0.3	0.3
2	0.61	0.6	0.2	0.2
4	0.10	0.3	<0.1	<0.1
3, 0	<0.03	<0.1	<0.1	<0.1
1	0.81	0.7	<0.1	0.2
2	2.17	1.1	0.5	0.5
4	0.77	1.0	<0.1	<0.1

NOTE. Values are means of duplicate determinations that deviated by <20%. AZT-MP (monophosphate), -DP (diphosphate), and -TP (triphosphate) concentrations are given in pmol/10^6 peripheral blood mononuclear cells. ND = not done.

enzyme and long incubation times are therefore required to obtain equimolar amounts of AZT from AZT-MP, -DP, and -TP. Recovery experiments showed the feasibility of our approach (table 1). AZT-TP concentrations found were always slightly above the expected value. Considering multiple sample manipulations and the lack of an internal standard, accuracy appears satisfactory.

Since the cell membrane is partly permeable for AZT-MP [13], and isolation of PBMC from whole blood takes ~1 h, we suspected a partial loss of intracellular AZT-MP may occur. However, no change in AZT-MP concentration and no hydrolysis of AZT-TP was observed when [^{3}H]AZT-labeled PBMC were harvested from cell cultures by ficoll-hypaque separation. Analyzing AZT nucleotide pools of PBMC grown in the presence of [^{3}H]AZT or AZT by either HPLC and liquid scintillation counting or HPLC-RIA yielded comparable results. AZT-TP concentrations measured in patient cells suggest a rapid decay in vivo (table 2). This contrasts with an in vitro study [7] using a leukemic T cell line in which an intracellular half-life for AZT-TP of 200 min was observed. A possible explanation may be the finding by Balzarini et al. [8] that intracellular metabolism of AZT is highly dependent on the cell species [8].

Stretcher et al. [14, 15] measured the concentration of total phosphorylated AZT (i.e., AZT-MP, -DP, and -TP) in PBMC of patients receiving AZT at a dose of 500 mg/day. In 14 samples obtained from 12 patients at various time points, concentrations of 0.3–4 pmol/10^6 cells were found. Pooled data from that population showed no decrease in total phosphorylated AZT over 3.5 h so the authors concluded that the decay of phosphorylated AZT was slow. Nevertheless, since AZT-MP is the most abundant phosphorylation product and the ratio between mono- and triphosphate appears inconstant (table 2), changes in the AZT-TP concentration will not necessarily be detected by that assay.

Assuming a mean cellular volume of 0.25 pl, the detection limit of 0.1 pmol/10^6 PBMC corresponds to an intracellular concentration of 0.4 μmol/l. Data from various studies indicate that AZT-TP may affect viral transcription at concentrations below the detection limit of our assay. Competitive inhibition of human immunodeficiency virus type 1 reverse transcriptase (HIV-RT) by AZT-TP has been studied by several groups [2–4]. Depending on the primer template used, K_i values ranging from 0.01 μmol/l (for HIV RNA) to 0.3 μmol/l (for activated calf thymus DNA) were reported, which suggests that considerable inhibition of HIV-RT occurs even at concentrations < 0.4 μmol/l.

Huang et al. [5] studied the incorporation of AZT-TP into a DNA primer by HIV-RT in vitro. They observed termination of primer elongation even when the AZT-TP concentration was 0.3 μmol/l, 100-fold less than that of thymidine 5′-triphosphate.

The data from this pilot study with three patients show that AZT nucleotides in PBMC can be measured with our method. Currently the assay is laborious and requires blood volumes that make frequent sampling unacceptable. Further modifications may improve its sensitivity and facilitate detailed studies of intracellular AZT nucleotide pharmacokinetics.

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