MATERNAL AND FETAL DISPOSITION OF ANTIVIRAL AGENTS IN

THE PREGNANT RAT

by

SUMMER R. LEWIS

(Under the Direction of Catherine A. White)

ABSTRACT

Human immunodeficiency virus type-1 (HIV) infection has increased dramatically in pregnant women, thus, exposing the fetus *in utero*. However, with the increasing use of combination therapies, drug-drug interactions causing significant health risks are becoming more common. Therefore, antiviral drugs are used therapeutically in pregnancy for the treatment of the mother and the fetus. 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. Drugs enter the fetal compartment through either passive diffusion and/or active transport across the placenta. Studies with single antiviral agents suggest that these drugs cross the placenta by passive diffusion. However, recent studies have identified several nucleoside transporters in the placenta. To date, very few studies have examined the fetal disposition of drugs administered in combination. To understand these interactions, the pharmacokinetics of these antiretroviral agents, alone and in combination, must be fully understood in both mother and fetus in order to successfully treat pregnant HIV positive women.

INDEX WORDS: Pharmacokinetics, HIV, Abacavir, Lamivudine, 3TC, Zidovudine, AZT, placental transport, antiviral, fetal disposition

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DEDICATION

This dissertation is dedicated with love and gratitude to my parents, Harold and Elaine Lewis.

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CHAPTER 1

INTRODUCTION

Human immunodeficiency virus type-1 infection has increased dramatically in women and adolescents of childbearing age. The proportion of all reported AIDS cases in adult and adolescent women increased from 7% in 1985 to nearly 50% as of December 2003 (13). Additionally, people in the 13 to 24 year age bracket account for approximately 50% of all new HIV cases. 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. *In vitro* and animal studies with single antiretroviral agents suggest that these drugs cross the placenta by passive diffusion. However, more recent work has identified several known transporters in the placenta for nucleosides. Saturation of placental uptake and transport and/or changes in maternal pharmacokinetics with combination therapies may occur and thus, fetal exposure to antiretroviral agents may be altered resulting in changes in therapeutic benefit/toxicity. With the increasing use of these combination therapies in the treatment of HIV, drug-drug interactions causing significant health risks are becoming more common. Also, physiological changes that occur during pregnancy alter the distribution and elimination of many drugs. Additionally, pregnant women are immune compromised which can significantly influence viral activity/viral load. These changes potentially increase the risk of adverse drug reactions and drug-drug interactions during

pregnancy for both the mother and fetus. To date, very few studies have examined the fetal disposition (distribution and elimination) of drugs under conditions of combination therapies.

Since HIV first appeared in the early 1980's, there has been an endless search for more effective treatments for this deadly virus. Although the virus was first prevalent mainly in the homosexual community, it rapidly spread into all populations. Early in the epidemic, it became apparent that the virus could be transmitted from pregnant women to their infant. AIDS Clinical Trial Group (ACTG) 076 was a landmark clinical study conducted in 1994 aimed at addressing this issue. The findings of this study were that a short course zidovudine (AZT) therapy regimen could drastically reduce the maternal-fetal transmission of HIV in infected pregnant women (6). In the decade since this first ACTG 076 study, successive trials have led to reductions in transmission to below 2% in the United States and Europe. All of these studies have led to the development of a set of consensus guidelines for the use of antiviral agents in pregnancy for the treatment of HIV. These guidelines were first published in 1998 (3) followed by several updates. These guidelines recommend that women receive treatment for HIV and that their pregnancy should not contraindicate treatment. The guidelines recommend that pregnant women continue on their current treatments, although the addition of zidovudine is recommended unless the patients current treatment involves stavudine (d4T) due to drug interactions involving the initial phosphorylation step of these two agents (8).

While there have been a significant number of clinical trials in pregnant women to treat HIV there are many combinations of agents that have not been extensively studied in pregnant populations. Combinations of zidovudine, lamivudine, and nevirapine appear to be able to reduce HIV transmission rates even when they are only used beginning with labor. However, other combinations such as abacavir, stavudine and didanosine have low efficacy (9). A

systematic study of these agents in appropriate *in vivo* and *in vitro* systems would have tremendous benefit in allowing for optimization of clinical trials for treating HIV positive pregnant women. In order to maximize information gained from these studies, pharmacokinetic models have been constructed in which the time course of drug concentration is mimicked in maternal plasma, placenta, fetus, and amniotic fluid by computer simulation. These models will provide a more complete understanding of the processes involved in the placental transport of antiviral agents.

The pregnant rat model has been used successfully in the study of the placental transfer of many compounds, including nucleoside analogs (1, 2, 4, 5, 7, 11) . Characteristics in placentation between the rat and human influence the efficiency and/or the rate of transfer of materials between the mother and embryo (12). Both the human and rodent placentas are hemochorial, in which trophoblastic cells are in direct contact with maternal blood without an intervening endothelium . There are several advantages to using the rat as a model, including easy handling, large litter size, ready availability on short notice, minimal housing requirements, and short gestation period (22 days) (10). The containment of each fetus, placenta, and amniotic fluid in individual fetal sacs allows for serial sampling of the pups, therefore providing a full concentration-time course.

DISSERTATION STRUCTURE

This dissertation is structured as follows: following a review of current literature, Chapters 3 and 4 contain the pharmacokinetic analysis of lamivudine alone and in combination with zidovudine; Chapter 5 describes the development and validation of an HPLC assay for the simultaneous determination of abacavir and AZT in rat tissues using HPLC-UV; Chapters 6 and

7 provide a pharmacokinetic analysis of abacavir alone and in combination with zidovudine; and Chapter 8 summarizes the final conclusions.

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CHAPTER 2

LITERATURE REVIEW

Acquired Immunodeficiency Syndrome

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 (9). 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. The total number of people living with human immunodeficiency virus (HIV), the virus that causes AIDS, rose in 2005 to reach an estimated 40.3 million people (69). Sub-Saharan Africa remains by far the worst affected region, with 25.8 million people living with HIV at the end of 2005. The global AIDS epidemic killed 3.1 million people in the past year, with over 25 million deaths since the first cases of AIDS were identified in 1981 (69). Despite increased funding, political commitment and progress in expanding access to HIV treatment, the AIDS epidemic continues to outpace the global response. (69).

The demographics have changed significantly since the virus was first identified 25 years ago, as it now becomes an epidemic of non-White populations, women, heterosexuals and injecting drug users (48). Women and children are experiencing growing rates of AIDS-related illness and death in many settings. As of December 2005, women accounted for 46% of all people living with HIV worldwide and for 57% of all people living with HIV in Sub-Saharan Africa (69). AIDS is affecting women most severely in places where heterosexual sex is a

dominant mode of HIV transmission, as is the case in Sub-Saharan Africa and Southeast Asia. HIV has become increasingly lodged among women who belong to marginalized sections of populations, including minorities, immigrants and sex workers. African American and Hispanic women, for example, represent less than one quarter of all women in the United States, but accounted for 80% of AIDS cases reported among women in 2000 (69).

Mother-to-Child Transmission

The increasing prevalence among women of childbearing age has been paralleled with an increase in mother-to-child transmission (MTCT) of HIV, with more than two million pregnancies in HIV-positive women each year. Just over half a million children (aged 15 and under) worldwide died from AIDS in 2005 and an estimated 700,000 children became newly infected with HIV (69). The vast majority of these children acquire the infection from their mother through vertical transmission. This can occur during pregnancy, during delivery, and postnatally during breastfeeding (39, 40). The fetus can get infected *in utero* through maternal blood, transplacental hemorrhage, and infection via umbilical cord or via the gastrointestinal mucosa while swallowing infected amniotic fluid. The contribution of each of these routes to overall transmission has not been quantified exactly, but it appears that a substantial proportion of infection occurs at the time of delivery or late in pregnancy (6, 21, 39). Many studies have reported that maternal plasma viral load appears to be a strong predictor of vertical transmission (8, 20, 23). However, transmission has been observed across the entire range of HIV viral load, indicating that viral load is not the only risk factor associated with vertical transmission. In addition to viral load, low CD4+ cell counts associated with advanced disease and genital tract secretions during labor and delivery increases the risk of HIV transmission to the infant (20, 31).

In the absence of any intervention, rates of vertical transmission can vary from 15 to 30 percent in developed countries and can reach as high as 30 to 45 percent in developing countries.

Current interventions employed in resource-rich countries include elective caesarean delivery, avoidance of breastfeeding, and antiretroviral therapy (74). Elective caesarean delivery in resource-constrained settings is seldom available and/or safe, and refraining from breastfeeding is often not acceptable, feasible, or safe. Therefore, efforts to prevent MTCT in resource-constrained settings have mostly focused on reducing MTCT around the time of labor and delivery, which accounts for one third to two thirds of overall transmission, depending on whether or not breastfeeding occurs (74). Two approaches are being sought with regard to antiretroviral therapy in pregnancy. In developed countries, the goal is to reduce maternal viral loads to below limits of detection using combination therapies and frequent monitoring. In developing countries, the goal is to obtain the shortest and most financially sustainable intervention that provides a cost-effective use of antiretroviral drugs (64).

Landmark Clinical Trials

In February 1994, the results of Pediatric AIDS Clinical Trials Group (PACTG) Protocol 076 demonstrated that administration of zidovudine (AZT) in three stages (antepartum, intrapartum, and postpartum to the neonate), reduced perinatal HIV-1 transmission from 25% to 8.5% (15). This trial was the pillar upon which many subsequent controlled trials and treatment guidelines have been based. Because of the expense and complexity of the complete PACTG 076 regimen, three studies, one in Thailand and two in Africa, were conducted to determine whether an abbreviated course of AZT was as effective (16, 58, 73). Antiviral therapy was provided at the time of greatest risk of transmission by giving AZT only late in the third trimester and intra-partum. The Thai study (no breastfeeding) demonstrated a 50% reduction in HIV transmission

(58), while the two African studies (breastfeeding) demonstrated a 38% reduced transmission (16, 73). None of these studies achieved efficacy rates equivalent to those seen in PACTG 076.

The PETRA trial compared HIV vertical transmission rates among breastfeeding women who received either a combination of AZT and lamivudine (3TC), or placebo (52). The rate of perinatal HIV-1 transmission with combined AZT and 3TC in PETRA arm B (intra-partum plus post-partum therapy) was less than half that of AZT monotherapy in a similar population (16, 26), thus supporting the use of combination antiretroviral therapy in pregnancy.

To date, the shortest and most cost-effective treatment is the use of nevirapine (NVP). The HIVNET 012 Study conducted in Uganda found NVP to reduce perinatal transmission 47% more effectively than AZT when initiated at the onset of labor and for the equivalent of 1 week postpartum in the neonate (26). Since 2000, the mainstay of the World Health Organization's (WHO) recommendations involves a two-dose regimen that includes a single dose of NVP to the mother in labor and a single dose to the infant within 48 hours. Concerns about the HIVNET 012 trial were raised in March 2002 when claims emerged that certain serious adverse events had not been properly reported. As a result, the Division of AIDS, National Institute of Allergy and Infectious Diseases at the National Institutes of Health conducted a reassessment of the trial procedures and results in the HIVNET 012 trial and determined that the NVP regimen was safe and effective (45). Another concern focused on the development of NVP resistance. Although the long elimination half-life of NVP provides convenient dosing, it may also facilitate viral drug resistance (38). Therefore, there has been an increased effort to evaluate the potential for effective and less expensive antiretroviral regimens, such as AZT and 3TC short course combinations (43, 53).

Current Antiviral Therapy

The need to delay the development of drug resistance and minimize potential dose-limiting side effects, has led to highly active antretroviral therapy (HAART) becoming the current standard of care for treatment of HIV infection (2). Three classes of antiretroviral drugs are currently available for the treatment of patients with HIV infection: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs). Since the first antiretroviral drug, 3'-azido-3'-deoxythymidine (zidovudine, AZT or ZDV) was introduced in 1987; twenty agents have been approved by the Food and Drug Administration (FDA) for the treatment of HIV infection. These drugs include eight NRTIs (zidovudine, didanosine, zalcitabine, emtricitabine, stavudine, lamivudine, tenofovir, and abacavir), three NNRTIs (nevirapine, delavirdine, and efavirenz), and nine PIs (saquinavir, ritonavir, indinavir, nelfinavir, atazanavir, fosamprenavir, tipranavir, lopinavir and amprenavir) (10).

NRTIs and NNRTIs inhibit the HIV-specific reverse transcriptase enzyme required for the transcription of viral RNA to proviral DNA, thus halting viral replication. The PIs inhibit HIV protease, the enzyme required for post-translational processing of HIV propeptides, and render virus particles noninfectious. Combination therapy with two NRTIs and a NNRTI or a PI is the currently recommended HAART regimen for use in adults.

Safety of Antiviral Therapy in Pregnancy

The current recommendations provided by the USA Public Health Service Task Force recommend the same treatment of HIV infection in pregnancy as those individuals who are not pregnant (12). However, recommendations regarding the choice of antiretroviral drugs for treatment of infected pregnant women are subject to unique considerations. These include

possible changes in dosing requirements resulting from physiologic changes associated with pregnancy, potential effects of antiviral drugs on the pregnant woman, and the potential short- and long-term effects of the antiviral drug on the fetus and newborn, which may not be known for certain antiviral drugs (12). During pregnancy, gastrointestinal transit time becomes prolonged; body water and fat increase throughout gestation and are accompanied by increases in cardiac output, ventilation, and liver and renal blood flow; plasma protein concentrations decrease; and renal sodium reabsorption increases (12). Placental transport of drugs and compartmentalization of drugs in the fetus and placenta also can affect drug pharmacokinetics in the pregnant woman. Additional considerations regarding drug use in pregnancy are the effects of the drug on the fetus and newborn and the pharmacokinetics and toxicity of transplacentally transferred drugs.

Information regarding the safety of drugs in pregnancy is derived from animal toxicity data, anecdotal experience, registry data, and clinical trials (12, 15, 16, 26, 39, 52, 58, 73). Data are limited for antiviral drugs, particularly when used in combination therapy. Therefore, more studies are needed to evaluate the placental transfer of these agents alone and in combination in order to successfully treat pregnant HIV infected women.

PLACENTAL TRANSPORT

Until the middle of the 20th century, the placenta was believed to provide a protective barrier to the fetus. This idea was questioned in 1961 when pregnant women who were administered thalidomide as an antianxiety agent gave birth to thousands of infants with limb defects (54). The 'thalidomide disaster' challenged the concept held previously, and it became clear that any drug or chemical substance administered to the mother is able to cross the placenta to some extent. Over recent years there has been a gradual rise in the use of drugs during pregnancy

despite the long-standing recognition that pregnant women are a special population (68). In some cases, the mother is continuously exposed to drugs during pregnancy based on the premise that the benefit to the mother outweighs the risk to the fetus. Therefore, an understanding of placental transfer of drugs has significant clinical relevance for both maternal and fetal welfare.

Placental Physiology and Drug Transfer

The human placenta is a discoid organ that separates the blood supply of the mother and the fetus, while being simultaneously perfused by both their circulations. It acts to supply the fetus with oxygen and nutrients and functions as an endocrine organ vital to a successful pregnancy (70). The placenta is endowed with transporters to facilitate the transport of nutrients to the fetus and the export of waste products. Some of the same transporters also mediate the passage of xenobiotics across the placenta. Nutrients such as glucose, amino acids and vitamins are transferred across the placenta via specific transport mechanisms present in the maternal-facing brush-border (apical) membrane and fetal-facing basal (basolateral) membrane of the syncytiotrophoblast, the functional unit of the placenta (22). Many of these transporters recognize xenobiotics as substrates, due to structural resemblance to physiological substrates, and mediate the transfer of these xenobiotics across the placenta (22). Therefore, the presence or absence of these transporters is important in determining whether a xenobiotic will accumulate in a tissue (34).

Transplacental movement of drugs is known to involve passive diffusion, facilitated diffusion, and active transport. Passive diffusion is the predominant form of exchange in the placenta and represents the permeation of a molecule down its concentration gradient. Passive diffusion does not require the input of energy, is not saturable and is not subject to competitive inhibition (61). Passive diffusion can be described by Fick's Law using the equation:

$$V_{diff} = \frac{D \times S \times (C_M - C_F)}{a}$$

where V_{diff} = rate of diffusion, D = diffusion coefficient, S = surface area of exchange, C_M = concentration in the maternal circulation, C_F = concentration in the fetal circulation, and a = thickness of the placenta. The amount of drug that crosses the placenta is comparable to other lipid membrane containing structures: drug transfer is dependent on the concentration of the drug in the maternal circulation, its physicochemical properties and the properties of the placenta. Passive diffusion is favored for unbound, low-molecular weight and highly lipid-soluble drugs that are predominantly un-ionized (43). Fetal exposure to a drug depends on various processes, including the rate of drug intake by the mother, the rate of drug elimination by the mother, the drug transfer rate across the placenta in both directions, and the drug distribution rates in mother and fetus (32). Facilitated diffusion requires the presence of a carrier substance within the placenta and the system can become saturated at high concentrations relative to the Michaelis-Menten constant (K_m). Transport by this mechanism does not require any input of energy. Ganciclovir, cephalexin, and the glucocorticoids are among those drugs that evidence has been found for carrier-mediated transport systems (61).

Active transport across the placental membrane by protein pumps requires energy, usually by adenosine triphosphate (ATP) hydrolysis or energy stored in the transmembrane electrochemical gradient provided by Na+, Cl- or H+. Active drug transporters are located either in the apical

membrane or in the basolateral membrane where they pump drugs into or out of the synctiotrophoblast (61).

Expression of Nucleoside Transporters in Placenta

To date, several active and facilitative transporters have been found to exist in the human and rat placenta. In addition, several of the same transporter families that exist in the human placenta have been shown to exist in the rat placenta (34). At least nine of the known transporters in the placenta have the ability to transport nucleoside antiviral agents or substrates that these transporters share in other tissues. A summary of these transporters is provided in Table 1.

Table 2.1. Nucleoside transporters in the placenta.

TRANSPORTER	MEMBRANE LOCALIZATION	DIRECTION	PHYSIOLOGICAL FUNCTION IN PLACENTA	REFERENCES
hENT1 rENT1	Apical	Influx/efflux	Mediate transport of purine and pyrimidine nucleosides	1, 34
hENT2 rENT2	Unknown	Influx/efflux	Mediate transport of purine and pyrimidine nucleosides	25, 36
hMRP rMRP	Apical or basolateral	Efflux	ATP-dependent efflux of organic anions	7, 34
hOAT4 rOAT1-3	Basolateral	Influx/efflux	Mediate Na+-independent uptake of organic anions	34, 60
hOCTN2 rOCTN1 and 2	Apical	Influx/efflux	Mediate passage of cationic substrates	34, 75
hOCT3 rOCT3	Basolateral	Influx	Mediate passage of cationic substrates	29, 34
hCNT1 and 2 rCNT1 and 2	Unknown	Influx/efflux	Mediate transport of purine and pyrimidine nucleosides	34, 70
hOATP rOATP	Basolateral	Influx/efflux	Mediate Na+-independent uptake of organic anions	34, 60
hBCRP rBCRP	Apical	Efflux	Efflux of chemotherapeutics	1, 18, 76

Efflux transporters include those belonging to the ATP-Binding Cassette transporter family, which function to remove xenobiotics from the placenta. The Multidrug-Resistance Proteins (MRP) mediate ATP-dependent transport of unconjugated, amphiphilic anions and of lipophilic compounds conjugated to glutathione, glucoronate and sulfate (60). The physiological function of the MRPs in the placenta remains speculative, although it seems likely that they could exert a feto-protective role by the removal of metabolic end products from the fetus to the mother (59). Three MRPs (MRP1-3) have been identified in basolateral membrane of the human placenta and the mRNA for six MRPs (MRP1-6) have been identified in the rat (34, 59). MRP1-3 are organic anion transporters which suggests that nucleoside antiviral agents are substrates. MRP4 and 5, although not yet identified in the human placenta, have been shown to transport nucleosides and nucleoside analogs (53). The Breast Cancer Resistance Proteins (BCRP) have been identified in the apical membranes of the human and rat placenta (1, 19, 76). Functional characterization in recent years has shown that BCRP can transport a wide range of substrates ranging from chemotherapeutic agents to organic anion conjugates. Wang et al. examined the cytotoxicity of the NRTIs, AZT and 3TC, in drug-resistant MT-4/DOX_{500} (BCRP-overexpressing cells) and MT-4 parental cells. They found that cytotoxicity of AZT and 3TC was reduced in MT-4/DOX_{500} cells compared with MT-4 cells, suggesting that AZT and 3TC may be BCRP substrates (71).

Transporters that are facilitative or equilibrative can function both as influx or efflux transporters depending on the directionality of the concentration gradient (68). Three organic cation transporters (OCT) have been identified in the rat (OCT3, OCTN1, and OCTN2) (34) and human (OCT2, OCT3, and OCTN2) (29, 75). OCTs and OCTNs are distinct subfamilies in the OCT group (60). OCT3 is thought to be located in the basal membrane where it actively

transports dopamine, norepinephrine and histamine as physiological substrates via a Na+ and Cl- independent system (22). The involvement of an OCT system has been suggested in the tubular secretion of AZT because cimetidine, and organic cation, reduces the renal clearance of AZT (13).

The organic anion-transporting polypeptide (OATP) and organic anion transporter (OAT) families are members of two distinct gene superfamilies that mediate the Na+ independent uptake of a host of organic anionic compounds (60). Of the eight OATPs and five OATS characterized, at least 2 OATPs (OATP-B and E) and one OAT member (OAT 4) are expressed in the human placenta (60). Minimal expression of OAT1-3 and eight OATP members were found in the rat placenta, with the exception of OATP12. ACV and AZT have been suggested as substrates of the OAT system because the renal excretion of these nucleosides analogs is reduced by probenecid, a typical inhibitor of organic anion transport (63). Estrone and nucleoside antiviral agents such as AZT are known to utilize the same OAT transporters. Therefore, nucleosides are likely substrates for OATPs since estrone is transported by OATP (46).

Two different Na+-independent equilibrative nucleoside transporters (ENT1 and ENT2) have been cloned from the human and rat placenta (25, 34). Both mediate the transport of purine and pyrimidine nucleosides, but differ in sensitivity to inhibition by nitrobenzylthioinosine. ENT1 is sensitive to inhibition whereas ENT2 is relatively insensitive to inhibition (22). ENT1 in both rats and humans is situated in the apical membrane, whereas the location of ENT2 is unknown (1, 25, 34). The concentrative nucleoside transporters (CNT1 and 2) are Na+ -dependent transporters that mediate active uptake of purine and pyrimidine nucleosides as well as nucleoside analogs by coupling to the inwardly directed Na+ gradient across the plasma membrane (71). The membrane localization in the rat and human placentas is unknown.

Methods of Studying Placental Drug Transport

Several *in vitro* and *in vivo* models have been established for studying the placental transfer of drugs and all have advantages and limitations. Some *in vitro* models include: 1) perfused placental cotyledon, 2) villus explants and monolayer cultures, 3) isolated trophoblast plasma membrane, and 4) isolated transporters and receptors (61). In particular, the perfused human placental cotyledon model has been used to investigate the mechanisms of anti-HIV dideoxynucleosides (66). These models resolve some of the ethical issues encountered within *in vivo* studies, however, they have several limitations. The extrapolation of the results from *in vitro* studies to pregnant women is problematic because the model involves a metabolically static system in contrast to the dynamic state of pregnancy (57).

In vivo methods to study the mechanisms of placental transfer of a drug involve the measurement of maternal-fetal clearance and fetal-maternal clearance of the drug (68). To determine these clearances, drug concentrations in both the maternal and fetal compartment must be measured (68). Since pregnant women are routinely excluded from clinical trials, the placental transfer of drugs has been investigated using animal models. Several animal species have been utilized to study the placental transfer of drugs, including non-human primates, rodents, and sheep (3, 4, 7, 14, 27, 47, 50, 51, 65-67, 56). Although the primates share the closest placental similarities to humans (19), 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 requirement of specialized housing and experimentation (27, 55).

Comparative Placental Structure

The pregnant rat model has been used successfully in the study of the placental transfer of many compounds, including nucleoside analogs (3, 4, 7, 14, 27, 35). Characteristics in placentation between the rat and human influence the efficiency and/or the rate of transfer of materials between the mother and embryo (62). Both the human and rodent placentas are hemochorial, in which trophoblastic cells are in direct contact with maternal blood without an intervening endothelium (33). However, there are some structural differences between human and rat placentas. In contrast to 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. (38, 62). Thus, the rat placenta is not structurally identical to that of humans, but it possesses the same types of cells and same basic hemochorial organization. In addition, studies using the pregnant rat model have yielded similar results to those reported in *in vitro* human placenta studies and in pharmacokinetics studies in monkeys (17, 27, 49).

There are several advantages to using the rat as a model, including easy handling, large litter size, ready availability on short notice, minimal housing requirements, and short gestation period (22 days) (55). The containment of each fetus, placenta, and amniotic fluid in individual fetal sacs allows for serial sampling of the pups, therefore providing a full concentration-time course. Another advantage is that their fetuses lack drug metabolizing capacity during most of their gestational life. Rodent fetuses are also unique in that due to their small size the amount of drug in the fetal body and the concentration in the plasma can be easily determined. The inability of the rodent fetus to eliminate drug and the analytical ability to determine both amount and

concentration of the drug in the fetus allows calculation of maternal/fetal disposition parameters without the need for fetal dosing (56).

In Vivo Placental Transport of Nucleoside Analogs

AZT has been the most widely used and studied antiviral in pregnancy. Several groups have investigated the placental transfer of AZT mono-therapy. AZT readily crosses the placenta by passive diffusion and is transferred extensively to fetal tissues. (17, 24, 49, 65). Although no accumulation was demonstrated in these studies, high concentrations in the amniotic fluid have been noted in several studies, suggesting that amniotic fluid serves as a slowly equilibrating reservoir.

Studies evaluating the potential interactions between nucleoside compounds in combination therapies have been conflicting. A series of studies by Unadkat show a lack of interaction between several antiviral agents, suggesting passive diffusion as the primary mechanism of transport (47, 51). Concurrent administration of d4T and AZT was reporterd to have no effect on the placental transfer of d4T in the pregnant pigtailed macque (47). In addition, no interaction was observed between ddI and AZT when the two drugs were coadministered in the pigtailed macaque (51). 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 (51). 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 (51).

Other studies observed a marked interaction between various antiviral agents (7, 22). Gallo et al. reported that ddI is capable of altering the pharmacokinetics of AZT, causing a reduction in the steady-state volume of distribution and the total clearance of AZT when ddI and AZT were coadministered to healthy monkeys. Also, a recent study by Brown et al. showed a significant alteration in the disposition of ACV and AZT when the two drugs were coadministered in pregnant rats. For AZT, decreases in exposure to all three tissues were seen in the presence of ACV. On the other hand, ACV showed a three-fold increase in drug exposure in amniotic fluid and fetal tissue with the combination therapy (7).

Compartmental Modeling of Pharmacokinetics During Pregnancy

To obtain a better understanding of the pharmacokinetic processes in the feto-maternal unit is to mimic the time course of drug concentrations by means of computer simulation, where mother and fetus function as one- or more-compartment systems with fast or slow pharmacokinetic characteristics. Several pharmacokinetic models on the transfer of nucleoside analogs between mother and fetus have been published (27, 47, 51, 56, 66). All models include two common compartments: maternal plasma and fetus. In addition, some models include a maternal peripheral compartment, fetal peripheral compartment , placenta, and/or amniotic fluid.

A simple model developed by Pereira et al., included a single compartment for maternal plasma, a single compartment for the fetus, and an amniotic fluid compartment (51). Huang et al. developed a five-compartment pharmacokinetic model that described AZT distribution in maternal plasma (central and peripheral), amniotic fluid, placenta, and fetal tissue of the pregnant rat (27). A series of studies by Unadkat's group included different pharmacokinetic models based on drug administration (47, 65, 66). Odinecs et al. administered bolus doses to the dam and fetus and characterized d4T concentrations in maternal and fetal plasma (central and

peripheral compartments), and amniotic fluid (47). The next study by Tuntland et al., administered bolus doses only to the dam and characterized ddC concentrations in the maternal plasma (central and peripheral compartments), fetal plasma, and amniotic fluid (65). A more recent study by Tuntland et al., administered bolus doses to the dam, fetus, and amniotic cavity, and characterized AZT concentrations in maternal and fetal plasma (central and peripheral), and amniotic fluid (66).

OBJECTIVES

Mother-to-child transmission remains the most important source of HIV infection in children. Over the past few years, considerable efforts have been made to introduce and expand programs to prevent mother-to-child transmission of HIV. Although progress has been made in implementing combination drug regimens, it is important to understand interactions at the placental level to optimize drug therapy. Evaluation of the pharmacokinetics and placental transport of antiviral drug combinations in pregnant women will further our knowledge about mechanisms involved in the fetal distribution of antiviral agents and lead to the optimization of treatment regimens for both mother and child. Therefore, the objectives of the current research are as follows:

Aim 1: To determine the pharmacokinetics and fetal disposition of AZT, 3TC, and abacavir in the pregnant rat.

Aim 2: To develop an assay for the determination of abacavir and AZT in maternal plasma, amniotic fluid, fetal and placental tissues using high-performance liquid chromatography with ultraviolet detection

Aim 3: To evaluate the interaction of 3TC/AZT and ABC/AZT in the fetal compartment to determine if purine and pyrimidine nucleoside antiretrovirals interact in the same manner.

Aim 4: To develop a pharmacokinetic model to characterize the placental transport of nucleoside analogs in combination in the pregnant rat and predict drug-drug interactions of other combinations of antiviral agents.

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CHAPTER 3

MATERNAL-FETAL PHARMACOKINETICS OF INTRAVENOUS LAMIVUDINE IN PREGNANT RATS[1]

[1]Lewis, S. R., Y. Alnouti, M. G. Bartlett, and C. A. White. To be submitted to *Antimicrobial Agents and Chemotherapy*.

ABSTRACT

The pharmacokinetics and placental transfer of lamivudine (3TC), a dideoxynucleoside analogue that has been shown to have potent activity against human immunodeficiency virus (HIV-1), was studied in the pregnant rat following IV administration. 3TC 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 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. Estimates for half-life ($t_{1/2}$), volume of distribution at steady state (V_{ss}), and clearance (CL) in maternal plasma were 1.9 ± 0.4 hr, 1.3 ± 0.1 L/kg, and 0.6 ± 0.1 L/hr-kg, respectively. Relative exposures of placenta, fetus and amniotic fluid for 3TC were 0.46 ± 0.07, 0.27 ± 0.08, and 0.28 ± 0.08, respectively. These values were consistent with the parameters obtained from the five compartment model. Significant differences were noted between the intercompartmental clearances between the maternal circulation and placenta, suggesting that active transport may play a role in the transplacental transfer of 3TC. However, further studies are needed to determine the role of these transporters in placental transport of 3TC.

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 the 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. (8, 10, 23).

Lamivudine (3TC) is a dideoxynucleoside analog of cytidine and is structurally similar to zalcitabine (ddC) (Figure 3.1). However, it differs from ddC in that it is a D-isomer versus an L-isomer and the 3'-carbon of the ribose ring is replaced with sulfur, forming an oxathiolane ring (14). Following i.v. administration in humans, 3TC is distributed into total body fluid (volume of distribution,Vss = 1.3 L/kg), and has low plasma protein binding (<36%) (26). Lamivudine triphosphate prevents HIV replication by competitively inhibiting viral reverse transcriptase and terminating the proviral deoxyribonucleic chain extension (26). 3TC has mostly been used in combination with zidovudine (AZT), given at a dose of 150 mg twice daily with 300 mg AZT (Combivir) (19).

There are several studies that have investigated the placental transport of AZT (6, 16, 21, 24, 27, 29); however, there remains a need for studies of other antiviral drugs, such as 3TC, and the placental transfer of these compounds alone and in conjunction with other therapeutic agents. 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 (9). 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 (13, 20). 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 agents (4-6, 16, 18).

Previous studies suggest that maternal-fetal transfer of single antiviral agents occurs through passive diffusion (3, 16, 22, 24, 27, 29). However, recent work found substantial interactions between antivirals in placental transport when using *in vitro* and animal models, supporting a transporter-mediated mechanism for placental transport of these compounds (6, 12). Continued study of these compounds alone and in combination is needed to gain further understanding of the placental transport processes of these agents. This study examines the placental transport and pharmacokinetics of 3TC during pregnancy.

MATERIALS AND METHODS

Chemicals and Reagents. 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%. Stavudine (d4T) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium sulfate was obtained from J.T Baker, Inc. (Philipsburg, NJ, 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 (n=7) (Harlan, Indianapolis, IN, USA) with an average weight of 328 ± 20 g were anesthetized intramuscularly on day 19 of pregnancy with ketamine:acepromazine:xylazine (50:3.3:3.4 mg/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 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 a Ultra-Turbax T8 (IKA Labortechnik, Germany). All samples were stored at –20 °C until analysis.

HPLC Analysis. 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 values for the individual tissues to the AUC value for the maternal plasma data.

Three five-compartment models were developed to characterize the distribution of 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 3.2). Model 1 (full 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. Model 2 (reduced model) incorporated bidirectional transfer between maternal plasma and placenta, placenta and fetus, placenta and amniotic fluid, and maternal plasma and tissue compartment. Model 3 (reduced model) incorporated bidirectional transfer between maternal plasma and placenta, placenta and fetus, fetus and amniotic fluid, and maternal plasma and tissue compartment. The model that best fit the 3TC data was determined by model selection criteria, such as parameter estimates and precision, residual plots, Akaike Information and Schwarz Criteria (AIC and SC, respectively), condition number, and the F-test. The differential equations were simultaneously fitted to the data. The equations for the best model are presented here (Equations 1-5).

$$\frac{dC_c}{dt} = \left(\frac{k_{pc} V_p}{V_b}\right) C_p + \left(\frac{k_{tc} V_t}{V_b}\right) C_t - \left(k_{cp} + k_{ct} + k_{c0}\right) C_c \qquad (1)$$

$$\frac{dC_p}{dt} = \left(\frac{k_{cp} V_b}{V_p}\right) C_c + \left(\frac{k_{fp} V_f}{V_p}\right) C_f - \left(k_{pc} + k_{pf}\right) C_p \qquad (2)$$

$$\frac{dC_f}{dt} = \left(\frac{k_{pf} V_p}{V_f}\right) C_p + \left(\frac{k_{af} V_a}{V_f}\right) C_a - \left(k_{fp} + k_{fa}\right) C_f \qquad (3)$$

$$\frac{dC_a}{dt} = \left(\frac{k_{fa} V_f}{V_a}\right) C_f - k_{af} C_a \qquad (4)$$

$$\frac{dC_t}{dt} = \left(\frac{k_{ct} V_b}{V_t}\right) C_c - k_{tc} C_t \qquad (5)$$

Using intercompartmental transfer rates, the model incorporates bidirectional transfer between maternal plasma and placenta (k_{cp} and k_{pc}), placenta and fetus (k_{pf} and k_{fp}), fetus and amniotic fluid (k_{fa} and k_{af}), and maternal plasma and tissue compartment (k_{ct} and k_{tc}). The elimination rate constant from the maternal plasma compartment is k_{c0}. Parameter estimates were generated by WINNONLIN and intercompartmental clearance values (CL) were calculated from the product of the intercompartmental rate constants and distribution volumes.

RESULTS AND DISCUSSION

Mean concentration-time profiles of 3TC in maternal plasma, placenta, fetus and amniotic fluid following intravenous administration of a 25 mg/kg bolus dose are shown in Figure 3.3. Maternal plasma 3TC concentrations 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 3.1. The maternal plasma pharmacokinetic parameters generated for 3TC in pregnant rats (volume of distribution at steady state, 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) (2, 26). These values for V_{ss} indicate that 3TC is extensively distributed, which is unexpected based on the hydrophilic nature of this drug, thus, suggesting that active transport may be involved. Compared to non-pregnant

rats (V_{ss} = 1.8 L/kg, $t_{1/2}$ = 1.04) (17), the V_{ss} was slightly lower, possibly due to the removal of pups at sample collection times (16). The clearance was significantly lower in pregnant rats resulting in a 2-fold increase in half-life as compared to non-pregnant rats. This decrease in clearance is consistent with results for other antivirals administered to pregnant rats (15, 16). A previous study reported that both pregnancy and anesthesia have been shown to result in decreased AZT clearance in rats; however, it was concluded that although the experimental procedures had some influence on the pharmacokinetics of AZT in the pregnant rats, the general pattern of AZT disposition in the placenta and fetus compared to the dam was similar to that shown previously for unanesthetized pregnant rats (15, 21).

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 pharmacokinetic parameters generated by noncompartmental analysis of the placenta, fetus and amniotic fluid data are shown in Table 3.2. The placenta declines in parallel with the maternal plasma (Figure 3.3). The uptake of 3TC into the placenta was rapid, with 3TC concentrations reaching a maximum at 5 minutes. 3TC uptake into the fetus was less rapid relative to the placental uptake, while the amniotic fluid showed a significantly slower uptake of 3TC. Interestingly, the concentration of 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 3TC. This phenomenon has previously been reported for 3TC and other antiviral agents (6, 16, 22, 25, 29). Mandelbrot et al., upon studying the maternal-

fetal transfer of 3TC between maternal plasma and fetal cord blood in humans, observed high concentrations of 3TC in the amniotic fluid compared to that of maternal blood (22). 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. RE values for the placenta, fetus and amniotic fluid are given in Table 3.2. RE for these tissues to 3TC range from 0.27 to 0.46 and are significantly lower than values reported for other antiviral agents (6). This is consistent with the lower lipophilicity of 3TC measured by its octanol-water partition coefficient (0.21) as compared with other antiviral agents (30).

The maternal plasma, placenta, fetus, and amniotic fluid were also simultaneously fitted to three five-compartment models (Figure 3.2). The models developed for 3TC maternal-fetal transport assumed no drug elimination from the fetus since metabolism of 3TC is a minor route of elimination, with only 5–10% of parent compound metabolized to the pharmacologically inactive trans-sulphoxide metabolite (14). Statistical analysis of the three pharmacokinetic models demonstrated that Model 3 best described 3TC concentrations in the maternal-placental-fetal unit (Table 3.3). Based on the condition number, examination of residual plots, parameter estimates and their precision, AIC, and SC, Model 3 was favored over the other models. In addition, by using the F-test to discriminate between the system of hierarchical models (full vs. reduced models), we found that the full model was superior to Model 2, but was not superior to Model 3, further supporting Model 3 as the best model. Model 3 incorporated bidirectional transfer between maternal plasma and placenta, placenta and fetus, fetus and amniotic fluid, and

maternal plasma and tissue compartment. The model-predicted 3TC concentration versus time profiles in maternal plasma, placenta, fetus, and amniotic fluid closely followed the observed data (Figure 3.2).

Intercompartmental clearances are presented in Table 3.3. The parameter estimates obtained using the five compartment model were consistent with observations obtained from the noncompartmental analysis and exhibited relatively low standard deviations compared to earlier studies using this model (16). The transfer of 3TC from maternal plasma to the placenta was rapid, consistent with the short T_{max}. The higher CL_{fp} pathway is, in part, responsible for the higher RE in the placenta as compared to the fetus and amniotic fluid. Peak concentrations in amniotic fluid were delayed compared to those in the fetus which is consistent with the 100-fold higher CL_{fp} as compared to CL_{fa}. Bidirectional transfer between amniotic fluid and fetus provided the best fit for 3TC data. 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 (28). 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 (29). The equivalent intercompartmental clearances for these two compartments are consistent with their REs. The bidirectional intercompartmental clearances for 3TC from amniotic fluid and placenta are not necessary for this drug since 3TC is polar and very little drug will diffuse from the amniotic fluid through umbilical cord membranes to the placenta.

Significant differences were noted between the intercompartmental clearances CL_{cp} and CL_{pc}, and between CL_{pf} and CL_{fp}, suggesting that active transport may play a role in the transplacental transfer of 3TC. These observations are supported by the recent identification of transporters in the placenta (13, 20). Since most antiviral agents are used in combination, there

is potential for competition for placental transporters and changes in maternal pharmacokinetics that may also impact fetal exposure. However, further studies are needed to determine the role of these transporters in placental transport of this agent.

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Table 3.1. Maternal plasma pharmacokinetic parameters for 3TC (mean ± standard deviation)

Parameter	Value
Half-life (hr)	1.9 ± 0.4
AUC (hr-mg/L)	43.0 ± 7.6
CL_T (L/hr-kg)	0.6 ± 0.1
V_{ss} (L/kg)	1.3 ± 0.1
C_{max}(mg/L)	47.4 ± 6.6

Table 3.2. Placental, Fetal and Amniotic Fluid Pharmacokinetic Parameters and Relative Exposures for 3TC (mean ± standard deviation)

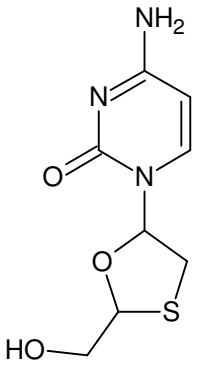
Parameter	**Placenta**	**Fetus**	**Amniotic Fluid**
Half-life (hr)	2.1 ± 0.3	3.3 ± 1.1	3.9 ± 1.2
AUC*	19.3 ± 1.9	11.2 ± 3.4	12.1 ± 3.5
Cmax**	9.0 ± 0.5	2.3 ± 0.9	1.7 ± 1.0
Tmax (hr)	0.1 ± 0	1.0 ± 0.6	3.6 ± 0.5
Relative Exposure***	0.46 ± 0.07	0.27 ± 0.08	0.28 ± 0.08

* 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 = $AUC_{tissue}/AUC_{maternal\ plasma}$

Table 3.3. Intercompartmental clearance estimates (ml/min) expressed as mean (standard deviation) of 3TC in pregnant rats (25 mg/kg).

Parameter	Model 1	Model 2	**Model 3**
CL_{cp}	0.26 ± 0.06	0.25 ± 0.04	0.25 ± 0.04
CL_{pc}	0.62 ± 0.20	0.61 ± 0.25	0.61 ± 0.25*
CL_{pf}	0.53 ± 0.20	0.51 ± 0.15	0.51 ± 0.15
CL_{fp}	1.12 ± 0.31	1.09 ± 0.23	1.09 ± 0.24*
CL_{pa}	0.005 ± 0.001	0.008 ± 0.001	
CL_{ap}	0.004 ± 0.003	0.01 ± 0.005	
CL_{ct}	4.68 ± 0.67	4.66 ± 0.73	4.67 ± 0.73
CL_{tc}	4.64 ± 0.82	4.70 ± 0.84	4.71 ± 0.85
CL_{fa}	0.007 ± 0.004		0.03 ± 0.002
CL_{af}	0.012 ± 0.006		0.03 ± 0.008
CL_{c0}	2.92 ± 0.48	2.95 ± 0.49	2.95 ± 0.49
Condition #	1361	372	359
WRSS	5.36	6.86	4.99
SBC	123	127	112
AIC	103	110	95
F-statistic		5.18[b]	1.27[a]

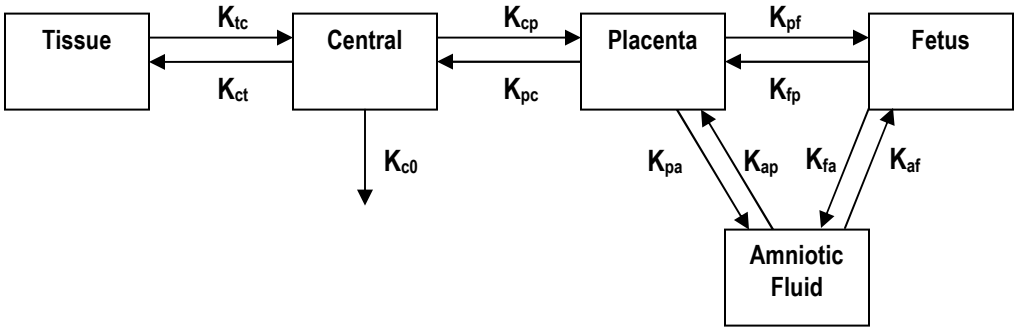
*Indicates significant differences between corresponding bidirectional intercompartmental clearances ($P < 0.05$).
[a] Significantly superior to the full model.
[b] Not significantly superior to the full model.



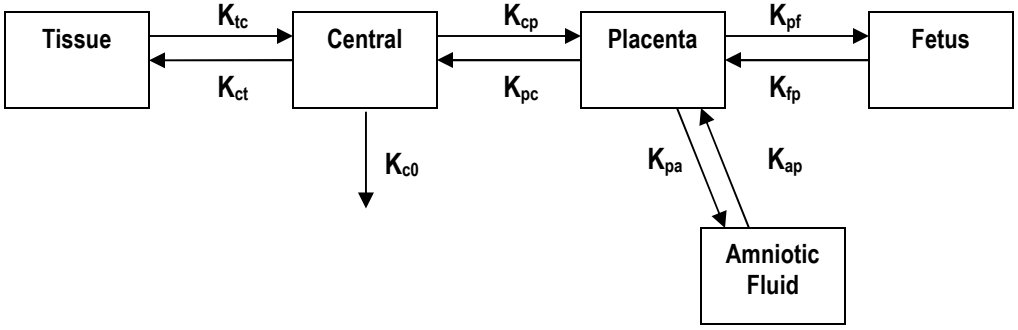
3TC

Figure 3.1. Chemical Structure of 3TC.

(a)



(b)



(c)

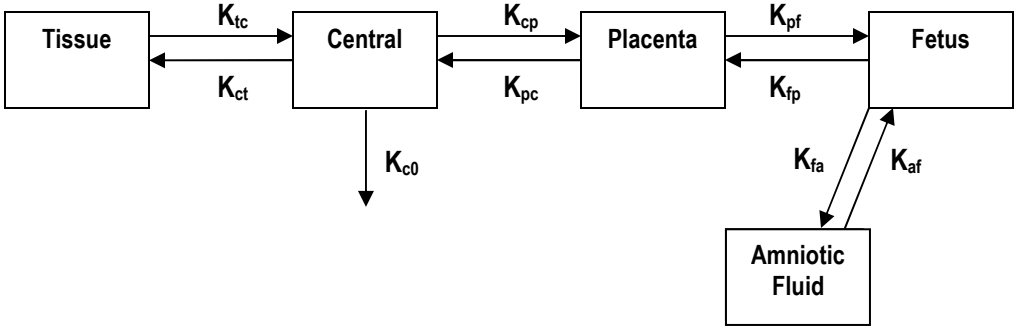


Figure 3.2. Schematic representations of the three pharmacokinetic models, Model 1 (a), Model 2 (b), and Model 3 (c), used to describe the disposition of 3TC in maternal central, maternal tissue, placental, fetal, and amniotic fluid compartments after iv injection to pregnant rats.

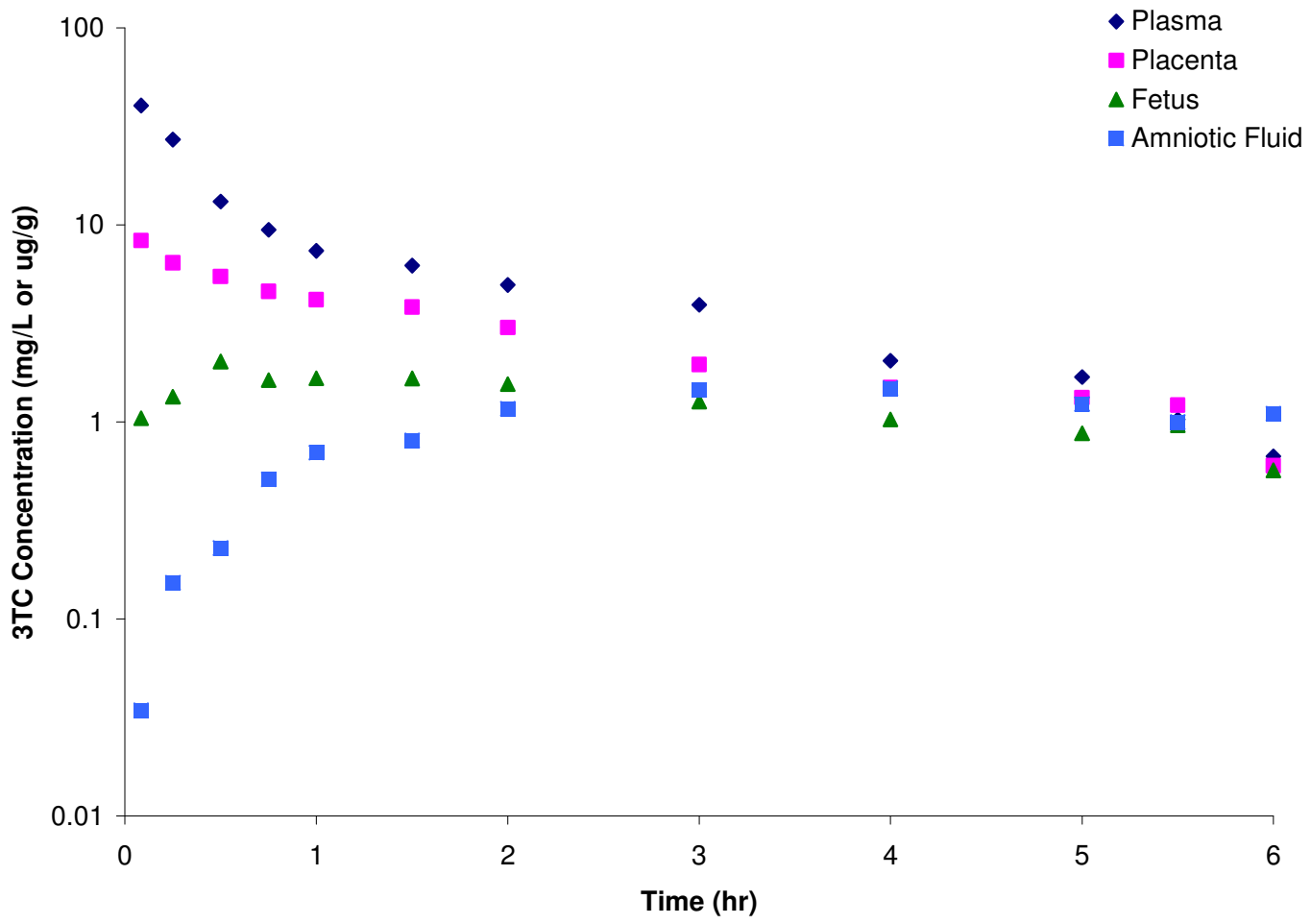


Figure 3.3. Concentration versus time profiles of 25 mg/kg dose of 3TC following IV bolus administration in maternal plasma, placenta, fetus, and amniotic fluid.

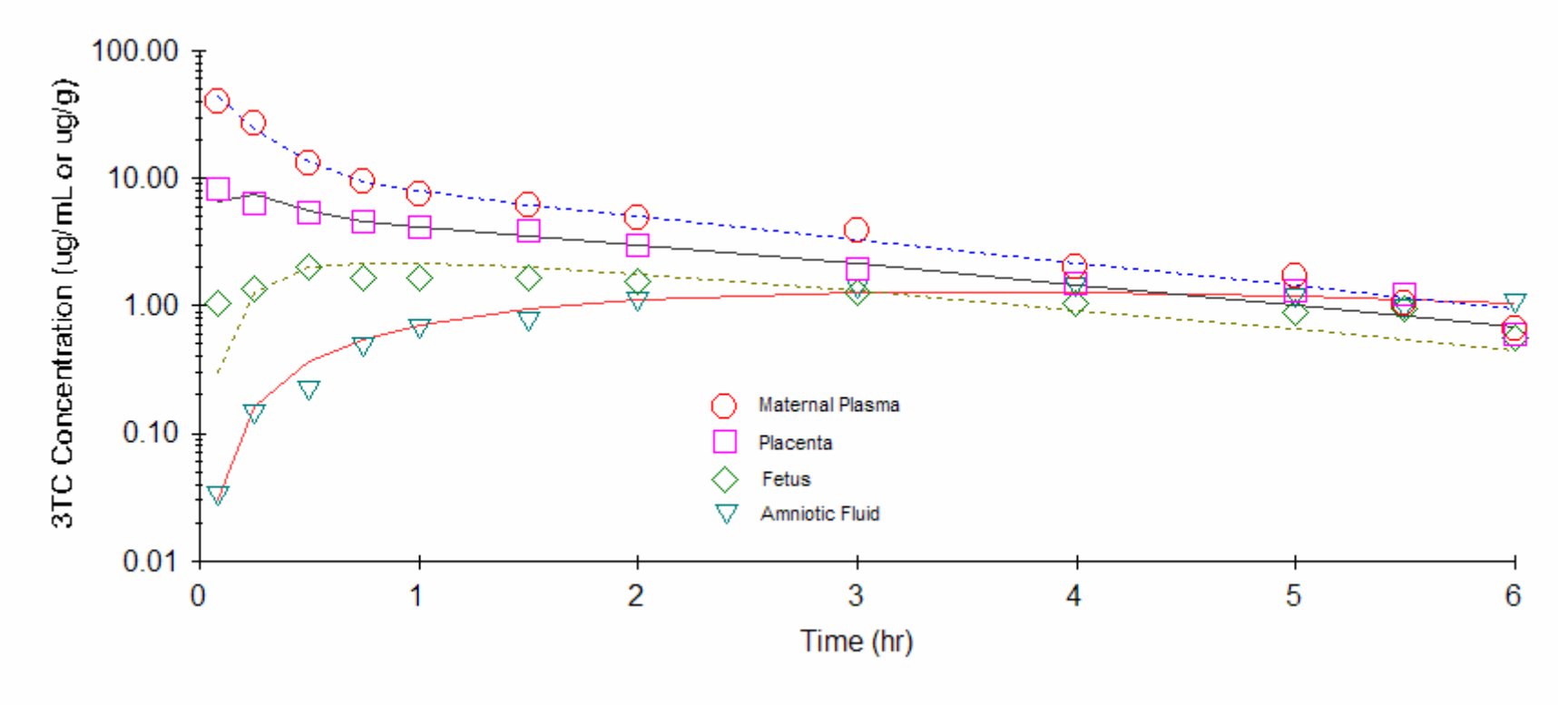


Figure 3.4. Measured 3TC concentration versus time data for maternal plasma, placenta, fetus, and amniotic fluid fitted simultaneously to the five-compartment model.

CHAPTER 4

INTERACTION OF LAMIVUDINE AND ZIDOVUDINE IN THE PREGNANT RAT[1]

[1]Lewis, S. R., Y. Alnouti, S. D. Brown, M. G. Bartlett, and C. A. White. To be submitted to *Journal of Pharmacology and Experimental Therapeutics*.

ABSTRACT

The pharmacokinetics and placental transfer of lamivudine (3TC) and zidovudine (AZT) monotherapy and lamivudine-zidovudine combination therapy were compared in the pregnant rat. Pregnant Sprague-Dawley rats were anesthetized on day 19 of pregnancy and a jugular catheter was implanted and laparotomy performed. Rats were dosed with 3TC, AZT or 3TC-AZT combination (25mg/kg, IV bolus, n=6). Serial blood, placenta, fetus and amniotic fluid (AF) samples were obtained. 3TC and AZT were quantitated by HPLC-MS-MS. A five-compartment model was fitted to the data and pharmacokinetic parameters and relative exposures (RE) ($AUC_{tissue}/AUC_{maternal\ plasma}$) were determined with WINNONLIN. The plasma pharmacokinetics of 3TC were not influenced by the co-administration of AZT. However, 3TC significantly increased clearance of AZT, which resulted in a 20% decrease in half-life (1.14 to 0.85 hr). No difference was noted in volume of distribution. RE of placenta and fetus for 3TC when AZT was coadministered significantly increased from 0.43 to 0.97 and 0.19 to 0.45, respectively. This is consistent with the two-fold decrease in the distribution clearance from placenta to maternal plasma for 3TC coadministered with AZT. RE of fetus and AF for AZT when 3TC was coadministered significantly decreased from 0.51 to 0.36 and 0.86 to 0.62, respectively. This is consistent with the increase in distribution clearance from AF to placenta. The changes noted in the placenta, fetus, and AF suggest that transporters play a complex role in the uptake of 3TC and AZT in these tissues. Thus, nucleoside drugs given in combination can dramatically influence fetal exposure to these agents.

INTRODUCTION

As of 2005, an estimated 40.3 million people are living with HIV/AIDS worldwide, of which 17.5 million are women (44). An increase in cases among women of childbearing age has resulted in an increase in cases among infants and children, most of who were infected during pregnancy, delivery, or breast feeding. It is estimated that 90% of HIV infections that occur among children are a result of vertical transmission from a mother to her infant (9). Zidovudine (AZT) was the first antiviral approved for treatment in pregnancy. Although zidovudine therapy alone has substantially reduced the risk for vertical transmission (14), due to the emergence of resistance, it is now used in combination with other antiviral drugs such as lamivudine (3TC). With the introduction of highly active antiretroviral therapy (HAART), a drastic decline in HIV mortality and morbidity has been observed in most western countries. Combination antiretroviral regimens of multiple antiretrovirals during pregnancy have demonstrated reductions in the rate of mother-to-child transmission to less than 2% (15, 27, 30). The combination most frequently used for this purpose is 3TC-AZT because it is shown to lower viral load and improve outcome in HIV-infected patients (24) (Figure 4.1). 3TC-AZT (Combivir) was approved by the FDA in 1997 for use in combination with other antiretroviral agents for the treatment of HIV infection, thus decreasing the rate of vertical transmission from 6.8% with AZT alone to 1.6% with combination therapy (29). However, little work has been done to characterize the placental transfer of these drugs when administered in combination.

The data about the safety of antiretroviral drugs in pregnancy are limited. A study conducted in France reported that several infants with *in utero* or neonatal exposure to either 3TC-AZT or AZT alone developed indications of mitochondrial dysfunction with the risk being higher among infants exposed to the combination of 3TC-AZT (3). Also, data from a study

published in 2006 suggests that 3TC-AZT combination treatment in mice produces greater mitochondrial DNA damage than either agent individually (11). However, there are conflicting data regarding whether mitochondrial disease is associated with perinatal antiretroviral exposure (15, 36). Although treatment with these compounds is efficacious in HIV-infected individuals and reduces the risk of vertical transmission during pregnancy, it may also inadvertently expose fetuses to these potentially toxic drugs. Therefore, it is important to study the placental transfer and pharmacokinetics in the fetal compartment in order to optimize therapeutic treatment regimens in pregnancy.

Pregnant women experience unique physiological changes such as changes in gastrointestinal transit time, cardiac output, and plasma protein concentrations, which may result in clinically significant alterations in drug pharmacokinetics (9, 38). Due to the difficulty and ethical concerns in studying placental and fetal drug distribution in humans, an appropriate animal model must be utilized. The pregnant rat has been chosen as a model because of their easy handling, large litter size (10-15 pups), and short gestation period. The containment of each fetus, placenta, and amniotic fluid in individual fetal sacs allows for serial sampling of the pups, therefore providing a full concentration-time course. The rat placenta also has many similarities the human placenta. Both the human and rat placentas are discoid-shaped, possess haemochorial placental membranes, and experience similar hemodynamic changes in pregnancy (16, 25). In addition, several of the same transporter families that exist in the human placenta have been shown to exist in the rat placenta (27). Successful use of the pregnant rat model in studying placental transfer has been demonstrated in several compounds, including antiviral compounds (5, 6, 8, 21, 22).

Previous studies suggest that maternal-fetal transfer of single antiviral agents occurs through passive diffusion (4, 31-35, 37, 39). However, recent work found substantial interactions between antivirals in placental transport when using *in vitro* and animal models, supporting a transporter-mediated mechanism for placental transport of these compounds (8, 19). Continued study of these compounds is needed to gain further understanding of the placental transport processes of these agents. This study examines the placental transport and pharmacokinetics of 3TC and AZT monotherapy and 3TC-AZT combination therapy during pregnancy.

MATERIALS AND METHODS

Chemicals and Reagents. Lamivudine (3TC), zidovudine (AZT) were 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%. The internal standards, FTC amd AZDU were synthesized as previously described (13, 23). The internal standard, stavudine (d4T) was purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile, sodium phosphate dibasic, and reagent-grade citric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium acetate, ammonium formate, formic acid and acetic acid were obtained from Aldrich (Milwaukee, WI, 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 (50:3.3:3.4 mg/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. Intravenous bolus doses (25 mg/kg) of each therapy group were prepared in 0.1 M NaOH in physiological saline and were administered via the jugular cannula.

Three dosing groups were used to complete the study: (i) 3TC monotherapy (25 mg/kg) (n = 6), (ii) AZT monotherapy (25 mg;/kg) (n = 6), and (iii) 3TC-AZT combination therapy (25 mg/kg each) (n = 7). 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 and AZT monotherapy. AZT concentrations were determined by an HPLC-UV method developed previously (8). Plasma and amniotic fluid samples were prepared by acid protein precipitation by adding 10 µl (50-µl spiked amniotic fluid sample) or 20 µl(100-µl spiked plasma sample) of 2 M perchloric acid. Placental and fetal tissue homogenates were processed by solid-phase extraction. Samples were loaded onto Sep-Pak C_{18} SPE cartridges (Waters Corp., Milford, MA, USA) pre-conditioned with methanol and mobile phase, followed by the sample load and a wash of the sample with deionized water and elution with methanol. Calibration curves were generated by using samples from spiked blank matrix to yield final calibration points of 0.1, 0.5, 1, 5, 10, 50, and 100 µg/ml. The internal standards, 3TC (plasma and amniotic fluid) and AZDU (placenta and fetus) yielded final concentration of 25 µg/ml and 10 µg/ml, respectively.

The chromatographic system consisted of a Hewlett-Packard (Agilent)1100 Series HPLC with a quaternary pump, degasser, autosampler, and variable-wavelength UV detector (Pal Alto, Calif.). Chromatographic separations were achieved by using an Agilent Eclipse XDB C-8 column (150 by 2.1 mm, 5 µl with a Phenomenex Security Guard C_{18} column (Torrance, CA, USA). The mobile phase consisted of a 30 mM acetate-citrate buffer at pH 3.08 and methanol. Under these conditions, AZT eluted at 15.9 min and 3TC eluted at 10.9 min in the plasma and amniotic fluid. In the fetus and placental tissues, AZT eluted at 19.8 min and AZDU at 18.4 min. This method has been previously validated to show acceptable precision and accuracy over the calibration range of 0.1 to 100 µg/ml.

HPLC Analysis and 3TC monotherapy. 3TC concentrations were determined by an HPLC-UV method developed previously (1). 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 has been 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.

HPLC Analysis and 3TC-AZT combination therapy. 3TC and AZT concentrations were determined by an HPLC-UV method developed previously (2). Samples were prepared by acetonitrile precipitation. 800 µl of acetonitrile was added to 100 µl of spiked samples. Samples were vortexed, 750 µl was aspirated and dried in vacuum. Finally, samples were reconstituted with a 100 µl of water: methanol (1:1) solution. Calibration curves were generated by using samples from spiked blank matrix to yield final calibration points of 25, 10, 1, 0.25, 0.1 and 0.05 µg/ml. The internal standard concentration was 0.5 µg/ml (FTC).

The chromatographic system consisted of a Shimadzu LC-10ADVP, SCL-10AVP system controller (Shimadzu, Tokyo, Japan) and autosampler. The mass spectrometer was a Sciex API-4000 triple quadrupole instrument with TurboIon Spray ESI source (Applied Biosystems,

Toronto, Canada). The entire LC-MS system is controlled by Analyst 2.1 Software (Applied Biosystems, Toronto, Canada). All chromatographic separations were performed with a Symmetry C_{18} column (5 μm, 150 x 3.9 mm I.D., Waters, Milford, MA, USA) equipped with a Phenomenex C-18 guard column (Torrance, CA, USA). The mobile phase consisted of methanol (mobile phase A) and 7.5 mM ammonium acetate pH 6.5 (mobile phase B) delivered from 2 separate LC pumps. The flow rate was 0.65 ml/min. Under these conditions, 3TC eluted at 2.9 min, AZT eluted at 6.1 min, and FTC (internal standard) at 3.2 min. This assay was validated to ensure both precision and accuracy in accordance with the Food and Drug Administration guidelines for bioanalytical method validation (44). The assay showed acceptable reproducibility (percent relative standard deviation < 15%) and accuracy (percent error < 15%) over the calibration range of 0.05 to 25 μg/ml.

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 generated from AZT monotherapy, 3TC monotherapy, and 3TC-AZT combination therapy animals. A 1/y-weighting scheme was used throughout the analysis. Amniotic fluid, placenta, and fetus data were subjected to noncompartmental analysis. Due to the inability to calculate accurate half-lives for 3TC-AZT in the tissues, the area under the curve was truncated at 6 hours for 3TC and 3TC-AZT in plasma, placenta, fetus, and amniotic fluid for the calculation of relative exposure. The relative exposure of each matrix was calculated by comparing the AUC values for the individual tissues to the AUC values for the corresponding plasma data.

Three five-compartment models were evaluated to characterize the distribution of 3TC and AZT in the maternal plasma (central) (C_c), maternal tissue (C_t), placental (C_p), fetal (C_f), and

amniotic fluid (C_a) compartments. Based on the goodness-of-fit criteria, the models that best described 3TC and AZT concentrations are shown in Figure 4.2. Model 1 (Equations 1-5) was used to characterize 3TC and 3TC-AZT concentrations and incorporates bidirectional transfer between maternal plasma and placenta, placenta and fetus, fetus and amniotic fluid, and maternal plasma and tissue compartment:

$$\frac{dC_c}{dt} = \left(\frac{k_{pc} V_p}{V_b}\right) C_p + \left(\frac{k_{tc} V_t}{V_b}\right) C_t - \left(k_{cp} + k_{ct} + k_{c0}\right) C_c \qquad (1)$$

$$\frac{dC_p}{dt} = \left(\frac{k_{cp} V_b}{V_p}\right) C_c + \left(\frac{k_{fp} V_f}{V_p}\right) C_f - \left(k_{pc} + k_{pf}\right) C_p \qquad (2)$$

$$\frac{dC_f}{dt} = \left(\frac{k_{pf} V_p}{V_f}\right) C_p + \left(\frac{k_{af} V_a}{V_f}\right) C_a - \left(k_{fp} + k_{fa}\right) C_f \qquad (3)$$

$$\frac{dC_a}{dt} = \left(\frac{k_{fa} V_f}{V_a}\right) C_f - k_{af} C_a \qquad (4)$$

$$\frac{dC_t}{dt} = \left(\frac{k_{ct} V_b}{V_t}\right) C_c - k_{tc} C_t \qquad (5)$$

Model 2 (Equations 6-10) was used to characterize AZT and AZT-3TC concentrations and incorporates bidirectional transfer between maternal plasma and placenta, placenta and fetus, placenta and amniotic fluid, and maternal plasma and tissue compartment:

$$\frac{dC_c}{dt} = \left(\frac{k_{pc} V_p}{V_b}\right) C_p + \left(\frac{k_{tc} V_t}{V_b}\right) C_t - \left(k_{cp} + k_{ct} + k_{c0}\right) C_c \qquad (6)$$

$$\frac{dC_p}{dt} = \left(\frac{k_{cp} V_b}{V_p}\right) C_c + \left(\frac{k_{fp} V_f}{V_p}\right) C_f - \left(k_{pc} + k_{pf} + k_{pa}\right) C_p \qquad (7)$$

$$\frac{dC_f}{dt} = \left(\frac{k_{pf} V_p}{V_f}\right) C_p - k_{cp} C_f \qquad (8)$$

$$\frac{dC_a}{dt} = \left(\frac{k_{pa} V_p}{V_a}\right) C_p - k_{ap} C_a \qquad (9)$$

$$\frac{dC_t}{dt} = \left(\frac{k_{ct} V_b}{V_t}\right) C_c - k_{tc} C_t \qquad (10)$$

In these models, k_{ct} and k_{tc} are the intercompartmental transfer rate constants between maternal central and tissue compartments, k_{cp} and k_{pc} are the intercompartmental transfer rate constants between maternal plasma and placental compartments, k_{pf} and k_{fp} are the intercompartmental transfer rate constants between placental and fetal compartments, k_{pa} and k_{ap} are the intercompartmental transfer rate constants between amniotic fluid and placental compartments, k_{fa} and k_{af} are the intercompartmental transfer rate constants between amniotic fluid and fetal compartments, and k_{c0}) is the elimination rate constant from the maternal plasma compartment. The differential equations were simultaneously fitted to the concentration-time data. Parameter estimates were generated by WINNONLIN and intercompartmental clearance values (CL) were calculated from the product of the intercompartmental rate constants and distribution volumes. The pharmacokinetic parameters generated for each dosing group and relative exposures were compared using the unpaired *t* test ($P < 0.05$) to identify statistically significant differences.

RESULTS

The concentration in maternal plasma-time profiles for 3TC and AZT are shown in Figure 4.3. The pharmacokinetic parameters generated from the compartmental analysis of the plasma data are presented in Table 4.1. For 3TC and AZT monotherapies, plasma concentrations declined in a bi-exponential manner, with an average terminal half-life of 1.9 ± 0.4 h and 1.1 ± 0.2 h, respectively. In combination, the plasma pharmacokinetics of 3TC remained unchanged.

However, the co-administration of 3TC resulted in a modest increase in the clearance of AZT, resulting in a decrease in half-life from 1.1 hr to 0.9 hr.

The concentration-time profiles of 3TC and AZT in placenta, amniotic fluid, and fetus are shown in Figs. 4.4 and 4.5. The pharmacokinetic parameters for these matrices are shown in Tables 4.2, 4.3, and 4.4. 3TC and AZT rapidly distributed into the placenta and fetus with peak concentrations observed within one hour of drug administration. A slower rate of uptake of 3TC and AZT is observed for amniotic fluid, with T_{max} observed at 3.6 ± 0.5 and 2.3 ± 0.6 hr, respectively. In comparison to 3TC, peak concentrations of AZT were 2- and 3-fold higher in the fetus and amniotic fluid, respectively. No significant differences were noted in the half-lives. The relative exposure of the placenta was similar for both AZT and 3TC; however, the relative exposure to AZT was 2-fold and 3-fold higher in fetus and amniotic fluid, respectively, as compared to 3TC.

C_{max} for AZT is consistently lower in the placenta, fetus, and amniotic fluid when given in combination with 3TC and occurred earlier in the amniotic fluid. The half-lives of AZT in the fetus and amniotic fluid are significantly shorter for AZT in the presence of 3TC. While not significant, an increase in half-life for AZT in the placenta was noted. These changes in tissue disposition resulted in significant decreases in RE for AZT in the fetus and amniotic fluid when administered with 3TC. The fitted 5-compartment model for AZT is shown in Figure 4.6. Fitted estimates of the distribution clearances are shown in Table 4.5. No significant differences among the placental, fetal and amniotic fluid distribution clearances between AZT alone and in combination with 3TC were noted.

While co-administration of AZT with 3TC did not alter the maternal pharmacokinetics of 3TC, dramatic changes were observed in the placenta, fetus and amniotic fluid. 3TC

concentrations peaked quickly and remained relatively high throughout the experiment. The half-life of 3TC increased over 2-fold in the placenta and fetus. Accurate estimates of the 3TC half-life were not possible since samples were only obtained for 6 hours. Peak 3TC concentrations were also significantly higher in the placenta and fetus when given in combination with AZT. These combined changes increased the RE of 3TC approximately 2-fold. 3TC concentrations in placenta and fetus were higher than plasma concentrations at 1.5 and 4 hours, respectively, suggesting sequestration of 3TC. A five compartment concentration-time curve for 3TC is shown in Figure 4.7. Fitted estimates of the distribution clearances are shown in Table 4.5. A significant decrease is CLpc was noted when 3TC was administered in combination with AZT. Additionally, CLfa (2-fold) and CLaf (10-fold) were significantly increased with combination therapy. These changes are consistent with the changes observed in RE. Comparison of the intercompartmental distributional clearances suggest that placental to maternal circulation and fetal to placenta pathways involve active transport. The placental to maternal circulation transport of 3TC appeared to be inhibited by AZT while the fetal to placental transport of 3TC was unaffected by AZT. Additionally, the movement of 3TC from fetus to amniotic fluid was altered by AZT resulting in a significant increase in transport of 3TC from the amniotic fluid to fetus.

DISCUSSION

In this study, we investigated the impact of combination therapy on the disposition of 3TC and AZT in the pregnant rat. Previous studies evaluating the potential interactions between nucleoside compounds have been conflicting. A series of studies by Unadkat et al. show a lack of interaction between several antiviral agents, suggesting passive diffusion as the primary

mechanism of transport (32, 36). Concurrent administration of ddI and zidovudine was reported to have no affect on the transfer of ddI across the placenta in the pigtailed macaque (32). In addition, no interaction was reported when zidovudine was coadministered with stavudine (36). However, other studies observed a marked interaction between various antiviral agents (8, 18). Gallo et al. reported that ddI is capable of altering the pharmacokinetics of AZT, causing a reduction in the steady-state volume of distribution and the total clearance of AZT when ddI and AZT were coadministered to healthy monkeys. Also, a recent study by Brown et al. showed a significant alteration in the disposition of ACV and AZT when the two drugs were coadministered in pregnant rats. There are no preclinical data investigating the combination of 3TC and AZT in pregnancy.

The maternal plasma pharmacokinetics of 3TC are unchanged in the presence of AZT. However, the co-administration of 3TC caused a significant increase in the clearance of AZT, resulting in a 20% decrease in half-life. Since neither 3TC nor AZT are significantly bound to plasma proteins a displacement interaction is unlikely to account for the increase in clearance. 3TC and AZT are both excreted primarily in the urine, so it is plausible that competition for the reabsorption of AZT may occur. The increase in AZT clearance could also be attributed to an upregulation of efflux transporters in the kidney. Studies have shown that a number of physiologically and pharmacologically important nucleosides are actively secreted by the kidneys, including AZT (12). This interaction noted in clearance may also influence fetal exposure since many transporters in the kidney have also been identified in the placenta.

3TC and AZT showed rapid distribution into the fetus and placenta, however, transfer of the drugs to the amniotic fluid occurred at a slower rate and reached a plateau, suggesting that the amniotic fluid compartment behaves as a slowly equilibrating compartment. The

accumulation of 3TC and AZT concentrations in the amniotic fluid is consistent with previously reported results (8, 10, 21, 31). The concentrations of 3TC in both the fetal and amniotic fluid were not as high as that of AZT, with AZT exhibiting 2- and 3- fold higher peak concentrations. This is consistent with the higher lipophilicity of AZT as measured by its octanol-water partition coefficient (0.98) compared to that of 3TC (0.21).

As indicated earlier, a pharmacokinetic model consisting of five compartments was fitted simultaneously to the observed 3TC and AZT i.v. bolus data. However, the models that provided the best fit to the data were slightly different. For 3TC the model without the placenta-amniotic fluid pathways provided the best fit, whereas for AZT, the model without the fetus-amniotic fluid pathways was superior. These differences may be related to differences in polarity for 3TC and AZT. Drug moves from the amniotic fluid through the umbilical cord membranes before it can re-enter the placenta. This movement is much slower for 3TC as compared to AZT. The deletion of the amniotic fluid-fetus transfer of AZT from the model is most likely due to the relatively low clearances between these two compartments as compared to the other intercompartment clearances (100-fold lower). The model-predicted concentration-versus-time profiles of 3TC and AZT in the maternal plasma, placenta, fetus, and amniotic fluid closely followed the observed data in most cases despite the complexity of the model. There was good agreement between the parameter estimates obtained from this compartmental analysis and the results obtained in the noncompartmental analysis. The combination of changes noted in CL_{pc}, CL_{fp}, and CL_{pf} coupled with the significant changes in CL_{ct} and CL_{c0} explain, in part, the 20-30% decrease in RE for AZT in fetus and amniotic fluid when given in combination with 3TC. Comparison of the intercompartmental distributional clearances suggest that placental to

maternal circulation and fetus to placenta pathways involve active transport of AZT. These transport pathways did not appear to be significantly altered by the coadministration of 3TC.

The increase in relative exposures observed in the fetal and placental tissues to 3TC is consistent with the decrease shown in the intercompartmental clearance from placenta to maternal plasma. This decrease suggests that the placental efflux transport of 3TC decreased in the presence of AZT. A possible mechanism of action could be saturation of an efflux transporter in the apical side of the placenta since AZT appears to have a higher affinity for these transporters as reflected in the 4-fold increase in CL_{pc}.

Active transport can be assumed when there is a significant difference between two intercompartmental clearances and/or when the distribution rate constants are large. For 3TC alone, the differences were noted between CL_{cp} and CL_{pc} and CL_{pf} and CL_{fp}, suggesting that active transport may play a role in the transplacental transfer of 3TC. The observation that CL_{cp} and CL_{pc} were not significantly different when 3TC was given in combination with AZT, suggests that AZT blocked the active transport pathway noted when 3TC was given alone. Cl_{fa} and CL_{af} were not significantly different for 3TC alone, suggesting that transfer between fetus and amniotic fluid occurs primarily by passive diffusion. However, a significant increase was observed in CL_{af} when 3TC was given in combination with AZT suggests an upregulation of transporters. For AZT, alone and in combination with 3TC, differences were not observed between corresponding pairs distribution clearances, CL_{cp} and $CL_{pc,}$ CL_{pf} and CL_{fp}, and CL_{pa} and CL_{ap}. This suggests that active transport plays a role in the transplacental transfer of AZT whether given alone or in combination with 3TC. Movement of AZT between placental and amniotic fluid was affected by 3TC administration but is a relatively minor pathway as compared to placenta-fetal distribution.

Several drug transporters have been found to exist in the placenta (20, 28). At least nine of the known transporters in the placenta transport nucleoside antiviral agents or substrates that these transporters share in other tissues (16, 28, 45). Among the efflux transporters include the ABC transporters such as the multidrug resistance proteins (MRPs) and the breast cancer resistance protein (BCRP), all of which function to move xenobiotics from the placenta to the maternal circulation. The role of MRP in the placenta has not been established, while studies investigating the role of BCRP in modulating the exposure of the fetus to drugs suggest a function for BCRP as a maternal-fetal barrier to passage of xenobiotics across the placenta (24). The bidirectional transporters can function both as influx or efflux transporters and 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). Qualitatively, the placental transporters in the rat mirror those found in the human placenta, which makes the pregnant rat an appropriate model for the further delineation of the role of placental transporters in the fetal uptake of antiviral agents.

The fetal disposition of 3TC in the pregnant rat was significantly altered when the two drugs were coadministered. The placental transport of 3TC decreased dramatically in the presence of AZT. This suggests that transporters, in addition to passive diffusion, play a role in the transport of these nucleoside analogs. Which specific transporters or how quantitatively these transporters affect *in vivo* disposition of therapeutic nucleoside analogs is unknown. Further studies using cell culture are necessary to identify which transporters are involved in the fetal uptake of these nucleoside analogs. The high concentrations found in the fetal compartment may have important clinical implications. The ability to provide prophylaxis in the fetal compartment provides a great benefit. However, there is concern regarding the potential toxicity

of perinatal exposure to nucleoside analogs (3). Further investigations will be needed to study whether high concentrations in these tissues will have an impact on the long-term safety of the fetus.

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Table 4.1. Pharmacokinetic parameters (mean plus or minus standard deviation) generated from compartmental analysis of plasma data collected from 3TC monotherapy, AZT monotherapy, and 3TC-AZT combination therapy in pregnant rats (25 mg/kg).

Drug Therapy	**Half-life (hr)**	**AUC (hr-mg/liter)**	**Clearance (liter/hr-kg)**	**V_{ss} (liter/kg)**	**C_{max} (mg/liter)**
3TC	1.94 ± 0.35	43.0 ± 7.6	0.60 ± 0.09	1.26 ± 0.14	47.4 ± 6.6
3TC-AZT	1.72 ± 0.26	38.1 ± 3.5	0.68 ± 0.07	1.29 ± 0.09	55.7 ± 6.9*
AZT	1.14 ± 0.15	42.2 ± 3.1	0.61 ± 0.05	0.87 ± 0.1	60.7 ± 21.2
AZT-3TC	0.85 ± 0.04*	35.0 ± 2.6	0.72 ± 0.05*	0.80 ± 0.04	57.1 ± 12.8

*Indicates significant difference between monotherapy and combination therapy ($P < 0.05$). V_{ss}, volume of distribution at steady state.

Table 4.2. Pharmacokinetic parameters for placenta generated by using noncompartmental analysis.

Drug Therapy	C_{max} (mg/liter)	T_{max} (hr)	Half-life (hr)	Relative Exposure**
3TC	8.9 ± 0.5	0.1 ± 0	2.1 ± 0.3†	0.43 ± 0.07
3TC-AZT	12.1 ± 1.5*	0.1 ± 0	7.1 ± 3.1*†	0.97 ± 0.18*
AZT	8.3 ± 0.7	0.2 ± 0.1	1.4 ± 0.4	0.44 ± 0.06
AZT-3TC	7.1 ± 0.9*	0.1 ± 0.1	2.0 ± 0.6	0.48 ± 0.14

*Indicates significant difference between monotherapy and combination therapy ($P < 0.05$).

**Relative exposure = $AUC_{tissue}/AUC_{maternal\ plasma}$

†Rough estimate of half-life

Table 4.3. Pharmacokinetic parameters for fetus generated by using noncompartmental analysis.

Drug Therapy	C_{max} (mg/liter)	T_{max} (hr)	Half-life (hr)	Relative Exposure**
3TC	2.3 ± 0.9	1.0 ± 0.6	3.3 ± 1.1[†]	0.19 ± 0.05
3TC-AZT	3.2 ± 0.3*	0.9 ± 0.2	6.9 ± 2.9*[†]	0.45 ± 0.01*
AZT	6.4 ± 1.1	0.5 ± 0.1	2.6 ± 0.78	0.51 ± 0.11
AZT-3TC	5.4 ± 0.5*	0.4 ± 0.2	1.7 ± 0.4*	0.36 ± 0.06*

*Indicates significant difference between monotherapy and combination therapy ($P < 0.05$).
**Relative exposure = $AUC_{tissue}/AUC_{maternal\ plasma}$
[†]Rough estimate of half-life

Table 4.4. Pharmacokinetic parameters for amniotic fluid generated by using noncompartmental analysis.

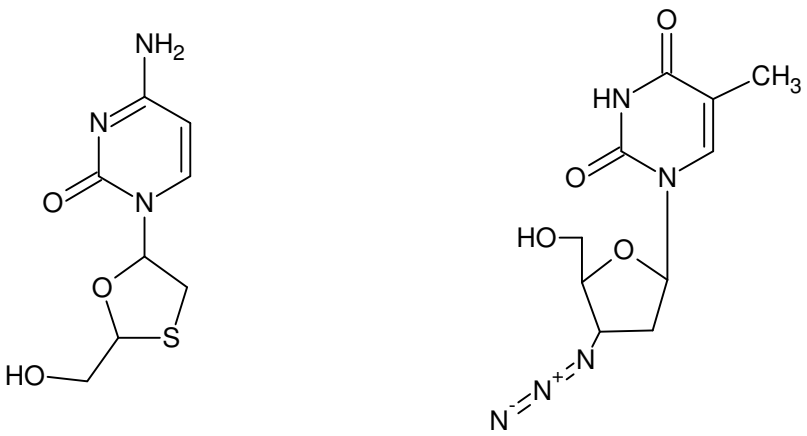
Drug Therapy	C_{max} (mg/liter)	T_{max} (hr)	Half-life (hr)	Relative Exposure**
3TC	1.7 ± 1.0	3.6 ± 0.5	3.9 ± 1.2†	0.14 ± 0.08
3TC-AZT	2.0 ± 0.2	1.8 ± 0.5*	4.4 ± 1.1†	0.20 ± 0.03
AZT	4.4 ± 0.6	2.3 ± 0.5	4.8 ± 0.5	0.86 ± 0.05
AZT-3TC	3.8 ± 0.9	1.4 ± 0.4*	3.2 ± 0.5*	0.62 ± 0.18*

*Indicates significant difference between monotherapy and combination therapy ($P < 0.05$).
**Relative exposure = $AUC_{tissue}/AUC_{maternal\ plasma}$

Table 4.5. Intercompartmental clearances (mean plus or minus standard deviation) of 3TC, AZT, and 3TC-AZT in pregnant rats (25mg/kg).

Drug Therapy	CL_{cp}	CL_{pc}	CL_{pf}	CL_{fp}	CL_{pa}	CL_{ap}	CL_{ct}	CL_{tc}	CL_{fa}	CL_{af}	CL_{c0}
3TC	0.25 (0.04)	0.61[b] (0.25)	0.51 (0.15)	1.09[b] (0.24)	-	-	4.67 (0.73)	4.71 (0.85)	0.03 (0.002)	0.03 (0.008)	2.95 (0.49)
3TC-AZT	0.26 (0.08)	0.24[a] (0.16)	0.52 (0.1)	1.03[b] (0.25)	-	-	5.17 (0.53)	4.42 (0.84)	0.05[a] (0.02)	0.10[ab] (0.05)	3.30 (0.38)
AZT	0.97 (0.39)	2.40[b] (1.00)	3.86 (0.87)	3.99 (0.97)	0.05 (0.02)	0.02[b] (0.004)	5.78 (1.96)	5.89 (1.93)	-	-	2.99 (0.49)
AZT-3TC	0.90 (0.31)	2.79[b] (1.14)	4.47 (0.99)	4.95 (1.53)	0.04 (0.01)	0.03[b] (0.01)	9.06[a] (2.51)	7.85 (2.81)	-	-	3.48[a] (0.21)

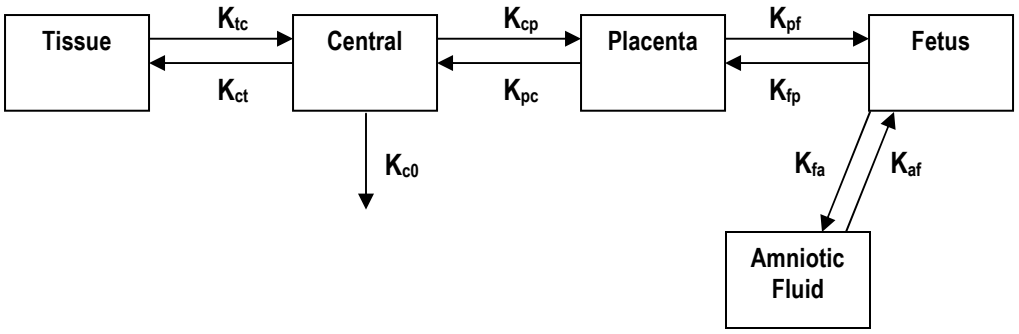
[a] Indicates significant difference between monotherapy and combination therapy ($P < 0.05$).
[b] Indicates significant differences between corresponding distribution clearances within each therapy group ($P<0.05$)



3TC AZT

Figure 4.1. Chemical Structures of 3TC and AZT.

(a)



(b)

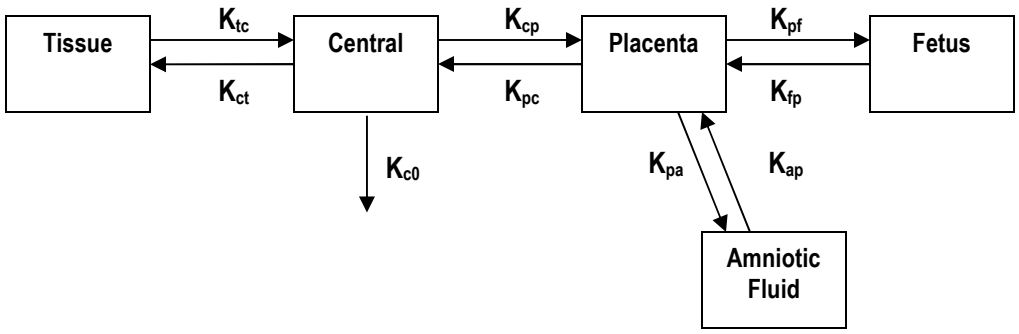
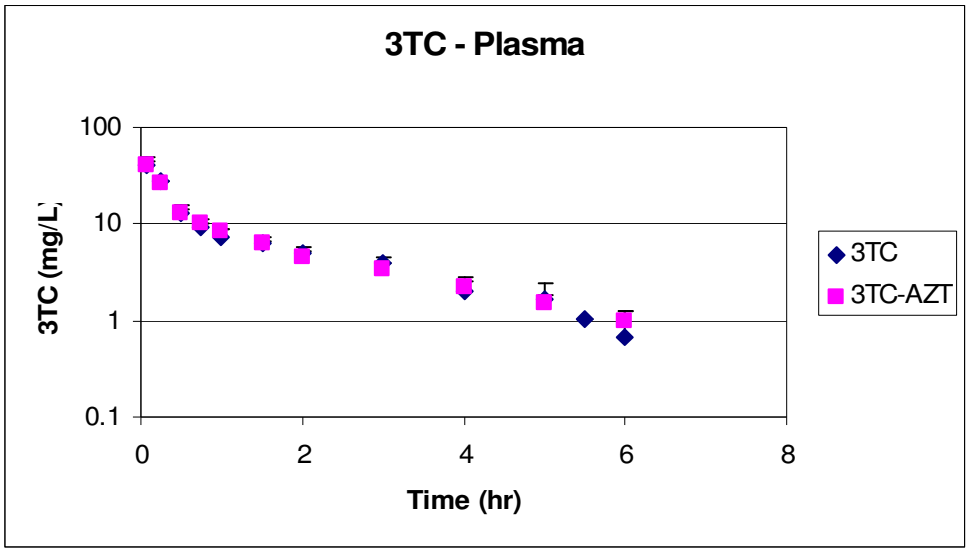


Figure 4.2. Schematic representation of the pharmacokinetic models used to describe the disposition of 3TC (a) and AZT (b) in maternal central, maternal tissue, placental, fetal, and amniotic fluid compartments after iv injection to pregnant rats.

(a)



(b)

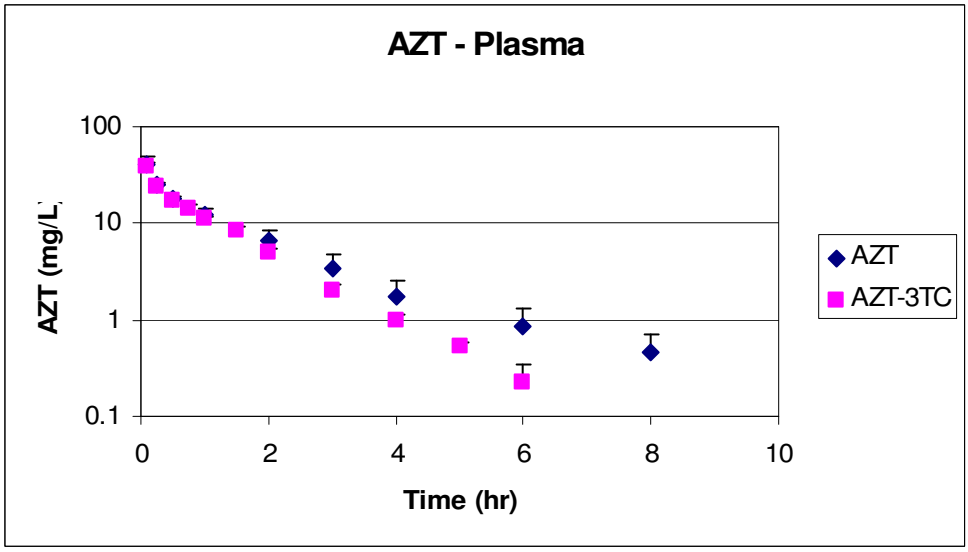
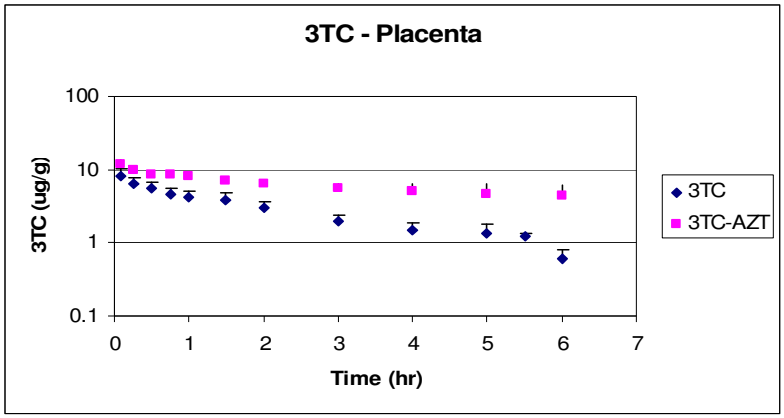
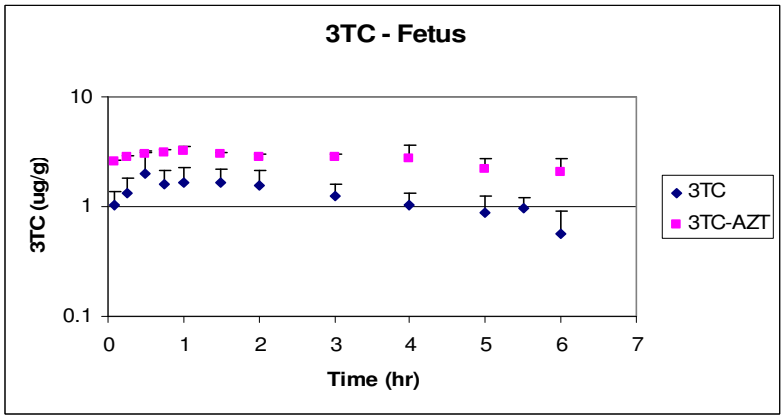


Figure 4.3. Concentration in plasma (mean plus standard deviation) versus time profiles for 3TC alone and in combination with AZT (a) and AZT alone and in combination with 3TC (b).

(a)



(b)



(c)

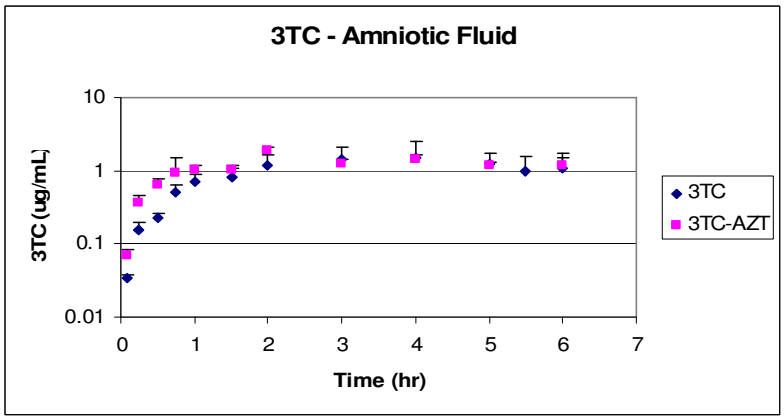
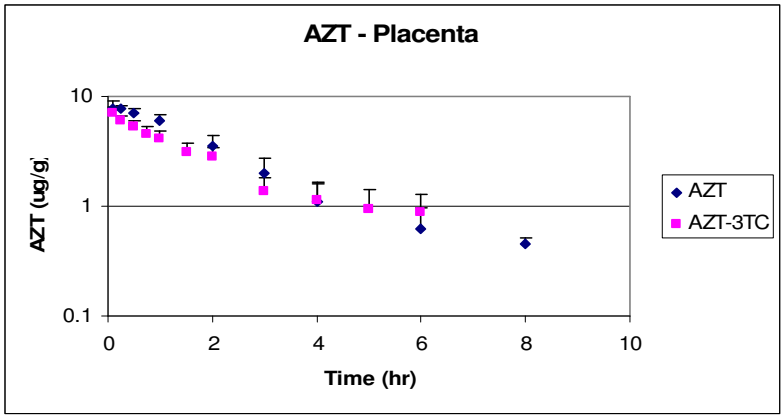
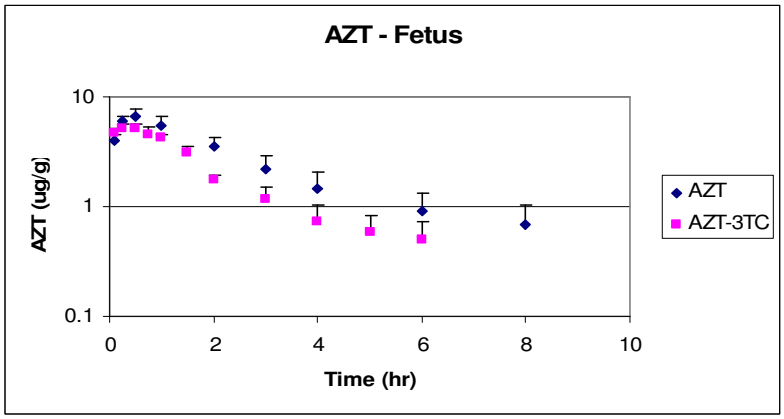


Figure 4.4. Concentration (mean plus standard deviation) versus time profiles of 3TC monotherapy and in combination with AZT from placenta (a), fetus (b), and amniotic fluid (c).

(a)



(b)



(c)

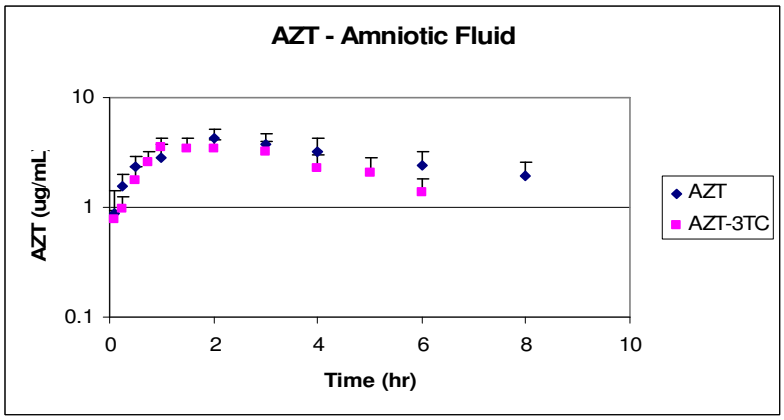
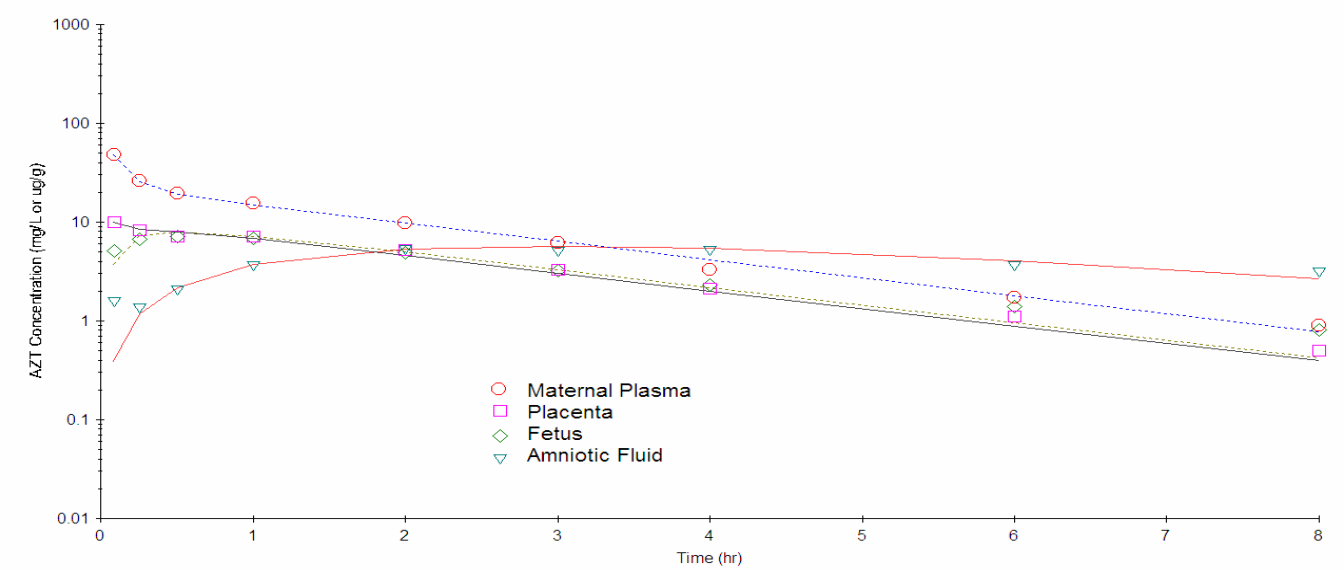


Figure 4.5 The concentration (mean plus standard deviation) versus time profile of AZT monotherapy and in combination with 3TC from placenta (a), fetus (b), and amniotic fluid (c).

(a)



(b)

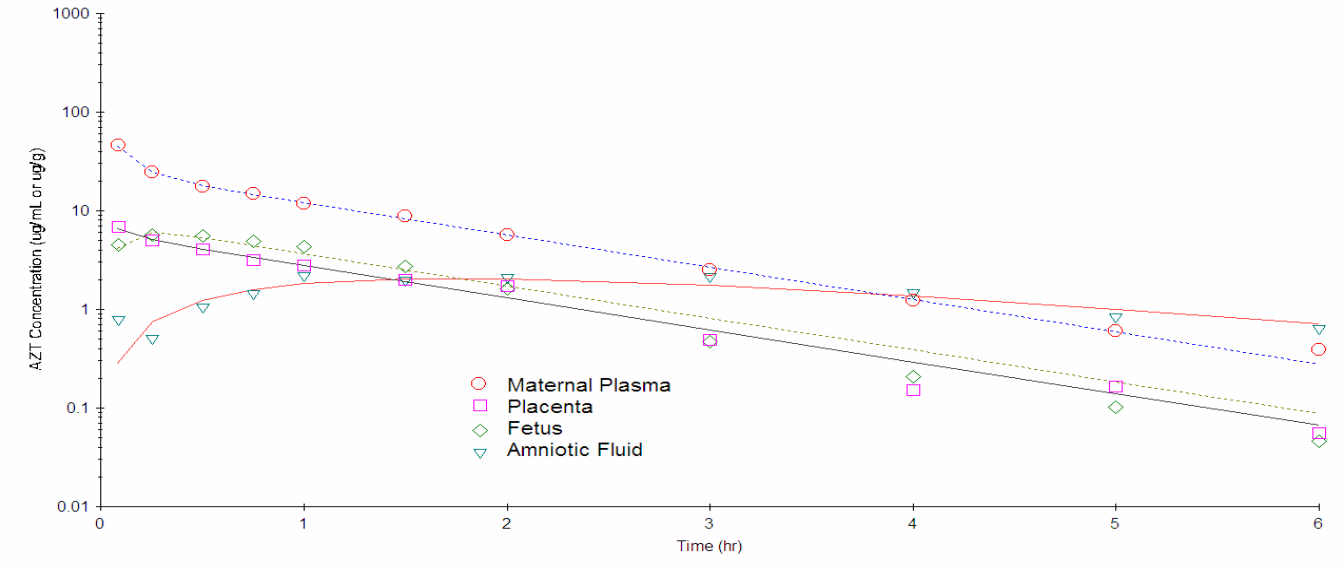
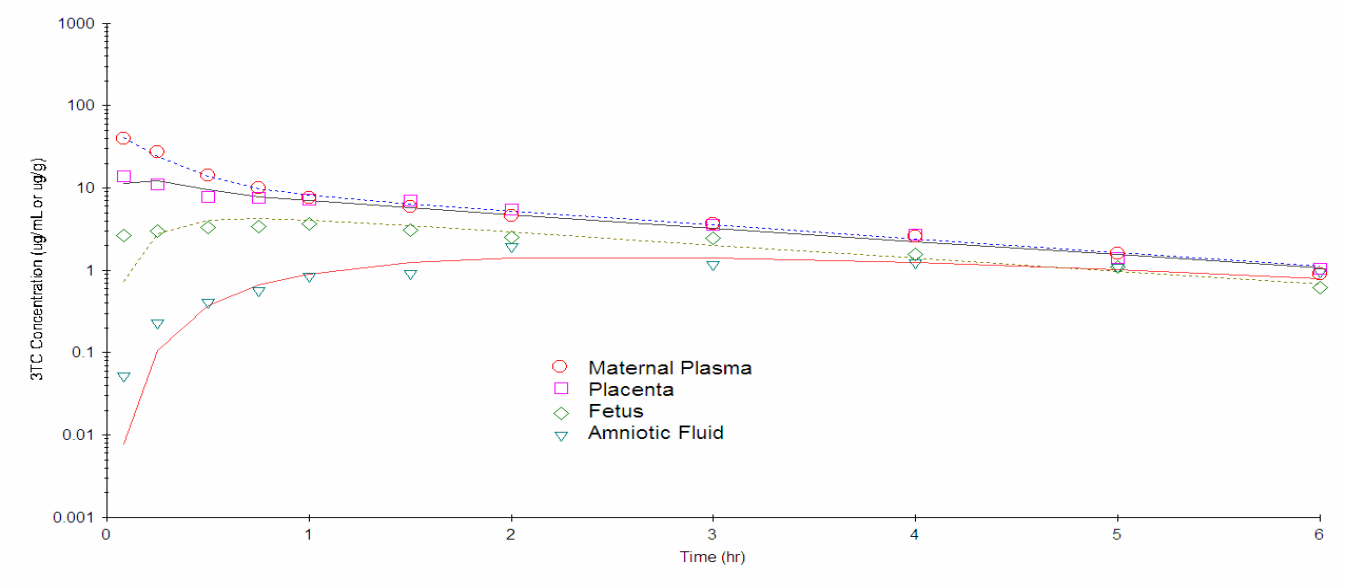


Figure 4.6. Measured AZT (a) and AZT-3TC (b) concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to the five compartment model.

(a)



(b)

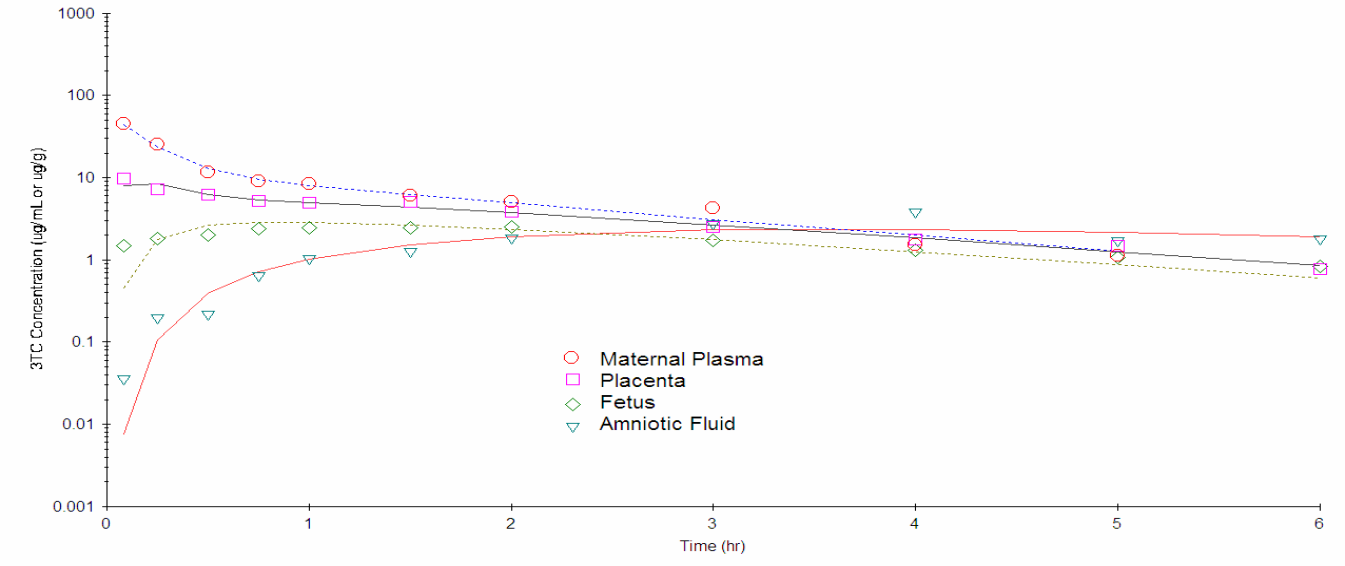


Fig 4.7. Measured 3TC (a) and 3TC-AZT (b) concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to the five compartment model.

CHAPTER 5

SIMULTANEOUS DETERMINATION OF ABACAVIR AND ZIDOVUDINE FROM RAT TISSUES USING HPLC WITH ULTRAVIOLET DETECTION[1]

[1]Lewis, S.R., C. A. White, and M. G. Bartlett. Submitted to *Journal of Chromatography B,* April 2006.

ABSTRACT

A simple high-performance liquid chromatography (HPLC) method has been developed and validated for the simultaneous determination of abacavir and zidovudine (AZT) in rat plasma, amniotic fluid, fetal, and placental tissues. Extraction of abacavir, AZT, and the internal standard, azidouridine (AZDU) in maternal plasma and amniotic fluid was carried out by protein precipitation. Extraction from fetal and placental homogenate was achieved by using a salting out technique. Chromatographic separation was performed using a C_8 column (150 x 4.6 mm, 5 μm). The mobile phase consisted of 12% acetonitrile in 25 mM sodium phosphate buffer (adjusted to pH 7 with sodium hydroxide) for the fetus, placenta, and amniotic fluid samples and 13% acetonitrile in 25 mM sodium phosphate buffer (adjusted to pH 7 with sodium hydroxide) for plasma at flow rates of 0.8 ml/min. The method was validated in the range of 0.05-50 μg/ml for both abacavir and AZT in the four biological matrices. The absolute recovery of abacavir ranged from 79 to 94%, while AZT recoveries ranged from 79 to 90% in the different biological matrices. The internal standard recovery ranged from 84 to 92%. Acceptable intra- and inter-day assay precision (< 10% R.S.D.) and accuracy (< 10% error) were observed over 0.05-50 μg/ml for all four matrices.

INTRODUCTION

In 2005, an estimated 700,000 children were newly infected with HIV worldwide and 540,000 children died from AIDS (1). Vertical transmission of HIV may occur early or late in pregnancy, during birth or postnatally through breastfeeding. In the absence of preventative measures, about 35% of children born to HIV-positive women will contract the virus. With the advent of potent antiretroviral therapies, a great reduction in morbidity and mortality has been achieved. Combination therapy or highly active antiretroviral therapy, generally consisting of 2 nucleoside reverse transcriptase inhibitors (NRTIs) and a nonnucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI), is currently the recommended standard treatment of HIV-infected nonpregnant adults in industrialized countries (2). In resource-poor settings, the approach to antiretroviral prophylaxis of vertical transmission of HIV is dramatically different in that the goal is to obtain effective, shorter, and less expensive antiretroviral regimens (3, 4).

Zidovudine (AZT) belongs to the NRTI class of antiretroviral agents and was the first anti-HIV agent to be approved by the FDA for the treatment of HIV-1 infection. In 1994, results from the PACTG protocol 076 clinical trial demonstrated that shortcourse AZT therapy reduced the maternal-fetal transmission of HIV infected pregnant women from 26% to 8% (5) As resistance to zidovudine begins to increase, emphasis has shifted to combination therapies. Combination therapies involving AZT can further reduce vertical transmission to less than 2% (6).

Abacavir is a novel nucleoside reverse transcriptase inhibitor (NRTI) used for the treatment of HIV and a synthetic analogue of guanine. It differs structurally from other NRTIs in that it is a carbocyclic nucleoside analogue rather than a dideoxynucleoside analogue. Abacavir sulfate was approved by the FDA in 1998 for use in combination with other

antiretroviral agents for the treatment of HIV-1 infection in adults and children (7). It is also prescribed in combination with lamivudine (3TC) and AZT, and marketed as Trizivir® for use in combination with a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor. Abacavir is classified by the FDA as a pregnancy category C drug, meaning that animal studies have shown an adverse effect on the fetus and there are no well-controlled studies in humans, but potential benefits may warrant use of this drug in pregnant women despite potential risks (8).

While the use of combinations of antiviral drugs is becoming increasingly common, the impact of such combination therapies on placental transport is largely unknown. A series of studies has reported the lack of interaction between several anti-HIV drugs, suggesting passive diffusion as the primary mechanism of placental transfer (9, 10). However, the combination of abacavir and AZT was not studied. Recent studies of other antiviral compounds found substantial interactions between antiviral compounds in placental transport (11, 12). Continued study of these compounds is necessary to gain further understanding of the mechanism of placental transport for this important class of therapeutic agents.

Due to ethical concerns, pregnant women are excluded from clinical trials, making it difficult to study placental and fetal distribution in humans (13). Therefore, an animal model must be utilized that will provide clinically useful information. The pregnant rat model has been proven successful for the investigation of the basic mechanisms involved in placental transfer of nucleoside analogs due to the structural similarities between rat and human placenta (14). The large litter size allows for serial sampling, providing a complete concentration versus time profile. The pregnant rat model has been utilized in maternal-fetal drug transfer studies of a variety of compounds, including nucleoside analogs (12, 15-21).

Several HPLC methods have been developed for abacavir and AZT analysis (23-25). However, none of these methods deal with the analysis of these compounds from complex matrices such as maternal plasma, amniotic fluid, placental and fetal homogenates. Also, the some of the methods use long run times and large sample volumes. This report describes the development and validation of a rapid HPLC assay for the simultaneous determination of abacavir and AZT in pregnant rat plasma, amniotic fluid, fetal and placental tissues. This method will be used in support of a comparative pharmacokinetic study to investigate the impact of abacavir and AZT combination therapy on placental transport.

EXPERIMENTAL

Chemicals and Reagents

Abacavir was obtained from GlaxoSmithKline. AZT and internal standard, 3'-azido-3'-deoxyuridine (AZDU), were obtained from Sigma (St. Louis, MO). HPLC-grade acetonitrile, sodium phosphate monobasic, and ammonium sulfate were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Preparation of Stock and Standard Solutions

Stock solutions of 1.0 mg/mL abacavir, AZT, and AZDU were individually prepared in deionized water. Standard solutions of abacavir and AZT were prepared by mixing and diluting the appropriate amounts from the individual stock solutions. The final concentrations of the standard solutions were 500, 250, 50, 25, 5, 2.5 and 0.5 μg/ml. A 25 μg/mL standard solution of AZDU was prepared with deionized water from the 1.0 mg/mL stock. Precision and accuracy standards with concentrations of 400, 35, 2, and 0.5 μg/mL were also prepared in the same manner. Stock solutions were kept refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis or validation.

Chromatographic System

The chromatographic analyses were performed using an HPLC system consisting of Agilent 1100 Series components including a quaternary pump, degasser, autosampler, and variable-wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Agilent Eclipse XDB C_8 column (150 x 4.6 mm, 5 µm) (Palo Alto, CA, USA) with a Phenomenex Security Guard C_{18} guard column (Torrance, CA, USA).

Chromatographic Conditions

The mobile phases used were 12% acetonitrile in 25 mM sodium phosphate buffer (adjusted to pH 7 with sodium hydroxide) for the fetus, placenta, and amniotic fluid samples and 13% acetonitrile in 25 mM sodium phosphate buffer (adjusted to pH 7 with sodium hydroxide) for plasma. The mobile phase flow rate was 0.8 ml/min and the detection wavelength was set at 270 nm. Under the chromatographic conditions described, abacavir, AZT and AZDU eluted at 8.3, 13.5, and 5.7 min (12 % acetonitrile), respectively for fetus, placenta, and amniotic fluid. Abacavir, AZT and AZDU eluted at 7.3, 10.3, and 5 min (13 % acetonitrile), respectively for plasma.

Calibration Curves

Blank plasma, amniotic fluid, placenta, and fetal tissue were collected from untreated anesthetized animals. The placental and fetal tissues were homogenized with 2 volumes of deionized water (w/v) using an Ultra-Turbax T8 tissue grinder (IKA Labortechnik, Germany). Plasma, placenta and fetus calibration points were prepared by spiking 100 µl of the biological matrices with 10 µl of each abacavir-AZT and AZDU standard solution. Amniotic fluid calibration points were prepared by spiking 50 µl of the biological matrix with 5 µl of each abacavir-AZT and AZDU standard solution. The calibration curves of all four matrices were in

the range of 0.05 – 50 μg/mL, with an internal standard concentration in each sample of 2.5 μg/mL. After each matrix was spiked, it was subject to further sample preparation before analysis.

Precision and Accuracy

This method was validated using four QC points for each calibration curve. Five replicates of each QC point were analyzed every day to determine the intra-day accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision. The concentrations of the QC points for all four matrices were 0.05, 0.2, 3.5, and 40 μg/mL.

Sample Preparation

Plasma and amniotic fluid samples were prepared with protein precipitation. After spiking, samples were vortexed briefly and 20μl of 2 M perchloric acid (plasma) or 10 μl of 2 M perchloric acid (amniotic fluid) was added. Samples were vortexed and centrifuged for 10 min at 10,000 rpm. The supernatant was removed and the pellet was discarded. Placental and fetal tissues were extracted using a salting out technique. Three hundred microliters of saturated ammonium sulfate solution and 400 μl acetonitrile were added to 100 μl of the biological matrices containing the analytes. The samples were vortexed and centrifuged at 10,000 rpm for 10 min. The upper organic layer was then evaporated to dryness in a vacuum centrifuge and the residues reconstituted in 100 μl deionized water. An injection volume of 20μl was used for all samples.

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 College of Pharmacy

animal facility (AALAC accredited). The environment was controlled with daily feedings of standard chow pellets and water ad libitum.

Timed pregnant female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing an average of 325 g were used. On day 19 of gestation rats were anesthetized using ketamine:acepromazine:xylazine (50:3.3:3.4 mg/kg) injected intramuscularly. A cannula was placed in the right jugular vein and a laparotomy was performed to allow concurrent serial sampling of blood and the fetal sac, each containing a fetus, placenta and amniotic fluid. The rats were administered an i.v. bolus dose of abacavir (25 mg/kg) and AZT (25 mg/kg) dissolved in 0.1 N NaOH in physiological saline (pH 7.4) via the jugular cannula. Individual blood and fetal sac samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 min after dosing and stored on ice until processed. Blood samples were collected in heparinized tubes and centrifuged at 10,000rpm for 10 min to enable plasma collection. Placental and fetal tissue samples were homogenized in two volumes of deionized water. All samples were stored at -20°C until analysis.

RESULTS AND DISCUSSION

The chemical structures for abacavir, AZT and AZDU are shown in Figure 5.1. Due to the differences in pKa values of the two analytes, it was necessary to maintain a pH value that was fairly neutral. Therefore, separation of the compounds from endogenous compounds was explored using various ratios of acetonitrile and buffer adjusted to pH 7. Baseline resolution was achieved at 12% acetonitrile in buffer for amniotic fluid, fetal and placental samples and at 13% acetonitrile for the plasma samples. Fig. 5.2a-d shows chromatographs of spiked abacavir and AZT (5 μg/ml) with the internal standard, AZDU. Several liquid-liquid extraction and protein precipitation methods were investigated for the extraction of abacavir, AZT, and the

internal standard, AZDU, from the different biological matrices. The complexity of the biological matrices limited the use of available techniques for the extraction of abacavir and AZT due to the wide variety of endogenous substances. Therefore, sample clean-up played a critical role in eliminating the overlapping of endogenous substances with peaks of interest. For fetal and placental homogenates, a salting out technique using saturated ammonium sulfate solution and acetonitrile provided the best extraction technique. This technique has been successfully used in the sample preparation of other antiviral compounds from the pregnant rat (25). For plasma and amniotic fluid, acid precipitation using perchloric acid achieved satisfactory separation of the analytes from the biological content.

The calibration curves for each day of validation and analysis showed acceptable linear response ($R^2 > 0.999$) through a range of 0.05 – 50 μg/mL. Linear regression equations were generated with JMP statistical software using a 1/x-weighting scheme for each day of validation. The range of concentrations was sufficient for use in calculating abacavir and AZT levels from samples taken from rats that were dosed with 25 mg/kg abacavir and 25 mg/kg AZT.

The extraction efficiencies for abacavir, AZT, and AZDU from various matrices are expressed in terms of absolute recovery. The recoveries are shown in Table 5.1. The absolute recoveries were calculated by comparing the peak areas of spiked plasma, amniotic fluid, fetal and placental homogenate samples to the corresponding peak areas of the untreated standard solutions (n=15). Abacavir recoveries ranged from 79 to 94%, while AZT recoveries ranged from 79 to 90% in the different biological matrices. The internal standard recovery ranged from 84 to 92%.

Intra-day (n=5) and inter-day (n=15) precision and accuracy were calculated for each matrix over 3 days. Intra-day precision and accuracy were calculated from the measurement of

five samples at each QC point on three separate days. Inter-day precision and accuracy were calculated from the pooled data from the 3 days. Four QC points of concentration 0.05 μg/mL (limit of quantitation, LOQ), 0.2, 3.5 and 40 μg/mL were selected to validate the method. Intra-day precision (% RSD) and accuracy (percentage error) for abacavir ranged from 0.28 to 7.42 and 0.04 to 9.86% , respectively, while for AZT it ranged from 0.24 to 9.75% and 0.01 to 8.52%, respectively. Inter-day precision and accuracy for abacavir ranged from 0.62 to 6.49% and 0.10 to 6.19%, respectively, while for AZT it ranged from and 0.49 to 8.69% and 0.28 to 6.45%, respectively. This validation data is compiled in Tables 5.2 and 5.3.

Stability testing was performed for abacavir, AZT, and AZDU at the concentration level of 0.2 μg/ml. Spiked matrix samples (20 samples) were subjected to three consecutive freeze/thaw cycles over the period of four days. Five samples were extracted and analyzed as described before. The remaining spiked matrix samples were stored at -20°C. Each of the following three consecutive days, spiked matrix samples were thawed, and five more were extracted and analyzed. The day-to-day measured peak areas of abacavir, AZT, and AZDU were compared and the results listed in Table 5.4. The % R.S.D. between the average peak areas of abacavir, AZT, and AZDU each day was less than 15%. The stability of extracted matrix samples in the autosampler was also evaluated. At time 0, three samples of each matrix were injected onto the HPLC column and analyzed. In another 24 hr, the same samples from each matrix were injected again. The peak areas for abacavir, AZT and AZDU in each injection were compared. The % R.S.D. between samples was less than 13% for all three compounds and no obvious decline in peak areas between each injection was observed.

To demonstrate the utility of this assay, a pregnant rat was given an i.v. bolus dose of abacavir and AZT (25 mg/kg each). Plasma, amniotic fluid, placental and fetal tissues were

processed and analyzed as mentioned. A calibration curve from each matrix was prepared on the day of analysis to calculate the concentrations of abacavir and AZT present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 2.5 μg/ml of the internal standard. In addition, real samples were spiked with the appropriate amounts of deionized water (10 μl for plasma, fetal, and placental homogenates, 5 μl for amniotic fluid) to account for the addition of abacavir and AZT in the calibration points. The sample peak area ratios of abacavir and AZT to the internal standard were used to calculate the concentrations of abacavir and AZT in each sample. The concentration-time profiles of abacavir and AZT in all matrices are shown in Figure 5.3. Plasma data was analyzed using a two-compartment model using WinNonlin (Pharsight, Mountain View, CA, USA). For abacavir, estimates for half-life ($t_{1/2}$), volume of distribution at steady state (V_{ss}), and clearance (CL) in maternal plasma were 213.4 min, 1.46 L/kg, and 5.83 mL/min-kg, respectively. For AZT, estimates for $t_{1/2}$, V_{ss}, and CL in maternal plasma were 85.7 min, 1.74 L/kg, and 17.17 mL/min-kg. These values were in close agreement with earlier reported data for abacavir and AZT (12, 26).

CONCLUSIONS

A sensitive and accurate method was developed and validated for the quantification of abacavir and AZT in rat maternal plasma, amniotic fluid, placental and fetal tissues. The use of acid precipitation and salting out techniques provided an inexpensive and convenient method of sample preparation. This method yielded high recoveries, good linearity, and precision and accuracy in the range of 0.05 – 50 μg/ml. This method will be useful for pharmacokinetic studies to investigate the fetal and maternal disposition of abacavir and AZT in the pregnant rat.

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Table 5.1. Absolute recoveries of ABC, AZT, and AZDU from plasma, amniotic fluid, placenta, and fetus (n=15)

Analyte	Concentration	Plasma	Amniotic Fluid	Placenta	Fetus
ABC	40	81.1 ± 2	78.5 ± 2.6	94.1 ± 3.5	89.6 ± 4.3
	3.5	84.8 ± 2.1	92.5 ± 2.8	89.5 ± 5.3	89.7 ± 5.5
	0.2	89.7 ± 1.5	85.6 ± 5.7	82.3 ± 4.2	92.3 ± 4.2
	0.05	87.3 ± 5.1	90.1 ± 5.4	87.5 ± 3.6	92.1 ± 4.9
AZT	40	83.8 ± 2.2	79.3 ± 2.7	80.7 ± 5.7	80.1 ± 3.8
	3.5	86.8 ± 2.6	88.6 ± 2.9	78.6 ± 4.8	82.7 ± 5.3
	0.2	87.3 ± 1.4	90.4 ± 4.9	85.5 ± 4	88.9 ± 5.7
	0.05	85.6 ± 5.6	89.1 ± 5.3	80.3 ± 4.7	83.2 ± 4.7
AZDU	2.5	83.5 ± 0.8	90.1 ± 1	91.7 ± 1.6	90.7 ± 1.5

Table 5.2. Intra-day (n=5) and inter-day (n=15) precision (% R.S.D.) and accuracy (% error) measured for QC points for abacavir from plasma, amniotic fluid, placental and fetal tissues. T.C denotes theoretical concentration and E.C denotes experimental concentration.

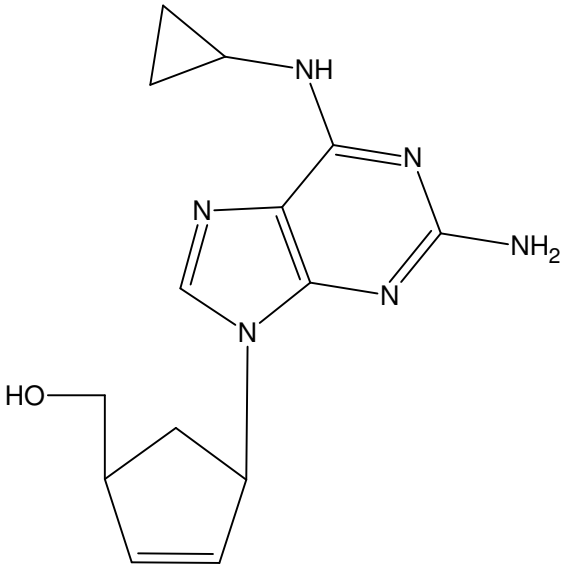
T.C	Day 1			Day 2			Day 3			Inter-day		
	E.C	Error	R.S.D.	E.C	Error	R.S.D	E.C	Error	R.S.D.	E.C.	Error	R.S.D.
Plasma												
40	40.1	0.32	0.36	39.9	0.33	0.47	39.3	1.84	0.28	39.8	1.01	0.62
3.5	3.51	0.15	0.66	3.39	3.01	1.50	3.49	0.34	0.38	3.46	1.70	1.07
0.2	0.20	0.89	2.84	0.20	0.69	1.33	0.20	0.37	1.77	0.20	0.07	2.06
0.05	0.051	2.52	7.42	0.050	0.04	5.61	0.051	2.88	6.46	0.051	6.19	1.78
Amniotic Fluid												
40	37.8	5.56	3.53	38.8	3.10	2.63	38.8	3.06	2.54	38.4	3.91	2.99
3.5	3.42	2.31	3.07	3.44	1.60	3.22	3.41	2.43	2.07	3.43	2.11	2.65
0.2	0.19	5.66	1.78	0.20	0.72	3.14	0.20	0.29	3.26	0.20	2.03	3.80
0.05	0.053	5.28	7.16	0.052	3.32	5.16	0.053	5.76	3.06	0.052	4.79	5.10
Placenta												
40	40.2	0.41	2.12	38.1	4.68	1.10	39.7	0.77	2.05	39.3	1.68	2.85
3.5	3.53	0.97	2.82	3.63	3.98	3.48	3.37	3.77	2.89	3.51	0.39	4.36
0.2	0.18	8.31	0.93	0.19	3.70	3.65	0.19	5.96	3.41	0.19	5.99	3.44
0.05	0.049	2.00	2.30	0.048	3.24	1.90	0.054	8.24	6.04	0.051	1.00	6.49
Fetus												
40	40.5	1.26	1.06	39.9	0.23	3.38	39.7	0.72	2.39	40.0	0.10	2.44
3.5	3.53	1.11	0.53	3.26	6.81	2.84	3.41	2.51	2.71	3.40	2.73	4.02
0.2	0.19	0.40	1.25	0.19	4.19	0.62	0.18	9.86	2.87	0.19	4.81	4.53
0.05	0.049	2.80	3.12	0.051	1.92	2.19	0.048	3.24	5.49	0.049	1.37	4.29

Table 5.3. Intra-day (n=5) and inter-day (n=15) precision (% R.S.D.) and accuracy (% error) measured for four QC points for AZT from plasma, amniotic fluid, placental, and fetal tissues. T.C denotes theoretical concentration and E.C denotes experimental concentration.

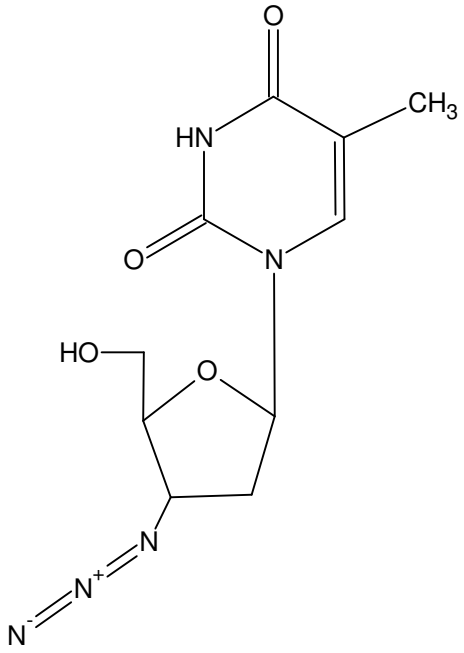
T.C	Day 1			Day 2			Day 3			Inter-day		
	E.C	Error	R.S.D.	E.C	Error	R.S.D	E.C.	Error	R.S.D.	E.C.	Error	R.S.D.
Plasma												
40	39.8	0.53	0.62	39.7	0.78	0.70	39.5	1.34	0.52	39.6	0.67	0.88
3.5	3.52	0.65	0.54	3.43	2.06	1.36	3.50	0.08	0.24	3.48	1.43	0.49
0.2	0.20	0.35	3.11	0.20	2.23	0.99	0.20	0.96	2.36	0.20	2.29	1.18
0.05	0.054	8.52	3.94	0.053	6.84	5.79	0.053	5.88	9.75	0.054	6.45	7.07
Amniotic Fluid												
40	39.2	2.08	3.59	38.7	3.14	2.66	38.9	2.85	2.38	38.9	2.69	2.75
3.5	3.44	1.66	3.13	3.49	0.40	1.89	3.38	3.52	1.41	3.43	1.86	2.50
0.2	0.20	0.46	2.90	0.20	0.27	3.93	0.19	3.81	2.37	0.20	1.33	3.46
0.05	0.053	7.04	3.08	0.052	4.44	8.18	0.054	8.12	1.61	0.053	6.53	4.91
Placenta												
40	39.0	2.53	2.63	39.1	2.15	2.88	38.9	2.66	2.40	39.0	2.44	2.46
3.5	3.46	1.17	1.08	3.56	1.92	3.76	3.43	1.85	3.25	3.49	0.36	3.23
0.2	0.18	7.72	5.43	0.21	5.16	5.60	0.19	4.48	9.36	0.20	2.34	8.69
0.05	0.051	2.00	4.78	0.053	6.28	6.43	0.054	0.64	7.67	0.051	2.97	6.39
Fetus												
40	40.0	0.01	1.42	40.7	1.81	4.12	38.9	2.66	2.40	39.9	0.28	3.29
3.5	3.51	0.28	1.38	3.51	0.20	7.15	3.43	1.86	3.25	3.48	0.45	4.40
0.2	0.20	1.78	2.33	0.20	0.55	4.38	0.20	4.48	9.36	0.19	2.27	5.84
0.05	0.051	2.20	4.82	0.052	4.20	5.33	0.052	0.64	7.67	0.05	2.34	5.78

Table 5.4. Results of freeze/thaw stability of abacavir, AZT, and internal standard, AZDU, in maternal plasma, amniotic fluid, placenta, and fetus, represented by area ± S.D. (n=5) of each day and % R.S.D. of the area between days.

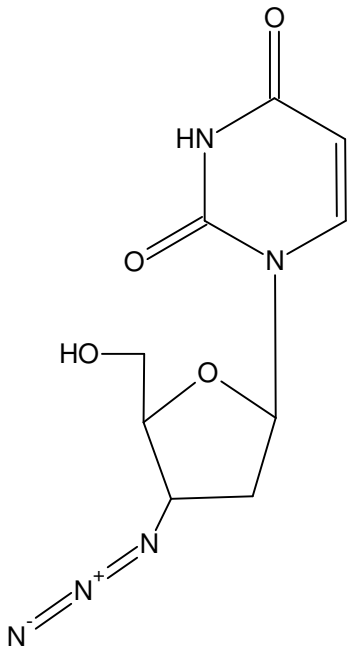
	Maternal plasma	Amniotic fluid	Placenta	Fetus
Abacavir				
Day 1	9.56 ± 0.98	10.8 ± 0.93	10.58 ± 1.45	9.16 ± 1.59
Day 2	9.94 ± 10.8	10.22 ± 1.39	8.22 ± 1.11	9.8 ± 0.76
Day 3	9.16 ± 0.69	9.18 ± 0.59	9.66 ± 1.29	8.24 ± 1.17
Day 4	9.98 ± 0.66	9.4 ± 0.32	10.54 ± 1.74	9.26 ± 0.36
% R.S.D.	8.8	8.1	14.3	10.6
AZT				
Day 1	5.28 ± 0.87	5.02 ± 0.7	3.66 ± 0.69	4.34 ± 0.42
Day 2	5.96 ± 0.74	5.62 ± 0.04	4.68 ± 0.49	5.66 ± 0.38
Day 3	5.06 ± 0.75	5.04 ± 0.32	5.52 ± 0.44	5.38 ± 0.42
Day 4	4.72 ± 0.48	5.04 ± 0.51	4.78 ± .2	5.56 ± 0.31
% R.S.D.	13.4	7.6	9.8	7.3
AZDU				
Day 1	94.78 ± 9.94	107.96 ± 3.8	98.56 ± 11.21	111.54 ± 14.08
Day 2	85.18 ± 5.9	107.3 ± 6.43	76.12 ± 16.71	104.38 ± 5.07
Day 3	99.22 ± 7.57	105.46 ± 4.71	72.9 ± 5.33	103.18 ± 5.81
Day 4	99.58 ± 4.77	112.02 ± 2.46	78.4 ± 13.63	109.64 ± 4.24
% R.S.D.	7.2	4.02	14.4	6.8



Abacavir



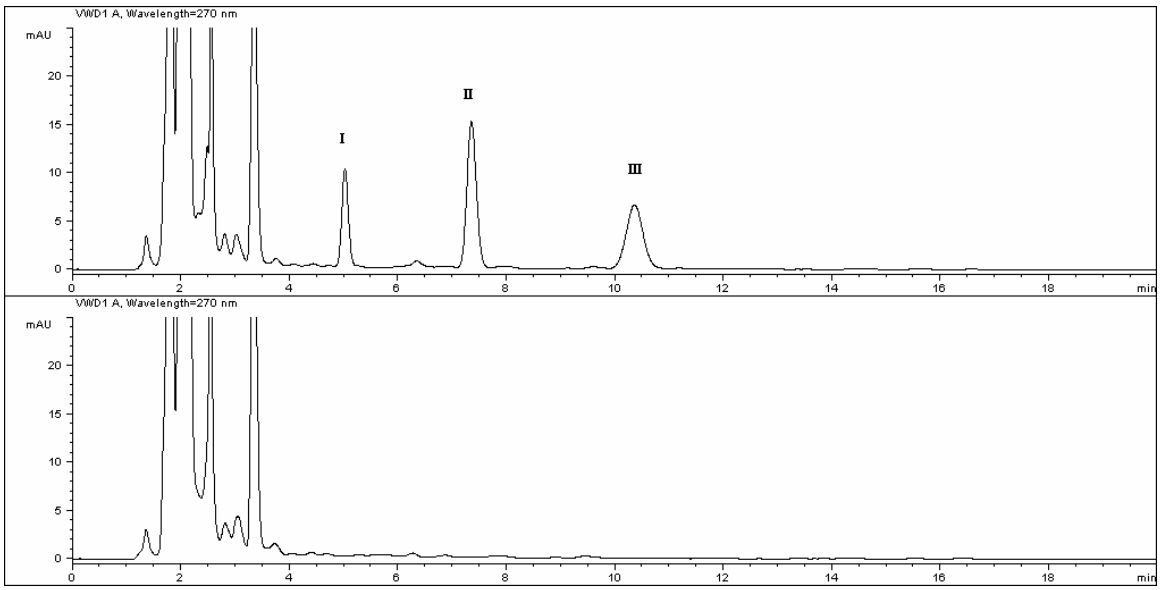
AZT



AZDU

Figure 5.1. Chemical structures of abacavir, zidovudine (AZT), and azidouridine (AZDU).

(a)



(b)

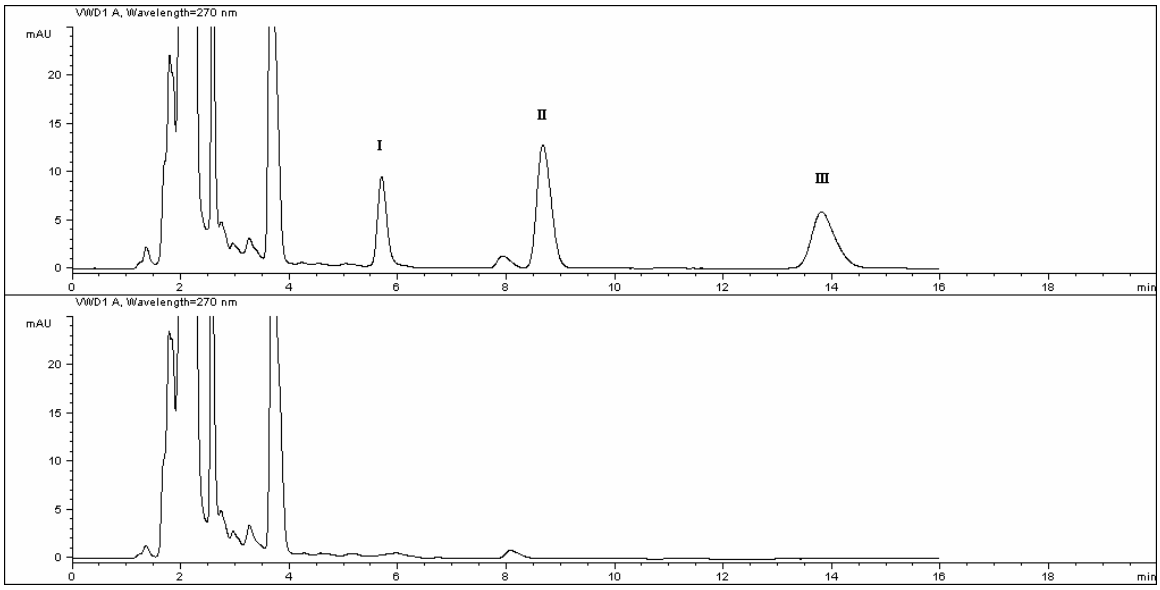
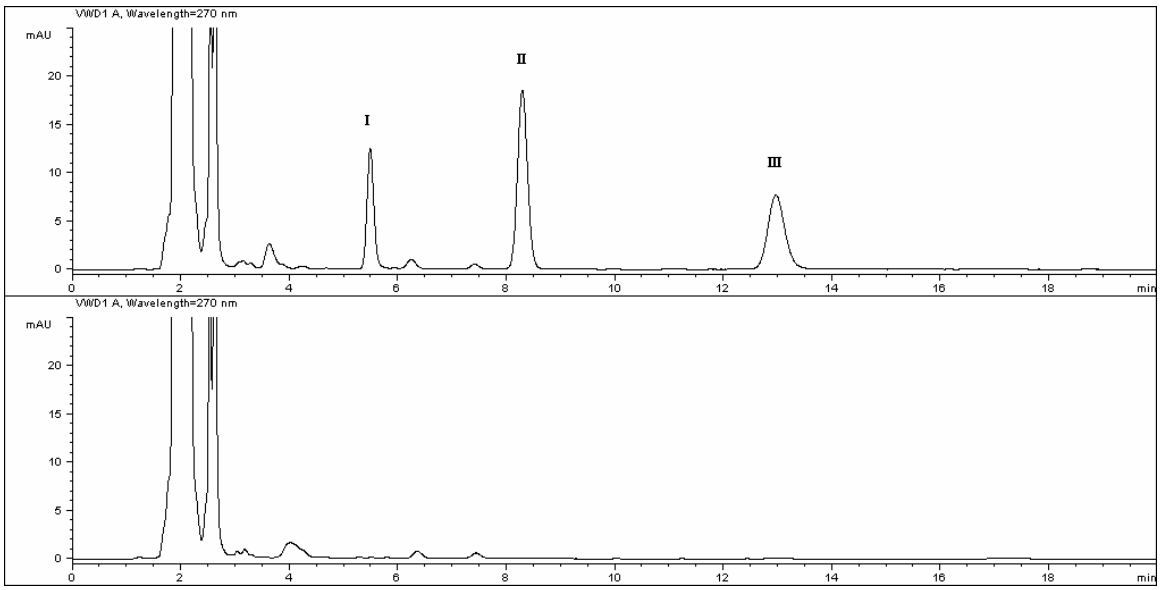
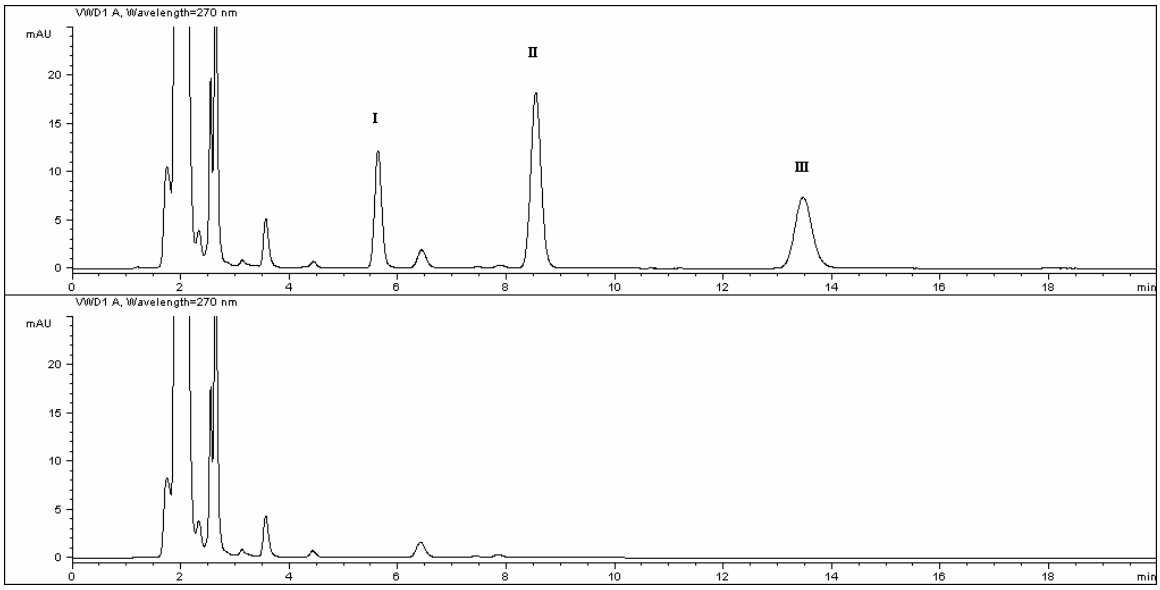


Figure 5.2. (a) Chomatographs of maternal plasma spiked with 5 µg/ml abacavir (II) and AZT (III) and 2.5 µg/ml (AZDU (I) (top) and blank maternal plasma (bottom). (b) Chromatographs of amniotic fluid spiked with 5 µg/ml abacavir (II) and AZT (III) and 2.5 µg/ml AZDU (I) (top) and blank amniotic fluid (bottom). (c) Chromatographs of fetal homogenate spiked with 5 µg/ml abacavir (II) and AZT (III) and 2.5 µg/ml AZDU (I) (top) and blank fetal homogenate (bottom). (d) Chromatographs of placental homogenate spiked with 5 µg/ml abacavir and AZT and 2.5 µg/ml AZDU (I) (top) and blank placental homogenate (bottom).

(c)

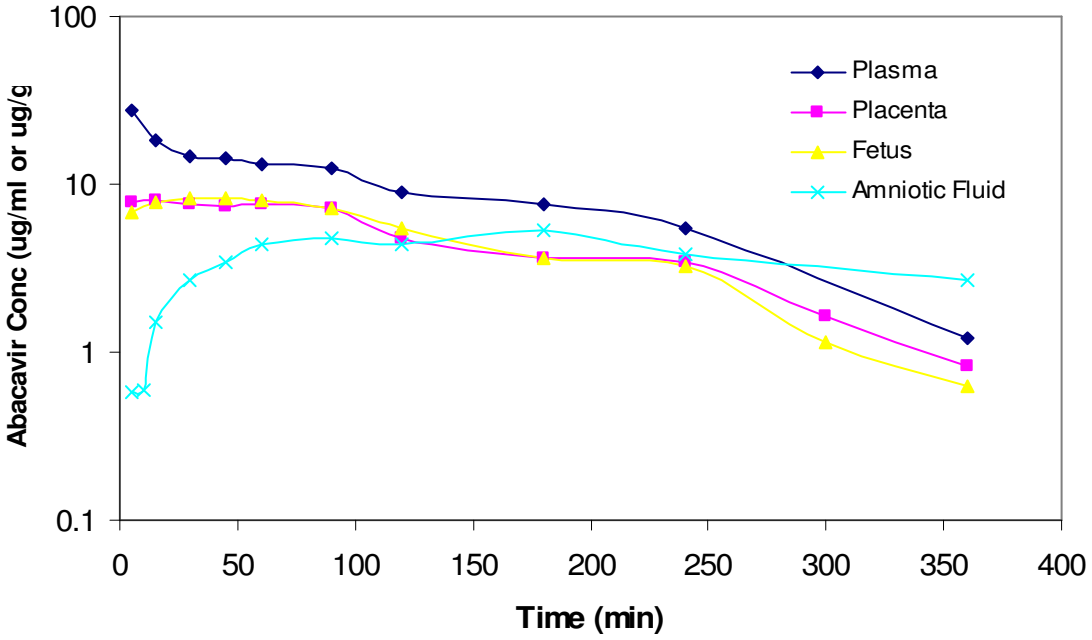


(d)



(Figure 5.2 Continued)

(a)



(b)

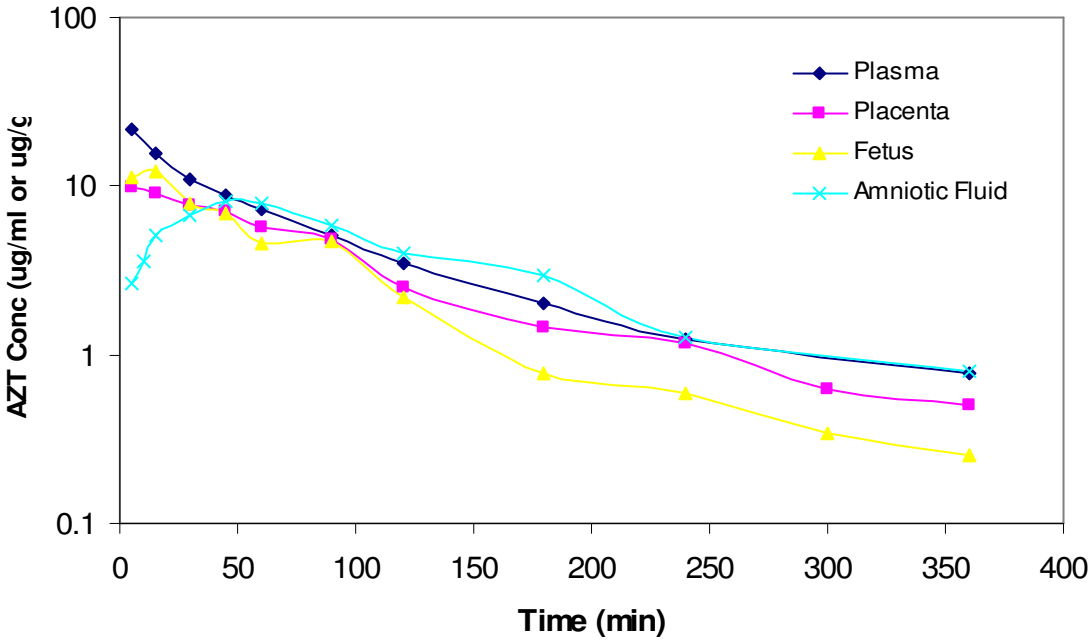


Figure 5.3. Concentration-versus-time profiles of abacavir (a) and AZT (b) in maternal plasma, amniotic fluid, placenta, and fetus after 25 mg/kg i.v. bolus doses of abacavir and AZT.

CHAPTER 6

TRANSPLACENTAL PHARMACOKINETICS OF INTRAVENOUS ABACAVIR IN PREGNANT RATS[1]

[1]Lewis, S. R., T. N. Clark, M. G. Bartlett, and C. A. White. To be submitted to *Antimicrobial Agents and Chemotherapy*.

ABSTRACT

The pharmacokinetics and placenta transfer of abacavir, a carbocyclic nucleoside analog, were studied in the pregnant rat. Abacavir 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 five hours post-dose. Concentrations in each matrix were determined by HPLC-MS-MS. 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 were determined. Relative exposures ($AUC_{tissue}/AUC_{maternal\ plasma}$) were calculated. Estimates for half-life ($t_{1/2}$), volume of distribution at steady state (V_{ss}), and clearance (CL) in maternal plasma were 0.9 ± 0.3 hr, 1.8 ± 0.8 L/kg, and 1.8 ± 0.3 L/hr-kg, respectively. Relative exposures of placenta, fetus and amniotic fluid for abacavir were 0.81 ± 0.20, 0.74 ± 0.27, and 1.24 ± 0.40, respectively. Significant differences were noted between the intercompartmental clearances CL_{pa} and CL_{ap}, suggesting that active transport may play a role in the amniotic fluid uptake of abacavir. The magnitude of k_{pc}, k_{pf}, and k_{fp} also suggest active transport at these interfaces. However, further studies are needed to elucidate the role of these transporters in the placental transport of this compound.

INTRODUCTION

Abacavir is a second-generation reverse transcriptase inhibitor, approved by the FDA in 1998 (9). It is currently marketed alone as Ziagen® (300 mg twice daily) and in combination with the reverse transcriptase inhibitors, zidovudine (AZT) and lamivudine (3TC), as Trizivir®. It was also recently approved for use in combination with 3TC only as Epzicom® in 2004 (9). Abacavir is a carbocyclic nucleoside analog that is converted by cellular enzymes to the active metabolite, carbovir triphosphate (12). Following iv administration in non-pregnant humans, The apparent volume of distribution after IV administration of abacavir was 0.86 L/kg, suggesting that abacavir distributes into extravascular space (12). Binding of abacavir to human plasma proteins is approximately 50%. Abacavir undergoes extensive hepatic metabolism in humans with less than 2% of the drug appearing unchanged in the urine (12). Abacavir is metabolized to a 5'-glucoronide and a 5'-carboxylate and while glucuronidation is not a major route of metabolism in rats, another metabolic pathway is active.

The guidelines that recommend treatment of HIV-infected pregnant women with antiviral therapies have been based on the belief that therapies of known benefit to women should not be withheld during pregnancy unless there are known adverse effects on the mother, fetus, or infant and unless these adverse effects outweigh the benefit to the woman (17). Physiologic changes that occur during pregnancy may affect the pharmacokinetics of a drug, thus affecting requirements for drug dosing and potentially altering the susceptibility of the pregnant woman to drug toxicity (5). During pregnancy, several changes occur, such as prolonged gastrointestinal transit time; increased body water and fat, accompanied by increases in cardiac output, ventilation, and liver and renal blood flow; and increased plasma protein concentrations to name a few. Placental transport of drugs, compartmentalization of drugs in the fetus and

placenta, and elimination of drugs by the fetus also can affect drug pharmacokinetics in the pregnant woman (5).

Most information regarding the safety of drugs in pregnancy is derived from animal toxicity data, anecdotal experience, registry data, and clinical trials (5). There are several studies that have investigated the placental transport of AZT (4, 13, 16, 18, 21, 30), however data are limited for other antiretroviral drugs. Pregnant women are rarely recruited, or even permitted, into clinical trials based on the principle that until benefit is clearly demonstrated it would be inappropriate to subject the fetus to risk (17). Therefore, animal models are frequently used to study the placental transfer of drugs in the fetal-placental unit.

We have chosen the pregnant rat as a model because rats and humans share the same type of placenta (haemochorial) and exhibit similar hemodynamic changes in pregnancy (5). The large litter size (10-15 pups) makes it possible for one fetal sac; which includes fetus, placenta, amniotic fluid; to be harvested at each time point. Thus, drug levels in each tissue can be determined such that the entire concentration-versus-time profile can be constructed from one rat. Furthermore, the similarities in rat and human transporters will allow for further study of the active transport of antiviral agents when given in combination therapies (11, 15). The pregnant rat model has been used previously in studying placental transfer has been demonstrated in several compounds, including antiviral compounds (1-4, 13, 14).

Continued study of antiviral agents alone and in combination with each other is needed to gain further understanding of the placental transport processes of these agents. The very limited information available for abacavir in pregnancy emphasizes the need for more studies on this important antiviral agent. The purpose of the present study was to examine fetal exposure to

abacavir by determining the placental transfer and fetal distribution of abacavir in the pregnant rat.

MATERIALS AND METHODS

Chemicals and Reagents. An abacavir (ABC) (Figure 6.1) standard was obtained from GlaxoSmithKline Pharmaceuticals, Inc. (Barnard Castle, Co. Durham, UK). Azidouridine (AZDU) was purchased from Sigma (St. Louis, MO, USA). For dosing, Ziagen was purchased from Cardinal Health (McDonough, GA, USA) from which the drug was extracted and recrystallized. HPLC-grade acetonitrile and optima water were purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA).

Animal Study. The use of animals in this study was approved by the University of Georgia Animal Use and Care Committee and studies were carried out in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health *Guide for the Care and Use of Laboratory* Animals. All rats were housed one per cage in the University of Georgia College of Pharmacy AALAC accredited animal facility with a controlled environment. The rat was fed standard chow pellets and allowed constant access to water.

Timed pregnant female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing an average of 321 ± 21 g were used throughout the study. On day 19 of gestation rats were anesthetized using a solution of ketamine:acepromazine:xylazine (50:3.3.:3.4 mg/kg) injected intramuscularly given in conjunction with a subcutaneous atropine (0.5 mg/kg). A cannula was surgically implanted in the right jugular vein prior to shipment by Charles River Laboratories to allow for IV administration of ABC and for serial sampling of maternal blood. On the day of sampling, a laparotamy was performed to allow for fetal, placental and amniotic

fluid sampling. Animals were maintained under anesthesia by periodic administration of the ketamine:acepromazine:xylazine solution IM. Body temperatures were monitored and held constant at 37°C using a heated surgical pad (Braintree Scientific, Braintree, MA, USA).

The rats were administered an IV bolus dose of 25 mg/kg of ABC dissolved in physiological saline via the jugular cannula followed by 1 ml of saline to rinse the cannula. Samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, and 300 minutes post-dose and stored on ice until processed. Blood samples of approximately 200 µl were collected via the jugular vein cannula and placed in heparinized microcentrifuge tubes. These tubes were centrifuged at 9,000 rpm for 10 minutes (Model 235V, Fisher Scientific, Pittsburgh, PA, USA) and the plasma was transferred to clean dry labeled tubes for storage. Approximately 300 µl of amniotic fluid sample was drawn directly from the fetal sac using an 18-gauge needle. The fluid was transferred to a labeled tube for storage. Fetal and placental samples were collected, rinsed, dried and placed in labeled vials. The samples were then homogenized in 2 volumes (v/w) of optima water using a Polytron PCU-2 tissue grinder (Kinematica GmbH, Luzern, Switzerland. All samples were stored at –20°C until analysis.

HPLC Analysis. ABC concentrations were determined by an HPLC-MS-MS method developed previously (6). Briefly, all samples were prepared using acetonitrile precipitation. Analyses were performed on an Agilent (Palo Alto, CA, USA) 1100 Series HPLC equipped with a quaternary pump, autosampler and column thermostatter coupled to a Micromass Quattro-LC triple quadrupole mass spectrometer equipped with a ZSpray® atmospheric pressure electrospray ionization (ESI) source (Waters-Micromass, Beverly, MA, USA). Chromatographic separations were achieved on a Zorbax Eclipse XDB C8 analytical column (5 µm, 2.1 x 150 mm, Agilent,

Wilmington, DE, USA) equipped with a Security-guard C18 guard column (2.0 x 4.0 mm, Phenomenex, Torrance, CA, USA).

The mobile phase used was 10 mM ammonium acetate (pH 6.3):acetonitrile under gradient conditions. The flow rate was held constant at 0.25 ml/min and was run at ambient temperature. Under the chromatographic conditions described, AZDU and ABC eluted at approximately 3.4 and 5.1 minutes, respectively. This assay was validated to ensure both precision and accuracy in accordance to recommended guidelines for bioanalytical method development and validation (20). The method demonstrated acceptable reproducibility (% RSD < 15%) and accuracy (% Error < 15%) in all matrices over the calibration range.

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. A 1/y-weighting scheme was used throughout the analysis. Amniotic fluid, placenta, and fetus data were subjected to noncompartmental analysis. The relative exposure of each matrix to ABC was calculated by comparing the AUC values for the individual tissues to the AUC value for the maternal plasma data.

In addition, a five-compartment model was used to characterize the distribution of ABC in the maternal plasma (central), maternal tissue, placental, fetal, and amniotic fluid compartments (Figure 6.2).

$$\frac{dC_c}{dt} = \left(\frac{k_{pc} V_p}{V_b}\right) C_p + \left(\frac{k_{tc} V_t}{V_b}\right) C_t - \left(k_{cp} + k_{ct} + k_{c0}\right) C_c \qquad (1)$$

$$\frac{dC_p}{dt} = \left(\frac{k_{cp} V_b}{V_p}\right) C_c + \left(\frac{k_{fp} V_f}{V_p}\right) C_f - \left(k_{pc} + k_{pf} + k_{pa}\right) C_p \qquad (2)$$

$$\frac{dC_f}{dt} = \left(\frac{k_{pf} V_p}{V_f}\right) C_p - k_{cp} C_f \qquad (3)$$

$$\frac{dC_a}{dt} = \left(\frac{k_{pa} V_p}{V_a}\right) C_p - k_{ap} C_a \qquad (4)$$

$$\frac{dC_t}{dt} = \left(\frac{k_{ct} V_b}{V_t}\right) C_c - k_{tc} C_t \qquad (5)$$

The model incorporates bidirectional transfer between maternal plasma and placenta ($k_{c\text{-}p}$ and $k_{p\text{-}c}$), placenta and fetus ($k_{p\text{-}f}$ and $k_{f\text{-}p}$), placenta and amniotic fluid ($k_{p\text{-}af}$ and $k_{af\text{-}p}$), and maternal plasma and tissue compartment ($k_{c\text{-}t}$ and $k_{t\text{-}c}$). The elimination rate constant from the maternal plasma compartment is $k_{c\text{-}0}$. The differential equations (1-5) were simultaneously fitted to the concentration-time data. Parameter estimates were generated by WINNONLIN and intercompartmental clearance values (CL) were calculated from the product of the intercompartmental rate constants and distribution volumes.

RESULTS AND DISCUSSION

Mean concentration-time profiles of ABC in maternal plasma, placenta, fetus and amniotic fluid following intravenous administration of a 25 mg/kg bolus dose are shown in Figure 6.3. Maternal plasma ABC concentrations decline in biexponential fashion after IV administration. The pharmacokinetic parameters generated from two-compartmental analysis of the maternal plasma data are presented in Table 6.1. The volume of distribution at steady state (V_{ss}) of ABC for the pregnant rat (V_{ss} = 1.84 L/kg) was similar to non-pregnant humans (V_{ss} = 1.78 L/kg) and rats (V_{ss} = 1.45 L/kg), indicating that ABC is extensively distributed (7, 12). The V_{ss} of ABC is higher than other antiviral agents in its class (i.e. AZT, log P = 0.09), which can be attributed to its significantly greater lipophilicity (log P = 1.2) (7). The clearance of ABC in the pregnant rat (1.82 L/hr-kg) was higher than that observed in humans (0.80 L/hr-kg) (12).

However, the clearance was similar to that in non-pregnant rats (1.8 L/kg-hr) (7), indicating that the behavior of the drug in the dam does not appear to be changed by pregnancy or anesthesia.

The pharmacokinetic parameters generated by noncompartmental analysis of the placenta, fetus and amniotic fluid data are shown in Table 6.2. The placenta, fetus, and amniotic fluid decline in parallel with the maternal plasma, indicated by their similar half-lives. The uptake of ABC into the placenta and fetus was rapid, with ABC concentrations reaching a maximum at 0.16 hr and 0.18 hr, respectively. ABC concentrations in the amniotic fluid reached their peak at 1 hour, indicating a relatively faster uptake as compared with other NRTIs (i.e. AZT, T_{max} = 2.33 hr) (1). In contrast to other antivirals we have studied thus far, ABC concentrations do not exhibit the characteristic slow rise and plateau in the amniotic fluid. Instead, concentrations rose relatively faster and showed an obvious decline, suggesting that the amniotic fluid does not act as a depot compartment for ABC. However, ABC concentrations in the amniotic fluid exceeded plasma levels from 45 minutes onwards, resulting in a higher AUC compared to that of the maternal plasma.

The relative exposure (RE) values for the placenta, fetus and amniotic fluid are given in Table 6.2. The RE for these tissues to 3TC range from 0.74 to 1.24 and are significantly higher than values reported for other antiviral agents at the same dose (i.e. AZT, RE range, 0.44 to 0.86) (1). The most significant difference was noted in the ABC concentrations in the amniotic fluid, with a RE of 1.24. Antiviral agents studied to date using this animal model have demonstrated amniotic fluid exposure levels of less than 0.2 (4). Again, this is consistent with the significantly higher lipophilicity of ABC compared with other antiviral agents (22).

The maternal plasma, placenta, fetus, and amniotic fluid were also simultaneously fitted to the five-compartment model. The selected model incorporated bidirectional transfer

between maternal plasma and placenta, placenta and fetus, placenta and amniotic fluid, and maternal plasma and tissue compartment. Fitted concentration-time curves are shown in Figure 6.4 and the intercompartmental clearances and rate constants are presented in Table 6.3. The parameter estimates obtained using the five-compartment model were consistent with observations obtained from the noncomparmental analysis. The transfer of ABC from maternal plasma to the placenta was rapid, consistent with the short T_{max} observed. Compared to other antiviral agents (i.e. AZT, k_{pa} = 0.04 hr^{-1}) (submitted), the magnitude of the transfer rate of ABC from placenta to amniotic fluid (k_{pa} = 1.31 hr^{-1}) was relatively high, indicating increased transfer into the amniotic fluid. This is consistent with the higher concentrations and shorter T_{max} of ABC observed in the amniotic fluid. Additionally, the k_{ap} was 2-fold higher than noted for other antivirals consistent with its shorter half-life in amniotic fluid.

Significant differences were noted between the intercompartmental clearances CL_{pa} and CL_{ap}, which suggests that active transport may play a role in the uptake of ABC into the amniotic fluid. Additionally, the magnitude of distribution rate constants, k_{pc}, k_{pf}, and k_{fp} indicate that active transport likely plays a role at the placenta-maternal, placenta-fetal and fetal-placenta interfaces. This is consistent with the literature, which indicates transporters are located in the apical and basolateral membranes of the placenta (11, 15).

Since humans and rats have similar transporters, it could have important clinical implications. In combination with other antiviral agents, there is a potential for increased fetal exposure to abacavir (via fetal swallowing and inhalation of amniotic fluid), providing increased protection of the fetus from contracting HIV. On the other hand, if active transport plays a significant role, drug interactions with other antiviral agents may potentially cause adverse effects to the developing fetus. Further studies are needed to determine which transporters are

involved in the placental transport of ABC and determine if drug interactions occur when ABC is administered in combination with other drugs.

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Table 6.1. Maternal plasma pharmacokinetic parameters for ABC (mean + standard deviation)

Parameter	Value
Half-life (hr)	0.90 ± 0.26
AUC (hr-mg/L)	14.1 ± 2.6
CL_T (L/hr-kg)	1.82 ± 0.32
V_{ss} (L/kg)	1.84 ± 0.76
C_{max}(mg/L)	47.5 ± 33.4

Table 6.2. Placental, Fetal and Amniotic Fluid Pharmacokinetic Parameters for ABC (mean ± standard deviation)

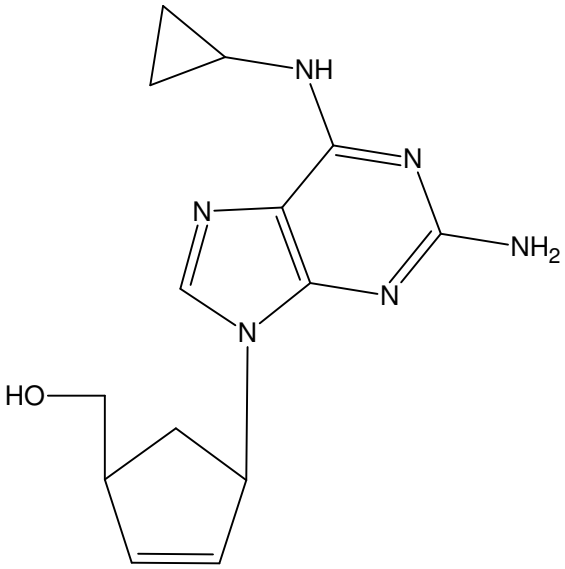
Parameter	Placenta	Fetus	Amniotic Fluid
Half-life (hr)	1.4 ± 0.5	1.4 ± 0.5	1.4 ± 0.4
AUC*	11.1 ± 1.8	10.3 ± 3.3	17.0 ± 4.0
Cmax**	7.7 ± 1.6	6.6 ± 1.2	6.6 ± 1.4
Tmax (hr)	0.18 ± 0.09	0.17 ± 0.08	1.0 ± 0.25
Relative Exposure***	0.81 ± 0.20	0.74 ± 0.27	1.24 ± 0.40

* 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 = $AUC_{Tissue}/AUC_{Maternal\ Plasma}$

Table 6.3. Intercompartmental transfer rates (hr^{-1}) and clearances (ml/min) expressed as mean (standard deviation) of ABC in pregnant rats (25 mg/kg).

k_{cp}	k_{pc}	k_{pf}	k_{fp}	k_{pa}	k_{ap}	k_{ct}	k_{tc}	k_{c0}
0.37 (0.13)	25.83 (11.84)	90.77 (49.44)	27.17 (14.19)	1.31 (0.43)	1.08 (0.31)	5.64 (3.93)	2.78 (1.08)	3.28 (2.02)
CL_{cp}	CL_{pc}	CL_{pf}	CL_{fp}	CL_{pa}	CL_{ap}	CL_{ct}	CL_{tc}	CL_{c0}
1.24 (0.75)	1.71 (0.80)	6.01 (3.36)	6.53 (3.8)	0.09 (0.03)	0.05* (0.02)	15.85 (10.99)	14.88 (11.98)	9.00 (1.79)

* indicates significant differences between corresponding bidirectional intercompartmental clearances ($P<0.05$)



Abacavir

Figure 6.1. Chemical Structure of Abacavir.

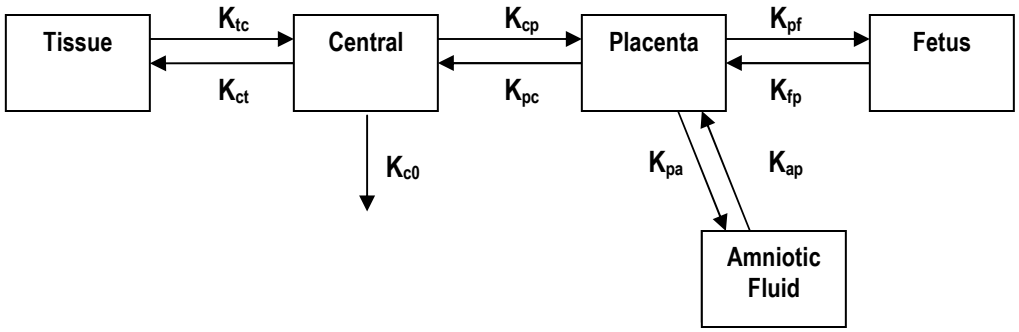


Figure 6.2. Schematic representation of the pharmacokinetic model used to describe the disposition of ABC in maternal central, maternal tissue, placental, fetal, and amniotic fluid compartments after iv injection to pregnant rats.

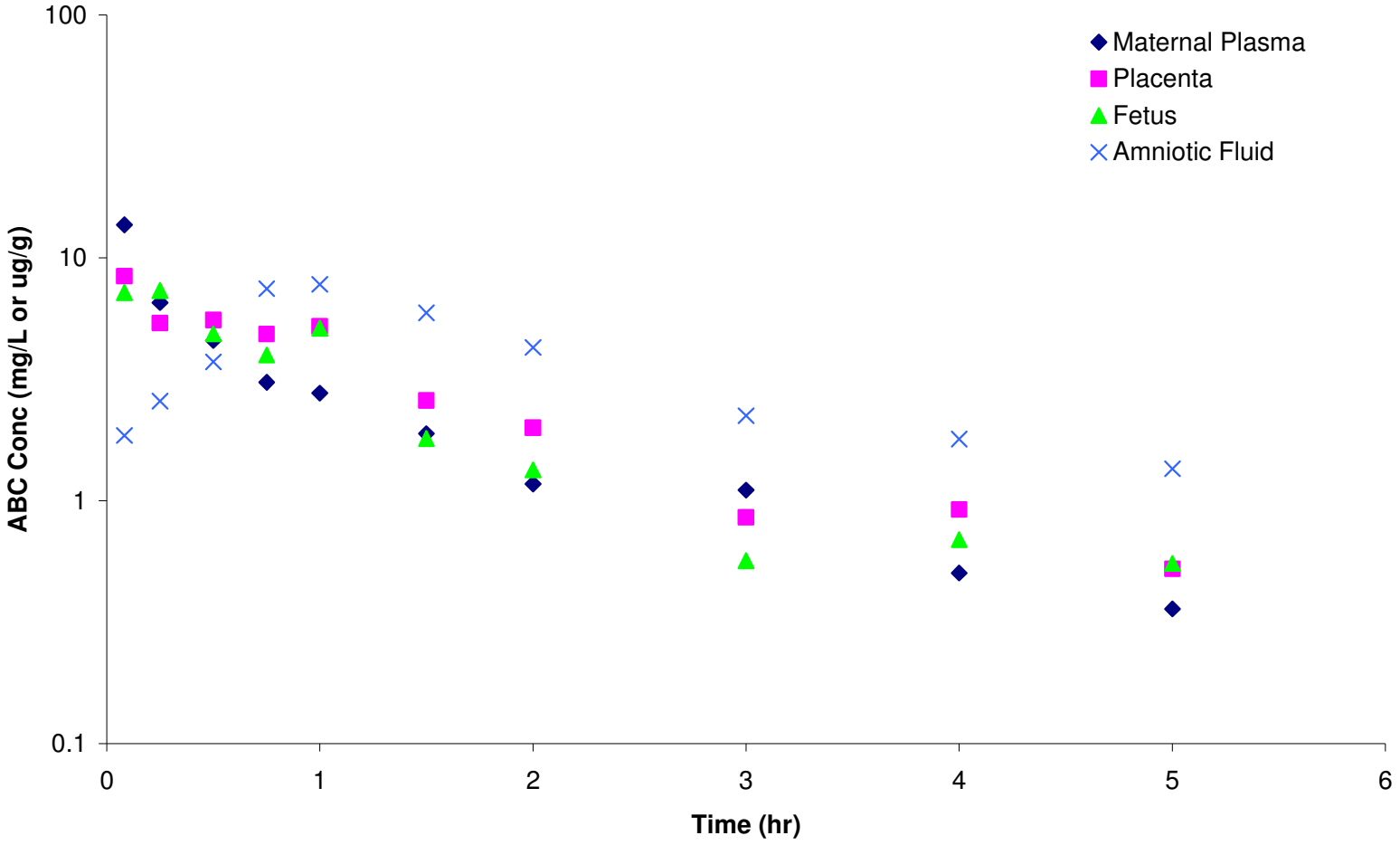


Figure 6.3. Concentration (mean + standard deviation) vs. time profiles of 25 mg/kg doses of ABC following IV bolus administration in (a) maternal plasma (b) placenta (c) fetus and (d) amniotic fluid.

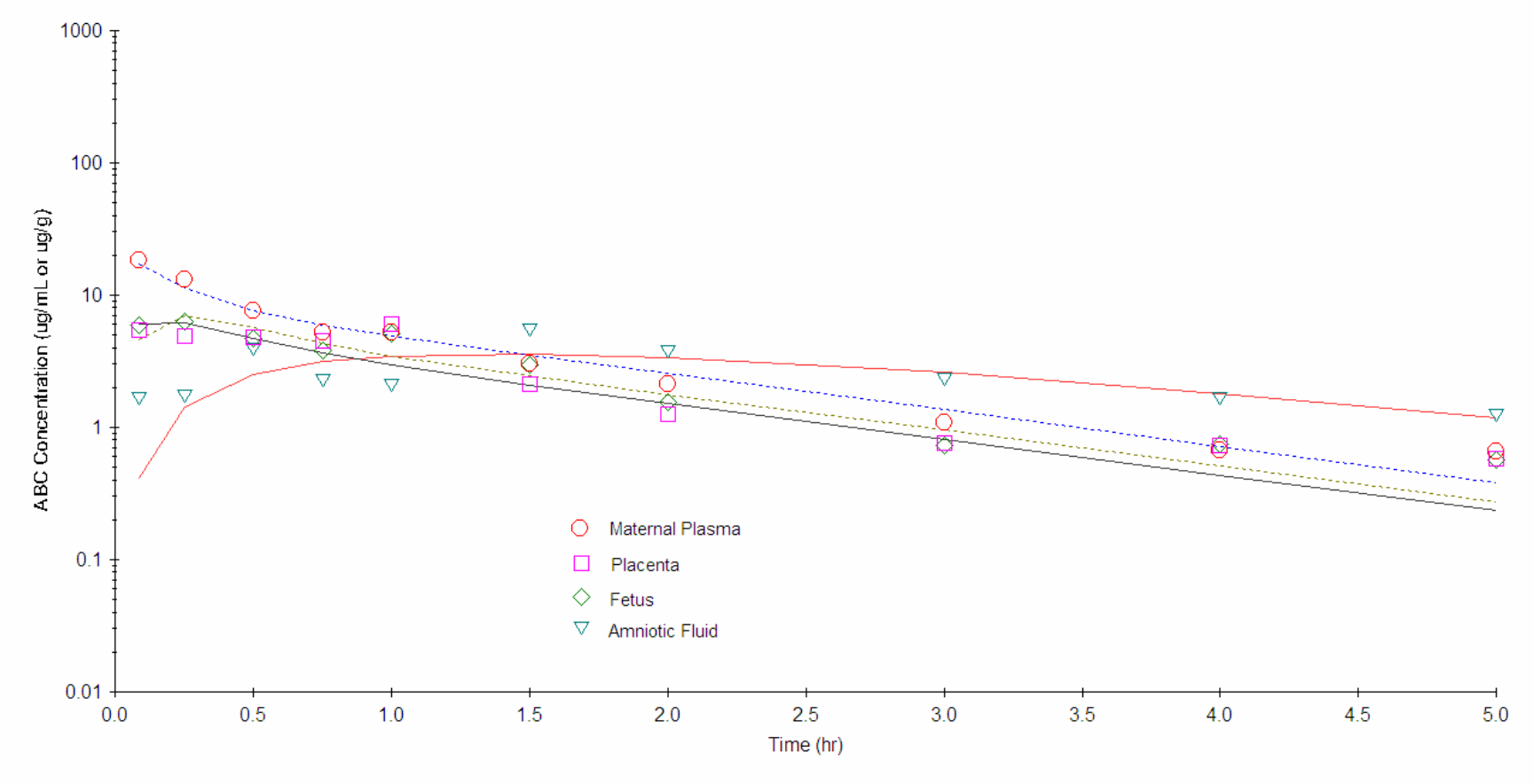


Figure 6.4. Measured ABC concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to the five compartment model.

CHAPTER 7

MATERNAL-FETAL DRUG-DRUG INTERACTIONS OF ABACAVIR AND ZIDOVUDINE IN THE PREGNANT RAT[1]

[1] Lewis, S.R., T.N. Clark, M.G. Bartlett, and C.A. White. To be submitted to *Pharmaceutical Research.*

ABSTRACT

The purpose of these experiments was to determine if abacavir (ABC) and zidovudine (AZT) interact at the maternal-placental interface. Thus, the pharmacokinetics and placental transfer of ABC and AZT monotherapy and ABC-AZT combination therapy were compared in the pregnant rat. Pregnant Sprague-Dawley rats were anesthetized on day 19 of pregnancy and a jugular catheter was implanted and laparotomy performed. Rats were dosed with ABC, AZT or ABC-AZT combination (25 mg/kg each, IV bolus, n=6). Serial blood, placenta, fetus and amniotic fluid (AF) samples were obtained. ABC and AZT were quantitated by HPLC-UV. A five-compartment model was fitted to the data and pharmacokinetic parameters and relative exposures (RE) ($AUC_{tissue}/AUC_{maternalplasma}$) were determined with WINNONLIN. Coadministration of AZT resulted in a 5-fold decrease in the clearance of abacavir. A significant increase in both half-life and AUC for abacavir was also noted. For AZT, the increases in clearance (0.61 to 1.03 L/hr-kg) and volume of distribution (0.87 to 1.74 L/kg) resulted in an increase in half-life (1.1 to 1.4 hr). Decreases in relative exposures of ABC to placenta, fetus, and amniotic fluid (12% to 67% decrease) were consistent with the net increase in the distribution clearance from placenta to maternal plasma. In contrast, relative exposures of placenta, fetus and AF for AZT when ABC was coadministered increased in all tissues (29% to 42% decrease), which is consistent with the significant increase in AZT Cmax. This change resulted from the net increases in distribution clearances from maternal plasma to placenta and fetus to placenta. The changes noted in the placenta, fetus, and AF suggest that transporters play a complex role in the uptake of nucleoside antiviral agents and may have different affinities for purine and pyrimidine analogs.

INTRODUCTION

Antiretroviral treatment of HIV-infected pregnant women is widely used to prevent mother-to-child HIV transmission and as primary therapy for HIV-infected mothers. The use of combination regimens of three or more drugs, referred to as highly active antiretroviral therapy (HAART) has led to dramatic improvements in HIV morbidity and mortality. Zidovudine (AZT) belongs to the nucleoside reverse transcriptase (NRTI) class of antiretroviral agents and was the first anti-HIV agent to be approved by the FDA for the treatment of HIV-1 infection. It was also the first drug shown to prevent mother-to-child transmission of HIV (2). The PACTG Protocol 076 demonstrated that administration of AZT in three stages (antepartum, intrapartum, and postpartum to the neonate), reduced perinatal HIV-1 transmission from 25% to 8.5% (11). The introduction of combination antiretroviral regimens has demonstrated further reductions in the rate of mother-to-child transmission to less than 2% (12, 23, 26).

Abacavir is a novel NRTI used in combination with other antiretroviral agents for the treatment of HIV and a synthetic analogue of guanine (Figure 7.1). It differs structurally from other NRTIs in that it is a carbocyclic nucleoside analogue rather than a dideoxynucleoside analogue (19). Due to the emergence of resistance by HIV to single antiviral agents, AZT is commonly combined with other antivirals drugs such as abacavir. Abacavir is currently prescribed in combination with 3TC and AZT, and marketed as Trizivir® for use in combination with a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor (18). Coadministration of abacavir and AZT in HIV-infected non-pregnant adults demonstrated only small and inconsistent effects on the steady-state pharmacokinetics of abacavir, with the most prominent effect seen at the lowest dose studied (200 mg TID) (27). The absence of statistically

significant influence of AZT on the pharmacokinetic parameters for higher doses of abacavir, suggested that any possible side effect was not of clinical significance (27).

While the use of combinations of antiviral drugs is becoming increasingly common, the impact of such combination therapies on placental transport is largely unknown. The physiological changes associated with pregnancy have a large impact on drug disposition, and changes in the pharmacokinetics of antiviral agents during pregnancy must be understood for these drugs to be used safely and effectively in pregnant women. Several studies reported the lack of interaction between several antiviral drugs, suggesting passive diffusion as the primary mechanism of placental transfer (29, 31). However, there is no current information available on abacavir and AZT combination therapy in pregnancy. A recent study has suggested that both abacavir and AZT can interact with a thymidine transporter identified in a continuous rat microglia cell line (MLS-9) (20). If these drugs interact competitively for transport sites at the placental level also, it is likely that at least one of the drugs would not reach its therapeutic concentration in the fetus and therefore would no longer effectively suppress viral replication (20). Studies of combinations of antiretroviral agents found substantial interactions between antiviral compounds in placental transport (6, 16). Continued study of these compounds is necessary to gain further understanding of the mechanism of placental transport for this important class of therapeutic agents. Since abacavir is increasingly being used in combination with AZT and other antivirals, we investigated the possibility of a pharmacokinetic interaction between these two drugs, particularly as it may apply to maternal-fetal transfer.

Due to the difficulty and ethical concerns in studying placental and fetal drug distribution in humans, an appropriate animal model must be utilized. We conducted this study using the pregnant rat model because it has been proven successful for the investigation of the basic

mechanisms involved in placental transfer of nucleoside analogs. The pregnant rat is also advantageous due to the structural similarities between rat and human placenta, low cost, and short gestation period (21). The containment of each fetus, placenta, and amniotic fluid in individual fetal sacs allows for serial sampling of the pups, therefore providing a full concentration-time course. In addition, several of the same transporter families that exist in the human placenta have been shown to exist in the rat placenta (24). The pregnant rat model has been utilized in maternal-fetal drug transfer studies of a variety of compounds, including nucleoside analogs. (3, 4, 6, 21, 22).

MATERIALS AND METHODS

Reagents and chemicals. Abacavir (ABC) was obtained from GlaxoSmithKline. AZT and internal standard, 3'-azido-3'-deoxythymidine (AZDU), were obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade and obtained from Fisher Scientific (Fair Lawn, NJ, 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 (50:3.3:3.4 mg/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. Intravenous bolus doses (25 mg/kg) of each therapy group were prepared in 0.1 M NaOH in physiological saline and were administered via the jugular cannula.

Three dosing groups were used to complete the study: (i) ABC monotherapy (25 mg/kg) (n = 7), (ii) AZT monotherapy (25 mg/kg) (n = 6), (iii) ABC-AZT combination therapy (25 mg/kg each) (n = 6). Blood samples of 150 to 250 μl were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 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 480 min. Duplicate 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 a Ultra-Turbax T8 (IKA Labortechnik, Germany). All samples were stored at –20 °C until analysis.

Analytical methods. Individual ABC and AZT concentrations in maternal plasma, placenta, fetus and amniotic fluid were determined by high-performance liquid chromatographic methods as described before (6, 10). ABC and AZT concentrations in biological matrices were determined simultaneously by a high-performance liquid chromatographic method using UV detection. Briefly, plasma and amniotic fluid samples were prepared by acid precipitation by

adding 10 µl (50 µl amniotic fluid) or 20 µl (100 µl plasma) of 2 M perchloric acid. Placental and fetal tissues were extracted using a salting out technique by adding 300 µl of saturated ammonium sulfate solution and 400 µl acetonitrile to 100 µl of the biological matrices containing the analytes. The chromatographic analyses were performed using and HPLC system consisting of an Agilent 1100 Series components including a quaternary pump, degasser, autosampler, and variable-wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Agilent Eclipse XDB C_{18} column (150 x 4.6 mm, 5 µm) (Palo Alto, CA, USA) with a Phenomenex Security Guard C_{18} guard column (Torrance, CA, USA). The mobile phases used were 12% acetonitrile in 25 mM sodium phosphate buffer (adjusted to pH 7 with sodium hydroxide) for the fetus, placenta, and amniotic fluid samples and 13% acetonitrile in 25 mM sodium phosphate buffer (adjusted to pH 7 with sodium hydroxide) for plasma. The mobile phase flow rate was 0.8 ml/min and the detection wavelength was set at 270 nm. Under the chromatographic conditions described, ABC, AZT and AZDU eluted at 8.3, 13.5, and 5.7 min (12 % acetonitrile), respectively for fetus, placenta, and amniotic fluid. ABC, AZT and AZDU eluted at 7.3, 10.3, and 5 min (13 % acetonitrile), respectively for plasma.

Data Analysis. 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 generated from AZT monotherapy, ABC monotherapy, and ABC-AZT combination therapy animals. A 1/y-weighting scheme was used throughout the analysis. Amniotic fluid, placenta, and fetus data were subjected to noncompartmental analysis, and the area under the concentration-time curve (AUC) was truncated at the last time point due to the inability to calculate an accurate elimination half-life for all tissues and amniotic fluid. The relative exposure of each matrix was

calculated by comparing the AUC values for the individual tissues to the truncated AUC values for the corresponding plasma data.

In addition to the analyses above, a five-compartment model was used to characterize the distribution of ABC and AZT in the maternal plasma (central) (C_c), maternal tissue (C_t), placental (C_p), fetal (C_f), and amniotic fluid (C_a) compartments (Figure 7.2). The model incorporates bidirectional transfer between maternal plasma and placenta, placenta and fetus, placenta and amniotic fluid, and maternal plasma and tissue compartment.

$$\frac{dC_c}{dt} = \left(\frac{k_{pc} V_p}{V_b}\right) C_p + \left(\frac{k_{tc} V_t}{V_b}\right) C_t - \left(k_{cp} + k_{ct} + k_{c0}\right) C_c \qquad (1)$$

$$\frac{dC_p}{dt} = \left(\frac{k_{cp} V_b}{V_p}\right) C_c + \left(\frac{k_{fp} V_f}{V_p}\right) C_f - \left(k_{pc} + k_{pf} + k_{pa}\right) C_p \qquad (2)$$

$$\frac{dC_f}{dt} = \left(\frac{k_{pf} V_p}{V_f}\right) C_p - k_{cp} C_f \qquad (3)$$

$$\frac{dC_a}{dt} = \left(\frac{k_{pa} V_p}{V_a}\right) C_p - k_{ap} C_a \qquad (4)$$

$$\frac{dC_t}{dt} = \left(\frac{k_{ct} V_b}{V_t}\right) C_c - k_{tc} C_t \qquad (5)$$

In this model, k_{ct} and k_{tc} are the intercompartmental transfer rate constants between maternal central and tissue compartments, k_{cp} and k_{pc} are the intercompartmental transfer rate constants between maternal plasma and placental compartments, k_{pf} and k_{fp} are the intercompartmental transfer rate constants between placental and fetal compartments, k_{pa} and k_{ap} are the intercompartmental transfer rate constants between amniotic fluid and placental compartments, and k_{c0} is the elimination rate constant from the maternal plasma compartment.

The differential equations 1-5 were simultaneously fitted to the concentration-time data. Parameter estimates were generated by WINNONLIN and intercompartmental clearance values (CL) were calculated from the product of the intercompartmental rate constants and distribution volumes. A 1/y weighting scheme provided the best fit of the model to the data. The index of relative exposure of the fetal tissues to ABC and AZT was calculated from the ratio of the area under the drug concentration-time curve for the tissue to that of the mother truncated at the last time point. 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 identify statistically significant differences.

RESULTS

The average concentration in plasma-time profiles for ABC and AZT are shown in Figure 7.3. The pharmacokinetic parameters generated from the compartmental analysis of the plasma data are presented in Table 7.1. For ABC and AZT monotherapies, plasma concentrations declined in a bi-exponential manner, with equivalent β-half-lives of roughly one hour, and α-half-lives of 0.11 h and 0.12 h, respectively. However the clearance and volume of distribution of acabavir were significantly higher as compared to AZT. Plasma pharmacokinetics changed significantly when the two drugs were given in combination. Coadministration of AZT resulted in a 5-fold decrease in the clearance of abacavir. Significant increases in both half-life and AUC for abacavir were also noted. No statistically significant changes were noted in the volume of distribution at steady state or maximum concentration of drug in serum (C_{max}) of abacavir when given in combination therapy. The proportional increases in clearance and volume of distribution (V_c and V_{ss}) resulted in insignificant changes in the half-life of AZT. However, these changes

resulted in significant changes in AUC and Cmax for AZT given in combination with abacavir. A 2-fold decrease in AZT AUC and Cmax was noted with the combination therapy.

The concentration-time profiles of ABC and AZT in placenta, amniotic fluid, and fetus are shown in Figures 7.4 and 7.5. The pharmacokinetic parameters for these matrices are shown in Tables 7.2, 7.3, and 7.4. Relative exposures are given in Table 7.5. The disposition of ABC and AZT in the placenta is the same for the monotherapies and the concentration-time profiles of both drugs mirrored the maternal plasma. In fact the concentration-time profiles of all placenta, fetus and amniotic fluid mirrored the terminal maternal plasma concentration-time profiles. The main difference between the drugs in the fetus is the longer half-life and increased AUC noted with AZT. In fact the half-life of AZT in the fetus is significantly longer than the maternal half-life. The disposition of AZT and ABC are quite different in the amniotic fluid. ABC had a higher C_{max} and shorter half-life as compared to AZT resulting in a higher AUC for AZT. Again the half-life of AZT in the amniotic fluid is significantly longer than the maternal half-life. ABC has a higher RE than AZT for the placenta, fetus and amniotic fluid. This increase in RE for the fetus and placenta is due to the difference in maternal pharmacokinetics rather than dispositional changes in these tissues.

In the combination therapy group, the C_{max} of AZT in all tissues increased approximately 50%. The half-lives of the placenta and fetus were not influenced by co-administration of ABC; however, the half-life of AZT in the amniotic fluid was significantly lowered in the presence of ABC. AUC of AZT was significantly lower in the fetus and amniotic fluid with co-administration of ABC. No change was noted for the placenta. These changes resulted in an approximate 20% increase in Re for the placenta, fetus and amniotic fluid with co-administration of ABC.

For ABC, an increase in the C_{max}, AUC and half-life in fetus and placenta is demonstrated in the combination therapy group. These changes coupled with the changes in maternal pharmacokinetics resulted in a 40% decrease in RE for the placenta and fetus with co-administration of AZT. Significant changes with combination therapy were also noted in amniotic fluid. ABC uptake into the amniotic fluid is significantly delayed resulting in a 4-fold increase in Tmax and a 30% decrease in C_{max}. The half-life of ABC in the amniotic fluid increased approximately 6-fold. It should be noted that this is an approximate value for half-life since sampling only occurred over one half-life. The RE of ABC in amniotic fluid decreased from 1.09 to 0.35 with co-administration of AZT.

The fitted 5-compartment concentration-time curves for ABC and AZT are shown in Figures 7.6 and 7.7. Estimates of the intercompartmental clearances are shown in Table 7.6. The model fitted the data quite well yielding accurate and precise estimates for the intercompartmental clearances. In combination with ABC, the intercompartmental distribution clearances of AZT for the fetal compartment (placenta, fetus and amniotic fluid) significantly increased. It should be noted that the differences are not of the same magnitude and ranged from 2- to 5-fold. ABC distribution within the fetal compartment was also influenced by the co-administration of AZT. CL_{cp} and CL_{pc} increased 3- and 4-fold in the presence of AZT; whereas, CL_{pa} and CL_{ap} both decreased. As observed with the 2 compartment analysis, the clearance of ABC was significantly decreased in the presence of AZT.

DISCUSSION

As part of an ongoing series of studies on nucleoside analogs in combination therapies, we studied the maternal and fetal disposition of ABC and AZT in combination. Previous studies

evaluating the potential interactions between nucleoside compounds have shown mixed results. Although several studies suggest passive diffusion as the primary mechanism of transport for combinations such as ddI-AZT and d4T-AZT (29, 31), there were also studies that found substantial interactions between various antiviral agents (6, 16). There have been several studies of the concurrent administration of ABC and AZT in non-pregnant adults. The pharmacokinetics of ABC, 3TC, and AZT were assessed after single oral administration of drugs, alone or 2 drugs combined or coadministration of the 3 drugs. It was demonstrated that abacavir led to slight alterations of AZT and 3TC concentrations: a 20% decrease in AZT C_{max}, a 40% increase in the AUC of its glucuronide metabolite, and a 35% and 15% decrease in 3TC C_{max} and AUC, respectively. This was considered to be related to interaction at absorption sites and renal excretion and not to be clinically significant (37). However, very little is known about how these drugs interact at the maternal-fetal interface.

This study shows significant differences in the disposition of ABC and AZT when the two drugs are coadministered in pregnant rats. The maternal plasma pharmacokinetics for both drugs were altered. The approximately 5-fold decrease in the clearance of ABC suggests that AZT resulted in an increase in the half-life and AUC of ABC when it was coadministered with AZT. This change in ABC clearance was unexpected since ABC is primarily eliminated by metabolism; whereas, AZT is primarily cleared by renal excretion in the rat. This decrease in clearance could be due, in part, to an increased efflux of ABC from hepatocytes. This is consistent with the observed increase of ABC efflux from the placenta to maternal circulation. Interestingly, the clearance of AZT increased with co-administration of ABC resulting in a decrease in AUC. This increase in clearance could result from an up-regulation of transporters in the kidney. This is supported by the observation of increased transport of AZT in the fetal

compartment with co-administration of ABC. Also noted was an increase in the volume of distributions of AZT which is consistent with the changes in intercompartmental distribution clearances observed with combination therapy. It is unlikely that this can be attributed to a change in plasma protein binding because of the low plasma protein binding of AZT (<36%).

The REs of ABC administered alone in the fetus, placenta, and amniotic fluid were higher than that of AZT monotherapy. This is consistent with the higher lipophilicity of ABC as measured by the log value of its octanol-water partition coefficient (1.2) compared to that of AZT (0.09). An interesting finding by Odinecs, et al. upon examination of fetal-maternal plasma drug concentration ratios in several NRTIS, suggests that lipophilicity is not the only determinant of the fetal-maternal plasma drug concentration ratio (29). In addition, a study examining the CSF penetration of pyrimidine nucleoside analogs, found that the CSF penetration appeared to be a function of the nucleobase rather than the degree of lipophilicity (28). In combination, there was a significant increase in the AUC for ABC in all tissues, indicating that the absolute exposure to the fetus increased whereas, the RE were decreased. This decrease in RE is a direct result of the change in maternal pharmacokinetics that resulted in higher maternal plasma levels of ABC. On the other hand, the AUC of AZT in all tissues significantly decreased, indicating a decrease in the absolute exposure of the fetus to AZT; whereas the RE increased. This increase in RE results from the changes in maternal pharmacokinetics of AZT coupled with the changes in tissue levels.

To further describe the transplacental transfer of ABC and AZT, a more complex and physiologically realistic model was used. The model-predicted ABC and AZT concentrations closely followed the observed data in most cases, with only small deviations apparent in the lower concentrations of the terminal elimination phase. The concentration-time profiles in

maternal plasma, placenta, and fetus were parallel in all drug therapy groups. The concentrations of ABC and AZT in the amniotic fluid, similar to other reported studies, rise and reach a plateau even though the ABC and AZT concentrations in the maternal compartment are decreasing. Therefore, the amniotic fluid acts as a slowly equilibrating compartment.

There was good agreement between the parameter estimates obtained from the five compartment analysis and those obtained in the noncompartmental analysis. The changes in elimination for ABC (decrease in CL_{c0}) and AZT (increase in CL_{c0}) when given in combination reflect the changes in clearance observed above. The pattern of drug distribution observed in the fitted concentration-time profiles illustrates the decrease in relative exposure of ABC to the fetal compartment when given in combination with AZT. The observed decrease in relative exposures of ABC to placenta, fetus, and amniotic fluid (12% to 67% decrease) when co-administered with AZT is consistent with the changes in the intercompartmental clearances between placenta and maternal plasma. When ABC was given in combination with AZT, a disproportionate increase in CL_{cp} and CL_{pc} was observed, with a greater increase in the redistribution of ABC from placenta to maternal plasma. This increase suggests saturation of uptake into the fetal compartment.

Conversely, the increase in relative exposures observed in the amniotic fluid, fetal and placental tissues to AZT (29% to 42% increase), is consistent with the net increase shown in the intercompartmental clearance from maternal plasma to placenta. This increase suggests that the placental transport of AZT increased in the presence of ABC. A possible mechanism of transport could be saturation of an efflux transporter out of the fetal compartment.

A recent study by Brown et al. showed marked differences in the maternal pharmacokinetics and fetal disposition of acyclovir and AZT when the two drugs were

coadministered (6). For AZT, a pyrimidine analog, decreases in exposure to all three tissues were seen in the presence of ACV. On the other hand, ACV, a purine analog, showed a three-fold increase in drug exposure in amniotic fluid and fetal tissue with the combination therapy (6). Interestingly, in this study, also involving a purine and pyrimidine, the opposite was observed. The pyrimidine analog, AZT, exhibited increases in exposure to all three tissues, while ABC, the purine analog, showed decreases in exposure to all three tissues. This suggests that other factors are involved in determining the fetal-maternal plasma drug concentration ratio.

Several drug transporters have been found to exist in the placenta (17, 24) that have been shown to transport nucleoside antiviral agents. Among those include the efflux transporters (MRP, BCRP), and influx/efflux transporters such as the concentrative and equilibrative nucleoside transporters and organic anion/cation transporters. Qualitatively, the placental transporters in the rat mirror those found in the human placenta, which makes the pregnant rat an appropriate model for the further delineation of the role of placental transporters in the fetal uptake of antiviral agents. The disposition of AZT and ABC in the pregnant rat was significantly altered when the two drugs were coadministered. The placental transport of ABC increased dramatically in the presence of AZT. Also, higher ABC plasma levels when given in combination with AZT did not correlate with increased distribution into the placenta, fetus and amniotic fluid. This supports the hypothesis that placental transporters play a significant role in the fetal distribution of nucleoside antiviral agents and may have different affinities for purine and pyrimidine analogs. Additional supportive evidence for active transport of the these drugs in the placenta is the differences in bi-directional distribution clearances observed for both ABC and AZT and the magnitude of the distribution rate constants (data not shown) for the maternal-placenta-fetal interfaces. Which specific transporters or how quantitatively these transporters

affect *in vivo* disposition of therapeutic nucleoside analogs is unknown. Further studies are necessary to identify which transporters are involved in the fetal uptake of these nucleoside analogs.

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Table 7.1. Pharmacokinetic parameters (mean plus or minus standard deviation) generated from compartmental analysis of plasma data collected from 3TC monotherapy, AZT monotherapy, and 3TC-AZT combination therapy in pregnant rats (25 mg/kg).

Parameter	ABC	ABC-AZT	AZT	AZT-ABC
β-Half-life (hr)	0.9 ± 0.26	3.6 ± 1.3*	1.14 ± 0.23	1.43 ± 0.31
α-half-life (hr)	0.11 ± 0.06	0.12 ± 0.06	0.12 ± 0.07	0.18 ± 0.05
AUC(hr-mg/liter)	14.1 ± 2.6	53.0 ± 15.2*	42.3 ± 8.4	24.6 ± 2.9*
Clearance (liter/hr-kg)	1.82 ± 0.32	0.35 ± 0.18*	0.61 ± 0.09	1.03 ± 0.13 *
C_{max} (mg/liter)	47.5 ± 33.4	44.9 ± 10.4	60.7 ± 21.3	29.0 ± 4.9*
V_1 (liter/kg)	0.77 ± 0.38	0.59 ± 0.15	0.46 ± 0.16	0.90 ± 0.16*
V_{ss} (liter/kg)	1.84 ± 0.76	1.46 ± 0.32	0.87 ± 0.12	1.74 ± 0.28 *

*indicates significant difference between monotherapy and combination therapy ($P < 0.05$). V_{ss}, volume of distribution at steady state.

Table 7.2. Pharmacokinetic parameters for placenta generated by using noncompartmental analysis.

Drug Therapy	C_{max} (mg/liter)	T_{max} (hr)	Half-life (hr)	AUC (hr-μg/g)
ABC	7.7 ± 1.6	0.18 ± 0.09	1.4 ± 0.5	11.1 ± 1.8
ABC-AZT	11.5 ± 3.3*	0.10 ± 0.04	2.8 ± 0.3*	34.6 ± 11.9*
AZT	8.3 ± 1.0	0.24 ± 0.15	1.4 ± 0.4	18.7 ± 4.2
AZT-ABC	12.1 ± 1.5*	0.15 ± 0.08	1.7 ± 0.1	19.6 ± 2.3

*indicates significant difference between monotherapy and combination therapy ($P < 0.05$).

Table 7.3. Pharmacokinetic parameters for fetus generated by using noncompartmental analysis.

Drug Therapy	C_{max} (mg/liter)	T_{max} (hr)	Half-life (hr)	AUC (hr-μg/g)
ABC	6.6 ± 1.2	0.17 ± 0.08	1.4 ± 0.5	10.3 ± 3.3
ABC-AZT	9.4 ± 1.8*	0.47 ± 0.27	2.1 ± 0.8*	31.2 ± 9.3*
AZT	6.5 ± 1.0	0.46 ± 0.10	2.6 ± 0.7	21.5 ± 6.1
AZT-ABC	10.0 ± 2.2*	0.21 ± 0.16*	2.7 ± 2.1	16.6 ± 1.7

*indicates significant difference between monotherapy and combination therapy ($P < 0.05$).

Table 7.4. Pharmacokinetic parameters for amniotic fluid generated by using noncompartmental analysis.

Drug Therapy	C_{max} (mg/liter)	T_{max} (hr)	Half-life (hr)	AUC(hr-μg/ml)
ABC	6.6 ± 1.4	1.0 ± 0.25	1.4 ± 0.4	17.0 ± 4.0
ABC-AZT	4.4 ± 1.1*	3.9 ± 0.8*	6.4 ± 1.4*	53.6 ± 9.7*
AZT	4.4 ± 0.7	2.3 ± 0.5	4.8 ± 0.6	36.7 ± 10.9
AZT-ABC	7.1 ± 1.3*	1.1 ± 0.3*	2.3 ± 0.6*	25.6 ± 3.9*

*indicates significant difference between monotherapy and combination therapy ($P < 0.05$).

Table 7.5. Relative exposure ($AUC_{tissue}/AUC_{maternal\ plasma}$) data (mean plus or minus standard deviation) from amniotic fluid, fetal tissue, and placental tissue generated from the noncompartmental analysis of tissue data collected from ABC monotherapy, AZT monotherapy, and ABC-AZT combination therapy rats (25 mg/kg).

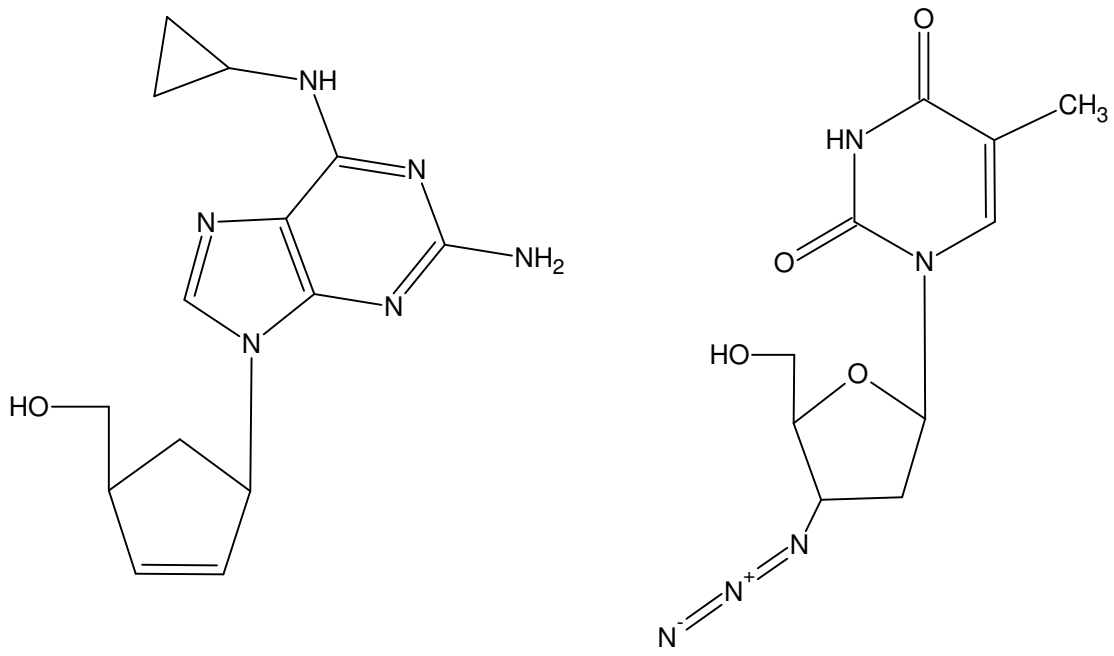
Tissue	$AUC_{tissue}/AUC_{maternal\ plasma}$			
	ABC	ABC-AZT	AZT	AZT-ABC
Placenta	0.76 ± 0.19	0.50 ± 0.06*	0.44 ± 0.05	0.80 ± 0.05*
Fetus	0.81 ± 0.20	0.52 ± 0.14*	0.51 ± 0.10	0.68 ± 0.10*
Amniotic Fluid	1.24 ± 0.40	0.67 ± 0.05*	0.86 ± 0.08	1.05 ± 0.19*

* indicates a significant difference between monotherapy and combination therapy ($P<0.05$).

Table 7.6. Intercompartmental clearances (ml/min) expressed as mean plus or minus standard deviation) of ABC, AZT, and ABC-AZT in pregnant rats (25 mg/kg).

Drug Therapy	CL_{cp}	CL_{pc}	CL_{pf}	CL_{fp}	CL_{pa}	CL_{ap}	CL_{ct}	CL_{tc}	CL_{c0}
ABC	1.24 (0.75)	1.71 (0.80)	6.01 (3.36)	6.53 (3.8)	0.09 (0.03)	0.05[b] (0.02)	15.85 (10.99)	14.88 (11.98)	9.00 (1.79)
ABC-AZT	3.06[a] (0.63)	6.08[ab] (1.34)	7.8 (4.68)	8.15 (5.16)	0.02[a] (0.01)	0.02[a] (0.01)	10.88 (4.55)	13.47 (5.63)	1.91[a] (0.97)
AZT	0.97 (0.39)	2.40[b] (1.00)	3.86 (0.87)	3.99 (0.97)	0.05 (0.02)	0.02[b] (0.004)	5.78 (1.96)	5.89 (1.93)	2.99 (0.49)
AZT-ABC	5.23[a] (1.55)	7.31[a] (2.30)	9.98[a] (5.36)	12.32[a] (6.48)	0.08[a] (0.02)	0.07[a] (0.01)	9.52 (5.86)	8.69 (4.50)	5.10[a] (0.52)

[a] Indicates significant difference between monotherapy and combination therapy ($P < 0.05$).
[b] Indicates significant differences between corresponding distribution clearances within each therapy group ($P<0.05$)



ABC AZT

Figure 7.1. Stuctures of ABC and AZT.

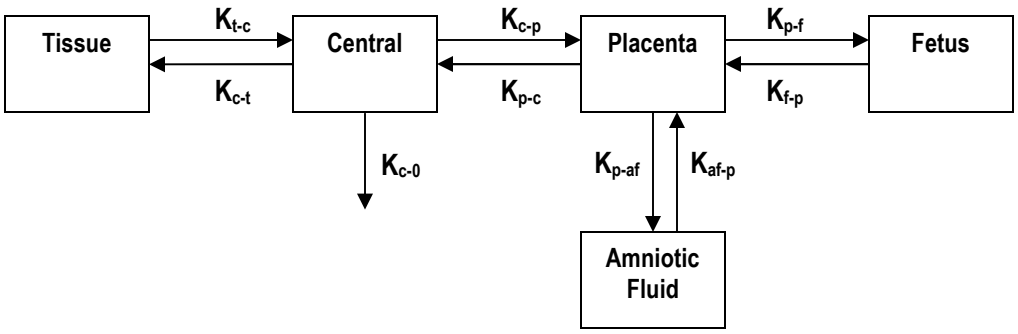
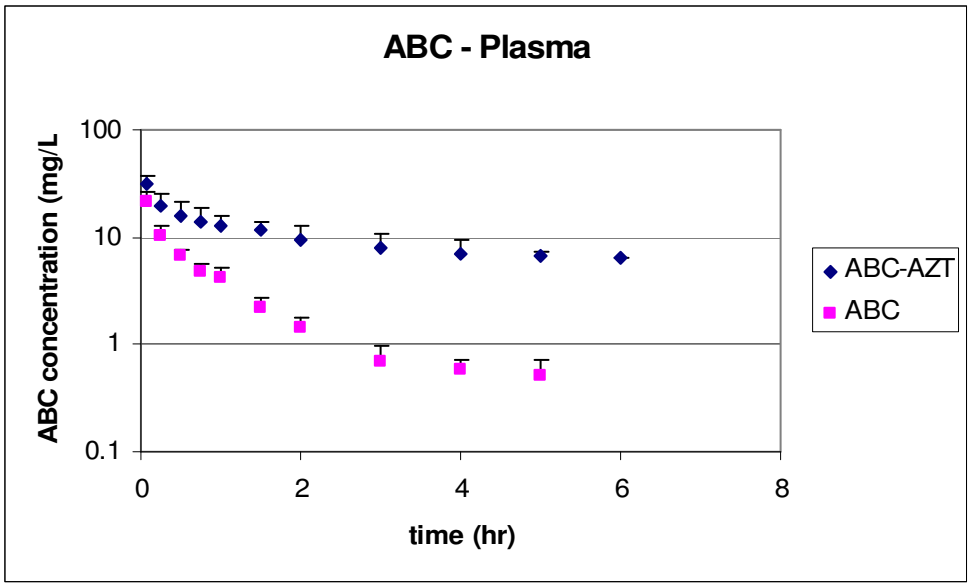


Figure 7.2. Schematic representation of the pharmacokinetic model used to describe the disposition of ABC and AZT in maternal central, maternal tissue, placental, fetal, and amniotic fluid compartments after iv injection to pregnant rats.

(a)



(b)

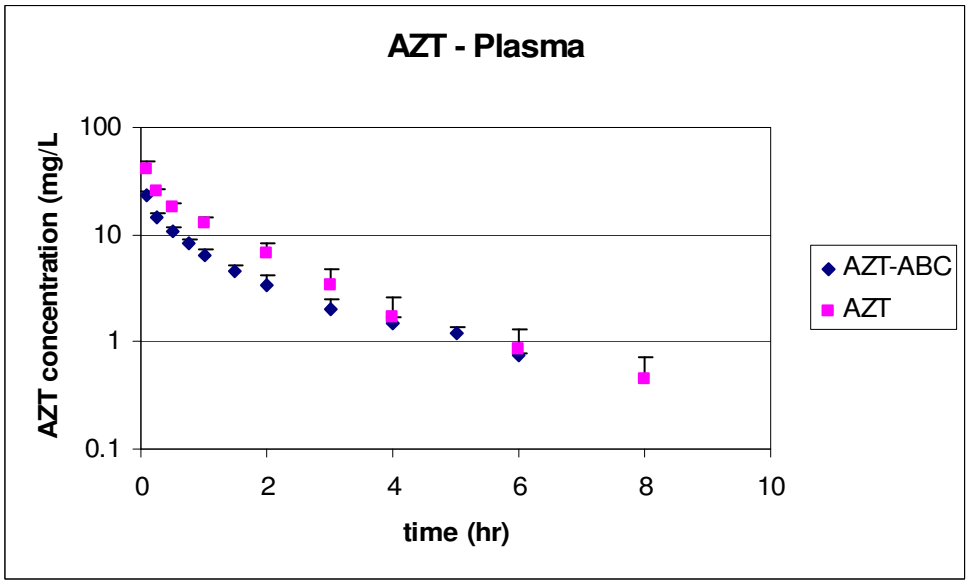
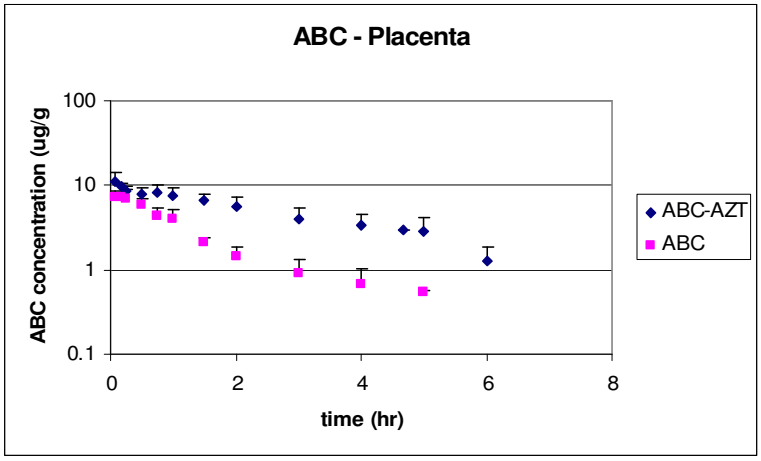
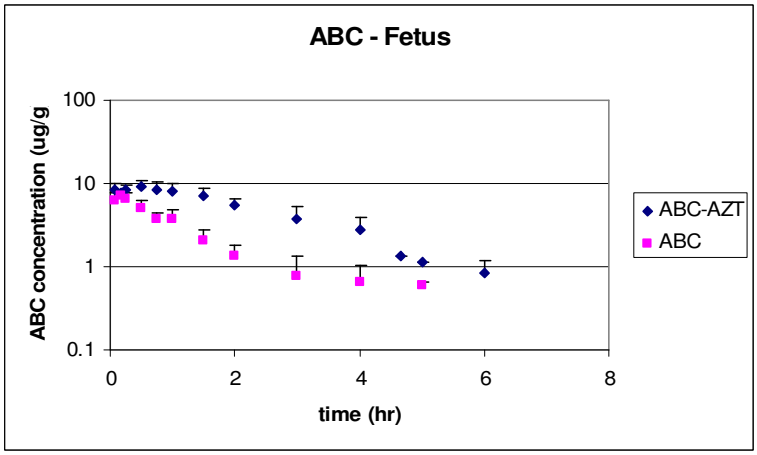


Figure 7.3. Concentration in plasma (mean plus standard deviation) versus time profiles for ABC alone and in combination with AZT (a) and AZT alone and in combination with ABC (b).

(a)



(b)



(c)

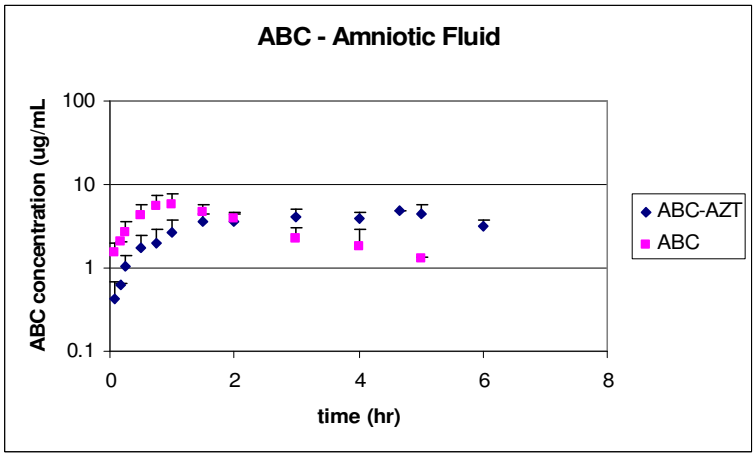
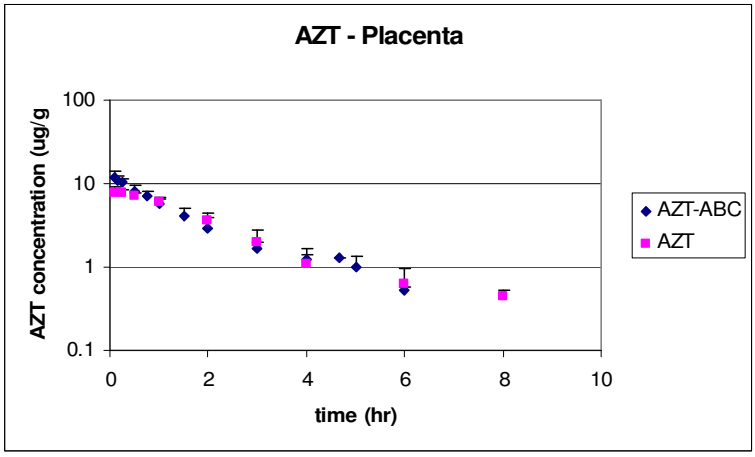
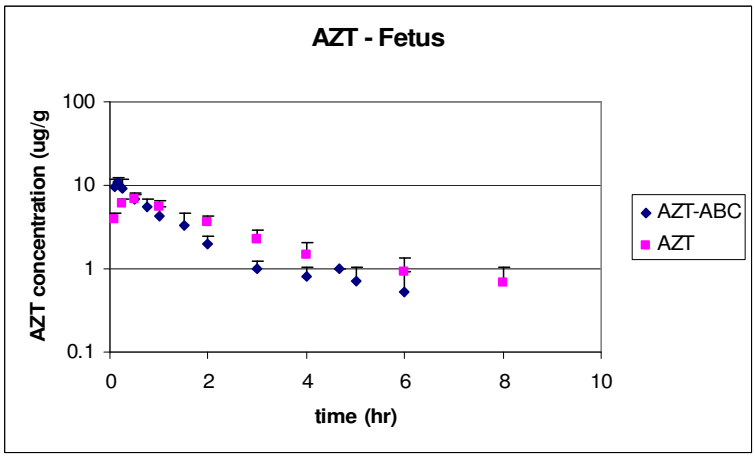


Figure 7.4. Concentration (mean plus standard deviation) versus time profiles of ABC monotherapy and in combination with AZT from placenta (a), fetus (b), and amniotic fluid (c).

(a)



(b)



(c)

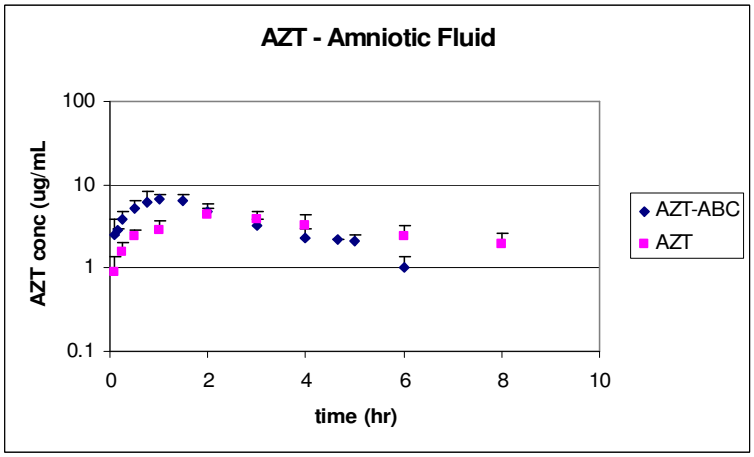
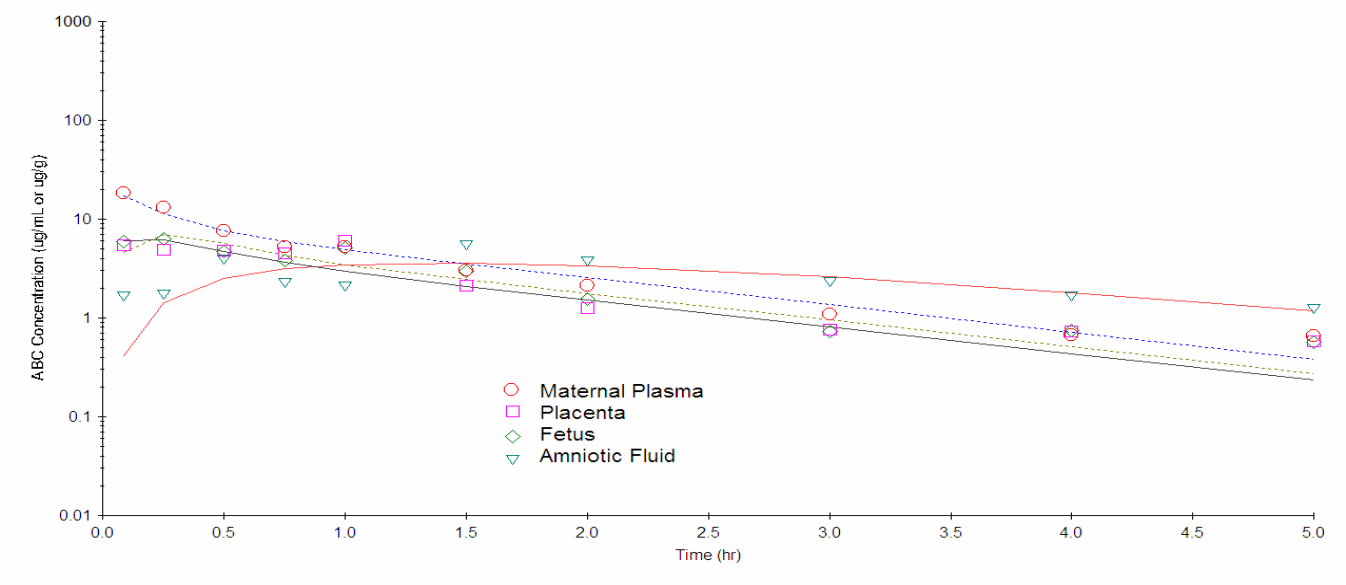


Figure 7.5. The concentration (mean plus standard deviation) versus time profile of AZT monotherapy and in combination with ABC from placenta (a), fetus (b), and amniotic fluid (c).

(a)



(b)

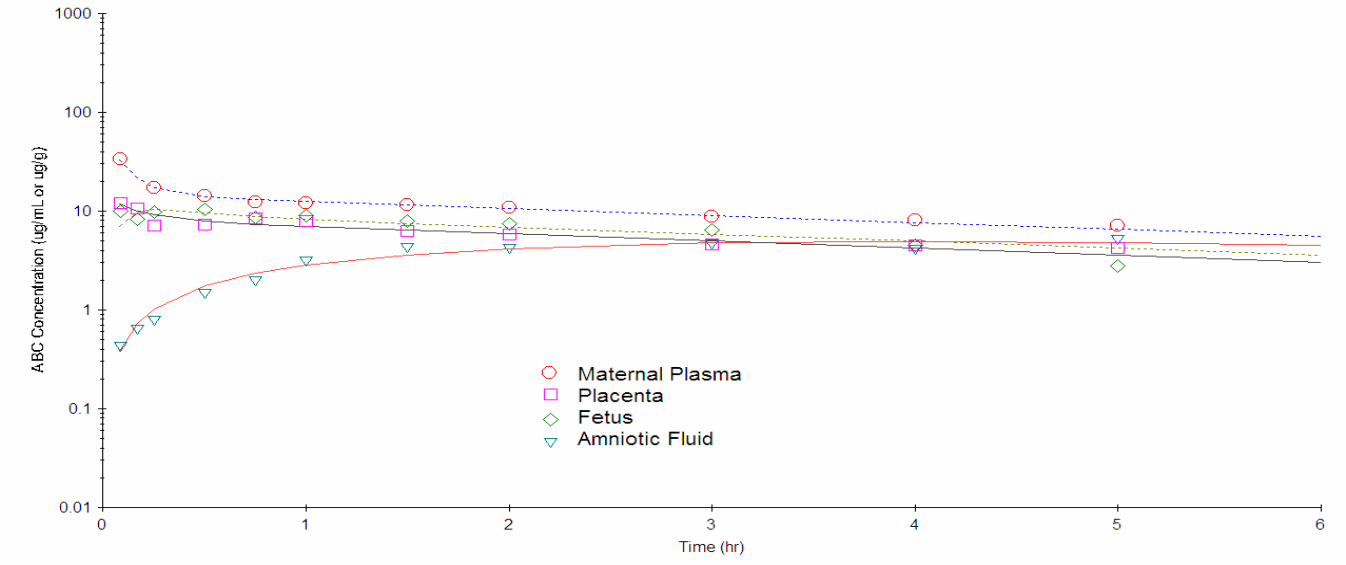
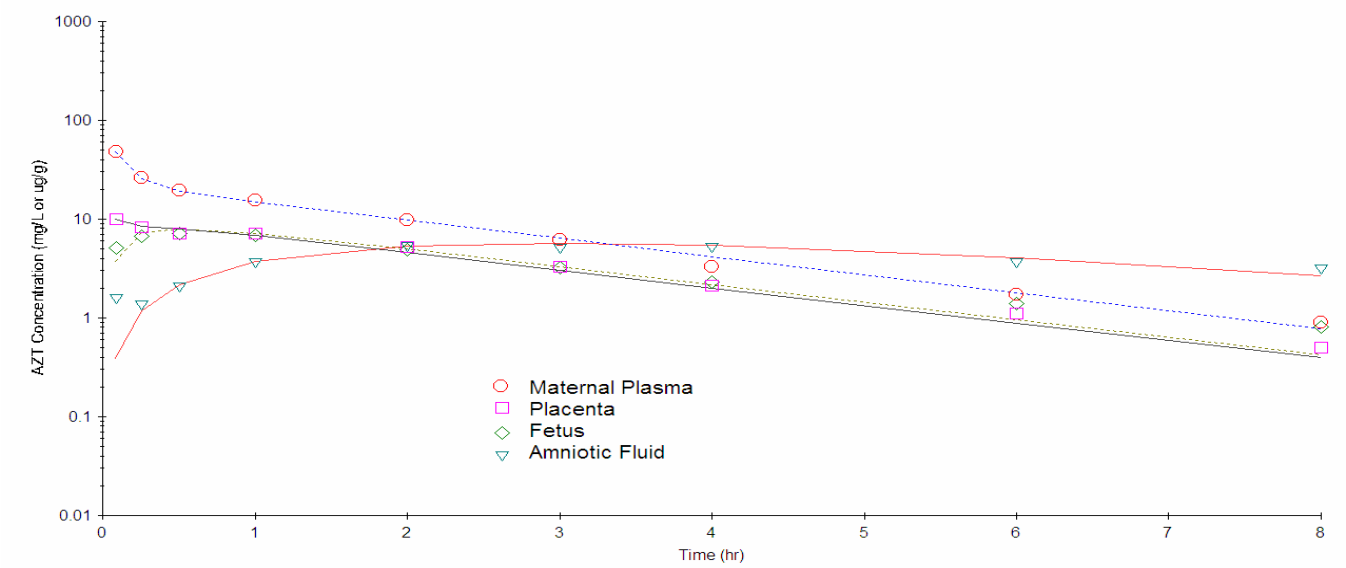


Figure 7.6. Measured ABC (a) and ABC-AZT (b) concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to the five compartment model.

(a)



(b)

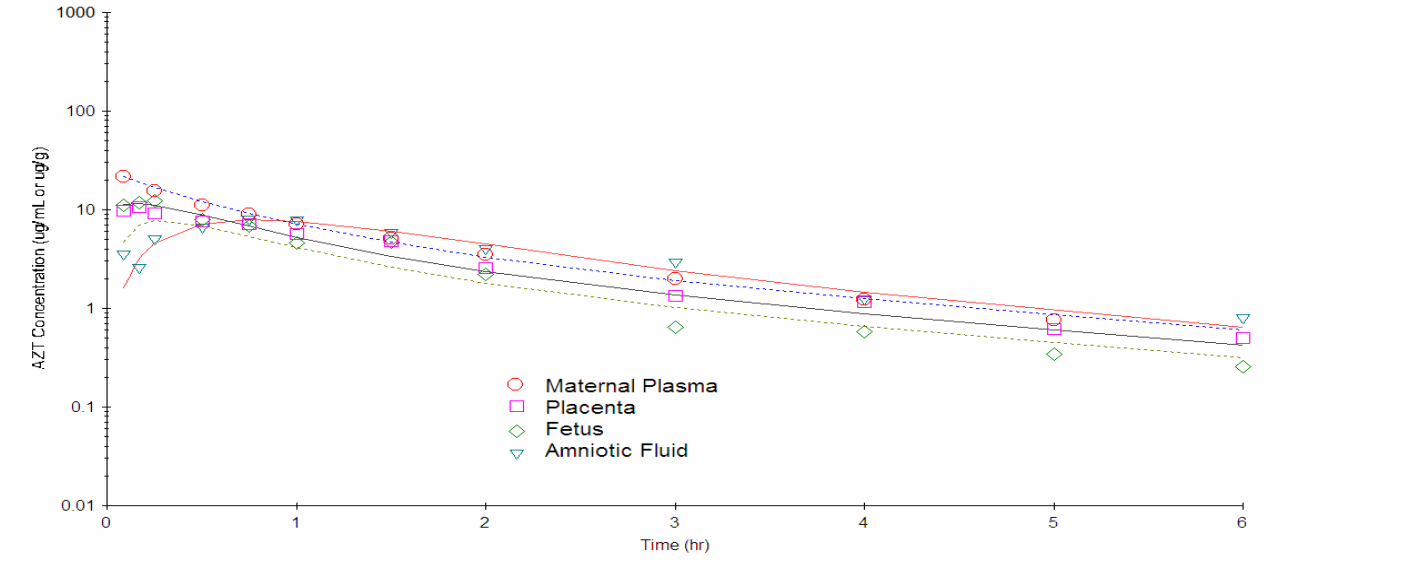


Figure 7.7. Measured AZT (a) and AZT-ABC (b) concentration versus time data for maternal plasma, placenta, fetal tissue, and amniotic fluid fitted simultaneously to the five compartment model.

CHAPTER 8

CONCLUSIONS

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. Although the placenta was once thought of as a barrier to xenobiotics, it is now well known that virtually all drugs ingested by the pregnant woman during pregnancy can cross the placenta to the fetus. Drug administration to pregnant women is common, so gaining a complete understanding of the extent of fetal exposure is important for safe drug therapy in pregnancy. However, the conduct of detailed studies on maternal-fetal drug disposition in pregnant women is limited by technical and ethical considerations. To overcome this drawback, animals models in conjunction with pharmacokinetic computer models have been proposed to allow a more complete understanding of the processes involved in the maternal-placental-fetal unit.

The purpose of these experiments was to identify the mechanisms involved in the placental transfer of several nucleoside antiviral agents alone and in combination with other agents and to determine if interactions are present at the maternal-fetal interface. Using a five-compartment model, our studies indicate significant differences in the transplacental clearances of single antiviral agents. For 3TC, transporters play a significant role in the movement of drug from maternal circulation to fetus. Data suggests transporters are involved at the maternal-placenta and placenta-fetal interfaces

and that movement of drug towards the maternal circulation is significantly higher than drug transport to the fetus. Clinically, this observation could be important because the goal for preventing maternal-fetal transmission of HIV depends on the ability to reach therapeutic concentrations in the fetus. When administered with AZT, the placental-maternal transport of drug was inhibited, yielding higher 3TC concentrations in the fetus and placenta. No differences were noted for AZT with co-administration of 3TC.

Similar findings for abacavir were noted. The data suggests that active transport was involved in the movement of drug between maternal circulation and the placenta and the placenta and fetus. However, the interaction with AZT was significantly different. AZT appeared to up-regulate transporters yielding 2- and 3-fold increases in the intercompartmental distribution clearances between maternal circulation and placenta. This resulted in a 50% increase in peak concentration and a 3-fold increase in AUC in both the placenta and fetus. Additionally, the transport of AZT was also increased at both the maternal-placenta and placenta-fetal interfaces. This resulted in a 50% increase in peak concentrations for the placenta and fetus. The clinical implications of this observation are two-sided. In combination with other antiviral agents, there is a potential for increased fetal exposure to abacavir providing increased protection of the fetus from contracting HIV. On the other hand, drug interactions with other antiviral agents may potentially cause adverse effects to the developing fetus due to the increased exposure to abacavir.

Since humans and rats have similar placental transporters, these findings could have important clinical implications. If active transport plays a large role in placental uptake, as our data suggest, co-administration of antiretroviral therapies will influence

delivery of these drugs to the fetus and alter the risk of mother to child transmission of HIV-1. Modification of dosing schedules in pregnant women may be needed to protect both mother and fetus. Further studies are needed to identify which transporters are involved in the placental transfer of these agents when given alone and in combination with other agents. Improved understanding of drug-drug interactions involved in antiretroviral therapies during pregnancy would lead to the development of safer treatment regimens, and improve outcomes for both mother and fetus.