Fluorescence Polarization Immunoassay for Zidovudine†

GEORGE G. GRANICH, MICHAEL R. EVELAND, AND DONALD J. KROGSTAD*

Departments of Medicine and Pathology, Divisions of Infectious Diseases and Laboratory Medicine, Washington University School of Medicine,* and Therapeutic Drug Monitoring Laboratory, Barnes Hospital, St. Louis, Missouri 63110

Received 29 December 1988/Accepted 19 April 1989

We report a fluorescence polarization immunoassay (FPIA) for zidovudine (azidothymidine; Retrovir). This assay is accurate and specific over the clinically relevant range of zidovudine concentrations in serum (from 1 to 1,250 ng/ml; from 0.004 to 4.8 μM) and is unaffected by potentially interfering compounds in the sera of patients with renal or hepatic failure. Cross-reactivity with structural analogs of zidovudine (including zidovudine glucuronide) is less than 0.05%, except for cross-reactivities of 0.2, 0.3, and 0.4% with 3-methylthymidine, 3',5'-dideoxythymidine, and A22U (the optical isomer of zidovudine), respectively. The FPIA for zidovudine is more sensitive and more specific than high-performance liquid chromatography (HPLC); it requires 50 to 60 or 200 versus 500 μl of serum and is faster to perform (45 specimens per h with the FPIA versus 3 specimens per h with HPLC). The zidovudine FPIA compares well with the radioimmunoassay. A correlation coefficient of 0.992 was observed with 31 serum specimens examined by both methods. All three assays (FPIA, radioimmunoassay, and HPLC) are unaffected by the heat treatment used to inactivate human immunodeficiency virus. The zidovudine FPIA should be particularly useful for analyzing specimens from large numbers of human immunodeficiency virus-infected patients receiving zidovudine in current clinical trials.

The nucleoside analog 3'-azido-3'-deoxythymidine (zidovudine; Retrovir) is a potent inhibitor of human immunodeficiency virus (HIV) replication in vitro (14). In studies of patients with the acquired immunodeficiency syndrome or acquired immunodeficiency syndrome-related complex, treatment with zidovudine has decreased viral antigenemia (4, 9), reduced the frequency of opportunistic infections (4), and increased survival (4). There is, however, significant hematologic toxicity associated with zidovudine, including anemia, neutropenia, pancytopenia, and bone marrow aplasia (3, 5, 16). The pharmacokinetics of zidovudine have been described previously (1, 8, 11, 12, 17). Although the relationship between levels in serum and toxicity or clinical outcome has not been defined, recent studies suggest that lower serum (and cerebrospinal fluid) zidovudine levels may increase the risk of HIV meningoencephalitis (7). The performance of such studies would be facilitated by a rapid and sensitive analytical method.

Zidovudine has been measured previously using high-performance liquid chromatography (HPLC) (6, 11). Although HPLC is sensitive, accurate, and precise, its disadvantages include a long analysis time (ca. 20 to 30 min), a complicated solid-phase extraction, the need for a relatively large sample size (500 μl), and interference from endogenous metabolites in the sera of patients with renal failure. This report describes a fluorescence polarization immunoassay (FPIA) for the measurement of zidovudine in serum and compares the FPIA with HPLC and radioimmunoassay (RIA). The FPIA has a rapid turnaround time, utilizes a simple extraction by protein precipitation, and requires a small sample volume (50 to 60 or 200 μl of serum). The FPIA for zidovudine is accurate and specific over the clinically relevant range of zidovudine concentrations in serum (1, 8, 11, 12, 17). The FPIA should be useful for laboratories performing large numbers of assays in clinical trials because of its short turnaround time and for pharmacokinetic studies with children because of the small sample size required.

(Part of this research was presented at the 28th Interscience Conference on Antimicrobial Agents and Chemotherapy, Los Angeles, Calif., 23 to 26 October 1988.)

MATERIALS AND METHODS

Reagents and samples. Zidovudine, zidovudine glucuronide, and the optical isomer of zidovudine (A22U) were obtained from Burroughs Wellcome Co., Research Triangle Park, N.C. The internal standard (3',5'-di-o-acetylatedexo-
apenosine), ammonium phosphate, monobasic and dibasic potassium phosphate, sodium chloride, β-glucuronidase, and the FPIA reagents were obtained from Sigma Chemical Co., St. Louis, Mo. HPLC-grade acetonitrile, methanol, and phosphoric acid (85%) were purchased from Fisher Scientific Co., Fairlawn, N.J. Extract-Clean C-18 solid-phase extraction columns (100 mg/1.0 ml) were obtained from Alttech Associates, Inc., Deerfield, Ill.

A stock solution of zidovudine, corrected for drug potency, was prepared at a concentration of 366.7 μg/ml (1,372.4 μM) in distilled water and stored at −20°C until needed. Calibration standards containing 0.19, 0.57, 1.69, 4.99, and 14.71 μM zidovudine (51, 152, 451, 1,332, and 3,928 ng of zidovudine per ml, respectively) were prepared by diluting the stock solution with calf serum (Sigma) and were used for the calibration curves. Reference samples containing 0.01, 0.1, 1.0, and 10.0 μg/ml zidovudine (2.7, 26.7, 267, and 2,670 ng of zidovudine per ml, respectively) were prepared similarly and were used to assess recovery and intrarun and interrun precision. Serum samples to which zidovudine had been added and serum samples from patients receiving zidovudine were analyzed by HPLC, FPIA, and RIA.

Preparation of serum specimens. Prior to study, serum specimens were heat treated (56°C for 30 min) to inactivate HIV (13).

* Corresponding author.
† Manuscript no. 2 from the Washington University AIDS Clinical Trials Group.
HPLC. The chromatographic system consisted of a U6K injector, two model 510 pumps, and a model 481 UV detector linked to an 810 Baseline Chromatography Workstation (Waters Associates, Inc., Milford, Mass.). The UV detector was set at 267 nm, with a sensitivity of 0.02 absorbance units full scale. Sample preparation and chromatographic analysis were performed as reported previously by Good et al. (6) and by Klecker et al. (11) with the following exceptions. Separation was achieved at ambient temperature on two µBondapak C-18 reversed-phase columns (0.4 by 30 cm; Waters) connected in series. The mobile phase consisted of acetonitrile and 20 mM ammonium phosphate buffer (20:80). The pH of the ammonium phosphate buffer was adjusted to 3.0 with 85% phosphoric acid (20:80), and the flow rate was 1.0 ml/min. The internal standard used was 3',5'-di-O-acetyldeoxyadenosine rather than A22U, the optical isomer of zidovudine (6).

C-18 Extract-Clean columns were washed with 2 column volumes of methanol followed by 2 column volumes of phosphate-buffered saline (0.2 g of KH2PO4, 1.2 g of KH2PO4, and 8.5 g of NaCl per liter, adjusted to pH 7.2). Serum samples were prepared by adding 0.1 ml of the internal standard solution (2.0 µg of 3',5'-di-O-acetyldeoxyadenosine per ml of phosphate-buffered saline) to 0.5 ml of the specimen. The peak height observed with the internal standard was reduced significantly if the internal standard was exposed to serum for ≥2 h at room temperature. This effect was not observed with heat-inactivated serum and presumably resulted from adenosine deaminase activity. For this reason, the mixture of serum sample plus internal standard was loaded onto the C-18 Extract-Clean column within 1 h of preparation and allowed to drain through the column. The column was then washed with 3 column volumes of phosphate-buffered saline and dried with a vacuum for 3 to 5 min. Zidovudine and the internal standard were eluted with 3 column volumes of methanol, evaporated to dryness under a stream of nitrogen, and reconstituted with 150 µl of distilled water. A 50-µl sample of the 150-µl reconstituted volume was injected into the HPLC system. The columns could be regenerated for further use (up to five times) by washing with 3 column volumes of methanol.

FPIA. The FPIA was performed as described by Jolley et al. (10), except that protein in the specimen was precipitated by the addition of an equal volume of extraction reagent (19.8 g of methanol, 1.28 g of 95% H2SO4, 6.27 g of 25% polyvinylsulfonic acid, 69.1 g of distilled water; Sigma). The FPIA utilizes fluorescein-labeled zidovudine (2 pmol per cuvette), which competes with the unlabeled zidovudine in the sample for the antibody. The fluorescent tracer was prepared by conjugating 3-(3-carboxypropyl)-3'-azido-3'-deoxythymidine to fluoresceinamine. The antisera was prediluted to provide approximately 160 to 170 milli-polarization units with 2 pmol of fluorescein-labeled zidovudine per cuvette in the absence of exogenous unlabeled zidovudine. Because the polarization of fluorescent light increases as the fluorescein-labeled zidovudine is bound by antibody, this assay provides a measure of bound and free labeled zidovudine in a competitive binding assay. The inverse relationship between the concentration of unlabeled zidovudine in the specimen and polarization was determined from the standard curve derived by testing the calibration standards (10, 15). The standard FPIA uses 50 to 60 µl of serum and standards containing 27, 94, 267, 534, and 1,356 ng of zidovudine per ml. For serum specimens containing less than 25 ng of zidovudine per ml, a second set of reagents was used with 200 µl of serum and standards containing 3, 10, 25, 60, and 140 ng of zidovudine per ml. Additional standards containing 0.18, 0.375, 0.75, 1.5, and 3.0 ng of zidovudine per ml were used to determine the lower limit of detection and the sensitivity of the second (more sensitive) FPIA.

RESULTS

Calibration curves. The FPIA calibration curves were nonlinear (Fig. 1) and were best described by a log-log transformation of the data, as with other FPIAs (10, 15). The HPLC calibration curve was linear; that is, the relationship between the zidovudine concentration and the peak height ratio (peak height of zidovudine divided by that of the internal standard) was linear (Fig. 2). The retention times for zidovudine and the internal standard were 7.0 ± 1.0 and 9.4 ± 1.2 min, respectively. However, the A22U peak was difficult to distinguish from that of zidovudine because its retention time (6.5 ± 0.8 min) was very close to that of zidovudine. For this reason, A22U was not used as the internal standard.

Limits of detectability and sensitivities. The limit of detectability was defined as the lowest concentration of zidovudine distinguishable from a control serum with no drug. The limit of detectability of the HPLC assay was 0.10 µM (27 ng/ml); the limits of detectability of the FPIAs were 0.002 µM (0.5 ng/ml) and 0.008 µM (2 ng/ml), respectively. Sensitivity was defined as the smallest measurable difference between drug concentrations. The sensitivity of the HPLC was 0.2 µM (54 ng/ml); the sensitivities of the FPIAs were 0.004 µM (1.0 ng/ml) and 0.019 µM (5 ng/ml).

Specificity. Forty-two commonly prescribed drugs were tested and found not to interfere with either the FPIA or the HPLC assay at a concentration of 10 µM. These drugs included four antiviral compounds (acyclovir, ganciclovir, 2',3'-dideoxyxycytidine, and 2',3'-dideoxyadenosine). Other drugs tested included six aminoglycosides (amikacin, gentamicin, netilmicin, kanamycin, streptomycin, and tobramycin), chloramphenicol, vancomycin, sulfamethoxazole, pentamidine, ketoconazole, amphotericin B, isoniazid, rifampin, pyrazinamide, digoxin, theophylline, seven anticon-
vulsants (diazepam, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid, and carbamazepine), four antiarrrhythmics (procainamide and its N-acetyl metabolite, quinidine, lidocaine, and disopyramide), four antidepressants (amitriptyline, nortriptyline, imipramine, and desipramine), three analgesics (acetaminophen, salicylate, and caffeine), cyclosporin A, methotrexate, and lithium. Two drugs (trimethoprim and sulfapyridine) interfered with the HPLC assay but not with the FPIA. No interferences were observed in the FPIA with serum specimens from patients with hepatic disease (bilirubin levels as high as 15.0 mg/dl), renal disease (creatinine levels as high as 7.0 mg/dl), hemolysis, or hyperlipidemia. Some interference was observed in the HPLC assay with serum specimens from patients with renal failure (serum creatinine, >3.0 mg/dl). The FPIA had cross-reactivities of 0.2, 0.3, and 0.4% with the zidovudine analogs 3-methylthymidine, 3', 5'-dideoxythymidine, and A22U (the optical isomer of zidovudine), respectively, but these analogs produced no interfering peaks in the HPLC assay. The other analogs tested (zidovudine glucuronide, adenosine, uridine, 5'-deoxythymidine, 2'-deoxyuridine, 5-bromouridine, thymidine, cytidine, inosine, 5-methyluridine, and 5-bromo-2'-deoxyuridine) had cross-reactivities of less than 0.05% in the FPIA and no detectable interfering peaks in the HPLC assay.

Effects of heat treatment. The heat treatment used to inactivate HIV had no effect on any of the three assays tested (FPIA, HPLC, or RIA).

Recovery. The absolute recoveries of the two methods were determined by adding zidovudine to serum and comparing the zidovudine concentrations measured in these serum specimens with those measured in aqueous specimens containing the same concentrations of zidovudine. Mean recoveries (n = 3) ranged from 93 to 100% for the FPIA and from 89 to 100% for the HPLC assay.

Precision. To define intrarun and interrun precision, each of the reference samples was tested 10 times in a single run and 10 times in separate runs (Table 1). Coefficients of variation ranged from 2.5 to 9% for the FPIA and from 8 to 33% for the HPLC assay.

Linearity. Linearity was assessed by comparing the values obtained for five serum samples to which zidovudine had been added with their theoretical concentrations (0.10, 0.34, 1.17, 4.11, and 14.37 μM zidovudine) using linear regression (2, 18). The equation of the regression line for the FPIA was y = 0.955x - 0.014, with a correlation coefficient of 0.999. The equation of the regression line for the HPLC assay was y = 0.938x + 0.129, with a correlation coefficient of 0.999.

Comparative accuracy. In a direct comparative study, 31 serum specimens from patients receiving zidovudine were assayed by FPIA, HPLC, and RIA. The concentrations determined by these three methods were compared by using linear regression (2, 18) and showed good agreement (Fig. 3 and 4). The equation of the regression line for FPIA versus HPLC was y = 1.037x + 0.078, with a correlation coefficient of 0.961. The equation of the regression line for FPIA versus RIA was y = 1.025x - 5.097, with a correlation coefficient of 0.992.

Glucuronidase treatment. As demonstrated by HPLC, β-glucuronidase treatment abolished the peak observed with

---

**TABLE 1.** Precision of FPIA and HPLC assay for zidovudine

<table>
<thead>
<tr>
<th>Measurement and assay</th>
<th>Precision (ng/ml) at zidovudine concn (μM) of*:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Intrainrun</td>
<td></td>
</tr>
<tr>
<td>FPIA</td>
<td>2.5</td>
</tr>
<tr>
<td>HPLC</td>
<td>ND</td>
</tr>
<tr>
<td>Interrun</td>
<td></td>
</tr>
<tr>
<td>FPIA</td>
<td>2.2</td>
</tr>
<tr>
<td>HPLC</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Data are presented as means, with the coefficients of variation in parentheses. A 1.0 μM concentration was 267 ng/ml. ND, Not done (below the limit of detection for HPLC).
zidovudine glucuronide and produced a new peak consistent with zidovudine (Fig. 5). The similar molar concentrations (20 μM) of these two compounds observed after incubation for 60 min at 37°C with HEPES (with or without β-glucuronidase) indicate that the hydrolysis of zidovudine glucuronide to zidovudine was complete when zidovudine glucuronide concentrations significantly greater than those typically found in the sera of patients receiving zidovudine treatment for HIV infection were used (12). Similar results were observed with both the FPIA and the RIA. No zidovudine was detected in specimens to which zidovudine glucuronide had been added when either the FPIA or RIA was used prior to hydrolysis with β-glucuronidase. Zidovudine alone (20 μM, 5,340 ng/ml) was detected after treatment with β-glucuronidase.

**DISCUSSION**

HPLC has been the method of choice for the quantitation of zidovudine in serum or plasma. Although HPLC is sensitive, accurate, and precise, it is impractical for laboratories performing large numbers of assays. The extensive sample preparation and the relatively long analysis time make this technique less desirable because they preclude a rapid turnaround time. The large sample size (500 μl) required renders HPLC less useful for measuring zidovudine in pediatric populations, and the technical expertise required typically restricts HPLC assays to large clinical or reference laboratories. In addition, endogenous interfering compounds compromise the HPLC assay for zidovudine in patients with renal failure.

In contrast to the HPLC assay, the FPIA is automated and rapid. Up to 45 tests may be performed per h (versus 3 with HPLC). Sample preparation is limited to a simple protein precipitation, and all subsequent operations are automated, thus reducing the technologist time required to perform the assay. Because the sample size for the FPIA can be as small as 50 μl, the FPIA should be useful for measuring levels of zidovudine in sera of children. Like the HPLC assay, the FPIA represents a significant investment if the machine must be purchased for this purpose alone. However, the equipment to run FPIAs is now commonly available, even in smaller clinical laboratories, and requires no special techni-

---

**Figures**

- **Fig. 3.** Comparison of FPIA and HPLC results for 31 clinical serum specimens from patients receiving zidovudine.
- **Fig. 4.** Comparison of FPIA and RIA results for clinical 31 serum specimens charted in Fig. 3.
- **Fig. 5.** Effects of β-glucuronidase treatment on the glucuronide metabolite of zidovudine. (A) A serum sample to which 40 μM zidovudine glucuronide (GZDV) had been added demonstrated an early peak (4.7 min) consistent with zidovudine glucuronide and a later peak consistent with the internal standard (IS). (B) After treatment with β-glucuronidase, the zidovudine glucuronide peak was no longer evident and there was a new peak consistent with zidovudine (ZDV) (7.0 min).
cal expertise to run. Because no interferences were observed in the FPIA with serum specimens from patients with renal disease, it should be possible to use the FPIA to measure levels of zidovudine in sera of patients with renal failure.

Because the FPIA for zidovudine correlates well with the RIA, either immunoassay should be suitable for the study of serum specimens from patients receiving zidovudine for HIV infection. However, neither immunoassay detects the principal metabolite of zidovudine (the 5'-glucuronide). This metabolite is difficult to measure by HPLC because it elutes rapidly (in 4.7 ± 0.8 min) from a C-18 column and is thus difficult to separate from early nonspecific peaks, especially in serum specimens from patients with renal or hepatic failure. For these reasons, we suggest that 5'-glucuronidase treatment followed by measurement of zidovudine with either the FPIA or RIA may provide a more accurate measure of the zidovudine glucuronide concentration than HPLC. With this approach, the amount of zidovudine glucuronide in a sample is the difference in the zidovudine concentration before and after hydrolysis with 5'-glucuronidase.

In this study, the FPIA for zidovudine was more sensitive and more specific than the HPLC assay and correlated well with HPLC and the RIA in the analysis of patient specimens. On the basis of these observations, we believe that the FPIA is an important alternative for the measurement of zidovudine in serum. In certain circumstances, such as pharmacokinetic studies of children or of patients with renal failure, the FPIA may be a much more useful method than HPLC.

ACKNOWLEDGMENTS

We thank Kenneth J. Stenglein and Dennis M. Murray of Sigma Chemical Co. for providing the antibody to zidovudine and for working with us in developing the FPIA; Steven S. Good of Burroughs Wellcome Co. for providing zidovudine, zidovudine glucuronide, and A22U; Larry Puckett of Incstar for providing the RIA kits; Gregory A. Storch and William G. Powderly for their thoughtful reviews of the manuscript; and J. William Campbell, Leonard Liebes, and Robert C. Packman for providing serum specimens from patients receiving zidovudine.

This work was supported in part by Public Health Service grant AI 25903 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED