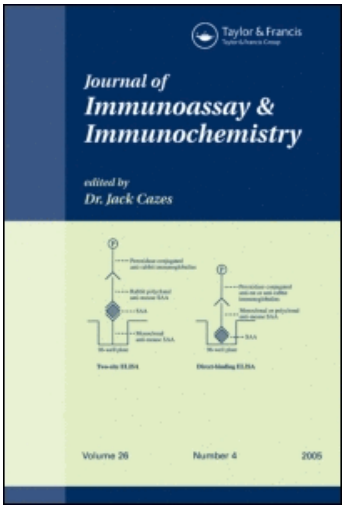

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Radioimmunoassay for Retrovir, An Anti-Human Immunodeficiency Virus Drug

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To cite this Article Quinn, Richard P. , Orban, Barbara and Tadepalli, Sarvamangala(1989) 'Radioimmunoassay for Retrovir, An Anti-Human Immunodeficiency Virus Drug', Journal of Immunoassay and Immunochemistry, 10: 2, 177 — 189
To link to this Article: DOI: 10.1080/01971528908053235
URL: http://dx.doi.org/10.1080/01971528908053235

JOURNAL OF IMMUNOASSAY, 10(2&3), 177-189 (1989)

RADIOIMMUNOASSAY FOR RETROVIR, AN ANTI-HUMAN IMMUNODEFICIENCY VIRUS DRUG

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ABSTRACT

A direct radioimmunoassay (RIA) for the quantitation of Retrovir® (zidovudine, azidothymidine, AZT) in biological fluids has been developed. The assay is sensitive with an I_{50} value of about 30 nM and with a lower limit of detection of about 3 nM. Intra-assay precision gave sample coefficients of variation that ranged from 1.77 to 8.65 % for the standard curve with human plasma. Inter-assay precision and accuracy were within acceptable limits. The RIA was validated by comparing results obtained from the analysis of rat plasma samples by both this RIA and a high-performance liquid chromatography method. None of the crossreactivities recorded should interfere with the assay system. The affinity constant of the antibody chosen for use was 1.4×10^9 L/mol. (KEY WORDS: Retrovir®, radioimmunoassay, antiviral, azidothymidine, AZT).

INTRODUCTION

The rapid evaluation and introduction of Retrovir®(AZT, 3'-azido-3'-deoxythymidine, BW A509U, zidovudine), the first FDA-approved drug for the treatment of HIV disease, necessitated the timely development of a classical, non-immunological technique for the determination of plasma and urine levels of this compound and its metabolites. The method chosen for these initial studies was high-performance liquid chromatography (HPLC) (1). It was apparent that a quicker assay system was needed to support the extensive preclinical and clinical evaluations of this drug, so the development of a radioimmunoassay (RIA) was initiated.

Because AZT is extensively metabolized to the 5'-O-glucuronide [GAZT] in man (2,3), the antisera chosen for the radioimmunoassay system needed to be able to detect the parent compound in serum or plasma, but be unaffected by GAZT. In addition, the antisera should not crossreact with any of the expected concurrent medications, while at the same time providing a sensitive assay. This paper reports the development of such an immunologically based assay system; it has some advantages over the HPLC method, including greater sensitivity and higher throughput.

MATERIALS AND METHODS

Chemicals and Reagents.

Compound BW A1183U (5-carboxyethyl-3'-azido-3'deoxythymidine) was prepared by Mr. A. Freeman and Dr. J. Rideout of the Dept. of Organic Chemistry, Wellcome Research Laboratories. Rabbit serum albumin (RSA) and labile enzyme-free bovine gamma globulin (LEF BGG) were purchased from Miles Laboratories. Other reagents were described previously (4,5).

Immunogen. 1183U-RSA

BW A1183U was coupled to RSA using the mixed anhydride procedure (4). A typical reaction is as follows: 300 mg of BW A1183U was dissolved in 3.0 ml of anhydrous

dimethylformamide (DMF) with stirring at room temperature. Ten minutes later 300 µl of triethylamine and 320 µl of isobutylchloroformate were added. The suspension was stirred at room temperature for 1.0 h ; the resulting mixed anhydride solution was chilled to 4° C. This solution was then slowly added dropwise to a prechilled RSA solution prepared by dissolving 250 mg of RSA in 25 ml of 0.1 N sodium bicarbonate buffer, pH 8.15. The addition took about 2.0 h. After the last addition, the solution was held at 4° C overnight. The conjugated product 1183U-RSA (5-carboxyethyl-3'-azido-3'-deoxythymidine-RSA) was isolated by chromatography on a Sephadex G-25 column (2.5 x 45 cm) using normal saline as the eluent. The first peak, which was the desired product, was identified and then concentrated at 50 psi using an Amicon Model 12 ultrafiltration cell fitted with a PM10 membrane. The concentrate was filter sterilized (0.22 microns), analyzed by UV spectroscopy, and then used for immunization. Hapten and protein concentrations were estimated simultaneously using two equations derived from the individual absorption spectra at 254 and 304 nm. The hapten-to-carrier ratio for the 1183U-RSA immunogens varied from 8.3 to 11.0 mol/mol. Protein recovery was generally better than 80%.

Immunizations and Bleedings.

Six rabbits were initially immunized s.c. with BW A1183U-RSA (2 mg total protein in two sites) in complete Freund's adjuvant. These animals were reimmunized one month later s.c. with the same quantity of immunogen in incomplete Freund's adjuvant and boosted again one month later s.c. (0.5 ml in 4 sites) with the same material. After this third immunization, the animals were bled and reimmunized s.c. periodically. An additional group of six rabbits was later immunized with this same immunogen, when it was found to give an adequate response.

Blood samples were collected and allowed to clot overnight. The resulting sera were heat-inactivated for 60 min at 60° C to destroy complement and esterase activity, and then were frozen at -20° C until used.

Preparation of Radioactive Antigens.

^{3}H-AZT was prepared by Dr. J. Hill of Chemical Development, Wellcome Research Laboratories [6]. The antigen was labelled at the 5'-position and had a specific activity of 12.5 Ci/mmol.

Binding Assays.

To evaluate the binding characteristics of the antisera, 100 µl of diluted anti-AZT serum was added to 300 µl of buffer (50 mM KH_2PO_4, 0.9% NaCl, 10 mM Na_2EDTA, and 0.01% ethylmercurithiosalicylic acid, pH 7.5) in 12 x 55 mm polypropylene tubes (W. Sarstedt, Inc.), followed by addition of 100 µl of diluted radioactive antigen (about 25,000 cpm). After mixing with a vortex apparatus, the tubes were incubated overnight at 4° C. After incubation, 50 µl of cold LEF-BGG (10 mg/ml) was added as the carrier protein, followed by 550 µl of cold saturated ammonium sulfate in water (pH 7.5 at 4° C). The tubes were mixed and held at 4° C for about 1 h. The tubes were then centrifuged for 20 min at 2230 x g and 4° C. The supernatant was decanted and discarded; any remaining fluid was carefully removed by aspiration. The precipitate was washed once with 1 ml of cold, half-saturated ammonium sulfate and, after recentrifugation, the supernatant was removed as above. The precipitate was dissolved in 0.1 ml of deionized water; 0.1 ml of 4N HCl was then added to each tube followed by 2.5 ml of Aquasol-2 scintillation counting fluid (NEN Research Products, Boston, Mass.). The tubes were capped, immediately mixed by vortex, placed in 20 ml scintillation vials (caps had an 0.5-in. hole to center the tubes accurately), and counted. Samples were assayed in triplicate.

RIA of AZT.

For the assay, 100 µl of diluted sample containing an unknown quantity of AZT was added to a 12 x 55 mm polypropylene tube, followed by 200 µl of buffer, 100 µl of diluted anti-AZT antiserum and 100 µl of radioactive antigen (^{3}H-AZT). Tubes were prepared in triplicate. No extraction or derivitization of the samples was required. The tubes were

then mixed using a vortex apparatus and were incubated at 4° C. The procedure from this point was the same as for the binding studies (see above). To obtain a standard curve, known amounts of AZT in buffer and 100 µl of normal plasma, urine or other fluid (diluted to the same extent as the unknowns) were added to the assay tubes, with the final incubation volume of 500 µl being maintained by adjustment of the buffer volume added. The scintillation-counting data were accumulated in a data acquisition system and then directed to a computer, where a standard curve was obtained by performing a log-logit transformation followed by iterative, weighted least-squares linear-regression analysis; unknown concentrations were calculated using the standard curve parameters obtained.

RESULTS

Response of Rabbits to the Immunogen.

The response of the first group of rabbits immunized with 1183U-RSA toward the tritiated antigen, ^{3}H-AZT, is shown in Figure 1. All six animals in this group responded with antibody by one month, although three had only minimal titers. The second group of six rabbits immunized with 1183U-RSA also responded quickly and gave moderate titers of antisera by one month [data not shown]. Two animals in this latter group with the highest initial titers (#9503 and #9505) were chosen for further evaluation. For the studies reported here, results were obtained using a pool of antisera obtained from the second, third and fourth bleedings. These bleedings were obtained after the fourth immunization.

RIA Standard Curve and Comparison with the HPLC Method.

A variety of conditions, including buffer composition and pH, time, temperature of incubation and other factors, were investigated [data not shown]. A typical standard curve is given in Figure 2, where antisera from rabbit #9505 was used with the tritiated antigen and with a 1:50 dilution of human plasma. A straight line was obtained using the log-logit

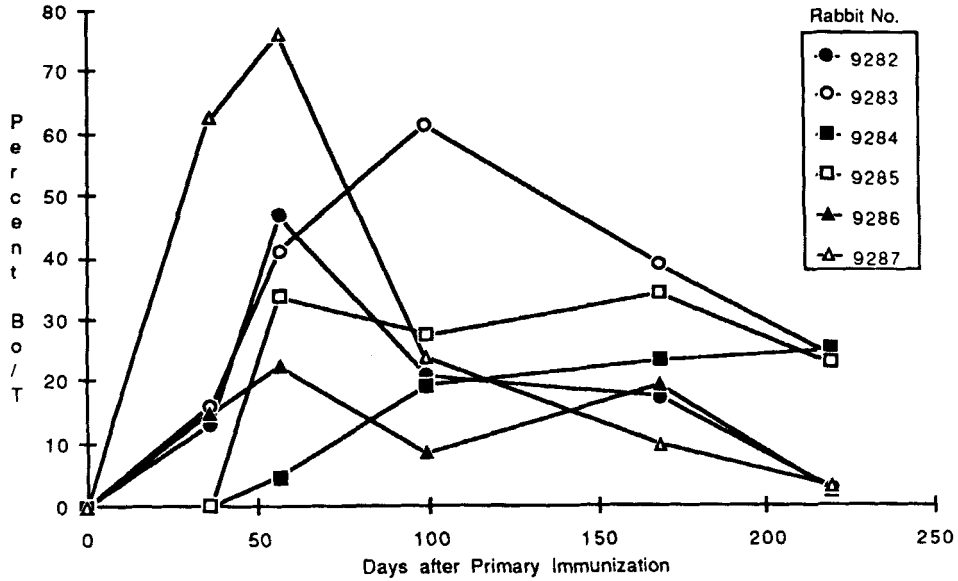

Figure 1. The Antibody Response [as Percent Bo/T] of Rabbits Immunized with 1183URSA as a Function of Time in Days after Primary Immunization. Antisera was diluted 1:10 for Analysis.

transformation of the data between 3 and 800 nM with an I_{50} of 30.6 nM. A coefficient of correlation (squared) of 0.9952 was obtained.

To validate the assay, a range of concentrations of AZT prepared in normal human plasma were analyzed by RIA [see below]. In addition, a number of plasma samples from rats dosed intravenously with AZT at 37.5 to 300 mg/kg were analyzed by both RIA and HPLC. A comparison of the results obtained using the two methods is given in Figure 3. A good correlation was obtained [R=0.989] with a slope of 0.769.

Intra- and Inter-assay Accuracy and Precision.

Results of a study designed to investigate the intra-assay precision and accuracy of the RIA using antisera from

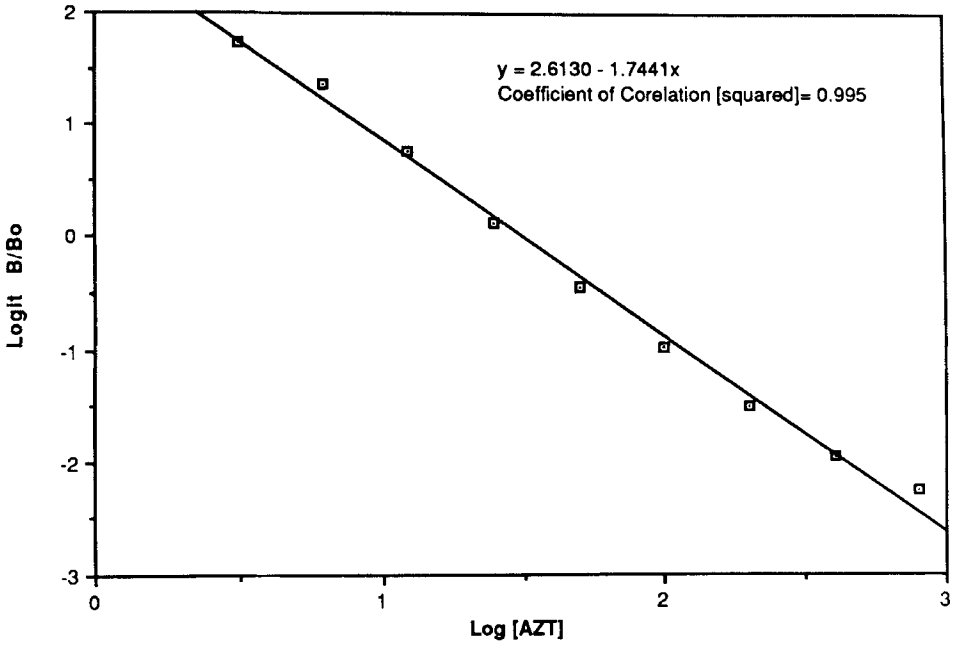

Figure 2. Standard Curve with Logit B/B_o plotted versus Ln (AZT) using Human Plasma at a 1:50 Dilution. Antisera #9505 at a 1:75 Dilution; ^{3}H-AZT as the Antigen (Specific Activity = 12.5 Curies/millimole). Standard Curve Parameters Obtained: B_o/T = 0.2259 Slope = -0.7938; R^2 = 0.9952; and I_{50} = 30.6 μM.

rabbit #9505 and ^{3}H-AZT are given in Table 1. Results from the quantitation of AZT in human plasma indicate good agreement between the prepared standard concentrations and the fitted values obtained from the log-logit analysis. Precision was also good, as judged from the CV values, which range from 1.77 to 8.65% for the standard curve samples and from 2.00 to 6.28% for the internal standard samples. A similar study was executed to obtain data on the inter-assay precision and accuracy, again using human plasma (Table 2). The agreement between the nominal and observed values for the internal standards indicates good accuracy for this system. The CV values obtained for the internal standards in this set of six experiments ranged from 6.83 to 27.39% .

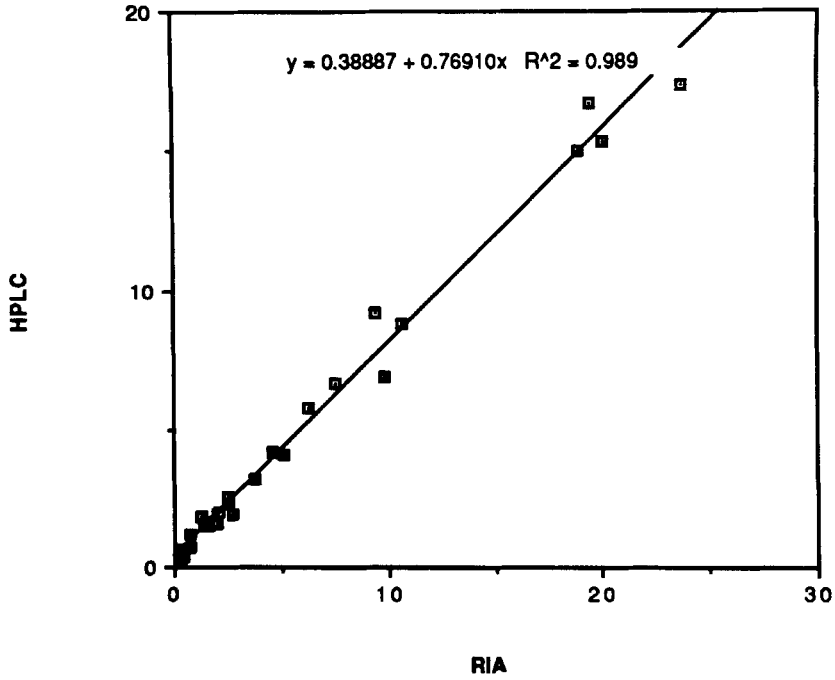

Figure 3. Comparison between High Performance Liquid Chromatography and Radioimmunoassay. Plasma samples were from rats which had received AZT intravenously at doses ranging from 37.5 to 300 mg/kg.

Specificity of the Assay.

The specificity of the antisera chosen for use in this assay is summarized in Table 3. Crossreactivities for antisera #9505 with ^{3}H-AZT are listed. The only appreciable crossreactivity seen were with the the analogue used to prepare the immunogen, BW A1183U. Very slight reactivity was seen with the known metabolites of AZT, 3'-amino-3'-deoxythymidine [the 5'-monophosphate of AZT] and GAZT. The crossreactivities seen with these materials should not limit the use of this assay for any study. This antigen demonstrated no other crossreactivities with a wide range of naturally occurring purines, other purines, thiopurines, a number of anti-coagulants and other materials.

TABLE 1

Intra-assay Precision and Accuracy for Human Plasma Analyses.
Antiserum #9505 at 1:75, ^{3}H-AZT as the Antigen, N=10.

Standard Curve Evaluation			
Standard Conc.	Value Found	%Deviation	CV (%)
160.0 pmoles	119.28	-25.45	2.98
80.0	66.21	-17.24	4.40
40.0	35.03	-12.42	2.73
20.0	21.81	+9.05	5.39
10.0	10.65	+6.50	3.70
5.0	5.574	+11.48	8.65
2.5	2.388	+4.48	6.96
1.25	1.119	+10.48	4.57
0.625	0.552	+10.40	1.77
0.3125	0.4154	+32.00	4.40
0.1562	0.1643	+5.15	2.86

Internal Standard Evaluation			
Nominal Value	Value found	% Deviation	CV (%)
20.0 pmoles	24.34	+21.70	6.28
10.0	10.86	+8.60	3.04
4.0	4.221	+5.52	2.00
2.0	2.030	+1.50	4.16
1.0	1.001	+0.05	3.66
0.5	0.552	+10.40	3.65

Standard Curve Parameters:

Bo/T:	0.190
Slope:	-0.7214 +/- 0.0104
I_{50} (nM)	24.456 +/- 0.501
Coefficient of Correlation:	0.989

Affinity Constant Determinations.

The affinity constant with ^{3}H-AZT was obtained with the antisera from rabbit #9505. The value was found to be 1.4 x 10^9 L/mol.

Table 2

Inter-assay Precision and Accuracy for the Radioimmunoassay for AZT. Antiserum #9505 at 1:75, ^{3}H-AZT as antigen, N=6.

Standard Curve Parameters:	Mean	Std. Dev.
Bo/T	0.2260	0.0200
Slope	-0.8280	0.1040
I_{50} (nM)	40.21	1.384
Coefficient of Correlation	0.9740	0.0240

Internal Standard Evaluation

Nominal Values	Value Found	%Deviation	CV (%)
20.0	18.84	-5.79	17.11
10.0	10.36	-3.61	6.83
5.0	5.578	-11.55	8.32
2.5	2.876	-15.04	11.85
1.0	1.055	-5.50	18.94
0.5	0.434	-13.22	27.39

DISCUSSION

The single-tube RIA for AZT reported here is sensitive, with an I_{50} value of about 30 nM and with a lower limit of detection of about 3 nM. This allows for a reasonable dilution [1:50] for the detection of trough values. If both peak and trough levels are needed, the wide range of the standard curve may be advantageous and allows one to detect both values with the same dilution for all of the members of a set. If only the peak level is of interest, then a higher dilution [1:500] may be more appropriate. Using the antigen and antibody described here, the assay system gives reproducible results as demonstrated by inter-assay and intra-assay precision.

The assay system is also very reliable as there are essentially no major crossreactivities. The only significant crossreactivity seen is with the material used to prepare the

TABLE 3

Crossreactivity of Various Metabolites and Other Materials, Including Potential Concurrent Medications with AZT in the Radioimmunoassay using ^{3}H-AZT as Antigen with Rabbit Antiserum #9505 at 1:75.

Compound[1]	% Crossreactivity [2]
A. Metabolites and Other Analogs:	
3'-Amino-3'-deoxythymidine	0.0021
AZT monophosphate	0.045
GAZT	0.031
BW A1183U	13.10
Thymidine	0.037
Uracil	<0.001
Uridine	<0.001
B. Potential Concurrent Medications:	
Acetominophen	<0.001
Acyclovir	<0.001
Ara A	<0.001
Aspirin	<0.001
BW B759U	<0.001
Chloramphenicol	<0.001
Dapsone	<0.001
Desciclovir	<0.001
Fanzil	<0.001
Isoniazid	<0.001
Penicillin	<0.001
Probenicid	<0.001
Pyrimethamine	<0.001
Ribavirin	<0.001
Rifampicin	<0.001
Trimethoprim	<0.001
Sulfamethoxazole	<0.001
Streptomycin	<0.001
Tetracycline	<0.001

[1] Compound Names: Desciclovir, 2-[(2-Amino-9H-purin-9-yl)methoxy]ethanol; GAZT, 3'-Azido-3'-deoxy-5'-o-ß-D-glucopyranosylthymidine; BW B759U, 9-[[2-Hydroxy-1-(hydroxymethyl) ethoxy]methyl]guanine; BW A1183U, 3-[1-(3-Azido-2,3-dideoxy-ß-D-erythro-pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidinyl] proprionic acid.

[2] Crossreactivity = I_{50} for AZT x 100 / I_{50} for Substance Tested

immunogen [BW A1183U]. That this crossreactivity is not even higher can be explained by the use of a radioligand which is significantly different, i. e. AZT, and thus this compound which was used to prepare the original immunogen might not be expected to compete as efficiently as non-radioactive AZT. The reactivity with GAZT is very low at 0.031%. Since GAZT plasma levels are typically only two to three times that of AZT [3], the error resulting from any GAZT interference should not exceed about 0.1%.

This RIA has now been used in both preclinical and clinical studies with AZT. Many sample types have been analyzed including plasma, urine and tissue-homogenate supernatants. Plasma samples from various species including the dog, duck, monkey, pigeon, rat, and woodchuck have been successfully analyzed. The affinity constant of antisera #9505 with the tritiated antigen was 1.4×10^9 L/mol.

ACKNOWLEDGEMENTS

We thank Steve Good and Dave Reynolds for their critical reading of the manuscript and Dr. Paulo de Miranda and Mrs. T. Burnette for the determination of the rat plasma AZT levels.

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Footnotes

1. Abbreviations Used: AZT, 3'-azido-3'-deoxythymidine [BW A509U, zidovudine]; B/Bo, ratio of the quantity of radioactive drug bound in the presence and absence of additional unlabelled drug; B/T, ratio of the quantity of radioactive drug bound to the total radioactive drug added; CV, coefficient of variation; GAZT, 3'-azido-3'-deoxy-5'-ß-D-glucopyranuronosylthymidine [5'-glucuronyl-AZT]; HPLC,

high-performance liquid chromatography; HIV, human immunodeficiency virus; LEF-BGG, labile enzyme-free bovine gamma globulin; DMF, dimethylformamide; RIA, radioimmunoassay; RSA, rabbit serum albumin.

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