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Scintillation proximity radioimmunoassay for the measurement of acyclovir

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Abstract

A homogeneous, single-tube scintillation proximity radioimmunoassay (SPRIA) to quantitate acyclovir (Zovirax®), ACV, (9-[(2[hydroxyethoxy)]methylguanine)} in human plasma is described. The reagents for the SPRIA are an anti-ACV monoclonal antibody (WACO4 MAb), tritiated ACV, and scintillation proximity reagent (goat anti-mouse immunoglobulin G (IgG) coupled to fluoromicrospheres). The ACV standard curve range in the SPRIA is from 0.7 ng ml^{-1} (3.0 nmol l^{-1}) to 90.0 ng ml^{-1} (0.4 μmol l^{-1}) with a 50% inhibitory concentration of 5.0 ng ml^{-1} (22.2 nmol l^{-1}). However, the lower limit of quantification is 7 ng ml^{-1} at 1:10 dilution of plasma. Analytical recovery of ACV in spiked human plasma controls ranges between 90–110%. Intra- and inter-assay relative standard deviations were $< 8\%$. This high throughput homogeneous assay is a rapid, convenient and simple alternative to the current radioimmunoassay that uses ammonium sulfate precipitation as the separation method. This technique is particularly attractive because it requires neither separation of bound from free drug nor use of scintillation fluid. The procedure was applied to quantitate ACV in samples from pre-clinical and clinical studies after the administration of valaciclovir, a prodrug of ACV (256U87, Valtrex®, L-valyl ester of ACV). Automation of this assay will further improve efficiency in processing a larger number of samples.

Keywords: Cross-reactivity; MAb; RIA; Scintillation proximity assay; Valaciclovir; L-Valyl ester of acyclovir

1. Introduction

Acyclovir [9-(2-hydroxyethoxy)methylguanine] (ACV, Zovirax®) (Fig. 1a) is an antiherpetic agent that has been in clinical use for treating a variety of herpes and varicella-zoster virus infections. The mechanism of action and pharmacokinetics of ACV has been well established for both intravenous and oral formulations of the drug [1,2]. More recently, a number of prodrugs of ACV has been investigated with the hope that they will provide better oral bioavailability of ACV [3–5]. Among these agents, valaciclovir (Fig. 1b) is a promising candidate drug that shows increased oral bioavailability and favorable toxicology [5]. Clinical studies show that valaciclovir is well absorbed and rapidly converted to ACV [5]. A variety of bioanalytical procedures to measure ACV were reported previously [6–8]. A significantly

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improved homogeneous radioimmunoassay using a commercially available scintillation proximity reagent is reported in this communication. This method offers technical simplicity and minimizes hands-on time for laboratory personnel.

Scintillation proximity radioimmunoassay (SPRIA), a homogenous immunoassay technique, utilizes Amersham Corporation's Scintillation Proximity Reagent beads. The solid phase fluoromicrospheres contain rare earth elements which emit light only when in close proximity to a radioactive material such as ^{3}H or ^{125}I. When radioactive components are free in solution they are not close enough to the bead to emit light. The beads used in this assay are coated with goat anti-mouse IgG (used as a second antibody which captures the primary antibody, mouse monoclonal antibody to ACV). Unlabeled ACV in the sample or in standards or controls competes with radioactive material for sites on the first antibody. The first antibody (with or without radioactive antigen) is bound to the beads by the second antibody. If radioactivity is present, then photons are emitted upon radioactive decay. The radioactive material used as the antigen is ^{3}H-ACV; the first antibody is mouse monoclonal antibody to ACV, and the second antibody is goat anti-mouse IgG which is coated on the SPA beads.

2. Materials and methods

ACV and valaciclovir were synthesized in the Organic Chemistry Division of Burroughs Wellcome Co. Tritiated ACV (25.0 Ci $mmol^{-1}$) was purchased from Du Pont (New England Nuclear Research Products, Boston, MA). Anti-ACV monoclonal antibody (MAb) was developed in the Wellcome Biotechnology Division of Wellcome Research Laboratories (UK). Anti-mouse scintillation proximity reagent (SPA reagent) was a product of Amersham Corp. (Arlington Heights, IL). The assay buffer was a phosphate buffer (0.05 M, pH 7.5) that included sodium chloride (0.15 M), sodium EDTA (0.01 M), thiomersal (0.10%) and bovine serum albumin (0.10%). All the chemicals used were of analytical grade.

2.1. Preparation of reagents

2.1.1. Primary antibody

WACO4 MAb was reconstituted in 500 μl of sterile water and stored frozen for use. WACO4 Mab was diluted to 1:1000 (SPRIA) and to 1:2000 (RIA) in RIA buffer for the assays.

2.1.2. Radioligand

^{3}H-labeled ACV was diluted with RIA buffer to a final dilution of approximately 20 000 cpm per 100 μl aliquot.

2.1.3. Normal human plasma

Normal human plasma (NHP) was diluted to the same extent as the control or unknown and was used to construct the standard curve for ACV.

2.1.4. Working standards

Standards for the curve were prepared by diluting (1:250) the stock solution of ACV [100 μmol l^{-1} (1 μmol $l^{-1} = 0.22521$ mg l^{-1})] with assay buffer to obtain a concentration of 90.0 ng ml^{-1}. The standard curve was generated from 90.0–0.7 ng ml^{-1} by serially diluting the 90.0 ng ml^{-1} standard.

2.1.5. Plasma ACV controls

Plasma controls were prepared using a 22.521 mg l^{-1} stock of ACV to give final concentrations

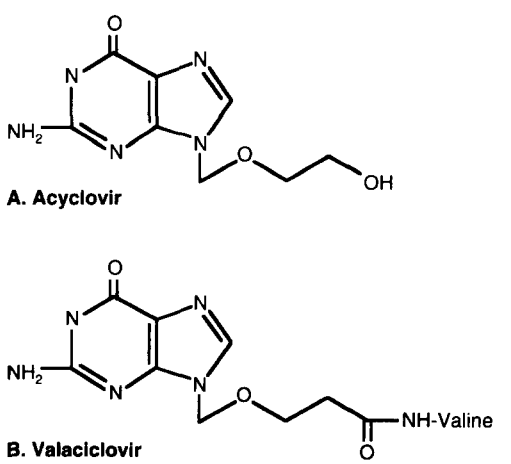

Fig. 1. Structures of (A) acyclovir and (B) valaciclovir.

of 45.0, 9.0 and 1.89 ng ml^{-1}. The controls were stored frozen in 0.5 ml aliquots at −20°C.

2.1.6. Anti-mouse IgG SPA reagent

SPA reagent was reconstituted with 20 ml of RIA buffer and the contents of the bottle were mixed by gentle inversion and resuspension. SPA reagent was magnetically stirred to ensure a homogenous suspension during the addition to assay tubes.

2.1.7. Clinical samples

Plasma samples (EDTA as anticoagulant) were collected from patients receiving ACV and were frozen at −20°C. After oral administration of valaciclovir, blood samples were collected and immediately kept on ice and centrifuged at 4°C. Plasma in two separate aliquots was frozen at −20°C to analyze for parent and for active drugs. Plasma from human immunodeficiency virus (HIV) positive individuals was prepared as above and the samples were heat-inactivated only for the analysis of total ACV.

2.2. Assay procedures

RIA using the ammonium sulfate precipitation method was carried out as previously described [6].

2.3. SPRIA for ACV

300 μl of RIA buffer was added to blank tubes, 200 μl to unknown or control or maximum binding (B_0) tubes, and 100 μl to standard tubes (Sarstedt plastic 12 mm × 55 mm tubes and 12.4 mm caps). 100 μl of diluted unknown sample, control or standard was added to each tube, followed by the addition of 100 μl of both antibody and ^{3}H-ACV solutions to each tube and 200 μl of SPA reagent. The tubes containing standards also received an appropriate dilution of NHP so that the protein concentration was equivalent. The contents of the tubes were mixed, they were then capped and incubated for 4 h at 25°C with constant, gentle mixing of the contents of tubes on a multi-tube mixer (Big Bill, Thermolyne, Sybron). 100 μl of 4 N HCl and 2.5 ml of scintillation fluid (Beckman Ready Safe®) were added to the total radioactivity tubes. When samples from patients who had received valaciclovir were analyzed, the assay procedure was carried out on ice until the tubes were placed on the shaker so that the VACV was not hydrolyzed [9]. After a 4 h incubation, the bound radioactivity in the tubes was counted in a scintillation counter for 2% 2σ errror and 10 min. No significant quenching was observed.

For the experiments on SPA reagent temperature, and length of incubation, a volume of 100 μl of SPA reagent (2 ×, SPA reagent reconstituted with 10 ml of buffer) was used.

2.4. Data analysis

For the earlier studies, either a log–logit weighted least-squares regression analysis or a four-parameter logistic transformation (MultiCalc™ software) was used to calculate concentrations of unknown samples from the standard curve parameters of the regression analysis. Equivalent results were obtained. For the later studies, including the precision and accuracy studies, the four-parameter logistic model was used for all subsequent analyses. The paired t-test was performed using an in-house generated spreadsheet analysis, using Microsoft Excel v.5.0.

3. Results

3.1. Sensitivity and limit of detection

A typical calibration curve of the SPRIA for ACV over a 125-fold concentration range is shown in Fig. 2. The curve indicates a linear dose response with a slope of 1.033. The lowest detection level of ACV (0.7 ng ml^{-1}) is the response at 90% binding of B_0 and the 50% inhibitory concentration is approximately 5.0 ng ml^{-1}.

3.2. Specificity of WACO4 MAb

WACO4 MAb with H^3-ACV was well characterized and its cross-reactivity with other metabolites of ACV and concomitant medications was

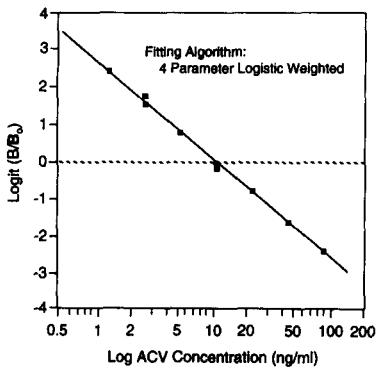

Fig. 2. Plasma standard curve for ACV with plot of logit B/B_0 versus log concentration (ng/ml^{-1}) of ACV. Slope = −1.033, intercept = 1.67, variance ratio = 8.84, IC − 20 = 19.52 ng ml^{-1}, IC − 50 = 5.1 ng ml^{-1} and IC − 80 = 1.33 ng ml^{-1}.

described elsewhere [10,11]. It was necessary to test the level of cross-reactivity of valaciclovir in the RIA to determine the concentrations of ACV after the administration of this prodrug. The parent drug cross-reacted 45% in the assay using WACO4 MAb. However, the parent analyte in plasma is found at very low levels even at high doses of prodrug after 2.0 h, since valaciclovir is rapidly converted to ACV. This is seen when the drug pharmacokinetic profile for each compound in plasma is generated (Fig. 3). ACV values were obtained by the SPRIA and valaciclovir concentrations were acquired by high performance liquid

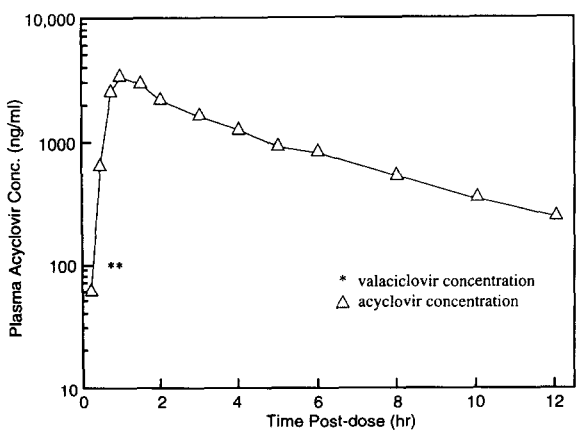

Fig. 3. Semilogarithmic plot of plasma acyclovir and 256U87 concentrations from a representative subject after single-dose administration of 500 mg 256U87. (*) 256U87 concentration post 0.75 and 1.0 h dose; (△) acyclovir concentration.

chromatography. The subject received a single dose of 500 mg valaciclovir. A rapid rise in the plasma ACV levels is seen, while the prodrug levels are very low (sub-micromolar) at 45 min and 1 h and undetectable thereafter. No interference was detected in either hemolyzed or lipemic plasma samples.

3.3. Inter- and intra-assay precisions and accuracy

Table 1 shows the reproducibility and recovery of ACV in plasma controls. The RSDs for precision of plasma controls for within- and day-to-day assays are < 8.0%. Accuracies (% bias) of control values are routinely within 10% of expected concentrations.

3.4. Dilution linearity

Serially-diluted plasma controls (1:10, 1:100 and 1:1000) in buffer or in normal human plasma also gave values that were within 10% of nominal concentrations (data not shown). Since the results with buffer and plasma were equivalent, no protein interference was noted.

3.5. Correlation between standard RIA and SPRIA

Data in Fig. 4 were generated from the analyses of 58 clinical samples for the analysis of acyclovir concentration by both assays. The two methods agreed well. The correlation coefficient (r) was 0.989, and the slope was 1.0530. When a paired t-test was performed on this data, a p value of 0.00004023 was obtained. A bias of 11.7% for the ammonium sulfate method versus the SPRIA method was observed.

3.6. Experiments with SPA reagent

The effect of assay volume on binding efficiency (Fig. 5) was investigated using different volumes of SPA reagent, and on the binding of WACO4 MAb at different temperatures and times of incubation (Fig. 6) with SPA reagent. The amount bound (% binding; B/T) was found to be inversely

Table 1
Intra- and Inter-assay precision and bias of ACV in plasma controls

Parameter		High control	Medium control	Low control
Nominal concentration of ACV control (ng ml^{-1})		45.04	9.0	1.89
Intra-assay SPRIA mean value (ng ml^{-1}) (n = 9)	± SD	43.58	8.09	1.87
		3.007	0.396	0.086
%RSD		6.9	4.9	4.6
%Bias		−3.25	−10.2	−0.95
Inter-assay SPRIA mean value (ng ml^{-1}) (n = 7)	± SD	44.32	8.39	1.87
		2.703	0.187	0.0012
%CV		6.1	7.1	1.2
%Bias		−1.6	−6.8	−1.07

proportional to the total final volume in the assay tube, suggesting that either better counting efficiency is obtained with more concentrated (lower total volume) SPA beads or that the antibody equilibrium is more favored under these conditions. Temperature also has a large effect on the binding, showing a decrease in binding as the temperatures increase. It was also noted that when samples were incubated at higher temperatures, lower binding was observed. However, reincubation of the same samples at lower temperatures resulted in higher binding. For convenience, incubation at 25°C for the assay was chosen, since the binding is not significantly compromised.

4. Discussion

Scintillation proximity reagent has been utilized to develop a specific and sensitive homogeneous radioimmunoassay (SPRIA) for ACV. The SPRIA for ACV is an improvement over the existing RIA which is based on ammonium sulfate precipitation to separate bound ACV from free ACV. The SPRIA is a single-tube assay, conveniently performed in a day without any separation or centrifugation procedures. An additional attractive feature of this assay is elimination of scintillant. The sensitivity of the SPRIA for ACV is equal to that of the standard RIA and the assay is quicker than the standard RIA. The RSDs of

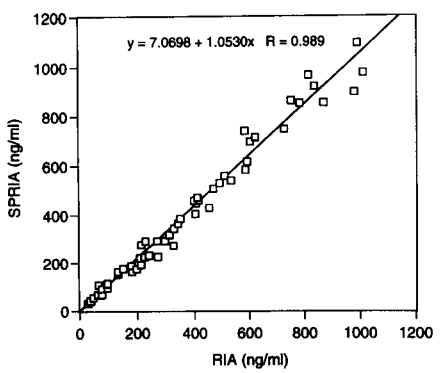

Fig. 4. Comparison of ACV concentrations determined using the standard RIA and the new method (SPRIA). Analysis of 58 clinical samples by both methods gave a slope of 1.053 and a correlation coefficient of 0.989.

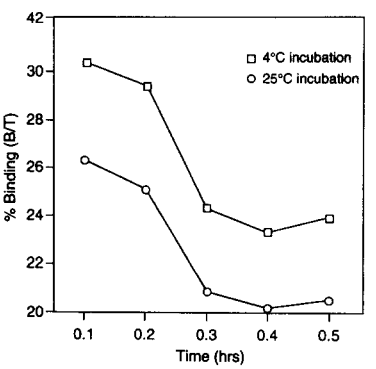

Fig. 5. Effects of final volume on binding in the SPRIA. Different volumes of SPA reagent containing an equal quantity of fluoromicrospheres were added to maximum binding tubes (B_0) and incubated at 4°C and 25°C to calculate efficiency of binding.

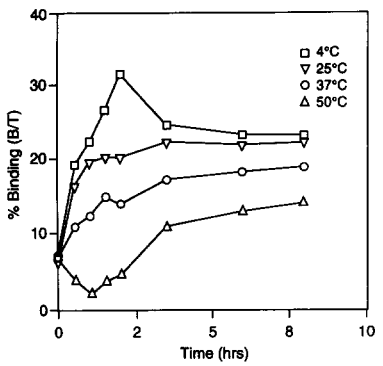

Fig. 6. Effects of temperature and time of incubation on the performance of SPA reagent. Binding of WACO4 MAb was evaluated in the SPRIA for H^3-ACV by incubating the assay with 100 μl of the SPA reagent for 0.5, 1.0, 2.0, 3.5 and 8.0 h at 4°C, 25°C, 37°C and 50°C.

inter- and intra-assay precision are good and acceptable analytical recoveries of ACV are obtained. The assay is specific in spite of cross-reactivity of WACO4 MAb (45%) with the parent drug, since valaciclovir levels (2 h post-dose) are only sub-micromolar in plasma even at high doses of the prodrug [5]. Plasma was prepared at 4°C and valaciclovir samples were immediately stored at −20°C to slow down further conversion of the prodrug to ACV and to maintain the integrity of the sample. Apparently, higher temperatures promote rapid conversion of valaciclovir to ACV [9].

Scintillation proximity reagents have been widely applied to the study of kinetics of receptor and ligand binding [12], transport of ions by cell membrane [13], and antigen and antibody reactions [14]. After Hart and Greenwald termed this procedure SPA, the reagent gained increasing popularity in immunoassay technology [15] because of several favorable features of the product. Although this new technology is applicable for detecting both large and small molecules, there are only limit reports of immunoassays for small molecule antigens of clinical importance.

SPA reagent was examined for its binding capacity at different temperatures, length of incubation period, and also the effect of volume on counting efficiency. It was observed that SPA reagent yielded higher binding when smaller volumes were used with the same total quantity of SPA beads at ambient and lower temperatures. Furthermore, lower binding obtained at higher temperatures is reversible, if the same sample is reincubated for a second time at a lower temperature.

This assay method has been extensively used to analyze acyclovir concentrations from all the clinical trial studies with valaciclovir. There is a slight bias of the older ammonium sulfate method over the SPRIA method. The difference obtained between the two methods should not significantly affect any of the pharmacokinetic parameters obtained. The SPRIA for ACV is a useful contribution to the therapeutic monitoring and clinical evaluation of ACV.

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