clinical diagnostic problem. Onishi et al. (7) reported that the bilirubin photoisomer in the E-configuration easily reverted to native bilirubin in bile. However, in our study, presumptive photobilirubin is still present in clinical samples and is stable for more than 24 h in serum.

Differential Assay of Zidovudine and Its Glucuronide Metabolite in Serum and Urine with a Radioimmunoassay Kit

Sarva M. Tadepalli, Larry Puckett, Steve Jees, Lewis Kanica, and Richard P. Quinn

We developed an ancillary procedure for the ZDV-Trac RIA (Incstar) to allow simultaneous determination of both zidovudine (3'-azido-3'-deoxythymidine, ZDV, AZT, Retrovir®) and its metabolite, the glucurononide of ZDV (3'-azido-3'-deoxy-5'-O-β-D-glucopyranuronosylthymidine, ZDVG, GAZT), in human serum and urine. Using the ZDV-Trac RIA, we measured ZDV concentrations before and after ZDVG in samples was hydrolyzed to ZDV by β-glucuronidase (EC 3.2.1.31); ZDV glucuronide concentration was calculated as the difference between the two results. This method enables rapid evaluation of a large number of samples with a total turn-around time of 6 h. The lower detection limit of the RIA was 0.27 μg/L; the measurements varied linearly with ZDV concentrations from 0.27 to 217 μg/L, with the 50% inhibitory concentration being ~10 μg/L. Analytical recoveries of in-house serum and urine controls for both ZDV and ZDVG exceeded 90%. Coefficients of variation (CVs) of serum controls were <6% for ZDV and <11% for ZDVG; for urine controls, CVs for both ZDV and ZDVG were <6%. Results for ZDVG concentrations obtained by HPLC and by the ZDV-Trac RIA system compared well: r = 0.978, slope 1.0, for serum samples, and r = 0.993, slope 1.09, for urine samples.

Additional Keyphrases: β-glucuronidase · monitoring therapy · azidodeoxythymidine (AZT) · acquired immunodeficiency syndrome

Zidovudine (ZDV, AZT, Retrovir®, 3'-azido-3'-deoxythymidine) is an anti-retroviral drug shown to be effective in the treatment of acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (1, 2). ZDV is extensively metabolized to 3'-azido-3'-deoxy-5'-O-β-D-glucopyranuronosylthymidine (ZDVG, GAZT), an inactive metabolite that is rapidly cleared from plasma with an apparent half-life of 1 h (3). The major adverse reaction associated with ZDV administration is bone-marrow suppression (4); thus it is important to assess the efficacy and toxicity of ZDV by monitoring ZDV and ZDVG in biological fluids.

Among the techniques described for quantifying ZDV are the HPLC method that measures both ZDV and ZDVG (5), other HPLC systems that measure only ZDV (6, 7), and, as we previously described, a [3H]ZDV-based radioimmunoassay (8), a time-resolved fluoroimmunoassay based on an anti-ZDV polyclonal antiserum, and an enzyme-linked immunosorbent assay based on an anti-ZDV monoclonal antibody (9). Granich et al. (10) recently reported a fluorescence polarization immunoassay for measuring ZDV.

None of the HPLC systems lends itself to high throughput. By contrast, immunoassays can be quick and efficient. A commercial immunoassay kit, ZDV-Trac RIA kit (Incstar Corp., Stillwater, MN), has been introduced for determinations of ZDV in human serum. Here we describe adaptation

References

1 Experimental Therapy Division, Burroughs Wellcome Co., 3050 Cornwallis Road, Research Triangle Park, NC 27709.
2 Incstar Corp., Stillwater, MN 55082.
3 Wellcome Research Laboratories, Beckenham, Kent, U.K. Received December 29, 1989; accepted March 30, 1990.
of the ZDV-Trac RIA kit so that both ZDV and ZDVG can be quantified in serum and urine samples. The ZDV-Trac RIA involves use of $^{125}$I-labeled ZDV ligand and polyclonal anti-ZDV antiserum. The ZDV-Trac RIA has certain advantages over the $^3$H-RIA, namely, shorter incubation time for the assay, and quicker and more efficient quantification by gamma counting instead of liquid-scintillation counting.

**Materials and Methods**

**Reagents**

ZDV and ZDVG were synthesized at Burroughs Wellcome Co. (Research Triangle Park, NC). $\beta$-Glucuronidase (EC 3.2.1.31, Type X, purified lyophilized powder; no. 7896) was purchased from Sigma Chemical Co., St. Louis, MO. ZDV-Trac RIA kits were supplied by Incstar Corp. The assay buffer for determining ZDV was 25 mmol/L phosphate buffer, pH 6.8, made up as follows: 0.85 mL of concentrated (85%) H$_3$PO$_4$ + 450 mL of de-ionized water, adjusted to pH 6.8 with KOH, and made up to 500 mL with de-ionized water.

**Clinical Specimens**

Serum and urine samples were collected from patients receiving ZDV for anti-human immunodeficiency virus (anti-HIV) therapy. Serum and urine samples were heat-inactivated in a water bath at 58°C for 60 min to ensure HIV inactivation (11) in patients' samples (and thereby decrease the health risk to those handling HIV samples) and frozen at −20°C until analysis. Both ZDV and ZDVG are stable after heat inactivation at 58°C for 1 h (5).

**Procedures**

**ZDV and ZDVG by HPLC:** HPLC analysis of samples for ZDV and ZDVG was carried out according to Good et al. (5).

Preparation of in-house serum and urine controls: We prepared 1 mmol/L stock solutions of ZDV and ZDVG and spectrophotometrically verified their concentrations by measuring their absorbances at 267 nm, using $\varepsilon = 7780$ for ZDV and $\varepsilon = 7650$ for ZDVG. Normal human serum controls containing a combination of ZDVG/ZDV were prepared by adding small aliquots of ZDV and ZDVG stock solutions to sera to give final concentrations of 4.0/2.0 μmol/L (ZDVG/ZDV) in the control. The other two controls were made by serially diluting that control with normal human serum to give final concentrations of 2.0/1.0 μmol/L and 0.5/0.25 μmol/L (ZDVG/ZDV). A pool of normal human urine was frozen at −20°C for 24 h, then thawed and filtered through a 0.22-μm (pore-size) filter (Corning disposable filtering unit system; Corning Glass Works, Corning, NY). We added to this known concentrations of ZDV and ZDV to give final concentrations of 400/80, 80/16, 16/3.2, 30/15, 10/5, and 3.75/1.25 μmol/L (ZDVG/ZDV), which were stored at −20°C in 1-mL aliquots. The molecular masses of ZDV and ZDVG are 267.2 and 443.4 Da, respectively, so to convert μmol/L to mg/L, multiply by 0.267 and 0.443, respectively.

Determination of ZDV by ZDV-Trac RIA kit: This RIA, which includes a polyclonal anti-ZDV antibody (supplied by Burroughs Wellcome Co.) and an $^{125}$I-radio-labeled tyramine derivative of ZDV ($^{125}$I-ZDV), was performed according to the protocol supplied by Incstar Corp. In brief, we diluted the patients' serum samples 10-fold with phosphate buffer, then mixed 200 μL of each sample in duplicate with 100 μL of anti-ZDV antibody and 100 μL of $^{125}$I-ZDV tracer in glass tubes and incubated for 2 h at room temperature. A preprecipitated double-antibody reagent (500 μL), goat anti-rabbit precipitating complex, was added to each tube and mixed thoroughly. After incubating these for 30 min at room temperature, we centrifuged the samples at 1000 × g for 20 min, decanted the supernates, and counted the radioactivity in the pellets with a gamma counter. Patients' urine samples and controls were diluted 1000-fold for ZDV analysis.

Hydrolysis of ZDV by $\beta$-glucuronidase to measure total ZDV in samples: $\beta$-Glucuronidase (25 kU) was reconstituted in 2.5 mL of the phosphate buffer. We combined 200 μL of 10-fold-diluted serum sample with 200 μL of the phosphate buffer and 100 μL (1 kU) of the $\beta$-glucuronidase reagent, mixed thoroughly, and incubated the samples in capped containers at 37°C in a water bath for 30 min. To assay ZDV in urine, we reconstituted 25 kU of $\beta$-glucuronidase in 1 mL of the phosphate buffer, then mixed 100 μL of this (25 kU of $\beta$-glucuronidase) with 100 μL of diluted urine sample and 500 μL of the phosphate buffer. We capped the tubes, vortex-mixed their contents, and incubated at 37°C in a water bath for 60 min. The final dilutions of serum and urine for total ZDV analysis were 20- and 2500-fold, respectively. We analyzed the hydrolyzed samples for total ZDV.

Analysis of data: Concentrations of unknowns were calculated by using the standard curve parameters derived from a log-log or a four-parameter logistic fit of transformed data. Both the ZDV and the ZDVG concentrations of unknowns were interpolated from the same standard curve. After correcting for dilutions, we subtracted the ZDV concentrations in nonhydrolyzed samples from those of the hydrolyzed samples (total ZDV), then multiplied by 1.66 (to account for the molecular mass difference between ZDV and ZDVG) to obtain ZDVG concentrations (μg/L).

**Results**

The serum concentration data presented in Figure 1 were from samples collected from 12 HIV antibody-positive patients who were receiving ZDV therapy. The investigators of the study were interested in quantifying the concentrations of both ZDV and ZDVG in serum to evaluate the...
safety and tolerance of ZDV administration (200 mg every 4 h, 1200 mg daily). The concentrations of ZDVG in serum were at least double those of ZDV throughout the study, as shown by the concentration–time curves for ZDV and ZDVG.

Dilution parallelism was tested by diluting the serum controls 10-, 20-, and 40-fold for ZDV analysis and 25-, 50-, and 100-fold for ZDVG analysis. The ZDV/ZDVG urine controls (30/10, 15/5, 3.75/1.25 μmol/L) were diluted 200-, 500-, and 1000-fold for ZDV analysis and 1000-, 2500-, and 5000-fold for ZDVG analysis. Both sets of in-house controls gave values within 10% of their expected concentrations for both ZDV and ZDVG at any given dilution (data not shown).

Reproducibility and analytical recovery of in-house controls: Both intra- and interassay precision and accuracy were assessed by analyzing in-house controls. Both serum and urine controls were tested in replicates of eight for intra-assay precision, and the values obtained from eight independent assays for both controls were used to determine the interassay precision. Both intra- and interassay CVs for analysis of in-house controls were low for ZDV and ZDVG analyses (Table 1). The analytical recoveries of in-house serum controls for ZDV estimation were 95% to 99%; for ZDVG, the recoveries ranged between 101% and 105%. In urine controls, analytical recovery of ZDV was between 95% and 97%; of ZDVG, between 97% and 99.9%.

Comparison of HPLC and ZDV-Trac RIA: Fifteen serum and urine samples from patients with renal insufficiency were analyzed by both HPLC (x) and ZDV-Trac RIA (y) to validate ZDV concentrations obtained by ZDV-Trac RIA. The data obtained from both methods were subjected to unweighted linear-regression analysis. For serum ZDV concentrations, the linear-regression equation was $y = 1.012x - 0.257$ mg/L ($r = 0.978$); for urine samples, the equation was $y = 1.093x + 1.676$ mg/L ($r = 0.9931$).

Discussion

Use of the supplementary hydrolysis procedure with the ZDV-Trac RIA kit, which facilitates the measurement of both ZDV and ZDVG in serum and urine, is an attractive alternative to HPLC methodology. This system is especially advantageous in analyzing small-volume samples (pediatric or neonatal samples) and allows for rapid evaluation of a large number of samples. The specificity of anti-ZDV antibody (8) is such that this RIA does not experience problems with interference by endogenous substances, a problem commonly encountered in HPLC analysis of samples from patients with renal insufficiency. Moreover, the major metabolite of ZDV, ZDVG, does not cross-react in this assay, a feature that enabled us to develop the differential analysis of ZDV and ZDVG after β-glucuronidase reaction with samples. Although HPLC has been the standard technique for the simultaneous analysis of ZDV and ZDVG in biological fluids, the ZDV-Trac RIA is another choice that offers technical simplicity and rapid turnaround time. Because most clinical and reference laboratories are equipped with gamma counters, they can conveniently use the ZDV-Trac RIA to determine ZDV and ZDVG in patients receiving ZDV therapy. In addition, HPLC analysis of ZDV concentrations is difficult, necessitating special care to discriminate among the early endogenous peaks co-eluted with ZDVG (5).

Our data indicate that hydrolysis of samples by β-glucuronidase to obtain ZDV concentrations is a reliable method of estimating the glucuronide metabolite of ZDV. Although the clinical need to monitor ZDV in serum or urine has not been established, we believe the differential analysis of ZDV and ZDVG in serum will aid in establishing a therapeutic range of ZDV, will allow assessments of the interactions of ZDV with other drugs, and can be used to assure compliance.

We are grateful to Dr. T. A. Krenitsky for his continued encouragement and support. We also thank Barbara Orban for the supply of anti-ZDV antiserum, David Reynolds for providing HPLC analysis, and Steve Weller for providing patients’ data.

References


Table 1. Intra- and Interassay Precisions and Analytical Recoveries of In-House Controls

<table>
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<tr>
<th>Expected connc of controls, μg/L</th>
<th>RIA, mean ± SD, μg/L</th>
<th>CV, %</th>
<th>% recovery</th>
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<td></td>
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<td>ZDVG</td>
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<td><strong>Intra-assay variation</strong></td>
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<tr>
<td>Serum</td>
<td>1770</td>
<td>534.4</td>
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<td>234±15.2</td>
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<td>2670</td>
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*n = 8 each.*
Use of Polymerase Chain Reaction to Detect Heterozygous Familial Hypercholesterolemia

Mauri Keinänen,1,4 Jukka-Pekka Oja,2 Eero Heve,2 Katrinla Aalto-Setälä,1,4 Kimmo Kontula,4,6 and Petri T. Kovanen1,6

We used a modification of the polymerase chain reaction (PCR), involving two pairs of oligonucleotide primers, to detect a mutation in the low-density lipoprotein (LDL) receptor gene, commonly occurring among patients with familial hypercholesterolemia (FH) in Finland. This mutation, called FH-Helsinki, involves a large (about 9500 base pairs, bp) deletion in the LDL receptor gene extending from intron 15 to exon 18. For the PCR, one pair of primers was designed to cover both sides of the deletion in its immediate vicinity. In the presence of the deletion, the primers were brought close enough to each other to allow the amplification and electrophoretic detection of a 300-bp amplification product. In the absence of the deletion, no amplification occurred and this band accordingly was not visible in the gel. To render the interpretation of the results unequivocal, we designed a second pair of oligonucleotide primers. This pair of primers allowed another amplification product (158 bp) to appear in samples containing a normal exon 17, i.e., in DNA specimens from healthy subjects and FH heterozygotes with or without the FH-Helsinki deletion. The technique is easy to perform, avoids the use of radioactive reagents, and is applicable to the detection of any extensive DNA deletion.

Additional Keyphrases: gene probes · low-density lipoprotein receptors · electrophoresis, agarose

A specific deletion in the low-density lipoprotein (LDL) receptor gene, designated FH-Helsinki, is found in at least one-third of Finnish patients heterozygous for familial hypercholesterolemia (FH) (1, 2). The 9.5-kb (kb = kilobase pairs) deletion leads to loss of exons 16 and 17 and part of exon 18. The mutation appears to give rise to a phenotype in which internalization of LDL particles by the cells is defective (2). The high prevalence of the deletion justifies the development of a convenient assay for detecting the mutation by using DNA techniques. The deletion can be identified by analysis of genomic DNA, with use of a conventional Southern blotting technique with radioactive probes (1).

Here we present another method for detecting the FH-Helsinki deletion, by using the polymerase chain reaction (PCR) (3). PCR allows rapid visual detection of DNA deletions in ethidium bromide-stained agarose or polyacrylamide gels (4, 5). Small deletions, from a few to several hundreds of base pairs (bp), can be identified in this way by means of the length differences between the amplified DNA fragments from controls and patients. However, longer deletions are likewise amenable to PCR detection. Thus, if the amplification primers are chosen to represent DNA stretches flanking the two ends of the deletion, the deletion brings the primers close enough to allow DNA amplification; in the absence of the deletion, this technique fails to show any bands, the upper limit for PCR amplification being in the range of 2000 bp (5). To avoid the problem of a false-negative result, we have designed two pairs of amplification primers and used a "duplex" PCR reaction (5, 7) to be able to verify with certainty the presence or absence of the FH-Helsinki mutation.

Materials and Methods

The samples for PCR were obtained from nine patients heterozygous for FH and from normal controls. The diagnosis of FH was based on typical clinical features, including a total serum cholesterol concentration >9.5 mmol/L, the presence of tendon xanthomas, and a positive family history (at least one first-degree family member with hypercholesterolemia), and was confirmed by establishing a...