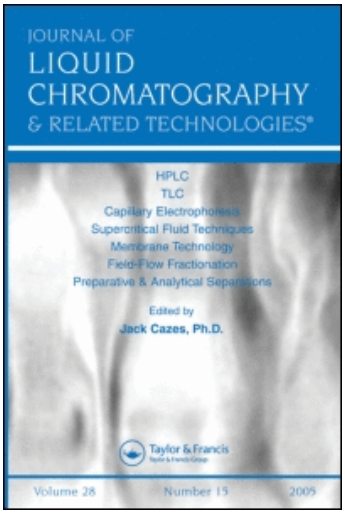

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Xu, Meng , White, Catherine A. and Bartlett, Michael G.(2008) 'Simultaneous Determination of Zalcitabine and Stavudine in Maternal Plasma, Amniotic Fluid, Placental, and Fetal Tissues using Reversed Phase on Silica Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 31: 4, 482 — 496
To link to this Article: DOI: 10.1080/10826070701812715
URL: http://dx.doi.org/10.1080/10826070701812715

Journal of Liquid Chromatography & Related Technologies®, 31: 482–496, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701812715

Simultaneous Determination of Zalcitabine and Stavudine in Maternal Plasma, Amniotic Fluid, Placental, and Fetal Tissues using Reversed Phase on Silica Liquid Chromatography

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Abstract: In order to study the placental transfer of nucleoside reverse transcriptase inhibitors, a quick and simple reversed phase high performance liquid chromatography (HPLC) method has been developed and validated using an underivatized silica column for the separation and analysis of DDC and D4T from rat plasma, amniotic fluid, placental, and fetal homogenate. Extraction of DDC, D4T, and their internal standard lamivudine (3TC) from the matrices was processed by liquid-liquid extraction enhanced by salting out the sample using a saturated solution of ammonium sulfate. 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 consisted of 3% methanol in 22 mM formic acid. The flow rate was 0.5 mL/min, and the detection wavelength was optimized at 265 nm. The calibration curves for each day of validation showed good linear response over the range from 0.1 μg/mL to 50 μg/mL. The absolute recoveries for all the drugs are all higher than 75%. All the intra- and inter-day assay precision and accuracy were better than 5% for all the matrices.

Keywords: Zalcitabine, Stavudine

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INTRODUCTION

According to a recent HIV/AIDS Surveillance Report, at the end of 2004, there were an estimated 123,405 adult women and adolescent girls and 6,804 children living with HIV or AIDS in 35 reporting areas (33 states, Guam, and the U.S. Virgin Islands).[1] The numbers for the entire United States and its territories are undoubtedly much higher.[2] It also stated that for children, 90% of the HIV infections are from vertical transmission from their mothers through blood, amniotic fluid, and/or breast milk.[1,3]

Over the past several years, use of multi-drug therapies has become the rule rather than the exception in the treatment of patients with human immunodeficiency virus (HIV) infections.[4] This has been propelled by the need to delay the development of drug resistance and minimize potential dose limiting side effects. These combination therapies have greatly enhanced the success of acquired immunodeficiency syndrome (AIDS) treatment. While the use of combinations of antiviral drugs is popular, the impact of such combination therapies on placental transport is largely unknown.[5] A series of studies by Unadkat and coworkers has reported the lack of interaction between several anti-HIV drugs when using the macaque as an animal model; their findings suggest passive diffusion as the primary mechanism for placental transport.[6–8] However, other studies showed substantial interactions between the antivirals AZT and acyclovir and between AZT and 3TC in placental transport when using the rat as the animal model.[5,9,10] The data from these studies support a transporter mediated mechanism for placental transport. The differences between these studies may be related to the animal models, experimental design, or may be specific to the agents studied. Continued study of these compounds is needed to gain further understanding of the mechanism of placental transport for this class of therapeutic agents.

2′,3′-Dideoxycytidine (Zalcitabine, 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.[11] DDC has been used in patients who cannot be maintained on AZT due to side effects (e.g., severe anemia).[12] 2′,3′-Didehydro-3′-deoxythymidine (D4T) is a powerful dideoxynucleoside analogue, which has shown powerful activity against HIV.[13] It inhibits HIV reverse transcriptase with relatively little inhibition of host cell DNA polymerases in various cell types.[14] Several HPLC methods have been developed to determine the concentrations of DDC and D4T.[15–22] Ding et al. have developed an HPLC-UV method to determine the drug concentrations of DDC in pregnant rat tissues already.[15] However, there is no existing HPLC method which can simultaneously determine the drug concentrations of DDC and D4T in pregnant rat tissues. In this

study, a rapid and sensitive HPLC method was developed and validated using reversed phase liquid chromatography on an underivatized silica column for the determination of concentrations in samples taken in a maternal fetal drug transfer study of DDC and D4T.

EXPERIMENTAL

Reagents and Chemicals

DDC and D4T were obtained from Sigma (St. Louis, MO, USA). The internal standard, 3TC, was obtained from GlaxoSmithKline (RTP, NC, USA). HPLC grade methanol, acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium sulfate 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).

Instrumentation

All HPLC experiments were performed on a Hewlett-Packard (Agilent) 1100 series HPLC equipped with a variable wavelength UV detector. Chromatographic separation was achieved on a Waters Spherisorb S3W silica column (4.6 × 3.0 mm, Milford, MA) equipped with a Phenomenex security guard C-18 guard column (4 × 3.0 mm, Torrance, CA).

The mobile phase consisted of Solvent A (22 mM formic acid) and Solvent B (acetonitrile) (97:3). The detection wavelength was set to be 265 nm. The flow rate was set to be 0.5 mL/min, and the injection volume was 40 μL. The HPLC run time was 22 min for each run.

Preparation of Standard Solutions

Individual DDC, D4T, and 3TC stock solutions were prepared in deionized water to give a final concentration of 1.0 mg/mL. Individual standard solutions of DDC and D4T with concentrations of 0.5, 1.25, 2.5, 5.0, 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, 10, and 200.0 μg/mL were also prepared in the same manner. A 10 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 and no degradation was observed during the period of this study. Fresh standard solutions were prepared for each day of analysis or validation.

Calibration Curves

Blank plasma was purchased from Harlan (Indianapolis, IN, USA). Blank amniotic fluid, placenta, and fetal tissues were collected from untreated animals. The placental and fetal tissues were homogenized with two volumes of distilled water (v/w). Plasma, placental, and fetal calibration points were prepared by spiking 100 μL of the biological matrices with 20 μL of each DDC and D4T standard solution and 10 μL of the 10 μg/mL 3TC solution. Amniotic fluid calibration points were prepared by spiking 50 μL of the biological matrices with 10 μL of each DDC and D4T standard solution and 10 μL of the 10 μg/mL D4T standard solution. The calibration curves of all the matrices were in the range of 0.1–50 μg/mL with individual calibration points of 50, 25, 10, 1, 0.5, 0.25, and 0.1 μg/mL. The internal standard concentration was 1 μg/mL for all samples.

Precision and Accuracy

This method was validated using four QC points for each calibration curve. Five replicates of each QC point were analyzed each day to determine the intra-day accuracy and precision. This process was repeated 3 times in 3 days to determine the inter-day accuracy and precision. The QC points for all of the four matrices were 0.1, 0.2, 2, and 40 μg/mL.

Sample Preparation

All the samples were prepared by the 'salting out' technique. A saturated ammonium sulfate solution of 200 μL and 1 mL of ice cold acetonitrile were added to the samples (100 μL for plasma, placental, and fetal homogenate, 50 μL for amniotic fluid). After being vortexed and centrifuged at 13,000 rpm for 10 min, the upper organic layer was aspirated and dried under vacuum. Samples were then reconstituted in 100 μL of distilled water for injection.

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 359 g, was anesthetized intramuscularly with ketamine:acepromazine: xylazine (50:3.3:3.4, mg/kg) and dosed on day 19 of gestation. 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 and D4T dissolved in 0.1 N NaOH in physiological saline (pH 7.4), via the jugular cannula. Blood samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, and 225 min after dosing into heparinized tubes and centrifuged at 10,000 rpm for 10 min to enable plasma collection. Amniotic fluid, placenta, and fetus samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, and 225 min. Placental and fetal tissue samples were homogenized in two volumes of deionized water. All samples were stored at -20°C until analysis. Data was analyzed using WinNonlin (Pharsight, Mountain View, CA, USA).

RESULTS AND DISCUSSION

Method Development

The chemical structures of DDC, D4T, and the internal standard used in this assay, 3TC, are shown in Figure 1. Separation of DDC, D4T, and 3TC from interfering matrix peaks was explored using different kinds of columns and mobile phases. Several liquid and solid phase extraction procedures were also investigated to extract DDC, D4T, and 3TC from the different biological matrices. It is a significant challenge to find suitable conditions to separate DDC and D4T in biological samples because DDC is a weak base and D4T is a weak acid and both analytes are very polar. We first tried phenyl columns and amino columns and were unsuccessful. Several reverse phase column such as C_{18} and C_8 columns were tried, but these phases do not retain the analytes well, especially DDC. HILIC (Hydrophilic interaction

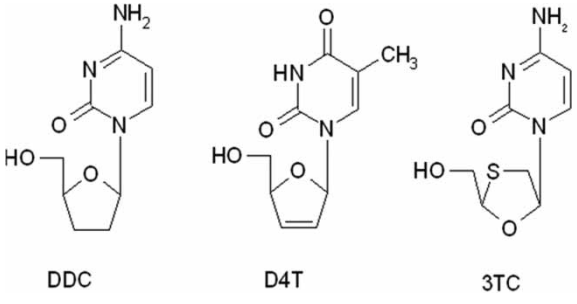

Figure 1. Chemical structures of DDC, D4T, and 3TC.

liquid chromatographic) (high organic on silica column) conditions were also tried, but we found that DDC and D4T were highly retained leading to long run times. Finally, we employed reversed phase conditions on silica and were able to generate a suitable separation. It is important to note that the pH values of the mobile phase were found to be an important factor in the separation. We tried a range of values and found that low pH values around 2 provided the best separation for these analytes. Figures 2–5 show chromatograms of each extracted blank matrix and extracted matrix spiked with DDC (2 μg/mL) and 3TC (1.0 μg/mL).

Calibration Curves

The calibration curves for each day of validation and analysis showed good linear response ($R^2 = 0.9995–0.9999$) over the range of 0.1–50 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.

Precision and Accuracy

Assay precision and accuracy were calculated for each matrix over 3 days. Precision, as expressed by %R.S.D., and accuracy as expressed by % error

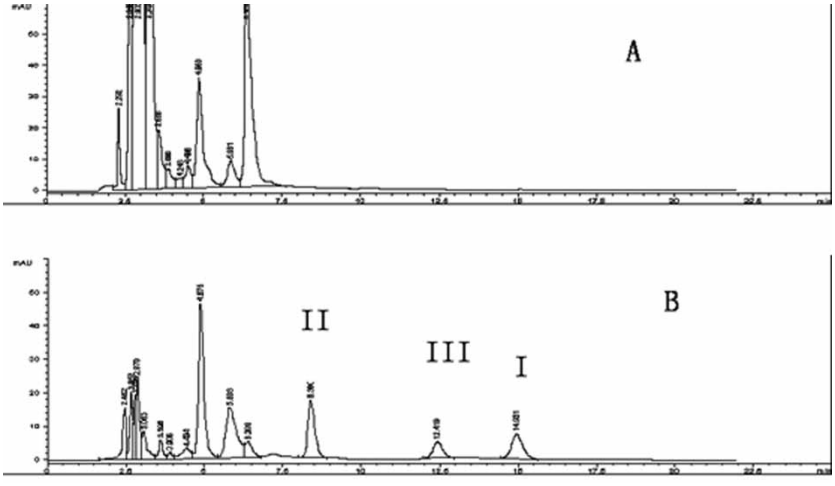

Figure 2. Chromatograms obtained from blank plasma (A) and plasma spiked with (I) DDC (2 μg/mL), (II) D4T (2 μg/mL) and (III) 3TC (1 μg/mL).

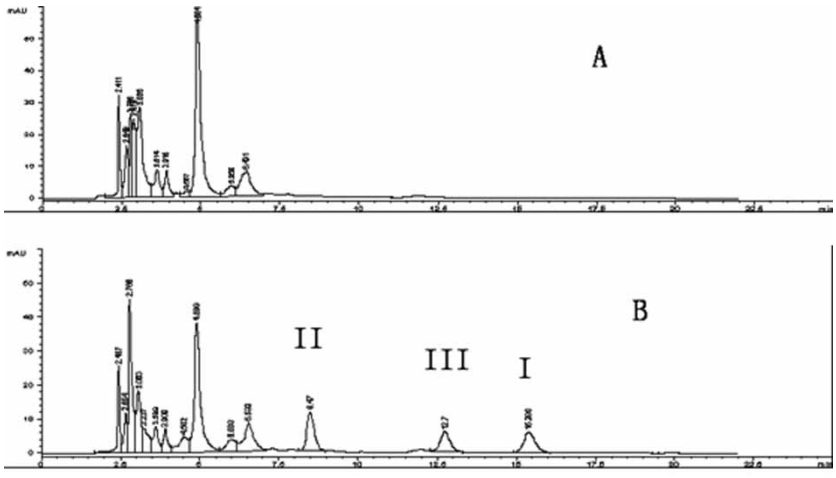

Figure 3. Chromatograms obtained from blank amniotic fluid (A) and amniotic fluid spiked with (I) DDC (2 μg/mL), (II) D4T (2 μg/mL), and (III) 3TC (1 μg/mL).

for DDC and D4T in the four biological matrices are shown in Table 2. Intra-day (n = 5) precision and accuracy were calculated from the measurement of five samples at each QC point on three separate days. Inter-day (n = 15) precision and accuracy were calculated from pooled data over 3 days. Four QC points of concentrations 40, 2, 0.2, and 0.1 μg/mL were used for these

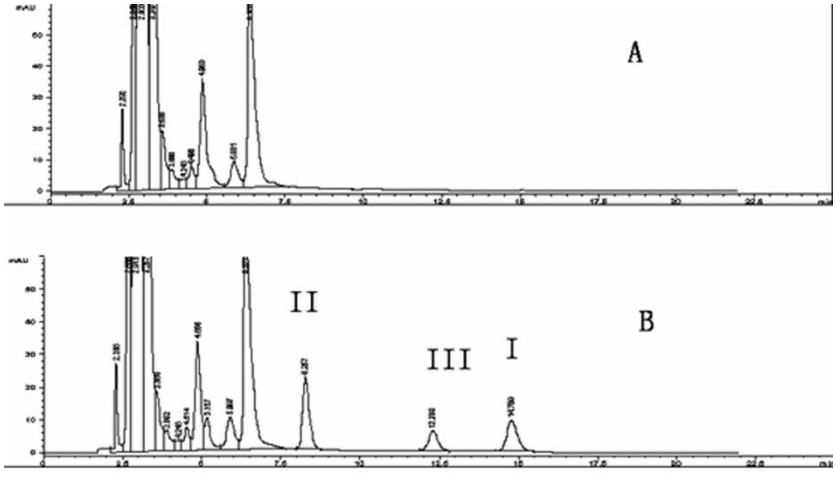

Figure 4. Chromatograms obtained from blank placental homogenate (A) and placental homogenate spiked with (I) DDC (2 μg/mL), (II) D4T (2 μg/mL), and (III) 3TC (1 μg/mL).

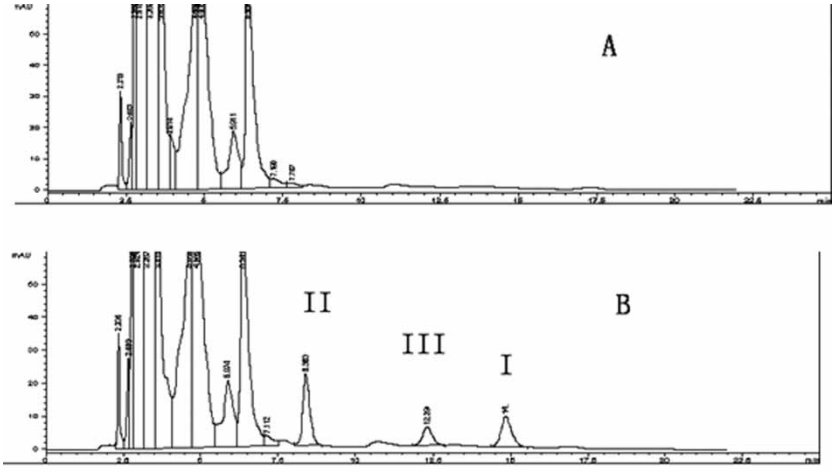

Figure 5. Chromatograms obtained from blank fetal homogenate (A) and fetal homogenate spiked with (I) DDC (2 μg/mL), (II) D4T (2 μg/mL), and (III) 3TC (1 μg/mL).

calculations. Intra-day precision (%R.S.D.) and accuracy (% error) of DDC ranged from 0 to 4.04 and 0.15 to 2%, respectively. Inter-day precision and accuracy of DDC ranged from 0.69 to 5 and 0 to 3.05%, respectively. Intra-day precision and accuracy of D4T ranged from 0.07 to 2.9 and 0.04 to 4.9%, respectively. Inter-day precision and accuracy of D4T ranged from 0.5 to 4.06 and 0 to 2%, respectively. Results are shown in Table 2 and Table 3.

Recovery Studies

The extraction efficiencies for DDC, D4T, and 3TC from the various matrices were expressed in terms of absolute recovery. Standard, spiked matrix samples of DDC and D4T at the 0.1, 0.2, 2.0, and 40.0 μg/mL levels, and the 3TC sample with a concentration of 1 μg/mL were extracted and analyzed

Table 1. Linear regression equations generated from validation data from each matrix, (n = 3, for each matrix)

R^2	Maternal plasma	Amniotic fluid	Placental homogenate	Fetal homogenate
DDC	0.9995 ± 0.0003	0.9999 ± 0.0000	0.9999 ± 0.0000	0.9995 ± 0.0002
D4T	0.9995 ± 0.0002	0.9999 ± 0.0001	0.9999 ± 0.0001	0.9998 ± 0.0001

Table 2. 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

	Intra-day (n = 5)			Inter-day (n = 15)		
Concentration DDC added (μg/mL)	Concentration DDC (μg/mL)	R.S.D. (%)	Error (%)	Concentration DDC found (μg/mL)	R.S.D. (%)	Error (%)
Maternal plasma						
0.1	0.102 ± 0.003	2.94	2	0.101 ± 0.002	1.98	1
0.2	0.196 ± 0.004	2.04	2	0.199 ± 0.005	2.51	0.5
2.0	1.993 ± 0.040	2.01	0.35	1.939 ± 0.081	4.18	3.05
40	39.78 ± 0.14	0.35	0.55	39.18 ± 1.02	2.6	2.05
Amniotic fluid						
0.1	0.098 ± 0.002	2.04	2	0.100 ± 0.004	4	0
0.2	0.202 ± 0.001	0.5	1	0.202 ± 0.002	0.99	1
2.0	2.011 ± 0.008	0.4	0.55	2.004 ± 0.016	0.8	0.2
40	40.52 ± 0.24	0.59	1.3	40.29 ± 0.28	0.69	0.73
Placental homogenate						
0.1	0.101 ± 0.001	0.99	1	0.099 ± 0.001	1.01	1
0.2	0.202 ± 0.002	0.99	1	0.202 ± 0.002	0.99	1
2.0	1.992 ± 0.003	0.15	0.4	1.987 ± 0.015	0.75	0.65
40	40.06 ± 0.00	0	0.15	40.01 ± 0.33	0.82	0.03
Fetal homogenate						
0.1	0.099 ± 0.004	4.04	1	0.100 ± 0.005	5	0
0.2	0.199 ± 0.004	2.01	0.5	0.201 ± 0.004	1.99	0.5
2.0	2.023 ± 0.033	1.63	1.15	1.998 ± 0.028	1.4	0.1
40	39.63 ± 0.22	0.56	0.93	40.20 ± 0.47	1.17	0.5

(n = 5). The drug solutions with the same concentration were yielded in the deionized water. The peak areas of these two sample sets were compared. DDC and 3TC recoveries from maternal plasma, amniotic fluid, placenta, and fetus ranged from 75.6% to 96.6%. The absolute recoveries for each individual matrix are displayed in Table 4.

Stability Studies

Stability testing was performed for DDC and D4T at 2 μg/mL concentration levels. Spiked matrix samples were subjected to three consecutive freeze/thaw cycles over the period of 4 days. Three samples were extracted and analyzed as described above. The remaining spiked matrix samples were stored at −20°C. Each of the following three consecutive

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 D4T in maternal plasma, amniotic fluid, placental and fetal homogenates

	Intra-day (n = 5)			Inter-day (n = 5)		
Concentration D4T added (μg/mL)	Concentration D4T found (μg/mL)	R.S.D. (%)	Error (%)	Concentration DD found (μg/mL)	R.S.D. (%)	Error (%)
Maternal plasma						
0.1	0.098 ± 0.001	1.02	2	0.100 ± 0.003	3	0
0.2	0.202 ± 0.004	1.98	1	0.201 ± 0.004	1.99	0.5
2.0	2.071 ± 0.060	2.9	3.55	2.018 ± 0.082	4.06	0.9
40	40.38 ± 0.48	1.2	0.95	4043 ± 0.59	1.46	1.08
Amniotic fluid						
0.1	0.098 ± 0.001	1.02	2	0.099 ± 0.003	3.03	1
0.2	0.201 ± 0.001	0.5	0.5	0.201 ± 0.004	1.99	0.5
2.0	2.003 ± 0.002	0.1	0.15	2.004 ± 0.016	0.8	0.2
40	40.05 ± 0.04	0.1	0.13	40.29 ± 0.28	0.69	0.73
Placental homogenate						
0.1	0.099 ± 0.002	1.67	0.4	0.099 ± 0.002	2.02	1
0.2	0.199 ± 0.003	1.43	0.2	0.199 ± 0.003	1.51	0.5
2.0	1.987 ± 0.002	0.11	0.64	1.999 ± 0.010	0.5	0.05
40	39.98 ± 0.026	0.07	0.04	39.98 ± 0.21	0.53	0.05
Fetal homogenate						
0.1	0.095 ± 0.003	2.72	4.9	0.098 ± 0.003	3.06	2
0.2	0.201 ± 0.003	1.3	0.6	0.201 ± 0.003	1.49	0.5
2.0	2.044 ± 0.012	0.59	2.18	2.021 ± 0.019	0.94	1.05
40	39.69 ± 0.23	0.59	0.78	39.97 ± 0.31	0.78	0.08

days, the spiked matrix samples were thawed, and three more were extracted and analyzed. The day-to-day measured peak areas of DDC and D4T were compared and the results listed in Table 5. The %R.S.D. between the average peak areas of DDC each day was less than 5.5%, and less than 5.9% for 3TC. There was no distinctive decline in peak areas for either DDC or 3TC over three consecutive freeze/thaw cycles at the 2 μg/mL level. The stability of extracted matrix samples in the auto-sampler was also evaluated. At time 0, one sample of each matrix was injected onto the HPLC column and analyzed. In another 24 h, the same sample from each matrix was injected again. The peak areas for DDC and D4T in each injection were compared. The %R.S.D. between each sample was $<8.0\%$ for both compounds, and there was no obvious decline in peak areas between each injection.

Table 4. The percent relative recovery ± S.D. (n = 5) of DDC, D4T and 3TC from maternal plasma, amniotic fluid, placental and fetal homogenates

n = 15 (%)	Maternal plasma	Amniotic fluid	Placental homogenate	Fetal homogenate
DDC (μg/mL)				
0.1	82.86 ± 2.07	80.77 ± 3.62	86.09 ± 2.81	80.87 ± 1.73
0.2	84.48 ± 3.07	79.20 ± 1.51	83.31 ± 2.76	78.91 ± 1.57
2.0	83.82 ± 2	79.53 ± 1.67	79.67 ± 1.08	75.63 ± 1.06
40	77.78 ± 2.3	75.72 ± 0.58	77.40 ± 0.96	77.87 ± 0.54
D4T (μg/mL)				
0.1	88.67 ± 3.62	91.16 ± 2.58	90.91 ± 2.01	85.69 ± 1.41
0.2	95.29 ± 3.67	83.42 ± 1.31	90.96 ± 1.56	85.12 ± 1.31
2.0	96.63 ± 1.54	87.11 ± 1.33	92.05 ± 0.94	86.97 ± 1.77
40	93.48 ± 1.47	83.67 ± 0.95	94.44 ± 1.04	90.92 ± 0.83
3TC (μg/mL)				
1.0	87.05 ± 2.41	78.28 ± 2.21	78.65 ± 2.23	78.89 ± 2.28

Animal Study

To demonstrate the utility of this assay, a pregnant rat was dosed with DDC and D4T 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

Table 5. 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 and D4T between days

n = 3	Maternal plasma	Amniotic fluid	Placental homogenate	Fetal homogenate
DDC (2 μg/mL)				
Day 1	235.74 ± 3.31	116.81 ± 1.74	223.54 ± 2.28	227.17 ± 1.27
Day 2	218.20 ± 1.07	102.46 ± 3.57	211.27 ± 3.57	213.06 ± 2.39
Day 3	212.53 ± 1.63	111.42 ± 0.78	224.82 ± 1.69	205.58 ± 1.45
Day 4	215.22 ± 1.32	106.29 ± 1.29	207.74 ± 3.39	200.64 ± 3.01
RSD (%)	4.75	5.71	3.97	5.46
D4T (2 μg/mL)				
Day 1	326.77 ± 3.73	159.96 ± 2.37	317.69 ± 1.93	325.42 ± 2.67
Day 2	325.29 ± 2.85	147.32 ± 1.68	301.12 ± 1.43	312.79 ± 2.31
Day 3	334.49 ± 1.30	148.64 ± 3.06	292.05 ± 0.74	299.75 ± 3.97
Day 4	303.62 ± 1.35	138.67 ± 4.95	294.54 ± 3.28	303.24 ± 3.83
RSD (%)	4.11	5.88	3.83	3.70

samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 1 μg/mL of the internal standard 3TC. Figure 6 shows the concentration time profile of DDC and D4T in all four biological matrices of the pregnant rat. WinNonlin (Pharsight, Mountain View, CA, USA) was used to fit a non-compartment IV bolus model to the plasma data. DDC has a half-life of 106.2 min, a steady state volume of distribution of 1.58l/kg, and total clearance of 0.75 l/h/kg based on the maternal plasma data. D4T has a half-life of 97.2 min, a steady state volume of distribution of 0.69 l/kg, and total clearance of 0.28 l/h/kg based on the maternal plasma data.

Animal studies with an IV bolus administration of a dose of 25 mg/kg of DDC alone were conducted for comparison to the DDC-D4T combination dose. When administered alone, DDC has a half-life of 120 min, a steady state volume of distribution of 1.90 l/kg, and total clearance of 1.00 l/h/kg in the maternal plasma. Therefore, there is little difference observed in maternal pharmacokinetics of DDC, both in single dose and in combination. This is similar to previous studies comparing the nucleoside antivirals

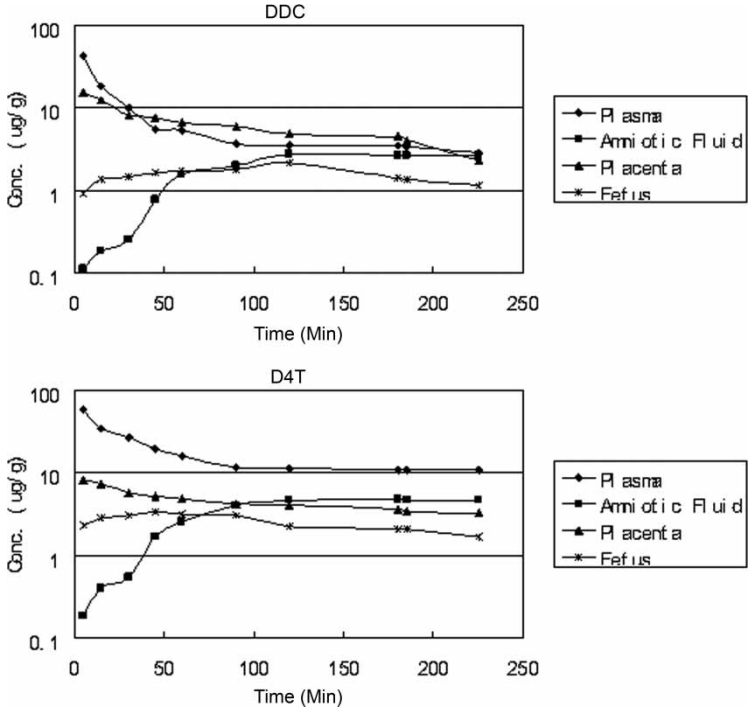

Figure 6. Concentration vs. time profile of DDC and D4T in maternal plasma, amniotic fluid, placenta, and fetus after 25 mg/kg i.v. bolus dose of DDC and D4T.

lamivudine and zidovudine. However, there are significant differences in relative exposures ($AUC_{tissue}/AUC_{maternal\ plasma}$) of the fetus to DDC when administered alone or in combination. For example, the relative exposure of the tissues was significantly lower in the combination doses. For the fetus, it was 0.6 in the single dose vs. 0.3 in the combination; for the placenta, it was 1.3 in the single dose vs. 0.7 in the combination. This preliminary data suggests that D4T decreases the fetal and placental exposure to DDC by perhaps as much as 50%. Additional animal studies will be needed to fully understand the interactions between these antivirals; however, it is clear that there is more than simple passive diffusion involved in the transport of the nucleoside antivirals between maternal and fetal circulations.

CONCLUSION

A sensitive, efficient, and accurate method was developed and validated for the simultaneous quantification of DDC and D4T in rat plasma, amniotic fluid, placental, and fetal tissues. This method is useful for pharmacokinetic studies to investigate the distribution of DDC and D4T in the maternal and fetal compartment of rats.

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Received August 1, 2007
Accepted September 10, 2007
Manuscript 6180