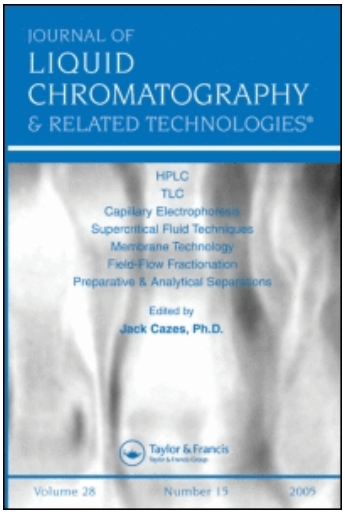

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 10 January 2002

To cite this Article Brown, Stacy D. , White, Catherine A. and Bartlett, Michael G.(2002) 'HPLC DETERMINATION OF ACYCLOVIR AND ZIDOVUDINE IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL, AND PLACENTAL TISSUES USING ULTRA-VIOLET DETECTION', Journal of Liquid Chromatography & Related Technologies, 25: 18, 2857 — 2871
To link to this Article: DOI: 10.1081/JLC-120014955
URL: http://dx.doi.org/10.1081/JLC-120014955

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JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES
Vol. 25, No. 18, pp. 2857–2871, 2002

HPLC DETERMINATION OF ACYCLOVIR AND ZIDOVUDINE IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL, AND PLACENTAL TISSUES USING ULTRA-VIOLET DETECTION

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ABSTRACT

A sensitive and reproducible HPLC assay has been developed and validated for the separation and analysis of acyclovir and zidovudine from plasma, amniotic fluid, placental homogenate, and fetal homogenate. Acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanosine, ACV) is the oldest and most widely used compound to treat episodes of genital herpes (herpes simplex virus-2, HSV-2). Zidovudine (3-azido-3′deoxythymidine, AZT) is the premier reverse transcriptase inhibitor released for the treatment of human immunodeficiency virus (HIV). Both of these drugs have been used in pregnant women to prevent the vertical (mother-to-child) transmission of their respective viruses. This gradient HPLC assay aids in the quantitation of these drugs from the matrices associated with pregnancy (maternal plasma, amniotic fluid, fetal tissue, and placenta). The mobile phase consists of 30 mM

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DOI: 10.1081/JLC-120014955
1082-6076 (Print); 1520-572X (Online)

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acetate/ citrate buffer (pH 3) and methanol. The plasma and amniotic fluid samples are prepared using a combination of protein precipitation and filtration, while the more complex tissues are prepared with the use of a solid-phase extraction (SPE). The method was validated in the calibration range of 0.1–100 μg/mL and showed precision (%Relative Standard Deviation; %RSD) and accuracy (%Error) of less than 15% for all matrices over three days. The assay was applied to a pharmacokinetic study involving the co-administration of ACV and AZT in the pregnant rat.

Key Words: HPLC; Acyclovir; Zidovudine; Pregnancy

INTRODUCTION

Acyclovir has been used to treat the symptoms and to suppress recurrent episodes of genital herpes for almost two decades. Genital herpes [herpes simplex virus-2 (HSV-2)] is not typically life threatening for adults, but it can be devastating for newborn infants. Newer statistics indicate that genital herpes may affect up to 25–30% of reproductive age women making it a real concern for perinatal transmission of the disease.[1,2] Neonatal herpes can manifest itself in three forms, affecting either the skin, eyes, and mucous membranes, the central nervous system, or major organ systems (disseminated disease).[3,4] All of these manifestations are associated with some degree of mortality with the disseminated infection being the most severe (20% survival rate).[3,4]

Zidovudine is the first reverse-transcriptase inhibitor released and is the only anti-viral approved for use in pregnant women. Pediatric AIDS is a leading cause of death in some developing nations, but the use of AZT, even if given only in the late stages of pregnancy, can reduce vertical (Human immuno deficiency virus (HIV) transmission by 51–68%.[5] Acyclovir and zidovudine (Fig. 1) have commonly been given in combination because of reports that ACV potentiates the in vitro activity of AZT.[6,7] Clinically, this drug combination has shown beneficial effects in patients with HIV, often by reducing the frequency of opportunistic infections in these patients.[8,9] Although, extreme fatigue has been reported as a side effect of this combination, the benefits often outweigh the risks when attempting to treat both viruses simultaneously.[10]

Mamede et al. conducted a study examining the effects of the ACV/AZT combination on maternal, fetal, and placental weights.[11] This study found that only the ACV mono-therapy group exhibited a decrease in maternal body weight over the controls. AZT had previously been shown to have no effect on rat maternal body weights, and this finding was replicated in the Mamede study.[11,12] Mamede proposed that AZT elicits a "protective effect" against the

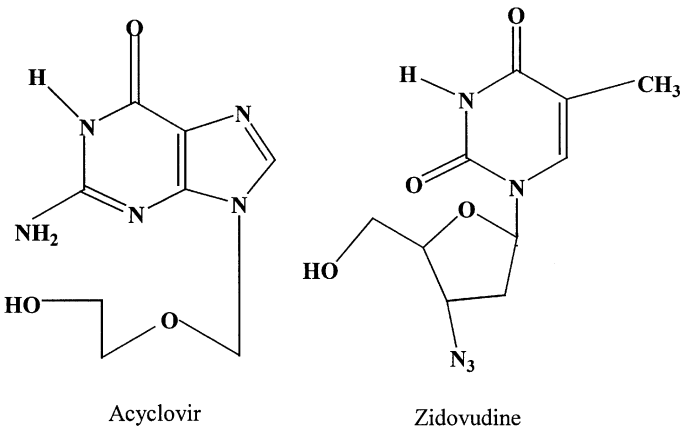


Figure 1. Structures of acyclovir and zidovudine.

ACV when they are administered in combination.[11] ACV also reduced the number of placentas and viable fetuses in the pregnant rats, but the addition of AZT to the therapy seemed to attenuate the abortive effect of ACV.[11]

This paper describes an HPLC assay used in the pharmacokinetic study of the ACV/AZT combination therapy in the pregnant rat. Because of the vast differences in the polarities of these two drugs, a gradient elution was used. Several assays exist for the quantitation of acyclovir[13–27] and zidovudine alone,[28–36] but none have examined the separation of this combination, especially in the complex biological matrices associated with pregnancy. This assay was used in conjugation with a previously developed assay for quantitating ACV, for comparing the pharmacokinetic differences between ACV and AZT mono-therapies, and the ACV/AZT combination therapy in pregnant rats.

EXPERIMENTAL

Reagents and Chemicals

Analytical standards of acyclovir and zidovudine were obtained from Sigma (St. Louis, MO). AZDU (3′-azido-3′-deoxythymidine), one of the internal standards used, was synthesized as previously described.[37] Lamivudine (3TC), also an internal standard, was recrystallized from Epivir® tablets because of the lack of availability of a commercially available standard. Reagent grade citric acid was acquired from Sigma. Reagent grade ammonium acetate and reagent grade octanesulfonic acid were bought from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile and methanol was purchased from Fisher Scientific (Fair Lawn,

NJ, USA). Sep-Pak Vac 1 cc C-18 cartridges were purchased from Waters (Milford, MA, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

Preparation of Stock and Standard Solutions

Stock solutions of ACV, AZT, AZDU, and 3TC were prepared in deionized water to yield final concentrations of 1.0 mg/mL drug. Acyclovir and zidovudine standard solutions were prepared with deionized water from the 1.0 mg/mL stocks to yield final concentrations of 750, 500, 100, 50, 25, 10, 5, 2.5, 1 μg/mL. One hundred micrograms per milliliter standard solutions of AZDU and 3TC were prepared with deionized water from the 1.0 mg/mL stocks. Stock solutions were kept refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis or validation.

Chromatographic System

The HPLC system consisted of Hewlett-Packard (Agilent) 1100 Series components, including a quaternary pump, degasser, autosampler, and variable wavelength UV detector (Palo Alto, CA). Chromatographic separations were achieved using an Agilent Eclipse XDB C-8 column (150 × 2.1 mm, 5 μm) (Palo Alto, CA) with a Phenomenex Security Guard C-18 guard column (Torrance, CA).

Chromatographic Conditions

The mobile phase used for the gradient was 30 mM acetate/citrate buffer (pH 3.1) : methanol. The gradient tables used can be seen in Tables 1 and 2. The chromatograms for each matrix can be seen in Fig. 2. The detection wavelength was fixed at 254 nm.

Table 1. Gradient Used for Plasma and Amniotic Fluid Samples (A = 30 mM Acetate/Citrate Buffer, pH 3.1, B = Methanol)

Time	%B	Flow Rate (mL/min)
5	0	0.150
16	100	0.250
17	0	0.150

Table 2. Gradient Used for Placenta and Fetal Tissue Samples (A = 30 mM Acetate/Citrate Buffer, pH 3.1, B = Methanol)

Time	%B	Flow Rate (mL/min)
5	0	0.150
20	100	0.150

Calibration Curves

Blank plasma, amniotic fluid, placenta, and fetal tissue were collected from untreated anesthetized animals. The placenta and fetal tissues were minced and homogenized with two volumes of deionized water (w/v) using a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH). Plasma calibration points were prepared by spiking 100 μL of plasma inside a 1.5 mL centrifuge tube with 10 μL of each ACV standard, 10 μL of each AZT standard, and 10 μL of the 250 μg/mL 3TC standard solution. Amniotic fluid calibration points were prepared by spiking 50 μL of fluid inside a 1.5 mL centrifuge tube with 5 μL of each ACV standard, 5 μL of each AZT standard, and 5 μL of the 250 μg/mL 3TC standard. Placental calibration samples were prepared using 150 μL of placental homogenate inside a 1.5 mL centrifuge tube spiked with 15 μL of each ACV standard, 15 μL of each AZT standard, and 15 μL of the 100 μg/mL AZDU solution. Fetal tissue calibration samples were prepared using 300 μL of fetal homogenate inside a 1.5 mL centrifuge tube with 30 μL of each ACV standard, 30 μL of each AZT standard, and 30 μL of the 100 μg/mL AZDU standard solution. Ultimately, the calibration concentrations of the analytes (ACV and AZT) in each matrix would be as follows: 0.1, 0.5, 1, 5, 10, 50, 100 μg/mL with an internal standard concentration in each sample of 10 μg/mL (AZDU) or 25 μg/mL (3TC). After each matrix was spiked, it was subject to further sample preparation before analysis.

Sample Preparation

Plasma and amniotic fluid samples were prepared with protein precipitation and filtration. After spiking, samples were vortexed briefly and 20 μL of 2 M perchloric acid (plasma) or 10 μL of 2 M perchloric acid (amniotic fluid) was added. The tubes were centrifuged for 10 min at 16,000 *g* using a Biofuge Pico Microcentrifuge (Heraeus Instruments, Hanau, Germany). After centrifuging, the supernatant was removed and filtered using either XPertek® syringe filters, 0.22 μm nylon filter (St.Louis, MO, USA) or CoStar SpinX® centrifuge tube filters, 0.22 μm nylon filter (Corning, NY, USA) and the pellet was discarded.

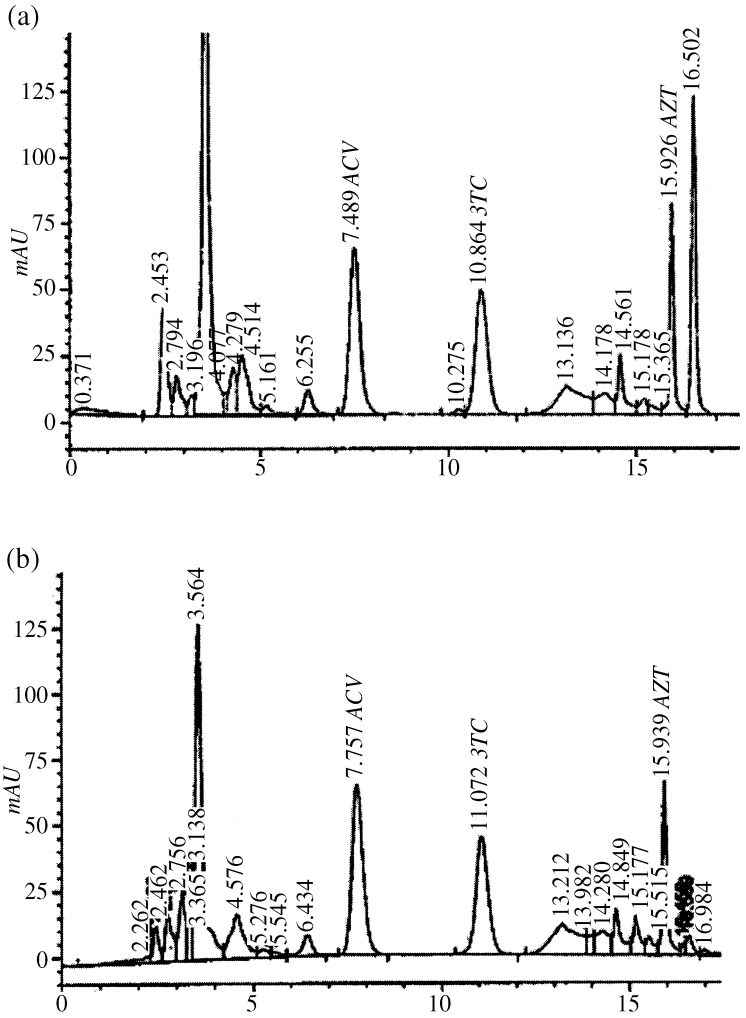
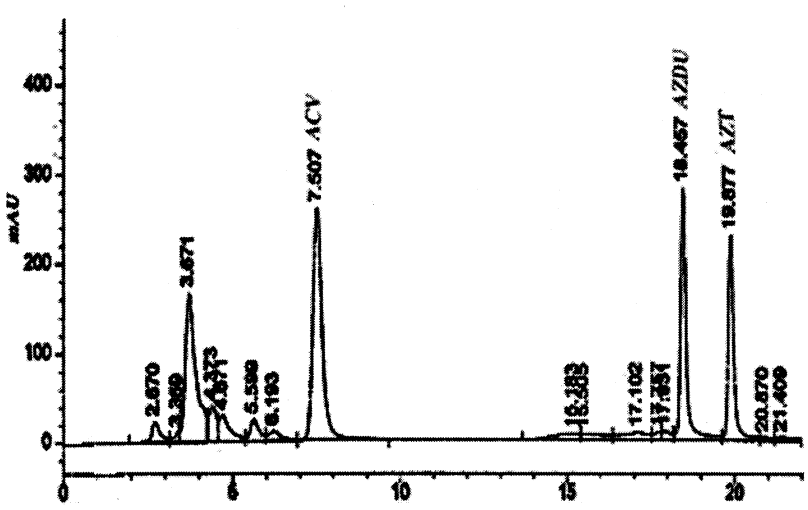


Figure 2. Chromatograms of ACV and AZT (10 μg/mL), and internal standards 3TC (25 μg/mL or AZDU (10 μg/mL) separation from (a) maternal plasma, (b) amniotic fluid, (c) placental homogenate, and (d) fetal homogenate.

Placental and fetal tissue samples were prepared using solid phase extraction (SPE). The homogenates were vortexed briefly after spiking and were pH adjusted using 30 mM acetate/citrate buffer with 5 mM octanesulfonic acid (pH 3.08) by adding 300 μL of mobile phase to the fetal homogenates and 150 μL to the placental homogenates. The tubes were vortexed again and

(c)



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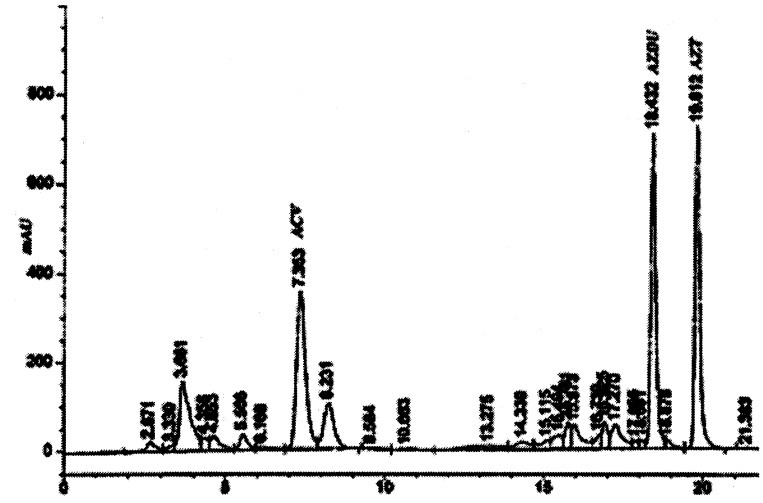

Figure 2. Continued.

centrifuged for 10 min at 16,000 *g*. Supernatants were loaded onto Sep-Pak C-18 SPE cartridges that had been preconditioned with 2 mL methanol followed by 2 mL of the acetate/citrate buffer with the ion-pair agent. The presence of the octanesulfonic acid helps the ACV stay retained to the cartridge long enough to facilitate clean-up of the sample. Samples were washed with 1 mL of deionized water/methanol (95 : 5 v/v) and eluted into clean culture tubes with 1 mL methanol. The eluents were evaporated to dryness in a vacuum centrifuge (Model SC110A, Savant Instruments Inc., Holbrook, NY) and the residues reconstituted in 100 μL (fetus) or 50 μL (placenta) of the aqueous component of the mobile phase. Reconstituted residues were then syringe filtered using 0.22 μm nylon syringe filters, or filtered using the CoStar centrifuge filters (also 0.22 μm). An injection volume of 10 μL was used for all samples.

Sample Collection

The use of animals in this study was approved by the University of Georgia Animal Use and Care Committee, and was conducted in accordance with the Animal Welfare Act and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.* The rats were housed, one animal per cage, in the University of Georgia College of Pharmacy animal facility (AALAC accredited). The environment was controlled (20–22°C, 14 hours of light per day) with daily feedings of standard chow pellets and water *ad libitum*.

Timed pregnant Sprauge–Dawley rats (Harlan, Indianapolis, IN, USA) weighing an average of 331 ± 34 g were anesthetized intramuscularly with ketamine : acepromazine (75 : 2.5 mg/kg) and dosed on day 19 of gestation. During anesthesia, animals were given atropine (0.5 mg/kg) subcutaneously. For dosing and blood sampling purposes, a cannula was surgically implanted in the right jugular vein. For sampling of the pups (amniotic fluid, placenta, and fetal tissues), a laparotomy was performed. The dose (60 mg/kg ACV + 60 mg/kg AZT) was given to the rats prepared as a 10 mg/mL solution in 0.1 M NaOH in physiological saline (pH 7.4). The rats were administered the dose intravenously via the jugular cannula, followed by 1 mL of phosphate buffered saline (pH 7.4) to rinse the cannula. Blood samples were collected at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480 min into heparinized tubes and centrifuged at 16,000 *g* for 10 min to enable plasma collection. Amniotic fluid, placenta, and fetus samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480 min. Placental and fetal tissue samples were homogenized in two volumes of deionized water. All samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

To achieve a timely separation between ACV and AZT, a gradient method had to be used. Lamivudine was initially chosen as the internal standard for this assay and was successfully used in the plasma and amniotic fluid samples. AZDU was chosen as the internal standard for the tissue samples because an endogenous peak in the tissue matrix interfered with the 3TC. Other anti-virals such as D4T (stavudine), DDI (didanosine), and DDC (zalcitabine) were run using the chromatographic conditions of this assay, and were found to pose no interference with the analytes. Figure 2 shows chromatograms of ACV, AZT, and the internal standards separated in plasma, amniotic fluid, placental, and fetal homogenates.

The calibration curves for each day of validation and analysis showed acceptable linear response ($R^2 > 0.99$) through a range of 0.1–100 μg/mL. Microsoft Excel® or JMP® statistical software was used to generate linear regression equations for all calibration curves. The range of 0.1–100 μg/mL was sufficient for use in calculating ACV and AZT levels from samples taken from rats that were dosed with 60 mg/kg ACV and 60 mg/kg AZT. Concentrations in the early plasma samples fell outside the range of the curve and had to be diluted prior to analysis.

The extraction efficiency for ACV and AZT from the various matrices is expressed in terms of relative recovery. Standard-spiked matrix samples at the 2.5 μg/mL level were extracted and analyzed ($n = 5$). An equal number of matrix blanks were extracted and spiked post-extraction. The peak areas of these two sample sets were compared showing high recoveries for ACV, AZT, AZDU, and 3TC. Acyclovir recovery from maternal plasma, amniotic fluid, placenta, and fetus ranged from 85% to 98%. AZT recoveries in each matrix ranged from 84% to 98%. The relative recoveries for each individual matrix can be found in Table 3.

Assay precision and accuracy was calculated for each matrix over a range of three days. Blanks from each matrix were spiked with ACV, AZT, and the appropriate internal standard to yield final concentrations corresponding with those in the calibration curve. Five replicates of blanks spiked with ACV and

Table 3. The Relative Recovery (±Standard Deviation, $n = 5$) of Analytes ACV and AZT (2.5 μg/mL) and Internal Standards 3TC (25 μg/mL) and AZDU (10 μg/mL)

Matrix	ACV	AZT	3TC	AZDU
Plasma	89.93 ± 5.01	91.87 ± 8.12	92.94 ± 1.69	n/a
Amniotic fluid	97.65 ± 2.43	98.39 ± 1.16	96.48 ± 0.63	n/a
Placenta	86.54 ± 8.60	93.16 ± 3.93	n/a	79.94 ± 13.1
Fetal tissue	84.87 ± 8.76	83.54 ± 4.13	n/a	83.14 ± 2.64

AZT concentrations of 0.25 μg/mL, 2.5 μg/mL, and 75 μg/mL were prepared for each validation day to test the precision (% Relative Standard Deviation, % RSD) and accuracy (%Error). This validation data is compiled in Tables 4 and 5.

To demonstrate the utility of this assay, a pregnant rat was dosed with ACV and AZT at the level of 60 mg/kg. Maternal plasma, amniotic fluid, placenta, and fetal samples were collected, extracted, and analyzed as described above. A calibration curve from each matrix was prepared on the day of analysis to calculate the concentrations of acyclovir and zidovudine present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 10 μg/mL of the appropriate internal standard (AZDU or 3TC). The sample peak area ratios of ACV and AZT to the internal standard were used to calculate the concentrations of ACV and AZT in each sample. Figures 3 and 4 show the concentration vs. time profiles for acyclovir and zidovudine in all four biological matrices of the pregnant rat. The half-life ($t_{1/2}$) for AZT, when given alone, was calculated from the plasma data using WinNonlin pharmacokinetics software. The $t_{1/2}$ was found to be 0.76 ± 0.07 hr, which correlates well with previously reported studies of AZT in the pregnant rat.[28] However, this and other pharmacokinetic parameters for AZT change

Table 4. The Precision (%RSD) and Accuracy (%Error) for ACV in Maternal Plasma, Amniotic Fluid, Fetal Tissue, and Placental Tissue over Three days ($n = 15$ for each Spike Point)

Concentration Added (μg/mL)	Concentration Found (μg/mL)	%RSD	%Error
Maternal plasma			
0.25	0.240 ± 0.028	11.6	10.3
2.5	2.68 ± 0.23	8.50	11.1
75	72.9 ± 4.5	6.30	5.22
Amniotic fluid			
0.25	0.244 ± 0.023	9.59	7.73
2.5	2.61 ± 0.21	7.89	7.76
75	72.2 ± 5.3	7.34	6.21
Placenta			
0.25	0.239 ± 0.027	11.1	9.55
2.5	2.49 ± 0.14	5.75	4.37
75	75.1 ± 5.9	7.79	6.48
Fetal tissue			
0.25	0.243 ± 0.033	13.5	12.4
2.5	2.56 ± 0.22	8.76	7.90
75	75.8 ± 5.3	7.00	5.34

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Table 5. The Precision (%RSD) and Accuracy (%Error) for AZT in Maternal Plasma, Amniotic Fluid, Fetal Tissue, and Placental Tissue over Three Days ($n = 15$ for each Spike Point)

Concentration Added (μg/mL)	Concentration Found (μg/mL)	%RSD	%Error
Maternal plasma			
0.25	0.240 ± 0.032	13.4	11.4
2.5	2.53 ± 0.21	8.14	6.23
75	70.5 ± 4.9	6.97	7.60
Amniotic fluid			
0.25	0.262 ± 0.025	9.46	9.05
2.5	2.67 ± 0.18	6.85	8.55
75	76.5 ± 5.4	7.00	6.19
Placenta			
0.25	0.231 ± 0.020	8.45	8.76
2.5	2.51 ± 0.19	7.47	5.63
75	73.4 ± 2.8	3.79	3.16
Fetal tissue			
0.25	0.254 ± 0.023	9.17	8.64
2.5	2.62 ± 0.15	5.68	6.87
75	77.2 ± 6.5	8.48	7.90

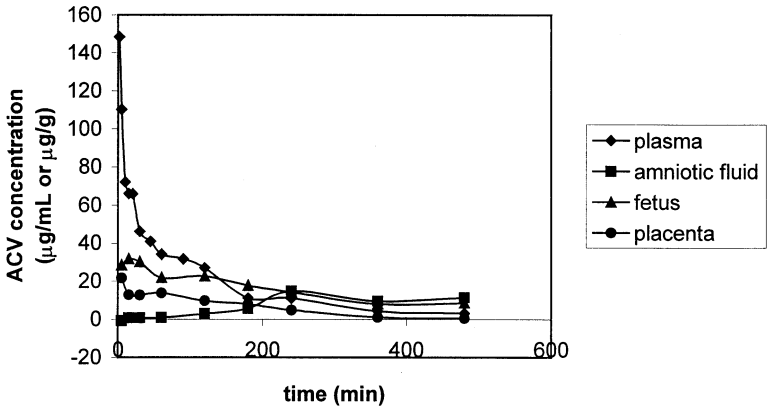

Figure 3. Concentration vs. time profile of ACV in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate from a rat dosed with ACV/AZT combination-therapy (60 mg/kg of each drug).

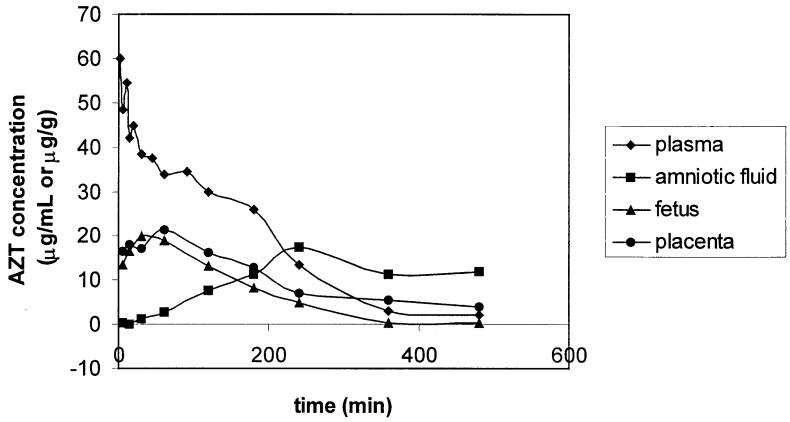

Figure 4. Concentration vs. time profile of AZT in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate from a rat dosed with ACV/AZT combination-therapy (60 mg/kg of each drug).

drastically when ACV is co-administered. Overall, maternal plasma exposure to AZT is prolonged when given with ACV, but the opposite is true for ACV. Both $t_{1/2}$ and area under the curve (AUC) of ACV is decreased when ACV and AZT are given in combination.

CONCLUSIONS

This assay has been validated for the separation and quantitation of acyclovir and zidovudine, two compounds very different in polarity. A balance had to be achieved between retaining the ACV (a highly polar drug) and eluting the AZT (a highly non-polar drug). This was done in four complex biological matrices using gradient elution and selective sample preparation techniques like solid-phase extraction. The method can be applied to the analysis of maternal plasma, amniotic fluid, placental tissue, and fetal tissue from rats that have been dosed with a combination of ACV and AZT. This assay can be used to analyze samples for the support of pharmacokinetic studies for comparing the placental transfer of ACV and AZT mono- and combination therapies.

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Received May 7, 2002
Accepted May 24, 2002
Manuscript 5875