Specific Routine Determination of 3’-Azido-3’-Deoxythymidine (AZT) in Plasma by Partly Automated Liquid Chromatography

Reinhard Kupferschmidt and Rainer W. Schmid

A simple isocratic HPLC method for determining azidothymidine (AZT) in serum and plasma of patients has been developed. The novel, specific, two-step, solid-phase extraction approach used for sample preparation gives a nearly quantitative recovery (95.3%) of AZT from the blood plasma matrix and requires only minimal handling of infectious clinical samples. Automatic “on-line” injection is achieved with an AASP™ system by switching a small cartridge, which retains the extracted analyte, into the HPLC stream. The overall HPLC procedure shows satisfying reproducibility with low standard deviation (CV: 2.1%). Because of the low detection limit (about 10 ng) and the possibility of concentrating AZT quantitatively in as much as 5 mL of plasma or serum, the method can be used in routine monitoring of AZT as well as in pharmacokinetic studies. Nevertheless, before establishing therapeutic drug monitoring for AZT, it still must be determined at what time after the last AZT dose blood specimens should be drawn for correct therapeutic interpretation of the concentration of AZT measured in blood.

Additional Keyphrases: acquired immune deficiency syndrome · human immunodeficiency virus · therapeutic drug monitoring

Recently, several nucleoside analogs have been reported to be potent antiviral inhibitors of human immunodeficiency virus-1 (HIV) replication in vitro (1). Until now, only 3’-azido-3’-deoxythymidine (AZT, zidovudin, Retrovir™) has been approved for human use and is, so far, the only anti-retroviral drug routinely given to patients for treating acquired immune deficiency syndrome (AIDS) (2). During therapy with AZT the immunological status of some AIDS patients improves dramatically and their concentrations of HIV antigen in serum are lowered significantly (3). Although drastic improvements in the health of AIDS patients may accompany therapy with AZT, it has several serious side effects. The most severe are the effects on the bone marrow, resulting in anemia and neutropenia (4). We assume that, by knowing the concentration of the drug in blood, it should be possible to optimize the efficacy of AZT therapy and to minimize toxic side effects. So far, however, the optimal therapeutic range for AZT in vivo is not known; all dosage recommendations are taken from biologically effective concentrations of AZT in in vitro experiments (5).

To be able to measure AZT in blood plasma or serum routinely, one must have a robust but selective analytical procedure. Besides such aspects as accuracy, specificity, and quick turnaround—generally required for any drug monitoring method—two further aspects have to be considered in clinical routine analysis of AZT. Because most AIDS patients are medicated with a wide variety of different drugs to treat their opportunistic infections, an analytical method for AZT in blood must be highly specific. Additionally, because of the serious consequences of HIV transmittance in blood of AIDS patients, the method has to be as safe as possible for the laboratory staff, and handling of the infectious blood samples has to be minimized.

Only two HPLC methods for the determination of AZT in blood have been described in the literature so far. Hedaya and Sawchuck (6) analyzed AZT in blood of healthy volunteers, using a C18 column for separation and liquid-liquid extraction for sample extraction. Because the sample-preparation procedure carries a high intrinsic potential of infection, these authors recommended the method only for assays of blood of non-HIV-infected individuals. Kiecker et al. (7) analyzed AIDS-patients’ samples by reversed-phase HPLC and used solid-phase extraction with C18 columns for sample preparation. They gave no further details about the specificity or the precision of the analytical method.

We have developed and investigated a new and specific HPLC method for routine measurement of AZT in plasma or serum of AIDS patients, which is based on a highly selective two-step, solid-phase extraction for sample preparation and chromatographic separation on a C18 column. Sample injection can be automated, minimizing handling of blood specimens.

Materials and Methods

Chemicals and Reagents

AZT and BW A22U, the internal standard, both in certified grade, were a gift from Wellcome Research Laboratory, Beckenham, U.K.

For the extraction step, we purchased analytical-grade chloroform and methanol from E. Merck, Darmstadt, F.R.G.

For HPLC analysis we used "HiPerSolv" methanol, acetoni-trile, and water from BDH Ltd., Poole, U.K.; 85% o-phosphoric acid ("p.a." grade) from Baker, Deventer, The Netherlands; triethylamine "Ionate" from Pierce Chemicals Co., Rockford, IL; and sodium hydroxide (p.a.) from E. Merck. All other chemicals used were of the highest quality available and were used without further purification.
Nucleosides, nucleotides, and purine and pyrimidine analogs (listed in Table 1) were all from Sigma Chemicals, Deisenhofen, F.R.G.

**Standard solutions.** Stock 1 g/L solutions of AZT and the internal standard, BW A22U, were prepared in an equi-volume mixture of methanol and water and stored at 4 °C. These standard solutions were stable at least for eight weeks. Standard curve samples were prepared by adding human drug-free control plasma to aliquots of the AZT stock solution to give final concentrations of 25, 50, 100, 333, 666, 1000, and 3000 μg/L.

**Blood Samples**

**Sample collection.** Venous blood from AIDS patients was collected in plain or heparinized tubes and centrifuged to obtain blood serum or plasma, respectively. To reduce the risk of infection during analysis, we incubated the plasma or serum samples in a water bath at 56 °C for at least 45 min, which reportedly inactivates HIV in blood (8). Nevertheless, for all further sample-handling steps, we still maintained the highest precautions and treated the heat-inactivated patients' samples as highly infectious material.

**Sample preparation.** To extract blood samples, we set aside a special working area in the laboratory, used only for this purpose. The working surfaces of the benchtops were always protected by a layer of plastic-backed absorbent pad and were cleaned with 700 mL/L ethanol after each working session. All used vials, pipette tips, and liquid waste from the extraction procedure were disposed of in the hospital's infectious waste immediately after sample extraction.

**Instrumentation**

For the HPLC analysis we used an isocratic HPLC system consisting of an FR-30 HPLC pump (Knauer, Berlin, F.R.G.) connected to an AASP-injection module (Varian Associates, Walnut Creek, CA) (Figure 1) and a Spectruminator D variable-wavelength detector and CI-10 integrator (both from LDC-Milton Roy, Riviera Beach, FL).

**Procedures**

**Extraction of AZT.** To extract AZT from blood plasma or serum we used a two-step, solid-phase extraction procedure. First, the AZT in plasma is concentrated by passage through a 1-mL C₁₈ Bond Elut extraction column (Analytichem Int., Harbor City, CA). Next the AZT is eluted from the Bond Elut column directly onto a silica-gel-filled AASP cartridge for on-line injection onto the HPLC column.

**Table 1. Commercially Available Purine and Pyrimidine Bases, Nucleosides, and Nucleotides Analogs That Do Not Interfere with the Assay**

<table>
<thead>
<tr>
<th>Bases</th>
<th>Nucleosides</th>
<th>Nucleotides</th>
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<tr>
<td>Adenine</td>
<td>Adenosine</td>
<td>Adenosine 2'- &amp; 3'-monophosphate</td>
</tr>
<tr>
<td>Cytosine</td>
<td>Cytidine</td>
<td>Cytidine 2'- &amp; 3'-monophosphate</td>
</tr>
<tr>
<td>Guanine</td>
<td>Guanosine</td>
<td>Guanosine 2'- &amp; 3'-monophosphate</td>
</tr>
<tr>
<td>Thymine</td>
<td>Thymidine</td>
<td></td>
</tr>
<tr>
<td>Uracil</td>
<td>Uridine</td>
<td>Uridine 2'- &amp; 3'-monophosphate</td>
</tr>
<tr>
<td>2'-Deoxynucleosides</td>
<td>Miscellaneous</td>
<td></td>
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<tr>
<td>2'-Deoxyadenosine</td>
<td>5-Methylcytosine</td>
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<tr>
<td>2'-Deoxyctidine</td>
<td>5-Methyld-2'-deoxycytidine</td>
<td></td>
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<tr>
<td>2'-Deoxyguanosine</td>
<td>Hypoxanthine</td>
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<tr>
<td>2'-Deoxythymidine</td>
<td>Uric acid</td>
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**Processing the extraction columns with a vacuum manifold (Vac-Elut, Analytichem Int.) allows simultaneous extraction of up to 10 samples.** The flow rate of liquids through the columns is adjusted to approximately 1–3 mL/min by regulating the negative pressure applied. Between the Vac-Elut manifold and the vacuum line we installed a 5-L flask, half-filled with chlorine bleach, to collect and inactivate the infectious sample waste. For AASP cartridge extractions, we connected the cassettes, equipped with an AASP reservoir, onto an AASP Vac-Elut station. Liquids were forced through the AASP cartridges either by applying positive pressure to the cartridges or by reducing the pressure on the AASP Vac-Elut station.

The procedure is as follows: Before starting the extraction procedure, condition the extraction columns by rinsing each column with one reservoir volume (~1.2 mL) of methanol, followed by the same volume of distilled water. Apply 1–3 mL of plasma sample and force it through the preconditioned C₁₈ extraction column. (To include an internal standard, add 50 μL (100 ng) of an aqueous solution of BW A22U to 1 mL of plasma or serum before the extraction.) Wash with one reservoir volume of water and dry the silica sorbent by aspirating air through the extraction columns for ~10 min.

Elute the AZT from the dried Bond Elut column by using a syringe connected to the column via a commercially available adapter (obtained from Analytichem Int.) to force two 1-mL portions of chloroform through the column into the reservoir of a SI AASP cartridge. Force the chloroform solution through the AASP cartridge and then dry the silica sorbent by pulling air through them for about 2 min. Then disconnect the cartridges from the "off-line" extraction manifold and place them into the AASP processor, ready for injection into the HPLC system. Place up to 10 AASP cassettes (1 cassette = 10 samples) in the AASP injection module. Start the injection process, which automatically seals each individual cartridge into a pneumatically driven stainless-steel chamber of the AASP station and process 125 μL of water through each cartridge ("pre-purge" setting: 5) to remove any air entrapped in the silica sorbent. Switch the chamber for 1 min into the eluant stream of the HPLC system ("valve-reset" setting: 1) to elute AZT from the cartridge onto the analytical column. After the chromatographic run, and before the next cartridge is processed, automatically flush the internal AASP fluid lines with 250 μL of HPLC-water ("after-purge")
setting: 10) to remove any remaining mobile phase before the next injection cycle.

Chromatographic conditions. To evaluate the chromatographic separation of AZT, we tried 110 × 4.6 mm cartridges of Partisil 5 C8 or C18 (Whatman, Clifton, NJ). However, we achieved better chromatographic resolution with a 200 × 4 mm column of Partisil 5 ODS-3 (Whatman) and isocratic elution with a mobile phase of acetonitrile/phosphate buffer (13/87 by vol). We operated the chromatograph at room temperature at a flow rate of 1 mL/min and detected AZT at its absorbance maximum, 267 nm.

The buffer was prepared by dissolving 8 mL of concentrated (85%) orthophosphoric acid in 1 L of water to give a concentration of about 120 mmol/L. After adding 2 mL of triethylamine we adjusted the pH of the buffer to pH 6.2 with 10 mol/L sodium hydroxide. After mixing the buffer with the organic modifier, acetonitrile, we degassed the eluant with helium before use.

Calculation of analytical recovery. Recovery of the extraction was checked by comparing peak areas for loop injection of 50 μL of aqueous standard containing 100 ng of AZT with peak areas for on-column injection of 1-mL plasma samples containing 50 μL of the same standard solution and extracted as described above.

Calculation of results. To prepare a standard curve, we used linear-regression analysis of measured peak areas vs AZT concentrations in drug-supplemented plasma samples and calculated the unknown AZT concentrations by use of the regression equation.

Stability Tests of AZT

To investigate the stability of AZT during inactivation and extraction of the plasma sample, we carried out several experiments.

Drug-free serum samples with added AZT were incubated in a water bath at 56 °C for 45 min, then frozen at −70 °C for 60 min. After repeating this procedure two times, we determined the concentration of AZT in the samples and in untreated AZT supplemented samples.

Patients' samples collected in plain tubes or in Vacutainer Tubes containing either lithium heparinate, sodium citrate, or EDTA (all from Becton Dickinson, Rutherford, NJ) were analyzed to check for any influence of anticoagulants on the measured AZT concentration.

We extracted AZT-supplemented pooled control plasma samples, using the two-step, solid-phase extraction procedure described. Before injection onto the HPLC system, we stored the AASP cartridges for up to 14 days at 4 °C and compared chromatograms with those obtained after injecting directly after the extraction procedure. This indicated whether the AZT on the silica AASP cartridge was at all degraded during storage.

Results

Chromatographic Separation of AZT

For good chromatographic separation of AZT, we used a reversed-phase column under isocratic conditions. Figure 2 illustrates the different selectivities for AZT, BW A22U, and 2'-deoxythymidine in a standard mixture injected onto a Partisil C8 or C18 column under otherwise identical chromatographic conditions. We chose 2'-deoxythymidine for comparison because of its structural similarities with AZT and with the endogenous nucleoside thymidine. From the retention times one can clearly see that the azido group, which is the only difference between AZT and 2'-deoxythymidi-
separation of AZT or the internal standard. When methanol is used as organic modifier, caffeine is eluted after AZT. However, when acetonitrile is the organic modifier of the mobile phase, caffeine is well separated from AZT and is eluted before it. This shorts time analysis time and ensures that all endogenous purine and pyrimidine analogs are eluted before AZT, early in the chromatogram, avoiding possible interferences from these compounds.

Extraction Procedure

Figure 4 shows chromatograms of plasma samples obtained during a routine analysis run. Control blood from a patient not being treated with AZT shows no compounds eluting in the retention window of AZT or of the internal standard (Figure 4, middle). The peak at about 6 min corresponds to a high content of caffeine in the plasma sample. For the next 30 min, no further peaks were eluted.

Figure 4 (right) shows the chromatogram of the control sample with 125 ng of AZT added per milliliter, and Figure 4 (left) shows that from a plasma sample extract of an AIDS patient being treated with AZT. There are no interferences in the elution window of AZT, not even in samples of patients concurrently being treated with as many as 15 other medications at the same time.

Despite the use of a two-step extraction approach, the absolute recovery of AZT and the internal standard from blood plasma is still almost quantitative: 95.3% (CV 2.1%) for AZT and 94.5% (CV 3.8%) for BW A22U (n = 10 each). This overall high recovery and the small standard deviation indicate that each of the extraction steps is little or not at all affected by small fluctuations during the procedure; i.e., the extraction method is robust.

Because the whole sample extract is injected on-line from the AASP silica cartridge onto the analytical column, and because of the low variability of the whole analytical procedure, we found routine use of an internal standard for the quantitative routine analysis of AZT in blood samples to be unnecessary.

Analytical Variables

Linearity and reproducibility. Analysis of AZT-supplemented plasma samples in the range from 10 to 3000 μg/L yielded a linear calibration curve for area vs AZT concentration. Typical regression calculations give a slope of 0.2620 and an intercept of 6.120 μg/L.

The overall reproducibility of the described method is very satisfactory: the between-assay variation (CV) was 2.1% when no internal standard was used (at AZT concentrations of 250 μg/L), 1.3% when BW A22U was added before the extraction procedure.

Specificity of the analytical procedure. Many often-prescribed clinical drugs were not expected to interfere with the assay because of their high lipophilicity, causing them to be eluted slowly from the reversed-phase column under our chromatographic conditions. It was much more important to determine whether the more-polar metabolites of these drugs would interfere with the assay. So far, after having measured AZT in a large number of blood samples from AIDS patients, we have encountered no drugs currently used in therapy of these AIDS patients that interfere in the separation of AZT (Table 2). Moreover, none of their possible metabolites that are present in blood under a steady-state medication were found to interfere.

Stability of AZT during sample preparation. We saw no change in AZT concentrations when plasma samples were inactivated by incubation at elevated temperatures before sample preparation. Even repeated freezing of AZT-supplemented plasma samples at −70 °C, followed by thawing and incubation at 56 °C in a waterbath for at least 60 min, did not result in any significant difference in AZT concentrations compared with samples not so treated.

After extraction from plasma, AZT shows considerable stability, also while retained on a silica sorbent. AASP silica cartridges have been kept stored after sample extraction for more than 14 days in a refrigerator at 4 °C without further precautions. After on-line injection of these cartridges, the same values for AZT were found as for AASP cartridges processed immediately after the sample extraction procedure.

Analysis of Patients' Samples

Using the described method, we measured steady-state AZT concentrations in blood of AIDS patients receiving

<table>
<thead>
<tr>
<th>Table 2. Drugs, Coadministered with AZT to AIDS Patients, Showing No Interferences in the HPLC Assay</th>
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<tbody>
<tr>
<td>Amphoterocin</td>
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<td>Sulfamethoxazole</td>
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<td>Trimethoprim</td>
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<td>Isoniazid</td>
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<td>Metronidazole</td>
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<td>Nefilimicin</td>
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<td>Ticarcolin</td>
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<td>Fioxacillin</td>
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<td>Ketocanazole</td>
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<td>Nystatin</td>
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<td>Dequanilium</td>
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<td>Loperamide</td>
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<td>Methadone</td>
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<td>Oxazepam</td>
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<td>Diazepam</td>
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<td>Prazepam</td>
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Fig. 4. Chromatograms of (left) a control plasma supplemented with AZT, 0.5 μmol/L; (middle) blood from a patient not being treated with AZT (caffeine peak); (right) blood from a patient receiving AZT at steady-state therapy, after two-step, solid-phase extraction of 1 mL of plasma

Chromatographic conditions as in Fig. 3
100–300 mg of AZT every 4 h. Because of the short biological half-life of AZT, and to get a better insight into the pharmacokinetic variability within individual AIDS patients, we followed the time course of AZT concentrations during one dosing interval in several patients. Figure 5 shows examples of concentration–time profiles of AZT in blood from four different AIDS patients. Peak values in these patients ranged between 1 and 3.5 mg/L. The preliminary measurements from these patients confirm the short half-life of AZT (6, 7). They also show that the peak concentrations of AZT may vary considerably, reaching maximum values within 40 to 50 min or as late as 120 min after the last AZT dose.

**Discussion**

Patients with AIDS seldom receive therapy only with AZT. Rather, they are usually treated with a wide range of other drugs, so that individual patients may be receiving as many as 15 co-medications. Therefore, a highly specific analytical method is an absolute necessity for measuring AZT in blood of AIDS patients. The observed $k'$-values of AZT in a reversed-phase separation system indicate that generally it is not the administered parent drugs but rather their (generally more polar) metabolites that must be regarded as potential interferences, having an elution behavior similar to that of AZT.

To deal with this problem and to achieve the best selectivity in the analysis for AZT in blood, we used a two-step, solid-phase extraction method for sample preparation. The C$_{18}$ column nonselectively retains most of the lipophilic, low-molecular-mass compounds from the blood plasma matrix. This initial C$_{18}$ extraction step offers a high sample-loading capacity, concentrating the AZT in as much as 5 mL of plasma or serum on the small 100-mg extraction column.

With chloroform as the eluent, only lipophilic compounds, which have good solubility in this nonpolar solvent, will be eluted from the reversed-phase silica, while the more-polar compounds are retained on the extraction column. In the chloroform eluate, only compounds with sufficient polarity will be retained in the subsequent extraction step and will be adsorbed on the unmodified silica gel of the AASP "SI" cartridge; all other substances will be flushed through the small column. Thus, most of the potentially interfering drugs are already removed during the sample preparation procedure and will not be injected onto the HPLC system. Only molecules that are sufficiently lipophilic to be retained in the first extraction step and sufficiently polar to be retained on silica in the second step will be recovered. Furthermore, the two-step extraction approach allows the use of an AASP injection module for automated on-line injection of a whole-sample extract of $\geq$1 mL of plasma. This significantly increases the sensitivity of the analytical method, allowing even very low concentrations of AZT in blood to be determined, as in pharmacokinetic studies. The two-step extraction procedure does not have to be substantially longer than a single-step extraction, because no off-line evaporation procedures are necessary.

Given the stability of the AZT retained on an extraction column, we propose that the first extraction step be carried out in the clinical routine laboratory next to the sampling area, where infectious blood specimens are routinely handled. The (noninfectious) extraction columns can then be sent to a specialized analytical laboratory for the chromatographic analysis, with no need for special precautions.

The described chromatographic method has been developed to be used for routine clinical assays of AZT in AIDS patients. Before establishing a therapeutic drug monitoring service, however, a number of pharmacokinetic factors must be considered. Not only does AZT show very rapid elimination, with a sharp maximum peak, but also the preliminary pharmacokinetic data from AIDS patients indicate that peaking of AZT concentrations in blood can vary considerably, complicating the choice of correct time for blood collection with respect to the last AZT dose. These aspects must be kept in mind before the therapeutic meaning of a single AZT blood value can be interpreted correctly. Additional kinetic studies are recommended to answer this question.

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**References**


![Fig. 5. Concentration–time profiles of AZT in blood of four AIDS patients under steady-state AZT therapy: patient 1 (○) received 500 mg daily, patient 2 (□) 1200, patient 3 (×) 700, patient 4 (△) 1000](image-url)