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# Determination of 3′-azido-2′,3′-dideoxyuridine in maternal plasma, amniotic fluid, fetal and placental tissues by high-performance liquid chromatography

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**Abstract**

3′-Azido-2′,3′-dideoxyuridine (AZDU, Azddu, CS-87) is a nucleoside analog of 3′-azido-3′-deoxythymidine (zidovudine, AZT) that has been shown to inhibit human immunodeficiency virus (HIV-1). AZDU is a potential candidate for treatment of pregnant mothers to prevent prenatal transmission of HIV/AIDS to their unborn children. A rapid and efficient high-performance liquid chromatography (HPLC) method for the determination of AZDU concentrations in rat maternal plasma, amniotic fluid, placental and fetal tissue samples has been developed and validated. Tissue samples were homogenized in distilled water, protein precipitated and extracted using a C-18 solid-phase extraction (SPE) method prior to analysis. Plasma and amniotic fluid samples were protein precipitated with 2 *M* perchloric acid prior to analysis. Baseline resolution was achieved using a 4.5% acetonitrile in 40 m*M* sodium acetate (pH 7) buffer mobile phase for amniotic fluid, placenta and fetus samples and with a 5.5% acetonitrile in buffer solution for plasma at flow-rates of 2.0 ml/min. The HPLC system consists of a Hypersil ODS column (150×4.6 mm) with a Nova-Pak C-18 guard column with detection at 263 nm. The method yields retention times of 6.2 and 12.2 min for AZDU and AZT in plasma and 8.3 and 17.6 min for AZDU and AZT in amniotic fluid, fetal and placental tissues. Limits of detection ranged from 0.01 to 0.075 μg/ml. Recoveries ranged from 81 to 96% for AZDU and from 82 to 96% for AZT in the different matrices. Intra-day ($n=6$) and inter-day ($n=9$) precision (% RSD) and accuracy (% Error) ranged from 1.48 to 6.25% and from 0.50 to 10.07%, respectively. 

*Keywords:* 3′-Azido-2′,3′-dideoxyuridine

## 1. Introduction

Since the onset of HIV/AIDS over 15 years ago, more than 47 million people worldwide have been infected. Over 2.2 million deaths due to the epidemic were reported in 1998, making HIV/AIDS the fourth leading cause of mortality globally [1]. The effects of AIDS among young children are serious and far-reaching. In the worst affected African countries it has, to date, doubled the infant mortality rate. Deaths among 1- to 5-year olds, the age group with the highest concentration of AIDS deaths, have risen even more sharply from 8 to 20 per 1000 patients. Mother-to-child (vertical) transmission is the largest

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source of HIV infection in children under the age of 15, accounting for 90% of the global infections in children [2,3]. The virus may be transmitted during pregnancy, childbirth or breastfeeding. In the absence of preventative measures, the risk of an infected mother transmitting the virus to her infant ranges from 15 to 35% [3]. It has been discovered that zidovudine (AZT) monotherapy in the mother during pregnancy has caused a steep decline in perinatally acquired AIDS [4]. Such treatments have prevented ~67% of prenatal HIV infections, causing the transmission rate to fall to ~8.3% [5].

AZT therapy does, however, cause serious side-effects such as gastrointestinal intolerance, bone marrow toxicity and myelosuppression [5,6]. Long-term administration of AZT in AIDS patients has also led to the generation of AZT-resistant HIV-1 strains [7]. A search for new and less toxic anti-HIV agents has caused a great deal of interest to be focused on other 2′,3′-dideoxynucleosides that have been reported to be effective in vitro [8–11]. Among compounds synthesized as analogs of AZT, AZDU has been shown to possess significant anti-HIV activity as well as reduced bone marrow toxicity [12–14]. The pharmacokinetics of AZDU and AZT are comparable at various doses, which could be expected due to the similarity of their chemical structures (i.e. they differ only by a methyl group at the 5 position of the pyrimidine ring). Total renal clearance for both drugs becomes dose-dependent with intravenous administration of doses greater than 250 mg/kg [15,16].

While clinical trials for AZDU use as an anti-HIV agent have got underway [17,18], no maternal–fetal pharmacokinetic studies have been conducted in order to determine the efficacy of AZDU in the prevention of prenatal HIV transmission. Due to ethical concerns, pregnant women are generally excluded from clinical trials [19], making it difficult to study placental and fetal drug distributions in humans. It is also very difficult to obtain fetal concentration data from humans. Therefore, an animal model must be utilized that will provide clinically useful mechanistic information. A pregnant rat model has been developed for the investigation of the basic mechanisms involved in the placental transfer of nucleoside analogs [20]. The rat model proves to be useful due to the similarities of the hemochorial placenta and hemodynamic pregnancy changes experienced in both rats and humans [21,22]. The large litter size allows for serial maternal blood, placental, fetal and amniotic fluid sampling, making it even more useful for pharmacokinetic studies. The pregnant rat model has been utilized in maternal–fetal drug transfer studies of a variety of compounds, including AZT [20,22–28].

Here we report a rapid and efficient HPLC method that has been developed and validated for the determination of AZDU concentrations in samples taken in a maternal–fetal drug transfer study of this potential anti-HIV agent. AZDU concentration levels were determined in maternal plasma, fetal, placental and amniotic fluid samples after extraction. Pharmacokinetic analysis of plasma data generated from the analysis of these samples is consistent with previously reported literature data, proving this to be a reliable assay for the determination of AZDU concentrations in these biological matrices.

## 2. Experimental

### 2.1. Reagents and chemicals

3′-Azido-2′,3′-dideoxyuridine (AZDU, CS-87) was synthesized as previously described [29]. The internal standard, 3′-azido-3′-deoxythymidine (zidovudine, AZT) was obtained from Glaxo-Wellcome (RTP, NC, USA). HPLC-grade acetonitrile, reagent grade acetic acid and sodium acetate trihydrate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sep-Pak Vac 1-cc C-18 cartridges were purchased from Waters (Milford, MA, USA).

### 2.2. Preparation of stock and standard solutions

Individual AZDU and AZT stock solutions were prepared in deionized water to give a concentration of 1.0 mg/ml. Individual standard solutions with concentrations of 500, 250, 50, 25, 5, 2.5 and 0.5 μg/ml were prepared by serial dilution with deionized water. Precision and accuracy standards with concentrations of 125 and 1.25 μg/ml were also prepared in the same manner. All stock and standard

solutions were refrigerated at 4°C and replaced every 2 weeks.

### 2.3. Chromatographic system

All HPLC experiments were performed on a chromatographic system consisting of a Waters (Milford, MA, USA) model 510 pump, model 717+ autosampler, and a model 486 variable wavelength UV detector operated remotely using Millennium 2010 software (version 2.0, Waters, Milford, MA, USA). Chromatographic separations were achieved on a Hypersil ODS analytical column (5 μm, 150 mm×4.6 mm I.D., Alltech, Deerfield, IL, USA) equipped with a Nova-Pak C-18 guard column (Waters, Milford, MA, USA).

### 2.4. Chromatographic conditions

The mobile phases used were 4.5% acetonitrile in 40 m*M* sodium acetate (adjusted to pH 7 with acetic acid) for the fetus, placenta and amniotic fluid samples and 5.5% acetonitrile in 40 m*M* sodium acetate (adjusted to pH 7 with acetic acid) for plasma. The mobile phase flow-rate was 2 ml/min and the detection wavelength was set at 263 nm. Under the chromatographic conditions described, AZDU eluted at 8.3 (4.5% acetonitrile) and 6.2 (5.5% acetonitrile) min and AZT eluted at 17.6 (4.5%) and 12.2 (5.5%) min, respectively.

### 2.5. Calibration curves

Blank placental and fetal tissue homogenates were prepared from untreated animals by homogenization with 2 vol. of deionized water (w/v) in a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). Calibration standards for all samples were prepared by spiking 100 μl of the tissue homogenate or biological fluid with 20 μl of AZDU standard solution and 10 μl of 20 μg/ml AZT internal standard solution to obtain AZDU concentrations of 0.1–100 μg/ml and 2.0 μg/ml AZT concentration. The spiked fetal and placental standards were then extracted from the biological matrices. All standards were prepared on the day of analysis. The extraction procedure is described in the following section.

### 2.6. Extraction procedure

In a 1.5-ml centrifuge tube 100 μl of the tissue homogenate, 10 μl of AZT internal standard (20 μg/ml) and 20 μl of an AZDU standard solution were combined and vortexed briefly. To precipitate proteins, 500 μl of ice cold acetonitrile was added to the tube. The tubes were vortexed for 30 s and centrifuged at 5000 rpm for 5 min in a microcentrifuge (Model 235 V, Fisher, Fairlawn, NJ, USA). The supernatant was evaporated at 40°C in a vacuum centrifuge (Model SC110A, Savant Instruments, Holbrook, NY, USA). The residue was reconstituted in 500 μl of 0.2% acetonitrile in water. The samples were then loaded onto a Sep-Pak C-18 solid-phase extraction cartridge preconditioned with 2 ml of acetonitrile followed by 2 ml of 0.2% acetonitrile in water. Samples were washed with 2 ml of the 0.2% acetonitrile in water solution. After discarding the eluent, the analytes were eluted with 2 ml of acetonitrile into clean culture tubes. Eluents from the cartridge were then dried at 40°C using the vacuum centrifuge and reconstituted in 150 μl of mobile phase. The samples were then transferred to injection vials where 50 μl of sample was injected onto the HPLC column.

In a 1.5-ml microcentrifuge tube 20 μl of an AZDU solution and 10 μl of the AZT internal standard were added to 100 μl of plasma or amniotic fluid. The tube was vortexed briefly before the addition of 20 μl of 2 *M* perchloric acid. The tubes were vortexed again for 30 s and then centrifuged at 9000 rpm for 10 min. The supernatants were then transferred to injection vials where 50 μl of sample was injected onto the HPLC column.

### 2.7. Sampling

Timed pregnant female Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) weighing an average of 325 g were used. On day 19 of gestation rats were anesthetized using a ketamine:acepromazine (75:2.5 mg/kg) solution injected intramuscularly. A cannula was placed in the right jugular vein and a laparotomy was performed to allow concurrent serial sampling of blood and the fetal sac, each containing a fetus, placenta and amniotic fluid. The rats were administered an i.v.

bolus dose (25 mg/kg) of AZDU dissolved in 0.1 N NaOH in physiological saline via the jugular cannula. Individual blood and fetal sac samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min post-dose and stored on ice until processed. Heparinized tubes were prepared prior to sampling by adding 20 μl of heparin (1000 U/ml) to 1.5-ml microcentrifuge tubes and allowing them to evaporate uncapped in a fume hood overnight. At each sampling interval, ~350 μl of blood was placed in a heparinized tube. These tubes were centrifuged at 9000 rpm for 10 min and the plasma was transferred to clean dry tubes for storage. Fetal and placental samples were collected, rinsed with deionized water, blotted dry and weighed. The samples were then homogenized in 2 vol. (w/v) of deionized water. Amniotic fluid samples, averaging ~200 μl in volume, were collected from the fetal sac using an 18-gauge needle with a 1-ml syringe. Samples were placed in pre-weighed microcentrifuge tubes. The sample tubes were reweighed and exact sample volumes were determined by assuming a density of 1 g/ml. All samples were stored at −20°C until analysis.

## 3. Results and discussion

The chemical structures for AZDU and the internal standard AZT are shown in Fig. 1. Separation of AZDU and AZT from interfering matrix peaks was explored using various ratios of acetonitrile and the buffer. Baseline resolution was achieved at 4.5% acetonitrile in buffer for amniotic fluid, fetal and placental samples and at 5.5% acetonitrile for the plasma samples. Fig. 2A–D shows chromatographs of spiked AZDU (1 μg/ml) with the internal standard, AZT.

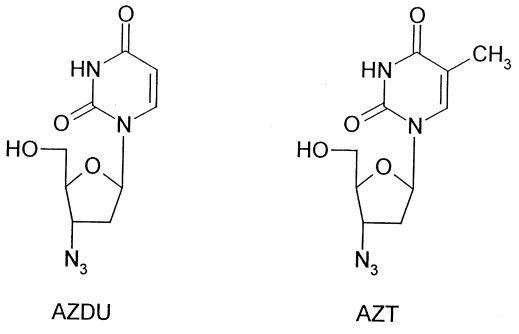



Fig. 1. Structures of AZDU and AZT.

The calibration curve showed a good linearity in the range of 0.1–100 μg/ml for plasma and in the range of 0.1–50 μl/ml for amniotic fluid and fetal and placental tissues ($r^2$=0.999–1.000). The range of concentrations encompasses the estimated range of post i.v. bolus dose (25 mg/kg) concentrations in the biological matrices.

The limits of detection (LOD) for AZDU in the biological matrices were determined by analysis of standard-spiked samples gradually decreasing in concentration. The LOD was determined as a concentration at which the signal/noise ratio was ~3 and were found to be <0.1 μl/ml for all four biological matrices. The LODs of AZDU were found to be 50 ng/ml for plasma, 25 ng/ml for fetus, 10 ng/ml for placenta and 50 ng/ml for amniotic fluid.

To investigate the extraction efficiency of AZDU and AZT from the various biological matrices (plasma, amniotic fluid, fetal and placental tissues), standard-spiked matrix samples were subjected to extraction and then analyzed. The resulting peak areas were compared to peak areas of samples containing equal amounts of analyte in mobile phase. The recoveries were high and reproducible, ranging from 81 to 96% for AZDU and from 82 to 96% for AZT. The recoveries for both AZDU and AZT in the four individual matrices are shown in Table 1.

Intra-day ($n$=6) and inter-day ($n$=9) precision and accuracy were calculated from standard curves constructed from each of the four biological matrices studied. The intra-day ($n$=6) precision and accuracy for AZDU (spiked concentrations 0.25 and 25 μg/ml) was in the range of 2.05–7.64% (RSD) and 1.73–10.2% (error), respectively. Inter-day ($n$=9) precision and accuracy for AZDU (unknown concentrations 0.25 and 25 μg/ml) ranged from 3.04 to 7.82% (RSD) and from 2.22 to 9.18% (error), respectively. These intra- and inter-day precision and accuracy data are tabulated in Table 2.

Acquired plasma, amniotic fluid, fetal and placental tissue samples were extracted and analyzed as described above. Sample peak area ratios of AZDU and AZT were used to determine AZDU concentrations from the regression equation obtained from standard-spiked samples prepared in blank fetus,

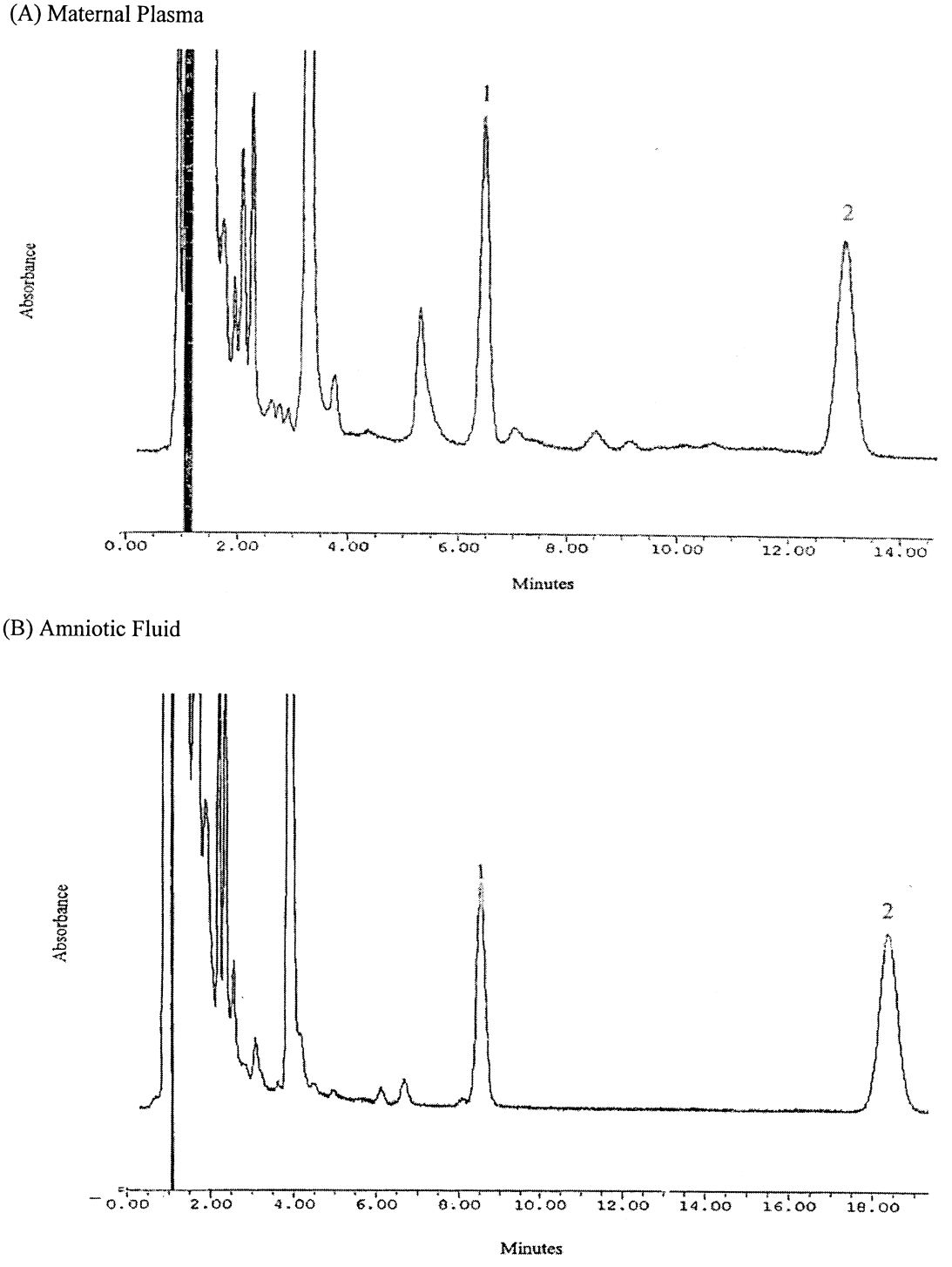
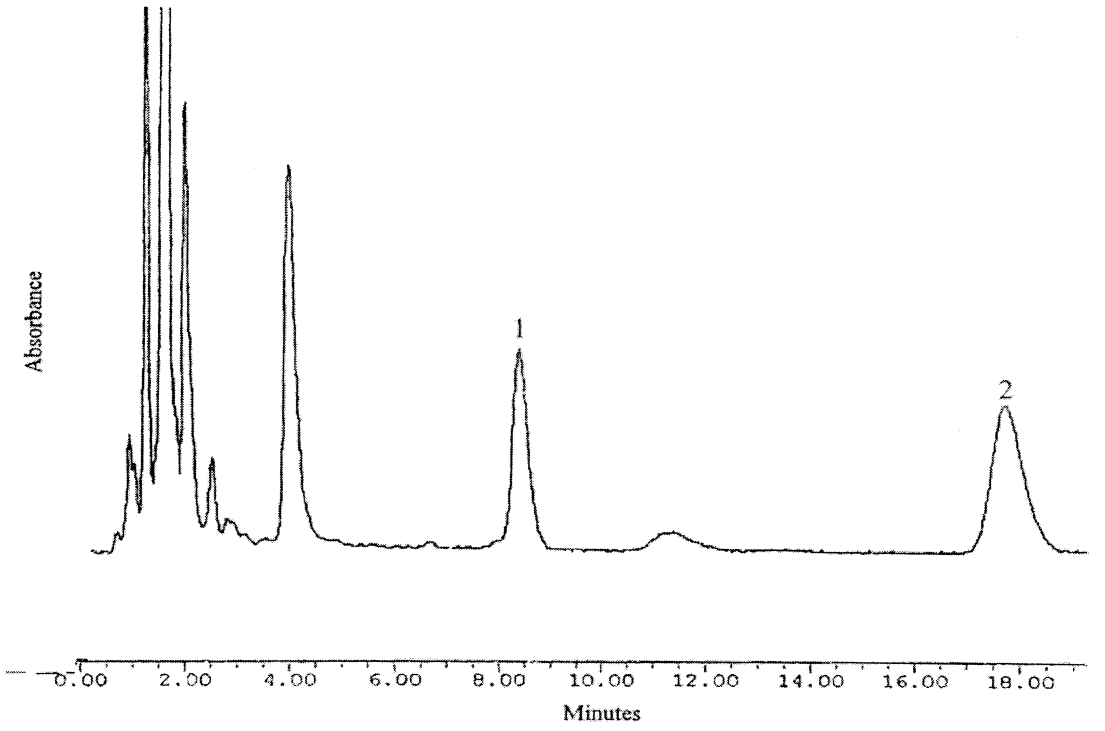



Fig. 2. Chromatographs of (1) AZDU (1 μg/ml) and (2) AZT (2 μg/ml) spiked (A) maternal plasma, (B) amniotic fluid, (C) placental homogenate and (D) fetal homogenate samples on a Hypersil ODS (5 μm, 150 mm×4.6 mm) analytical column in a 40 m*M* sodium acetate (pH 7):acetonitrile buffer with detection at 263 nm.

(C) Placental Homogenate




(D) Fetal Homogenate

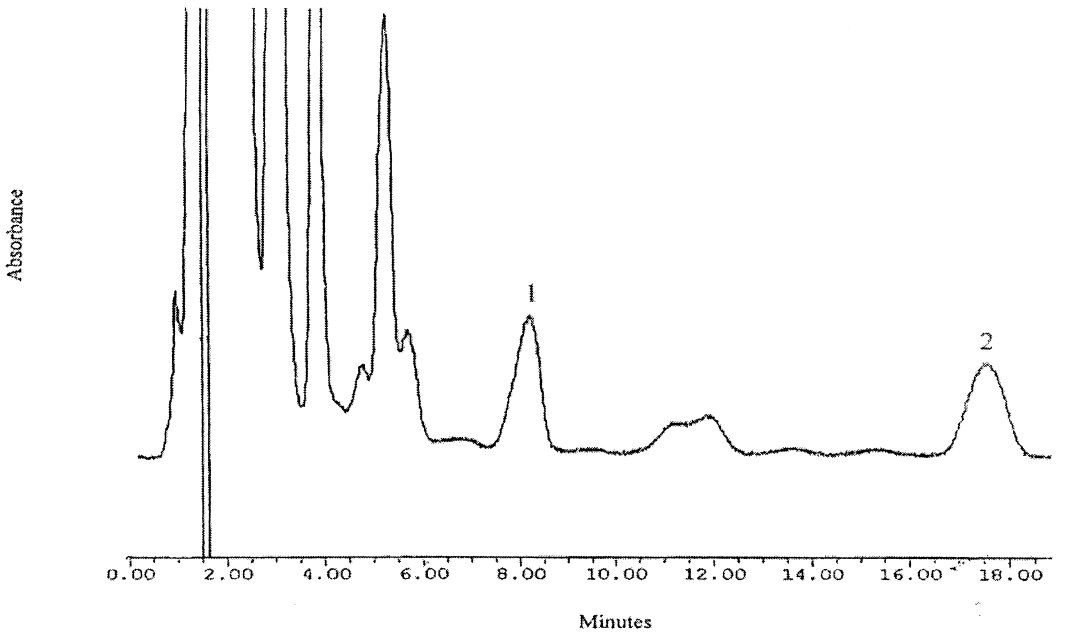



Fig. 2. (*continued*)

Table 1
The % recovery±SD ($n$=5) of AZDU and AZT from maternal plasma, amniotic fluid, placental homogenate and fetal homogenate

| Analyte | % Extraction efficiency (±SD) | | | |
|---|---|---|---|---|
| | Maternal plasma | Amniotic fluid | Placental homogenate | Fetal homogenate |
| AZDU | 96.47±1.49 | 95.26±2.03 | 81.16±2.79 | 85.42±5.78 |
| AZT | 95.87±3.19 | 88.38±2.07 | 92.16±3.38 | 81.99±4.08 |

Table 2
The intra- and inter-day precision (% RSD) and accuracy (% Error) of AZDU in maternal plasma, amniotic fluid, placental homogenate and fetal homogenate

| Concentration added (μg/ml) | Intra-day ($n$=6) | | | Inter-day ($n$=9) | | |
|---|---|---|---|---|---|---|
| | Concentration found (μg/ml) | RSD (%) | Error (%) | Concentration found (μg/ml) | RSD (%) | Error (%) |
| Maternal plasma | | | | | | |
| 0.25 | 0.235±0.019 | 6.12 | 5.90 | 0.260±0.013 | 5.14 | 4.49 |
| 25.0 | 24.93±0.61 | 2.45 | 1.73 | 25.16±0.77 | 3.04 | 2.22 |
| Amniotic fluid | | | | | | |
| 0.25 | 0.225±0.005 | 2.05 | 10.2 | 0.240±0.019 | 7.82 | 7.38 |
| 25.0 | 22.77±0.78 | 3.43 | 8.93 | 23.40±1.15 | 4.92 | 6.84 |
| Placental homogenate | | | | | | |
| 0.25 | 0.225±0.011 | 4.71 | 10.07 | 0.228±0.012 | 5.34 | 9.18 |
| 25.0 | 23.37±1.52 | 6.51 | 7.33 | 24.40±1.58 | 6.45 | 5.17 |
| Fetal homogenate | | | | | | |
| 0.25 | 0.266±0.011 | 4.25 | 6.53 | 0.252±0.017 | 6.69 | 5.11 |
| 25.0 | 24.50±1.87 | 7.64 | 5.87 | 24.59±1.33 | 5.39 | 4.31 |

placenta, plasma and amniotic fluid. Fig. 3 shows the concentration versus time curve of the AZDU in the pregnant rat in all four biological matrices. An AZDU half-life of 1.3 h and steady state volume of distribution of 0.73 l/kg and total clearance of 0.32 l/h per kg were calculated from maternal plasma. All values were in agreement with previously reported literature values [15,30].

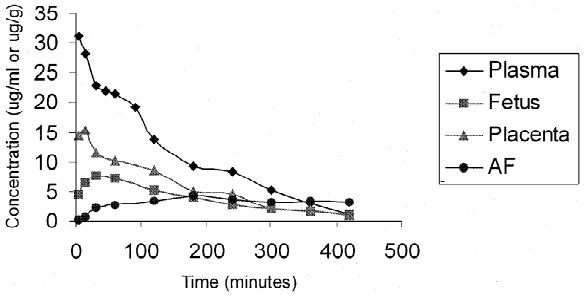



Fig. 3. Concentration versus time curve of AZDU in maternal plasma, amniotic fluid, placental homogenate and fetal homogenate.

## 4. Conclusions

A sensitive and efficient method for the extraction and analysis of AZDU in rat plasma, amniotic fluid, fetal and placental tissues has been developed and validated. This method yields high recoveries, shows good linearity, precision and accuracy within the range of 0.1–100 μg/ml. The solid-phase extraction, although not necessary for concentrating our samples, provides excellent sample clean-up. Due to usage of the entire fetus or placenta, very complex sample matrices, a sample clean-up step that would

dramatically reduce possible interferences was needed. Combining acetonitrile protein precipitation with solid-phase extraction afforded optimal tissue sample clean-up, allowing for a decrease in sample analysis time. The estimated pharmacokinetic parameters from the analysis of collected samples were comparable to literature data, further validating the reliability of this particular method for the determination of AZDU concentrations in biological samples. This method allows for a pharmacokinetic investigation for the determination of placental transport of AZDU and will predict relative efficacy by comparison to other antiviral agents examined with the same animal model.

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