QUANTITATIVE ANALYSIS AND PHARMACOKINETICS OF ANTIVIRAL AGENTS IN THE PREGNANT RAT

by

MENG XU

(Under the Direction of Michael G. Bartlett)

ABSTRACT

Antiviral drugs are used therapeutically in pregnancy for treatment of both mother and fetus. Antiviral drugs are presumed to prevent viral transmission from mother to fetus by decreasing maternal viral load and/or accumulation of the drugs in the fetal compartment. Due to a number of reasons, pregnant women are generally not used during clinical trials, so very little is known about the behavior of therapeutic agents during pregnancy. A pregnant rat model has been developed to investigate the pharmacokinetics and placental transport of antivirals during pregnancy. Presented here are validated analytical methods for the extraction and quantitation of the nucleoside reverse transcriptase inhibitors zalcitabine (DDC) and stavudine (D4T) in the various matrices needed to support the maternal-fetal pharmacokinetic studies. Also presented here are the pharmacokinetics of DDC and D4T, using the pregnant rat model.

INDEX WORDS: Analytical, Pharmacokinetics, HIV, Zalcitabine, DDC, Stavudine, D4T, Lamivudine, 3TC, placental transport, fetal disposition

QUANTITATIVE ANALYSIS AND PHARMACOKINETIC S OF ANTIVIRAL AGENTS IN THE PREGNANT RAT

by

MENG XU

B.S., University of Sci. & Tech. of China, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2008

© 2008

Meng Xu

All Rights Reserved

QUANTITATIVE ANALYSIS AND PHARMACOKINETIC S OF ANTIVIRAL AGENTS IN THE PREGNANT RAT

by

MENG XU

Major Professor:	Michael G. Bartlett
Committee:	Catherine A. White
	James V. Bruckner
	J. Warren Beach
	Randall L. Tackett

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2008

DEDICATION

This dissertation is dedicated with love and gratitude to my parents, Yuejin Xu and Yingying Wang.

ACKNOWLEDGEMENTS

Firstly, I would like to thank my parents for their love and support throughout my long journey of schooling. To Pingping Wang, my uncle, thank you for introducing me into this field and without your support, I would never have accomplished anything I have today.

I would like to thank my major professor, Dr. Michael G. Bartlett. Thank you for everything you taught me in the lab and the effort you have made to prepare me for a career in bioanalytical chemistry. I thank for Dr. Catherine A. White for the countless hours spent in the lab dosing pregnant rats and data analysis. I also wish to thank the remaining members of my committee, Dr. James V. Bruckner, Dr. J. Warren Beach, Dr. Randall L. Tackett, for their helpful ideas and advice throughout my graduate career. I would like to especially thank my labmates, Shonetta, Leah, Guodong, Yongzhen, Buyun and Feng for their knowledge, encouragement, and stimulating conversations; and also Summer, Shawn, and Liang for their help with pharmacokinetics. Lastly, but certainly not least, I would like to thank everyone who helped me at UGA. I could not have fulfilled my goals without your sincere support.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

CHAPTER

1 INTRODUCTION 1

2 LITERATURE REVIEW: PLACENTAL TRANSFER OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS 9

3 SIMULTANEOUS DETERMINATION OF ZALCITABINE AND STAVUDINE IN . MATERNAL PLASMA, AMNIOTIC FLUID, PLACENTAL AND FETAL TISSUES USING REVERSED PHASE ON SILICA LIQUID CHROMATOGRAPHY 33

4 COMPARISON OF THE FETAL DISPOSITION OF ZALCITABINE AND LAMIVUDINE IN THE PREGNANT RAT 60

5 STAVUDINE AFFECTS THE PLACENTAL TRANSFER OF ZALCITABINE IN THE PREGNANT RAT 87

6 SIMULTANEOUS DETERMINATION OF ZALCITABINE AND STAVUDINE IN MATERNAL PLASMA, AMNIOTIC FLUID, PLACENTAL AND FETAL TISSUES USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETY 110

7 CONCLUSIONS.....138

APPENDIX

1 DETERMINATION OF DIISOPROPYLFLUOROPHOSPHATE (DFP) IN RAT PLASMA AND BRAIN TISSUE BY HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY MASS SPECTROMETRY.....141

2 STABILITY OF LOW CONCENTRATION CEFTAZIDIME IN NORMAL SALINE AND BALANCED SALT SOLUTIONS IN PLASTIC SYRINGES UNDER VARIOUS STORAGE CONDITIONS.....173

CHAPTER 1

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) within the United States. (1) The numbers for the entire United States and its territories are undoubtedly much higher. (2) The current common treatment strategy for HIV-infected patients by highly active antiretroviral therapy (HAART) containing two nucleoside reverse transcriptase inhibitors (NRTIs) combined with one non-nucleoside reverse transcriptase inhibitors (NNRTIs) or one protease inhibitors (PIs) or both. (3) Because the HIV virus can cross the placenta, there has been a coordinate increase in the number of children born to HIV positive mothers. Antiretroviral therapy is thought to prevent transmission of HIV from mother to child by decreasing maternal viral load and/or accumulation of drugs in the fetal compartment. (4) 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 have 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 zidovudine and acyclovir and between zidovudine and lamivudine in the placenta when using the rat as an 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.

ANALYTICAL METHODS

1. Sample Preparation

The extraction of the NRTIs from biological matrices such as plasma, serum, urine, and amniotic fluid, fetal and placental tissues is the most important step in method development. This step is needed in order to provide a sample that is reproducibly clean, homogenous and ready for direct injection onto the analytical column. (11) Protein precipitation, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are often used.

a) Protein precipitation

Protein precipitation is the simplest procedure to remove proteins from biological samples. Briefly, inorganic or organic solvents such as perchloric acid (PCA), trichloroacetic acid (TCA) and acetonitrile (ACN) are used to precipitate proteins in

biological samples. The mixtures are then centrifuged to remove the denatured proteins. (12)

b) Liquid-liquid extraction

Liquid-liquid extraction, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase. The commonly used organic solvents to extract chemicals from biological samples include dichloromethane, ethyl acetate and isopropyl ether.

c) Solid phase extraction

LLE is a simple and efficient method for the separation and concentration of relatively hydrophobic compounds. (12) However, for more polar compounds, LLE may not be an effective extraction procedure. SPE is a more effective extraction procedure for many NRTIs. SPE is a separation process that is used to remove solid or semi-solid compounds from a mixture of impurities based on their physical and chemical properties. The separation ability of solid phase extraction is based on the preferential affinity of desired or undesired solutes in a liquid, mobile phase for a solid, stationary phase through which the sample is passed. Impurities in the sample are either washed away while the analyte of interest is retained on the stationary phase, or vice-versa. Analytes that are retained on the stationary phase can then be eluted from the solid phase extraction

cartridge with the appropriate solvent. The most frequently used SPE materials are C_{18} and polymeric cartridges.

2. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention times vary depending on the interactions between the stationary phase, molecules being analyzed, and the solvent(s) used. Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With these stationary phases, retention times are longer for molecules which are more non-polar, while polar molecules elute more readily. The retention time can be increased by adding a polar solvent to the mobile phase or decreased by adding a more hydrophobic solvent. Reversed phase chromatography (RPC) is so commonly used that it is not uncommon for it to be incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RPC to qualify drugs before their release. Normal phase HPLC (NP-HPLC) separates analytes based on polarity; it was the first kind of HPLC chemists developed. NP-HPLC uses a polar stationary phase and a non-polar mobile phase, and works effectively for relatively polar analytes. The polar analyte associates with and is retained by the polar stationary phase. Adsorption strength

increases with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength not only depends on the functional groups in the analyte molecule, but also on steric factors. The effect of sterics on interaction strength allows this method to resolve (separate) structural isomers.

3. Analyte Detection

a) Ultraviolet (UV) detection

Ultraviolet detectors are the mainstay of routine HPLC analysis. The detector monitors the column effluent at a specified wavelength. All compounds that have some absorption at this wavelength will generate a response. (13) The advantages of UV detection include – ease of operation, low background with many HPLC solvents, relatively high sensitivity, and the ability to recover the original sample. Disadvantages include that the analyte must have absorbance at the specified wavelength and the analyte must be chromatographically resolved from other compounds that absorb at the same wavelength. (14)

b) Mass spectrometry

Mass spectrometry is an analytical technique that measures the mass-to-charge ratio of charged particles. All mass spectrometers have three basic components: (i) an ionization source, (ii) a mass analyzer and (iii) a detector. In the field of pharmaceutical bioanalysis, especially in the area of drug quantitation, the overwhelming choice for ionization involves atmospheric pressure ionization (API) techniques such as atmospheric

pressure chemical ionization (APCI) and electrospray ionization (ESI). Both APCI and ESI are "soft" ionization techniques. ESI produces multiply charged ions which allows for the analysis of very large molecules in an instrument with a limited mass range. (15) APCI achieves analyte ionization by collision with reagent ions without excessive energy transfer. (15)

DISSERTATION STRUCTURE

This dissertation is structured as follows: following a review of current literature in Chapter 2, Chapter 3 describes the development and validation of an HPLC assay for the simultaneous determination of DDC and D4T in rat tissues using HPLC-UV; Chapter 4 provides a comparison of the fetal disposition of DDC and 3TC in the pregnant rat; Chapter 5 contains the pharmacokinetic analysis of DDC alone and in combination with D4T; Chapter 6 describes the development and validation of an LC-MS/MS assay for the simultaneous determination of DDC and D4T in rat tissues using HPLC-UV, it also gives a comparison of the pharmacokinetics between pregnant rats with high dose and low dose DDC/D4T combination; and Chapter 8 summarizes the final conclusions. In the Appendix, a SPME GC-MS method developed for the analysis of diisopropylfluorophosphate (DFP) in rat plasma and brain tissue and stability studies of low concentration ceftazidime in normal saline and balanced salt solutions in plastic syringes under various storage conditions are included.

REFERENCES

1. Centers for Disease Control and Prevention. HIV/AIDS surveillance report: Cases of HIV infection and AIDS in the United States, 2004. Atlanta: The Centers; 2005. Vol.

16. (Available at http://www.cdc.gov/hiv/topics/surveillance/resources /reports/2004report/default.htm.)

2. Cibulka, N.J. Mother-to-Child Transmission of HIV in the United States. *Am. J. Nurs.* 2006, 106 (7), 56-63.

3. Beach, J.W. Chemotherapeutic Agents for Human Immunodeficiency Virus Infection: Mechanism of Action, Pharmacokinetics, Metabolism, and Adverse Reactions. *Clin. Therapeu.* 1998, 20 (1), 2-25.

4. Hansen, M. *Pathophysiology: Foundations of Disease and Clinical Intervention*, W.B. Saunders Company, Philadelphia, United States, 1998.

5. Alnouti, Y.; Lewis, S.R.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zidovudine and Lamivudine from Rat Tissues by Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 2005, 19, 503–508.

6. Pereira, C.M.; Nosbisch, C.; Baughman, W.L.; Unadkat, J.D. Effect of Zidovudine on Transplacental Pharmacokinetics of ddI in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 1995, 39, 343-345.

7. Odinecs, A.; Nosbisch, C.; Unadkat, J.D. Zidovudine does not Affect Transplacental Transfer or Systemic Clearance of Stavudine (2', 3'-didehydro-3'-deoxythymidine) in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 1996, 40, 1569-1571.

8. Tuntland, T.; Nosbisch, C.; Baughman, W.L.; Massarella, J.; Unadkat, J.D.; Mechanism and Rate of Placental Transfer of Zalcitabine (2',3'-dideoxycytidine) in Macaca nemestrina. *Am. J. Obstet. Gynecol.* 1996, 174, 856-863.
9. Alnouti, Y.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zidovudine and Lamivudine from Rat Plasma, Amniotic Fluid and Tissues by HPLC. *Biomed. Chromatogr.* 2004, 18, 641–647.
10. Brown, S.D.; Bartlett, M.G.; White, C.A. Pharmacokinetics of Intravenous Acyclovir, Zidovudine, and Acyclovir-Zidovudine in Pregnant Rats. *Antimicrob. Agents Chemother.* 2003, 47(3), 991–996.
11. Snyder, L.R.; Kirkland J.J.; Glajch J.L. Practical HPLC Method Development, 2nd Ed. John Wiley & Sons, Inc., New York, NY. 1997.
12. Lai, J.; Wang J.; Cai, Z. Nucleoside Reverse Transcriptase Inhibitors and Their Phosphorylated Metabolites in Human Immunodeficiency Virus-infected Human Matrices. *Journal of Chromatography B*. 2008, 868(1-2), 1-12.
13. Chamberlain, J. The Analysis of Drugs in Biological Fluids, 2nd Ed. CRC Press, Boca Raton, FL. 1995.
14. Cunico, R.L.; Gooding, K.M.; Wehr, T. Basic HPLC and CE of Biomolecules. Bay Bioanalytical Laboratory, Inc., Richmond, CA. 1998.
15. Watson, J.T. Introduction to Mass Spectrometry, 3rd Ed. Lippincott-Raven Publishers, Philadelphia, PA. 1997.

CHAPTER 2

LITERATURE REVIEW: PLACENTAL TRANSFER OF NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS

---

[1]Meng Xu, Michael G. Bartlett, Catherine A. White. To be submitted to *Antimicrobial Agents and Chemotherapy*.

ABSTRACT

Human immunodeficiency virus type-1 (HIV) infection has increased dramatically in pregnant women, thus, exposing the fetus *in utero*. Antiviral drugs are presumed to prevent the transmission of infections from mother to fetus by decreasing maternal viral load and/or accumulation of drugs in the fetal compartment. Some nucleoside reverse transcriptase inhibitors (NRTIs), the first class of antiretroviral drugs developed, have been clinically shown to prevent vertical transmission in pregnancy. Thus, it is essential to determine the comparative placental transfer of these drugs. This paper reviews the different methods using to study the placental transfer of NRTIs.

Keywords: Zalcitabine, Stavudine, Lamivudine, Zidovudine, Didanosine, Abacavir, Emtricitibine, nucleoside reverse transcriptase inhibitors.

INTRODUCTION

In June of 1981, the Centers for Disease Control and Prevention reported unusual occurrences in which cases of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma were observed in young, homosexual men (1). All of these individuals shared a profound immunodeficiency, the hallmark of which was a depletion of CD4-positive, or T-helper, lymphocytes. Today, Acquired Immunodeficiency Syndrome (AIDS) is an epidemic that reaches far beyond what was ever predicted. 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 the 35 reporting areas (33 states, Guam, and the U.S. Virgin Islands) of the United States. (2) The numbers for the entire United States and its territories are undoubtedly much higher. (3) It also stated that for children, 90% of HIV infections are from vertical transmission from their mothers through blood, amniotic fluid, and/or breast milk. (2, 4) With these statistics, it is obvious that vertical transmission of HIV is an important problem which needs to be addressed. In early 1994, a pediatric clinical trial group (PACTG 076) investigated the ability of the reverse transcriptase inhibitor zidovudine (AZT) to reduce the rate of HIV transmission from a pregnant woman to her child. The regimen consisted of initiation of oral zidovudine between week 14 and 34 of gestation which was continued through the reminder of the pregnancy, intravenous administration of zidovudine throughout the labor and delivery process and oral administration of zidovudine to the newborn for the first six weeks post-partum. (5) This particular therapy was able to reduce the risk of transmission

by 70%. (6) With the number of HIV infections increasing, it is essential to determine the placental transfer of other antivirals because of the increased resistance of HIV to zidovudine. (7, 8) The use of these drugs is important in maintenance of maternal health through pregnancy and in possible prevention of vertical transmission of HIV. (7-11)

Over the past several years, use of multidrug therapy has become the rule rather than the exception in the treatment of patients with HIV infection. This has been propelled by the need to delay the development of drug resistance and minimize potential dose-limiting side effects. (12) Nucleoside reverse transcriptase inhibitors (NRTIs) were the first class of antiretroviral drugs developed. The active form of NRTIs is not the drug itself but its triphosphorylated (TP) metabolites, which competitively inhibit HIV reverse transcriptase following incorporation into the proviral DNA, resulting in DNA chain termination and prevention of viral replication. (13) To date, seven nucleoside reverse transcriptase inhibitors (NRTIs), abacavir (ABC), didanosine (DDI), lamivudine (3TC), stavudine (D4T), zalcitabine (DDC), zidovudine (AZT) and emtricitibine (FTC) have been approved by the U.S. Food and Drug Administration (FDA). (Figure 1) (14) Currently, several NRTIs are used in pregnancy to treat HIV infection and several studies concerning placental transfer of NRTIs have been conducted by different groups. This paper reviews the different methods people have been using to study the placental transfer of NRTIs. Since emtricitibine was the most recent antiviral to be approved by FDA very little is known about its placental transport; therefore, this paper does not discuss emtricitibine.

Clinical studies in pregnant women

The most straightforward way to study placental transfer would be to conduct the clinical studies in pregnant women. However, because of ethical considerations, *in vivo* human placental transfer studies have been restricted to single paired maternal and umbilical cord blood and amniotic fluid samples obtained at the time of delivery. Because of this limitation most studies of placental drug transfer have used the ratio between concentrations in the fetal and maternal compartments after a single dose of drug to the mother as an index of relative fetal drug exposure. (15) Mathematical models with simulations of maternal and fetal plasma level profiles have demonstrated that the maternal-fetal drug ratio depends largely on the timing of the sample with respect to the maternal dose. (15, 16)

When using the comparison of cord-to-maternal blood drug concentration ratios, the results are obtained at delivery and thus cannot be extrapolated to earlier gestational ages. (17) Moreover, cord-to-maternal blood ratios offer data at only a single point in time. The measured maternal drug concentrations depend on the time between the last maternal dose and delivery, which is usually long. (17) In this situation, the useful data generated for placental transfer using pregnant women is quite limited.

Mandelbrot et al. first conducted a clinical study of the placental transfer of 3TC in HIV- infected pregnant women in 1997. (15) The dose was 150 mg twice daily.

At a median of 8.5 hours after the last maternal oral dose of 3TC, median maternal and fetal plasma concentrations were tested. Individual maternal and fetal concentrations

were strongly correlated, and their median ratio was about 1, which showed that 3TC appeared to cross the placenta by simple diffusion. They then conducted another clinical study of placental transfer of 3TC, AZT, ABC, D4T and DDI in 100 HIV- infected pregnant women between 1999 and 2002. (17) Every woman received the drugs intravenously during labor for prevention of mother to child transmission of HIV. The dose used was 2 mg/kg of body weight intravenously over a 1-h period, followed by a continuous infusion of 1 mg/kg/h intravenously until delivery. Maternal blood samples and amniotic fluid were obtained during delivery or cesarean section, and paired cord blood samples were obtained by venipuncture immediately after delivery. A significant relationship between concentrations in maternal and cord plasma samples was found for AZT, 3TC, D4T, and DDI. For AZT, 3TC, and D4T, the ratio of the drug concentration in the fetal plasma to that in the maternal plasma was about 1, which suggests that these drugs freely crossed the placenta through a passive diffusion mechanism. For DDI, the ratio of the concentration in the fetal plasma to that in the maternal plasma was the lowest, and the author claimed that low concentrations of DDI in the maternal circulation at delivery may be largely due to once-a-day dosing. The placental transfer of ABC was also high, but no detailed data was generated from the literature. Other clinical studies all showed that the concentrations of the drug in the fetal blood were higher or equaled those found in the maternal blood, which suggests passive diffusion. (18, 19)

Placental transfer in primates

Pigtailed macaque (*Macaca nemestrina*) are sometimes chosen as a representative animal model for studying placental transfer of NRTIs primarily because they have a discoid placenta with multivillous fetal-maternal interdigitation and a hemo-monochorionic barrier similar to that found in humans. (20, 21) In addition, the macaque is an excellent model because both HIV-2 and SIV are transmitted from the mother to the fetus in this species. (22, 23) However, although the primates share the closest placental similarities to humans (20), several disadvantages make it difficult to use this species and other large, long gestation species (sheep, baboon, etc). Some of these limitations include cost, small sample size, long gestation period, and the requirement of specialized housing and experimentation (24, 25).

Unadkat et al. have studied the placental transfer of AZT, DDC, D4T and DDI using the pigtailed macaque model. (26-35) Each of the 4 drugs was tested in 3 or 4 near-term pregnant pigtailed macaques. The macaques underwent long-term catheterization at 125 to 130 days of gestation. For each dideoxynucleoside, the drug was administered as an intravenous bolus or as a 30-hour infusion to the dam via the femoral vein or to the fetus via the carotid artery. The doses used were designed to achieve maternal plasma drug concentrations within the therapeutic range. To determine whether the transfer of the dideoxynucleoside was saturable, they also infused the drug to the dam at a rate designed to achieve steady-state maternal plasma concentration within the therapeutic range and within tenfold of the therapeutic range. Blood samples were collected from the dam via

the femoral artery, from the fetus via the jugular vein, and from the amniotic fluid at regular intervals after dosing. They expressed the transplacental clearance of each dideoxynucleoside as a fraction of the transplacental clearance of antipyrine determined during the same experiments within an animal. The mechanism of *in vivo* placental transfer of drugs was elucidated by comparing the antipyrine-normalized maternal-fetal clearance of drug with the normalized fetal-maternal clearance. Active maternal-fetal transport is suggested when the normalized maternal-fetal clearance is significantly greater than normalized fetal-maternal clearance, whereas active fetal-maternal transport is suggested when normalized fetal-maternal clearance is significantly greater than normalized maternal-fetal clearance. Passive diffusion across the placenta is indicated when the 2 normalized clearances are not significantly different from each other. (26) Their findings were that the mechanism of placental transfer of AZT, D4T, DDC and DDI was passive in the *in vivo* pregnant macaque model. (26) They also studied the effects of AZT on DDI and D4T, which showed no significant difference with the profiles with DDI and D4T dosed alone. (32, 34) However, the ratio of the AZT concentration in amniotic fluid to that in the fetus after coinfusion with DDI was significantly higher than the corresponding ratio reported for AZT alone (34). The investigators hypothesized that this increase in accumulation could be related to the saturable absorption mechanism involved in the absorption of AZT, suggesting that the presence of DDI in the amniotic fluid may interfere with the reabsorption of AZT from the amniotic fluid by the fetus. Thus, the accumulation of AZT would give rise to a

higher ratio of the concentration in amniotic fluid to that in fetal plasma than that when AZT was administered alone (34).

Placental transfer in rodents

The pregnant rat model has been used successfully in the study of the placental transfer of many compounds, including nucleoside analogs (24, 36-51). Characteristics in placentation between the rat and human influence the efficiency and/or the rate of transfer of materials between the mother and embryo (52). Both the human and rodent placentas are hemochorial, in which trophoblastic cells are in direct contact with maternal blood without an intervening endothelium, and the hemodynamic changes present in the pregnant rat are similar to those seen in a human pregnancy (36, 53, 54). However, there are some structural differences between human and rat placentas. In contrast to the human placenta that contains one syncytiotrophoblast, the rat placenta consists of syncytiotrophoblast layers I and II, which are interconnected through gap junctions to facilitate the transport of nutrients, such as glucose. (52, 55) The pregnant rat model is ideal for pharmacokinetic studies because of the short gestation time and the containment of each fetus, placenta, and amniotic fluid in individual fetal sacs that allows for concurrent serial sampling of the pups. (39)

Huang et al. have studied the disposition of AZT in nearterm (day 20) pregnant rats after intravenous bolus administration of AZT at 50 mg/kg. A compartmental pharmacokinetic model was developed to describe AZT concentrations in maternal

plasma (i), placenta (ii), fetus (iii), amniotic fluid (iv), and the maternal tissue compartment (v). (38) Intercompartmental distributional clearances suggest that the mechanism of maternal-placental, placental-fetal, and fetal-amniotic fluid transfer of AZT was by passive diffusion. (38) However, Brown et al. studied the pharmacokinetics and placental transfer of acyclovir and zidovudine monotherapies and acyclovir/zidovudine combination in timed-pregnancy Sprague-Dawley rats with a dose of 60 mg of each drug/kg of body weight in monotherapy and in combination therapy by intravenous bolus administration. Significant alteration in the disposition of ACV and AZT were noticed when the two drugs were coadministered in pregnant rats. For AZT, decreases in exposure to all three tissues (amniotic fluid, placenta and fetus) were seen in the presence of ACV. Conversely, ACV showed a three-fold increase in drug exposure in amniotic fluid and fetal tissue with the combination therapy (39). The changes noted in the placenta and fetus suggest that transporters, in addition to passive diffusion, play a role in the uptake of both ACV and AZT in these tissues. Nucleoside, organic cation, and organic anion transporters could possibly contribute to the placental transfer of these two compounds. (39, 52, 56, 57)

In vitro perfusion through human placenta

There are several *in vitro* methods for studying the placental transfer of drugs, which includes the perfused placental cotyledon, villus explants and monolayer cultures, isolated trophoblast plasma membrane, and isolated transporters and receptors. (52) In

particular, the perfusion through human placenta has been used in studying the placental transfer of NRTIs. (26, 58-65) In those experiments, 3 or 4 term placentas were obtained from healthy women who had uncomplicated pregnancies. Immediately after delivery, a distinct lobe of the placenta was perfused by means of cannulas placed in the maternal intervillous space and in a fetal chorionic artery and vein. The unbound steady-state drug concentrations in samples taken from the inward-flowing and outward-flowing perfusates were used to determine the maternal-fetal and the fetal-maternal transplacental clearances. The antipyrine-normalized transplacental clearance, either maternal-fetal or fetal-maternal, often referred to in the literature as the clearance index (Ci), is indicative of the rate of placental transfer. (26)

Several studies with DDC, D4T, AZT, 3TC, D4T and ABC were conducted using this perfused human placenta model. These studies indicated there were no significant changes in the Ci when DDI, DDC, D4T, and 3TC were used in combination with therapeutic and 10x concentrations of AZT. When endogenous bases and the equilibrative nucleoside transporter 1 (ENT1) inhibitor dipyridamole were added to placental perfusion studies with these anti-HIV nucleoside inhibitors, there were no changes in the Ci, suggesting no transport system and no adverse effects of the combination. Thus, the compounds cross from the maternal to the fetal compartment by simple diffusion. (64) Studies with ABC showed that abacavir is the first nucleoside inhibitor studied with a high Ci of about 50% that of antipyrine. This suggests that

abacavir is highly lipophilic and is readily transferred across the human placenta by simple diffusion. (65)

DISCUSSION

Most of the data generated from pregnant women, *in vivo* studies from primates and the *in vitro* perfusion model suggests the placental transfer of the NRTIs is by simple diffusion. However, some problems exist in all of these studies. For *in vitro* studies, the biggest disadvantage is that the *in vitro* perfused human placenta model lacks the fetus, a potential site of elimination of drugs, thus it is widely believed not to be informative regarding the extent of *in vivo* transfer of drugs across the placenta. (26) For human studies, the data is quite limited since pregnant women are usually excluded from the pharmacokinetic studies for fear of toxic effects of the drugs on fetuses. (26) What's more, the data collected from the human studies are mostly from one time sampling during delivery, thus are not informative in studying the placental transfer of these drugs during pregnancy. The pigtailed macaque is a good animal model since it has a discoid placenta with multivillous fetal-maternal interdigitation and a hemo-monochorionic barrier similar to that found in humans.

In addition, both HIV-2 and the simian immunodeficiency virus can be transmitted from the mother to the fetus in this animal species. (26) However, some problems are related with this animal model too. First, in several studies, the fetal levels and the amniotic fluid drug levels were only collected at the time of delivery. Second, mostly, only steady-state

drug concentrations were monitored in these studies. Lastly, only a few data points were taken in these studies, and there is a lack of full pharmacokinetic profiles in both the maternal and the fetal compartments. The pregnant rat model is good for pharmacokinetic studies because of the short gestation time and the containment of each fetus, placenta, and amniotic fluid in individual fetal sacs that allows for concurrent serial sampling of the pups. Thus, a full pharmacokinetic profile can be generated for both the maternal and the fetal compartments. The data generated from the pregnant rat model shows interactions between the NRTIs which suggests active transport may play a role in the placental transfer of these drugs. However, more studies are needed to further support this hypothesis.

CONCLUSION

This paper reviews the placental transfer of NRTIs using both *in vivo* and *in vitro* methods. Although passive diffusion is suggested by many of the studies, there is building evidence that active transport processes are involved.

REFERENCES

1. **CDC**. 1993. Pregnancy Outcomes Following Systemic Prenatal Acyclovir Exposure -- June 1, 1984-June 30, 1993. MMWR. **42**(41):806-809.
2. **CDC**. 2005. HIV/AIDS Surveillance Report: Cases of HIV Infection and AIDS in the United States, 2004. Atlanta: The Centers; 2005. Vol.16. (Available at

http://www.cdc.gov/hiv/topics/surveillance/resources/reports/2004report/default.htm.)

3. **Cibulka, N.J.** 2006. Mother-to-Child Transmission of HIV in the United States. Am. J. Nurs. **106** (7), 56-63.
4. **Hansen, M.** 1998. Pathophysiology: Foundations of Disease and Clinical Intervention, W.B. Saunders Company, Philadelphia, United States.
5. **Sperling, R.S., D.E. Shapiro, R.W. Coombs, J.A. Todd, S.A. Herman, G. McSherry, M.H. O'Sullivan, R.B. Wan Dyke, E. Jimenez; C. Rouzioux, P.M. Flynn, J.L. Sullivan. 1996.** Maternal Viral Load, Zidovudine Treatment, and the Risk of Transmission of Human Immunodeficiency Virus Type 1 from Mother to Infant. N Engl J Med **335**: 1621-1629.
6. **Wilfert, C.M.** 1996. Beginning to Make Progress against HIV. N Engl J Med **335**:1678-1680.
7. **Pacifici, G. M.** 2005. Transfer of Antivirals across the Human Placenta. Early Human Development. **81**: 647-654.
8. Recommendations for Use of Antiretroviral Drugs in Pregnant HIV-1-infected Women for Maternal Health and Interventions to Reduce Perinatal HIV-1 Transmission in the United States. December 17, 1994. From the internet: http://aidsinfo.nih.gov.

9. **Fiscus, S.A., A.A. Adimora, M.L. Funk, V.J. Schoenbach, D. Tristram, W. Lim, et al.** 2002. Trends in Interventions to Reduce Perinatal Human Immunodeficiency Virus Type 1 Transmission in North Carolina. Pediatr Infect Dis J. **21**:664–668.

10. **Lallemant, M., G. Jourdain , S. Le Coeur, S. Kim, S. Koetsawang, A.M. Comeau, et al.** 2000. A Trial of Shortened Zidovudine Regimens to Prevent Mother-to-child transmission of Human Immunodeficiency Virus type 1. Perinatal HIV Prevention Trial (Thailand) Investigators. N Engl J Med. **343**:982– 91.

11. **Tuomala, R.E., D.E. Shapiro, L.M. Mofenson, Y. Bryson, M. Culnane, M.D. Hughes, et al.** 2002. Antiretroviral Therapy during Pregnancy and the Risk of an Adverse Outcome. N Engl J Med. **346**:1863– 70.

12. **Beach, J.W.** 1998. Chemotherapeutic Agents for Human Immunodeficiency Virus Infection: Mechanism of Action, Pharmacokinetics, Metabolism, and Adverse Reactions. Clin. Ther. **20** (1):2-21.

13. **Rodman, J.H., B. Robbins, P.M. Flynn, A. Fridland.** 1996. A Systemic and Cellular Model for Zidovudine Plasma Concentrations and Intracellular Phosphorylation in Patients. J Infect Dis. **174**(3):490-499.

14. **Lai, J., J. Wang, Z. Cai.** 2008. Nucleoside Reverse Transcriptase Inhibitors and their Phosphorylated Metabolites in Human Immunodeficiency Virus-infected Human Matrices. J. of Chromatogr. B. **868**(1-2): 1-12

15. **Mandelbrot, L., G. Peytavin, G. Firtion, R. Farinotti.** Maternal-Fetal Transfer and Amniotic fluid Accumulation of Lamivudine in Human Immunodeficiency Virus-Infected Pregnant Women. 2001. Am J Obstet Gynecol. **184**:153-158.

16. **Anderson, D.F., T.M. Phernetton, J.H. Rankin.** 1980. Prediction of Fetal Drug Concentrations. Am J Obstet Gynecol. **137**:735-8.

17. **Chappuy, H., J.M. Tréluyer, V. Jullien, J. Dimet, E. Rey, M. Fouché, G. Firtion, G. Pons, L. Mandelbrot.** 2004. Maternal-Fetal Transfer and Amniotic Fluid Accumulation of Nucleoside Analogue Reverse Transcriptase Inhibitors in Human Immunodeficiency Virus-Infected Pregnant Women. Antimicrob Agents Chemother. **48**(11): 4332-4336.

18. **Gillet, J.Y., A. Bongain, D. Abrar, R. Garraffo, P. Lapalus.** 1990. Preliminary Study on the Transport of AZT (Retrovir-zidovudine) Through the Placenta. J Gynecol Obstet Biol Reprod (Paris). **19**(2):177-80.

19. **Siu, S.S., j.H. Yeung, M.W. Pang, P.Y. Chiu, T.K. Lau.** 2005. Placental Transfer of Zidovudine in First Trimester of Pregnancy. Obstet Gynecol. **106**(4):824-827.

20. The European Collaborative Study. 1999. AIDS. **13**:1377-1385.

21. **King, B.F.** 1986. Morphology of the Placenta and Fetal membranes, in Comparative Primate Biology: Reproduction and Development. pp. 311–331, Alan R. Liss Inc., New York.

22. **Ho, R.J.Y., M.B. Agy, W.R. Morton, M. Scheibel, J. McClure, A. Watson, S.L. Hu, C. Nosbisch, N. Dorofeeva, J.D. Unadkat JD.** 1996. Development of a

Chronically Catheterized Maternal-Fetal Macaque Model to Study *in utero* Mother-to-fetus HIV Transmission: A preliminary report. J Med Primatol. **25:**218–224.

23. **Ochs, H.D., W.R. Morton, C.C. Tsai, M.E. Thouless, Q. Zhu, L.D. Kuller, Y.P. Wu, R.E. Benveniste.** 1991. Maternal-Fetal Transmission of SIV in Macaques: Disseminated Adenovirus Infection in an Offspring with Congenital SIV Infection. J Med Primatol**. 20:**193–200.
24. **Ibrahim, S.S., F.D. Boudinot.** 1989. Pharmacokinetics of 2',3'-Dideoxycytidine in Rats: Application to Interspecies Scale-up. J Pharm Pharmacol. **41**:829-834.
25. **Samtani, M.N., M. Schwab, P.W. Nathanielsz, W.J. Jusko.** Area/Moment and Compartmental Modeling of Pharmacokinetics during Pregnancy: Applications to Maternal/Fetal Exposures to Corticosteroids in Sheep and Rats. 2004. Pharm. Res. **21**:2279-2291.
26. **Tuntland, T., A. Odinecs, C.M. Pereira, C. Nosbisch, J.D. Unadkat.** 1999. In Vitro Models to Predict the *in vivo* Mechanism, Rate, and Extent of Placental Transfer of Dideoxynucleoside Drugs Against Human Immunodeficiency Virus. Am J Obstet Gynecol. **180**:198-206.
27. **Tuntland, T., A. Odinecs, C. Nosbisch, J.D. Unadkat.** 1998. *In vivo* Maternal–Fetal–Amniotic Fluid Pharmacokinetics of Zidovudine (AZT) in the Pigtailed Macaque: Comparison of Steady-State and Single Dose Regimens. J Pharmacol Exp Ther. **258**:54-62.

28. Tuntland, T., C. Nosbisch, W.L. Baughman, J.W. Massarella, J.D. Unadkat. 1996. Mechanism and Rate of Placental Transfer of Zalcitabine (2′, 3′-dideoxycytidine) in *Macaca nemestrina*. Am J Obstet Gynecol. **174**:856-63.

29. Tuntland, T., C. Nosbisch, J.D. Unadkat. 1997. Effect of Pregnancy, Mode of Administration and Neonatal Age on the Pharmacokinetics of Zalcitabine (2′,3′-dideoxycytidine) in the Pigtailed macaque (*Macaca nemestrina*). J Antimicrob Chemother. **40**:687-93.

30. Odinecs, A., C. Nosbisch, R.D. Keller, W.L. Baughman, J.D. Unadkat. 1996. *In vivo* Maternal-Fetal Pharmacokinetics of Stavudine (2′,3′-Didehydro- 3′-Deoxythymidine) in Pigtailed Macaques (*Macaca nemestrina*). Antimicrob Agents Chemother. **40**:196-202.

31. Odinecs, A., C. Pereira, C. Nosbisch, J.D. Unadkat. 1996. Prenatal and Postpartum Pharmacokinetics of Stavudine (2′,3′-Didehydro-3′- Deoxythymidine) and Didanosine (dideoxyinosine) in Pigtailed Macaques (*Macaca nemestrina*). Antimicrob Agents Chemother. **40**:2423-5.

32. Odinecs, A., C. Nosbisch, J.D. Unadkat. 1996. Zidovudine Does Not Affect Transplacental Transfer or Systemic Clearance of Stavudine (2′,3′- didehydro-3′-deoxythymidine) in the Pigtailed Macaque (*Macaca nemestrina*). Antimicrob Agents Chemother. **40**:1569-71.

33. Pereira, C.M., C. Nosbisch, H.R. Winter, W.L. Baughman, J.D. Unadkat. 1994. Transplacental Pharmacokinetics of Dideoxyinosine in Pigtailed Macaques. Antimicrob Agents Chemother. **38**:781-6.

34. Pereira, C.M., C. Nosbisch, W.L. Baughman, J.D. Unadkat. 1995. Effect of Zidovudine on Transplacental Pharmacokinetics of Dideoxyinosine in the Pigtailed Macaque *(Macaca nemestrina)*. Antimicrob Agents Chemother. **39**:343-5.

35. Lopez-Anaya, A., J.D. Unadkat, L.A. Schumann, A.L. Smith. 1990. Pharmacokinetics of Zidovudine (azidothymidine). I. Transplacental Transfer. J Acquir Immune Defic Syndr. **3**:959-964.

36. Boike, G. M., G. Deppe, J. D. Young, N. L. Gove, S. F. Bottoms, J. M. Malone, Jr., V. K. Malviya, and R. J. Sokol. 1989. Chemotherapy in a Pregnant Rat Model. 1. Mitomycin-C: Pregnancy-Specific Kinetics and Placental Transfer. Gynecol. Oncol. **34:**187–190.

37. Boike, G. M., G. Deppe, J. D. Young, J. M. Malone, Jr., V. K. Malviya, and R. J. Sokol. 1989. Chemotherapy in a Pregnant Rat Model. 2. 5-Fluorouracil: Nonlinear Kinetics and Placental Transfer. Gynecol. Oncol. **34:**191–194.

38. Huang, C. S.-H., F. D. Boudinot, and S. Feldman. 1996. Maternal-Fetal Pharmacokinetics of Zidovudine in Rats. J. Pharm. Sci. **85:**965–970.

39. Brown, S.D., M.G. Bartlett, C.A. White. 2003. Pharmacokinetics of Intravenous Acyclovir, Zidovudine, and Acyclovir-Zidovudine in Pregnant Rats. Antimicrob Agents Chemother. **47**(3):991-996.

40. **Brown, S.D., C.A. White, M.G. Bartlett.** 2002. Hydrophilic Interaction Liquid Chromatography/Electrospray Mass Spectrometry Determination of Acyclovir in Pregnant Rat Plasma and Tissues. Rapid Commun Mass Spectrom. **16**(19):1871-6.

41. **Brown, S.D., C.A. White, C.K. Chu, M.G. Bartlett.** 2002. Determination of Acyclovir in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography. J Chromatogr B. **772**(2):327-334.

42. **Xu, M, C.A. White, and M.G. Bartlett.** 2008. Simultaneous Determination of Zalcitabine and Stavudine in Rat Plasma, Amniotic Fluid, Placental and Fetal Tissues using High Performance Liquid Chromatography. J. Liq. Chromatogr. **31**: 482–496.

43. **Lewis, S.R., C.A. White, and M.G. Bartlett.** 2007. Simultaneous Determination of Abacavir and Zidovudine from Rat Tissues using HPLC with Ultraviolet Detection. J Chromatogr B. **850**(1-2):45-52.

44. **Ding, Y., L.N. Williamson LN, C.A. White, and M.G. Bartlett.** 2004. Determination of 2',3'-Dideoxycytidine in Maternal Plasma, Amniotic Fluid, Placental and Fetal Tissues by High-Performance Liquid Chromatography. J Chromatogr B. **811**(2):183-189.

45. **Alnouti, Y., S.R. Lewis, C.A. White, M.G. Bartlett.** 2005. Simultaneous Determination of Zidovudine and Lamivudine from Rat Tissues by Liquid Chromatography/Tandem Mass Spectrometry. Rapid Commun Mass Spectrom. **19**(4):503-508.

46. **Alnouti, Y., C.A. White, M.G. Bartlett.** 2004. Simultaneous Quantitation of Zidovudine and Zidovudine Monophosphate from Plasma, Amniotic Fluid and Tissues by Micellar Capillary Electrophoresis. Biomed Chromatogr. **18**(8):523-531.

47. **Alnouti, Y., C.A. White, M.G. Bartlett.** 2004. Simultaneous Determination of Zidovudine and Lamivudine from Rat Plasma, Amniotic Fluid and Tissues by HPLC. Biomed Chromatogr. **18**(9):641-647.

48. **Alnouti, Y., C.A. White, M.G. Bartlett.** 2004. Determination of Lamivudine in Plasma, Amniotic Fluid, and Rat Tissues by Liquid Chromatography. J Chromatogr B. **803**(2):279-84.

49. **Clark, T. N., C. A. White, and M. G. Bartlett.** 2006. Determination of Didanosine in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography-Tandem Mass Spectrometry. Biomed Chromatogr. **20**(6-7):605-611.

50. **Clark, T. N., C. A. White, C. K. Chu, and M. G. Bartlett.** 2001. Determination of 3'-Azido-2', 3'-Dideoxyuridine in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography. J. Chromatogr. B **755:**165–172.

51. **Clark, T. N., C. A. White, and M. G. Bartlett.** 2004. Determination of Abacavir in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by a Polarity Switching Liquid Chromatography/Tandem Mass Spectrometry Method. Rapid Commun Mass Spectrom. **18**(4):405-11.

52. **Takeda, M., S. Khamdang, S. Narikawa, H. Kimura, Y. Kobayashi, T. Yamamoto, S. Cha, T. Sekine, and H. Endou.** 2002. Human Organic Anion Transporters and Human Organic Cation Ttransporters Mediate Renal Antiviral Transport. J Pharm Exper Ther. **300**:918-924.

53. **Faber, J. J., and K. L. Thornburg.** 1983. Placental physiology: Structure and Function of Fetomaternal Exchange. Raven, New York, N.Y.

54. **Leazer, T.M., and C.D. Klaasen.** 2003. The Presence of Xenobiotic Transporters in Rat Placenta. Drug Metabolism and Disposition. **31**:153-167.

55. **Mills, J.L.** 1995. Protecting the Embryo from X-Rated Drugs. N Engl J Med. **333**(2):124-5.

56. **Ganapathy, V., P. D. Prasad, M. E. Ganapathy, and F. H. Leibach.** 2000. Placental Transporters Relevant to Drug Distribution across the Maternal-Fetal Interface. J. Pharmacol. Exp. Ther. **294:**413–420.

57. **Wada, S., M. Tsuda, T. Sekine, S. H. Cha, M. Kimura, Y. Kanai, and H. Endou.** 2000. Rat Multispecific Organic Anion Transporter 1(rOAT1) Transports Zidovudine:Acyclovir, and Other Antiviral Nucleoside Analogs. J. Pharmacol. Exp. Ther. **294:**844–849.

58. **Liebes, L., S. Mendoza, D. Wilson, J. Dancis.** 1990. Transfer of Zidovudine (AZT) by Human Placenta. J Infect Dis. **161**:203-207.

59. Schenker, S., R.F. Johnson, T.S. King, R.S. Schenken, G.I. Henderson. 1990. Azidothymidine (zidovudine) Transport by the Human Placenta. Am J Med Sci. **299**:16-20.

60. Bawdon, R.E., S. Sobhi, J. Dax. 1992. The Transfer of Anti-Human Immuno-Deficiency Virus Nucleoside Compounds by the Term Human Placenta. Am J Obstet Gynecol. **167**:1570-1574.

61. Bawdon, R.E., S. Kaul, S. Sobhi. 1994. The Ex Vivo Transfer of the Anti- HIV Nucleoside Compound D4T in the Human Placenta. Gynecol Obstet Invest. **38**:1-4.

62. Dancis, J., J.D. Lee, S. Mendoza, S. Liebes. 1993. Transfer and Metabolism of Dideoxyinosine by the Perfused Placenta. J Acquir Immune Defic Syndr. **6**:2-6.

63. Henderson, G.I., A.B. Perez, Y. Yang, R.L. Hamby, R.S. Schenken, S. Schenker. 1994. Transfer of Dideoxyinosine across the Human Isolated Placenta. Br J Clin Pharmacol. **38**:237-42.

64. Bawdon, R.E. 1997. Ex Vivo Human Placental Transfer of Anti-Human Immunodeficiency Virus Compounds. Infect. Dis. Obstet. Gynecol. **5**:310-315.

65. Bawdon, R.E. 1997. The Ex Vivo Human Placental Transfer of the Anti-HIV Nucleoside Inhibitor Abacavir and the Protease Inhibitor Amprenavir. Infect. Dis. Obstet. Gynecol. **6**:244-246.

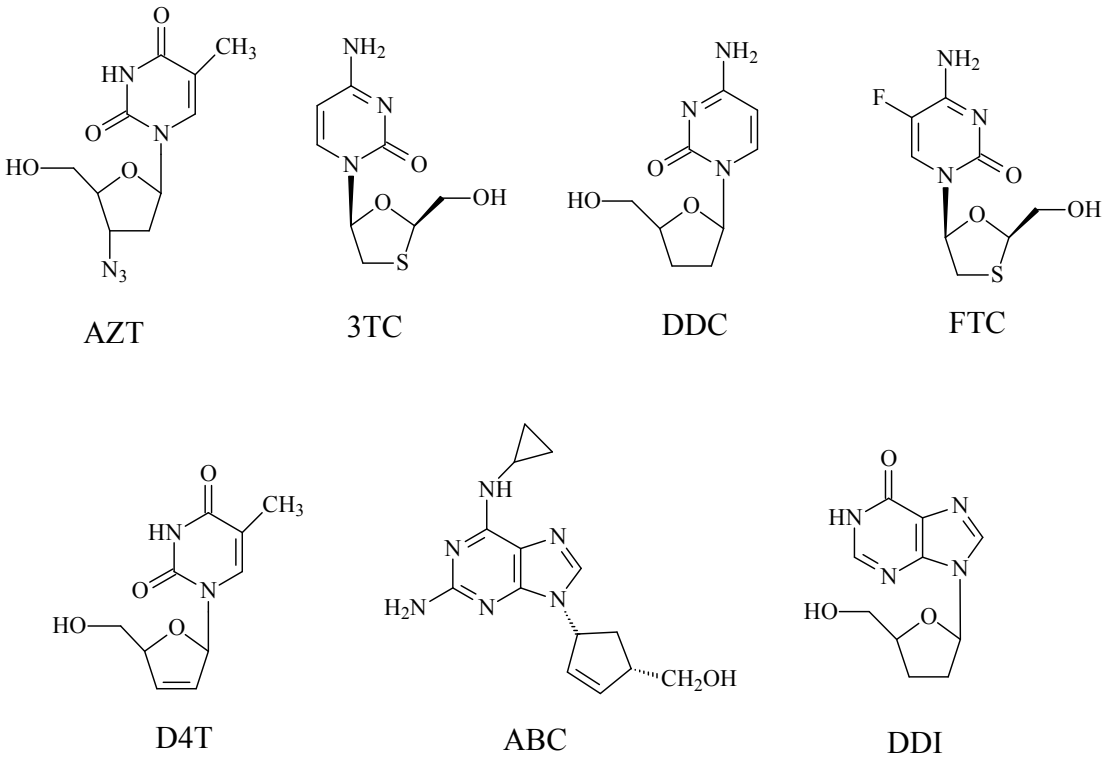


Figure 2.1 Chemical structures of the NRTIs

CHAPTER 3

SIMULTANEOUS DETERMINATION OF ZALCITABINE AND STAVUDINE IN MATERNAL PLASMA, AMNIOTIC FLUID, PLACENTAL AND FETAL TISSUES USING REVERSED PHASE ON SILICA LIQUID CHROMATOGRAPHY[1]

---

[1]Meng Xu, Catherine A. White, Michael G. Bartlett. 2008. *Journal of Liquid Chromatography & Related Techniques*. 31: 482–496. Reprinted here with permission of publisher.

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 (4.6mm × 100mm) equipped with a Phenomenex guard column. The mobile phase consisted of 3% methanol in 22mM formic acid. The flow rate was 0.5 ml/min, and the detection wavelength was optimized at 265nm. The calibration curves for each day of validation showed good linear response over the range from 0.1μg/ml to 50μg/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

1. 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 multidrug 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.

2. Experimental

2.1. 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).

2.2. 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 (4.6×3.0mm, Milford, MA) equipped with a Phenomenex security guard C-18 guard column (4×3.0mm, Torrance, CA).

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

2.3. 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 250.0μg/ml were prepared by serial dilution with deionized water. Precision and accuracy standards with concentrations of 1.0, 10 and 200.0μg/ml were also prepared in the same manner. A 10μg/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.

2.4. 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 20μl 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 10μl of each DDC and D4T standard solution and 10 μl of the 10μg/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 1μg/ml for all samples.

2.5. 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 matrics were 0.1, 0.2, 2 and 40 μg/ml.

2.6. Sample preparation

All the samples were prepared by the 'salting out' technique. 200μl of a saturated ammonium sulfate solution and 1ml 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.

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^{o}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 359g, 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 0.1N NaOH in physiological saline (pH 7.4) via the jugular cannula. Blood samples were collected at 5, 15, 30, 45, 60, 90, 120, 180 and 225min 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 225min. 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).

3. Results and Discussion

3.1. Method development

The chemical structures of DDC, D4T and the internal standard used in this assay, 3TC, are shown in Fig. 1. Separation of DDC, D4T and 3TC from interfering matrix peaks was explored using different kind 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 were unsuccessful. Several reverse phase column such as C18 and C8 columns were tried but these phases do not retain the analytes well, especially DDC. HILIC (Hydrophilic interaction 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. Fig. 2-5 show chromatograms of each extracted blank matrix and extracted matrix spiked with DDC (2μg/ml) and 3TC (1.5μg/ml).

3.2. 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 0.1–50μg/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.

3.3. 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 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 0.1μg/ml were used for these 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.

3.4. 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 40.0μg/ml levels, and the 3TC sample with a concentration of 1μg/ml were extracted and analyzed (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.

3.5. Stability studies

Stability testing was performed for DDC and D4T at 2μg/ml concentration level. 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 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 4. 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 2μg/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 24h, 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.

3.6. 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 samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 1μg/ml of the internal standard 3TC. Fig. 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 106.2min, 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 97.2min, a steady state volume of

distribution of 0.69l/kg, and total clearance of 0.28l/h/kg based on the maternal plasma data.

Animal studies with an IV bolus administration of a dose of 25mg/kg of DDC alone were conducted for comparison to the DDC-D4T combination dose. When administered alone, DDC has a half-life of 120min, a steady state volume of distribution of 1.90l/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 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 fetus, it was 0.6 in the single dose vs. 0.3 in the combination; for 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.

4. 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.

References

1. Centers for Disease Control and Prevention. HIV/AIDS surveillance report: cases of HIV infection and AIDS in the United States, 2004. Atlanta: The Centers; 2005. Vol. 16. (Available at http://www.cdc.gov/hiv/topics/surveillance/resources/reports/2004report/default.htm.)

2. Cibulka, N.J. Mother-to-Child Transmission of HIV in the United States. *Am. J. Nurs.* 2006, 106 (7), 56-63.

3. Hansen, M. *Pathophysiology: Foundations of Disease and Clinical Intervention*, W.B. Saunders Company, Philadelphia, United States, 1998.

4. Beach, J.W. Chemotherapeutic Agents for Human Immunodeficiency Virus Infection: Mechanism of Action, Pharmacokinetics, Metabolism, and Adverse Reactions. *Clin. Therapeu.* 1998, 20 (1), 2-25.

5. Alnouti, Y.; Lewis, S.R.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zidovudine and Lamivudine from Rat Tissues by Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 2005, 19, 503–508.

6. Pereira, C.M.; Nosbisch, C.; Baughman, W.L.; Unadkat, J.D. Effect of Zidovudine on Transplacental Pharmacokinetics of ddI in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 1995, 39, 343-345.

7. Odinecs, A.; Nosbisch, C.; Unadkat, J.D. Zidovudine does not Affect Transplacental Transfer or Systemic Clearance of Stavudine (2', 3'-didehydro-3'-deoxythymidine) in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 1996, 40, 1569-1571.

8. Tuntland, T.; Nosbisch, C.; Baughman, W.L.; Massarella, J.; Unadkat, J.D.; Mechanism and Rate of Placental Transfer of Zalcitabine (2',3'-dideoxycytidine) in Macaca nemestrina. *Am. J. Obstet. Gynecol.* 1996, 174, 856-863.

9. Alnouti, Y.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zidovudine and Lamivudine from Rat Plasma, Amniotic Fluid and Tissues by HPLC. *Biomed. Chromatogr.* 2004, 18, 641–647.

10. Brown, S.D.; Bartlett, M.G.; White, C.A. Pharmacokinetics of Intravenous Acyclovir, Zidovudine, and Acyclovir-Zidovudine in Pregnant Rats. *Antimicrob. Agents Chemother.* 2003, 47(3), 991–996.

11. Children with AIDS, U.S. Food and Drug Administration, 1990 (available at http://www.fda.gov/bbs/topics/CONSUMER/CON00038.html).

12. Simonds, R.J.; Steketee, R.; Nesheim, S.; Matheson, P.; Palumbo, P.; Alger, L.; Abrams, E.J.; Orloff, S.; Lindsay, M.; Bardeguez, A.D.; Vink, P.; Byers, R.; Rogers, M. Impact of Zidovudine Use on Risk and Risk Factors for Perinatal Transmission of HIV. *AIDS.* 1998, 12 (3), 301-308.

13. Mitsuya, H.; Broder, S. Inhibition of the in Vitro Infectivity and Cytopathic Effect of Human T-Lymphotrophic Virus type III/Lymphadenopathy-Associated Virus (HTLV-

III/LAV) by 2', 3'-Dideoxynucleosides. *Proc. Natl. Acad. Sci. U.S.A.* 1986, 83 (6), 1911-1915.

14. Ferrua, B.; Tran, T.T.; Quaranta, J.F.; Kubar, J.; Roptin, C.; Condom, R.; Durant, J.; Guedj, R. Measurement of the Anti-HIV Agent 2′,3′-Didehydro-2′,3′-Dideoxythymidine (D4T) by Competitive ELISA. *J. Immunolog. Meth.* 1994, 176(1), 103-110.

15. Ding, Y.; Williamson, L.N.; White, C.A.; Bartlett, M.G. Determination of 2', 3'-Dideoxycytidine in Maternal Plasma, Amniotic Fluid, Placental and Fetal Tissues by High-Performance Liquid Chromatography. *J. Chromatogr. B.* 2004, 811, 183–189.

16. Contreras, J.; Gonzalez, H.M.; Menendez, R.; Lopez, M. Development and Validation of a Reversed-Phase Liquid Chromatographic Method for Analysis of D4T (Stavudine) in Rat Plasma. *J. Chromatogr. B.* 2004, 801(2), 199-203.

17. Fan, B.; Bartlett, M.G.; Stewart, J.T. Determination of Lamivudine/Stavudine/ Efavirenz in Human Serum Using Liquid Chromatography/Electrospray Tandem Mass Spectrometry with Ionization Polarity Switching. *Biomed. Chromatogr.* 2002, 16(6), 383-389.

18. Frijus-Plessen, N.; Michaelis, H.C.; Foth, H.; Kahl, G.F. Determination of 3'-Azido-3'-Deoxythymidine, 2',3'-Dideoxycytidine, 3'-Fluoro-3'-Deoxythymidine and 2',3'-Dideoxyinosine in Biological Samples by High-Performance Liquid Chromatography. *J. Chromatogr.* 1990, 534, 101-107.

19. Bezy, V.; Morin, P.; Couerbe, P.; Leleu, G.; Agrofoglio, L. Simultaneous Analysis of Several Antiretroviral Nucleosides in Rat Plasma by High Performance Liquid Chromatography with UV Using Acetic Acid / Hydroxylamine Buffer: Test of this New Volatile Medium-pH for HPLC-ESI-MS/MS. *J. Chromatogr. B.* 2005, 821(2), 132-143.

20. Kapoor, N.; Khandavilli, S.; Panchagnula, R. Simultaneous Determination of Lamivudine and Stavudine in Antiretroviral Fixed Dose Combinations by First Derivative Spectrophotometry and High Performance Liquid Chromatography. *J. Pharmaceut. Biomed. Anal.* 2006, 41(3), 761-765.

21. Huang, Y.; Zurlinden, E.; Lin, E.; Li, X.; Tokumoto, J.; Golden, J.; Murr, A.; Engstrom, J.; Conte, J. Liquid Chromatographic-Tandem Mass Spectrometric Assay for the Simultaneous Determination of Didanosine and Stavudine in Human Plasma, Bronchoalveolar Lavage Fluid, Alveolar Cells, Peripheral Blood Mononuclear Cells, Seminal Plasma, Cerebrospinal Fluid and Tonsil Tissue. *J. Chromatogr. B.* 2004, 799(1), 51-61.

22. Wong, S.L.; Sawchuk, R.J. High-Performance Liquid Chromatographic Determination of 2',3'-Didehydro-3'-Deoxythymidine (d4T) in Human and Rabbit Plasma and Urine and its Application to Pharmacokinetic Studies in the Rabbit. *Pharm. Res.* 1991, 8(5), 619-623.

Table 3.1 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 3.2 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

Concentration DDC added	Intra-day (n=5)			Inter-day (n=15)		
(μg/ml)	Concentration DDC found (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

Table 3.3 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

Concentration D4T added	Intra-day (n=5)			Inter-day (n=15)		
(μg/ml)	Concentration D4T found (μg/ml)	R.S.D. (%)	Error (%)	Concentration DDC 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	40.43±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

Table 3.4 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 (μ g/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 (μ g/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 (μ g/ml)				
1.0	87.05±2.41	78.28±2.21	78.65±2.23	78.89±2.28

Table 3.5 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 μ g/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 μ g/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

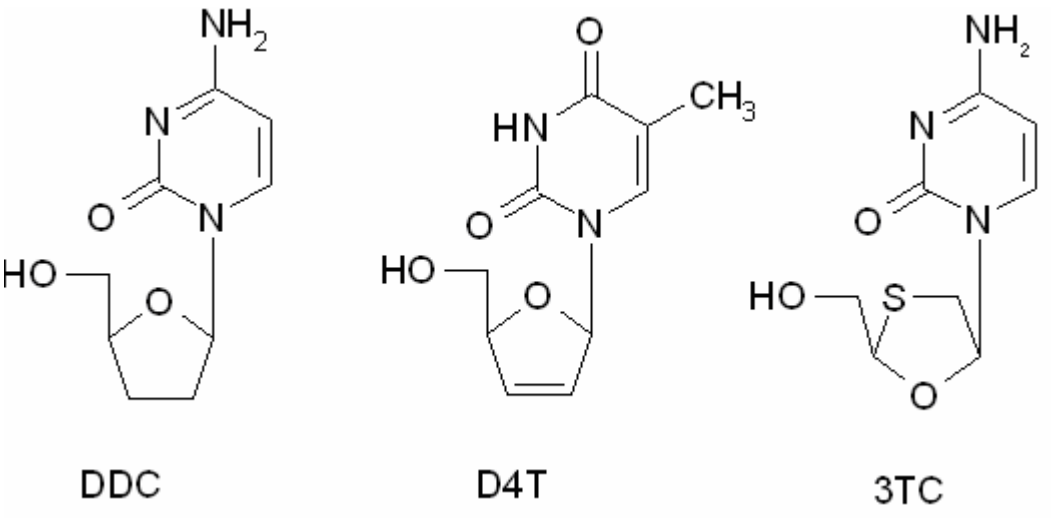


Fig. 3.1 Chemical structures of DDC, D4T and 3TC

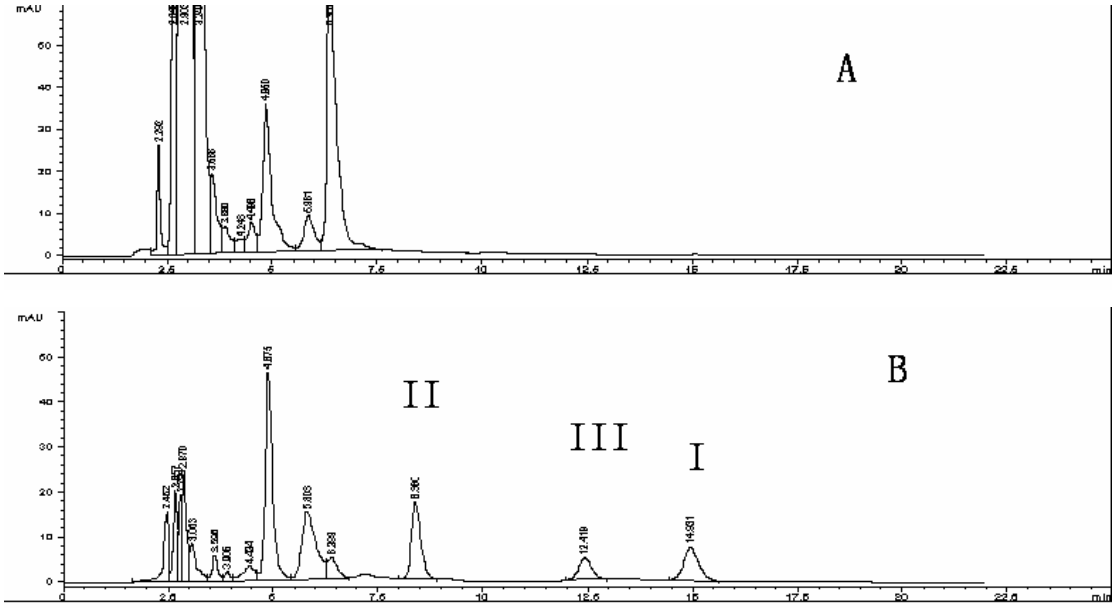


Fig.3.2 Chromatograms obtained from blank plasma (A) and plasma spiked with DDC (I, 2μg/ml), D4T (II, 2μg/ml) and 3TC (III, 1μg/ml)

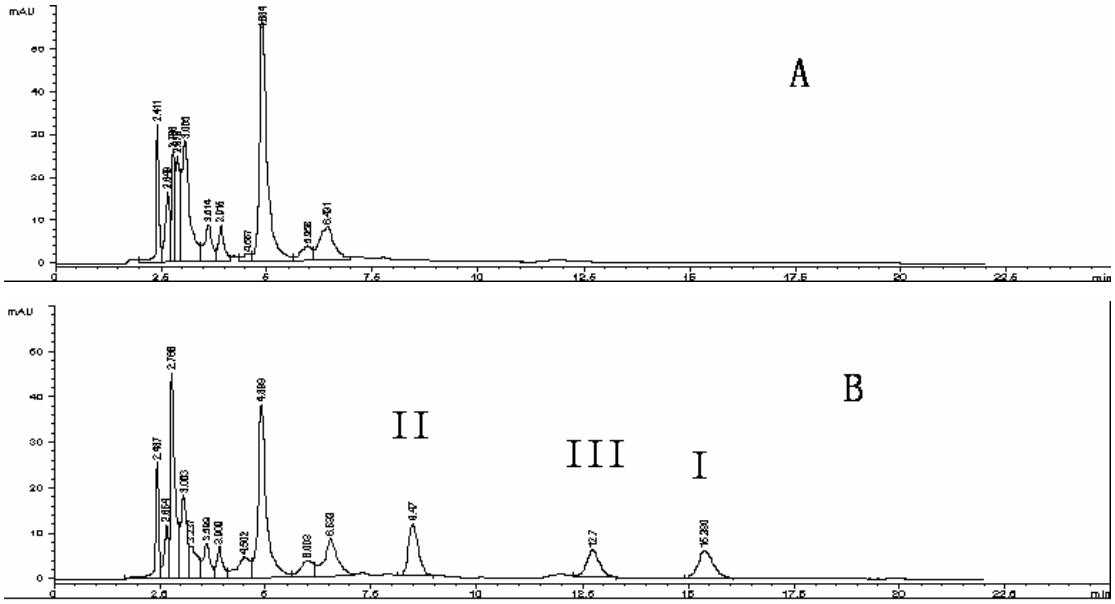


Fig.3.3 Chromatograms obtained from blank amniotic fluid (A) and amniotic fluid spiked with DDC (I, 2μg/ml), D4T (II, 2μg/ml) and 3TC (III, 1μg/ml)

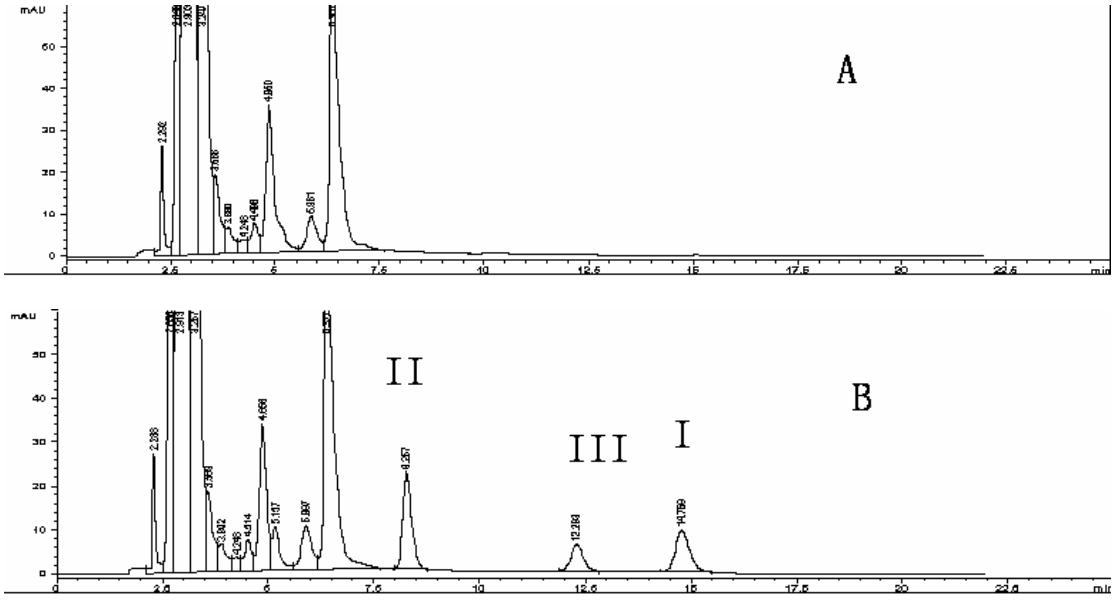


Fig.3.4 Chromatograms obtained from blank placental homogenate (A) and placental homogenate spiked with DDC (I, 2μg/ml), D4T (II, 2μg/ml) and 3TC (III, 1μg/ml)

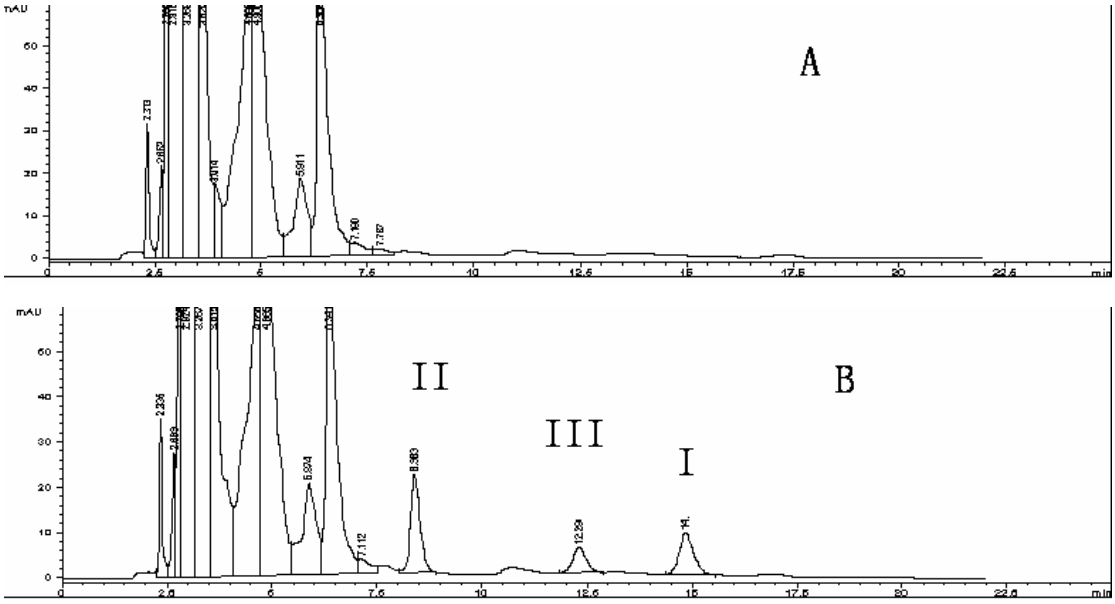
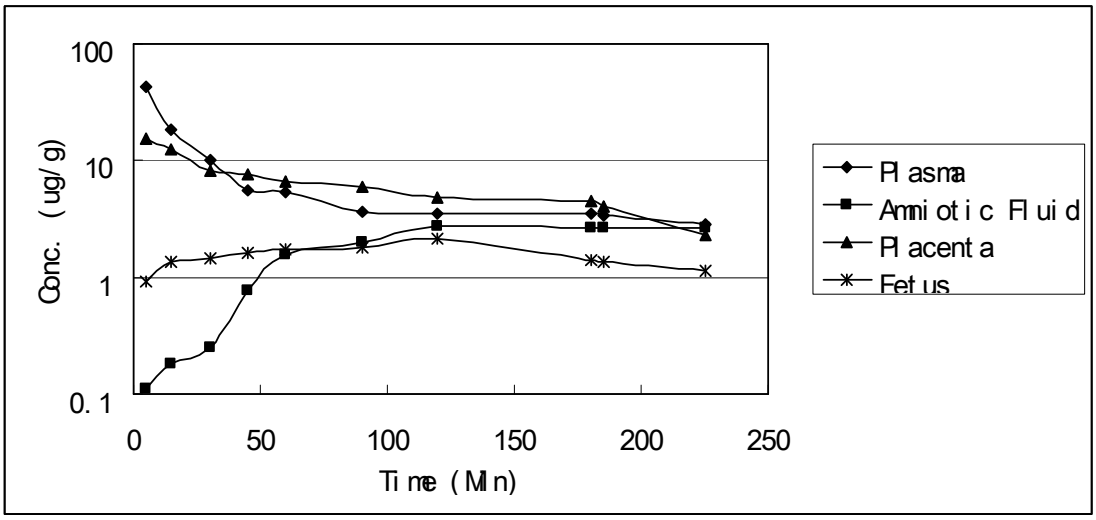


Fig.3.5 Chromatograms obtained from blank fetal homogenate (A) and fetal homogenate spiked with DDC (I, 2μg/ml), D4T (II, 2μg/ml) and 3TC (III, 1μg/ml)

DDC



D4T

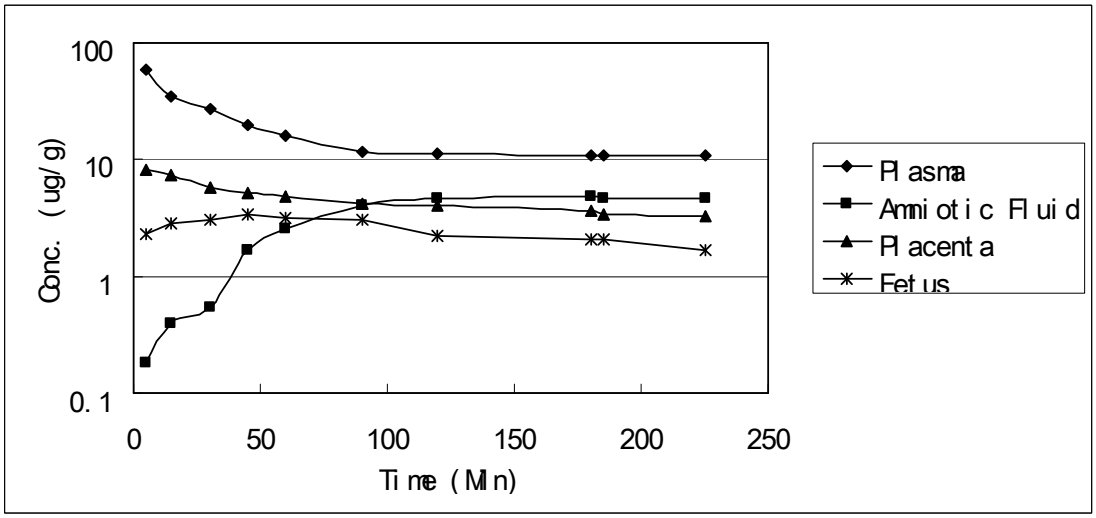


Fig. 3.6 Concentration vs. time profile of DDC and D4T in maternal plasma, amniotic fluid, placenta and fetus after 25 mg/kg i.v. bolus dose of DDC and D4T

CHAPTER 4

COMPARISON OF THE FETAL DISPOSITION OF ZALCITABINE AND LAMIVUDINE IN THE PREGNANT RAT[1]

---

[1]Meng Xu, Summer R. Lewis, Michael G. Bartlett, Catherine A. White. To be submitted to *Antimicrobial Agents and Chemotherapy*.

ABSTRACT

Both zalcitabine (DDC) and lamivudine (3TC) are nucleoside reverse transcriptase inhibitors that have been shown to inhibit the human immunodeficiency virus (HIV). Although they have similar structures and physical-chemical properties, the major structural difference is that DDC is a d-isomer and 3TC is an l-isomer. In this paper, the pharmacokinetics and placental transfer of DDC and 3TC were studied and compared in the pregnant rat following IV administration. The drugs were administered IV bolus separately at a dose of 25 mg/kg to timed-pregnant Sprague-Dawley rats on day 19 of gestation via a jugular cannula. Maternal plasma, placenta, fetus and amniotic fluid samples were collected over a period of six hours post-dose. Concentrations in each matrix were determined by HPLC-UV. All pharmacokinetic parameters were determined using WINNONLIN. A two compartment model with first order elimination was used to fit all maternal plasma data. All tissue data was analyzed by noncompartmental analysis. In addition, a five-compartment model was fitted to the data and pharmacokinetic parameters and relative exposures ($AUC_{tissue}/AUC_{maternal\ plasma}$) were determined.

Maternal peak plasma concentrations were similar for DDC (47.8 ± 4.4 mg/L) and 3TC (47.4 ± 6.6 mg/L). However, the clearance of 3TC (0.6 ± 0.1 l/hr-kg) was significantly lower than that of DDC (1.0 ± 0.1 l/hr-kg) resulting in a significantly higher AUC for 3TC. With both drugs the placental half-life was equivalent to the maternal plasma half-life; whereas, the fetal and amniotic fluid concentrations declined with a

significantly longer half-life (2-fold increase). Relative exposures for 3TC in the placenta (0.5 ± 0.1), fetus (0.3 ± 0.1), and amniotic fluid (0.3 ± 0.1) indicate no significant accumulation of 3TC. Relative exposures for DDC in the placenta (1.3 ± 0.1) and fetus (0.6 ± 0.1) were significantly higher than those observed for 3TC. No difference was noted in relative exposures of amniotic fluid. These values were consistent with the parameters obtained from the five compartment model. These significant differences suggest that active transport may play a role in the transplacental transfer of both DDC and 3TC. However, further studies are needed to determine the role of these transporters in placental transport of the antiviral drugs.

Keywords: Lamivudine; Zalcitabine

INTRODUCTION

Viral infections have increased dramatically in pregnant women, thus, exposing the fetus in utero. Therefore, antiviral drugs are used therapeutically in pregnancy for treatment of the mother and fetus. Antiviral drugs are presumed to prevent viral transmission from mother to fetus by decreasing maternal viral load and/or accumulation of drugs in the fetal compartment. Several studies of mother-to-infant transmission of HIV have demonstrated a correlation between the maternal virus load at delivery and risk of HIV transmission to the child. (9, 13, 25).

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 (36). DDC has been used in patients who cannot be maintained on AZT due to side effects (e.g. severe anemia) (33). Lamivudine (3TC) is a dideoxynucleoside analog of cytidine and is structurally similar to DDC (Figure 1). However, it differs from DDC in that the 3'-carbon of the ribose ring is replaced with sulfur, forming an oxathiolane ring (16). Although they have similar structures and physical-chemical properties, the major structural difference is that 3TC is an l-isomer and DDC is a d-isomer. 3TC has mostly been used in combination with zidovudine (AZT), given at a dose of 150 mg twice daily with 300 mg AZT (Combivir) (21). Both DDC and 3TC triphosphate prevent HIV replication by competitively inhibiting viral reverse transcriptase and terminating the proviral deoxyribonucleic chain extension (30).

There are several studies that have investigated the placental transport of AZT (6, 18, 23, 28, 31, and 34); however, there remains a need for studies of other antiviral drugs, such as DDC and 3TC. The physiological changes experienced by pregnant women, such as changes in gastrointestinal transit time, cardiac output, and plasma protein concentrations, may result in clinically significant alterations in drug pharmacokinetics (7). Therefore, an appropriate animal model must be used to study the placental transfer of drugs in the fetal-placental unit since pregnant women are generally excluded from clinical trials. The pregnant rat has been chosen as a model because of the similarities to humans in placental structure and hemodynamic changes in pregnancy (12). Their easy handling, large litter size (allowing for serial sampling), and short gestation period make the rat more convenient and economical than other large animal species. In addition, the similarities in rat and human transporters will allow for further study of the active transport of antiviral agents (15, 22). Most *in vivo* human placental transfer studies are restricted to single paired maternal and umbilical blood and amniotic fluid samples obtained at the time of delivery. Thus, the ability to obtain the entire concentration versus time profile from the pregnant rat makes it a more reliable model for the study of placental transfer. The pregnant rat model has been used previously in studying placental transfer of several compounds, including antiviral agent (4-6, 8, 10, 14, 17-19, 26, 27, 35).

The effect of pregnancy on the placental transfer of DDC and 3TC has not been investigated and limited data are available on the influence of pregnancy on the

pharmacokinetics of these drugs (11). This study provides a thorough comparison on the placental transfer and pharmacokinetics of DDC and 3TC.

MATERIALS AND METHODS

Reagents and chemicals. DDC and Stavudine (D4T) were obtained from Sigma (St. Louis, MO, USA). Lamivudine (3TC) was extracted from commercially available tablets. The purity of the extracts was determined by comparison to reference standards provided by the manufacturer (GlaxoSmithKline, RTP, NC, USA) and found to be greater than 98%. HPLC-grade methanol, acetonitrile and sodium phosphate dibasic 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).

Animal study. 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 hr of light per day) with daily feedings of standard chow pellets and water.

Timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) with an average weight of 328 ± 20 g were anesthetized intramuscularly on day 19 of pregnancy with ketamine:acepromazine:xylazine (50:3.3:3.4mg/kg) given in conjunction with

subcutaneous atropine (0.5 mg/kg). Subsequent doses of anesthesia were administered as needed. Body temperature was monitored with a Cooper Instrument Corporation temperature probe (model TC 100A; Cooper, Middlefield, Conn.) and maintained with heated surgical pads and incandescent lights. Prior to dosing, a laporatomy was performed and a small incision was made in the uterine wall to allow for sampling of the pups and a cannula was surgically implanted in the right jugular vein. The blood supply to the individual fetus was tied off prior to removal to minimize bleeding. An intravenous bolus dose (25 mg/kg) of DDC or 3TC was prepared in 0.1 M NaOH in physiological saline and administered via the jugular cannula.

Blood samples of 150 to 250 µl were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 min after dosing into heparinized tubes and centrifuged at 10,000 rpm for 10 min using a Micro-centrifuge Model 235V (Fisher Scientific, USA) to allow for collection of plasma. Pups were harvested at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min. Duplicate and triplicate pups were sampled at the same time point from individual pregnant rats throughout the study to ensure that each fetal sac had similar concentrations at any given time. Amniotic fluid samples were pulled from the fetal sacs with an 18-gauge needle and deposited into clean Eppendorf tubes. Placental and fetal tissues were homogenized in two volumes of deionized water (wt/vol) using an Ultra-Turbax T8 (IKA Labortechnik, Germany). All samples were stored at –20 °C until analysis.

HPLC analysis. DDC concentrations were determined by an HPLC-UV method

developed previously (37). The samples were analyzed 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 (4.6×3.0mm, Milford, MA) equipped with a Phenomenex security guard C-18 guard column (4×3.0mm, Torrance, CA). The mobile phase consisted of Solvent A (22mM formic acid) and Solvent B (acetonitrile) (97:3). The detection wavelength was set to be 265nm. The flow rate was set to be 0.5ml/min, and the injection volume was 40µl. The HPLC run time was 22min for each run. The calibration curves of plasma, amniotic fluid, placental and fetal homogenates were in the range of 0.1 – 50 µg/ml. The internal standard concentration was 1µg/ml (3TC) for all samples. All the samples were prepared by the 'salting out' technique. 200µl of a saturated ammonium sulfate solution and 1ml of ice cold acetonitrile were added to the samples (100µl for plasma, placental and fetal homogenate, 50µl 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 100µl of distilled water for injection.

3TC concentrations were determined by an HPLC-UV method developed previously (1). Briefly, plasma and amniotic fluid samples were prepared using 15 µl of 2 M perchloric acid. Fetal and placental samples were processed using a salting out technique using 180 µl saturated ammonium sulfate solution and 360 µl of cold acetonitrile. The calibration curves of plasma and amniotic fluid were in the range of 0.1 – 50 µg/ml and 0.2 – 50 µg/ml for placental and fetal homogenates. The internal standard concentration

was 5 μg/ml (d4T) for all samples.

The chromatographic system consisted of Waters (Milford, MA, USA) 510 pump, 717 autosampler and 486 UV detector operated with Millennium 2010 data system. A YMC phenyl column (5 μm, 150 mm×2 mm i.d., Waters, Milford, MA, USA) equipped with a Phenomenex C18 guard column (Torrance, CA, USA) was used to achieve all the chromatographic separations. The mobile phase consisted of 5% methanol in 20 mM dibasic sodium phosphate (pH 6), pH was adjusted using phosphoric acid and NaOH concentrated solutions. The flow rate was 0.2 ml/min and the detection wavelength was 256 nm. This method was previously validated to show acceptable precision and accuracy over the calibration range 0.1 – 50 μg/ml for plasma and amniotic fluid and 0.2 – 50 μg/ml for placental and fetal homogenates.

Data analysis. Initially, the plasma data was subjected to compartmental analysis using WinNonlin (Pharsight, Mountain View, CA, USA). A two-compartment intravenous bolus model with first-order elimination was used to fit the plasma data. Amniotic fluid, placenta, and fetus data were subjected to noncompartmental analysis. The relative exposure (RE) of each matrix was calculated by comparing the AUC for the individual tissues to the AUC for the maternal plasma data.

A five-compartment model was used to characterize the distribution of DDC and 3TC in the maternal plasma (central) (C_c), maternal tissue (C_t), placental (C_p), fetal (C_f), and amniotic fluid compartments (C_a) (Figure 2). This model incorporated bidirectional transfer between maternal plasma and placenta, placenta and fetus, placenta and amniotic

fluid, fetus and amniotic fluid, and maternal plasma and tissue compartment.

RESULTS AND DISCUSSION

Mean concentration-time profiles of DDC and 3TC in maternal plasma, placenta, fetus and amniotic fluid following intravenous administration of a 25 mg/kg bolus dose are shown in Figure 3. Maternal plasma concentrations of both DDC and 3TC decline in a biexponential fashion after IV administration. The pharmacokinetic parameters generated from two-compartmental analysis of the maternal plasma data are presented in Table 1. DDC has a half-life of 2.0 hr, a steady state volume of distribution of 1.9 L/kg (V_{ss}), and total clearance of 1.0 L/h/kg based on the maternal plasma data. These values were in close agreement with previously reported values for DDC pharmacokinetics in rats (19, 20). The maternal plasma pharmacokinetic parameters generated for 3TC in pregnant rats (V_{ss} = 1.3 L/kg, $t_{1/2}$ = 1.9 hr) are similar to previously reported values observed in humans (V_{ss} = 1.3 L/kg, $t_{1/2}$ = 2 – 4 hrs), and rhesus monkeys (V_{ss} = 1.2 L/kg, $t_{1/2}$ = 1.4 hr) (3, 30). These values for V_{ss} indicate that both DDC and 3TC are extensively distributed, which is unexpected based on the hydrophilic nature of the drugs, thus, suggesting that active transport may be involved. Based on these data, we found that maternal peak plasma concentrations were similar for 3TC (47.4 ± 6.6 mg/L) and DDC (47.8 ± 4.4 mg/L). However, the clearance of 3TC (0.6 ± 0.1 l/hr-kg) was significantly lower than that of DDC (1.0 ± 0.1 l/hr-kg) resulting in a significantly higher AUC for 3TC.

The pharmacokinetic parameters generated by noncompartmental analysis of the

placenta, fetus and amniotic fluid data are shown in Table 2. DDC concentrations in the placenta exceeded maternal levels after 30 minutes. However, the concentration of DDC in fetal or amniotic fluid did not demonstrate a big increase as placental levels exceeded maternal plasma values. This phenomenon suggests that saturation of active/facilitative transport occurs during this process. Unlike DDC, the placenta concentration of 3TC declines in parallel with the maternal plasma. The uptake of both DDC and 3TC into the placenta was rapid, with the concentrations reaching a maximum at 5 minutes. For both drugs, the uptake into the fetus and amniotic fluid was less rapid relative to the placental uptake. Interestingly, the concentration of DDC and 3TC in the amniotic fluid slowly rises and reaches a plateau, rather than declining with the maternal plasma, suggesting that the amniotic fluid compartment could serve as a depot for the two drugs. This phenomenon has previously been reported for 3TC and other antiviral agents (6, 18, 24, 29, and 34). Investigators suggested that the high concentrations in amniotic fluid could be attributed to recirculation of the compound due to fetal swallowing and micturition. Although the half-lives of 3TC in the fetus and amniotic fluid were longer than the half-life observed in the maternal plasma, the AUC and C_{max} values for all tissues were significantly lower than that of maternal plasma. To summarize, with both drugs the placental half-life was equivalent to the maternal plasma half-life; whereas, the fetal and AF concentrations declined with a significantly longer half-life (2-fold increase). Generally, the relative exposures for DDC are generally higher for 3TC in all tissues, with a significant difference in placenta and fetus and a not significant difference in

amniotic fluid. For 3TC, all the exposures range from 0.3 to 0.5; but for DDC, the relative exposure in placenta is higher than that in fetus, which almost equals to that in amniotic fluid, showing that the placenta has the protective effect for DDC.

Duplicate and triplicate pups were sampled at the same time point from individual pregnant rats throughout the study to ensure that each fetal sac (placenta, fetus, and amniotic fluid) had similar concentrations at any given time. Low coefficients of variation were observed among fetal sacs removed at the same time point in individual dams (6.3% in fetal tissue, 7.8% in placenta, and 8.4% in amniotic fluid), indicating good reliability of this data.

The maternal plasma, placenta, fetus, and amniotic fluid were also simultaneously fitted to a five-compartment model (Figure 2). The model developed for DDC and 3TC maternal-fetal transport assumed no drug elimination from the fetus since metabolism of both DDC and 3TC is a minor route of elimination (2, 16). The model-predicted DDC and 3TC concentration versus time profiles in maternal plasma, placenta, fetus, and amniotic fluid closely followed the observed data (Figure 4).

Intercompartmental clearances are presented in Table 3. The transfer of both drugs from maternal plasma to the placenta was rapid, consistent with the short T_{max}. Significant differences existed between CL_{cp} and CL_{pc} for 3TC, which shows that active transporters played a role in transplacental transfer of 3TC by pumping 3TC from placenta back into maternal plasma; however, there was no deference between CL_{cp} and CL_{pc} for DDC, showing passive diffusion. Significant differences existed between CL_{pf}

and CL_{fp} for both DDC and 3TC, and CL_{fp} was about 3-fold higher than CL_{pf} for DDC and 2-fold higher for 3TC. These findings showed that transporters helped pumping both DDC and 3TC back to placenta from fetus. This result was consistent with our findings with the two-compartment and noncompartment modeling, which showed a relative high RE in placenta for DDC but not for 3TC. Not many differences were noticed between CL_{pa} and CL_{ap} and between CL_{ct} and CL_{tc} for either DDC or 3TC, suggesting passive diffusion. However, significant differences existed between CL_{pa} and CL_{ap} for both DDC and 3TC, suggesting that the transporters tended to pump drugs from fetus back to amniotic fluid. For CLT, it was significant higher for DDC than 3TC, which was consistent with the results from the 2-compartment modeling. (4.66 ml/min vs. 1.0 l/hr-kg for DDC, 2.92 ml/min vs. 0.6 l/hr-kg)

Previous studies have shown that drug can be transferred to the amniotic fluid from the fetus via urination and lung secretion and to the fetus via inhalation and swallowing (32). Besides, the drugs can diffuse from the amniotic fluid through umbilical cord membranes to the placenta. Although the transfer rates are small relative to transfer rates from other compartments, omission of these compartments could lead to different estimates of transplacental clearances (34). The equivalent intercompartmental clearances for these two compartments are consistent with their REs. These observations are supported by the recent identification of transporters in the placenta (15, 22). However, further studies are needed to determine the role of these transporters in placental transport of this agent.

To summarize, although DDC and 3TC have very similar structures and chemical and physical properties, their pharmcokinetics in placental transfer are very different. We assume that maybe these differences due to their L-D differences. Thus, we assume that some transporters may play a role here, and they have different steroselectivities. However, further studies are needed to prove these suggestions.

REFERENCES

1. **Alnouti, Y, C.A .White, and M.G. Bartlett.** 2004. Determination of Lamivudine in Plasma, Amniotic Fluid, and Rat Tissues by Liquid Chromatography. J Chrom B. **803**:279-284.
2. **Beach, J.W.** 1998. Chemotherapeutic Agents for Human Immunodeficiency Virus Infection: Mechanism of Action, Pharmacokinetics, Metabolism, and Adverse Reactions. CLINICAL THERAPEUTICS. **20**: 2-25.
3. **Blaney, S. M., M. J. Daniel, A.J. Harker, K. Godwin, and F.M. Balis.** 1995. Pharmacokinetics of Lamivudine and BCH-189 in Plasma and Cerebrospinal Fluid of Nonhuman Primates. Antimicrob. Agents Chemother. **39**:2779-2782.
4. **Boike, G.M., G. Deppe, J.D. Young, J.M. Malone, Jr,, V.K. Malviya, and R.J. Sokol.** 1989. Chemotherapy in a Pregnant Rat Model. 1. Mitomycin-C: Pregnancy-Specific Kinetics and Placental Transfer. Gynecol Oncol. **34**: 187-190

5. **Boike, G.M, G. Deppe, J.D. Young, N.L. Gove, S.F. Bottoms, J.M. Malone, Jr,, V.K. Malviya, and R.J. Sokol.** 1989. Chemotherapy in a Pregnant Rat Model. 2. 5-Flouorouracil: Nonlinear Kinetics and Placental Transfer. Gynecol Oncol. **34**: 191-194.

6. **Brown, S.D., M.G. Bartlett, and C.A. White.** 2003. Pharmacokinetics of Intravenous Acyclovir, Zidovudine, and Acyclovir-Zidovudine in Pregnant rats. Antimicrob. Agents. Chemother. **47**:991-996.

7. **CDC**. 2002. USPHS Task Force Recommendations for Use of Antiretroviral Drugs in Pregnant HIV-1-Infected Women for Maternal Health and Interventions to Reduce Perinatal HIV-1 Transmission in the United States. MMRW. **51**(RR-18): 1-38. (updates at http://AIDSinfo.nih.gov)

8. **Clark, T. N., C. A. White, C. K. Chu, and M. G. Bartlett.** 2001. Determination of 3'-Azido-2', 3'-Dideoxyuridine in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography. J. Chromatogr. B **755:**165–172.

9. **Coll, O., M. Hernandez, C. A. Boucher, C. Fortuny, B. M. De Tejada, Y.Canet, I. Caragol, J. Tijnagel, J.M. Bertran, and T. Espanol.** 1997. Verical HIV-1 Transmission Correlates with a High Maternal Viral Load at Delivery. J Acquir Immune Defic Syndr Hum Retrovirol. **14**:26-30.

10. **Dawson, B. V., P. D. Johnson, S. J. Goldberg, and J. B. Ulreich.** 1990. Cardiac Teratogenesis of Trichloroethylene and Dichloroethylene in a Mammalian Model. J. Am. Coll. Cardiol. **16:**1304–1309.

11. **Ding, Y., L.N. Williamson, C.A. White, and M.G. Bartlett.** 2004. Determination of 2', 3'-Dideoxycytidine in Maternal Plasma, Amniotic Fluid, Placental and Fetal Tissues by High-Performance Liquid Chromatography. J. of Chromatogr. B. **811**:183–189.

12. **Faber, J.J., K.L. Thornburg.** 1983. Placental Physiology: Structure and Function of Fetomaternal Exchange. Raven: New York. 1-32.

13. **Fang, G.,H. Burger, R. Grimson, P. Tropper, S. Nachman, D. Mayers, O. Weislow, R. Moore, C. Reyelt, N. Hutcheon, D. Baker, and B. Weiserk.** 1995. Maternal Plasma Human Immunodeficiency Virus Type 1 RNA Level: a Determinant and Projected Threshold for Mother-to-Child Transmission. Proc Natl Acad Sci. USA. **92**:12100-12104.

14. **Fujinaga, M., J. M. Baden, A. Suto, J. K. Myatt, and R. I. Mazze.** 1991. Preventative Effects of Phenoxybenzamine on Nitrous Oxide-Induced Reproductive Toxicity in Sprague-Dawley Rats. Teratology **43:**151–157.

15. **Ganapathy, V., P.D. Prasad, M.E. Ganapathy, and F.H. Leibach.** 2000. Placental Transporters Relevant to Drug Distribution across the Maternal-Fetal Interface. J Pharmacol Exper Ther. **294**:413-420.

16. **GlaxoSmithKline.** Epivir Product Information. 2003. Available at http://us.gsk.com/products/assets/us_epivir.pdf. [Last accessed March 29, 2006].

17. **Heffez, D. S., J. Aryanpur, G. M. Hutchins, and J. M. Freeman.** 1990. The Paralysis Associated with Myleomeningocele: Clinical and Experimental Data Implicating a Preventable Spinal Cord Injury. Neurosurgery **26:**987–992.

18. **Huang, C.S., F.D. Boudinot, and S. Feldman.** 1996. Maternal-Fetal Pharmacokinetics of Zidovudine in Rats. J Pharm Sci. **85**:965-970.

19. **Ibrahim, S.S. and F.D. Boudinot.** 1989. Pharmacokinetics of 2', 3'-Dideoxycytidine in Rats: Application to Interspecies Scale-up. J Pharm Pharmacol. **41**:829-834.

20. **Ibrahim, S.S., F.D. Boudinot**. 1991. Pharmacokinetics of 2', 3'-Dideoxycytidine after High-dose Administration to Rats. J. Pharm. Sci. **80**: 36-38.

21. **Johnson, M. A., K. H. Moore, G. J. Yuen, A. Bye, and G. E. Pakes.** 1999. Clinical Pharmacokinetics of Lamivudine. Clin Pharmacokinetics. **36**:41-66.

22. **Leazer, T.M. and C.D. Klaasen.** 2003. The Presence of Xenobiotic Transporters in Rat Placenta. Drug Metabolism and Disposition. **31**:153-167.

23. **Little, B.B., R.E. Bawdon, J.T. Christmas, S. Sobhi, and L.C. Gilstrap.** 1989. Pharmacokinetics of Azidothymidine during Late Pregnancy in Long-Evans Rats. Am J Obstet and Gynecol. **161**:732-734.

24. **Mandelbrot, L, G Peytavin, G Firtion, and R Farinotti.** 2001. Maternal-Fetal Transfer and Amniotic Fluid Accumulation of Lamivudine in Human

Immunodeficiency Virus-Infected Pregnant Women. Am J Obstet Gynecol. **184**:153-158.

25. **Mayaux, M.J., E. Dussaix, J.Isope,C.Rekacewicz,L.Mandelbrot,N. Ciraru-Vigneron, M.C. Allemon, V. Chambrin, C. Katlama, J.F. Delfraissy, and J. Puel.** 1997. Maternal Virus Load during Pregnancy and Mother-to-Child Transmission of Human Immunodeficiency Virus Ttype 1: The French Perinatal Cohort Studies. J Infect Dis. **175**:172-175.

26. **Ostrea, E. M., Jr., A. Romero, D. K. Knapp, A. R. Ostrea, J. E. Lucena, and R. B. Utarnachitt.** 1994. Postmortem Drug Analysis of Meconium in Earlygestation Human Fetuses Exposed to Cocaine: Clinical Implications. J. Pediatr. **124:**477–479.

27. **Patel, B. A., C. K. Chu, and F. D. Boudinot.** 1989. Pharmacokinetics and Saturable Renal Tubular Secretion of Zidovudine in Rats. J. Pharm. Sci. **78:**530– 534.

28. **Patterson, T.A., Z.K. Binienda, G.W. Lipe, M.P. Gillam, W. Slikker, and J.A. Sandberg.** 1997. Transplacental Pharmacokinetics and Fetal Distribution of Azidothymidine, Its Glucoronide, and Phosphorylated Metabolites in Late-Term Rhesus Macaques after Maternal Infusion. Drug Metab Dispos. **25**:453-459.

29. **Pereira, C.M., C. Nosbisch, H.R. Winter, W.L. Baughman, and J.D.** Unadkat. 1994. Transplacental Pharmacokinetics of Dideoxyinosine in Pigtailed Macaques. Antimicrob. Agents. Chem. **38**:781-786.

30. **Perry, C.M., D. Faulds.** 1997. Lamivudine: A Review of Its Antiviral Activity, Pharmacokinetic Properties and Therapeutic Efficacy in the Management of HIV Infection. Drugs. **53**: 657-680.

31. **Schenker, S, R.F. Johnson, T.S. King, R.S. Schenken, and G.I. Henderson.** 1990. Azidothymidine (Zidovudine) Transport by the Human Placenta. Am J Med Sci. **299**:16-20.

32. **Schmidt,W.** The Amniotic Fluid Compartment: the Fetal Habitat. 1992. Adv Anat Embryol Cell Biol. **127**: 1-100.

33. **Simonds, R.J., R. Steketee, S. Nesheim, P. Matheson, P. Palumbo, L. Alger, E.J. Abrams, S. Orloff , M. Lindsay, A.D. Bardeguez, P. Vink, R. Byers, and M. Rogers.** 1998. Impact of Zidovudine Use on Risk and Risk Factors for Perinatal Transmission of HIV. AIDS 12: 301-308.

34. **Tuntland, T, A. Odinecs, C. Nosbisch, and J.D. Unadkat.** 1998. In Vivo Maternal-Fetal-Amniotic Fluid Pharmacokinetics of Zidovudine in the Pigtailed Macaque: Comparison of Steady-State and Single-Dose Regimens. J Pharmacol Exper Ther. **285**:54-62.

35. **Unadkat, J. D., J. P. Wang, D. Pulham, and R. L. O. Semmes.** 1989. Doseranging Pharmacokinetics of Zidovudine (Azidothymidine) in the Rat. Pharm. Res. **6:**734–736.

36. **U.S. Food and Drug Administration.** 1990. Children with AIDS. (available at http://www.fda.gov/bbs/topics/CONSUMER/CON00038.html).

37. **Xu, M, C.A. White, and M.G. Bartlett.** 2008. Simultaneous Determination of Zalcitabine and Stavudine in Rat Plasma, Amniotic Fluid, Placental and Fetal Tissues using High Performance Liquid Chromatography. J Liq.Chromatogr. **31**: 482–496.

Table 4.1. Maternal plasma pharmacokinetic parameters (mean ± standard deviation)

Parameter	Value	
	DDC	**3TC**
Half-life (hr)	2.0± 0.4	1.9 ± 0.4
AUC (hr-mg/L)	23.8± 1.6	43.0 ± 7.6[a]
CL_T (L/hr-kg)	1.0± 0.1	0.6 ± 0.1[a]
V_{ss} (L/kg)	1.9± 0.2	1.3 ± 0.1[a]
C_{max}(mg/L)	47.8 ± 4.4	47.4 ± 6.6

a indicates significant difference between the values for DDC and 3TC.

Table 4.2. Placental, Fetal and Amniotic Fluid Pharmacokinetic Parameters and Relative Exposures (mean ± standard deviation)

Parameter	Placenta		Fetus		Amniotic Fluid	
	DDC	3TC	DDC	3TC	DDC	3TC
Half-life (hr)	2.3± 0.9	2.1 ± 0.3	4.9± 2.9	3.3 ± 1.1	5.2± 2.0	3.9 ± 1.2
AUC*	30.2± 7.2	19.3 ± 1.9[a]	15.4 ± 3.2	11.2 ± 3.4	12.4± 3.4	12.1 ± 3.5
Cmax**	18.2 ± 1.8	9.0 ± 0.5 [a]	2.0 ± 0.6	2.3 ± 0.9	1.5 ± 0.2	1.7 ± 1.0
Tmax (hr)	0.1 ± 0	0.1 ± 0	1.7± 0.6	1.0 ± 0.6	2.3 ± 0.5	3.6 ± 0.5[a]
Relative Exposure***	1.3± 0.4	0.5 ± 0.1[a]	0.6± 0.1	0.3 ± 0.1[a]	0.5± 0.1	0.3 ± 0.1

* Expressed as hr·mg/kg for placenta and fetus and as hr·µg/mL for amniotic fluid
** Expressed as µg/g for placenta and fetus and as µg/mL for amniotic fluid
***Relative Exposure = $AUC_{tissue}/AUC_{maternal\ plasma}$
a indicates significant difference between the values for DDC and 3TC.

Table 4.3. Intercompartmental clearance estimates (ml/min) of DDC and 3TC in pregnant rats (25 mg/kg).

Parameter	DDC	3TC
CL_{cp}	0.67± 0.13	0.26 ± 0.06[a]
CL_{pc}	0.79± 0.22	0.62 ± 0.20[b]
CL_{pf}	0.42± 0.09	0.53 ± 0.20
CL_{fp}	1.21± 0.30[b]	1.12 ± 0.31[b]
CL_{pa}	0.006± 0.002	0.005 ± 0.001
CL_{ap}	0.006± 0.004	0.004 ± 0.003
CL_{ct}	7.23± 2.65	4.68 ± 0.67
CL_{tc}	7.71± 2.23	4.64 ± 0.82
CL_{fa}	0.006± 0.005	0.007 ± 0.004
CL_{af}	0.021± 0.002[b]	0.012 ± 0.006[a]
CL_{T}	4.66± 0.52	2.92 ± 0.48[a]

a indicates significant difference between the values for DDC and 3TC.
b Indicates significant differences between corresponding distribution clearances within each therapy group.

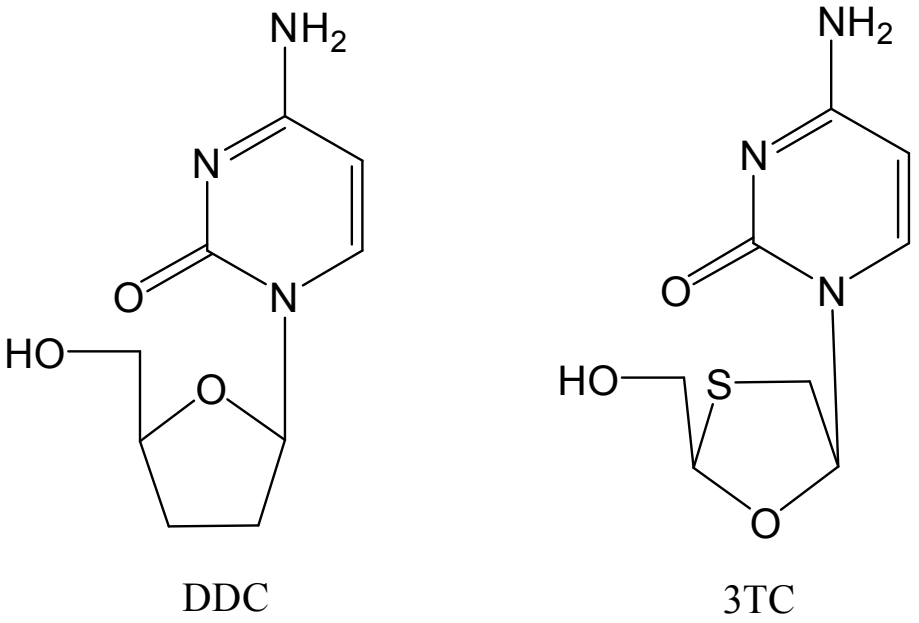


Figure 4.1. Chemical structures of DDC and 3TC

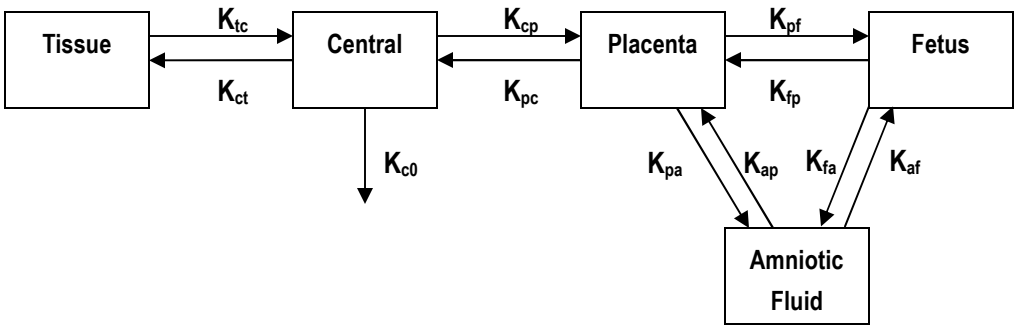
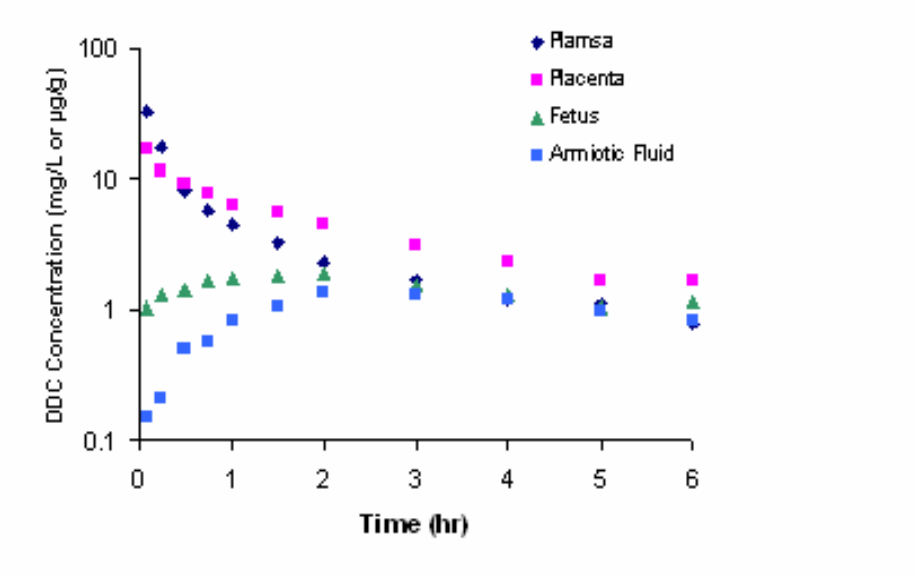


Figure 4.2. Schematic representation of the pharmacokinetic model, used to describe the disposition of DDC and 3TC in maternal central, maternal tissue, placental, fetal, and amniotic fluid compartments after iv injection to pregnant rats.

(a)



(b)

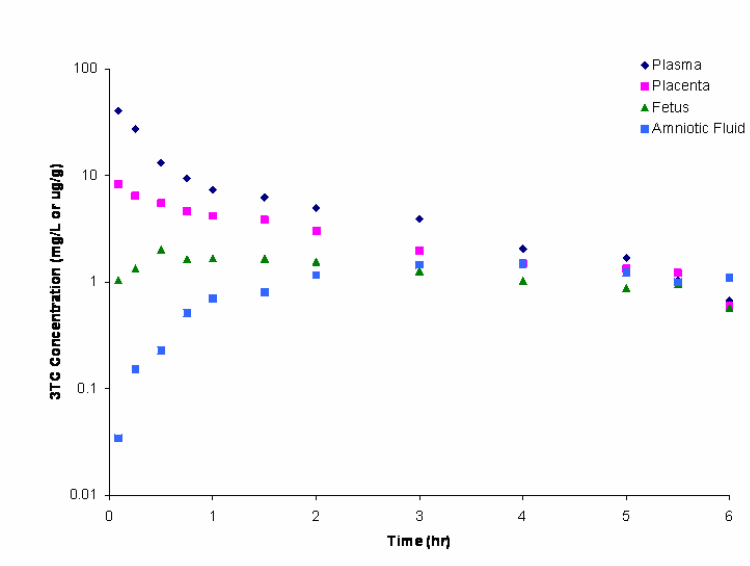


Figure 4.3. Concentration versus time profiles of 25 mg/kg dose of (a) DDC (b) 3TC following IV bolus administration in maternal plasma, placenta, fetus, and amniotic fluid. (n=6 for DDC, n=7 for 3TC)

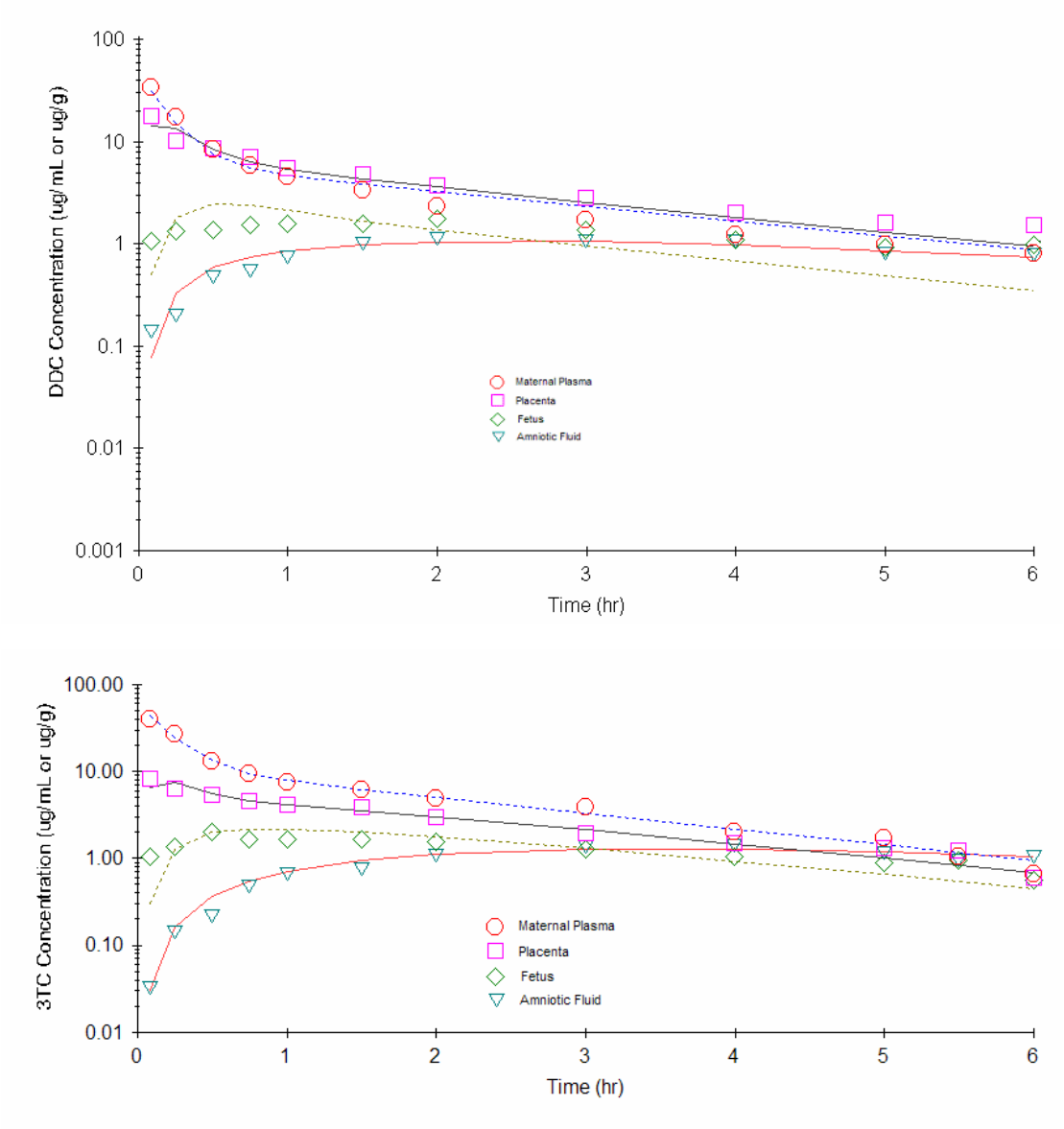


Figure 4.4. Measured DDC and 3TC concentration versus time data for maternal plasma, placenta, fetus, and amniotic fluid fitted simultaneously to the five-compartment model.

CHAPTER 5

STAVUDINE AFFECTS THE PLACENTAL TRANSFER OF ZALCITABINE IN THE PREGNANT RAT[1]

---

[1]Meng Xu, Michael G. Bartlett, Catherine A. White. To be submitted to *the Journal of Pharmacology & Experimental Therapeutics*.

ABSTRACT

Both zalcitabine (DDC) and stavudine (D4T) are nucleoside reverse transcriptase inhibitors that have been shown to inhibit the human immunodeficiency virus (HIV). The effects of D4T on DDC were evaluated during placental transfer in the pregnant rat. DDC alone or in combination with D4T was administered IV bolus at a dose of 25 mg/kg to timed-pregnant Sprague-Dawley rats on day 19 of gestation via a jugular cannula. Maternal plasma, placenta, fetus and amniotic fluid samples were collected over a period of seven hours following drug administration. Concentrations in each matrix were determined by HPLC-UV. All pharmacokinetic parameters were determined using WINNONLIN. A two compartment model with first order elimination was used to fit all maternal plasma data. All tissue data was analyzed by noncompartmental analysis and relative exposures (RE, $AUC_{tissue}/AUC_{maternal\ plasma}$) were determined.

Maternal pharmacokinetics were similar for DDC alone and DDC in combination with D4T, with a C_{max} 47.8± 4.4 vs. 43.1± 9.1 mg/L, AUC 23.8± 1.6 vs. 21.8± 2.4 hr-mg/L, Cl_T 1.0± 0.1 vs 1.2± 0.1 L/hr-kg, Vss 1.9± 0.2 vs. 1.6 ± 0.1 L/kg, half life 2.0± 0.4 vs. 1.9± 0.6 hr. However, the RE for DDC given in combination in the placenta (0.6±0.1 vs. 1.3±0.4) and fetus (0.2±0.1 vs. 0.6±0.1) were significantly lower than DDC alone, which suggests that D4T either blocks the movement of DDC into placenta, or has a upregulation of efflux transporters which transfer DDC from placenta back into plasma.

Keywords: Zalcitabine, Stavudine

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is an epidemic that has far exceeded early predictions. For children, about 90% of the human immunodeficiency virus (HIV) infections are due to vertical transmission from the infected mothers through blood, amniotic fluid, and/or breast milk. (CDC, 2005; Hansen, 1998) Therefore, antiviral drugs are used therapeutically in pregnancy for treatment of the mother and the fetus, which are presumed to prevent viral transmission from mother to fetus by decreasing maternal viral load and/or accumulation of drugs in the fetal compartment. (Coll et al., 1997; Fang et al., 1995; Mayaux et al., 1997) Nucleoside reverse transcriptase inhibitors (NRTIs) compose the first class of antiretroviral drugs developed. The active forms of NRTIs are not the drug, as administered, but its triphosphorylated (TP) metabolites, which competitively inhibit HIV reverse transcriptase following incorporation into the proviral DNA. (Rodman et al., 1996) However, the mechanism associated with placental transfer of NRTIs is largely unknown.

DDC was one of the earliest NRTI used for AIDS therapy and was the first drug approved under FDA's accelerated drug review policy. (FDA, 1990) DDC has been used in patients who cannot be maintained on AZT due to side effects (e.g. severe anemia). (Simonds et al., 1998) D4T is a powerful dideoxynucleoside analogue which has shown powerful activity against HIV. (Mitsuya et al., 1986) It inhibits HIV reverse transcriptase with relatively little inhibition of host cell DNA polymerases in various cell types. (Ferrua et al., 1994)

Since pregnant women are usually excluded from the pharmacokinetic studies for fear of toxic effects of the drugs on the fetus, an appropriate animal model must be used to study the placental transfer of drugs in the fetal-placental unit. (Tuntland et al., 1999) The pregnant rat model has been used successfully in the study of the placental transfer of many compounds, including nucleoside analogs (Ibrahim et al., 1989; Boike et al., 1989a, 1989b; Huang et al., 1996; Clark et al., 2001, 2004, 2006; Brown et al., 2002a, 2002b, 2003; Ding et al., 2004; Alnouti et al., 2004a, 2004b, 2004c, 2005; Lewis et al., 2007; Xu et al., 2008). The rat placenta is similar to the human placenta since both are hemochorial, in which trophoblastic cells are in direct contact with maternal blood without an intervening endothelium. In addition, the hemodynamic changes present in the pregnant rat are similar to those seen in a human pregnancy (Faber et al., 1983; Boike et al., 1989a; Leazer et al., 2003). Finally, the pregnant rat model is ideal for pharmacokinetic studies because of the short gestation time and the containment of each fetus, placenta, and amniotic fluid in individual fetal sacs which allows for concurrent serial sampling of the pups. (Brown et al., 2003)

A series of studies by Unadkat and coworkers has reported the lack of interaction between several anti-HIV drugs (D4T and AZT; DDI and AZT) when using the macaque as an animal model; their findings suggest passive diffusion as the primary mechanism for placental transport. (Pereira et al., 1995; Odinecs et al., 1996; Tuntland et al., 1996) However, our previous studies have shown substantial interactions between AZT and acyclovir, and between AZT and 3TC. (Brown et al., 2003; Alnouti et al., 2004b) 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 placental transport processes of these agents. This study examines the effects of D4T on the placental transfer of DDC in pregnant rats.

MATERIALS AND METHODS

Reagents and chemicals. DDC and D4T were obtained from Sigma (St. Louis, MO, USA). Lamivudine (3TC) was extracted from commercially available tablets. The purity of the extracts was determined by comparison to reference standards provided by the manufacturer (GlaxoSmithKline, RTP, NC, USA) and found to be greater than 98%. HPLC-grade methanol and 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).

Animal study. 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 hr of light per day) with daily feedings of standard chow pellets and water.

Timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) with an

average weight of 330g were anesthetized intramuscularly on day 19 of pregnancy with ketamine:acepromazine:xylazine (50:3.3:3.4mg/kg) given in conjunction with subcutaneous atropine (0.5 mg/kg). Subsequent doses of anesthesia were administered as needed. Body temperature was monitored with a Cooper Instrument Corporation temperature probe (model TC 100A; Cooper, Middlefield, Conn.) and maintained with heated surgical pads and incandescent lights. Prior to dosing, a laporatomy was performed and a small incision was made in the uterine wall to allow for sampling of the pups and a cannula was surgically implanted in the right jugular vein. The blood supply to the individual fetus was tied off prior to removal to minimize bleeding. An intravenous bolus dose (25 mg/kg) of DDC and DDC/D4T was prepared in 0.1 M NaOH in physiological saline and administered via the jugular cannula.

Blood samples of 150 to 250 µl were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 and 420 min after dosing into heparinized tubes and centrifuged at 10,000 rpm for 10 min using a Micro-centrifuge Model 235V (Fisher Scientific, USA) to allow for collection of plasma. Pups were harvested at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 and 420 min. Duplicate and triplicate pups were sampled at the same time point from individual pregnant rats throughout the study to ensure that each fetal sac had similar concentrations at any given time. Amniotic fluid samples were pulled from the fetal sacs with an 18-gauge needle and deposited into clean Eppendorf tubes. Placental and fetal tissues were homogenized in two volumes of deionized water (wt/vol) using an Ultra-Turbax T8 (IKA Labortechnik, Germany). All samples were stored at –20 °C until

analysis.

HPLC analysis. DDC concentrations were determined by an HPLC-UV method developed previously (Xu et al., 2008). The samples were analyzed on an Agilent 1100 series HPLC equipped with a variable wavelength UV detector. Chromatographic separation was achieved on a Waters Spherisorb S3W silica column (4.6×3.0mm, Milford, MA) equipped with a Phenomenex security guard C-18 guard column (4×3.0mm, Torrance, CA). The mobile phase consisted of Solvent A (22mM formic acid) and Solvent B (acetonitrile) (97:3). The detection wavelength was set to be 265nm. The flow rate was set to be 0.5ml/min, and the injection volume was 40µl. The HPLC run time was 22 min for sample. The calibration curves of plasma, amniotic fluid, placental and fetal homogenates were in the range of 0.1 – 50 µg/ml. The internal standard concentration was 1 µg/ml (3TC) for all samples. All samples were prepared by the 'salting out' technique. 200µl of a saturated ammonium sulfate solution and 1ml of ice cold acetonitrile were added to the samples (100µl for plasma, placental and fetal homogenate, 50µl 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 100µl of distilled water for injection.

Data analysis. Initially, the plasma data was subjected to compartmental analysis using WinNonlin (Version 5.2, Pharsight, Mountain View, CA, USA). A two-compartment intravenous bolus model with first-order elimination was used to fit the plasma data. Amniotic fluid, placenta, and fetus data were subjected to

noncompartmental analysis. Due to the inability to calculate accurate half-lives for DDC in the tissues, the area under the curve was truncated at 6 hours in plasma, placenta, fetus, and amniotic fluid for the calculation of relative exposure. The relative exposure (RE) of each matrix was calculated by comparing the AUC values for the individual tissues to the AUC value for the maternal plasma data. The pharmacokinetic parameters generated for each dosing group and the relative exposure numbers were compared by using the unpaired *t* test ($P < 0.05$) to detect statistically significant differences.

RESULTS AND DISCUSSION

Duplicate and triplicate pups were sampled throughout the whole operation at the same time point from individual pregnant rats throughout the study to ensure that each fetal sac (placenta, fetus, and amniotic fluid) had similar concentrations at any given time. Low coefficients of variation were observed among fetal sacs removed at the same time point in individual dams (5.9% in fetal tissue, 6.2% in placenta, and 4.4% in amniotic fluid), indicating good reliability of this data.

The average concentration in plasma vs. time profile for DDC dosed alone and in combination with D4T is shown in Fig. 2 (n=6 for DDC alone, n=5 for DDC/D4T combination). The pharmacokinetic parameters generated from the compartmental analysis of the plasma data are presented in Table 1. Maternal pharmacokinetics were similar for DDC alone and DDC in combination with D4T, with a C_{max} 47.8± 4.4 vs. 43.1± 9.1 mg/L, AUC 23.8± 1.6 vs. 21.8± 2.4 hr-mg/L, Cl_T 1.0± 0.1 vs 1.2± 0.1 L/hr-kg,

Vss 1.9± 0.2 vs. 1.6 ± 0.1 L/kg, half life 2.0± 0.4 vs. 1.9± 0.6 hr. The maternal plasma concentration of DDC in both situation declined in a biexponential fashion following IV administration. The high value for V_{ss} indicates that in both situations, DDC is extensively distributed, which was unexpected based on the hydrophilic nature of DDC, and suggested that active transport may be involved.

The concentration versus time profiles of DDC dosed alone and in combination with D4T from amniotic fluid, placenta, and fetus are compared in Fig. 3, and the pharmacokinetic parameters for these matrices generated from noncompartmental analysis are tabulated in Table 2. The half-life of DDC in the placenta was significantly shorter for DDC in the presence of D4T. While not significant, a decrease in half-lives for DDC in the fetus and amniotic fluid was also noted. The AUCs of DDC in placenta, fetus and amniotic fluid were significantly lower with the presence of D4T, and the C_{max} was significant lower in fetus and amniotic fluid for the combination. These changes in tissue disposition resulted in significant decreases in RE for DDC in the placenta and fetus, and a non-significant decrease in amniotic fluid when administered with D4T.

The uptake of DDC into the placenta was rapid, with the concentration reaching a maximum at 5 minutes. One interesting observation concerning DDC monotherapy was that DDC concentrations in the placenta exceeded maternal levels after 30 minutes. However, the concentration of DDC in fetal or amniotic fluid did not demonstrate a significant increase as placental levels exceeded maternal plasma values. This phenomenon suggests that saturation of active/facilitative transport occurs during this

process. Unlike the monotherapy, the placental concentration of DDC in combination with D4T declined with the maternal plasma with a faster half life, resulting in a significant lower RE in the placenta (0.6 vs. 1.3). However, this significant decrease in placental RE resulted in a significant decrease in RE in fetus but not in the amniotic fluid. The concentration of DDC in the amniotic fluid slowly rose and reached a plateau, rather than declining with the maternal plasma, suggesting that the amniotic fluid compartment could serve as a depot for DDC. This phenomenon has previously been reported for other antiviral agents (Pereira et al., 1995; Huang et al., 1996; Tuntland et al., 1998; Mandelbrot et al., 2001; Brown et al., 2003). Investigators suggested that the high concentrations in amniotic fluid could be attributed to recirculation of the compound due to fetal swallowing and micturition. In both situations, the AUC for DDC in the amniotic fluid and in the fetus almost equals, which suggested that the co-administration of D4T won't affect the distribution of DDC between amniotic fluid and fetus.

A significant decrease in placental transfer of DDC was noticed when coadministered with D4T, which suggests that D4T either blocks the movement of DDC into placenta, or has an upregulation of efflux transporters which transfer DDC from placenta back into maternal plasma. However, which specific transporters are involved is not known. There are several drug transporters which exist in the placenta, and at least nine of the known transporters in the placenta transport nucleoside antiviral agents or substrates that these transporters share in other tissues (Culnane et al., 1999; St-Pierre et al., 2000; Ganapathy et al., 2000; Leazer et al., 2003; Lewis, 2006). Efflux transporters include those belonging

to the ABC transporter family, which function to remove xenobiotics from the placenta. (Lewis, 2006) Among which, the breast cancer resistance protein (BCRP), modulates the exposure of the fetus to drugs by acting as a maternal-fetal barrier to passage of xenobiotics across the placenta; while the function of multidrug resistance proteins (MRPs) is not known (Jonker et al., 2000; Tudor-Williams et al., 1999) Transporters that are facilitative or equilibrative can function both as influx or efflux transporters depending on the directionality of the concentration gradient, which are referred to as bidirectional transporters. (UNAIDS, 2005; Yasuda et al., 2005) This group of transporters include the equilibrative and concentrative nucleoside transporters (ENT and CNT), the organic anion transporter (OAT 4), organic anion transporting polypeptides (OATPs), and the organic cation transporters (OCT 2, OCT 3, and OCTN 2). (Kitchen et al., 1995; Syme et al., 2004; UNAIDS, 2005) Thus, it is important to conduct further studies using cell culture models to identify which transporters are involved in the fetal uptake of these nucleoside analogs.

CONCLUSION

The disposition of DDC in the pregnant rat was significantly altered when it was dosed in combination with D4T. The placental transport of DDC decreased dramatically in the presence of D4T. This suggests that transporters, in addition to passive diffusion, play a role in the transport of these nucleoside analogs. However, which specific transporters or how these transporters affect *in vivo* disposition of therapeutic nucleoside

analogs is unknown. Further studies using cell culture or other *in vitro* techniques are necessary to identify which transporters are involved in the fetal uptake of these nucleoside analogs.

REFERENCES

Alnouti Y, White CA, Bartlett MG (2004a) Simultaneous Quantitation of Zidovudine and Zidovudine Monophosphate from Plasma, Amniotic Fluid and Ttissues by Micellar Capillary Electrophoresis. *Biomed Chromatogr* 18(8):523-531.

Alnouti Y, White CA, Bartlett MG (2004b) Simultaneous Determination of Zidovudine and Lamivudine from Rat Plasma, Amniotic Fluid and Tissues by HPLC. *Biomed. Chromatogr.* 18:641–647.

Alnouti Y, White CA, Bartlett MG (2004c) Determination of Lamivudine in Plasma, Amniotic Fluid, and Rat Tissues by Liquid Chromatography. *J Chromatogr B* 803(2):279-84.

Alnouti Y, Lewis SR, White CA, Bartlett MG (2005) Simultaneous Determination of Zidovudine and Lamivudine from Rat Tissues by Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Commun Mass Spectrom* 19(4):503-508.

Boike GM, Deppe G, Young JD, Gove NL, Bottoms SF, Malone JM Jr., Malviya VK, and Sokol RJ (1989a) Chemotherapy in a Pregnant Rat Model. 1. Mitomycin-C: Pregnancy-Specific Kinetics and Placental Transfer. *Gynecol. Oncol.* 34**:**187–190.

Boike GM, Deppe G, Young JD, Malone JM Jr., Malviya VK, and Sokol RJ (1989b) Chemotherapy in a Pregnant Rat Model. 2. 5-Fluorouracil: Nonlinear Kinetics and Placental Transfer. *Gynecol. Oncol.* 34**:**191–194.

Brown SD, White CA, Bartlett MG (2002a) Hydrophilic Interaction Liquid Chromatography/Electrospray Mass Spectrometry Determination of Acyclovir in Pregnant Rat Plasma and Tissues. *Rapid Commun Mass Spectrom* 16(19):1871-6.

Brown SD, White CA, Chu CK, Bartlett MG (2002b) Determination of Acyclovir in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography. *J Chromatogr B* 772(2):327-334.

Brown SD, Bartlett MG, White CA (2003) Pharmacokinetics of Intravenous Acyclovir, Zidovudine, and Acyclovir-Zidovudine in Pregnant Rats. *Antimicrob. Agents Chemother* 47(3):991–996.

CDC. 2005. HIV/AIDS Surveillance Report: Cases of HIV Infection and AIDS in the United States, 2004. Atlanta: The Centers; 2005. Vol.16. (Available at http://www.cdc.gov/hiv/topics/surveillance/resources/reports/2004report/default. htm.)

Clark TN, White CA, Chu CK, and Bartlett MG (2001) Determination of 3'-Azido-2', 3'-Dideoxyuridine in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography. *J. Chromatogr. B* 755:165–172.

Clark TN, White CA, and Bartlett MG (2004) Determination of Abacavir in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by a Polarity Switching Liquid

Chromatography/Tandem Mass Spectrometry Method. *Rapid Commun Mass Spectrom* 18(4):405-411.

Clark, T. N., C. A. White, and M. G. Bartlett (2006) Determination of Didanosine in Maternal Plasma, Amniotic Fluid, Fetal and Placental Tissues by High-Performance Liquid Chromatography-Tandem Mass Spectrometry. Biomed Chromatogr. 20(6-7):605-611.

Coll O, Hernandez M, Boucher CA, Fortuny C, De Tejada BM, Canet Y, Caragol Y, Tijnagel J, Bertran JM, and Espanol T (1997) Verical HIV-1 Transmission Correlates with a High Maternal Viral Load at Delivery. J Acquir *Immune Defic Syndr Hum Retrovirol* 14:26-30.

Culnane M, Fowler M, Lee SS, McSherry G, Brady M, O'Donnell K, Mofenson L, Gortmaker SL, Shapiro DE, Scott G, Jimenez E, Moore EC, Diaz C, Flynn PM, Cunningham B, and Oleske J (1999) Lack of Long-Term Effects of in Utero Exposure to Zidovudine among Uninfected Children Born to HIV-Infected Women. *J. Amer. Med. Ass.* 281(2):151-157.

Ding Y, Williamson LN, White CA, and Bartlett MG (2004) Determination of 2',3'-Dideoxycytidine in Maternal Plasma, Amniotic Fluid, Placental and Fetal Tissues by High-Performance Liquid Chromatography. *J Chromatogr B* 811(2):183-189.

Faber JJ, and Thornburg KL (1983) Placental physiology: Structure and Function of Fetomaternal Exchange. Raven, New York, N.Y.

Fang G, Burger H, Grimson R, Tropper P, Nachman S, Mayers D, Weislow O, Moore R, Reyelt C, Hutcheon N, Baker D, and Weiserk B **(**1995) Maternal Plasma Human Immunodeficiency Virus Ttype 1 RNA Level: a Determinant and Projected Threshold for Mother-to-Child Transmission. *Proc Natl Acad Sci* 92:12100-12104.

FDA (1990) Children with AIDS, U.S. Food and Drug Administration, 1990 (available at http://www.fda.gov/bbs/topics/CONSUMER/CON00038.html).

Ferrua, B.; Tran, T.T.; Quaranta, J.F.; Kubar, J.; Roptin, C.; Condom, R.; Durant, J.; Guedj, R. Measurement of the Anti-HIV Agent 2′,3′-Didehydro-2′,3′- Dideoxythymidine (D4T) by Competitive ELISA. *J. Immunolog. Meth.* 1994, 176(1), 103-110.

Ganapathy V, Prasad PD, Ganapathy ME, and Leibach FH (2000) Placental Transporters Relevant to Drug Distribution across the Maternal-Fetal Interface. *J Pharmacol Exper Ther* 294(2):413-420.

Hansen M (1998) Pathophysiology: Foundations of Disease and Clinical Intervention, W.B. Saunders Company, Philadelphia, United States.

Huang CS, Boudinot FD, and Feldman S (1996) Maternal-Fetal Pharmacokinetics of Zidovudine in Rats. *J. Pharm. Sci.* 85:965–970.

Ibrahim SS, FD Boudinot (1989) Pharmacokinetics of 2',3'-Dideoxycytidine in Rats: Application to Interspecies Scale-Up. *J Pharm Pharmacol* 41:829-834.

Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH, and Schinkel AH (2000) Role of Breast Cancer Resistance Protein in the Bioavailability and Fetal Penetration of Topotecan. *J Natl Cancer Inst* 92(20):1651-1656.

Kitchen VS, Skinner C, Ariyoshi K (1995) Safety and Activity of Saquinavir in HIV Infection. *Lancet* 345:952-955.

Leazer TM, and Klaasen CD (2003) The Presence of Xenobiotic Transporters in Rat Placenta. Drug Metabolism and Disposition. 31:153-167.

Lewis SR (2006) Dissertation: Maternal and Fetal Disposition of Antiviral Agents in the Pregnant Rat. University of Georgia.

Lewis SR, White CA, and Bartlett MG (2007) Simultaneous Determination of Abacavir and Zidovudine from Rat Tissues using HPLC with Ultraviolet Detection. *J Chromatogr B* 850(1-2):45-52.

Mandelbrot L, Peytavin G, Firtion G, and Farinotti R (2001) Maternal-Fetal Transfer and Amniotic Fluid Accumulation of Lamivudine in Human Immunodeficiency Virus-Infected Pregnant Women. *Am J Obstet Gynecol* 184:153-158.

Mayaux MJ., Dussaix E, Isope J, Rekacewicz C, Mandelbrot L, Ciraru-Vigneron N, Allemon MC, Chambrin V, Katlama C, Delfraissy JF, and Puel J (1997) Maternal Virus Load during Pregnancy and Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1: The French Perinatal Cohort Studies. *J Infect Dis* 175:172-175.

Mitsuya, H.; Broder, S. Inhibition of the in Vitro Infectivity and Cytopathic Effect of Human T-Lymphotrophic Virus type III/Lymphadenopathy-Associated Virus (HTLV-III/LAV) by 2', 3'-Dideoxynucleosides. *Proc. Natl. Acad. Sci. U.S.A.* 1986, 83 (6), 1911-1915.

Odinecs A, Nosbisch C, Unadkat JD (1996) Zidovudine does not Affect Transplacental Transfer or Systemic Clearance of Stavudine (2', 3'-Didehydro-3'-Deoxythymidine) in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother* 40:1569-1571.

Pereira CM, Nosbisch C, Baughman WL, Unadkat JD (1995)Effect of Zidovudine on Transplacental Pharmacokinetics of DDI in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 39:343-345.

Rodman JH, Robbins B, Flynn PM, Fridland A (1996) A Systemic and Cellular Model for Zidovudine Plasma Concentrations and Intracellular Phosphorylation in Patients. *J Infect Dis* 174(3):490-499.

Simonds, R.J.; Steketee, R.; Nesheim, S.; Matheson, P.; Palumbo, P.; Alger, L.; Abrams, E.J.; Orloff, S.; Lindsay, M.; Bardeguez, A.D.; Vink, P.; Byers, R.; Rogers, M. Impact of Zidovudine Use on Risk and Risk Factors for Perinatal Transmission of HIV. *AIDS.* 1998, 12 (3), 301-308.

St-Pierre MV, Serrano MA, Marcias RI, Dubs U, Hoechli M, Lauper U, Meier PJ, and Marin JJ (2000) Expression of Members of the Mutildrug Resistance Protein Family in Human Term Placenta. *Am J Physiol Reg Integ Comp Phsiol* 279:R1495-R1503.

Syme MR, Paxton JW, and Keelan JA (2004) Drug Transfer and Metabolism by the Human Placenta. *Clin Pharmacokinetics* 43: 487-514.

Tudor-Williams G, and Lyall E (1999) Mother to Infant Transmission of HIV. *Curr Opin Infect Dis* 12:21-26.

Tuntland T, Nosbisch C, Baughman WL, Massarella J, Unadkat JD (1996) Mechanism and Rate of Placental Transfer of Zalcitabine (2',3'-dideoxycytidine) in Macaca nemestrina. *Am. J. Obstet. Gynecol.* 174:856-863.

Tuntland T, Odinecs A, Nosbisch C, and Unadkat JD (1998) In Vivo Maternal-Fetal-Amniotic Fluid Pharmacokinetics of Zidovudine in the Pigtailed Macaque: Comparison of Steady-State and Single-Dose Regimens. *J Pharmacol Exper Ther* 285:54-62.

Tuntland T, Odinecs A, Pereira CM, Nosbisch C, Unadkat JD (1999) In Vitro Models to Predict the In Vivo Mechanism, Rate, and Extent of Placental Transfer of Dideoxynucleoside Drugs Against Human Immunodeficiency Virus. *Am J Obstet Gynecol* 180:198-206.

UNAIDS. AIDS Epidemic Update: December 2005. Geneva, 2005. Available at http://data.unaids.org/Publications/IRC-pub06/epi_update2005_en.pdf. [Last accessed April 2, 2006]

Xu M, White CA, and Bartlett MG (2008) Simultaneous Determination of Zalcitabine and Stavudine in Rat Plasma, Amniotic Fluid, Placental and Fetal Tissues using High Performance Liquid Chromatography. *J. Liq. Chromatogr.* 31: 482–496.

Yasuda S, Itagaki S, Hirano T, Iseki K (2005) Expression level of ABCG2 in the Placenta Decreases from the Mid Stage to the End of Gestation. *Biosci. Biotechnol. Biochem* 69: 1871-1876.

Table 5.1. Maternal plasma pharmacokinetic parameters (mean ± standard deviation) for DDC when dosed alone or in combination with D4T

Parameter	Value	
	DDC	**DDC-D4T**
Half-life (hr)	2.0± 0.4	1.9 ± 0.6
AUC (hr-mg/L)	23.8± 1.6	21.8 ± 2.4
CL_T (L/hr-kg)	1.0± 0.1	1.2 ± 0.1
V_{ss} (L/kg)	1.9± 0.2	1.6 ± 0.1
C_{max}(mg/L)	47.8 ± 4.4	43.1 ± 9.1

Table 5.2. Placental, Fetal and Amniotic Fluid Pharmacokinetic Parameters and Relative Exposures (mean ± standard deviation) for DDC when dosed alone and in combination with D4T

Parameter	Placenta		Fetus		Amniotic Fluid	
	DDC	DDC-D4T	DDC	DDC-D4T	DDC	DDC-D4T
Half-life (hr)	2.3± 0.9	1.1 ± 0.1*	4.9± 2.9 [d]	3.1 ± 0.6 [d]	5.2± 2.0	2.6 ± 1.4
AUC [a]	30.2± 7.2	13.9 ± 1.2*	15.4 ± 3.2	4.7 ± 1.5*	12.4± 3.4	4.9 ± 0.9*
Cmax [b]	18.2 ± 1.8	15.3 ± 2.1	2.0 ± 0.6	0.9 ± 0.3*	1.5 ± 0.2	1.1 ± 0.4
Tmax (hr)	0.1 ± 0	0.1 ± 0	1.7± 0.6	1.0 ± 0.6	2.3 ± 0.5	3.8 ± 1.1
Relative Exposure [c]	1.3± 0.4	0.6 ± 0.1*	0.6± 0.1	0.2 ± 0.1*	0.5± 0.1	0.3 ± 0.1

a Expressed as hr·mg/kg for placenta and fetus and as hr·μg/mL for amniotic fluid
b Expressed as μg/g for placenta and fetus and as μg/mL for amniotic fluid
c Relative Exposure = $AUC_{tissue}/AUC_{maternal\ plasma}$
d Estimates of half life are based on a limited number of data points in the elimination phase.
* Indicates significant difference between DDC dosed alone and in combination with D4T ($P < 0.05$)

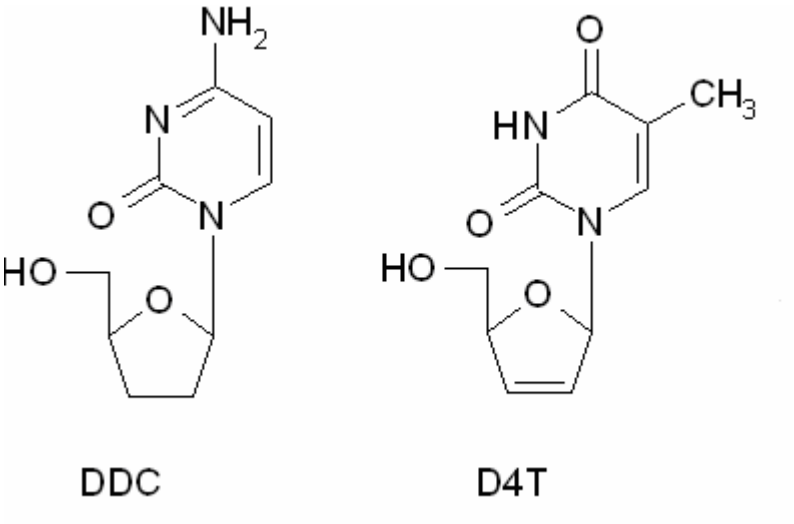
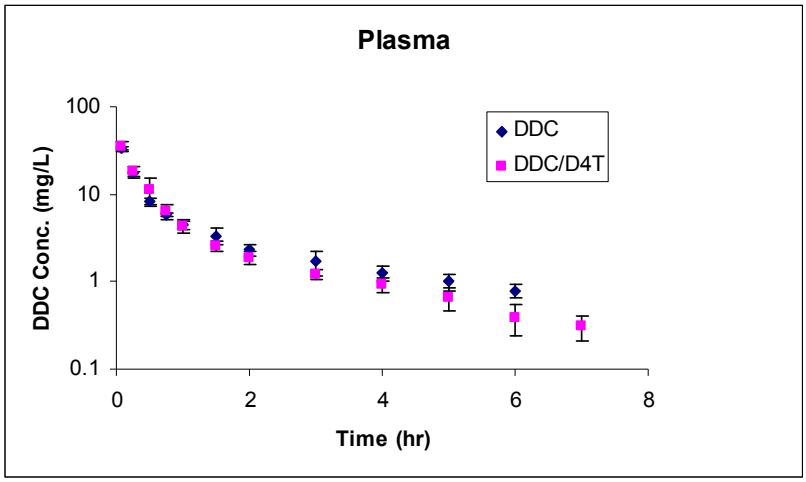
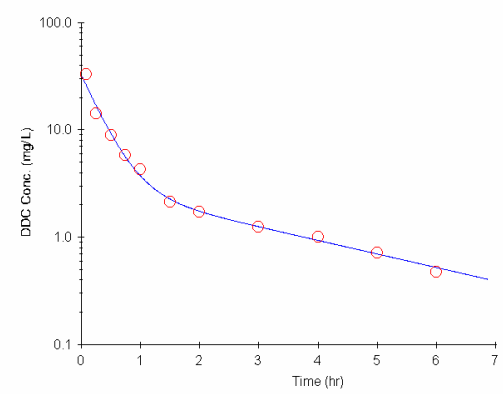


Figure 5.1 Chemical structures of DDC and D4T

(a)



(b)



(c)

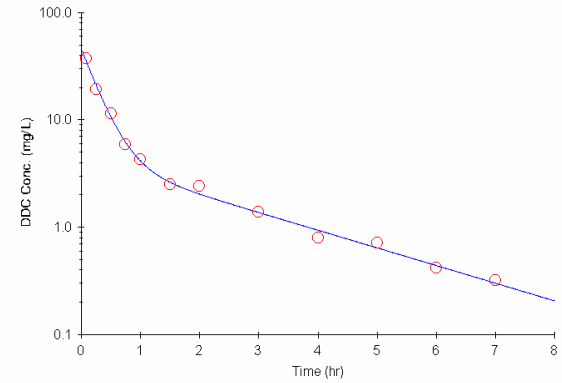
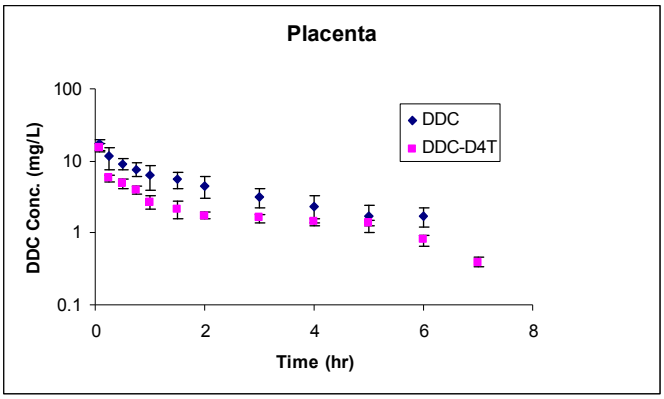
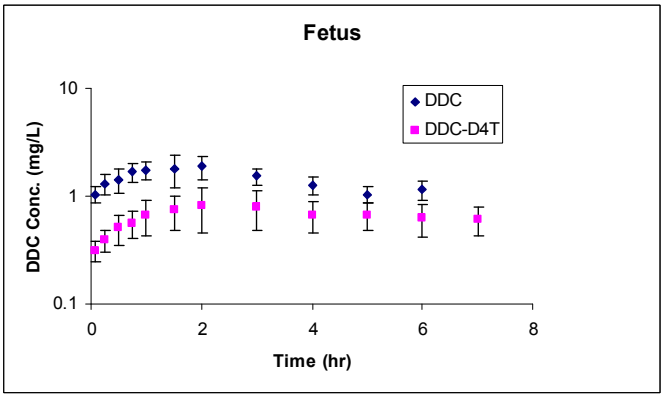


Figure 5.2. (a)Concentration in plasma versus time profiles for DDC alone and in combination with D4T; Fitted plasma versus time profiles for DDC alone (b) and in combination with D4T (c)

(a)



(b)



(c)

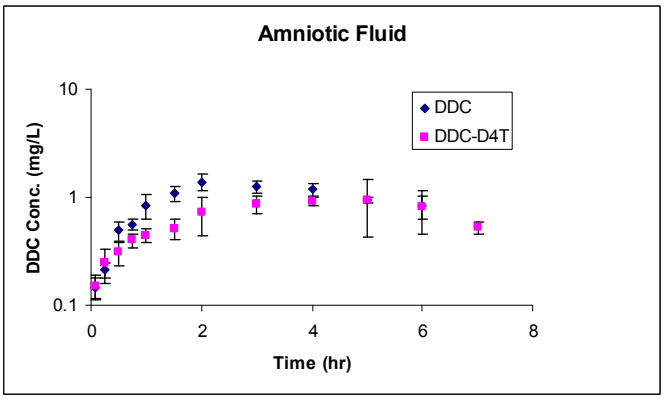


Figure 5.3. Concentration (mean plus standard deviation) versus time profiles of DDC alone and in combination with D4T from placenta (a), fetus (b), and amniotic fluid (c)

CHAPTER 6

SIMULTANEOUS DETERMINATION OF ZALCITABINE AND STAVUDINE IN MATERNAL PLASMA, AMNIOTIC FLUID, PLACENTAL AND FETAL TISSUES USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETY[1]

---

[1]Meng Xu, Catherine A. White, Michael G. Bartlett. To be submitted to *Journal of Liquid Chromatography & Related Techniques*.

Abstract:

In order to study the placental transfer of nucleoside reverse transcriptase inhibitors, a quick and simple reversed phase high performance liquid chromatography with tandem mass spectrometry 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 protein precipitation using ice-cold acetonitrile. Chromatographic separation was achieved on a Waters Spherisorb S3W silica column (4.6mm × 100mm) equipped with a Phenomenex guard column. The mobile phase consisted of 20% methanol in 22mM formic acid. The flow rate was 0.4 ml/min, and MRM was used for detection. The calibration curves for each day of validation showed good linear response over the range from 2 ng/ml to 2000 ng/ml. The absolute recoveries for all the drugs are all higher than 70%, and the matrix effects are all lower than 20%. All the intra- and inter- day assay precision and accuracy were better than 10% for all the matrices.

Keywords: Zalcitabine; Stavudine

1. Introduction

For children, about 90% of the human immunodeficiency virus (HIV) infections are due to vertical transmission from the infected mothers through blood, amniotic fluid, and/or breast milk. [1, 2] This perinatal transmission of HIV occurs as a result of transplacental dissemination of the virus and intrapartum exposure to infected blood in genital tract. [3-5]

With the number of HIV infections increasing, it is essential to determine the placental transfer of antivirals because of the increased resistance of HIV to zidovudine. [6, 7] In addition, the use of multidrug therapies has become the rule rather than the exception in the treatment of patients with HIV infections.[8]The use of these drugs is important in maintenance of maternal health through pregnancy and in possible prevention of vertical transmission of HIV. [7] While the use of combinations of antiviral drugs is popular, the impact of such combination therapies on placental transport is largely unknown.

Our previous studies showed substantial interactions between the antivrals AZT and acyclovir, AZT and 3TC and between Zalcitabine (DDC) and Stavudine (D4T). [9-12] The data from these studies support a transporter-mediated mechanism for placental transport. However, 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.[13-15] The differences between these studies may be related to the animal

models, experimental design, or may be specific to the agents studied. More important, the dose used in our previous studies is several times higher than the dose used in human beings or the doses used in macaques from other studies. In order to obtain pharmacokinetic data which is more directly comparable to other studies, we decided to conduct a pharmacokinetic study in rats with a dose of 5 mg/kg of the DDC/ D4T combination. Several HPLC methods have been developed to determine the concentrations of DDC and D4T.[16-23] An HPLC-UV method was developed previously for the simultaneous determination of DDC and D4T in the pregnant rat model within a linear range from 0.1μg/ml to 50μg/ml. [12] However, these methods are either not sensitive enough or do not include all the necessary matrices we are going to use. Thus, a sensitive and specific analytical method was needed for this pharmacokinetic study. In this study, a rapid and sensitive HPLC- tandem MS 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.

2. Experimental

2.1. 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).

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. Instrumentation

An Agilent 1100 series HPLC system, consisting of a degasser, binary pump, autosampler, and thermostatted column compartment, was used in this study (Agilent, Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Quattro Micro triple-quadrupole mass spectrometer equipped with a Z-spray ion source (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters, Beverly, MA, USA). Chromatographic separation was achieved on a Waters Spherisorb S3W silica column (4.6×100.0mm, Milford, MA) equipped with a Phenomenex security guard C-18 guard column (4×3.0mm, Torrance, CA).

2.3. Liquid chromatographic and mass spectrometric conditions

The mobile phase consisted of Solvent A (22mM formic acid) and Solvent B (methanol) (80:20). The flow rate was set to be 0.4 ml/min, and the injection volume was 50µl. The HPLC run time was 13 min for each run. The LC flow was diverted to waste from 0 to 3 minutes using a six-port switching valve. The mass spectrometer was run in positive ion ESI mode using multiple-reaction monitoring (MRM) to monitor the mass transitions. Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 l/h with a temperature of 375 $^{\circ}$C. The cone gas flow was set to 0 l/h. Argon was the collision gas and the collision cell pressure was 2.4×10^{-3} mbar. The precursor to product

ion transitions along with the cone voltage and collision energy for each analyte and the internal standard were as follows: DDC *m*/*z* 212→*m*/*z* 112, 13V, 7 eV; D4T *m*/*z* 247→*m*/*z* 149 (sodium adducts), 15V, 10 eV; 3TC *m*/*z* 230→*m*/*z* 112, 13V, 7 eV. The source temperature and capillary voltage were set at 130 ◦C and 2.5 kV, respectively.

2.4. Preparation of standard solutions

Individual DDC, D4T and 3TC stock solutions were prepared in deionized water to give a final concentration of 10 mg/ml. Individual standard solutions of DDC and D4T with concentrations of 10, 50, 100, 500, 1000, 5000 and 10000 ng/ml were prepared by serial dilution with deionized water. Precision and accuracy standards with concentrations of 25, 250 and 2500 ng/ml were also prepared in the same manner. A 1μg/ml 3TC standard solution was prepared with deionized water from the 10 mg/ml 3TC stock solution. The 10 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.

2.5. Calibration curves

Blank plasma was purchased from Innovative Research (Novi, MI, 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 100μl of the

biological matrices with 20µl of each DDC and D4T standard solution and 10µl of the 1µg/ml 3TC solution. Amniotic fluid calibration points were prepared by spiking 50µl of the biological matrices with 10µl of each DDC and D4T standard solution and 10µl of the 1µg/ml 3TC standard solution. The calibration curves of all the matrices were in the range of 2–2000 ng/ml with individual calibration points of 2, 10, 20, 100, 200, 1000 and 2000 ng/ml.

2.6. 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 four matrics were 2, 5, 50 and 500 ng/ml.

2.7. Sample preparation

All samples were prepared using protein precipitation. 1 ml of ice cold acetonitrile was added to the samples (100µl for plasma, placental and fetal homogenate, 50µl for amniotic fluid). After being vortexed and centrifuged at 13,000 rpm for 10 min, the solid was discarded and the liquid was aspirated and dried under vacuum. Samples were then reconstituted in distilled water for injection. (100µl for plasma, placental and fetal homogenate, 60µl for amniotic fluid)

2.8. 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^{o}C, 14 h of light per day) with daily feedings of standard chow pellets and water ad libitum.

Three timed pregnant Sprauge–Dawley rat (Harlan, Indianapolis, IN, USA), weighing from 320 to 380 g, were 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 (5 mg/kg) of 5 mg/ml DDC and D4T 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, and fetus samples were collected at 5, 15, 30, 45, 60, 90, 120, 180 and 240, 300 and 360 min. Placental and fetal tissue samples were homogenized in two volumes of deionized water. All samples were stored at −20 $^{\circ}$C until analysis. Data was analyzed using WinNonlin (Version 5.2, Pharsight, Mountain View, CA, USA).

3. Results and Discussion

3.1. Method development

Hydrophilic interaction chromatography (HILIC) is characterized by the presence of a high initial concentration of organic modifier to favor hydrophilic interaction between

the solute and the hydrophilic stationary phase. [24] Briefly, HILIC based on silica columns is normal-phase chromatography utilizing conventional reversed-phase (RP) mobile phases. Thus, the retention times of highly polar compounds are increased as their hydrophilicity increases. Since both DDC and D4T are highly hydrophilic compounds, HILIC is a good choice for retention. However, although D4T worked well under HILIC conditions (90% to 80% organic); DDC was permanently retained on the silica column when the percentage of organic solvent was higher than 30 percent. On the other hand, both DDC and D4T were well retained on the silica column with a high aqueous (10% to 20%) mobile phase. Thus, we chose to use 20% methanol on the silica column as our chromatographic conditions which was different from both traditional reverse phase conditions and HILIC conditions.

During optimization of MS conditions, we found that the intensity of D4T plus sodium peak was 10-fold higher than the M+H peak, since D4T is a weak acid. Although it is rare to use adduct ions for quantitation, since the intensity of many are highly variable. Besides, our method meets validation requirements under FDA guideline. [25] Fig. 1-4 shows the representative chromatograms of each extracted blank matrix and extracted matrix spiked with DDC (20 ng/ml), D4T (20 ng/ml) and 3TC (100 ng/ml).

3.2. Calibration curves

The calibration curves for each day of validation and analysis showed good linear response (R^2 = 0.992–0.995) over the range of 2–2000 ng/ml for both drugs in all matrices. Microsoft Excel or JMP statistical software was used to generate linear

regression equations for all calibration curves. A $1/x^2$-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.

3.3. 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 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 2, 5, 50, and 500 ng/ml were used for these calculations. Intra-day precision (% R.S.D.) and accuracy (% error) of DDC ranged from 1 to 7.5% and 0.48 to 9.46%, respectively. Inter-day precision and accuracy of DDC ranged from 1.32 to 9.6% and 0.06 to 6.2%, respectively. Intra-day precision and accuracy of D4T ranged from 0.5 to 7.2% and 0.4 to 8.11%, respectively. Inter-day precision and accuracy of D4T ranged from 1 to 9.2% and 0.48 to 6.69%, respectively. These results are shown in Table 2 and Table 3.

3.4. Recovery and matrix effect

Absolute recovery, relative recovery and matrix effect for DDC, D4T and 3TC are summarized in Table 4. Samples spiked with a drug concentration of 100 ng/mL were used for these calculations. Absolute recoveries ranged from 70.8% to 86.0%, and relative recoveries ranged from 84.8% to 98.5%. The suppression or the enhancement by

the matrix was lower than 20% for all compounds.

3.5. Stability studies

Stability testing was performed for DDC and D4T at 100 ng/ml concentration level. 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 days, the spiked matrix samples were thawed, and three more were extracted and analyzed. The freeze/thaw stability tests indicate both DDC and D4T were stable over three consecutive freeze/thaw cycles. (See Table 5) 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 24h, the same sample from each matrix was injected again. The peak areas for DDC and D4T in each injection were compared. The error between each sample was <10% for both compounds and there was no obvious changes in peak areas between each injection. (See Table 6)

3.6. Animal study

To demonstrate the utility of this assay, 3 pregnant rats were dosed with DDC and D4T at the level of 5 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 and D4T present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 100 ng/ml of the internal standard

3TC. Fig. 5 shows the mean concentration–time profile of DDC and D4T in all four biological matrices of the pregnant rats. 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 155min, a steady state volume of distribution of 1.38 l/kg, and total clearance of 0.64 l/h/kg based on the maternal plasma data. D4T has a half-life of 77 min, a steady state volume of distribution of 1.82 l/kg, and total clearance of 0.88 l/h/kg based on the maternal plasma data. Animal studies with an IV bolus administration of a dose of 25mg/kg of DDC-D4T were also conducted for comparison to the DDC-D4T combination low dose. In that study, DDC has a half-life of 112 min, a steady state volume of distribution of 1.6 l/kg, and total clearance of 1.2 l/h/kg in the maternal plasma; D4T has a half-life of 113 min, a steady state volume of distribution of 0.6 l/kg, and total clearance of 0.34 l/h/kg in the maternal plasma. Therefore, the clearance for DDC at the higher dose was about 2-fold higher than at the lower dose but it was the opposite for D4T, which showed a 2-fold decrease in clearance at the higher dose. The steady state volume of distribution for D4T also decreased approximately 2-3 fold at the higher dose. This preliminary data showed that D4T may be up regulating transporters for DDC in the kidney; on the other hand, DDC may inhibit the active tubular secretion of D4T in the kidney. Significant differences in relative exposures ($AUC_{tissue}/AUC_{maternal\ plasma}$) of the placenta were also noticed for DDC and D4T at the two doses. The relative exposure of the placenta was significantly lower for DDC at the lower dose (0.28 with the lower dose vs. 0.6 with the higher dose). However, the opposite effect was observed for

D4T, which had a higher relative exposure for the placenta at the lower dose. (0.46 with the lower dose vs. 0.29 with the higher dose). No significant difference was noted in the relative exposures of the amniotic fluid for DDC and D4T. The relative exposures of both DDC and D4T in the fetus were increased with the increase in dose. The results suggested that with the increase in dose, there is an increase in the distribution of DDC into the placenta and fetus. The increase in fetal levels of D4T may due to the saturation of the efflux transporters from fetus to placenta. This preliminary data suggests that the interactions between DDC and D4T are not linear at different doses, which suggests that active transporters may play a role in placental transfer for both DDC and D4T. The fact that the transport of nucleosides appears to be non-linear also suggest that the differences may be related to regulation of the transporters. However, additional in vivo and in vitro studies will be needed to fully elucidate this mechanism.

4. Conclusion

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

References

1. Centers for Disease Control and Prevention. HIV/AIDS Surveillance Report: Cases of HIV Infection and AIDS in the United States, 2004. Atlanta: The Centers; 2005. Vol. 16. (Available at http://www.cdc.gov/hiv/topics/surveillance/resources /reports /2004report/default.htm.)
2. Hansen, M. *Pathophysiology: Foundations of Disease and Clinical Intervention*, W.B. Saunders Company, Philadelphia, United States, 1998.
3. Duff, P. HIV Infection in Women. *Prim Care Update Ob Gyns.* 1996, 3, 45-49.
4. Bryson, Y. Perinatal HIV-1 Transmission: Recent Advances and Therapeutic Interventions. *AIDS.* 1996, 10, S33-42.
5. John, G.C.; Kreiss, J. Mother-to-Child Transmission of HIV. *Epidemiol Rev.* 1996, 18, 149-157.
6. Recommendations for Use of Antiretroviral Drugs in Pregnant HIV-1 Infected Women for Maternal Health and Interventions to Reduce Perinatal HIV-1 Transmission in the United States, 1994. (Available at http://aidsinfo.nih.gov)
7. Pacifici, G.M. Transfer of Antivirals across the Human Placenta. 2005, 81, 647-654.
8. Beach, J.W. Chemotherapeutic Agents for Human Immunodeficiency Virus Infection: Mechanism of Action, Pharmacokinetics, Metabolism, and Adverse Reactions. *Clin. Therapeu.* 1998, 20 (1), 2-25.

9. Alnouti, Y.; Lewis, S.R.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zidovudine and Lamivudine from Rat Tissues by Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 2005, 19, 503–508.

10. Alnouti, Y.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zidovudine and Lamivudine from Rat Plasma, Amniotic Fluid and Tissues by HPLC. *Biomed. Chromatogr.* 2004, 18, 641–647.

11. Brown, S.D.; Bartlett, M.G.; White, C.A. Pharmacokinetics of Intravenous Acyclovir, Zidovudine, and Acyclovir-Zidovudine in Pregnant Rats. *Antimicrob. Agents Chemother.* 2003, 47(3), 991–996.

12. Xu, M.; White, C.A.; Bartlett, M.G. Simultaneous Determination of Zalcitabine and Stavudine in Rat Plasma, Amniotic Fluid, Placental and Fetal Tissues using High Performance Liquid Chromatography. *J Liq Chromatogr.* 2008, 31, 482–496.

13. Pereira, C.M.; Nosbisch, C.; Baughman, W.L.; Unadkat, J.D. Effect of Zidovudine on Transplacental Pharmacokinetics of ddI in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 1995, 39, 343-345.

14. Odinecs, A.; Nosbisch, C.; Unadkat, J.D. Zidovudine does not Affect Transplacental Transfer or Systemic Clearance of Stavudine (2', 3'-didehydro-3'-deoxythymidine) in the Pigtailed Macaque (Macaca nemestrina). *Antimicrob. Agents Chemother.* 1996, 40, 1569-1571.

15. Tuntland, T.; Nosbisch, C.; Baughman, W.L.; Massarella, J.; Unadkat, J.D.; Mechanism and Rate of Placental Transfer of Zalcitabine (2',3'-dideoxycytidine) in Macaca nemestrina. *Am. J. Obstet. Gynecol.* 1996, 174, 856-863.

16. Ding, Y.; Williamson, L.N.; White, C.A.; Bartlett, M.G. Determination of 2', 3'-Dideoxycytidine in Maternal Plasma, Amniotic Fluid, Placental and Fetal Tissues by High-Performance Liquid Chromatography. *J. Chromatogr. B.* 2004, 811, 183–189.

17. Contreras, J.; Gonzalez, H.M.; Menendez, R.; Lopez, M. Development and Validation of a Reversed-Phase Liquid Chromatographic Method for Analysis of D4T (Stavudine) in Rat Plasma. *J. Chromatogr. B.* 2004, 801(2), 199-203.

18. Fan, B.; Bartlett, M.G.; Stewart, J.T. Determination of Lamivudine/Stavudine/ Efavirenz in Human Serum Using Liquid Chromatography/Electrospray Tandem Mass Spectrometry with Ionization Polarity Switching. *Biomed. Chromatogr.* 2002, 16(6), 383-389.

19. Frijus-Plessen, N.; Michaelis, H.C.; Foth, H.; Kahl, G.F. Determination of 3'-Azido-3'-Deoxythymidine, 2',3'-Dideoxycytidine, 3'-Fluoro-3'- Deoxythymidine and 2',3'-Dideoxyinosine in Biological Samples by High-Performance Liquid Chromatography. *J. Chromatogr.* 1990, 534, 101-107.

20. Bezy, V.; Morin, P.; Couerbe, P.; Leleu, G.; Agrofoglio, L. Simultaneous Analysis of Several Antiretroviral Nucleosides in Rat Plasma by High Performance Liquid Chromatography with UV Using Acetic Acid / Hydroxylamine Buffer: Test of this

New Volatile Medium-pH for HPLC-ESI-MS/MS. *J. Chromatogr. B.* 2005, 821(2), 132-143.

21. Kapoor, N.; Khandavilli, S.; Panchagnula, R. Simultaneous Determination of Lamivudine and Stavudine in Antiretroviral Fixed Dose Combinations by First Derivative Spectrophotometry and High Performance Liquid Chromatography. *J. Pharmaceut. Biomed. Anal.* 2006, 41(3), 761-765.
22. Huang, Y.; Zurlinden, E.; Lin, E.; Li, X.; Tokumoto, J.; Golden, J.; Murr, A.; Engstrom, J.; Conte, J. Liquid Chromatographic-Tandem Mass Spectrometric Assay for the Simultaneous Determination of Didanosine and Stavudine in Human Plasma, Bronchoalveolar Lavage Fluid, Alveolar Cells, Peripheral Blood Mononuclear Cells, Seminal Plasma, Cerebrospinal Fluid and Tonsil Tissue. *J. Chromatogr. B.* 2004, 799(1), 51-61.
23. Wong, S.L.; Sawchuk, R.J. High-Performance Liquid Chromatographic Determination of 2',3'-Didehydro-3'-Deoxythymidine (d4T) in Human and Rabbit Plasma and Urine and its Application to Pharmacokinetic Studies in the Rabbit. *Pharm. Res.* 1991, 8(5), 619-623.
24. Naidong, W. Bioanalytical Liquid Chromatography Tandem Mass Spectrometry Methods on Underivatized Silica Columns with Aqueous/Organic Mobile Phases. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2003, 796(2), 209-224.
25. F.D.A. U.S. Department of Health and Human Services, 2001.

(A) (B)

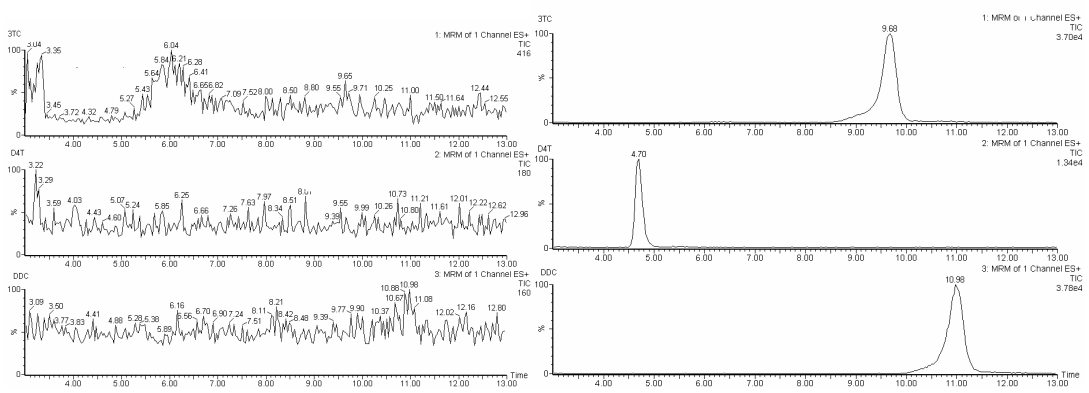


Fig.6.1 Chromatograms obtained from blank plasma (A) and plasma spiked with DDC (20 ng/ml), D4T (20 ng/ml) and 3TC (100 ng/ml) (B)

(A) (B)

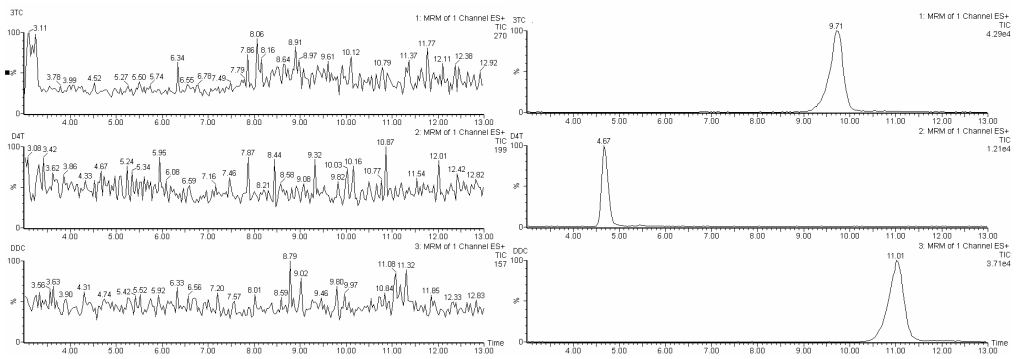


Fig.6.2 Chromatograms obtained from blank amniotic fluid (A) and plasma spiked with DDC (20 ng/ml), D4T (20 ng/ml) and 3TC (100 ng/ml) (B)

(A) (B)

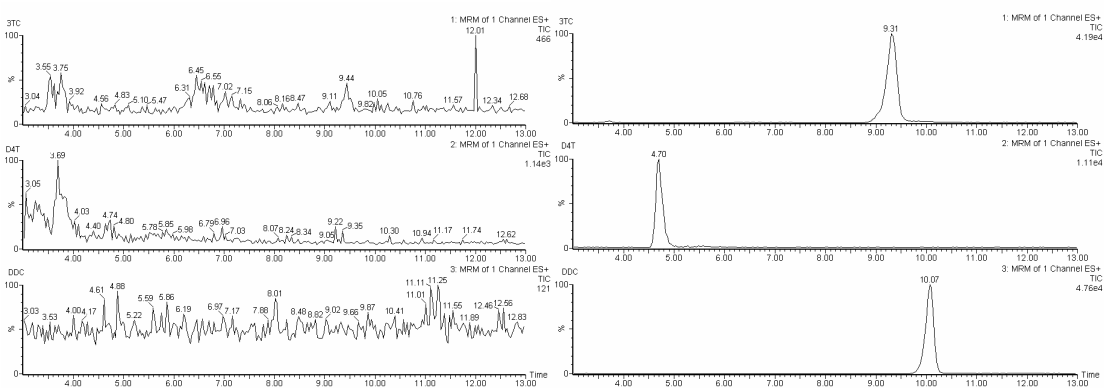


Fig.6.3 Chromatograms obtained from blank placental homogenate (A) and plasma spiked with DDC (20 ng/ml), D4T (20 ng/ml) and 3TC (100 ng/ml) (B)

(A) (B)

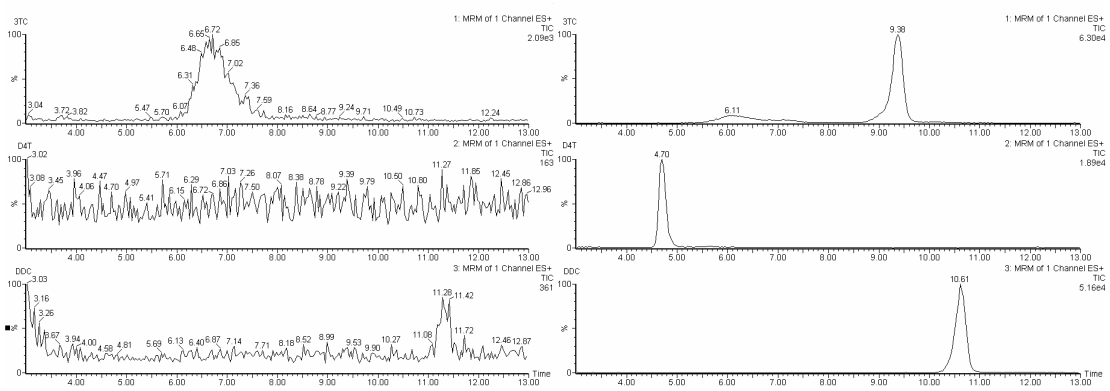
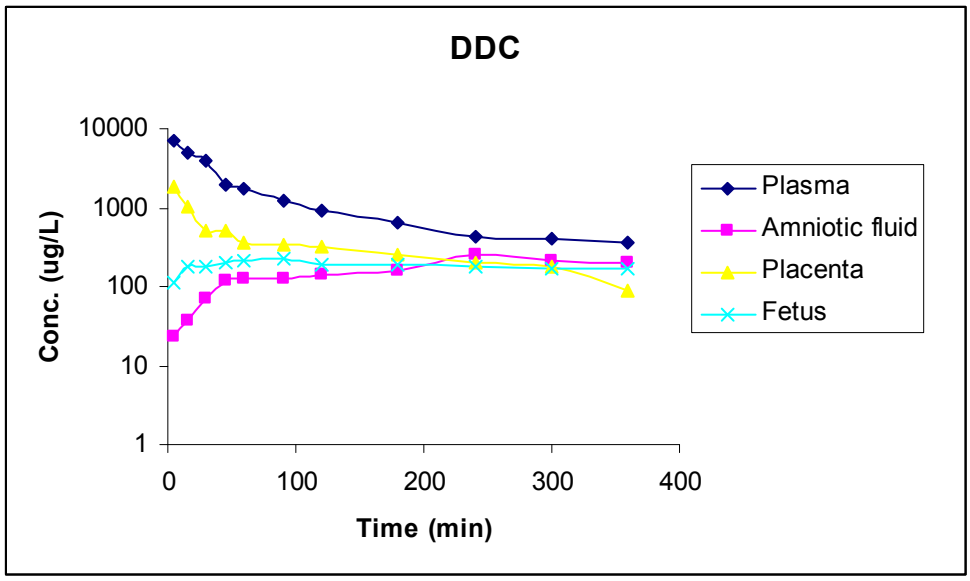


Fig.6.4 Chromatograms obtained from blank fetal homogenate (A) and plasma spiked with DDC (20 ng/ml), D4T (20 ng/ml) and 3TC (100 ng/ml) (B)



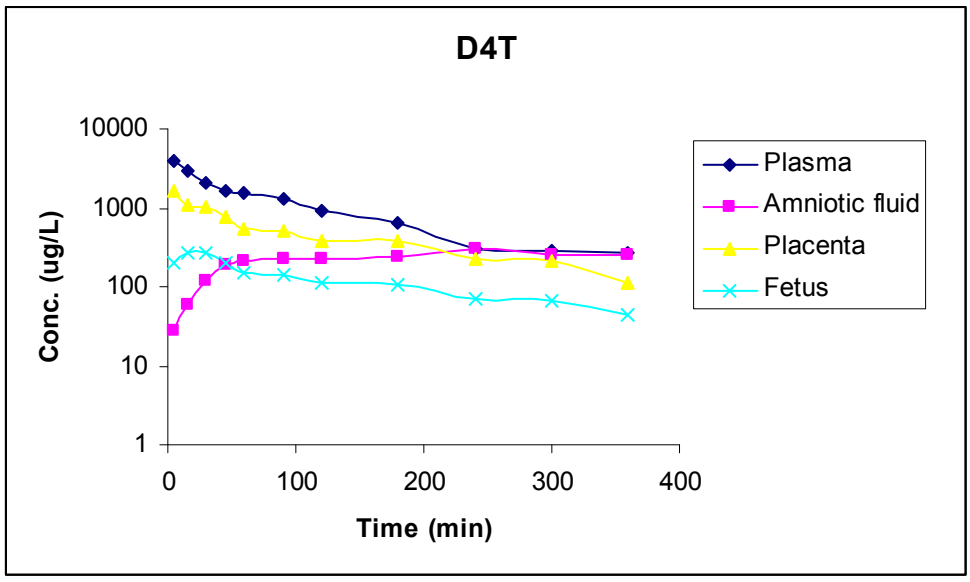


Fig. 6.5 Concentration vs. time profile of DDC and D4T in maternal plasma, amniotic fluid, placenta and fetus after 5 mg/kg i.v. bolus dose of DDC and D4T (n=3)

Table 6.1 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				
	0.993±0.002	0.995±0.003	0.992±0.001	0.993±0.001
D4T				
	0.995±0.001	0.994±0.001	0.994±0.001	0.992±0.001

Table 6.2 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 LC-MS/MS method used to quantitate DDC in maternal plasma, amniotic fluid, placental and fetal homogenates

Concentration DDC added	Intra-day (n=5)			Inter-day (n=15)		
(ng/ml)	Concentration DDC found (ng/ml)	R.S.D. (%)	Error (%)	Concentration DDC found (ng/ml)	R.S.D. (%)	Error (%)
Maternal plasma						
2	2.14±0.08	4	7	2.06±0.15	7.5	3
5	4.87±0.05	1	2.6	5.13±0.12	2.4	2.6
50	51.64±2.56	5.12	3.28	50.93±3.43	6.86	1.86
500	509.24±7.82	1.56	1.85	506.14±7.92	1.58	1.23
Amniotic fluid						
2	2.05±0.04	2	2.5	2.10±0.06	3	5
5	5.19±0.08	1.6	3.8	5.31±0.16	3.2	6.2
50	52.93±3.45	6.9	5.86	51.42±4.21	8.42	2.84
500	514.01±6.76	1.35	2.8	516.73±8.02	1.6	3.35
Placental homogenate						
2	2.11±0.02	1	5.5	1.99±0.19	9.5	0.5
5	5.08±0.07	1.4	1.6	5.26±0.48	9.6	5.2
50	49.76±0.59	1.18	0.48	49.97±3.91	7.82	0.06
500	479.70±5.97	1.19	4.06	497.44±6.59	1.32	0.51
Fetal homogenate						
2	1.87±0.15	7.5	6.5	2.10±0.12	2.4	5
5	4.79±0.24	4.8	4.2	5.01±0.26	5.2	0.2
50	50.04±1.98	3.96	0.8	49.68±3.25	6.5	0.64
500	485.27±9.16	1.83	9.46	496.66±9.60	1.92	0.67

Table 6.3 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 LC-MS/MS method used to quantitate D4T in maternal plasma, amniotic fluid, placental and fetal homogenates

Concentration D4T added	Intra-day (n=5)			Inter-day (n=15)		
(ng/ml)	Concentration D4T found (ng/ml)	R.S.D. (%)	Error (%)	Concentration D4T found (ng/ml)	R.S.D. (%)	Error (%)
Maternal plasma						
2	2.15±0.03	1.5	7.5	2.04±0.03	1.5	2
5	5.31±0.08	1.6	6.2	5.06±0.10	2	1.2
50	52.77±1.64	3.28	5.54	51.62±2.59	5.18	3.24
500	519.28±5.12	1.02	3.86	512.38±16.27	3.25	2.48
Amniotic fluid						
2	2.06±0.01	0.5	3	2.13±0.18	9	6.5
5	5.08±0.04	0.8	1.6	5.10±0.36	7.2	2
50	51.69±4.25	8.5	3.38	50.24±3.55	7.1	0.48
500	508.43±3.96	0.79	1.69	506.64±20.47	4.09	1.32
Placental homogenate						
2	1.96±0.16	8	2	1.89±0.17	8.5	5.5
5	5.02±0.13	2.6	0.4	4.85±0.32	6.4	3
50	47.98±3.47	6.94	2.02	47.69±2.56	5.12	4.62
500	468.74±15.28	3.06	6.25	487.66±18.86	3.77	2.47
Fetal homogenate						
2	1.94±0.04	2	3	1.88±0.02	1	6
5	4.85±0.36	7.2	3	4.75±0.44	8.8	5
50	49.09±0.87	1.74	1.82	48.45±4.60	9.2	3.1
500	459.44±12.68	2.54	8.11	466.56±25.99	5.2	6.69

Table 6.4. The absolute, relative recovery ± S.D. (n = 3) and matrix effect of DDC, D4T and 3TC from maternal plasma, amniotic fluid, placental and fetal homogenates

Drugs and matrices	Absolute recovery (%)	Relative recovery (%)	Matrix effect(%)
100 ng/ml spiked Maternal plasma			
3TC	78.6±2.3	90.9±2.6	-15.6
DDC	86.0±3.7	98.5±4.2	-14.5
D4T	76.8±1.0	86.5±1.3	-12.7
Amniotic fluid			
3TC	76.9±1.5	92.1±4.8	-19.8
DDC	84.6±4.4	91.7±3.1	-16.5
D4T	75.8±3.2	89.3±0.9	-17.8
Placental homogenate			
3TC	70.8±4.2	82.9±2.2	-17.1
DDC	72.9±3.8	85.8±1.4	-17.7
D4T	70.8±1.9	84.8±4.5	-19.8
Fetal homogenate			
3TC	79.6±0.8	93.7±4.7	-17.5
DDC	78.0±1.3	87.6±2.4	-12.3
D4T	77.9±3.6	86.1±2.8	-10.6

Table 6.5 Results of freeze/thaw stability of DDC and D4T in maternal plasma, amniotic fluid, placental and fetal homogenates (n=3).

Freeze-thaw Stability (3 cycles)	Observed conc. ± S. D. (ng/ml)	RSD (%)	Error (%)
100 ng/ml spiked Maternal plasma			
DDC	102.5±4.3	4.3	2.5
D4T	106.2±2.8	2.8	6.2
Amniotic fluid			
DDC	105.7±6.1	6.1	5.7
D4T	104.0±8.4	8.4	4.0
Placental homogenate			
DDC	112.6±6.8	6.8	12.6
D4T	113.2±4.4	4.4	13.2
Fetal homogenate			
DDC	110.8±8.3	8.3	10.8
D4T	108.9±9.8	9.8	9.8

Table 6.6 Results of autosampler stability of DDC and D4T in maternal plasma, amniotic fluid, placental and fetal homogenates (n=3).

Autosampler stability (24h)	Observed conc. ± S. D. (ng/ml)	RSD (%)	Error (%)
100 ng/ml spiked Maternal plasma			
DDC	102.1±3.3	3.3	2.1
D4T	101.2±2.6	2.6	1.2
Amniotic fluid			
DDC	101.4±4.5	4.5	1.4
D4T	100.9±5.2	5.2	0.9
Placental homogenate			
DDC	105.8±6.6	6.6	5.8
D4T	109.6±4.8	4.8	9.6
Fetal homogenate			
DDC	106.7±4.0	4.0	6.7
D4T	103.9±3.6	3.6	3.9

CHAPTER 7

CONCLUSIONS

With the number of HIV infections increasing, preventing the vertical transmission of HIV from an infected mother to her unborn children has become more essential. Antiviral agents are presumed to prevent the transmission of infections from mother to fetus by decreasing maternal viral load and/or accumulation of drugs in the fetal compartment. The use of therapeutic agents in pregnant women has always presented a challenge to the clinician, trying to balance benefit of a therapeutic agent to both the mother and her fetus against the risk of drug-induced adverse effects on the developing fetus or mother. Nucleoside reverse transcriptase inhibitors (NRTIs) compose the first class of antiretroviral drugs developed, and these drugs have been clinically shown to prevent vertical transmission during pregnancy. Thus, gaining a complete understanding of the extent of fetal exposure of the NRTIs is important for safe drug and therapy. However, the conduct of detailed studies on maternal-fetal drug disposition in pregnant women is limited by technical and ethical considerations. Thus, it is important to employ a suitable animal model to gain a greater understanding of the processes involved in the maternal-placental-fetal system. The purpose of these experiments was to identify the factor involved in the placental transfer of several nucleoside antiviral agents alone and in combination with other agents and to determine if interactions were present

at the maternal-fetal interface.

Pharmacokinetic studies cannot be conducted without a validated analytical method. First, an HPLC-UV method that simultaneously determined DDC and D4T in maternal plasma, amniotic fluid, placental and fetal tissues was developed and validated. This method was utilized to study the pharmacokinetics of DDC dosed alone and in combination with D4T at a dose of 25 mg/kg. Then, a sensitive LC-MS/MS method was developed to study the pharmacokinetics of DDC and D4T with a dose of 5 mg/kg.

Although DDC and 3TC have similar structures and physical-chemical properties, the pharmacokinetics and placental transfer of DDC and 3TC were quite different. The clearance of 3TC was significantly lower than that of DDC resulting in a significantly higher AUC for 3TC. With both drugs the placental half-life was equivalent to the maternal plasma half-life; whereas, the fetal and amniotic fluid concentrations declined with a significantly longer half-life (2-fold increase). The relative exposure for DDC in the placenta and fetus were significantly higher than those observed for 3TC. These significant differences suggest that active transport may play a role in the transplacental transfer of both DDC and 3TC.

The effects of D4T on DDC during placental transfer in pregnant rat were also studied. The maternal pharmacokinetics were similar for DDC alone and DDC in combination with D4T, however, the relative exposure for DDC given in combination in the placenta and fetus were significantly lower than DDC alone, which suggested that D4T either blocked the movement of DDC into placenta, or increased the efflux of DDC

from the placenta back into the maternal plasma. These findings suggest that active transport may play a role in the transplacental transfer of DDC.

The pharmacokinetics with animals dosed with 25 mg/kg and 5 mg/kg of DDC/D4T combination were compared. The preliminary data suggests that the interactions between DDC and D4T were not linear at different doses and that active transporters may play a role in placental transfer for both DDC and D4T. The fact that the transport of nucleosides appears to be non-linear also suggest that the differences may be related to regulation of the transporters.

APPENDIX 1

DETERMINATION OF DIISOPROPYLFLUOROPHOSPHATE (DFP) IN RAT PLASMA AND BRAIN TISSUE BY HEADSPACE SOLID-PHASE MICROEXTRACTION GAS CHROMATOGRAPHY MASS SPECTROMETRY[1]

---

[1]Meng Xu, Alvin V. Terry Jr., and Michael G. Bartlett. Submitted to *Rapid Communications in Mass Spectrometry*, 05/08

ABSTRACT

A simple, sensitive and rapid method for the determination of diisopropylfluorophosphate (DFP) in rat plasma and brain tissue using headspace solid-phase microextraction (HS-SPME) and gas chromatography/mass spectrometry (GC/MS) is presented. A 65 μm polydimethylsiloxane/ divinylbenzene (PDMS/DVB) fiber was selected for sampling, and the main parameters affecting the SPME process such as extraction and desorption temperature, extraction and desorption time, salt addition, and fiber preheating time were optimized in each matrix to enhance the extraction efficiency and the sensitivity of the method. The lower limit of quantitation for DFP in plasma and brain tissue was 1 ng/ml and 3 ng/g, respectively. The method showed good linearity over the range from 1-100 ng/ml in plasma and 3-300 ng/g in brain tissue with correlation coefficient (R^2) values higher than 0.995. The precision and accuracy for intra-day and inter-day were less than 10%. The relative recoveries in plasma and brain for DFP were greater than 50%. Stability tests including autosampler and freeze and thaw were also investigated. This validated method was successfully applied to study the neurobehavioral effects of low-level OP (organophosphate) exposures.

INTRODUCTION

Organophosphates (OP) are used both as insecticides or chemical warfare agents. They inhibit cholinesterases by phosphorylation at the active-site serine and acute or chronic exposure to OP agents increases activity in CNS regions and peripheral organs served by acetylcholine (ACh)-containing neurons.[1-2] A considerable number of human and animal studies have focused on the long-term consequences of acute organophosphate (OP) exposure, however, relatively little attention has been given to the subject of chronic, "low-level" exposures that are not associated with acute cholinergic symptoms. [3] This may be a particular concern given the widespread use of OP insecticides (and consequent human exposure) in household, agricultural, and commercial environments worldwide and the ever-increasing threat of intentional OP poisoning by terrorists. During chronic exposure to low levels of OP agents, symptoms of overt toxicity (e.g., gastrointestinal disturbance, muscle fasciculations) may occur, but often subside with continued low level OP exposure.[4-6] Additionally, there is now significant evidence to suggest that chronic exposure to OPs is associated with impairments in attention, memory, and other domains of cognition and further, that these symptoms cannot be explained exclusively by cholinesterase inhibition.[3, 7-20] Thus, it is very critical to address the potential threat to society posed by chronic low-level OP exposure and alterations in cognitive function, as well as identify novel molecular mechanisms associated with these cognitive changes.

Diisopropylfluorophosphate (DFP) is a prototypical alkylphosphate organophosphate

that was originally developed as a chemical warfare agent by the British in 1941. It possesses a great deal of structural homology with other highly toxic nerve agents such as Sarin and Soman. However, it exhibits markedly reduced potency (in terms of lethality) compared to these compounds and as a consequence is one of the most extensively studied alkylphosphates.[21] DFP is known to bind to the active site of acetylcholinesterase and neurotoxic esterase and to produce an irreversibly inhibited enzyme by a mechanism known as "aging". These factors may contribute to the histopathologic lesions in the CNS, profound ataxia, and neurologic difficulties produced by DFP. However, these interactions (in particular, the degree of cholinesterase inhibition) are not necessarily predictive of the level of cognitive impairment observed in animals chronically exposed to the compound. [9, 22] Further, prolonged cognitive deficits were also observed in rats after exposure to relatively small doses of DFP for 14 days.[9] Therefore, it appears that the prolonged effects of DFP may not solely rely on inhibition of acetylcholinesterase or interactions with neurotoxic esterase. In order to look into these problems thoroughly, a rapid and sensitive analytical method which can determine DFP in both plasma and brain tissue is needed.

Solid-phase microextraction (SPME) is a miniaturized and solvent-free sample preparation technique for chromatographic-spectrometric analysis by which the analytes are extracted from a gaseous or liquid sample by absorption in, or adsorption on, a thin polymer coating fixed to the solid surface of a fiber, inside an injection needle or inside a capillary.[23] All steps of the conventional liquid-liquid extraction (LLE) such as

extraction, concentration, (derivatization) and transfer to the chromatograph are integrated into one step and one device, considerably simplifying the sample preparation procedure.[24] SPME has several advantages over classical GC techniques.[25] Firstly, SPME can pre-concentrate the samples due to the high affinity of the analyte for the fiber coating. Secondly, sample handling is minimized, which also helps to minimize the loss of volatile analytes and reduces potential error. Furthermore, since the biological molecules present in the sample do not partition into the headspace, matrix effects can be reduced by headspace SPME methods.[25]

In this study, a rapid and sensitive HS-SPME GC-MS method for the determination of DFP in rat plasma and brain tissue was developed. The lower limit of quantitation (LLOQ) for DFP is 1 ng/ml in plasma and 3 ng/g in brain tissue.

EXPERIMENTAL

Chemicals and reagents

Analytical grade DFP and ammonium sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, sodium fluoride, sodium carbonate and sodium sulfate were purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade isopropanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water used in experiments was generated from a Continental deionized water system (Natick, MA, USA). Ultra high purity (UHP) helium was purchased from National Welders (Charlotte, NC, USA).

Instrumentation

The analyses were carried out on an Agilent 6890 gas chromatograph coupled with a model 5973 mass selective detector (Palo Alto, CA, USA). The GC was equipped with a 0.75 mm i.d. SPME liner. Separation of the analytes was obtained on a ZB-5MS column (Phenomenex, Torrance, CA, USA: 30m×0.25mm i.d., 0.25μm film thickness) using helium as carrier gas (flow rate, 1ml/min). The GC injection port and interface transfer line were maintained at 250 and 280 ºC, respectively. During the fiber desorption process, the splitless mode of injection was used. After 2.5 min, the split vent valve was opened to sweep any residual vapors from the liner. The oven temperature was initially held at 60 ºC for 1 min, then increased to 100 ºC at 5 ºC /min, and held for 1 min. The mass spectrometer was operated in the positive electron ionization (EI) mode with an electron energy of 70 eV. Quantitation of DFP was performed using selected-ion monitoring (SIM) mode of m/z 101 (quantitation ion) and m/z 127 (confirmation ion). A solvent delay of 2.5 min was used to protect the filament from oxidation.

Preparation of standard solutions

A stock solution of DFP was prepared in isopropanol to yield a final concentration of 10 mg/ml. Standard solutions for the calibration curve were prepared from the stock solution at the following concentrations: 10, 25, 50, 100, 250, 500 and 1000 ng/ml. Standards used to assess precision and accuracy were prepared in isopropanol from the stock solution at concentrations of 10, 40, 400 and 800 ng/ml. All stock and standard solutions were refrigerated at 4 ºC during the day of use and were prepared fresh daily.

Sample preparation

Prior to extraction, brain tissue was homogenized with two volumes of deionized water (w/v) using a homogenizer (Polytron®, Brinkman, Switzerland). Samples for the calibration curves and QCs were prepared by adding 20.0 µl of the DFP standard into 200 µl blank plasma or brain homogenates. This yielded calibration standard concentrations of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml in plasma, 3.0, 7.5, 15.0, 30.0, 75.0, 150.0 and 300.0 ng/g in brain tissue. The final concentrations of QCs were 1.0, 4.0, 40.0, and 80.0 ng/ml in plasma, and 3.0, 12.0, 120.0 and 240.0 ng/g in brain tissue. Before adding any of the biological samples, approximately 0.01 g of NaF was added into a 2.0 ml SPME vial. All the autosampler vials were quickly sealed with PTFE-coated silicone septa and aluminum caps. The vials were vortexed for 5 min and set in the autosampler for analysis. Injections of blank matrices were run before each batch of samples to insure that there was not a detectable background level for DFP before beginning.

Headspace SPME procedure

Headspace SPME sampling was performed using a 65 µm polydimethylsiloxane/ divinylbenzene (PDMS/DVB) fiber (Sulpelco, Bellefonte, PA, USA) mounted on a Combi/Pal System autosampler (CTC Analytics, Zwingen, Switzerland). Fibers were conditioned at 250 ºC for 30 min prior to use. Sample vials were preheated in the agitator for 10 min before analysis and then the SPME fiber was exposed to the headspace by piercing the septum with the needle of the fiber assembly. After extraction for 15 min at 30 ºC under agitation, the fiber was withdrawn into the needle and immediately desorbed

at 250 ºC for 1 min into the GC injection port.

Method validation

The methods were validated for linearity, recovery, accuracy and precision. Calibration curves were generated by linear regression analyses of the peak area of DFP against the concentration applying a weighting of $1/x^2$. Precision (expressed as % relative standard deviation, RSD) and accuracy (expressed as % error) were calculated for four quality control (QC) samples. Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over three days in order to determine the inter-day accuracy and precision. Relative recoveries for the plasma method were calculated for spiked samples at different concentrations (n=5) by dividing the peak area for DFP spiked in the biological matrices by peak area for an equal concentration of DFP in deionized water. Because DFP was volatile, every precaution was taken to ensure it remained stable during analysis. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls. [26] The autosampler stability was evaluated over a period of 24 hours to determine if there was any loss of signal due to the time a sample spends in the autosampler prior to analysis. Freeze/thaw stability was investigated by comparing the samples following three freeze/thaw cycles against freshly spiked samples. Stability testing was performed at 5 ng/ml in plasma and 15ng/g in brain tissue.

Sampling

Male albino Wistar rats (Harlan, Indianapolis, IN) 2-3 months old were doubly housed in a temperature controlled room (25^{O}C), maintained on a reversed 12-hour light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI). All procedures employed during this study were reviewed and approved by the Medical College of Georgia Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines.

Each experimental group received subcutaneous injections of vehicle (Kroger® Pure Peanut Oil) or DFP 1.0, 0.75, 0.50 and 0.25 mg/kg dissolved in vehicle in a volume of 0.7 ml/kg body weight every other day over a 30 day treatment period.

For the plasma study, blood sampling occurred weekly throughout the DFP treatment regimen and during a subsequent two-week washout period. Rats were anesthetized by intraperitoneal injection (1 ml/kg body weight) of a cocktail containing ketamine (40 mg/ml) and xylazine (8 mg/ml). Blood was collected from the jugular vein using a 1.0 cc syringe fitted with a 25 G needle; 0.7 ml of blood was immediately added to a Microtainer® Plasma Separator Tube containing lithium heparin (BD catalog t#365958). This tube was inverted eight times, and then centrifuged according to the BD protocol. A small amount of the resulting plasma (40ul) was aliquoted into 0.5 ml tubes while the remaining plasma was added to 1.5 ml tubes. The aliquots were snap frozen in liquid nitrogen, and stored at −70° C until analyzed. For the brain study, at the end of the 30

day treatment, half of the rats were anesthetized by intraperitoneal injection (1 ml/kg body weight) of a cocktail containing ketamine (40 mg/ml) and xylazine (8 mg/ml); brains were harvested, cut in half sagittally and snap frozen in dry ice-chilled isopentane, and then stored at −70°C. The rest of the rat brains were harvested after the one week drug-free washout period.

RESULTS AND DISCUSSION

Optimization of the HS-SPME conditions

In order to optimize HS-SPME sampling conditions, we evaluated the effect of several parameters which were known to affect extraction efficiency, including fiber coatings, fiber position in the sample vial and GC-injector, sample preheating time, extraction time and temperature, desorption time and temperature and salt additives. The optimization was performed in both matrices, and the results were similar. In this paper, all the representative graphs were from brain tissue.

The first step in the development of any SPME methodology is the selection of a fiber coating for extracting the analyte of interest in the biological sample. [27] Three types of SPME fiber coatings (PDMS, PDMS/DVB and CAR/PDMS) were evaluated in this study for their efficiency in extracting DFP. PDMS/DVB and CAR/PDMS fibers work better than PDMS since DFP is a fairly polar compound. However, PDMS/DVB fiber was chosen because it shows a cleaner background than CAR/PDMS in the biological matrices since fewer peaks showed up in the chromatographs for the blank matrices. For

HS-SPME, the depth of the fiber in the vial should be adjusted so that the fiber rod is above the meniscus of the liquid phase. The penetration length of the fiber in the autosampler vials were changed from 22-31 mm (Fig. 1), it was found that the sample response at 22 mm was slightly higher than that at other lengths. Therefore, 22 mm was selected as the fiber penetration position. The optimized position of the fiber in the GC injector normally resides in the middle of the liner. The optimization results (Fig. 2) illustrated that 54 mm was the optimum length of the fiber exposed in the GC injector. The optimization results were similar to previous studies with the volatile compound trichloroethane (TCE) and its metabolites.[28-30]

Figure 3 shows the effect of the preheating time on the peak area of DFP. From 1-10 minutes, DFP response increased with increased preheating time since the preheating process improved the mass transfer of analyte from the liquid sample to the headspace. After 10 minutes, there did not appear to be any significant difference on sample response. As a result, 10 minutes was chosen as the preheating time.

Decreasing the water solubility of organic compounds by adding salt is known as the "salting out" effect.[31-32] The addition of a salt to an aqueous sample increases the ionic strength of the sample, thereby increasing the partitioning of organic compounds into the SPME fiber coating. In the present study, Na_2SO_4, Na_2CO_3, $(NH_4)_2SO_4$, NaCl and NaF were tested to determine their effect on the extraction efficiency of DFP from the biological matrices. The results showed that NaF and $(NH_4)_2SO_4$ worked better than other salts which may due to their influence on protein precipitation properties since DFP is

approximately 60% protein bound in plasma. The amount of the salts was also studied, and finally 0.01g NaF was selected.

Headspace SPME is dependent on the equilibrium between the aqueous phase of the sample, the sample headspace and the solid-phase fiber coating, and thus extraction time and temperature critically impact the headspace SPME process. [33] The effects of extraction time (5–30 min) and temperature (30 to 60 °C) and on the extraction of DFP are shown in Fig. 4. Although SPME has maximum sensitivity at the equilibrium point, full equilibration is not necessary for accurate and precise analysis due to the linear relationship between the amount of analyte adsorbed by the SPME fiber and its initial concentration in the sample matrix under non-equilibrium conditions. [34] It was therefore necessary to establish the best compromise between extraction efficiency, extraction temperature and extraction time. Comparing the extraction time profiles obtained at different temperatures revealed that higher sampling temperatures increased the speed of equilibrium but lowered the amount of extracted analyte on the fiber due to the decreased fiber coating/headspace partition coefficient. [33] Based on the profile, an extraction time of 15 min and a temperature of 30 ºC were selected as the optimum extraction conditions.

Finally, the desorption time and temperature were optimized. The desorption temperature was examined over the range from 100-270 ºC and the desorption time was examined from 30-300 seconds. (Fig. 5 and Fig. 6) The response generally increased with the increase of the temperature. However, when the temperature is higher than 250 ºC, the sensitivity no longer improved but more coatings from the SPME fiber were removed

resulting in reduced fiber life times. Therefore, 250 ºC was then selected as the optimum desorption temperature with the maximum SPME fiber lifetime. At this temperature, 60 seconds was found to be sufficient for optimum recovery and complete analyte desorption. No carry-over was observed throughout the analysis.

Validation of the method

After optimization the method was validated according to internationally accepted criteria.[26] The parameters validated were selectivity, calibration curves, precision and accuracy, limits of quantitation, recovery and stability.

The selectivity of the method was evaluated by analysis of blank matrices and matrices spiked with DFP standard. Figure 7 shows the representative chromatograms obtained from each blank matrix and matrix spiked with the LLOQ standard (1 ng/ml or 3 ng/g). No interfering peaks from endogenous compounds were observed at the retention times of DFP. Utilization of selected-ion monitoring (SIM) mode enhanced the mass spectrometric selectivity by eliminating the need to scan a large range of masses.

The calibration curves for each day of validation in different matrices showed good linear response (R^2>0.995) over the range from 1 to 100 ng/ml or 3 to 300 ng/g. SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation for each matrix. The lower limit of quantitation, LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 1 ng/ml for DFP in

plasma and 3 ng/g in brain tissue as shown in Table 1. A signal-to-noise (S/N) ratio > 10 at the LLOQ was observed for DFP in both matrices.

Assay precision and accuracy for DFP in each matrix were established at the LLOQ, low, medium, and high concentration levels over a range of 3 days. Table 1 summarizes the accuracy and precision data that were collected. The intra-day precision and accuracy (n=5) were better than 6.39% and 5.06% for DFP in both matrices. The inter-day precision and accuracy were determined by pooling all of the validation assay (n=15) QC samples. The values for the inter-day precision and accuracy were lower than 6.45% and 5.44%.

While recovery studies are normally performed in bioanalytical method validation the values are not normally reported for SPME studies. However, relative recovery is a good parameter for evaluating the matrix effect during method validation. The relative recoveries for DFP were calculated by comparing the amount extracted by the HS-SPME method in different biological matrices with the amount extracted in water. The obtained values are summarized in Table 2. Relative recoveries for DFP in plasma ranged from 50.8-58.4%, and from 59.5-72.9% in brain tissue. As the plasma protein binding of DFP is about 60%, thus, the mass transfer of DFP from the aqueous phase to the headspace may be hindered in plasma, which explains why the relative recovery of DFP in plasma is a little lower than brain tissue.

Stability testing is very important for validated methods in biological samples. Samples may sit in the autosampler for many hours. DFP is a volatile compound, so

examining its loss during the sample analysis is critical. Besides, DFP is relatively unstable in water and can decompose upon exposure to moisture forming hydrogen fluoride. Autosampler stability was evaluated at 5 ng/ml in plasma and 15 ng/g in tissue samples. Blank plasma and brain tissue homogenate were spiked with DFP and were left on the autosampler at room temperature for 24 hours, and they were injected at 0, 6, 12, 18 and 24 hrs. These samples were compared with samples prepared freshly. The obtained values for precision were less than 9.10%, and the relative error was less than 12.80% (Table 3). Both matrices can be stable on the autosampler for 6 hours. At 12 hours, less than 85% of DFP were recovered from the sample, and at 24 hours, about 30% of DFP was lost from the samples. Freeze and thaw stability was also evaluated in both matrices at the same concentration level (n=5). Blank matrices were spiked with 5 ng/ml or 15 ng/g of DFP, and these aliquots were stored at -20 ºC for 24 hr. After three complete freeze and thaw cycles, the samples were compared to those prepared freshly, and the obtained value for precision and relative error was less than 9.61% and 3.13%, respectively.

Applications

This method was applied to the plasma and brain samples from rats dosed with low-dose of DFP. The concentration data are presented in Table 4 and Table 5. The representative chromatograms resulting from the analysis of DFP in plasma and brain tissue from rats dosed with 1 mg/kg DFP are shown in Figure 8. DFP is a polar compound and as such would be expected to have difficulty crossing the blood-brain barrier. This is supported

by measurement of brain levels which were approximated 0.5-1% of corresponding plasma levels. Since concentrations of DFP in the brain were similar at doses between 1-0.25 mg/kg, the transport of these compounds may be saturable. However, this will require further investigation to determine.

CONCLUSIONS

A simple, specific, rapid and sensitive SPME-GC/MS method for the determination of DFP in rat plasma and brain has been developed and validated. It is the first published GC-MS method to validate DFP in biological samples. The use of headspace-SPME prevents the use of expensive and toxic organic solvents and greatly decreases the time and effort for sample preparation. During the SPME process, several influential parameters such as preheating time, extraction time, extraction temperature, salt effects, fiber conditions and desorption conditions were investigated and optimized. This validated method also yields good linearity, precision and accuracy. This method was also successfully applied to study the plasma and brain levels of DFP following low levels of exposure.

REFERENCES

1. Aldrich WN, Reiner E, *Enzyme inhibitors as substrates*, 1972.
2. Hutson DG, Roberts TR, *Insecticides*, 1985.
3. Ray DE, Richards PG, *Toxicol Lett* 2001; **120**:343.

4. Brodeur J, Dubois KP, *Arch Int Pharmacodyn Ther* 1964; **149**:560.

5. Costa LG, Schwab BW, Murphy SD, *Toxicology* 1982; **25**:79.

6. Overstreet DH, Russell RW, Vasquez BJ, Dalglish FW, *Pharmacol Biochem Behav* 1974; **2**:45.

7. Terry AV Jr., Silks LA 3rd, Dunlap RB, Odom JD, Kosh JW, *Gen Pharmacol* 1992; **23**:689.

8. Stone JD, Terry AV Jr., Pauly JR, Prendergast MA, Buccafusco JJ, *Brain Res* 2000; **882**:9.

9. Prendergast MA, Terry AV Jr., Buccafusco JJ, *Neurotoxicol Teratol* 1998; **20**:115.

10. Prendergast MA, Terry AV Jr., Buccafusco JJ, *Psychopharmacology (Berl)* 1997; **129**:183.

11. Patterson TA, Terry AV Jr., Kosh JW, *Br J Pharmacol* 1989; **97**:451.

12. Abdulla FA, Calaminici MR, Stephenson JD, Sinden JD, *Psychopharmacology (Berl)* 1993; **111**:508.

13. McDonald BE, Costa LG, Murphy SD, *Toxicol Lett* 1988; **40**:47.

14. Gershon S, Shaw FH, *Lancet* 1961; **1**:1371.

15. Metcalf DR, Holmes JH, *Ann N Y Acad Sci* 1969; **160**:357.

16. Bartus RT, Dean RL 3rd, Beer B, Lippa AS, *Science* 1982; **217**:408.

17. Warburton DM, Wesnes K, *Neuropsychobiology* 1984; **11**:121.

18. Buccafusco JJ, Aronstam RS, *J Pharmacol Exp Ther* 1986; **239**:43.

19. Buccafusco JJ, Heithold DL, Chon SH, *Toxicol Lett* 1990; **52**:319.

20. Buccafusco JJ, Jackson WJ, Terry AV Jr., Marsh KC, Decker MW, Arneric SP, *Psychopharmacology (Berl)* 1995; **120**:256.

21. Hobbiger FE, Pharmacology of anticholinesterase drugs. *Handbook of experimental pharmacology*, 1972.

22. Bushnell PJ, Padilla SS, Ward T, Pope CN, Olszyk VB, *J Pharmacol Exp Ther* 1991; **256**:741.

23. Pragst F*, Anal Bioanal Chem* 2007; **388**:1393.

24. Vas G, Vekey K, *J Mass Spectrom* 2004; **39**:233.

25. Alpendurada MF, *J Chromatogr A* 2000; **889**:3.

26. F.D.A. U.S. Department of Health and Human Services, 2001.

27. Kuriki A, Kumazawa T, Lee X-P, Hasegawa C, Kawamura M, Suzuki O, Sato K. *J Chromatogr B*, 2006; **844**:283–291

28. Liu Y, Muralidhara S, Bruckner JV, Bartlett MG. *Rapid Commun Mass Spectrom*. 2008; **22**(7):1023-31.

29. Liu Y, Muralidhara S, Bruckner JV, Bartlett MG. *Rapid Commun Mass Spectrom*. 2008; **22**(6):797-806.

30. Liu Y, Muralidhara S, Bruckner JV, Bartlett MG.. *J Chromatogr B* 2008; **863**(1):26-35.

31. Kumazawa T, Seno H, Lee X-P, Ishii A, Suzuki O, Sato K, *Chromatographia* 1996; **43**:393.

32. Hall BJ, Brodbelt JS, *J. Chromatogr. A* 1997; **777**:275.

33. Lord HL, Pawliszyn J*, Anal. Chem.* 1997; **69**:3899.

34. Ai J, *Anal. Chem.* 1997; **69**:3260.

Table A1.1. The intra-day (n=5) and inter-day (n=15) precision (%R.S.D.) and accuracy (% error) of the HS-SPME-GC/MS method used to quantitate DFP in rat plasma and brain tissue

Concentration DFP added	Intra-day (n=5)			Inter-day (n=15)		
(ng/ml or ng/g)	Concentration DFP found	R.S.D. (%)	Error (%)	Concentration DFP found	R.S.D. (%)	Error (%)
Plasma (ng/ml)						
1	1.02±0.03	3.13	1.51	1.03±0.06	5.80	2.93
4	3.99±0.18	4.44	0.39	3.99±0.15	3.81	0.38
40	38.90±2.23	5.72	2.75	38.17±2.25	5.89	4.57
80	75.95±1.83	2.40	5.06	75.65±1.89	2.50	5.44
Brain homogenate (ng/g)						
3	2.94±0.09	3.19	2.13	2.92±0.14	4.85	2.97
12	12.30±0.32	2.60	2.52	11.66±0.74	6.36	3.29
120	116.58±7.44	6.39	2.86	112.83±7.29	6.45	1.35
240	228.36±2.19	0.96	4.85	224.10±11.04	4.92	5.04

Table A1.2. The relative recovery (%) (mean ± S.D.) of DFP from rat plasma and brain homogenate (n=5)

Concentration (ng/ml or ng/g)	Plasma
Plasma (ng/ml)	
1	50.8 ± 1.2
4	52.5 ± 1.5
40	58.4 ± 1.2
80	50.8 ± 4.2
Brain homogenate (ng/g)	
3	59.5 ± 5.0
12	68.6 ± 5.0
120	72.9 ± 3.9
240	69.5 ± 6.0

Table A1.3. Stability testing of DFP in rat plasma and brain homogenate (n=5)

Stability	Spiked conc. (ng/ml or ng/g)	Observed conc. ± S.D. (ng/ml or ng/g)	R.S.D. (%)	Relative error (%)
Plasma				
Three freeze-thaw cycle	5ng/ml	4.89 ± 0.47	9.61	2.20
Autosampler stability (6h)	5ng/ml	4.51 ± 0.41	9.10	9.80
Brain homogenate				
Three freeze-thaw cycle	15ng/g	14.52 ± 1.11	7.65	3.13
Autosampler stability (6h)	15ng/g	13.08 ± 1.02	7.80	12.80

Table A1.4. DFP concentrations in plasma study (n=5)

	1	2	3	4	5	6	7
Control	ND	ND	ND	ND	ND	ND	ND
1.0	ND	1053.41	82.26	413.50	203.84	ND	ND
0.75	ND	912.62	234.73	634.62	192.04	ND	ND
0.50	ND	383.97	150.09	390.01	200.93	ND	ND
0.25	ND	438.24	107.24	243.62	128.76	ND	ND

1 was taken before dosing; 2 and 4 were taken on the day of dosing; 3 and 5 were taken on dose intervals; 6 was taken after one week washout and 7 was taken after two week washout. From 2 to 5, the samples were diluted 10 times with blank rat plasma before analyzing.

Table A1.5. DFP concentrations in brain study (n=4)

	30 day dosing	One week washout
Control	ND	ND
1.0	4.42	ND
0.75	5.32	ND
0.50	5.22	ND
0.25	3.11	ND

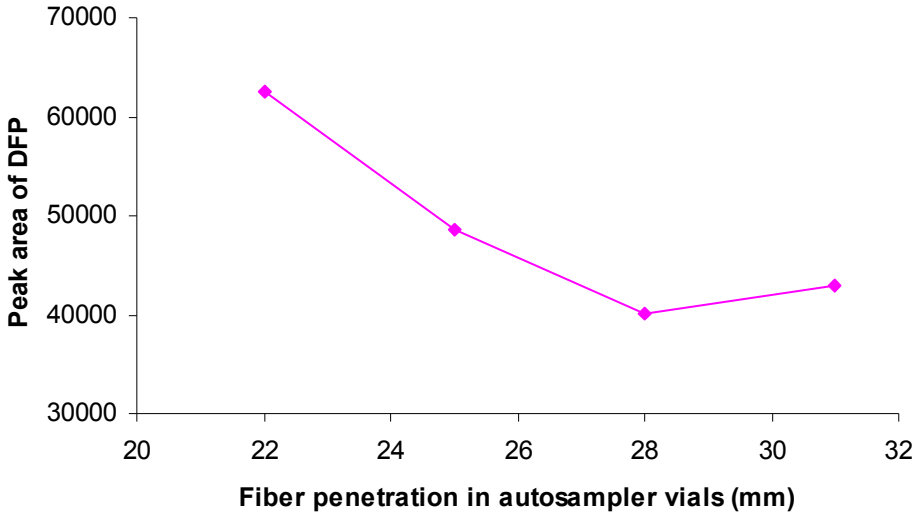


Figure A1.1. Effect of SPME fiber penetration in autosampler vials on efficiency

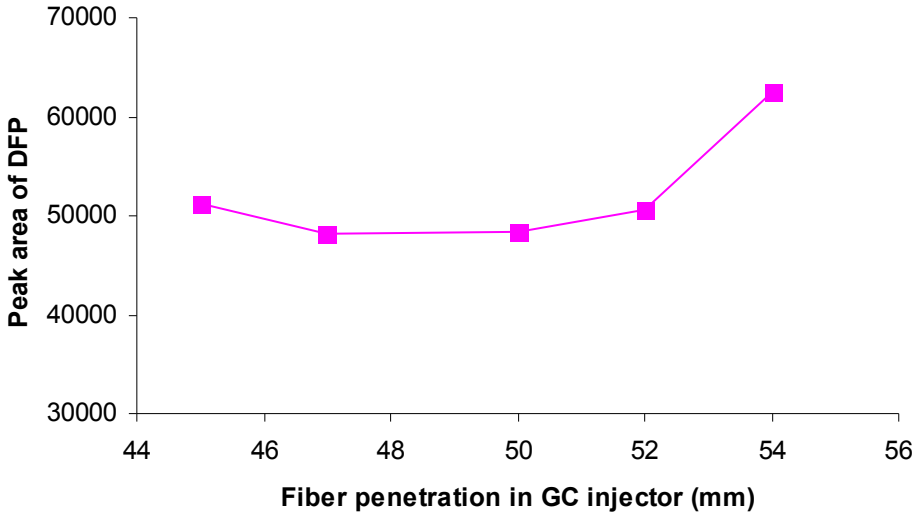


Figure A1.2. Effect of SPME fiber penetration in GC injector on efficiency

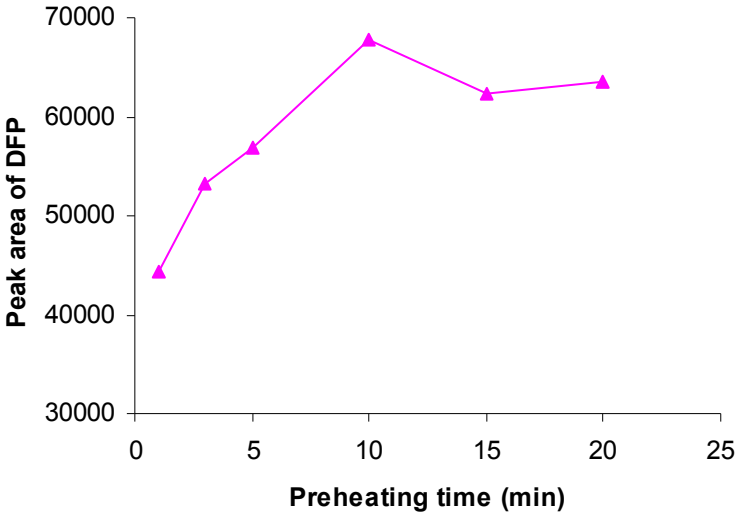


Figure A1.3. Effect of sample preheating time on SPME efficiency

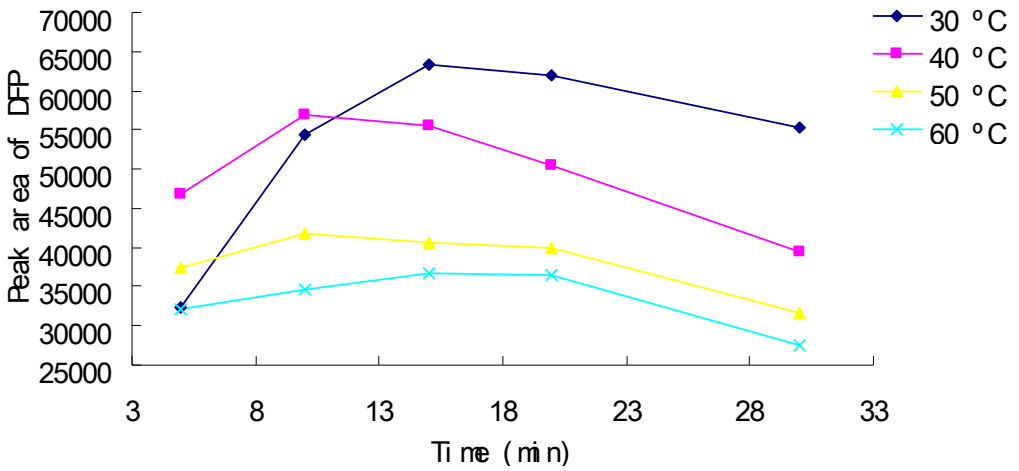


Figure A1.4. Plot of DFP peak area versus extraction time obtained on different extraction temperature (30, 40, 50 and 60 ºC). Conditions: preheat 10 min, 0.01g of NaF added.

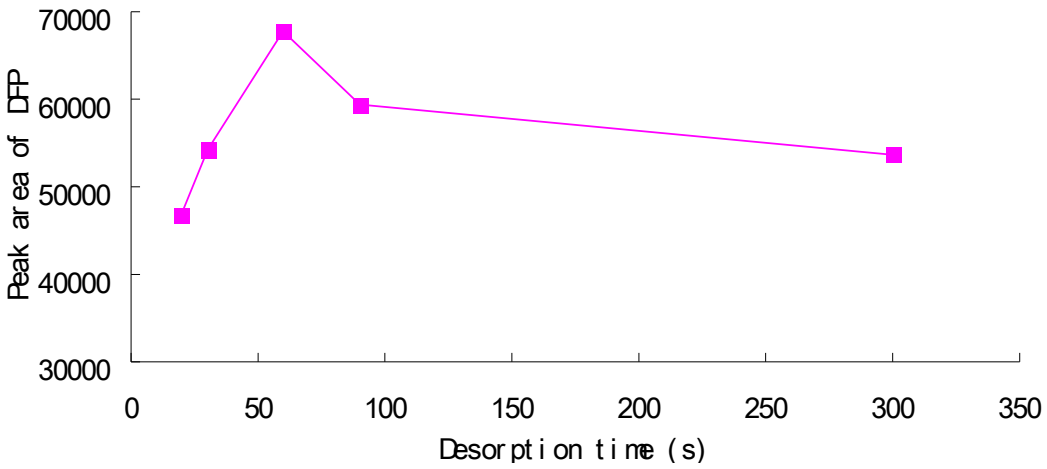


Figure A1.5. Effect of SPME fiber desorption time on efficiency.

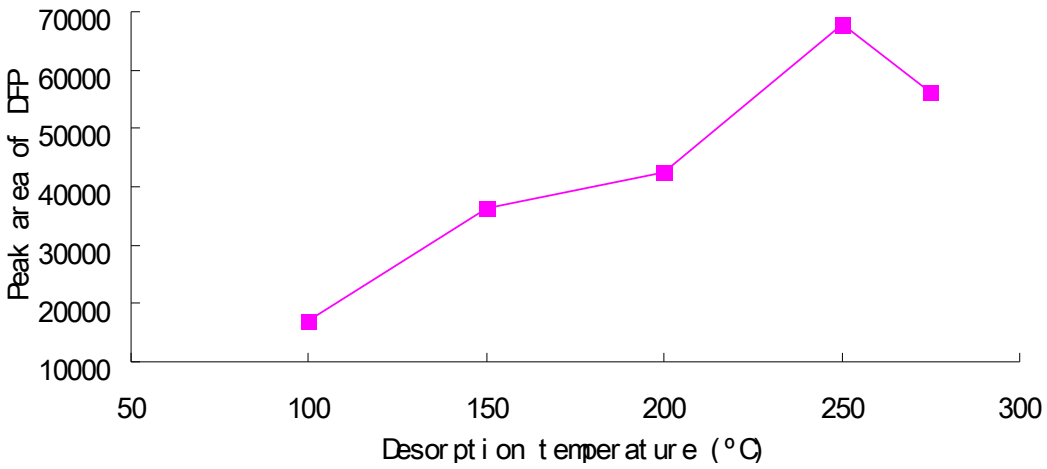


Figure A1.6. Effect of SPME fiber desorption temperature on efficiency.

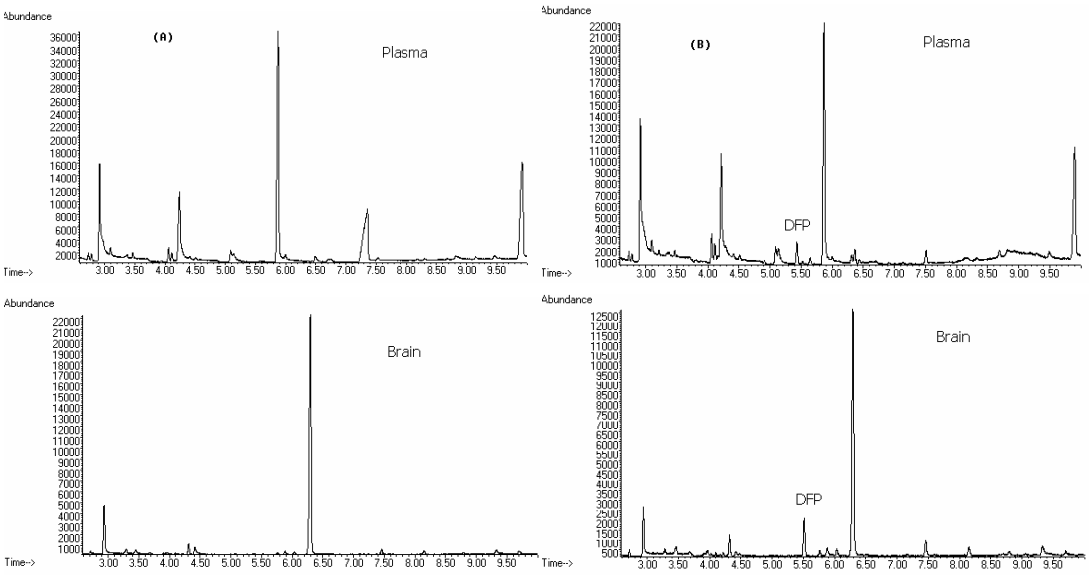


Figure A1.7. Representative chromatograms obtained from blank plasma and brain tissue(A); plasma and brain tissue spiked with the LLOQ (1 ng/ml in plasma or 3 ng/g in brain tissue) concentration of DFP.

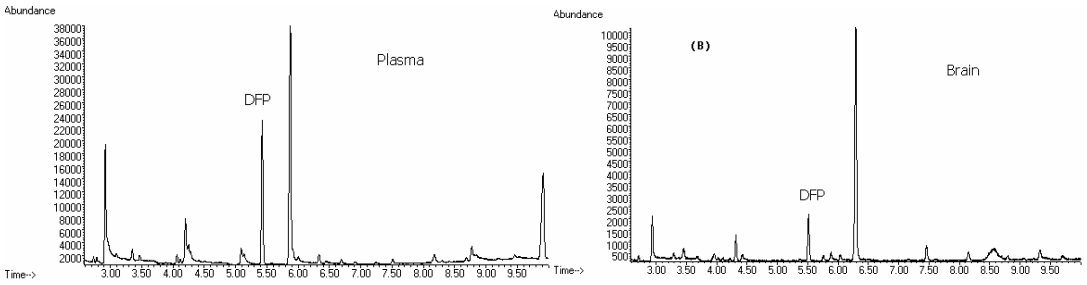


Figure A1.8. Representative chromatograms of (A) plasma; (B) brain samples from rat dosed with 1 mg/kg DFP

APPENDIX 2

STABILITY OF LOW CONCENTRATION CEFTAZIDIME IN NORMAL SALINE AND BALANCED SALT SOLUTIONS IN PLASTIC SYRINGES UNDER VARIOUS STORAGE CONDITIONS[1]

[1]Meng Xu, Flynn N. Warren, Michael G. Bartlett. Submitted to *International Journal of Pharmaceutical Compounding*, 05/08.

ABSTRACT

The objective of this study was to access the stability of ceftazidime in normal saline and balanced salt solutions. Ceftazidime was dissolved in normal saline or balanced salt solutions (BSS) at a concentration of 1 mg/ml. The solutions were then stored under three different sets of conditions, ambient temperature (25^{o}C), refrigerated (4^{o}C), or frozen (-20^{o}C). The concentration of ceftazidime was determined at various intervals using a USP high performance liquid chromatography method. Based on our results, ceftazidime was found to be stable (>90%) up to 72 h in both saline and BSS at ambient temperature. Ceftazidime was stable in the refrigerator for 17 days in both solutions. No significant degradation was noticed for up to 60 days when the solutions were kept in the freezer.

INTRODUCTION

Ceftazidime is a third-generation cephalosporin antibiotic which has broad spectrum activity against Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa* (1). Low concentration ceftazidime is often mixed with 0.9% sodium chloride injection (normal saline) and balanced salt solution (BSS) in hospitals for ophthalmic solutions (2, 3). While several papers have reviewed the stability of ceftazidime at higher concentrations in plastic syringes, glass vials and pump reservoirs (2, 4-9), no study has evaluated the stability of ceftazidime prepared at lower concentrations (1 mg/mL) and stored in plastic syringes.

Drug losses can occur over time by several mechanisms. The compound itself may be chemically unstable. The compound may precipitate from solution due to pH changes or interaction with various salts in the reconstituting solution. Finally, the drug may be lost through non-specific adsorption with the surfaces of containers. Adsorption losses are normally low but as the concentration of a drug is reduced they may become significant.

The purpose of these investigations was to determine the physiochemical stability of ceftazidime in normal saline and BSS injections at low concentrations. New regulations from the Joint Commission on Accreditation of Healthcare Organizations (JCAHO) has led hospital pharmacies to investigate creating batches of medications that can be left in the hospital wards or maintained in the pharmacy to be used as the need arises. However, there is normally little information available regarding many medications after they have been reconstituted. Most package inserts recommend immediate use with no storage of the reconstituted product. Therefore, there is a need to study these products under a wide variety of concentrations and storage conditions. In this study, we determined the stability of low concentration ceftazidime under three different common storage conditions including, ambient temperature (25^{o}C), refrigeration (4^{o}C), and freezing (-20^{o}C).

MATERIALS AND METHODS

Materials

Ceftazidime standard was obtained from Sigma (St. Louis, MO, USA). Ceftazidime for injection and 0.9% sodium chloride (normal saline) were obtained from Hospira Worldwide, Inc. (Lake Forest, IL, USA). Balance salt solution was bought from Baxter Healthcare Corporation (Deerfield, IL, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Phosphoric acid and triethylamine were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sodium phosphate dibasic was from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

Methods

Two bulk glass vials each containing ceftazidime powder 1.0g were reconstituted with 1L of normal saline and 1L of BSS. Bulk vials were inverted 10 times to ensure homogeneity before samples were removed. Immediately after preparation, approximately 2mL of solution was drawn from each vial into a syringe. Thus, 500 syringes of 1mg/mL ceftazidime in normal saline and BSS were prepared. For the stability of ceftazidime

solutions under ambient temperature, the samples were tested on the same day they were prepared, and for refrigerated and frozen samples, each sample was given 30 min to reach ambient temperature before analysis.

Analytical methods. All HPLC experiments were performed on an Agilent 1100 series HPLC equipped with a variable wavelength UV detector. Chromatographic separation was achieved on an Agilent Zorbax Eclipse XDB-C8 column (4.6×150 mm, Santa Clara, CA). The mobile phase consisted of Solvent A (50mM sodium phosphate + 0.2% triethylamine; the pH was adjusted to 3.0 using phosphoric acid) and Solvent B (acetonitrile) (90:10). The detection wavelength was set to 280 nm. The flow rate was set to 0.8 ml/min, and the injection volume was 10 µl. The HPLC run time was 8 min. This method followed the current method of the United States Pharmacopeia. (10)

Concentrations of ceftazidime were determined using a similar approach to Stewart and co-workers (4). Briefly, ceftazidime concentrations were determined by comparison to freshly prepared standards at 1 mg/ml, which represented 100% of the original dose. Each time point was determined by comparing the average from six samples to the average of the reference samples from the same batch.

Method integrity was demonstrated daily by analysis of a freshly prepared standard of ceftazidime with a concentration of 1 mg/ml. This sample was injected 6 times, and again after every 6 runs throughout each batch of samples. The RSD of all the standards run on the same day was less than 1%. An RSD of less than 1% for the standard was considered an acceptance criteria for each batch of samples.

RESULTS AND DISCUSSION

The retention time for ceftazidime was 4.7 min. The system suitability requirements of the assay published in the USP were consistently met. These included a tailing factor

between 0.75 – 1.5 and a relative standard deviation of no more than 1.0% for peak areas of replicate injections of ceftazidime. The assay was linear across the concentration range from 1.0 mg/ml – 0.1 mg/ml.

The current study has significantly more time points and a much larger sample size relative to earlier studies (4-9). The previous studies were all conducted at concentrations 30 – 200 times higher than the current study as well. The results of the current study are summarized in table 1 and table 2. Overall, the stability of ceftazidime in normal saline and BSS appear to be equivalent under all storage conditions. This result is similar to Walker et al. who found that ceftazidime stability in saline and 5% dextrose were equivalent (7).

Ceftazidime degraded rapidly at 25 °C, with approximately 10% lost after 3 days. Previous studies had not followed ceftazidime for more than 24 h at room temperature. Therefore, the current study extends the current 24 h shelf-life to 72 hrs at the lower concentration. In addition, Zhou and Notari predicted a shelf-life of 1.7 days at 30 °C, which is consistent with our study (9).

When refrigerated at 4 °C, more than 90% of the original concentration remained after 17 days. Previous studies followed ceftazidime for 7-10 days and did not show stability for any longer (5-8). The sole exception was the study of Stewart and co-workers which found ceftazidime concentrations to have decreased by only 2-3% after 28-days (4). It should be noted that their study was conducted at 100-200 times the concentration of the current study and suggests that absorptive loses to the surfaces of plastic syringes at lower concentrations may decrease their stability. At -20 °C, ceftazidime showed limited degradation after 60 days. Similar results were observed in earlier studies out to 90 days (4-6).

Figures 1 and 2 showed the high performance liquid chromatograms of the ceftazidime solutions on day 0 and day 4 under ambient temperature in saline and BSS. The peak for ceftazidime is observed at 4.7 min and is well resolved from the observed degradation products. This retention time is consistent with the earlier report of Stewart and co-workers (4). Many new peaks representing chemical degradation were clearly observed after 3 days. Barnes A.R. *et al.* reported that pyridine was formed as part of this degradation process of ceftazidime, and that pyridine formation follows apparent zero-order kinetics (3, 8, 9). We observed pyridine at 3.1 min and found this was the major degradation process in the current studies.

CONCLUSION

Ceftazidime (1mg/mL), in both normal saline and BSS, was found to be stable for 72 h at ambient temperature, 17 days at 4^{o}C or at least 60 days at -20 ^{o}C. The results of this study showed that syringes prepared at low concentrations of ceftazidime were less stable when refrigerated when compared to higher concentrations of ceftazidime.

REFERENCES

1. O'Callaghan CH, Acred P, Harper PB et al. GR 20263, a new broad-spectrum cephalosporin with anti-pseudomonal activity. *Antimicrob Agents Chemother* 1980; 17: 876-883.
2. Das Gupta V, Bethea C, de la Torre M. Chemical stabilities of cefoperazone sodium and ceftazidime in 5% dextrose and 0.9% sodium chloride injections. *Journal of Clinical Pharmacy and Therapeutics* 1988; 13: 199–205.
3. Barnes AR, Nash S. Preservative efficacy in ceffuroxime and ceftazidime eye drop formulations. *Journalof Clinical Pharmacy and Therapeutics* 1994; 19: 327–332.

4. Stewart JT, Warren FN, Johnson SM et al. Stability of ceftazidime in plastic syringes and glass vials under various storage conditions. *American Journal of Hospital Pharmacy* 1992; 49: 2765-2768.
5. Nahata MC, Morosco MC, Fox JL. (1992) Stability of ceftazidime (with arginine) stored in plastic syringes at three temperatures. *American Journal of Hospital Pharmacy* 1992; 49: 2954–2956.
6. Stiles ML, Allen LV Jr, Fox JL. Stability of ceftazidime (with arginine) and of cefuroxime sodium in infusion-pump reservoirs. *American Journal of Hospital Pharmacy*, 1992; 49: 2761–2764.
7. Walker SE, Dranitsaris G. Ceftazidime stability in normal saline and dextrose in water. *Canadian Journal of Hospital Pharmacy* 1988; 41: 65-70.
8. Barnes AR, Nash S. Stability of ceftazidime in a viscous eye drop formulation. *Journal of Clinical Pharmacy and Therapeutics*, 1999; 24: 299–302.
9. Zhou M, Notari RE. Influence of pH, temperature, and buffers on the kinetics of ceftazidime degradation in aqueous solutions. *Journal of Pharmaceutical Sciences* 1995; 84: 534–538.
10. U.S. Pharmacopeia & National Formulary. 2000: 350-352.

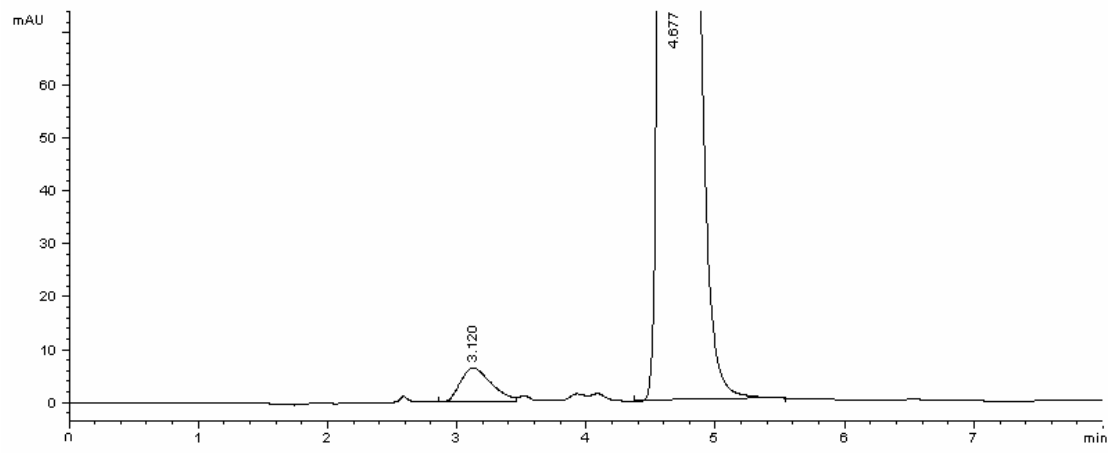
Table A2.1. Assay results of ceftazidime in 0.9% sodium chloride (n=6)

Days	25°C	4°C	-20°C
	Percentage		(%)
0	100	100	100
0.5	98.5	-	-
1	94.2	98.9	100.7
1.5	93.4	-	-
2	93.8	95.7	100.3
2.5	92.7	-	-
3	91.3	94.7	100.6
3.5	89.6	-	-
7	-	94.3	98.5
14	-	92.8	99.1
16	-	90.3	-
17	-	90.2	-
18	-	88.9	-
28	-	-	99.4
42	-	-	98.1
60	-	-	100.6

Table A2.2. Assay results of ceftazidime in balanced salt solution (n=6)

Days	25°C	4°C	-20°C
	Percentage		(%)
0	100	100	100
0.5	98.5	-	-
1	98.3	95.1	100.3
1.5	96.8	-	-
2	95.3	95.7	99.9
2.5	92.8	-	-
3	90.6	95.6	100.1
3.5	89.2	-	-
7	-	93.9	98.1
14	-	90.9	97.4
16	-	91.5	-
17	-	90.2	-
18	-	89.3	-
28	-	-	99.1
42	-	-	98.7
60	-	-	100.5

a)



b)

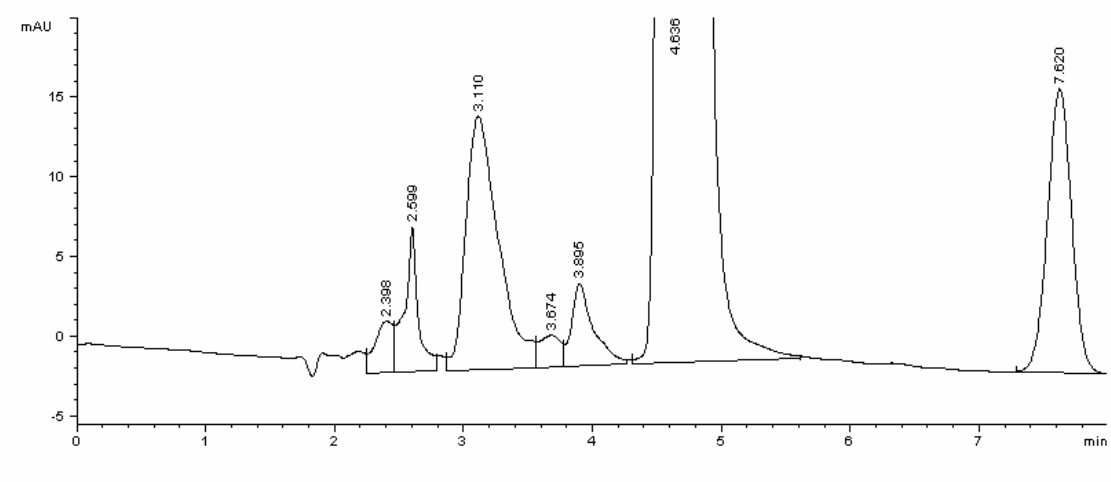
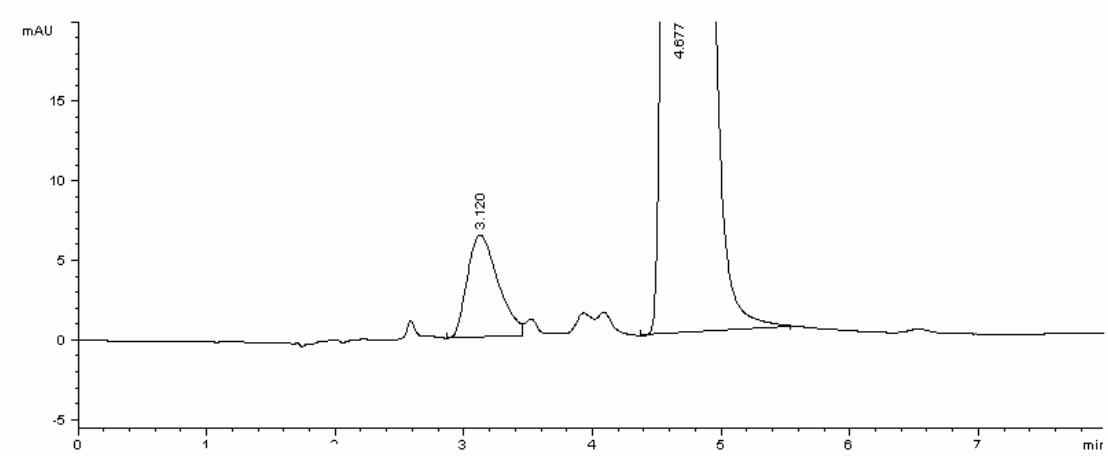


Figure A2.1. Representative chromatograms of ceftazidime in normal saline a) Day 0 b) Day 4

a)



b)

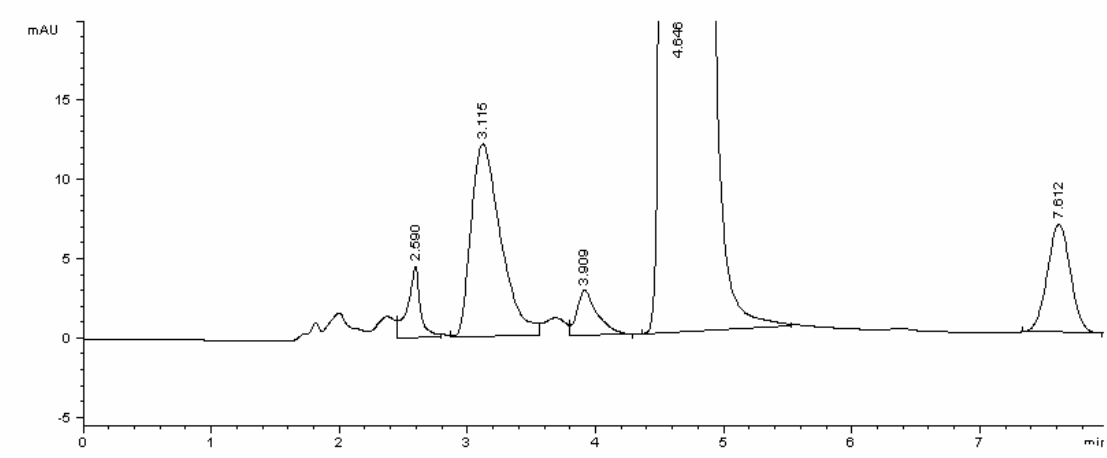


Figure A2.2 Representative chromatograms of ceftazidime in BSS a) Day 0 b) Day 4