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Determination of acyclovir in maternal plasma, amniotic fluid, fetal and placental tissues by high-performance liquid chromatography

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Abstract

Acyclovir {9-[(2-hydroxyethoxy)-methyl]-guanosine, Zovirax, ACV} is a synthetic purine nucleoside analog active against herpes simplex virus types 1 (HSV-1), 2 (HSV-2), and varicella zoster virus. Acyclovir has frequently been used in HSV-2 seropositive mothers to prevent prenatal transmission of herpes virus to their unborn children. A fast and reproducible HPLC method for the determination of the highly polar acyclvoir in maternal rat plasma, amniotic fluid, placental tissue, and fetal tissue has been developed and validated. Plasma and amniotic fluid samples were prepared by protein precipitation using 2 *M* perchloric acid and syringe filtering. Tissue samples were homogenized in distilled water, centrifuged, and extracted using a C_{18} solid-phase extraction method prior to analysis. Baseline resolution was achieved for acyclovir and the internal standard gancyclovir, an anti-viral of similar structure to acyclovir, using an Agilent Eclipse XDB C_8 column (150×2.1 mm, 5 μm). The mobile phase used for the plasma and amniotic fluid was 10 m*M* acetate/citrate buffer–3.7 m*M* aqueous octanesulfonic acid (87.5:12.5, v/v) at a flow-rate of 0.2 ml/min. The mobile phase used for the tissue samples was 30 m*M* acetate/citrate buffer with 5 m*M* octanesulfonic acid–acetonitrile (99:1, v/v). Both aqueous mobile phase portions were pH adjusted to 3.08. All separations were done using an Agilent 1100 Series HPLC system with UV detection of 254 nm. The assay was validated for each matrix over a range of 0.25–100 μg/ml over 3 days using five replicates of three spiked concentrations. The relative standard deviation and percent error for each validation data set was <15% for middle and high quality control (QC) points and <20% for all low QC points. All calibration curves showed good linearity with an R^2>0.99. The extraction efficiency for recovery of acyclovir from all matrices was >80%.

Keywords: Acyclovir

1. Introduction

Herpes simplex virus-2 (HSV-2), also known as genital herpes, is one of the most common viral infections in humans. HSV-2 affects 20–25 million people in the United States, with approximately 500,000 new cases reported each year [1]. In adults of reproductive age, this accounts for a seroprevalence of HSV-2 of 16–22% [2]. HSV-2 is characterized by cycles of viral latency and subsequent reactivation that remain with the infected individual for the duration of his or her life [2,3]. Although there is no cure for genital herpes, several anti-viral compounds have been introduced which decrease the frequency of episodes of active lesions. Acyclovir, 9-[(2-hydroxyethoxy)-methyl]-guanosine, is the most

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widely used of these anti-virals either in its original form (Zovirax) or as the pro-drug valacyclovir (Valtrex) because it has been shown to be effective in the treatment of HSV-1, HSV-2, and varicella zoster virus [4]. It is widely tolerated in different populations and disease states, and has a high therapeutic index, possibly due to its highly selective biological activity [3,4].

Although acyclovir has not been officially approved for use in pregnancy, many obstetricians prescribe oral acyclovir for HSV-2 positive mothers to reduce the possibility of an episode immediately preceding delivery or to help prevent in utero transmission. Since 85% of neonatal herpes cases are acquired as a result of passage through an infected birth canal, most HSV-2 pregnant women undergo a cesarean section instead of a vaginal delivery [2,5]. However, due to the numerous case studies reporting the successful use of acyclovir to suppress HSV-2 during pregnancy without evidence of toxicity to the newborn, many physicians feel the risks of cesarean delivery are much greater than those associated with the use of acyclovir [2,5–9].

Although the safety and efficacy of acyclovir use during pregnancy has been demonstrated though case studies and the *Acyclovir in Pregnancy Registry*, little is known about the placental transfer of acyclovir [5–9]. Even at the clinical trial stage of acyclovir, placental and fetal drug distribution data is not obtained because pregnant women are excluded from clinical trials [10]. Some groups have attempted to characterize acyclovir transfer using the perfused human placenta model [11,12]. Although the results of these studies are interesting, they do not necessarily translate well to in vivo drug behavior. If human data from ACV dosed pregnant women was collected, the matrices gathered for analysis would be limited to maternal plasma, placenta, and possibly amniotic fluid, but a sample of fetal tissue could never be included. For this reason, an animal model that accurately represents the placental mechanisms of humans must be utilized. Previously, a pregnant rat model was developed and used in the study of the placental transfer of nucleoside analogs as well as a variety of other compounds [13–21]. This model is relevant because of the similar changes seen in the hemochordial placenta and the hemodynamic pregnancy for rats and humans [14,22]. The containment of each rat pup in an individual fetal sack and the large litter size also make it a useful model for serial sampling in pharmacokinetic studies.

Several high-performance liquid chromatography (HPLC) methods exist for the quantitation of ACV from plasma, serum, and urine [23–35]. Some of these methods require more specialized equipment like fluorimetric detection [30] or extremely large sample volumes [23,25,26,34]. Depending upon the internal standard chosen for the method, run time can also be lengthy [31]. Solid-phase extraction (SPE) is commonly used as a sample clean-up technique, but may not always be necessary for relatively simple matrices [24,30,33]. Radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays can also be found for acyclovir [35–37]. While sensitive, these assays require specialized reagents and can be lengthy. This paper reports an efficient and reproducible HPLC–UV method that has been developed and validated for quantitating acyclovir from maternal plasma, amniotic fluid, fetal tissue, and placental tissue collected during a maternal–fetal drug transfer study. The assay reported here is the first to report quantitation of acyclovir from such complex tissue matrices. It requires small plasma sample volumes in order to maximize the number of pharmacokinetic time points that can be collected from the rat model. Sample preparation for the plasma and amniotic fluid samples is a simple protein precipitation, thus saving both time and money. This study utilized the pregnant rat model where all samples of four biological matrices were collected at various time-points to get a complete profile of the drug's distribution across the placenta.

2. Experimental

2.1. Reagents and chemicals

Analytical standards of acyclovir and the internal standard, gancyclovir (GAN), were obtained from Sigma (St. Louis, MO, USA). Reagent-grade citric acid was acquired from Sigma as well. 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).

2.2. Preparation of stock and standard solutions

Appropriate amounts of gancyclovir and acyclovir were weighed and added to deionized water to yield final stock solution concentrations of 1.0 mg/ml. Acyclovir standard solutions were prepared with deionized water from the 1.0 mg/ml ACV stock to yield final concentrations of 750, 500, 100, 50, 25, 10, 5, 2.5, 1 μg/ml. A 100 μg/ml gancyclovir standard solution was prepared with deionized water from the 1.0 mg/ml GAN stock. Stock solutions were kept refrigerated when not in use and replaced on a bi-weekly basis. The stock solutions were assumed to be stable over a period of 2 weeks due to the low degree of variability (<5% RSD) during that time. Fresh standard solutions were prepared for each day of analysis or validation.

2.3. 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, USA). Chromatographic separations were achieved using an Agilent Eclipse XDB C_8 column (150×2.1 mm, 5 μm) (Palo Alto, CA, USA) with a Phenomenex Security Guard C_{18} guard column (Torrance, CA, USA).

2.4. Chromatographic conditions

The mobile phase used for the plasma and amniotic fluid matrices was 10 m*M* acetate/citrate buffer–3.7 m*M* aqueous octanesulfonic acid (87.5:12.5, v/v) adjusted to pH 3.08 with phosphoric acid. The retention times under these conditions were ~8 min for GAN and ~11 min for ACV (see Fig. 2). The mobile phase used for the placental and fetal tissue samples was 30 m*M* acetate/citrate buffer with 5 m*M* octanesulfonic acid (pH 3.08)–acetonitrile (99:1, v/v). Under these conditions, GAN eluted at ~10 min and ACV eluted at ~12 min. A different mobile phase was required for the tissue matrices due to the greater number of endogenous peaks present that had to be separated from the analytes. All flow-rates were kept at a constant 0.200 ml/min and the detection wavelength was fixed at 254 nm.

2.5. Calibration curves

Blank plasma, amniotic fluid, placenta, and fetal tissue was 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, USA). Plasma calibration points were prepared by spiking 100 μl of plasma inside a 1.5-ml centrifuge tube with 10 μl of each acyclovir standard and 10 μl of the 100 μg/ml gancyclovir 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 acyclovir standard and 5 μl of the 100 μg/ml gancyclovir standard. Placental calibration samples were prepared using 200 μl of placental homogenate inside a 1.5 ml centrifuge tube spiked with 20 μl of each acyclovir standard and 20 μl of the 100 μg/ml gancyclovir solution. Finally, fetal calibration standards were prepared using 300 μl of fetal homogenate inside a 1.5 ml centrifuge tube with 30 μl of each acyclovir standard and 30 μl of the 100 μg/ml gancyclovir standard solution. Ultimately, the calibration concentrations of acyclovir 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. After each matrix was spiked, it was subject to further sample preparation before analysis.

2.6. 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.

Placental and fetal tissue samples were prepared using SPE. The homogenates were vortexed briefly after spiking and were pH adjusted using the aqueous portion of the mobile phase (30 m*M* acetate citrate buffer with 5 m*M* octanesulfonic acid, pH 3.08) by adding 300 μl of mobile phase to the fetal homogenates and 200 μl to the placental homogenates. The tubes were vortexed again and 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 aqueous portion of the tissue mobile phase. Samples were washed with 1 ml of deionized water and eluted into clean culture tubes with 3 ml methanol. The eluents were evaporated to dryness in a vacuum centrifuge (Model SC110A, Savant Instruments, Holbrook, NY, USA) and the residues reconstituted in 100 μl of mobile phase. Reconstituted residues were then syringe filtered using 0.22 μm nylon syringe filters. An injection volume of 10 μl was used for all samples.

2.7. Sample collection

The use of animals in this study was approved by the University of Georgia Animal Use and Care Committee. The rats were housed one animal per cage in the University if Georgia College of Pharmacy animal facility (AALAC accredited). The environment was controlled (20–22 °C, 14 h 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 333 g (±22 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 laparotamy was performed. The dose of acyclovir given to the rats was prepared as a 10 mg/ml solution of acyclovir in 0.1 *M* NaOH in physiological saline (pH 7.4). The rats were administered the i.v. bolus dose of acyclovir (60 mg/kg) 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.

3. Results and discussion

The structures for acyclovir and the internal standard used in this assay, gancyclovir, are shown in Fig. 1. To achieve baseline resolution of ACV and GAN from each other as well as interfering matrix peaks, the levels of octanesulfonic acid and the concentrations of the buffer were altered until the desired separation was achieved. Fig. 2a–d shows chromatograms of each matrix spiked with ACV (2.5 μg/ml) and GAN (10 μg/ml). Other anti-virals including AZT (zidovudine), AZDU, 3TC (lamivudine), D4T (stavudine), DDI (didanosine),

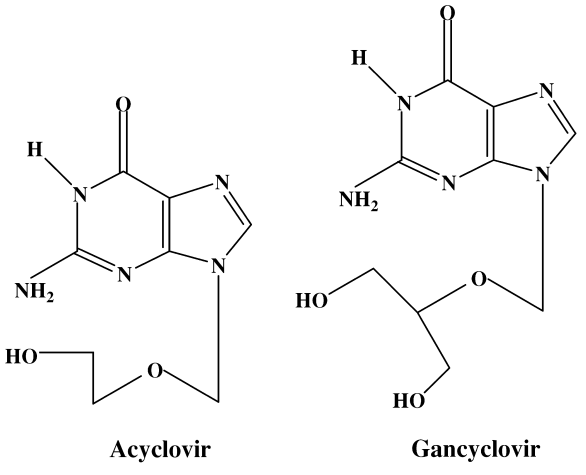

Fig. 1. Structures of acyclovir and gancyclovir.

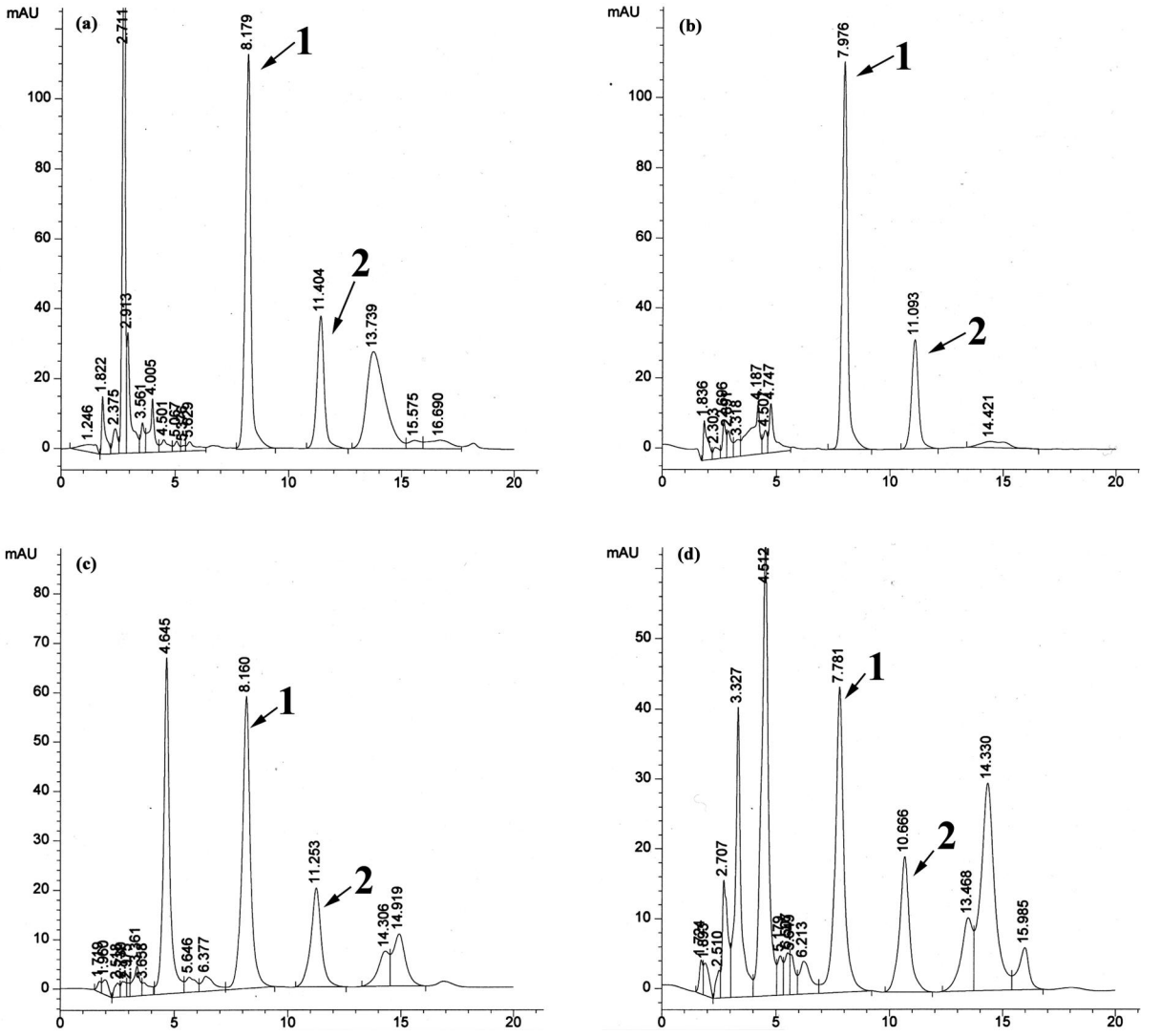

Fig. 2. Chromatograms of (1) gancyclovir (~8 min retention time, 10 μg/ml) and (2) acyclovir (~11 min retention time, 2.5 μg/ml) spiked into (a) maternal plasma, (b) amniotic fluid, (c) placental homogenate, and (d) fetal homogenate.

and DDC (zalcitabine) were run using this method to show that they did not have any interfering peaks.

The calibration curves for each day of validation and analysis showed acceptable linear response ($R^2 >$ 0.99) through a range of 0.25–100 μg/ml. Microsoft Excel or JMP statistical software was used to generate linear regression equations for all calibration curves. Calibration curves for the different matrices are displayed in Table 1. The range of 0.25–100 μg/ml was sufficient for use in calculating

Table 1
Linear regression equations generated from validation data from each matrix; slope±SD, intercept±SD and correlation coefficient±SD (n=3 for each matrix)

Matrix	Slope	Intercept	R^2
Plasma	0.077±0.009	0.047±0.04	0.995±0.004
Amniotic fluid	0.074±0.011	0.084±0.06	0.997±0.002
Fetus	0.140±0.021	0.0093±0.009	0.997±0.003
Placenta	0.170±0.006	0.163±0.05	0.998±0.003

ACV levels from samples taken from rats that were dosed with 60 mg/kg ACV. 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 GAN 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 both ACV and GAN for all matrices. Acyclovir recovery from maternal plasma, amniotic fluid, placenta, and fetus ranged from 82% to 90%. The relative recoveries for each individual matrix can be found in Table 2.

Assay precision and accuracy was calculated for each matrix over a range of 3 days. Blanks from each matrix were spiked with ACV and GAN to yield final concentrations corresponding with those in the calibration curve. Five replicates of blanks spiked with ACV concentration of 0.25 μg/ml (limit of quantitation, LOQ), 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). According to the analytical method validation criteria set forth by the United States Food and Drug Administration, the assay precision and accuracy was within acceptable limits for each matrix over 3 days [38]. This validation data is compiled in Table 3. The intra-day and inter-day variability of the GAN response (peak area) was less than 10% for the course of the validation.

To demonstrate the utility of this assay, a pregnant rat was dosed with ACV 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 concentration of acyclovir 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 internal standard gancyclovir. The sample peak area ratios of ACV to GAN were used to calculate the concentration of ACV in each sample. Fig. 3 shows the concentration–time profile of acyclovir in all four biological matrices of the pregnant rat. Fig. 4 shows a closer look at the tissue profile of acyclovir in the rat pup. Using WinNonlin, the ACV half-life was calculated to be 45.7 min. The volume of distribution at steady state of 1.1 l/kg, the total clearance of 1.03 ml/min, and the area under the curve of 14.7 μ*M* l were also calculated from maternal plasma. All values were in

Table 3
The precision (RSD, %) and accuracy (% error) ($n=15$ at each spike concentration) of the HPLC–UV assay used to quantitate acyclovir in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate

Concentration ACV added (μg/ml)	Concentration ACV found (μg/ml)	RSD (%)	Error (%)
Maternal plasma			
0.25	0.269±0.034	12.7	12.7
2.5	2.57±0.273	10.6	9.7
75	70.3±6.39	9.1	8.9
Amniotic fluid			
0.25	0.260±0.045	17.2	14.5
2.5	2.42±0.343	14.2	12.2
75	71.3±6.95	9.7	8.9
Placental homogenate			
0.25	0.247±0.030	12.1	10.1
2.5	2.38±0.190	8.0	7.7
75	70.1±5.32	7.6	9.1
Fetal homogenate			
0.25	0.268±0.0351	13.1	12.2
2.5	2.54±0.321	12.6	11.4
75	71.3±7.81	11.0	9.7

Table 2
The % relative recovery±SD ($n=5$) of acyclovir and gancyclovir from maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate

	Maternal plasma	Amniotic fluid	Placental homogenate	Fetal homogenate
Acyclovir	87.11±8.58	86.73±4.63	90.02±7.14	82.33±12.3
Gancyclovir	75.83±11.7	82.97±5.16	69.27±6.41	45.86±8.39

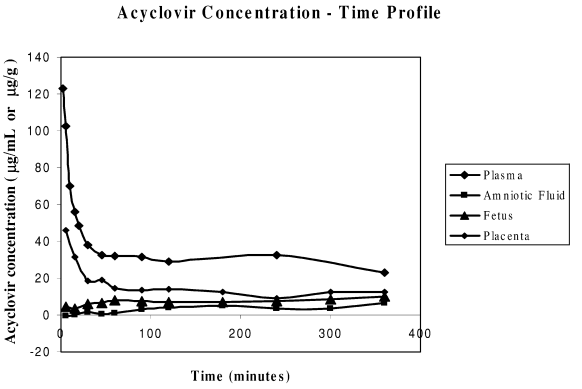

Fig. 3. Concentration versus time curve of acyclovir in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate.

close agreement with previously reported literature values for acyclovir pharmacokinetics in rats [39,40]. Fig. 4 shows that there is little evidence for extensive clearance of acyclovir from the amniotic fluid. This low clearance from the rat amniotic fluid may correlate with previously reported observations of acyclovir accumulation in human amniotic fluid [12].

4. Conclusions

Extracting and analyzing such highly polar compounds out of complex biological matrices poses some unique problems. The HPLC assay reported here combats these problems by combining the sample clean-up power of SPE with the prolonged retention ability acquired with an isocratic separation and an ion-pair mobile phase additive. This assay for the determination of acyclovir from plasma, amniotic fluid, placental homogenate, and fetal homogenate is sensitive, reproducible, and efficient. The extraction methods used yield high recoveries for acyclvoir, and the assay shows good linearity, precision, and accuracy in the calibration range of 0.25–100 μg/ml in all four complex biological matrices. The initial pharmacokinetic parameters generated from the maternal plasma showed good correlation with the reported literature values thus demonstrating the utility of this method for pharmacokinetic studies. Further pharmacokinetic investigations will be used to determine the efficiency of which acyclovir crosses the placental for the prevention of perinatal HSV.

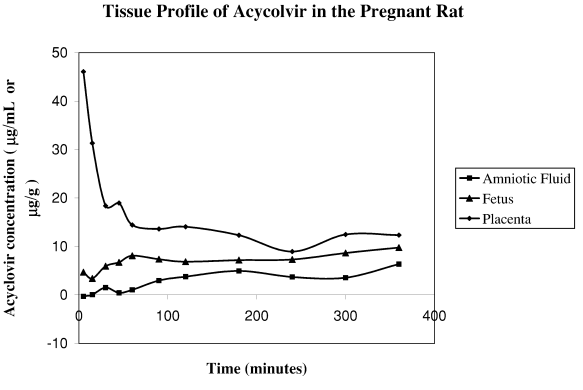

Fig. 4. Concentration–time profile of acyclovir accumulations in fetal tissue, placenta, and amniotic fluid.

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