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

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Abstract

2′,3′-Dideoxycytidine (DDC) is a nucleoside reverse transcriptase inhibitor that has been shown to inhibit the human immunodeficiency virus (HIV). DDC is a candidate for treatment of pregnant women to prevent prenatal transmission of HIV/AIDS to their unborn children. A quick and simple high-performance liquid chromatography (HPLC) method has been developed and validated for the determination of DDC concentrations in samples collected from a pregnant rat model (maternal plasma, amniotic fluid, placental and fetal tissues). Extraction of DDC and its internal standard 2′,3′-dideoxy-3′-thiacytidine (3TC) in plasma and amniotic fluid was carried out by protein precipitation. Extraction from placental and fetal homogenates was achieved by solid phase extraction using Waters Oasis HLB solid phase extraction cartridges. Chromatographic separation was achieved on a Waters Spherisorb S3W silica column (4.6 mm × 100 mm) equipped with a Phenomenex guard column. The mobile phase used was 10% methanol in water with 22 mM formic acid. The flow rate was 0.5 ml/min, and the detection wavelength was optimized at 275 nm. Under these chromatographic conditions, DDC eluted around 12 min, and 3TC eluted around 10 min. The calibration curves for each day of validation and analysis showed good linear response through the range of 0.15–75.0 μg/ml in each of the four matrices. The relative recovery for DDC in each of the matrices ranged from 87.8% to 103.0%. Acceptable intra- and inter-day assay precision (<15% R.S.D.) and accuracy (<15% error) were observed over 0.15–75.0 μg/ml for all four matrices.

Keywords: 2′,3′-Dideoxycytidine

1. Introduction

Approximately 1.2% of the adult population in the world today is infected with human immunodeficiency virus (HIV). Among these, half are women and over three million are children. In 2001, there were more than three million deaths due to HIV infection. In the same year, the United States had 15,000 deaths from acquired immunodeficiency syndrome (AIDS) and has more than 890,000 adults living with HIV [1].

For children, 89% of HIV infections are from vertical transmission from their mothers through blood, amniotic fluid, and/or breast milk [2]. In the absence of preventative measures, the risk of a pregnant HIV-infected woman to transmit the disease to her fetus is between 20% and 50% with increasing risk in subsequent pregnancies [3]. According to the Centers for Disease Control (CDC), the average age of diagnosis of AIDS in infants is 9 months; they often die shortly, thereafter [4].

Several lines of investigation support the hypothesis that in utero anti-HIV therapy using 3′-azido-3′-deoxythymidine (AZT) may be of clinical benefit to the infected fetus [5–7]. Because of these findings, an AIDS clinical trial was initiated where AZT was administered to pregnant women in

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their second or third trimester. The results of this study were a dramatic reduction in the HIV transmission rate to the fetus. However, there remains a need for studies of other anti-HIV drugs and the placental transfer properties of these compounds alone and in conjunction with other therapeutic agents [8].

2′,3′-Dideoxycytidine (DDC) was one of the earliest nucleoside reverse transcriptase inhibitors (NRTI) used for AIDS therapy. DDC was the first drug approved under the principles and procedures of FDA's proposed accelerated drug review policy, endorsed by the White House Council on Competitiveness and announced by the Vice President on 9 April 1992 [4]. DDC has been used in patients who cannot be maintained on AZT due to side effects (e.g. severe anemia) [9].

No maternal–fetal pharmacokinetic studies have been conducted in order to determine the efficacy of DDC in the prevention of prenatal HIV transmission. Due to ethical concerns, pregnant women are generally excluded from clinical trials, 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 information. A pregnant rat model has been developed for the investigation of the basic mechanisms involved in the placental transfer of nucleoside analogs. 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 [10]. 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 nucleoside antivirals, including 3′-azido-3′-deoxythymidine (AZT), 9-[(2-hydroxyethoxy)-methyl]-guanosine (ACV), and 3′-azido-2′,3′-dideoxyuridine (AZDU) [11–14].

Several HPLC methods have been developed for DDC analysis, but none of them deal with the analysis of DDC from complex matrices such as, maternal plasma, amniotic fluid, placental and fetal homogenates [15,16]. Also most of the HPLC methods developed are not readily transferable to mass spectrometry-based detectors, because ion-pairing agents are used. Some of the methods use large sample volumes, long run times, or relatively high percentages of expensive organic solvents. In this study, a rapid and sensitive HPLC method was developed and validated using an underivatized silica column for the determination of DDC concentrations in samples taken in a maternal–fetal drug transfer study of DDC. By using a silica column under reverse-phase chromatographic conditions, lower percentages of organic solvents can be used to obtain sufficient retention of DDC and its internal standard 2′,3′-dideoxy-3′-thiacytidine (3TC). This form of chromatography is referred to as hydrophilic interaction liquid chromatography (HILIC) and has been recently increasing in use for the analysis of small polar molecules [13,17–19].

2. Experimental

2.1. Reagents and chemicals

2′,3′-Dideoxycytidine (DDC) was obtained from Sigma (St. Louis, MO, USA). The internal standard, 2′,3′-dideoxy-3′-thiacytidine (3TC), was obtained from GlaxoSmithKline (RTP, NC, USA). HPLC-grade methanol was purchased from J.T. Baker (Phillipsburg, NJ, USA). Reagent grade formic acid was from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

2.2. Preparation of stock and standard solutions

Individual DDC and 3TC stock solutions were prepared in deionized water to give a final concentration of 1.0 mg/ml. Individual standard solutions with concentrations of 0.5, 1.25, 2.5, 3.75, 5.0, 12.5, 25.0, 50.0, 125.0 and 250.0 μg/ml were prepared by serial dilution with deionized water. Precision and accuracy standards with concentrations of 1.0, 7.5 and 200 μg/ml were also prepared in the same manner. A 7.5 μg/ml 3TC standard solution was prepared with deionized water from the 1.0 mg/ml 3TC stock solution. The 1.0 mg/ml stock solutions were kept refrigerated when not in use and replaced on a weekly basis. Fresh standard solutions were prepared for each day of analysis or validation.

2.3. Chromatographic system

All HPLC experiments were performed on a Hewlett-Packard (Agilent) Series II 1090 liquid chromatography (Palo Alto, CA, USA). The detector used was a Waters Lambda-Max Model 481 LC Spectrophotometer (Milford, MA, USA). The data processing device was a Hewlett-Packard (Agilent) 3395 Integrator. The software used was Hewlett-Packard (Agilent) ChemStation for LC Rev. A.04.01. Chromatographic separation was achieved on a Waters Spherisorb® S3W silica column (4.6 mm × 100 mm) from Waters (Milford, MA, USA) equipped with a Phenomenex C-184 mm × 3 mm i.d. guard column (Torrance, CA, USA)

2.4. Chromatographic conditions

The mobile phase used was water with 22 mM formic acid–methanol (9:1). The mobile phase flow rate was 0.5 ml/min and the detection wavelength had been optimized and set at 275 nm. Under the chromatographic conditions described, DDC eluted at ∼12 min and 3TC eluted at ∼10 min, respectively.

2.5. Calibration curves

Blank amniotic fluid, placenta, and fetal tissue were collected from untreated anesthetized rats. The placenta and fetal tissues were minced and homogenized with two volumes of

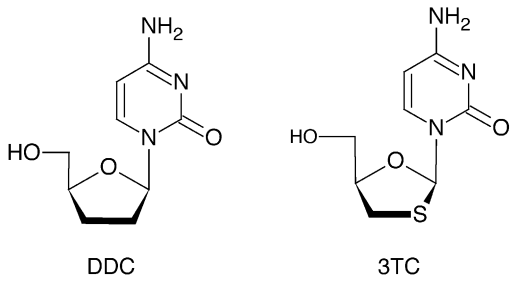

Fig. 1. Chemical structures of 2′,3′-dideoxycytidine (DDC) and 2′,3′-dideoxy-3′-thiacytidine (3TC).

deionized water (w/v) using a Tekmar tissue grinder (Model SDT-1810, Cincinnati, OH, USA).

Plasma, placenta, and fetal tissue calibration points were prepared by spiking 100 μl of biological fluid, or tissue samples inside a 1.5 ml centrifuge tube with 30 μl of each DDC standard and 20 μl of the 7.5 μg/ml 3TC standard solution. Amniotic fluid calibration points were prepared by spiking 50 μl of the fluid inside a 1.5 ml centrifuge tube with 15 μl of each DDC standards and 10 μl of the 7.5 μg/ml 3TC standard solution. Ultimately, the calibration concentrations of DDC in each matrix would be as follows: 0.15, 0.375, 0.75, 1.125, 1.5, 3.75, 7.5, 15.0, 37.5 and 75.0 μg/ml with an internal standard concentration in each sample of 1.5 μg/ml. The final concentrations of precision and accuracy points were 0.15, 0.3, 2.25 and 60.0 μg/ml. After each matrix was spiked, it was subject to further sample preparation before analysis.

2.6. Extraction procedure

Plasma and amniotic fluid samples were prepared with protein precipitation and filtration. After spiking, samples were vortexed for 10 s and 4.5 volumes of each matrix of ice-cold acetonitrile (450 μl for plasma, 225 μl for amniotic fluid) was added. The tubes were vortexed for 60 s and centrifuged for 10 min at 13,000 rpm using a Biofuge Pico Microcentrifuge (Heraeus Instruments, Hanau, Germany). The supernatant was evaporated under a gentle stream of nitrogen until reaching dryness. The pellet was reconstituted in either 150 μl for plasma or 75 μl of deionized water for amniotic fluid. The reconstituted solution was vortexed for 60 s and sonicated for 5 min, vortexed again for 30 s and centrifuged for 5 min at 13,000 rpm using the same microcentrifuge. The

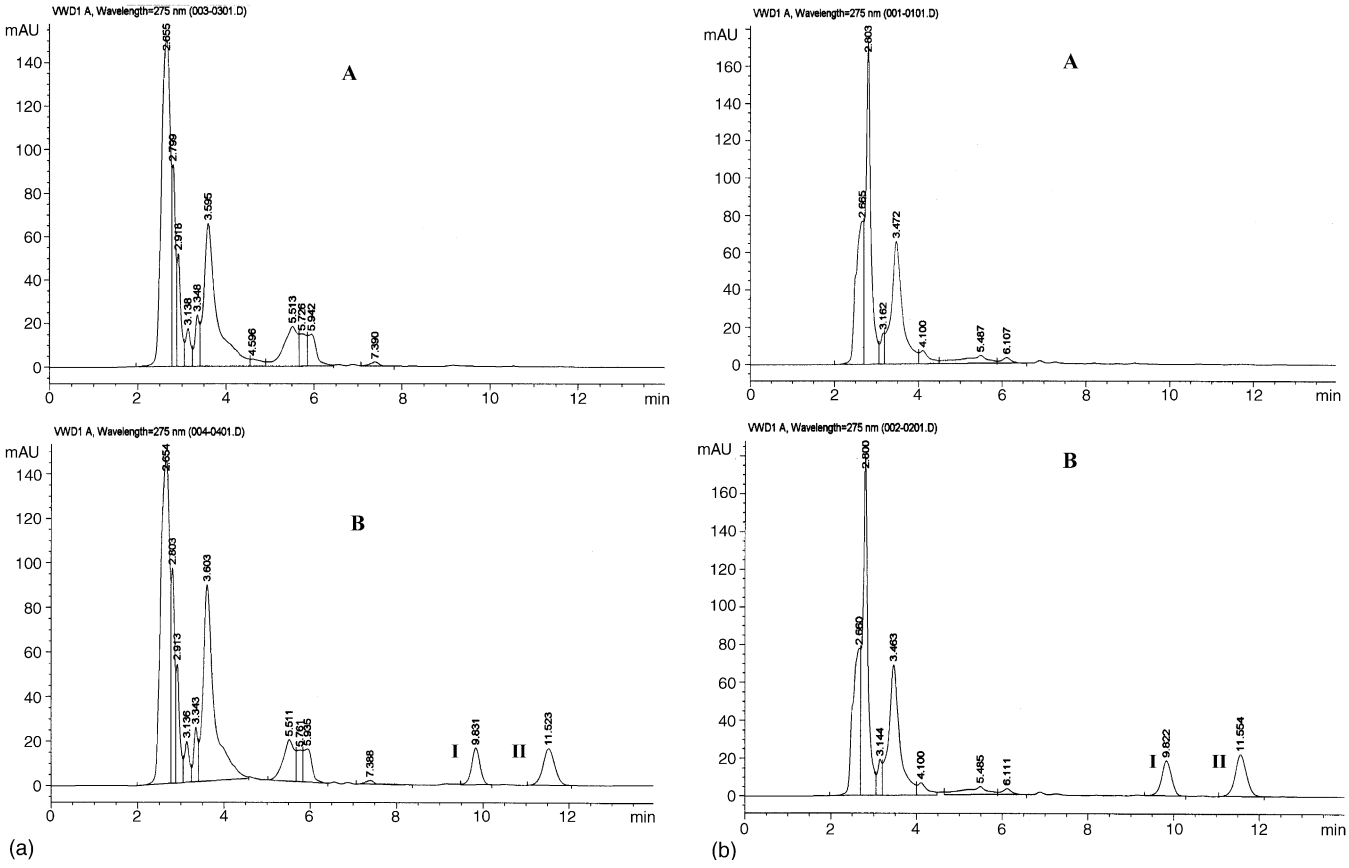

Fig. 2. (a) Chromatographs of (A) blank maternal plasma and (B) maternal plasma spiked with 2.25 μg/ml DDC (II) and 1.5 μg/ml 3TC (I). (b) Chromatographs of (A) blank amniotic fluid and (B) amniotic fluid spiked with 2.25 μg/ml DDC (II) and 1.5 μg/ml 3TC (I). (c) Chromatographs of (A) blank placental homogenate and (B) placental homogenate spiked with 2.25 μg/ml DDC (II) and 1.5 μg/ml 3TC(I). (d) Chromatographs of (A) blank fetal homogenate and (B) fetal homogenate spiked with 2.25 μg/ml DDC (II) and 1.5 μg/ml 3TC (I).

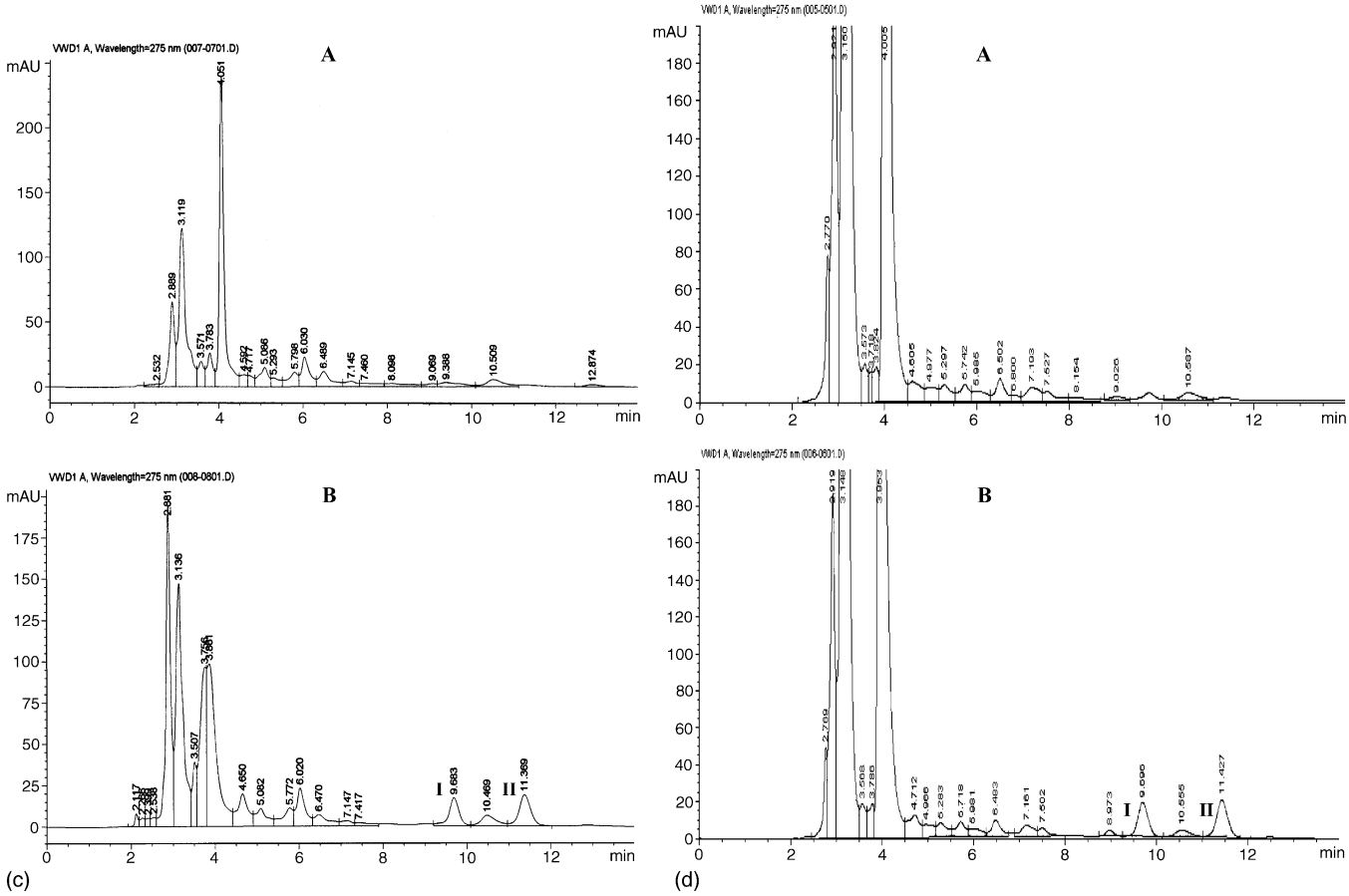

Fig. 2. (*Continued*).

resulting solution was 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 homogenate samples were prepared using solid phase extraction. The homogenates were vortexed for 10 s after spiking and 850 μl of a 10 mM ammonium acetate (pH 9) buffer was added to the homogenates. The tubes were vortexed for 60 s and centrifuged for 10 min at 13,000 rpm using the above microcentrifuge. Supernatants were loaded onto Waters Oasis HLB cartridges that had been preconditioned with 1 ml of methanol followed by 1 ml of deionized water. Samples were washed with 0.5 ml of 10 mM ammonium acetate (pH 9) buffer and eluted into clean culture tubes with 1 ml of methanol. The eluents were transferred to 1.5 ml centrifuge tubes and were evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted in 150 μl of deionized water. The reconstituted solution was vortexed for 60 s and sonicated for 5 min, vortexed again for 30 s and centrifuged for 5 min at 13,000 rpm using the above microcentrifuge. The resulting solution was filtered using XPertek syringe filters, 0.22 μm nylon fliter (St. Louis, MO, USA) and the pellet was discarded. An injection volume of 40 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 of 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.

A timed pregnant Sprauge–Dawley rat (Harlan, Indianapolis, IN, USA), weighing 335 g, was anesthetized intramuscularly with ketamine:acepromazine:xylazine (50:3.3:3.4, mg/kg) and dosed on day 18 of gestation. During anesthesia, the animal was 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 rats were administered an i.v. bolus dose (25 mg/kg) of 25 mg/ml DDC dissolved in 0.1N NaOH in physiological saline (pH 7.4) via the jugular cannula. Blood samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min after dosing into heparinized tubes and centrifuged at 10,000 rpm for 10 min to enable plasma collection. Amniotic fluid, placenta,

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

Matrix	Slope	Intercept	R^2
Plasma	0.677 ± 0.021	−0.007 ± 0.004	0.99988 ± 0.00005
Amniotic fluid	0.707 ± 0.058	−0.001 ± 0.008	0.99995 ± 0.00001
Placental homogenate	0.671 ± 0.037	0.014 ± 0.009	0.99918 ± 0.00044
Fetal homogenate	0.670 ± 0.027	−0.016 ± 0.017	0.99975 ± 0.00022

and fetus samples were collected at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 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 chemical structures of DDC and the internal standard used in this assay, 3TC, are shown in Fig. 1. Separation of DDC and 3TC from interfering matrix peaks was explored using different kind of columns and mobile phases. Different UV wavelengths were tried and the wavelength that gave the maximum response for DDC was used. Fig. 2a–d shows chromatograms of each extracted blank matrix and extracted matrix spiked with DDC (2.25 μg/ml) and 3TC (1.5 μg/ml).

The calibration curves for each day of validation and analysis showed good linear response (R^2 = 0.99870–0.99997) through the range of 0.15–75.0 μg/ml. Microsoft Excel or JMP statistical software was used to generate linear regression equations for all calibration curves. A 1/x-weighting scheme was used for each day of the validation and analysis for all four matrices. Calibration curves for the different matrices are displayed in Table 1. The range of 0.15–75.0 μg/ml encompasses the estimated range of post i.v. bolus dose concentrations in all the biological matrices.

The extraction efficiencies for DDC and 3TC from the various matrices were expressed in terms of relative recovery. Standard-spiked matrix samples at the 0.3, 6.0 and 60.0 μg/ml levels 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. DDC and 3TC recoveries from maternal plasma, amniotic fluid, placenta, and fetus ranged from 70.9% to 100.5%. The relative recoveries for each individual matrix are displayed in Table 2.

Assay precision and accuracy for DDC was calculated for each matrix over a range of 3 days. Blanks from each matrix were spiked with DDC and 3TC to yield final concentrations of DDC corresponding with those used in the calibration curve (0.15, 0.375, 0.75, 1.125, 1.5, 3.75, 7.5, 15.0, 37.5, 75.0 μg/ml). Five replicates of blanks spiked with DDC concentrations of 0.15 μg/ml (limit of quantitation, LOQ), 0.3, 2.25 and 60.0 μg/ml were prepared for each validation day to test the precision (relative standard deviation, %R.S.D.) and accuracy (% error). The intra-day (n = 5) precision and accuracy for DDC (spiked concentrations: 0.15, 0.3, 2.25 and 60.0 μg/ml) were in the range of 0.7%–14.5% (%R.S.D.) and 0.9%–14.6% (% error), respectively. The inter-day (n = 15) precision and accuracy for DDC (spiked concentrations: 0.15, 0.3, 2.25 and 60.0 (g/ml) ranged from 1.8% to 12.9% (%R.S.D.) and from 1.4% to 12.2% (% error), respectively. These intra- and inter-day precision and accuracy data are shown in Table 3.

Stability testing was performed for DDC and 3TC at 0.3 μg/ml concentration level. Spiked matrix samples (20 samples) were subjected to three consecutive freeze/thaw cycles over the period of 4 days. Five samples were extracted and analyzed as described above. The remaining spiked matrix samples were stored at −20 °C. Each of the following three consecutive days, the spiked matrix samples were thawed, and five more were extracted and analyzed. The day-to-day measured peak areas of DDC and 3TC were compared and the results listed in Table 4. The %R.S.D. between the average peak area of DDC each day was less than 12.0%, and less than 7.0% for 3TC. There was no distinctive decline in peak areas for either DDC or 3TC over three consecutive freeze/thaw cycles at 0.3 μg/ml level. The stability of extracted matrix samples in the autosampler was also evaluated. At time 0, one sample of each matrix was injected onto the HPLC column and analyzed. In another 22 h, the same sample from each matrix was injected again. The peak areas for DDC and 3TC in each injection were compared. The %R.S.D. between each sample was <15.0% for both compounds and there was no obvious decline in peak areas between each injection.

In humans, more than 70% of DDC is excreted unchanged in the urine [20]. The cytidine ring on DDC undergoes oxidative deamination to produce dideoxyuridine (DDU), although the amount of DDU produced is quite low. DDC also undergoes some phase II metabolism producing a glucoronide conjugate. However, glucoronide conjugates are normally not ob-

Table 2
The percent relative recovery ± S.D. (n = 5) of DDC and 3TC from maternal plasma, amniotic fluid, placental and fetal homogenates

	Maternal plasma	Amniotic fluid	Placental homogenate	Fetal homogenate
DDC (μg/ml)				
0.3	93.9 ± 11.9	100.5 ± 5.1	87.1 ± 4.6	84.9 ± 1.4
6.0	93.2 ± 1.4	97.2 ± 2.2	97.8 ± 1.3	71.5 ± 2.4
60.0	93.5 ± 0.7	98.4 ± 1.1	90.0 ± 1.1	70.9 ± 6.8
3TC (μg/ml)				
1.5	93.8 ± 1.0	98.3 ± 2.2	97.6 ± 11.8	94.6 ± 5.2

Table 3
The intra-day (n = 5, at each spiked concentration) and inter-day (n = 15, at each spiked concentration) precision (%R.S.D.) and accuracy (% error) of the HPLC–UV method used to quantitate DDC in maternal plasma, amniotic fluid, placental and fetal homogenates

Concentration DDC added (μg/ml)	Intra-day			Inter-day		
	Concentration DDC found (μg/ml)	R.S.D. (%)	Error (%)	Concentration DDC found (μg/ml)	R.S.D. (%)	Error (%)
Maternal plasma						
0.15	0.162 ± 0.008	4.884	7.882	0.164 ± 0.007	4.558	9.229
0.3	0.319 ± 0.015	4.610	6.352	0.320 ± 0.011	3.416	6.789
2.25	2.234 ± 0.043	1.906	1.530	2.328 ± 0.221	9.503	5.181
60.0	58.430 ± 1.304	2.231	2.720	58.962 ± 1.338	2.270	2.276
Amniotic fluid						
0.15	0.152 ± 0.017	11.074	8.425	0.146 ± 0.013	9.085	7.757
0.3	0.309 ± 0.022	7.070	6.191	0.303 ± 0.019	6.354	4.941
2.25	2.207 ± 0.090	4.070	3.308	2.202 ± 0.115	5.233	3.249
60.0	60.376 ± 0.630	1.044	0.856	60.340 ± 1.063	1.762	1.424
Placental homogenate						
0.15	0.148 ± 0.021	14.153	10.438	0.136 ± 0.017	12.241	12.210
0.3	0.278 ± 0.039	14.141	10.181	0.283 ± 0.032	11.347	10.195
2.25	2.209 ± 0.048	2.189	2.378	2.232 ± 0.075	3.377	2.808
60.0	59.169 ± 0.873	1.475	1.467	58.259 ± 1.542	2.646	3.014
Fetal homogenate						
0.15	0.172 ± 0.007	4.307	14.414	0.162 ± 0.011	6.585	8.450
0.3	0.321 ± 0.030	9.326	7.633	0.293 ± 0.038	12.902	10.553
2.25	2.289 ± 0.023	1.008	1.721	2.278 ± 0.077	3.380	2.715
60.0	57.957 ± 0.703	1.214	3.405	58.381 ± 1.161	1.989	2.844

served in the rat. Therefore, we would not anticipate any interference from metabolites in this assay. Fig. 3 shows a sample from a dosed rat with no evidence for any additional signals.

To demonstrate the utility of this assay, a pregnant rat was dosed with DDC at the level of 25 mg/kg. Maternal plasma, amniotic fluid, placenta and fetal tissue 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 DDC present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 1.5 μg/ml of the internal standard 3TC. The sample peak area ratios of DDC to 3TC were used to calculate the concentration of DDC in each sample. Fig. 4 shows the concentration–time profile of DDC in all four biological matrices of the pregnant rat. WinNonlin (Pharsight, Mountain View, CA, USA) was used to fit a two-compartment IV bolus model to the plasma data. DDC has a half-life of 82.9 min, a steady state volume of distribution of 1.41 l/kg, and total clearance of 1.1 l/h/kg based on the maternal plasma data. These values were in close agreement with previously reported values for DDC pharmacokinetics in rats [21,22].

Table 4
Results of freeze/thaw stability of DDC in maternal plasma, amniotic fluid, placental and fetal homogenates, represented by area ± S.D. (n = 5) of each day and %R.S.D. of the area of DDC between days

	Maternal plasma	Amniotic fluid	Placental homogenate	Fetal homogenate
DDC				
Day 1	7256.2 ± 292.2	6810.0 ± 275.9	7155.0 ± 798.6	6454.6 ± 682.7
Day 2	6689.0 ± 635.7	7325.6 ± 290.3	6692.2 ± 497.8	5239.0 ± 249.7
Day 3	6631.4 ± 131.3	7472.0 ± 321.2	6112.0 ± 644.4	5329.0 ± 254.9
Day 4	6797.2 ± 299.8	7319.8 ± 295.3	5420.0 ± 602.0	5525.2 ± 145.0
%R.S.D.	4.1	4.0	11.8	9.9
3TC				
Day 1	34048.4 ± 1247.8	35759.6 ± 477.9	31659.6 ± 1129.6	33831.2 ± 86.9
Day 2	33594.6 ± 2666.0	37010.8 ± 351.6	31568.6 ± 1038.4	36335.8 ± 1239.9
Day 3	35929.6 ± 358.7	36626.4 ± 434.7	32346.8 ± 295.8	38187.2 ± 379.6
Day 4	35303.8 ± 1743.7	36159.6 ± 1040.3	31442.2 ± 2928.2	38743.0 ± 663.8
%R.S.D.	3.1	1.5	1.3	6.0

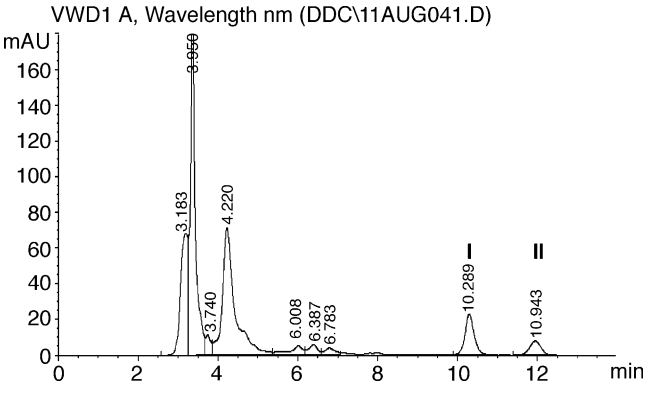

Fig. 3. Chromatogram from amniotic fluid taken 30 min after dosing at 25 mg/kg.

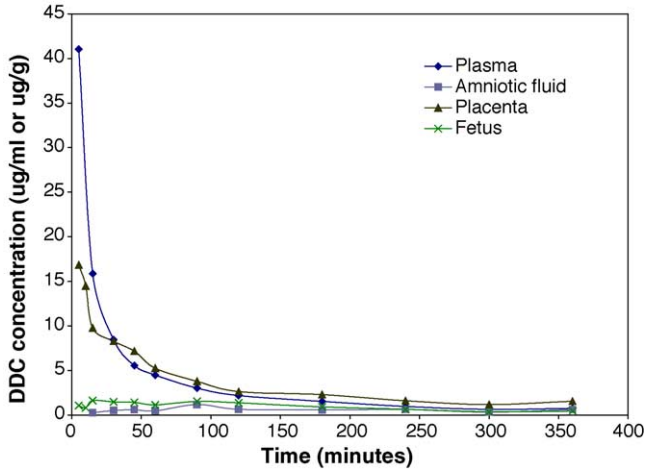

Fig. 4. Concentration vs. time curve of DDC in maternal plasma, amniotic fluid, placenta and fetus after 25 mg/kg i.v. bolus dose of DDC.

4. Conclusions

Extracting and analyzing such highly polar compounds out of complex biological matrices poses some unique problems. By the use of a highly polar column and a low percentage of organic solvent, separation of the matrix interferences, DDC, and 3TC were achieved while providing adequate retention for both DDC and 3TC. This assay for the determination of DDC from plasma, amniotic fluid, placental and fetal homogenates is sensitive and efficient. The retention of such highly polar compounds was achieved under reversed-phase chromatography condition without use of ion-pairing reagents, making this method readily transferable to an LC–MS platform. This method yielded high recoveries, showed good linearity, precision and accuracy within the range of 0.15–75.0 μg/ml. The estimated pharmacokinetic parameters from the analysis of collected samples were comparable to literature data, further validating the reliability of this method for the determination of DDC in a placental transport study.

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