QUANTATIVE ANALYSIS AND PHARMACOKINETICS OF REVERSE TRANSCRIPTASE INHIBITORS IN THE PREGNANT RAT

by

TERESA NICOLE CLARK

(Under the Direction of Michael G. Bartlett)

ABSTRACT

For over two decades there has been a ceaseless search for more effective treatments of HIV/AIDS. Today there are a number of different therapies that fall into one of three categories, based on their mechanism of action. All currently marketed anti-HIV drugs are classified as either 1) nucleoside reverse transcriptase inhibitors, 2) non-nucleoside reverse transcriptase inhibitors, or 3) protease inhibitors. Each of these compounds has gone through FDA-regulated clinical trials to prove safety and efficacy. Due to a number of reasons, pregnant women are generally not used during clinical trials, so very little is known about the behavior of drugs during pregnancy. A pregnant rat model has been developed to investigate the pharmacokinetics and placental transport of drugs during pregnancy. Presented here are validated analytical methods for the extraction and quantitation of the nucleoside reverse transcriptase inhibitors azidouridine, didanosine and abacavir in the various matrices needed for maternal-fetal pharmacokinetic studies. Also presented here are the pharmacokinetics of two of these compounds, azidouridine (as compared to zidovudine) and abacavir, using a pregnant rat model.

INDEX WORDS: Azidouridine, AZDU, Zidovudine, AZT, Abacavir, ABC, Didanosine, DDI, High Performance Liquid Chromatography, HPLC-UV, LC/MS/MS, Pharmacokinetics

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TERESA NICOLE CLARK

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TERESA NICOLE CLARK

Major Professor: Michael G. Bartlett

Committee: James T. Stewart
Catherine A. White
J.Warren Beach
Ronald Orlando

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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## DEDICATION

*This is dedicated to the memory of my Granny, Lillian McLawhorn Anderson, the first in an ever-growing line of independent Anderson women. Although she is no longer with us, her spirit lives on in her daughters and granddaughters.*

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# INTRODUCTION

## Prologue

Every year roughly 2 billion prescriptions are filled in the United States. Taking a prescription medication has become so commonplace that most people think nothing of it. Most prescription drug users have little to no understanding of the long road their little pill or capsule took to get to their local pharmacy. It is a complicated process that costs an average of $359 million [1]. Along with the medicinal chemists who originally synthesize the new drug, the pharmacokineticists who study the drug's behavior in the body, the toxicologists who investigate the potential toxicity of the drug, the analytical and bioanalytical chemists play an important role in this process.

The analytical chemists team up with the medicinal chemists in the structural characterization and confirmation of their synthesized products. The bioanalytical chemists work alongside the pharmacokineticists and toxicologists to measure the amounts of the drug in the various required biological fluids and tissues. They also identify and quantitate any metabolites that are present. Once the drug makes it through to manufacturing, the analytical chemists come in again to study formulation development and perform stability testing. They also identify and characterize unknown impurities in raw and intermediate manufactured drug substances. It is safe to say, that none of the prescription medications that we so often take for granted would be at our disposal if it weren't for the work of analytical chemists.

If drug substances are to be analyzed from any given biological matrix, whole blood, plasma, urine, bile, or tissue to name a few, some type of sample clean-up is generally required. A way of carefully extracting the drug of interest from the particular matrix without losing a significant portion of the analyte in the process is not always an easy task. Usually the sample has to be subjected to some other means of separating the analyte from the matrix residue that made it through the extraction technique as well. Using a variety of chromatographic techniques, mainly gas chromatography (GC) and high performance liquid chromatography (HPLC), the analyte can be effectively separated, or resolved, from the remaining matrix components. When these chromatographic techniques are coupled with a detection device, such as an ultraviolet detector (UV) or mass spectrometer (MS), the chemists can get a true measure of the amount of the drug present in the sample.

**Extraction of Analyte**

As it often goes, the most important step in the analysis of compounds from biological matrices generally proves to be the most difficult. Before a biological sample can be analyzed for drug content, the sample must be pretreated to remove as much of the endogenous matrix compounds as possible. This step is needed in order to provide a sample that is reproducibly clean, homogenous and ready for direct injection onto the analytical column [2]. If a sample is clean upon analysis, the measurements become more reproducible, the chromatography becomes less complicated, the lifetime of the analytical column is longer and the overall sample analysis process becomes more straightforward and simple. Therefore, sample preparation/extraction is a “necessary

evil" that must be dealt with in a manner that allows for the cleanest sample possible with the minimal amount of time and cost. Here we discuss the sample preparation techniques of protein precipitation/denaturation and solid phase extraction (SPE).

*Protein Precipitation*

The main feature of biological matrices, especially plasma and serum, is the presence of large amounts of proteins. Often there is a strong affinity between these proteins and the drug of interest. Therefore, removal of the proteins by methods such as filtration or dialysis would also remove the drug that is bound to these proteins. When drug content is measure, there would be an inaccurate reporting of the true amount of drug originally present in the sample. The simplest and oldest method of removing proteins from biological samples is to precipitate them out and isolate the filtrate. Once a protein is denatured by precipitation, its drug-binding ability is destroyed. This releases the total amount of the drug present into the filtrate [3]. The specific approach to the precipitation depends on the particular compound of interest and the sensitivity of the analytical method.

A favored method for removal of proteins is through the use of acidic reagents. The most common acids used for precipitation are trichloroacetic acid and perchloric acid. Due to the "strong" nature of these acids, a very small amount has to be added in order to precipitate/denature all proteins present in a given sample. This is very beneficial in that the sample volume is not increase dramatically in the process. Once the acid has been added to the sample, it is then mixed and subjected to centrifugation to pellet out the proteins. The supernatant can then be transferred to injection vials and

injected directly onto the system. Such strong acids can also have detrimental effects on the drug of interest, however. If a drug is not stable under extremely acidic conditions, then acid precipitation should not be used [3].

For drugs in which acid protein precipitation is not appropriate, organic solvents can be used to denature and precipitate proteins. The organic solvents of choice include methanol, ethanol and acetonitrile. At least two volumes of methanol or ethanol and equal volumes of acetonitrile are required to precipitate out the proteins present [3]. Generally, three volumes of solvent, regardless of organic strength, are added to the sample to ensure total removal of all proteins. Once the solvent is added, the sample is vortex mixed and then centrifuged to pellet out the proteins. The supernatant can then be injected, diluted or evaporated, depending on the chromatographic conditions as well as the need for sample concentration. If a concentration step is needed due to lack of sensitivity in the analytical method, the supernatant can be evaporated and the residue reconstituted in a lesser volume of a solvent compatible with the mobile phase.

*Solid Phase Extraction (SPE)*

Solid phase extraction uses a small disposable plastic syringe-like cartridge packed with varying amounts of a given sorbent. The sorbent is usually a reverse-phase material, such as $C_{18}$ silica and is held in place by frits in the same manner as in an HPLC column. Generally, the separation principles, phase selections and method development for SPE are closely related to that for HPLC [2].

Before the sample is added to the cartridge it must first undergo a conditioning step. This step removes any impurities and solvates (wets) the sorbent. The conditioning

step involves running several cartridge-volumes of an organic solvent through followed by an equal amount of an aqueous wash. The aqueous wash prepares the cartridge for the addition of the more "aqueous" sample and allows for greater retention of the analyte. Once the cartridge is properly conditioned, the sample is loaded and washed with an appropriate (usually aqueous) solution that will retain the analyte while removing the majority of interferences from the biological matrix. The analyte is then eluted off the column using an organic solvent. The eluent can then be directly injected, diluted or evaporated if a concentration step is required [2].

Due to the theoretical similarities with HPLC, many of the phases found in HPLC columns can also be used as SPE sorbents. Therefore, the wide assortment of packing materials, the variety of organic solvents and aqueous solutions that can be used in sample elution, makes solid phase extraction considerably more versatile than all other methods of biological sample preparation [3]. This versatility, coupled with the ability for automation, a more complete removal of interferences and greater analyte recovery makes SPE a technique of choice for biological sample preparation.

**Chromatographic Separation**

It was a century ago in 1903 that a young Russian botanist by the name of Michial Semenovich Tswett presented a paper entitled "*On a New Category of Adsorption Phenomena and Their Application to Biochemical Analysis*" at a meeting of the Biological Section of the Warsaw Society of Natural Sciences [4]. Little did Tswett or the other scientists present at the meeting realize that his adsorption separation technique

outlined in this paper would grow to become one of the most widely used analytical techniques of our time. Chromatography, as it would be called in later papers, was born.

In the many years since the first reports of chromatographic separation, the technique has grown to encompass more than liquids passing through a simple column filled with fine calcium carbonate. Liquid chromatographic methods based upon a wide variety of separation principles have also been proposed, such as partition, ion exchange and size-exclusion. With the recognition of the high speed of LC in the 1960's following the development of surface-coated stationary phase packings and smaller spherical packing sizes, modern day high performance liquid chromatography began taking shape [5].

Although, drug analysis encompasses a wide variety of separation techniques such as gas chromatography, supercritical fluid chromatography and capillary electrophoresis, high performance liquid chromatography (HPLC) is the most widely used of these techniques. For this reason we discuss the separation technique of HPLC.

*High Performance Liquid Chromatography (HPLC)*

High performance liquid chromatography involves the passing of a liquid mobile phase through a column packed with some type of porous stationary phase. In reverse-phase HPLC, the type most commonly used for drug analysis, the mobile phase consists of some type of aqueous component (water, buffer, an organic modifier (methanol, acetonitrile, etc.) and a column, generally of some type of derivitized silica. The types of aqueous and organic components as well as their ratio is optimized for each compound to allow for proper retention and to effectively separate (resolve) them from other analytes

and/or other interferences. The column packing material is also chosen on the basis of retention and selectivity. More polar columns (phenyl, cyano, amino) columns generally yield better chromatography for more polar analytes. In the same manner, more nonpolar columns ($C_{18}$, $C_8$) work well for nonpolar compounds. Along with mobile phase composition and column packing, flow rate, pH, temperature, column length and column diameter are other parameters used to optimize chromatographic separation [2].

**Analyte Detection**

The analytical column may be the heart of an HPLC separation, but a detector is needed in order to see the results of the chromatography. One can develop the most efficient and fast separation, but without a means of seeing the analytes, all efforts are in vain. Unlike flame ionization and thermal conductivity detectors in gas chromatography, HPLC has no sensitive universally applicable detector. Therefore, the detector chosen for an HPLC application is based on needed levels of sensitivity, structural characteristics of the analyte and availability of access to a particular detection method. There are several characteristics that a detector should possess, however. The ideal detector should have good stability and reproducibility, provide a linear response to solutes extending over several orders of magnitude, have a short response time that is independent of flow rate, and be highly reliable and easy to use. It should also have minimal internal volume to reduce band broadening [6].

There are many types of detectors fitting the above criteria that are used with HPLC. They include Ultraviolet/Visible (UV/Vis) absorbance, fluorescence, electrochemical, refractive index, and mass spectrometry, to name a few [6]. In most

cases, detection is carried out with ultraviolet (UV) detection with a variable-wavelength or diode-array detector [2]. One of the fastest growing detection methods, especially within the pharmaceutical industry, is the use of mass spectrometry (MS). For the widespread use and the industrial applicability, we will discuss ultraviolet and mass spectrometric detection.

*Ultraviolet (UV) Detection*

Ultraviolet detectors are the mainstay of routine HPLC analysis. The detector monitors the column effluent at a specified wavelength. All compounds that have some absorption at this wavelength will generate a response [3]. Ultraviolet detectors are considered to be selective detectors because only compounds with the appropriate molecular structure can be detected. UV detection is based on the basic principles of Beer's Laws.

$$A = \varepsilon bC = \log(I_o/I)$$

Analyte concentration (C) in the flow cell is related to the absorbance (A), analyte molar absorptivity (ε) and the flow cell length (b) as well as the ratio of original light intensity ($I_o$) to the sample's light intensity ($I$) [7]. Usually the light from the lamp is directed to a reference diode to obtain a measure of the original light intensity [2]. The flow cell length and molar absorptivity for a particular compound are constant in a given detector. Therefore, absorbance is only dependent on analyte concentration [7].

Although there are several types of lamps that generate light at a variety of wavelengths, most HPLC applications are carried out using wavelengths under 400 nm. Therefore, the deuterium lamp is the most common light source [2].

The advantages of UV detection include – ease of operation, low background with many HPLC solvents, relatively high sensitivity, and nondestruction of samples. Disadvantages include – analyte must have absorbance at the specified wavelength and analyte must be chromatographically resolved from other compounds that absorb at the same wavelength [7].

*Mass Spectrometry*

Shortly after Tswett introduced the idea of chromatography to the world, a physicist named J.J. Thomson introduced his parabolic mass spectroscope in 1907 [8,9]. Since then a variety of mass spectrometers have been produced and found utility in every facet of science.

Mass spectrometry has, in fact, emerged to be one of the most important analytical tools of our time. The advances in the field of mass spectrometry in recent decades are almost too numerous to count. With its ability to measure almost infinitely small amounts of analyte with almost as small sample sizes under a variety of conditions and the instrumentation and software becoming very "user-friendly", a common misconception about the technique has arisen. Ideas like "any monkey can press a button and get data" and that a mass spectrometer is a "magic" box that spits out the exact data that you need without any effort at all, reflects the general scientific public's view of mass spectrometry.

All mass spectrometers have three basic components: (1) an ionization source, (2) a mass analyzer and (3) a detector. Ion formation is the most critical part in all of mass spectrometry. Without the formation of ions in the source, mass analysis, regardless of

the type of analyzer used, is impossible [10]. In the field of pharmaceutical bioanalysis, especially in the area of drug quantitation, the overwhelming choice for ionization involves the atmospheric pressure ionization (API) techniques of atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Both APCI and ESI are "soft" ionization techniques. Unlike the harder ionization techniques that bombard the sample with large amounts of energy that can blast it apart, APCI and ESI achieve analyte ionization by collision with reagent ions without the excessive energy transfer [9]. The result is that the analytes predominantly remain intact upon ionization, even those held together by weaker noncovalent bonds [11]. If fragmentation does occur, it is to a much smaller degree than seen with the harder techniques.

*Atmospheric Pressure Chemical Ionization (APCI)*

Atmospheric pressure chemical ionization (APCI) is a special type chemical ionization in which, as the name implies, the ionization process occurs at atmospheric pressure. In APCI a corona discharge is used for ionization. The primary ions formed undergo a series of ion molecule collisions to give abundant $H_3O^+$ ions from water or $NH_4^+$ ions from traces of ammonia. These reagent ions then protonate anything more basic than themselves or form adduct ions. The analyte enters the ionization region from a liquid stream that exits an LC column sheathed by a stream of nitrogen gas in order to produce a fine spray. APCI requires a sufficient amount of heat be added to the ionization region to vaporize both the solvent droplets and analyte. This is accomplished by the addition of a drying gas, usually nitrogen [12]. The analyte ions that are formed at atmospheric pressure are then sampled through a skimmer interface that allows for the

maintenance of the vacuum in the mass spectrometer [9]. Negative ions can also be formed in APCI. Negative ionization occurs through the use of reagent ions such as solvated $O^{2-}$ rather than through electron attachment reactions. APCI works well for most compounds with molecular weights less that 1000 Daltons and often provides fragment ions that can assist in interpretation. APCI is easily interfaced with a liquid chromatography system and can accommodate flow rates from 0.2 – 2 ml/min [12].

*Electrospray Ionization (ESI)*

Electrospray dates back to the work of Zeleny in 1917 [13] and actually refers to the dispersion of liquids into small charged droplets using electrostatic fields. It was in the late 1960's that Malcolm Dole proposed using electrospray as an ionization technique for the mass spectrometric analysis of large molecules. Due to a variety of experimental difficulties, Dole abandoned his experiments in the early 1970's and electrospray was shelved until the 1980's [11]. In the mid-1980's John Fenn and colleagues demonstrated the ability to use Dole's principles for ESI sample transfer and ionization with a mass spectrometer [14-16].

In the electrospray ion source, the liquid flow from the inlet is converted to charged liquid droplets by the presence of an electric field. The droplets are excessively positively or negatively charged through redox chemistry, depending on the capillary bias polarity, and are attracted to the mass spectrometer inlet [9]. The liquid solvent is evaporated from the surface of the droplets before they enter the mass spectrometer by application of a drying gas, heat or both. As the droplet size decreases, its surface electric field density increases and the mutual repulsions of like charges on the surface

become so large that it exceeds the forces of surface tension. Once this happens the ions begin to leave the droplet by what is known as a "Taylor cone". The ions are then directed into the mass spectrometer [17].

Electrospray ionization allows for the analysis of very large molecules in an instrument with a limited mass range. This is due to its ability to produce multiply charged molecules. It is also readily interfaced with HPLC and capillary electrophoresis (CE) in that it requires a continuous inlet flow of liquid sample [9].

Once the sample is ionized and suitable for analysis, it passes from the source into the mass analyzer. As with the ionization method, there are numerous choices when it comes to mass analyzers. When interfacing with HPLC for bioanalytical quantitation, the single or triple quadrupole is the overwhelming choice [17].

*Quadrupole Mass Analyzer*

As the name indicates, quadrupole mass analyzers are four precisely parallel rods, arranged to have a hyperbolic cross section, to which direct current (DC) voltage and a radio-frequency (RF) potential are applied [17]. According to quadrupole theory, the specific symmetric arrangement of the rods allows for the production of hyperbolic fields [18-20]. Opposing rods are connected electrically to each other in pairs. One pair of rods receives a positive DC potential and a specific RF potential. The other pair of rods receives a negative DC potential and an RF voltage of the same specific potential, but $180^{o}$ out of phase [21]. The field imposed on the quadrupoles acts as a mass filter, selecting which ions are allowed to pass through and reach the detector. As ions move into the field region, they oscillate based on their mass-to-charge ratio (*m/z*) [17]. By

ramping the RF amplitude and DC voltages at a fixed ratio, the quadrupole filters out ions with different *m/z*. Ion trajectories through the space in the center of the rods are complicated. Only the ions possessing a specific *m/z* value adopt stable oscillations and pass successfully through the center of the rods for any given set of RF and DC voltages. All other ions will have unstable trajectories and will collide with rod surfaces and not pass through to the detector [9,21]. The stable trajectory of the ions that pass successfully through the quadrupole, avoiding collision with the rods, has a value that corresponds to a bounded solution to the Mathieu equation [9,18,19].

Triple quadrupole mass spectrometers are a tandem arrangement of three quadrupoles in which the first and third quadrupoles act as mass selective filters (*Q*) and the second quadrupole operates in RF-only mode, serving as a collision cell (*q*). The first quadrupole is used to select the "precursor ion", or ion which will be fragmented. This precursor ion then passes into the second, RF-only, quadrupole where it collides with an appropriately stable gas and produces product ions. The "product ions" then pass into the third quadrupole where they are analyzed [9].

Quadrupole mass analyzers have a tolerance for relatively high pressures, giving them the capacity to interface with electrospray ionization. Also, quadrupoles are now able to routinely analyze up to an *m/z* of 4000. This makes them useful for analysis of proteins and other biomolecules when coupled with electrospray ionization which commonly produces a charge distribution below *m/z* 4000 for these compounds [17].

The final component of the mass spectrometer is the ion detector. The detector measures the current due to the analyzed ion beam and generates a signal by either producing secondary electrons, which are then further amplified, or by initiating a current

generated by the moving charge [17]. The most important criteria for any ion detector are sensitivity, accuracy, resolution and response time [21]. There are a variety of ion detectors available for use with mass spectrometry. They include the Faraday cup, scintillation counters, electron multiplier, high-energy dynodes, arrays and Fourier transform-ion cyclotron resonance detectors. Among these, the electron multipliers are the most commonly used detectors. These detectors work by converting the kinetic energy of ions in the analyzed beam into secondary electrons [17]. For applications outlined in this manuscript, a form of an electron multiplier detector known as the Daly (photomultiplier conversion dynode or scintillation counting) detector was used. Therefore, we will discuss the general characteristics of electron multipliers and the specifics of the Daly detector.

*Electron Multiplier Detectors*

Electron multipliers consist of a series of dynodes (generally 16-20) connected together and maintained at increasing potentials by voltage dividers. The ion beam from the mass analyzer strikes the first dynode (the conversion dynode), resulting in the release of secondary electrons in direct proportion to the number of ions striking the dynode surface. The secondary electrons are then accelerated and focused on the second dynode where more secondary ions are produced. This process continues through the course of the series of dynodes, ultimately causing a cascade effect of electron production. The final dynode (the anode) is connected to a conventional amplifier. Electron multipliers exhibit fast response times, high sensitivities and high gains (typically a magnitude of

$10^6$). The lifetime of electron multipliers are limited to only a few years due to surface contamination from incident ions from the mass analyzer or from poor vacuum [9,17,21].

*Daly Detectors*

Daly detectors, also known as photomultiplier conversion dynodes or scintillation counters, are similar to the electron multipliers in that the ion beam from the mass analyzer initially strikes a conversion dynode, in this case a Daly knob, held at a very large negative potential to produce secondary electrons. Instead of accelerating towards another dynode, the electrons produced at the Daly knob are directed to a scintillator (a phosphorous screen) where photons are released upon electron strikes. The photons are then amplified in the usual manner by a conventional photomultiplier [17].

Daly detectors offer significant advantages over similar electron multiplier detectors. The primary advantage is that all parts, except conversion dynode, are located outside the vacuum chamber and are therefore unexposed to the internal environment of the mass spectrometer. This prevents damage from initial beam electrons and allows for servicing of the detector without disturbing the pressure in the mass spectrometer. Another advantage is that the large potential difference between the Daly knob and the scintillator is advantageous for detecting high molecular weight ions. A decelerating lens can be installed in front of the screen to allow for the differentiation between stable and metastable ions. The sensitivity of Daly detectors is comparable to electron multipliers, but they have a longer lifespan, generally 5 years or more [9,17].

**Preface to Antiviral Placental Transport Studies**

On June 5, 1981, the Centers for Disease Control (CDC) issued its weekly report of outbreaks of illness and unusual deaths in the United States. It was in this report that doctors reported a parasitic lung infection, *Pneumocystis carinii pneumonia*, had shown up in five young, homosexual men in Los Angeles. The report went on further to say that three out of three of the men tested had inexplicable suppression of their immune system [22]. This unexplained illness was later determined to be caused by a virus. The virus was then isolated and given the name human immunodeficiency virus (HIV) and the illness was given the name acquired immunodeficiency syndrome (AIDS). Thus began an outbreak of an epidemic of epic proportions. Today, every country in the world is affected, some more than others, by this killer without a cure. The United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) report that at the end of 2002 38.6 million adults and 3.2 million children worldwide were living with HIV. In 2002 alone, approximately 800,000 children under the age of 14 became infected with HIV. Of these, more than 90% were babies born to HIV infected mothers [23].

When AIDS was first discovered in the early 1980's there were no effective treatments. Therefore, the life expectancy of an infected individual was months to a couple of years once full-blown AIDS developed. In 1986, the Burroughs Wellcome Company became the first pharmaceutical manufacturer to offer a hope for AIDS victims. Following a whirlwind development and clinical trial process, zidovudine, better known as AZT, became the first effective treatment for the disease [24]. Today there are 16 antivirals currently approved for use alone or in combination to battle HIV. These

antivirals fall into three different categories, nucleoside analog reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and protease inhibitors [25]. While the death rate due to AIDS infections continues to plummet in developed nations, the focus of the worldwide community has shifted to finding solutions to the ever-growing infection rates in sub-Saharan Africa and Southeast Asia. Of particular interest is the prevention of the transmission of HIV from an infected woman to her unborn or newborn child.

In 1994, a clinical study, ACTG 076, demonstrated the ability to reduce the transmission rate from mother to child (vertical transmission). In this landmark study, it was discovered that if an HIV positive expecting mother was given a zidovudine (AZT) monotherapy regimen in the later stages of her pregnancy, the vertical transmission rate could be greatly reduced. Using the AZT monotherapy, mother-to-child transmission rates dropped from an average 25% to 8% [26]. Today, with the widespread use of HIV testing and highly active antiretroviral therapy (HAART), prenatal and perinatal infection rates in the US are even lower.

Even in developed nations such as the US, infected women are still becoming pregnant and in most cases the women are already on some type of antiviral therapy. It is recommended that for women who are already on a specific therapy regimen that it not be changed unless there are known adverse effects on the mother or fetus and that these effects outweigh the risks of ending or modifying the therapy [27]. For many of the approved antivirals, very little is known about their behavior in a pregnant woman, especially when given in combination [28].

Placental transfer rate of drugs is based largely on molecular size, lipophilicity, extent of ionization and protein binding [29]. The transplacental transport of compounds occurs by passive diffusion, facilitated diffusion or active transport [30,31]. Physiological changes that occur during pregnancy may affect absorption, distribution, biotransformation and elimination and therefore, may affect dosing requirements and susceptibility of the mother to drug toxicity. Placental transport of drugs, compartmentalization of the drugs in the fetus and placenta as well as the kinetics of the drugs in these compartments can also affect the maternal pharmacokinetics [28]. For these reasons it is important to have an understanding of the behavior of administered antivirals during pregnancy.

Due to ethical and social concerns, pregnant women are generally excluded from clinical trials [32]. Although several other models have been developed to study the placental transfer and maternal-fetal pharmacokinetics of a variety of compounds, the pregnant rat model has proven to be ideal for the study of numerous compounds, including nucleoside analogs [33-42]. Rodents, like humans, develop a single discoid-shaped placenta per fetus. Humans and rodents also share the hemochorial feature of the placenta, in that the single barrier between the maternal and fetal systems is of fetal origin [30]. These physiological similarities, coupled with the short gestation period (approximately 21 days), large litter size (10-15 pups) and individual fetal sacs per pup (each containing a fetus, a placenta and amniotic fluid) makes the pregnant rat model ideal for studying placental transfer and maternal-fetal kinetics.

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# CHAPTER 1

# DETERMINATION OF 3'-AZIDO-2',3'-DIDEOXYURIDINE (AZDU) IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL AND PLACENTAL TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

1Clark, T. Nicole, Catherine A. White, Chung K. Chu, Michael G. Bartlett. 2001. *Journal of Chromatography B: Biomedical Applications*. 755 (1-2), 165-172. Reprinted here with permission of publisher.

**Abstract**

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

## Introduction

Since the onset of HIV/AIDS over 15 years ago, more than 47 million people worldwide have been infected. Over 2.2 million deaths due to the epidemic were reported in 1998, making HIV/AIDS the fourth leading cause of mortality globally [1]. The effects of AIDS among young children are serious and far-reaching. In the worst affected African countries it has, to date, doubled the infant mortality rate. Deaths among one to five year olds, the age group with the highest concentration of AIDS deaths, have risen even more sharply from 8 to 20 per 1000 patients. Mother-to-child (vertical) transmission is the largest source of HIV infection in children under the age of 15, accounting for 90% of the global infections in children [2,3]. The virus may be transmitted during pregnancy, childbirth or breastfeeding. In the absence of preventative measures, the risk of an infected mother transmitting the virus to her infant ranges from 15-35% [3]. It has been discovered that Zidovudine (AZT) monotherapy in the mother during pregnancy has caused a steep decline in perinatally acquired AIDS [4]. Such treatments have prevented approximately 67% of prenatal HIV infections, causing the transmission rate to fall to around 8.3% [5].

AZT therapy does, however, cause serious side effects such as gastrointestinal intolerance, bone marrow toxicity and myelosuppression [5,6]. Long term administration of AZT in AIDS patients has also led to the generation of AZT-resistant HIV-1 strains [7]. A search for new and less toxic anti-HIV agents has caused a great deal of interest to be focused on other 2',3'-dideoxynucleosides that have been reported to be effective in vitro [8-11]. Among compounds synthesized as analogs of AZT, AZDU has been shown to possess significant anti-HIV activity as well as reduced bone marrow toxicity [12-14].

The pharmacokinetics of AZDU and AZT are comparable at various doses, which could be expected due to the similarity of their chemical structures (*i.e.* – they differ only by a methyl group at the 5 position of the pyrimidine ring). Total renal clearance for both drugs becomes dose-dependent with intravenous administration of doses greater than 250 mg/kg [15,16].

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

Here we report a rapid and efficient HPLC method that has been developed and validated for the determination of AZDU concentrations in samples taken in a maternal-fetal drug transfer study of this potential anti-HIV agent. AZDU concentration levels were determined in maternal plasma, fetal, placental and amniotic fluid samples after

extraction. Pharmacokinetic analysis of plasma data generated from the analysis of these samples is consistent with previously reported literature data, proving this to be a reliable assay for the determination of AZDU concentrations in these biological matrices.

## Experimental

*Reagents and Chemicals*

3'-azido-2',3'-dideoxyuridine (AZDU, CS-87) was synthesized as previously described [29]. The internal standard, 3'-azido-3'-deoxythymidine (Zidovudine, AZT) was obtained from Glaxo-Wellcome (RTP, NC, USA). HPLC-grade acetonitrile, reagent grade acetic acid and sodium acetate trihydrate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sep-Pak Vac 1cc C-18 cartridges were purchased from Waters (Milford, MA, USA).

*Preparation of Stock and Standard Solutions*

Individual AZDU and AZT stock solutions were prepared in deionized water to give a concentration of 1.0 mg/ml. Individual standard solutions with concentrations of 500, 250, 50, 25, 5, 2.5 and 0.5 μg/ml were prepared by serial dilution with deionized water. Precision and accuracy standards with concentrations of 125 and 1.25 μg/ml were also prepared in the same manner. All stock and standard solutions were refrigerated at $4^{o}$C and replaced every two weeks.

*Chromatographic System*

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

*Chromatographic Conditions*

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

*Calibration Curves*

Blank placental and fetal tissue homogenates were prepared from untreated animals by homogenization with two volumes of deionized water (w/v) in a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). Calibration standards for all samples were prepared by spiking 100 μl of the tissue homogenate or biological fluid with 20 μl of

AZDU standard solution and 10 μl of 20 μg/ml AZT internal standard solution to obtain AZDU concentrations of 0.1-50 μg/ml and 2.0 μg/ml AZT concentration. The spiked fetal and placental standards were then extracted from the biological matrices. All standards were prepared on the day of analysis. The extraction procedure is described in the following section.

*Extraction Procedure*

In a 1.5 ml centrifuge tube 100 μl of the tissue homogenate, 10 μl of AZT internal standard (20 μg/ml) and 20 μl of an AZDU standard solution were combined and vortexed briefly. To precipitate proteins, 500 μl of ice cold acetonitrile was added to the tube. The tubes were vortexed for 30 seconds and centrifuged at 5000 rpm for 5 minutes in a microcentrifuge (Model 235 V, Fisher, Fairlawn, NJ, USA). The supernatant was evaporated at $40^0$C in a vacuum centrifuge (Model SC110A, Savant Instruments Inc., Holbrook, NY, USA). The residue was reconstituted in 500 μl of 0.2% acetonitrile in water. The samples were then loaded onto a Sep-Pak C-18 solid phase extraction cartridge preconditioned with 2 ml of acetonitrile followed by 2 ml of 0.2% acetonitrile in water. Samples were washed with 2 ml of the 0.2% acetonitrile in water solution. After discarding the eluent, the analytes were eluted with 2 ml of acetonitrile into clean culture tubes. Eluents from the cartridge were then dried at $40^o$C using the vacuum centrifuge and reconstituted in 150 μl of mobile phase. The samples were then transferred to injection vials where 50 μl of sample was injected onto the HPLC column.

In a 1.5 ml microcentrifuge tube 20 μl of an AZDU solution and 10 μl of the AZT internal standard were added to 100 μl of plasma or amniotic fluid. The tube was

vortexed briefly before the addition of 20 µl of 2M perchloric acid. The tubes were vortexed again for 30 seconds and then centrifuged at 9000 RPM for 10 minutes. The supernatants were then transferred to injection vials where 50 µl of sample was injected onto the HPLC column.

*Sampling*

Timed pregnant female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing an average of 325 g were used. On day 19 of gestation rats were anesthetized using a ketamine:acepromazine (75:2.5 mg/kg) solution injected intramuscularly. A cannula was placed in the right jugular vein and a laparotamy was performed to allow concurrent serial sampling of blood, fetus, placenta and amniotic fluid. The rats were administered an IV bolus dose (25 mg/kg) of AZDU dissolved in 0.1 N NaOH in physiological saline via the jugular cannula. Samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 minutes post-dose and stored on ice until processed. Blood samples were placed in heparinized microcentrifuge tubes. These tubes were centrifuged at 9000 rpm for 10 minutes and the plasma was transferred to clean dry tubes for storage. Fetal and placental samples were collected and homogenized in 2 volumes of deionized water. All samples were stored at –20$^{o}$C until analysis.

**Results and Discussion**

The chemical structures for AZDU and the internal standard AZT are shown in Fig. 1.1. Separation of AZDU and AZT from interfering matrix peaks was explored using various ratios of acetonitrile and the buffer. Baseline resolution was achieved at

4.5% acetonitrile in buffer for amniotic fluid, fetal and placental samples and at 5.5% acetonitrile for the plasma samples. Figure 1.2A-D shows chromatographs of spiked AZDU (1 μg/ml) with the internal standard, AZT.

The calibration curve showed a good linearity in the range of 0.1 to 100 μg/ml for plasma and in the range of 0.1 to 50 μg/ml for amniotic fluid and fetal and placental tissues. The range of concentrations encompasses the estimated range of post IV bolus dose (25 mg/kg) concentrations in the biological matrices.

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

To investigate the extraction efficiency of AZDU and AZT from the various biological matrices (plasma, amniotic fluid, fetal and placental tissues), standard-spiked matrix samples were subjected to extraction and then analyzed. The resulting peak areas were compared to peak areas of samples containing equal amounts of analyte in mobile phase. The recoveries were high and reproducible, ranging from 81 to 96% for AZDU and from 82 to 96% for AZT. The recoveries for both AZDU and AZT in the four individual matrices are shown in Table 1.1.

Intra-day (n=6) and inter-day (n=9) precision and accuracy were calculated from standard curves constructed from each of the four biological matrices studied. The intra-day (n=6) precision and accuracy for AZDU (spiked concentrations 0.25 and 25 μg/ml)

was in the range of 2.05-7.64% (RSD) and 1.73-10.2% (error), respectively. Inter-day (n=9) precision and accuracy for AZDU (unknown concentrations 0.25 and 25 μg/ml) ranged from 3.04-7.82% (RSD) and 2.22-9.18% (error), respectively. These intra- and inter-day precision and accuracy data are tabulated in Table 1.2.

Acquired plasma, amniotic fluid, fetal and placental tissue samples were extracted and analyzed as described above. Sample peak area ratios of AZDU and AZT were used to determine AZDU concentrations from the regression equation obtained from standard-spike samples prepared in blank fetus, placenta, plasma and amniotic fluid. Figure 1.3 shows the concentration vs. time curve of the AZDU in the pregnant rat in all four biological matrices. An AZDU half-life of 1.3 hours and steady state volume of distribution of 0.73 L/kg and total clearance of 0.32 L/hr·kg were calculated. All values were determined to be in agreement with literature values previously reported. [15,30]

**Conclusions**

A sensitive and efficient method for the extraction and analysis of AZDU in rat plasma, amniotic fluid, fetal and placental tissues has been developed and validated. This method yields high recoveries shows good linearity, precision and accuracy within the range of 0.1-100 μg/ml. The solid phase extraction, although not necessary for concentrating our samples, provides excellent sample cleanup. Due to usage of the entire fetus or placenta, very complex sample matrices, a sample cleanup step that would dramatically reduce possible interferences was needed. Combining acetonitrile protein precipitation with solid phase extraction afforded optimal tissue sample cleanup, allowing for a decrease in sample analysis time. The dose response results from the analysis of

collected samples were comparable to literature data, further validating the reliability of this particular method for the determination of AZDU concentrations in biological samples. This method allows for a pharmacokinetic investigation for the determination of the effectiveness of AZDU in the prevention of perinatal HIV transmission from a pregnant mother to her unborn child.

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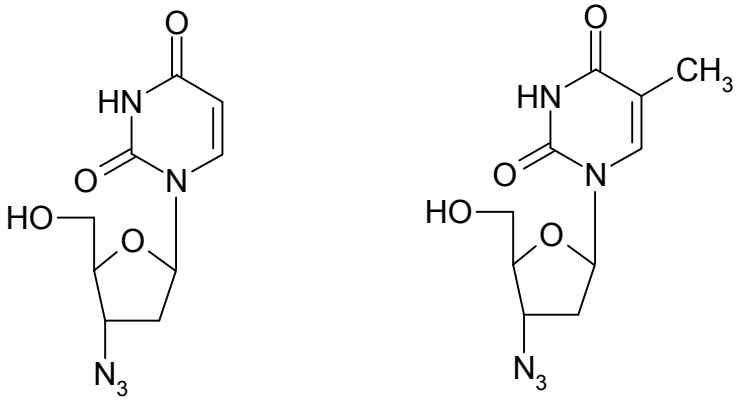
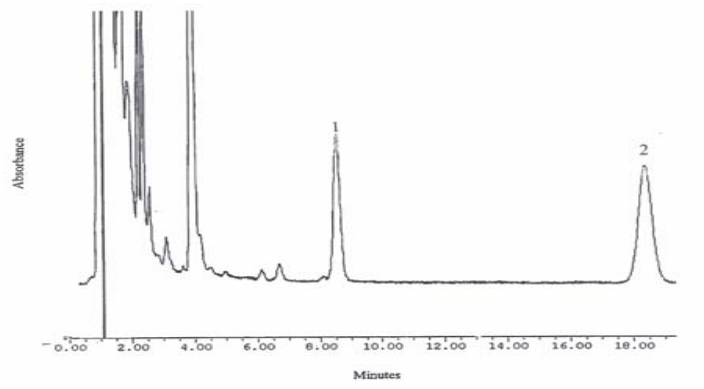
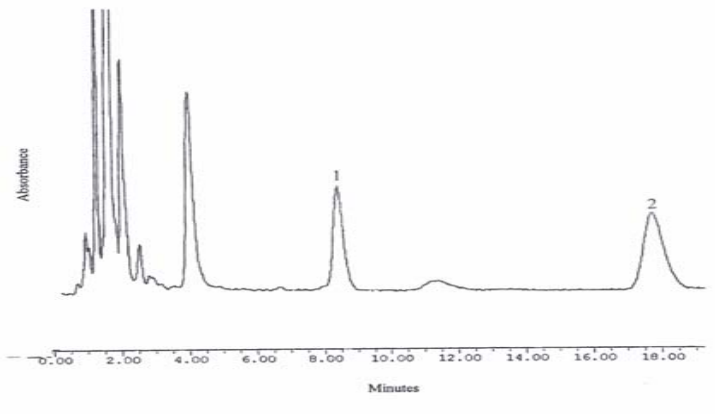



**Figure 1.1.** Structures of AZDU and AZT

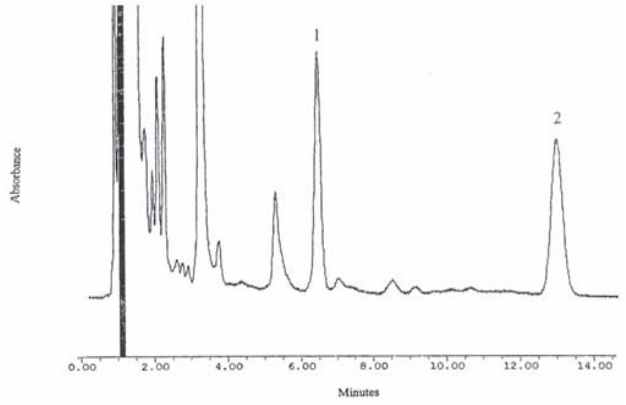
(A) Maternal Plasma




(B) Amniotic Fluid




(C) Placental Homogenate




(D) Fetal Homogenate

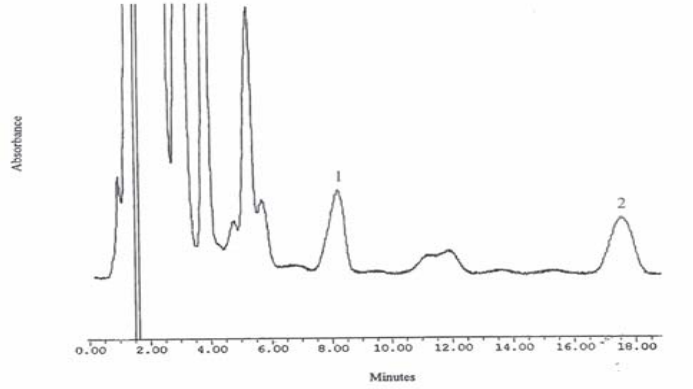



**Figure 1.2.** Chromatographs of (1) AZDU (1 μg/ml) and (2) AZT (2 μg/ml) spiked (A) maternal plasma, (B) amniotic fluid, (C) placental homogenate and (D) fetal homogenate samples

**Table 1.1.** The % recovery ± standard deviations (n=5) of AZDU and AZT from maternal plasma, amniotic fluid, placental homogenate and fetal homogenate

| Analyte | % Extraction Efficiency (±SD) | | | |
|---|---|---|---|---|
| | Maternal Plasma | Amniotic Fluid | Placental Homogenate | Fetal Homogenate |
| AZDU | 96.47 ± 1.49 | 95.26 ± 2.03 | 81.16 ± 2.79 | 85.42 ± 5.78 |
| AZT | 95.87 ± 3.19 | 88.38 ± 2.07 | 92.16 ± 3.38 | 81.99 ± 4.08 |

**Table 1.2.** The intra- and inter-day precision (% RSD) and accuracy (% Error) of AZDU in maternal plasma, amniotic fluid, placental homogenate and fetal homogenate

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

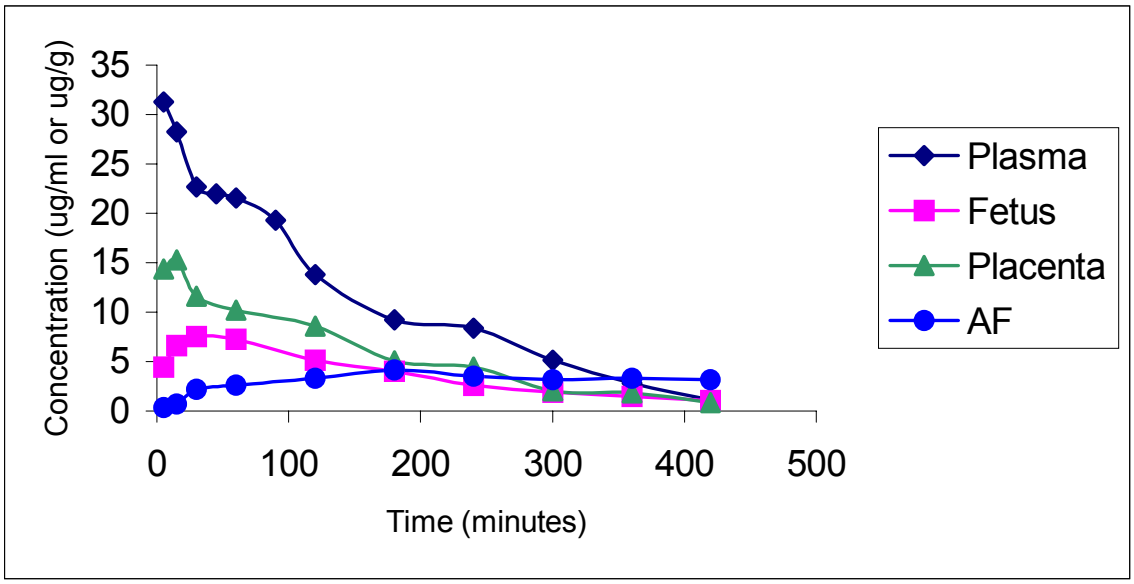



**Figure 1.3.** Concentration versus time curve of AZDU in maternal plasma, amniotic fluid, placental homogenate and fetal homogenate

# CHAPTER 2

# COMPARATIVE PHARMACOKINETICS OF ZIDOVUDINE AND AZIDOURIDINE IN THE PREGNANT RAT[1]

[1]Clark, T. Nicole, Michael G. Bartlett, Chung K. Chu, A. Timothy Eley, Catherine A. White. To be submitted to *Biopharmaceutics and Drug Disposition.*

**Abstract**

The pharmacokinetics and placental transport of zidovudine (3'-azido-3'-deoxythymidine, AZT) and azidouridine (3'-Azido-2',3'-dideoxyuridine, AZDU), nucleoside analogues that have been shown to inhibit human immunodeficiency virus (HIV-1), were studied in the pregnant rat following IV administration. Despite the widespread use of antiviral agents during pregnancy, very little is known about fetal exposure and *in vivo* placental transport mechanisms. AZT and AZDU were administered IV bolus to female 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 eight hours post-dose. Concentrations in each matrix were determined by HPLC-UV. All data was analyzed using WinNonlin®. Both AZT and AZDU demonstrate first-order kinetics and are characterized by a maternal plasma half-life of 0.95 and 1.12 hours, a volume of distribution of 0.84 and 0.82 L/kg and clearance values of 0.63 and 0.56 L/hr/kg, respectively. The determined pharmacokinetic parameters for AZDU are comparable to those for AZT except for relative fetal exposures. An infusion study determined that placental uptake of AZDU occurs mainly by passive diffusion, while fetal uptake occurs by passive diffusion as well as a saturable carrier mediated process.

KEYWORDS: AZIDOURIDINE, ZIDOVUDINE, PHARMACOKINETICS, PLACENTAL TRANSPORT, PREGNANT RATS

## Introduction

Although the human immunodeficiency virus (HIV), the virus that causes AIDS, may have been first introduced to humans from infected animals in the early parts of the twentieth century, it did not successfully spread into the global human population until the 1970's. In industrialized nations, the AIDS epidemic was first manifested in among groups of people with a common exposure risk. In the United States, homosexual men were among the first to demonstrate HIV infection. This group was followed by individuals receiving infected blood or blood products, injection drug users, and eventually children of infected mothers.[1] Infection rates in women represent an increasing number of reported cases in the US, accounting for almost 25% of adult cases reported between July 1998 and June 1999.[2] This makes the potential transmission of HIV from an infected woman to her unborn/newborn child a real issue of concern.

A French study conducted in the late 1980's determined that perinatal transmission of HIV can occur in utero, during labor and delivery or post-partum through breastmilk.[3] Vertical transmission rates average between 25 and 30%,[4] but are affected by the stage of HIV infection in the mother, the use of an antiviral therapy regimen by the mother before and during pregnancy, the length of the labor and delivery process, the decision to or not to breastfeed, among others.[2]

In early 1994, a pediatric clinical trial group (PACTG 076) investigated the ability of the reverse transcriptast inhibitor zidovudine (AZT) to reduce the rate of HIV transmission from a pregnant woman to her child. The regimen consisted of initiation of oral zidovudine between week 14 and 34 of gestation which was continued through the remainder of the pregnancy, intravenous administration of zidovudine throughout the

labor and delivery process and oral administration of zidovudine to the newborn for the first six weeks post-partum. This particular therapy regimen was able to reduce the risk of transmission by 70%.[5]

Serious side effects such as gastrointestinal disruption, bone marrow toxicity and myelosuppression are associated with long-term zidovudine therapy.[6,7] Because AZT has been used for almost 20 years, a number of drug-resistant strains of HIV have arisen.[8] For these and other reasons, the search for new, less toxic anti-HIV agents has focused on other nucleoside analogs that have been shown to possess antiviral activity in vitro.[9-12] Among these compounds, azidouridine, a structural analog of zidovudine, has demonstrated significant anti-HIV activity as well as a decrease in bone marrow toxicity.[13-15] The pharmacokinetics of AZDU and AZT were explored at various doses in mice. As expected, the behavior of these two drugs that differ only by the presence/absence of a methyl group on the pyrimidine ring is similar at all doses explored.[16]

Although a number of groups have investigated the placental transfer of AZT using animal or in vitro models,[17-21] no maternal-fetal pharmacokinetic studies have been conducted in order to determine the behavior of AZDU during pregnancy or to explore its potential use to prevent the vertical transmission of HIV from an infected mother to her child.

Pregnant women generally do not participate in clinical trials, making it difficult to obtain data on drug behavior in the mother during pregnancy and placental and fetal drug distributions. Therefore, an animal model must be utilized that will provide clinically useful mechanistic information. Huang et. al. have developed a pregnant rat

model that provides clinically useful mechanistic information the investigation of the basic mechanisms involved in the placental transfer of nucleoside analogs.[18] The presence of a hemochorial placenta and similarities in physiological changes experienced during pregnancy by both rats and humans make a pregnant rat a suitable model for investigating drug behavior during pregnancy.[19,20] The large litter size (10-15 pups) and presence of individual fetal sacs containing a fetus, placenta and amniotic fluid that allow for serial sampling, make it useful for pharmacokinetic studies. The pregnant rat model has been used for the investigation of maternal-fetal drug transfer studies of a variety of compounds, including antivirals.[18,21-29] Here we report the results of a comparative study of the pharmacokinetics of azidouridine and zidovudine in the pregnant rat following intravenous administration of a 25 mg/kg dose.

## MATERIALS AND METHODS

### Chemicals

Azidouridine (3'-azido-2',3'-dideoxyuridine,AZDU) was synthesized as previously described.[30] Zidovudine (3'-azido-3'-deoxythymidine, AZT) was obtained from Glaxo-Wellcome (RTP, NC, USA). The structures of the AZDU and AZT are shown in Figure 2.1. HPLC-grade acetonitrile, reagent grade acetic acid and sodium acetate trihydrate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Sep-Pak Vac 1cc $C_{18}$ cartridges were purchased from Waters Corp. (Milford, MA,USA).

**Animals**

Timed pregnant female Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing an average of 325 g were used throughout the study. All surgical procedures and sampling were performed on day 19 of gestation. The gestation period for the rat is approximately 21 days, so in order to mimic the late-term administration of antivirals for the prevention of vertical-HIV transmission, a late gestation day was chosen for our model. Animal studies received prior approval from the University of Georgia Animal Care and Use Committee and 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 and had free access to food and water.

**Pharmacokinetic Studies**

On day 19 of gestation rats were anesthetized using a ketamine:acepromazine (75:2.5 mg/kg) solution injected intramuscularly. At the same time, the rats received a 0.5 mg/kg dose of atropine in order to prevent pulmonary edema. A cannula was surgically placed in the right jugular vein and a laparotamy was performed to allow concurrent serial sampling of blood, fetus, placenta and amniotic fluid. Animals were maintained under anesthesia by periodic administration of the ketamine:acepromazine solution IM. Body temperatures were monitored and held constant at 37$^{o}$C using a heated surgical pad (Braintree Scientific, Braintree, MA, USA).

IV Bolus Study

The rats were administered an IV bolus dose at 25 mg/kg of AZDU or AZT dissolved in 0.1 N NaOH in physiological saline (pH 7.4) via the jugular cannula followed by 1 ml of physiological saline (pH 7.4) to rinse the cannula. Samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 minutes post-dose and stored on ice until processed. Blood samples of approximately 200-300 μl were collected via the jugular vein cannula and placed in heparinized microcentrifuge tubes. These tubes were centrifuged at 9000 rpm for 10 minutes (Model 235V, Fisher Scientific, Pittsburgh, PA, USA) and the plasma was transferred to clean dry labeled tubes for storage. Amniotic fluid samples were 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 (w/v) of deionized water using a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). Urine was collected throughout the sampling process and the pooled sample volume was recorded. All samples were stored at –20$^{o}$C until analysis.

IV Infusion Study

Following administration of anesthesia and atropine, both the jugular and femoral veins were cannulated and a laparotamy was performed to allow for sampling of fetus, placenta and amniotic fluid. Animals were maintained under anesthesia by periodic administration of the ketamine:acepromazine solution. Body temperatures were

monitored and held constant at 37°C using a heated surgical pad (Braintree Scientific, Braintree, MA, USA).

Each rat was infused to 4 steady state concentrations by administration of a loading dose and an infusion of AZDU in saline at 20 μl/min for 45 minutes via the jugular cannula. Once steady state was reached, blood, fetus, placenta and amniotic fluid samples were obtained via the femoral vein and laporatomy. In order to insure that ample time had passed to allow steady state to be reached, two blood sample and three fetal, placental, and amniotic fluid samples were collected at each timepoint. $C_{ss,tissue}/C_{ss,plasma}$ were determined at each steady state concentration range of 3 to 48 mg/kg to assess placental and fetal uptake of AZDU.

**Analytical Method**

Plasma and amniotic fluid samples were protein precipitated by adding 20 μl of 2M perchloric acid to 100 μl of plasma or amniotic fluid. Following centrifugation, the supernatant was injected onto the HPLC. Fetal and placental tissue homogenates were first protein precipitated by adding 500 μl of ice cold acetonitrile to 100 μl of homogenate. The supernatant was evaporated and reconstituted in 500 μl of 0.2% acetonitrile in water. The reconstitute was then subjected to solid phase extraction using Waters Sep-Pak Vac $C_{18}$ SPE cartridges. The SPE method involves conditioning the cartridge with 2 ml each of acetonitrile and 0.2% acetonitrile in water. The samples were loaded and then washed with 2 ml of 0.2% acetonitrile in water. The analytes were eluted with 2 ml of acetonitrile. The eluents were evaporated, reconstituted in mobile phase and injected onto the HPLC. The internal standard, AZT for analysis of AZDU

and AZDU for the analysis of AZT, was also spiked into each sample to yield a final concentration of 2 μg/ml. Calibration curves were generated using samples from spiked blank matrix to yield final concentrations of 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml for plasma and 0.1, 0.5, 1, 5, 10 and 50 μg/ml for amniotic fluid, placental and fetal tissue homogenates.

The chromatographic system consisted of a Waters (Milford, MA, USA) model 510 pump, model 717+ autosampler and a model 486 variable wavelength UV detector. Chromatographic separations were achieved using a Hypersil ODS analytical column (150 x 4.6 mm, 5 μm, Alltech, Deerfield, IL, USA) equipped with a Nova-Pak $C_{18}$ guard column (Waters, Milford, MA,USA).

The mobile phase for the fetus, placenta and amniotic fluid consisted of 40 mM sodium acetate (pH 7):acetonitrile (94.5:5.5 v/v) run isocratically at 2 ml/min with the detector wavelength set at 263 nm. Under these conditions, AZDU eluted at approximately 6.2 minutes and AZT eluted at approximately 12.2 minutes. The mobile phase used for the plasma had to be adjusted slightly to resolve the AZDU from a co-eluting endogenous compound. The plasma mobile phase consisted of 40 mM sodium acetate (pH 7):acetonitrile (95.5:4.5 v/v) run isocratically under the same conditions reported above. Under these conditions, AZDU eluted at approximately 8.3 minutes and AZT eluted at approximately 17.6 minutes. Both methods for determining AZDU and AZT concentrations have been previously validated to show acceptable precision and accuracy for the quantitation of these analytes in the specified ranges.[31,32]

**Data Analysis**

Maternal plasma pharmacokinetic parameters were calculated from concentration-time data using WinNonlin®. Initial parameter estimates were generated through noncompartmental analysis with an IV bolus input model. Using the generated estimates, further compartmental analysis was performed with a 1-compartment IV bolus model with first order elimination. Placental, fetal and amniotic fluid data were subjected to noncompartmental analysis only, using a noninstantaneous input model. The pharmacokinetic parameters generated for each dosing group and the relative exposure numbers were compared using Student's t-test ($\alpha < 0.05$) to detect statistically significant differences. All statistical calculations were performed using Microsoft Excel.

**RESULTS**

AZDU and AZT concentration-time profiles in maternal plasma, placenta, fetus and amniotic fluid after 25 mg/kg IV bolus dose administrations are shown in Figure 2.2. AZT rapidly distributed into the placental and fetal compartments with maximum concentrations being observed within one hour of intravenous administration. Placental and fetal concentrations were consistently lower than maternal plasma levels yielding peak concentrations 4- and 5-fold lower in the placenta and fetus, respectively. The plasma, placental and fetal concentration-time profiles of AZT had equivalent terminal half-lives. This indicates that AZT did not accumulate in these tissues and rapidly reached equilibrium with the plasma compartment. Amniotic fluid peak concentrations were achieved at 120 minutes. Unlike the placental and fetal AZT levels, which steadily declined with the same half life as plasma once the maximum concentration was reached,

the amniotic fluid AZT half-life increased by approximately 4-fold as compared to the plasma half-life.

AZDU was also rapidly distributed into the placental and fetal compartments with maximum concentrations being observed within one hour of intravenous administration. Placental and fetal concentrations were consistently lower than maternal plasma levels with peak concentrations 2- and 7-fold lower in the placenta and fetus, respectively. The plasma, placental and fetal concentration-time profiles of AZDU had equivalent terminal half-lives. As with AZT, AZDU did not accumulate in these tissues and rapidly reached equilibrium with the plasma compartment. Amniotic fluid peak concentrations were achieved on the average at approximately 120 minutes. Unlike the placental and fetal AZDU levels, which steadily declined once the maximum concentration was reached, the amniotic fluid concentrations remained relatively steady for 4 hours and then gradually declined with an apparent elimination half-life of 6 hours.

A comparison of the average AZT and AZDU maternal pharmacokinetic parameters is shown in Table 2.1. No significant differences were noted between the plasma pharmacokinetics of AZT and AZDU. However, AZDU concentrations in the maternal plasma were consistently higher in the elimination phase. A comparison of the placental, fetal and amniotic fluid pharmacokinetic parameters is shown in Table 2.2. Again, there is very little difference in the behavior of these to drugs in these compartments. The AZDU placenta $C_{max}$ value is the only parameter that shows any statistical difference from AZT, however the fetal levels were 16% lower with AZDU. The relative exposures, expressed as $AUC_{tissue}/AUC_{plasma}$, for the fetal and placental tissues are shown in Table 2.3. The placental ($0.42 \pm 0.04$) and fetal ($0.50 \pm 0.20$)

relative exposure values are almost identical for AZT. Significant differences are observed between the placental and fetal exposure of AZDU. The placental exposure ($0.63 \pm 0.2$) is twice that observed for the fetus ($0.30 \pm 0.1$). This suggests that AZDU uptake into the fetal compartment is not solely by passive diffusion.

The infusion study with AZDU over a 12-fold concentration range was employed to determine if active/facilitated transport is responsible for transfer of drug into the placenta and fetus. Table 2.4 shows the concentrations for each matrix at the respective steady-state concentrations in a single rat. The values were similar for all other rats dosed. Figure 2.3 shows normalized plots of fetal and placental concentrations versus maternal plasma concentration values. Placental uptake of AZDU was independent of steady state concentration indicating no saturation of uptake at the concentration range studied. The normalized fetal data was fit to a Michaelis-Menton model and yielded $V_{max}$ and $k_m$ values of 0.263 and 6.901 mg/L, respectively. Both values exhibited low coefficient of variation values of 5.71% for $V_{max}$ and 18.77% for $k_m$. Fetal uptake of AZDU was saturated at higher concentrations suggesting a carrier mediated process was involved in the transport of drug from the placenta to the fetus.

**DISCUSSION**

The pharmacokinetic parameters generated for both AZDU and AZT from maternal plasma are similar to previously reported values for AZT in the rat.[31] Therefore, the behavior of the drugs in the maternal compartment is not changed by the pregnancy status of the female rat. The similarity in the parameters between the two compounds is

to be expected in that the two drugs differ only by the absence of a methyl group in the AZDU molecule.

From the concentration-time profiles, AZT and AZDU levels in amniotic fluid compartment were shown to peak after approximately two hours and remain constant until after 4 hours post-dose. These observations suggest that the drug is accumulating in this compartment and is therefore not in equilibrium with the central plasma compartment. This is in contrast to the placental and fetal profiles which indicate these tissues are in rapid equilibrium with the maternal plasma.

There is a major difference in the fetal uptake of the two drugs. Previous studies on the maternal-fetal pharmacokinetics of AZT with a 50 mg/kg IV bolus dose administration using the same pregnant rat model suggests that passive diffusion is solely responsible for the transport of AZT across the placenta into the fetal compartment.[19] This is also demonstrated in our study with a 25 mg/kg dose of AZT. With AZDU, we are seeing that the relative fetal exposure is significantly less than that of AZT. The relative exposure of the placental tissues with AZDU is approximately twice as much as that of the fetus after IV bolus administration. Coupled with the data obtained from the infusion studies, this leads us to conclude that uptake of AZDU into the placenta occurs mainly by passive diffusion at the concentrations studied. However, transport into the fetus appears to occur by both passive diffusion and a carrier mediated process(es). This conclusion is supported by the data showing the fetal concentrations leveling off at higher maternal plasma concentrations, suggesting that fetal uptake is saturable at these higher AZDU levels. Previous studies on maternal-fetal pharmacokinetics of AZT and acyclovir, a common anti-herpes simplex virus drug, administered in combination using

this model also revealed the potential for facilitated transport of antivirals across the placenta.[22] The presence of transporters for nucleosides in the placenta has been documented,[36] solidifying these conclusions.

Further studies are needed in order to determine the exact transporter mechanism(s) involved in the movement of AZDU from the placenta to the fetus. The clinical impact of active transport will only be significant when using multidrug therapy in which there could be an impact on maternal plasma and fetal pharmacokinetics and where competition for transporters is present.

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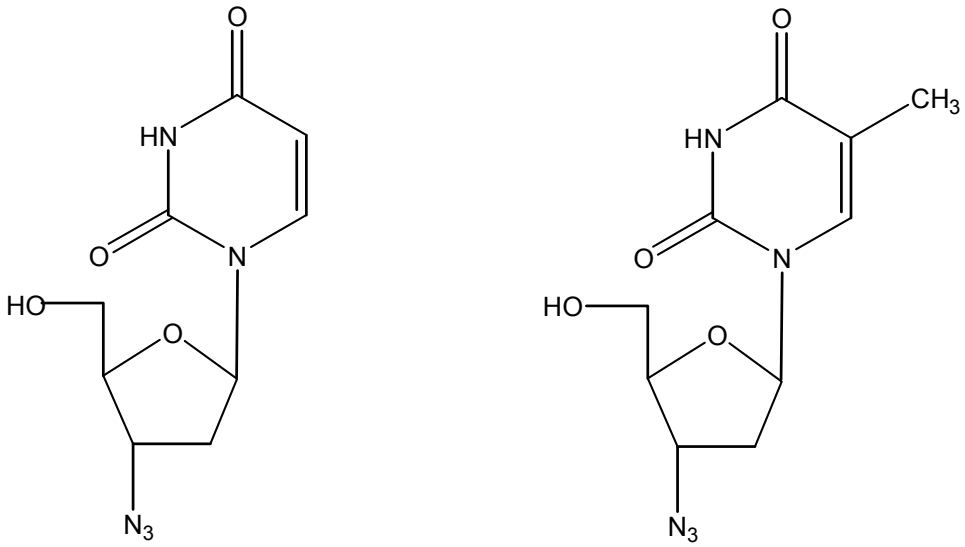



Azidouridine Zidovudine

**Figure 2.1. Structures of Azidouridine and Zidovudine**

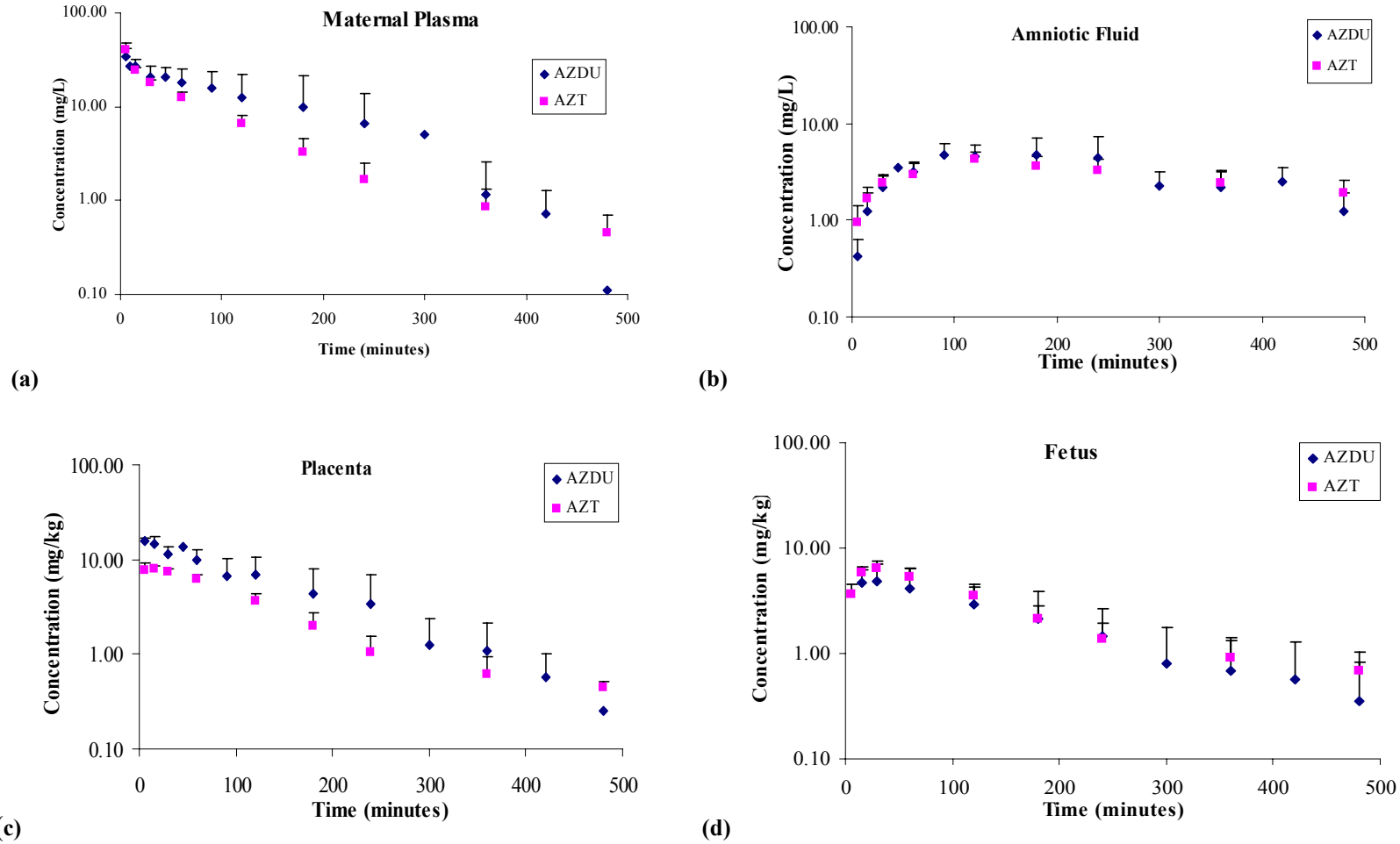



**Figure 2.2. Concentration vs. Time Profiles of 25 mg/kg Doses of AZDU and AZT Following IV Bolus Administration in (a) Maternal Plasma, (b) Amniotic Fluid, (c) Placenta and (d) Fetus**

**Table 2.1. Comparison of Maternal Pharmacokinetic Parameters (mean ± standard deviation)**

| Parameter | AZT | AZDU |
|---|---|---|
| Half-life (hr) | 1.0 ± 0.1 | 1.0 ± 0.1 |
| $V_{ss}$ (L/kg) | 0.8 ± 0.1 | 0.8 ± 0.02 |
| $Cl_T$ (L/hr/kg) | 0.6 ± 0.1 | 0.6 ± 0.3 |
| AUC (hr·mg/L) | 43.8 ± 8.1 | 48.9 ± 23.1 |

**Table 2.2. Comparison of Placental, Fetal and Amniotic Fluid Pharmacokinetic Parameters (mean ± standard deviation)**

| | Placenta | | Fetus | | Amniotic Fluid | |
|---|---|---|---|---|---|---|
| **Parameter** | **AZT** | **AZDU** | **AZT** | **AZDU** | **AZT** | **AZDU** |
| Half-life (hr) | 1.3 ± 0.4 | 1.1 ± 0.5 | 1.9 ± 0.7 | 1.6 ± 0.6 | 4.1 ± 0.7 | 3.5 ± 4.8 |
| AUC* | 18.6 ± 3.9 | 28.9 ± 10.6 | 22.3 ± 8.8 | 15.6 ± 9.7 | 32.0 ± 4.7 | 43.1 ± 28.8 |
| $C_{max}$** | 8.4 ± 0.9† | 17.1 ± 2.5† | 6.3 ± 1.1 | 5.4 ± 1.9 | 4.3 ± 0.7 | 5.4 ± 0.9 |
| $T_{max}$ (hr) | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 2.1 ± 0.7 | 2.2 ± 0.8 |

***** Expressed as hr·mg/kg for placenta and fetus and as hr·mg/L for amniotic fluid
****** Expressed as mg/kg for placenta and fetus and as mg/L for amniotic fluid
†Statistically significant differences at $p<0.05$

**Table 2.3. Relative Fetal and Placental AZT and AZDU Exposure Following IV Bolus Dosing (mean ± standard deviation)**

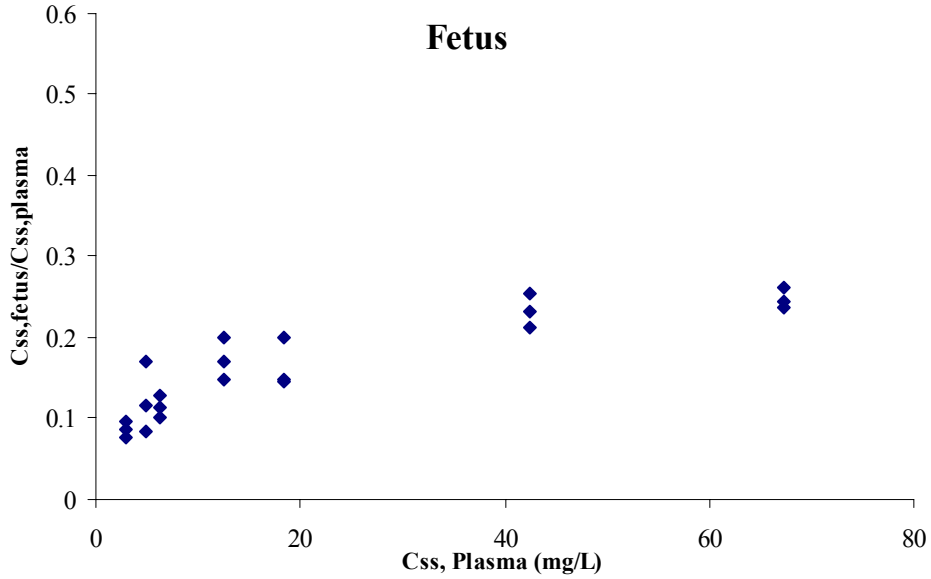
| Tissue | AZT Relative Exposure* | AZDU Relative Exposure* |
|---|---|---|
| Fetus | $0.50 \pm 0.2^{\dagger}$ | $0.30 \pm 0.1^{\dagger}$ |
| Placenta | $0.42 \pm 0.04$ | $0.63 \pm 0.2$ |

*Relative Exposure = $AUC_{Tissue}/AUC_{Plasma}$
†Statistically significant differences at $p<0.05$

**Table 2.4. Steady-State Maternal Plasma, Placental, Fetal and Amniotic Fluid Concentrations Following IV Infusion with Loading Dose**

| Estimated Plasma Steady-State Concentration (mg/L) | Measured Plasma Steady-State Concentration (mg/L) | Measured Placenta Steady-State Concentration (mg/kg) | Measured Fetus Steady-State Concentration (mg/kg) | Measured Amniotic Fluid Steady-State Concentration (mg/L) |
|---|---|---|---|---|
| 3.0 | 3.19 | 0.98 ± 0.08 | 0.26 ± 0.03 | 0.19 ± 0.17 |
| | 2.72 | | | |
| 6.0 | 6.40 | 2.49 ± 0.17 | 0.72 ± 0.08 | 1.49 ± 0.23 |
| | 6.21 | | | |
| 18.0 | 19.39 | 8.53 ± 1.58 | 3.05 ± 0.56 | 3.71 ± 1.57 |
| | 17.55 | | | |
| 48.0 | 73.33 | 26.95 ± 1.83 | 16.71 ± 0.8 | 14.87 ± 3.95 |
| | 61.42 | | | |

(a)




(b)

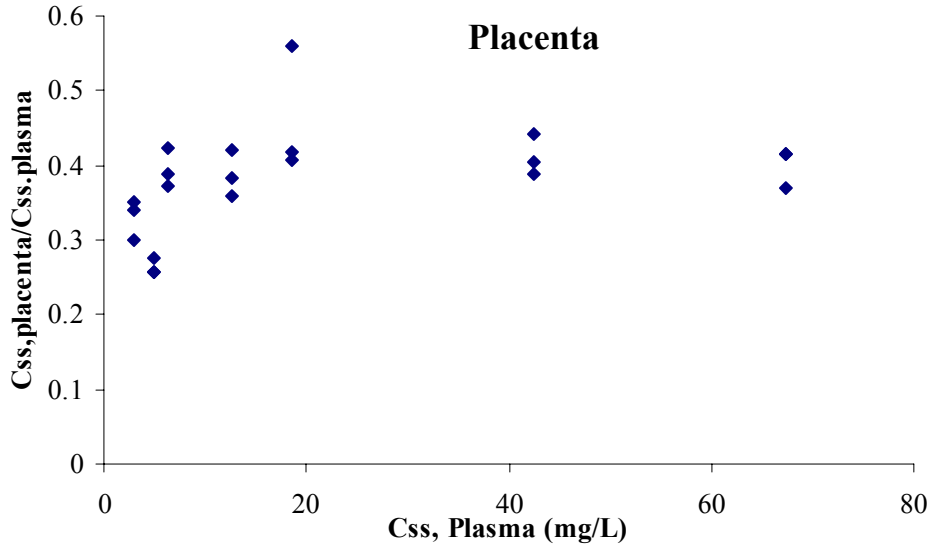



**Figure 2.3. Normalized Plots of (a) Fetal and (b) Placental vs. Maternal Plasma Steady State Concentrations of AZDU Following IV Infusion**

# CHAPTER 3

# DETERMINATION OF ABACAVIR IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL AND PLACENTAL TISSUES BY A POLARITY SWITCHING LC/MS/MS METHOD[1]

[1]Clark, T. Nicole, Catherine A. White, Michael G. Bartlett. Submitted to *Rapid Communications in Mass Spectrometry.* 07/03

**Abstract**

A rapid and efficient high performance liquid chromatography (HPLC)-tandem mass spectrometry method for the determination of the carbocyclic nucleoside antiviral abacavir concentrations in maternal rat plasma, amniotic fluid, placental and fetal tissue samples has been developed and validated. All tissue samples were homogenized in water prior to analysis and all samples were prepared by protein precipitation with acetonitrile followed by dilution with HPLC grade water. An Agilent 1100 Series HPLC coupled to a Micromass Quattro-LC mass spectrometer was used for all analyses. Separation of the analyte and internal standard from the matrices was achieved on a Zorbax Eclipse XDB $C_8$ analytical column (2.1 x 150 mm, 5 μm particle size) equipped with a Phenomenex Security-guard $C_{18}$ (4.0 x 2.0 mm) guard cartridge. The mobile phase consisted of 10 mM ammonium acetate:acetonitrile using a gradient method at a flow rate of 0.25 ml/min for all matrices. The method yields retention times of approximately 3.4 and 5.1 minutes for the internal standard Azidouridine and Abacavir. For all matrices the limit of detection was approximately 1 ng/ml. Recoveries ranged from 53-87% for abacavir and from 69-84% for azidouridine in the different matrices. Within- and between-run precision (%RSD) and accuracy (%Error) were under 15% for all matrices.

KEYWORDS: ABACAVIR, AZIDOURIDINE, POLARITY SWITCH, QUANTITATION, LC/MS/MS

## Introduction

Since the onset of HIV/AIDS in the early 1980's, there has been an endless search for more effective treatments for this deadly virus. Although the virus first appeared in the homosexual community, it rapidly spread into all populations.[1] Eventually, the vertical transmission of HIV from a pregnant woman to her unborn/newborn child became a serious issue. A landmark clinical study in 1994 demonstrated the ability of a shortcourse zidovudine (AZT) therapy regimen to drastically reduce the maternal-fetal transmission of HIV in infected pregnant women.[2] Once AZT gained approval for use in pregnancy, the vertical transmission rate among infected mothers dropped to approximately 8%.[3]

The decision to administer a therapeutic regimen to a pregnant woman is largely based on the therapeutic benefit of the regimen to the mother and fetus versus the perceived risk to the fetus.[4] Pregnancy should not prevent the administration of optimal therapeutic regimens. Treatment recommendations for HIV-infected pregnant women are based on the belief that therapeutic regimens known to benefit the woman should not be withheld during pregnancy unless there are known adverse effects on the mother, fetus or infant. These effects must outweigh the benefits of the therapy to the woman.[5] However, there are several factors that must be taken into consideration when determining the optimal therapeutic regimen. These include – 1) potential dose changes due to physiological changes associated with pregnancy, 2) potential effects of the antiviral drugs on the pregnant woman and 3) potential short- and long-term effects of the antiviral drugs on fetus and newborn. These issues have not been addressed for many of the antivirals.[6]

Abacavir (ABC) is a second-generation reverse transcriptase inhibitor that received FDA approval in December 1998 and was first administered in 1999.[7] It is currently marketed alone as Ziagen® and in combination with two other reverse transcriptase inhibitors, zidovudine (Retrovir®) and lamivudine (Epivir®), as Trizivir® by GlaxoSmithKline. Unlike previous RTI's, abacavir is a carbocyclic nucleoside analog. The absence of the glycosidic linkage on the sugar of ABC means it is not subject to the phosphorylases that cleave N-glycoside linkages, in turn making it more chemically stable.[8-10]

Very little is known about the behavior of abacavir during pregnancy. Due to ethical concerns, pregnant women generally do not participate in clinical trials.[11] This, coupled with the difficulty in obtaining fetal concentration data, makes it difficult to study placental and fetal drug distributions in humans. In order to gain insight into the behavior of drugs during pregnancy, an animal model must be utilized that will provide clinically useful mechanistic information. A pregnant rat model has been developed for the investigation of the basic mechanisms involved in the placental transfer of nucleoside analogs.[12,13] This rat model proves to be useful due to physiological similarities of pregnancy in both rats and humans.[14,15] The large litter size allows for serial maternal blood, placental, fetal and amniotic fluid sampling, making it even more useful for pharmacokinetic studies. The pregnant rat model has been utilized in maternal-fetal drug transfer studies of a variety of compounds.[12,13,15-21]

Here we present an analytical method that will be used in support of a placental transport/fetal exposure study of this second generation antiviral. Abacavir concentration levels were determined in maternal plasma, fetal, placental and amniotic fluid samples

after protein precipitation. Following preparation, samples are then analyzed using a polarity switching LC/MS/MS method. Polarity switching methods are not uncommon when analyzing for multiple compounds. Generally there are two or more analytes of interest with a stable-labeled internal standard available for each. Therefore even though the polarities are switched during the run, the analyte and its internal standard are run in the same "mode". The polarity switching technique presented here involves the analysis of the internal standard, azidouridine (AZDU), in the negative mode and the analyte, ABC, in the positive mode. We find that although the compounds are run under different polarities, the method is highly reproducible and efficient.

## EXPERIMENTAL

### Reagents and Chemicals

Abacavir (ABC) was obtained from GlaxoSmithKline Pharmaceuticals,Inc. (Barnard Castle, Co. Durham, UK) and the internal standard, azidouridine (AZDU) was purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile and optima water was purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA).

### Instrumentation and Supplies

All 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 Z-Spray® atmospheric pressure electrospray ionization (ESI) source (Waters-Micromass,

Beverly, MA, USA). Chromatographic separations were achieved on a Zorbax Eclipse XDB $C_8$ analytical column (5 μm, 2.1 x 150 mm, Agilent, Wilmington, DE, USA) equipped with a Security-guard $C_{18}$ guard column (2.0 x 4.0 mm, Phenomenex, Torrance, CA, USA). Tissues were homogenized using a Polytron PCU-2 tissue grinder (Kinematica GmbH, Luzern, Switzerland). All sample centrifugation was conducted in a Biofuge Pico microcentrifuge (Heraeus Instruments, Hanau, Germany). All rats used were purchased from Charles River Laboratories (Wilmington, MA, USA).

**Method Development**

The chemical structures for abacavir and the internal standard azidouridine are shown in Figure 3.1. Full scan mass spectra were obtained operating in positive mode for abacavir and negative mode for azidouridine. Abacavir showed the protonated molecule, $[M+H]^+$, at *m/z* 287 and azidouridine showed the deprotonated molecule, $[M-H]^-$, at *m/z* 252. Product ion spectra (Figure 3.2) were obtained for both compounds with the most abundant ions being the protonated base, *m/z* 191, for abacavir and the neutral loss of 43 Da, *m/z* 209, for azidouridine. The product ion of abacavir is a result of the loss of the sugar moiety to give the purine base. The product ion of azidouridine is a result of the breakdown of the pyrimidine ring yielding the loss of HCNO as previously described.[22] As a result of this experiment, the mass spectrometer was set up in multiple-reaction monitoring (MRM) mode to monitor the transitions from the precursor ions (*m/z* 287 for ABC and *m/z* 252 for AZDU) to the product ions (*m/z* 191 for ABC and *m/z* 209 for AZDU). Blanks of each matrix were examined under these conditions and no peaks from endogenous compounds were detected in either transition.

Due to the fact that the two analytes were being monitored in two different polarities, it was necessary to achieve baseline resolution of at least 30 seconds to allow for polarity switching. Several analytical columns were investigated for the separation of ABC and AZDU including $C_8$ and $C_{18}$ stationary phases in a variety of different column lengths and particle sizes. Due to the differences in the pKa values of the two analytes, it was necessary to maintain a fairly neutral pH. Therefore, HPLC grade water and buffers with ranges falling around pH 7 were evaluated. Various levels of organic modifiers (acetonitrile and methanol) were investigated for aiding in the resolution of the two compounds while still maintaining a reasonable run time.

A previously reported method for the determination of abacavir in human plasma used perchloric acid precipitation for sample pretreatment.[23] Under the chromatographic conditions necessary for the separation of our two analytes, this sample preparation method did not prove useful and further precipitation techniques were evaluated for sample pretreatment. Protein precipitation with ice cold acetonitrile followed by dilution with water to match initial mobile phase conditions was chosen. Due to the fact that we did not need to quantitate at extremely low levels, a dilution step allowed us to easily see our limit of quantitation (LOQ).

**Sample Preparation**

Calibration curves were prepared by spiking blank plasma, amniotic fluid and tissues obtained from a non-dosed pregnant rat. Blank placental and fetal tissue homogenates were prepared from untreated animals by homogenization with two volumes of optima water (v/w). All samples were prepared in the following manner.

In a 0.65 ml centrifuge tube 40 µl of plasma or tissue homogenate, 10 µl of AZDU internal standard (50 µg/ml) and 10 µl of a ABC standard solution were combined and vortexed. 150 µl of ice cold acetonitrile was added and the samples were vortexed for 30 seconds. The samples were then centrifuged at 13 000 rpm for 5 minutes. 150 µl of the supernatant was transferred to a clean 1.5 ml tube and diluted to 1 ml with 850 µl of optima water. The samples were vortexed mixed for 30 seconds and 100 µl was transferred to injection vials where 20 µl of sample was injected.

Amniotic fluid samples were prepared in a similar fashion. In a 0.65 ml centrifuge tube 20 µl of amniotic fluid, 5 µl of AZDU internal standard (50 µg/ml) and 5 µl of a ABC standard solution were combined and vortexed. The tubes were then centrifuged at 13 000 rpm for 5 minutes in the microcentrifuge. 75 µl of the supernatant was transferred to a clean 1.5 ml tube and diluted to 500 µl with 425 µl of optima water. The samples were vortexed mixed for 30 seconds and 100 µl was transferred to injection vials where 20 µl of sample was injected.

**Chromatographic and Mass Spectrometric Conditions**

The mobile phase used was 10 mM ammonium acetate (pH 6.3):acetonitrile under gradient conditions (see Table 3.1). 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. Baseline resolution of at least 0.5 min was needed in order to allow for an effective and reproducible polarity switch for the determination of the two analytes.

The ESI source of the Quattro-LC was operated in the negative ion mode for AZDU and in the positive ion mode for ABC. Once the AZDU had eluted the polarity of the source was switched to the positive ion mode to allow for analysis of abacavir. The capillary voltage was held constant at 3.00 kV while the cone voltage was set at 25 V for AZDU and at 29 V for ABC. The source temperature was set at 90$^{o}$C and the desolvation temperature was held at 250$^{o}$C. For quantitation of abacavir and the internal standard azidouridine, the transitions *m/z* 287 (Q1) → *m/z* 191 (Q3) (abacavir) and *m/z* 252 (Q1) → *m/z* 209 (Q3) (azidouridine) were monitored using the MRM experiment with collision energies of 20 eV and 11 eV for ABC and AZDU, respectively.

**Ionization Suppression Study**

Regions of ionization suppression were determined using a post-column infusion of a 25 μg/ml solution of ABC and AZDU while injecting a "blank" of each matrix following a method previously described.[24] This experiment was repeated three times with three different blanks of each of the four matrices.

**Validation and Recovery Study**

Individual ABC and AZDU stock solutions were prepared in optima water to give a concentration of 1.0 mg/ml. Calibration and quality control standards of ABC were prepared by serial dilution in optima water to obtain concentrations of 0.25, 0.5, 2.5, 5, 12.5, 25, 50, and 125 μg/ml. A stock solution of AZDU at a concentration of 50 μg/ml was prepared in optima water. Calibration samples were prepared on each day of analysis by spiking 40 μl of the tissue homogenate or plasma and 20 μl of amniotic fluid

with appropriate volumes of ABC and AZDU standard solutions to obtain ABC concentrations of 50 ng/ml - 25 μg/ml and 10 μg/ml AZDU concentration. The spiked samples were then prepared according to the extraction procedure described above.

Quantitation was performed using the peak area ratios between ABC and the internal standard, AZDU. AZDU concentrations were held constant at 10 μg/ml for all samples. A calibration curve including the points: 50 ng/ml, 100 ng/ml, 500 ng/ml, 1 μg/ml, 2.5 μg/ml, 5 μg/ml, 10 μg/ml and 25 μg/ml ABC was prepared in each matrix on each day of validation. A calibration curve was run at the beginning and end of each validation day and plotted together to obtain an average calibration curve. Calibration curves for each day of validation (and of sample analysis) were weighted by a factor of 1/y using JMP IN statistical software.

Five replicate samples of each of the following concentrations: 50 ng/ml, 150 ng/ml, 1.25 μg/ml and 22.5 μg/ml were prepared on each day validation to assess precision and accuracy. Precision was calculated as the relative standard deviation (%RSD) and accuracy was reported as percent error. All precision and accuracy calculations were performed using Microsoft Excel.

Recovery was expressed as the percent difference between samples spiked with ABC and AZDU pre- and post-extraction. Five replicates of each matrix were spiked to concentrations of 1.25 μg/ml ABC and 10 μg/ml AZDU and extracted. The peak areas of the extracted samples were compared with peak areas of five replicates of blank samples that had been extracted and spiked to 1.25 μg/ml ABC and 10 μg/ml AZDU following extraction.

Limits of detection were determined by analysis of samples with decreasing abacavir concentrations. The limit of detection is defined as the abacavir concentration at which the analyte signal to noise ratio is 3.

**Stability**

Although both azidouridine and abacavir elute within approximately 5 minutes, there is a 10 minute column reequilibration period needed after each injection. This yields a total duty cycle time of 15 minutes per sample. During validation as many as 44 samples were run at any given time, meaning that a sample could sit at room temperature in an autosampler vial for up to 11 hours before being injected onto the HPLC. For this reason, autosampler stability of prepared samples was investigated. A single sample at a concentration of 1.25 μg/ml was prepared in each matrix. 100 μl aliquots of each sample were transferred to individual injection vials, giving a total of 10 samples per matrix and a total of 40 samples overall. A single sample from each matrix was injected once every hour for a period of 10 hours, with each sample being injected only once. Following analysis, area counts of both abacavir and the internal standard, azidouridine for the samples were compared to those of the first sample injected.

**RESULTS AND DISCUSSION**

The $C_8$ analytical column with a length of 150 mm was chosen because it provided adequate retention of the two analytes while affording a baseline resolution of over 1 minute under the developed chromatographic conditions. Ammonium acetate at a concentration of 10 mM was chosen because it buffers within the range desired and has a

pH of approximately pH 6.3. This allows for the prepping of the buffer solution without addition of any acidic or basic modifier. The chromatographic conditions were established to allow for the most efficient separation and elution of the two analytes while still maintaining the baseline resolution needed for the polarity switch. A gradient method was used in which the acetonitrile composition was increased, allowing for a faster elution of abacavir while also improving the peak shape. Due to the large dead volume in the mixing cell of the Agilent 1100 series quaternary pumps, there is an extreme gradient delay when running at lower flow rates. Therefore, although the gradient conditions may appear too extreme, there is a lag period of approximately 4 minutes for the mobile phase conditions to go from the initial conditions of lower organic content to the higher organic composition. Likewise, there is an approximate 4 minute delay in returning to initial conditions for column re-equilibration. Figure 3.3 shows chromatograms of ABC (50 ng/ml) and AZDU (10 μg/ml) from the four matrices.

Small ionization suppression regions are present at 1.2-1.4 minutes and 5.25-5.5 minutes for the four matrices (Figure 3.4). The elution of AZDU at 3.4 minutes and ABC at 5.0 minutes was adequate to avoid the effects of any ion suppression areas.

Results from recovery experiments can be seen in Table 3.2. Recovery for all matrices was greater than approximately 70% for all matrices except fetus. The abacavir recovery in the fetal tissue homogenate was only slightly greater than 50%. Abacavir is highly protein bound, therefore complicating extraction from this matrix. Although recoveries in plasma, placenta and fetus are around 70%, with a limit of detection of 1 ng/ml and a limit of quantitation of 50 ng/ml, sufficient amounts of drug were reproducibly extracted to allow for quantitation.

Table 3.3 shows the within- and between-run validation data over three days with an n = 5 for within-run data and an n = 15 for between-run data at each validation point. All % RSD and % error values for each matrix were below 15%. All validation data generated was in compliance with the suggested criteria for bioanalytical method development.[25]

Autosampler stability experiments were performed in order to determine stability of a prepared sample over the duration of a validation run. Comparisons of the area counts for abacavir and azidouridine between the first and last samples revealed that the area counts do not deviate by more than $\pm$8.58% for abacavir and $\pm$7.34% for azidouridine. The % RSD values with an n=10 for ABC and AZDU in each matrix ranged from 4.97-13.05% and 2.76-8.37%, respectively. Therefore, the compounds were determined to be stable in autosampler injection vials throughout the entire course of the validation run.

**Application of Method**

To verify the utility of the method for determining abacavir concentrations in the specified matrices in support of a fetal exposure study, a pregnant rat was dosed with abacavir and samples were taken as specified for this particular pregnancy model. Animal use was approved by the University of Georgia's Institutional Animal Care and Use Committee (IACUC). The rat was housed at the University of Georgia College of Pharmacy animal facility (AALAC accredited) until the day of use. The animal facility environment is controlled to maintain a temperature of 20-22$^{o}$C with 14 hours of light each day. The rat was fed standard chow pellets and allowed constant access to water.

A timed pregnant female Sprague-Dawley rat on day 19 of gestation weighing 344 g was used. The rat was anesthetized using a ketamine:acepromazine:xylazine (75:2.5 mg/kg) solution injected intramuscularly. In order to prevent pulmonary edema, atropine was administered sub cutaneously throughout the experiment. A cannula was placed in the right jugular vein prior to shipment by Charles River Laboratories. This allows for IV administration of abacavir and for serial sampling of maternal blood. On the day of the surgery, a laparotamy was performed to allow concurrent serial sampling of fetus, placenta and amniotic fluid. The rat was administered an IV bolus dose (25 mg/kg) of ABC dissolved in phosphate buffered saline via the jugular 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 were placed in heparinized microcentrifuge tubes. These tubes were centrifuged at 5000 rpm for 10 minutes and the plasma was transferred to clean dry tubes for storage. Fetal and placental samples were collected and homogenized in 2 volumes of optima water. Amniotic fluid samples were placed in clean dry microcentrifuge tubes. All samples were stored at –20$^{o}$C until analysis.

On the day of sample analysis, calibration curves were generated and QC samples were prepared for each matrix as previously described. Prior to extraction, each collected sample was spiked with the appropriate amount of AZDU to yield a final concentration of 10 μg/ml. Figure 3.5 shows the concentration vs. time curve of the ABC in the pregnant rat in all four biological matrices. An ABC half-life of 0.82 hours, steady state volume of distribution of 1.78 L/kg and total body clearance of 1.86 L/hr/kg were calculated. All values were similar to previously reported literature values.[26]

## CONCLUSIONS

This is the first validation method for the quantitation of abacavir from complex matrices such as amniotic fluid and fetal and placental tissue homogenates. Using a gradient HPLC method allows for sufficient retention and resolution of the internal standard and analyte. The acetonitrile precipitation is fast and simple, therefore saving time and money. The recoveries are approximately 70% or greater for all matrices except fetus using this method. However, even with this decreased recovery, validation data falls well under the suggested regulations. The assay is accurate and reproducible yielding low values for % RSD and % error over the validation range. The dose response results from the analysis of collected samples were comparable to literature data, further validating the reliability of this particular method for the determination of ABC concentrations in biological samples. This method allows for a pharmacokinetic investigation for the determination of the maternal-fetal pharmacokinetics as well as placental/fetal uptake of ABC during pregnancy.

A polarity switching method was employed in order to determine the effectiveness of analyzing for compounds that ran under different mass spectrometric conditions. As a rule, most methods involve the analysis of analytes in the positive mode. However, several antivirals give higher responses when run in the negative mode, azidouridine being one of these. Future plans are to study not only the maternal-fetal pharmacokinetics of single-therapy abacavir, but to investigate the changes in the pharmacokinetics of the mother and fetus when it is given in combination with other antivirals. The combined therapies may require a polarity switching method in order to

obtain the desired limits of detection. Here we prove that this can be accomplished effective and efficiently with no decrease in signal or increase in error.

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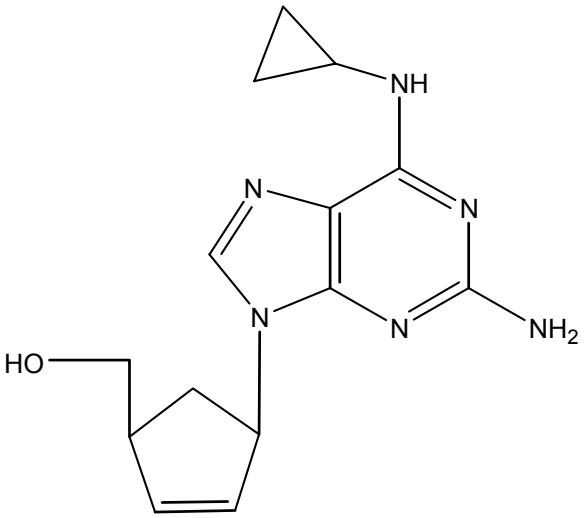
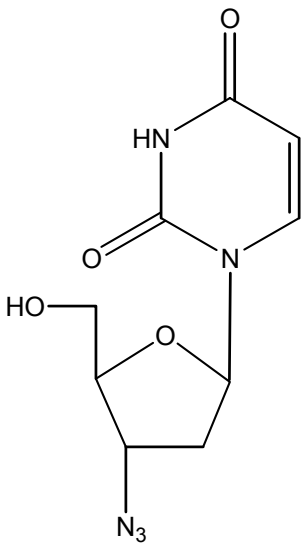


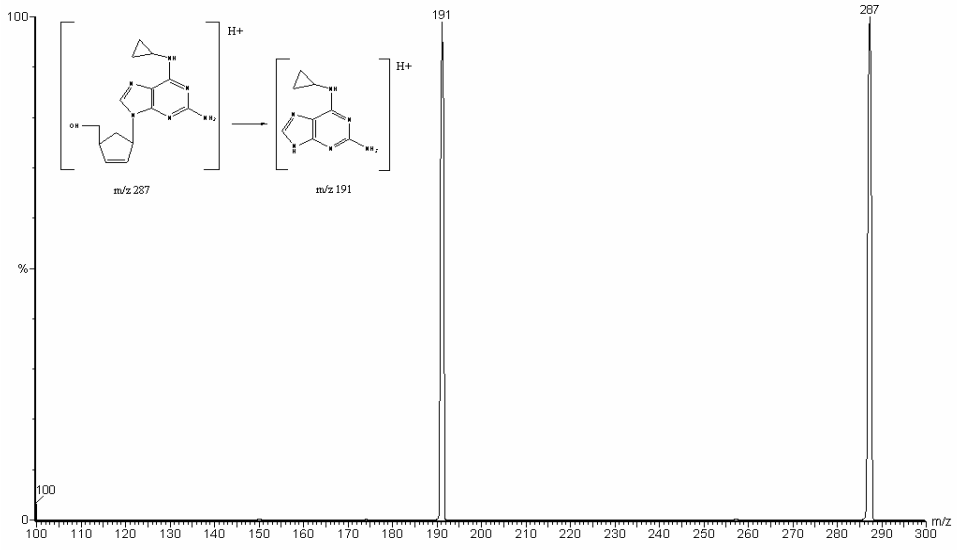

(a) Abacavir




(b) Azidouridine

**Figure 3.1.** Molecular structures of (a) Abacavir and (b) Azidouridine

(a)




(b)

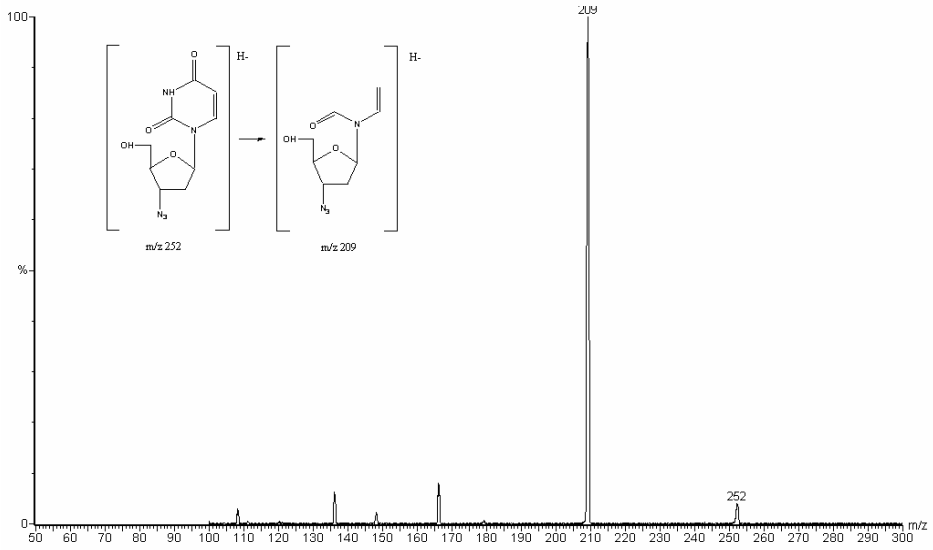
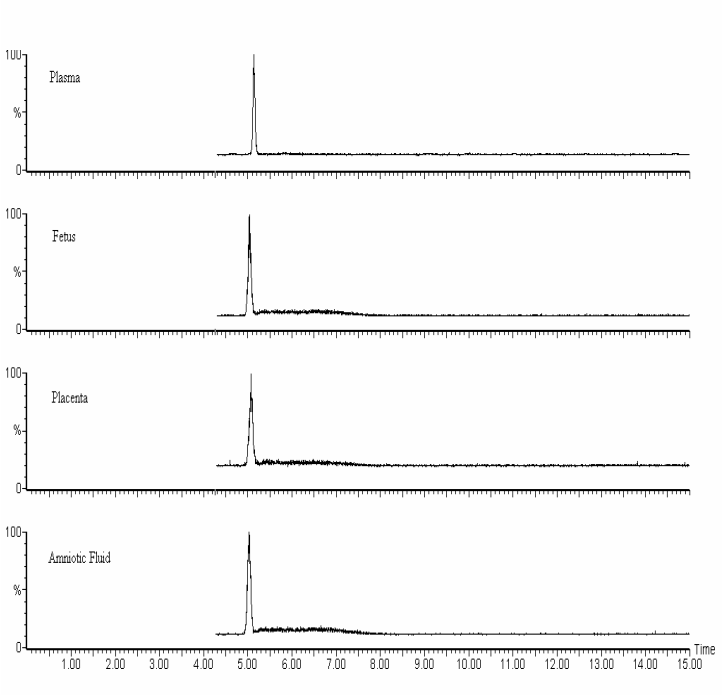



**Figure 3.2.** Product ion mass spectra of (a) Abacavir and (b) Azidouridine

(a)




(b)

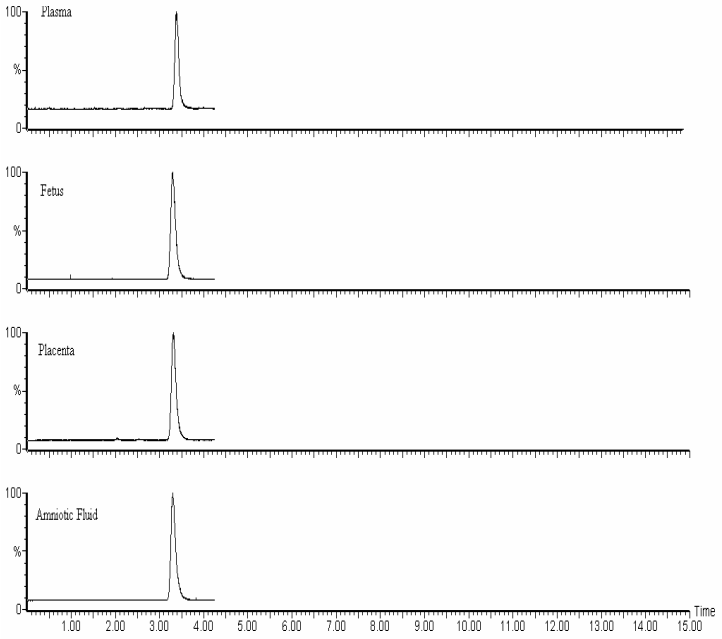
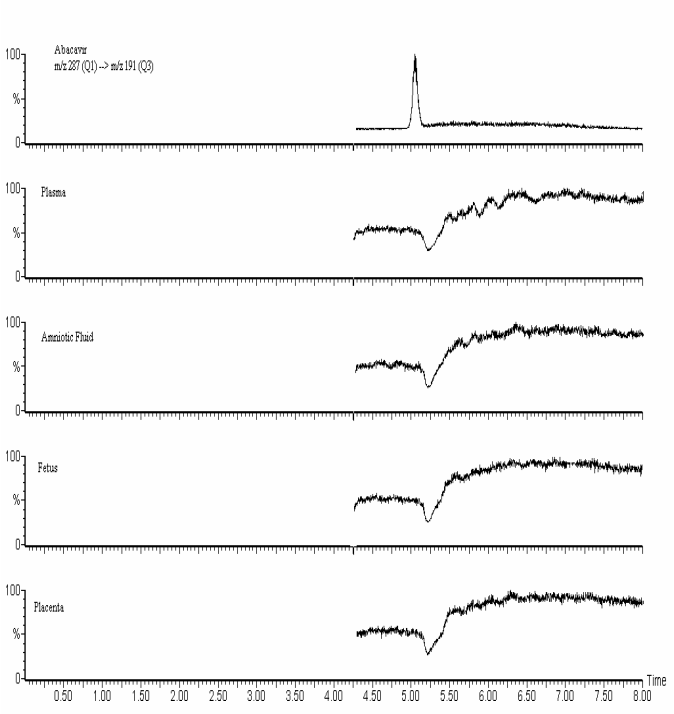



**Figure 3.3.** Chromatograms of (a) ABC (50 ng/ml) and (b) AZDU (10 μg/ml) generated from extracted plasma, amniotic fluid, placental and fetal tissues

(a)




(b)

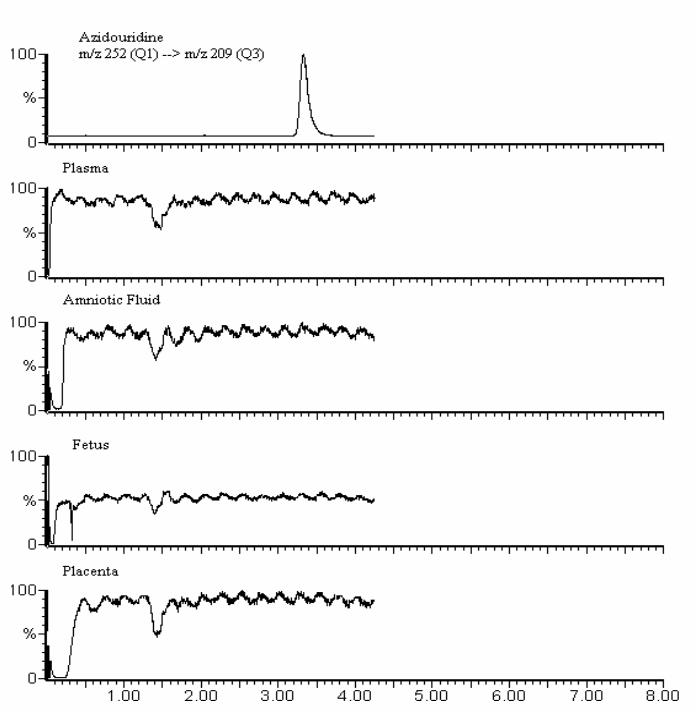



**Figure 3.4.** Regions of ionization suppression in maternal plasma, amniotic fluid, placental and fetal tissues for (a) abacavir and (b) azidouridine

**Table 3.1.** Gradient Conditions for Chromatographic Separation of Azidouridine and Abacavir

| Time (minutes) | % Buffer | % Acetonitrile |
|---|---|---|
| 0.0 | 85 | 15 |
| 0.1 | 30 | 70 |
| 1.5 | 30 | 70 |
| 2.0 | 85 | 15 |
| 15.0 | 85 | 15 |

**Table 3.2.** Percent relative recovery ± standard deviation (n=5) of ABC (1.25 μg/ml) and AZDU (10 μg/ml) from maternal plasma, amniotic fluid, placental and fetal tissues

| Analyte | % Relative Recovery (±SD) | | | |
|---|---|---|---|---|
| | **Maternal Plasma** | **Amniotic Fluid** | **Placenta** | **Fetus** |
| **ABC** | 68.3 ± 8.3 | 87.2 ± 4.3 | 78.7 ± 16.4 | 52.8 ± 12.2 |
| **AZDU** | 68.5 ± 9.6 | 74.4 ± 8.2 | 73.8 ± 5.4 | 83.7 ± 13.9 |

**Table 3.3.** The within- and between-run precision (% RSD) and accuracy (% Error) of ABC in maternal plasma, amniotic fluid, placenta and fetus

| Theoretical Concentration | Within-run (n=5) | | | Between-run (n=15) | | |
|---|---|---|---|---|---|---|
| | Concentration Found (μg/ml) | RSD (%) | Error (%) | Concentration Found (μg/ml) | RSD (%) | Error (%) |
| **Maternal Plasma** | | | | | | |
| 0.05 μg/ml | 0.053 ± 0.001 | 1.92 | 5.61 | 0.050 ± 0.003 | 6.56 | 5.49 |
| 0.15 μg/ml | 0.150 ± 0.008 | 5.60 | 3.95 | 0.142 ± 0.011 | 7.53 | 6.94 |
| 1.25 μg/ml | 1.44 ± 0.11 | 7.74 | 14.80 | 1.36 ± 0.15 | 10.98 | 12.15 |
| 22.5 μg/ml | 23.03 ± 0.99 | 4.31 | 3.76 | 23.31 ± 2.66 | 11.40 | 8.81 |
| **Amniotic Fluid** | | | | | | |
| 0.05 μg/ml | 0.046 ± 0.005 | 9.77 | 11.80 | 0.050 ± 0.005 | 10.95 | 8.70 |
| 0.15 μg/ml | 0.141 ± 0.013 | 8.91 | 8.40 | 0.155 ± 0.016 | 10.29 | 9.22 |
| 1.25 μg/ml | 1.15 ± 0.12 | 10.29 | 9.33 | 1.26 ± 0.18 | 13.99 | 11.08 |
| 22.5 μg/ml | 21.84 ± 0.49 | 2.26 | 3.00 | 23.41 ± 2.13 | 9.11 | 7.41 |
| **Placenta** | | | | | | |
| 0.05 μg/ml | 0.049 ± 0.001 | 2.71 | 2.63 | 0.050 ± 0.006 | 11.24 | 8.90 |
| 0.15 μg/ml | 0.160 ± 0.006 | 3.50 | 7.07 | 0.155 ± 0.011 | 7.07 | 6.46 |
| 1.25 μg/ml | 1.31 ± 0.07 | 5.24 | 6.32 | 1.25 ± 0.09 | 7.27 | 6.37 |
| 22.5 μg/ml | 23.53 ± 1.53 | 6.51 | 6.48 | 22.70 ± 1.65 | 7.25 | 6.37 |
| **Fetus** | | | | | | |
| 0.05 μg/ml | 0.047 ± 0.003 | 7.41 | 8.52 | 0.047 ± 0.006 | 11.83 | 9.56 |
| 0.15 μg/ml | 0.154 ± 0.014 | 7.22 | 9.14 | 0.150 ± 0.020 | 13.17 | 10.43 |
| 1.25 μg/ml | 1.23 ± 0.08 | 6.48 | 5.32 | 1.24 ± 0.11 | 9.23 | 7.45 |
| 22.5 μg/ml | 21.56 ± 1.97 | 9.16 | 7.22 | 23.65 ± 3.00 | 11.99 | 12.70 |

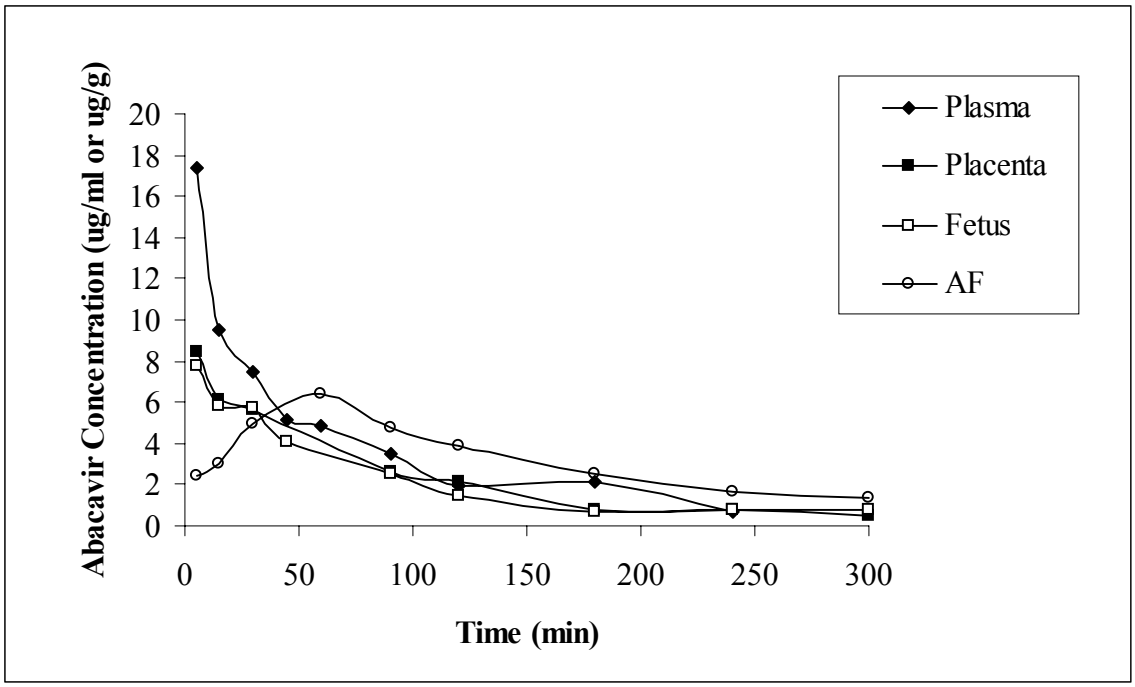



**Figure 3.5.** Concentration versus time profile of ABC in maternal plasma, amniotic fluid, placenta and fetus

# CHAPTER 4

# PHARMACOKINETICS OF INTRAVENOUS ABACAVIR IN THE PREGNANT RAT[1]

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1Clark,T. Nicole, Michael G. Bartlett, Catherine A. White. To be submitted to *Antimicrobial Agents and Chemotherapy.*

**Abstract**

The pharmacokinetics and placental transfer of Abacavir, a carbocyclic nucleoside analogue that has been shown to inhibit human immunodeficiency virus (HIV-1), were studied in the pregnant rat following IV administration. 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 LC/MS/MS. All data was analyzed 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. Abacavir is characterized by a maternal plasma half-life of 0.85 hours, a volume of distribution of 1.90 L/kg and a clearance value of 1.88 L/hr/kg.

KEYWORDS: ABACAVIR, PHARMACOKINETICS, PREGNANT RAT, PLACENTAL TRANSPORT

## Introduction

The decision to administer a therapeutic regimen to a pregnant woman is a very precarious one. It is largely based on weighing the therapeutic benefit of the regimen to the mother and/or fetus against the perceived risk to the fetus. Due to the fact that human safety data in pregnancy is lacking for a large number of drugs, clinicians are generally advised to refrain from prescribing drugs to pregnant patients. However, in some clinical situations, the benefits severely outweigh any perceived risk (13). One such situation is when a pregnant woman is infected with HIV (human immunodeficiency virus).

For women with HIV, pregnancy should not prevent the administration of an optimal therapeutic regimen. Recommendations for the treatment of HIV-infected pregnant women are based on the belief that therapeutic regimens known to benefit the woman should not be withheld during pregnancy unless it is known that there will be adverse effects on the mother, fetus or infant. In all cases, these effects must outweigh the benefits of the therapy to the woman. An antiviral therapy regimen is also beneficial to the fetus in that it reduces the risk of HIV transmission from the mother (23).

There are numerous physiological changes that occur in a woman's body during pregnancy that can affect the pharmacokinetics of drugs. Such changes include an increase in gastrointestinal transit time, decreased plasma protein concentrations, increased renal reabsorption of sodium, changes in hepatic metabolic pathways and increases in body water and fat accompanied by an increase in cardiac output, ventilation, liver and renal blood flow (5,9,25,26). The placental transport, compartmentalization in the placenta and embryo/fetus, metabolism by the fetus and placenta and elimination of

the drug by the fetus also have an effect on the behavior of a drug in a pregnant woman (25).

Abacavir (Figure 4.1) is a novel, second-generation reverse transcriptase inhibitor that received FDA approval for the treatment of HIV in December 1998 (29). It is currently marketed alone as Ziagen® and in combination with two other reverse transcriptase inhibitors, zidovudine (Retrovir®) and lamivudine (Epivir®), as Trizivir® by GlaxoSmithKline. Unlike previous RTI's, abacavir is a carbocyclic nucleoside analog. The absence of the glycosidic linkage on the sugar of ABC means it is not subject to the phosphorylases that cleave N-glycoside linkages, in turn making it more chemically stable (7,19,27). Although the pharmacokinetics of abacavir have been explored in several different populations (8,16,17,18,20,21,30,31), the effect of pregnancy on the behavior of this drug has not been investigated.

The pregnant rat has been successfully used for investigating the placental transport of a variety of compounds, including a number of nucleoside analogs (1,2,3,4,6,11,14,15,24,T. N. Clark, M. G. Bartlett, and C. A. White, submitted for publication). Many similarities exist in placental development and physiological changes between humans and rodents during pregnancy. This combined with a short gestation time and the presence of a large litter and individual fetal sacs containing a fetus, a placenta and amniotic fluid that allow for serial sampling of each matrix, makes the pregnant rat an ideal model for pharmacokinetic studies (3,10). Here we present the results of a pharmacokinetic study of abacavir during pregnancy using a pregnant rat model.

## MATERIALS AND METHODS

### Chemicals and Reagents

An abacavir (ABC) standard was obtained from GlaxoSmithKline Pharmaceuticals,Inc. (Barnard Castle, Co. Durham, UK) and the internal standard, azidouridine (AZDU) was purchased from Sigma (St. Louis, MO, USA). For dosing, a prescription of abacavir sulfate tablets (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 Studies

Animal studies received prior approval from the University of Georgia Animal Care and Use Committee and 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. The animal facility environment is controlled to maintain a temperature of 20-22$^{o}$C with 14 hours of light each day. 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 $\pm$ 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. At the

same time, the rats received a subcutaneous dose of atropine (0.5 mg/kg) in order to prevent pulmonary edema. A cannula was surgically placed in the right jugular vein prior to shipment by Charles River Laboratories to allow for IV administration of abacavir 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$^{o}$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$^{o}$C until analysis. Upon completion of the sampling timecourse, animals were euthanized using pentobarbital (150 mg/kg).

**Analytical Method**

On the day of sample analysis, calibration and QC samples were prepared for each matrix. Calibration curves were prepared by spiking blank plasma, amniotic fluid and tissues obtained from a non-dosed pregnant rat to obtain final concentrations of 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, and 25.0 μg/ml for abacavir and 2.5 μg/ml for azidouridine. Blank placental and fetal tissue homogenates were prepared from untreated animals by homogenization with two volumes of optima water (w/v). All samples were prepared in the following manner.

In a 0.65 ml centrifuge tube 50 μl of plasma or tissue homogenate and 10 μl of AZDU internal standard (25 μg/ml) and were combined and vortexed. 150 μl of ice cold acetonitrile was added and the samples were vortexed for 30 seconds. The samples were then centrifuged at 13 000 rpm for 5 minutes. 150 μl of the supernatant was transferred to a clean 1.5 ml tube and diluted to 1 ml with 850 μl of optima water. The samples were vortexed mixed for 30 seconds and 100 μl was transferred to injection vials where 20 μl of sample was injected.

Amniotic fluid samples were prepared in a similar fashion. In a 0.65 ml centrifuge tube 250 μl of amniotic fluid and 5 μl of AZDU internal standard (25 μg/ml) were combined and vortexed. The tubes were then centrifuged at 13 000 rpm for 5 minutes in the microcentrifuge. 75 μl of the supernatant was transferred to a clean 1.5 ml tube and diluted to 500 μl with 425 μl of optima water. The samples were vortexed mixed for 30 seconds and 100 μl was transferred to injection vials where 20 μl of sample was injected.

All 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 Z-Spray® atmospheric pressure electrospray ionization (ESI) source (Waters-Micromass, Beverly, MA, USA). Chromatographic separations were achieved on a Zorbax Eclipse XDB $C_8$ analytical column (5 μm, 2.1 x 150 mm, Agilent, Wilmington, DE, USA) equipped with a Security-guard $C_{18}$ 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.

The ESI source of the Quattro-LC was operated in negative ion mode for AZDU and in positive ion mode for ABC. Once the AZDU had eluted the polarity of the source was switched to the positive mode to allow for analysis of abacavir. The capillary voltage was held constant at 3.00 kV while the cone voltage was set at 25 V for AZDU and at 29 V for ABC. The source temperature was set at 90°C and the desolvation temperature was held at 250°C. For quantitation of abacavir and the internal standard azidouridine, the transitions *m/z* 287 (Q1) → *m/z* 191 (Q3) (abacavir) and *m/z* 252 (Q1) → *m/z* 209 (Q3) (azidouridine) were monitored using the MRM experiment with collision energies of 20 eV and 11 eV for ABC and AZDU, respectively.

This assay was validated to ensure both precision and accuracy in accordance to recommended guidelines for bioanalytical method development and validation (28). The method demonstrated acceptable reproducibility (% RSD < 15%) and accuracy (% Error

< 15%) in all matrices over the calibration range (T. N. Clark, C. A. White, and M. G. Bartlett, submitted for publication).

**Data Analysis**

Maternal plasma pharmacokinetic parameters were calculated from concentration-time data using WinNonlin®. The plasma data from all rats was subjected to compartmental analysis using a two-compartment IV-bolus model with first order elimination. Placental, fetal and amniotic fluid data were subjected to noncompartmental analysis only, using an extravascular adminstration model. Relative exposure values for each tissue matrix were expressed by comparing the extrapolated area under the curve (AUC) values for the individual tissue to the AUC values from the corresponding plasma data.

## RESULTS AND DISCUSSION

Mean (± standard deviation) concentration-time profiles of abacavir in maternal plasma, placenta, fetus and amniotic fluid following intravenous administration of a 25 mg/kg bolus dose are shown in Figure 4.2. Maternal plasma ABC concentrations decline in biexponential fashion after IV administration. The amniotic fluid concentrations were significantly higher than maternal plasma, placenta and fetus from the 45 minute timepoint to the end of the timecourse at 5 hours. Abacavir is the first antiretroviral agent evaluated in this animal model to demonstrate this behavior. Placental and fetal concentrations were not significantly higher than the maternal plasma concentration throughout the study.

The pharmacokinetic parameters generated from two-compartmental analysis of the maternal plasma data are presented in Table 4.1. The maternal plasma pharmacokinetic parameters generated for abacavir are similar to previously reported values observed in mice (12). Therefore, the behavior of the drug in the maternal compartment does not appear to be changed by the pregnancy status of the female rat. The maternal plasma elimination phase half-life of 0.85 hr is similar to that observed with zidovudine and azidouridine at this dose using the pregnant rat model (half-lives of approximately 1.0 hr each) (T. N. Clark, M. G. Bartlett, and C. A. White, submitted for publication). However, the volume of distribution and clearance values are different from what has been observed with other reverse transcriptase inhibitors using the pregnant rat model (4,15,T. N. Clark, M. G. Bartlett, and C. A. White, submitted for publication). The steady-state volumes of distribution ($V_{ss}$) for azidouridine and zidovudine were reported as being 0.8 L/kg for both compounds, which is similar to total body water (0.7 L/kg) for the rat. The $V_{ss}$ for abacavir is 1.78 L/kg, indicating more extensive distribution of ABC as compared to AZT and AZDU. The 2-3 fold increase in volume of distribution can be attributed to the lipophilicity of abacavir. While zidovudine is a relatively lipophilic compound with an octanol/water partition coefficient (log p) value of 0.9, the partition coefficient value for abacavir is 1.2 (22). Abacavir is a moderate to high clearance drug with a value of 1.79 L/hr/kg, which is three times greater than the total body clearance of 0.6 L/hr/kg observed with azidouridine and zidovudine. In humans, abacavir is extensively metabolized to a 5'-glucuronide and a 5'-carboxylate with less than 2% of the dose excreted unchanged in the urine (20). While glucuronidation is not a major route of metabolism in rats, the other metabolic pathway is

active. Even though the volume of distribution of abacavir is higher than that reported for other antivirals studied with this model, the dramatic increase in clearance causes no net change in the maternal plasma half life.

The pharmacokinetic parameters generated by noncompartmental analysis of the placenta, fetus and amniotic fluid data are shown in Table 4.2. As expected, the placenta and fetus had similar $T_{max}$ values of 0.17 hours, indicating rapid uptake of abacavir into these tissues. This is consistent with $T_{max}$ values for zidovudine and azidouridine (0.2-0.4 hr). Amniotic fluid reached its $C_{max}$ at approximately 1 hour, which is two-fold lower than the $T_{max}$ observed for AZT and AZDU. This decrease in $T_{max}$ for amniotic fluid is consistent with the increased lipophilicity of ABC as compared to AZT and AZDU. The half-life of ABC in the placenta, fetus and amniotic fluid is significantly longer than the half-life observed in the maternal plasma. The relative increase in half-life for the placenta and fetus is consistent with other antiviral agents. However, the relative increase in amniotic fluid half-life (1.5-fold) is significantly lower than that observed for AZT (4-fold) and AZDU (6-fold).

The relative exposure values for the placenta, fetus and amniotic fluid are given in Table 4.3. The relative exposure for these tissues to abacavir range from 0.68 to 1.23 and are significantly higher than values reported for other antiviral agents at the same dose. The most significant change is seen in the amniotic fluid where the ABC relative exposure value was greater than 1.0. This results from the amniotic fluid abacavir concentration exceeding the plasma levels after 45 minutes. Other antiviral agents studied to date using this model demonstrated amniotic fluid exposure levels of less than 0.2.

The data in the fetal compartment (fetus, placenta and amniotic fluid) indicate and increased exposure to abacavir. If this pattern persists in humans, increased protection of the fetus from the vertical transmission of HIV may be observed when abacavir is added to a therapeutic regimen. On the other hand, if an infected woman becomes pregnant while on a therapeutic regimen containing abacavir, dosing adjustments may need to be made during the pregnancy to prevent potential adverse effects to the mother and/or developing fetus.

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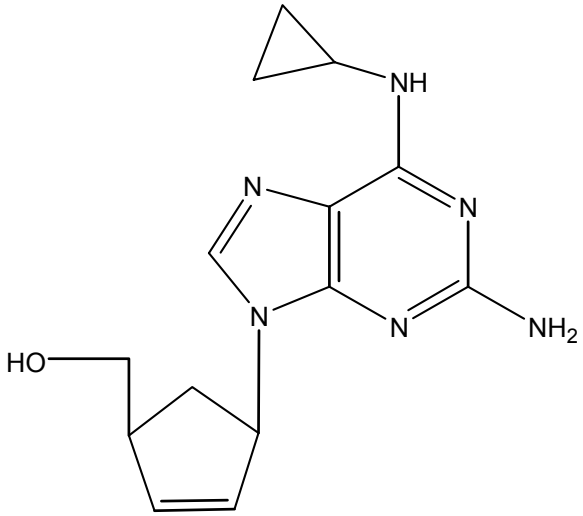


Abacavir

**Figure 4.1. Molecular Structure of Abacavir**

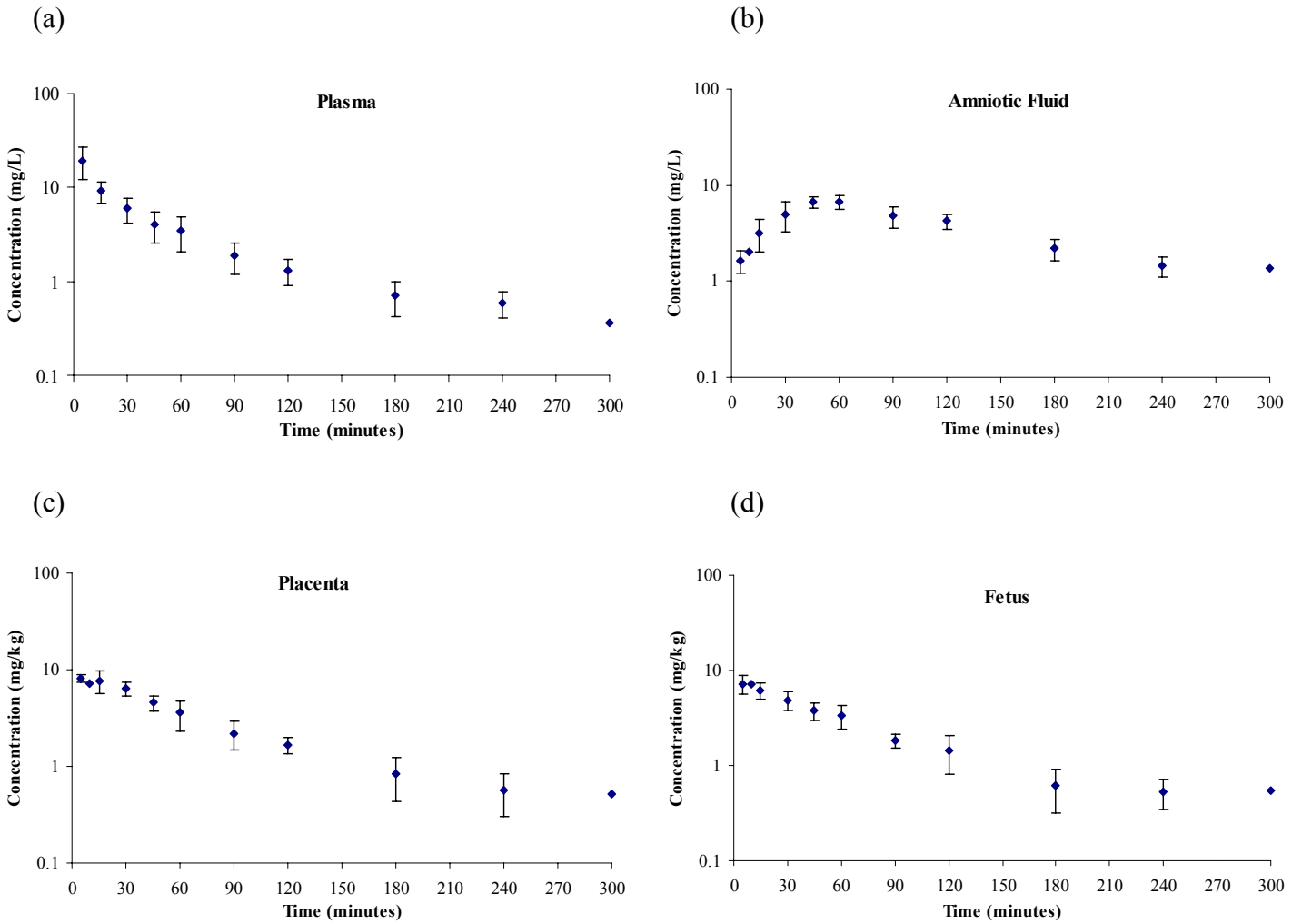


**Figure 4.2. Concentration (mean ± standard deviation) vs. Time Profiles of 25 mg/kg Doses of Abacavir Following IV Bolus Administration in (a) Maternal Plasma (b) Amniotic Fluid (c) Placenta and (d) Fetus**

**Table 4.1. Maternal Plasma Pharmacokinetic Parameters (mean ± standard deviation)**

| Parameter | Value |
|---|---|
| Half-life (hr) | 0.85 ± 0.28 |
| $V_{ss}$ (L/kg) | 1.78 ± 0.95 |
| $CL_T$ (L/hr/kg) | 1.79 ± 0.43 |
| AUC (hr·mg/L) | 13.82 ± 3.17 |
| $C_{max}$ (mg/L) | 46.18 ± 35.02 |

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

| Parameter | Placenta | Fetus | Amniotic Fluid |
|---|---|---|---|
| Half-life (hr) | 1.41 ± 0.27 | 1.24 ± 0.41 | 1.38 ± 0.39 |
| AUC* | 10.97 ± 1.86 | 9.09 ± 2.18 | 16.12 ± 4.03 |
| $C_{max}$** | 8.73 ± 1.41 | 6.55 ± 1.30 | 7.45 ± 1.11 |
| $T_{max}$ (hr) | 0.17 ± 0.10 | 0.17 ± 0.10 | 0.94 ± 0.13 |

***** Expressed as hr·mg/kg for placenta and fetus and as hr·mg/L for amniotic fluid
** Expressed as mg/kg for placenta and fetus and as mg/L for amniotic fluid

**Table 4.3. Relative Fetal, Placental and Amniotic Fluid Abacavir Exposure Following IV Bolus Dosing (mean ± standard deviation)**

| Tissue | Relative Exposure* |
|---|---|
| Fetus | 0.68 ± 0.24 |
| Placenta | 0.83 ± 0.26 |
| Amniotic Fluid | 1.23 ± 0.52 |

*Relative Exposure = $AUC_{Tissue}/AUC_{Plasma}$

# CHAPTER 5

# DETERMINATION OF DIDANOSINE IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL AND PLACENTAL TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY[1]

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1Clark, T. Nicole, Catherine A. White, Michael G. Bartlett. Submitted to *Journal of Mass Spectrometry.* 07/03

**Abstract**

A rapid and efficient high performance liquid chromatography (HPLC)-tandem mass spectrometry method for the determination of didanosine concentrations in maternal rat plasma, amniotic fluid, placental and fetal tissue samples has been developed and validated. Tissue samples were homogenized in optima water and centrifuged. The supernatant was subjected to solid phase extraction (SPE) prior to analysis. Plasma and amniotic fluid samples were extracted without pretreatment. An Agilent 1100 Series HPLC coupled with a Micromass Quattro II triple quadrupole mass spectrometer was used for all analyses. Chromatographic resolution was achieved on a Nova-Pak phenyl analytical column (2.0 x 150 mm, 4 μm particle size) equipped with a Phenomenex Security-guard phenyl guard cartridge (2.0 x 4.0 mm) using 60% methanol in 10 mM ammonium acetate buffer mobile phase for all matrices at a flow rate of 0.15 ml/min. The method yields retention times of 2.9 minutes for didanosine and 3.0 minutes for the internal standard, stavudine. Limits of detection were 1 ng/ml for all matrices. Recoveries were 70% or greater for both compounds in the different matrices. Within- and between-run precision (% RSD) and accuracy (% Error) was less than 15% for all matrices.

KEYWORDS: DIDANOSINE, STAVUDINE, LC/MS/MS, QUANTITATION, ANTIVIRALS

## Introduction

Since AIDS 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.[1] Eventually, the vertical transmission of HIV from an infected pregnant woman to her unborn/newborn child became a serious concern. A landmark clinical study conducted in 1994 demonstrated the ability of a shortcourse zidovudine (AZT) therapy regimen to drastically reduce the maternal-fetal transmission of HIV in infected pregnant women.[2] Once AZT gained FDA approval for use in pregnancy, the vertical transmission rate among infected mothers in the US dropped to approximately 8%.[3]

In industrial nations such as the United States, many infected women are still becoming pregnant, either by accident or by choice. In most cases the women are already on some type of therapy regimen. It is recommended that unless there are serious known adverse effects on the mother or fetus that outweigh any risks of discontinuing or modifying a current regimen, then a woman's therapy should not be changed due to her pregnancy.[4] For many of the antiviral compounds on the market, very little known about their behavior during pregnancy, making it difficult to determine if a therapeutic regimen needs to be altered.[5]

Didanosine (DDI) is a potent anti-HIV drug that has been approved for use for almost 15 years.[6] Didanosine, marketed as Videx® by Bristol-Meyers Squibb, was one of the first nucleoside analog reverse transcriptase inhibitors approved to treat infected individuals. Even though there are a variety of treatments available today, DDI is still widely used. Due to chemical stability issues, didanosine was previously administered

several times daily in combination with an antacid.[7] In 2000, a once-daily extended release formulation of DDI received FDA approval.[8]

Due to ethical concerns, pregnant women are generally excluded from clinical trials.[9] This makes it difficult to study placental and fetal drug distributions in humans. Therefore, an animal model must be utilized that will provide clinically useful mechanistic information. A pregnant rat model has been developed and used for the investigation of the basic mechanisms involved in the placental transfer of nucleoside analogs.[10,11] The rat model proves to be useful due to the similarities of the hemochorial placenta and hemodynamic pregnancy changes experienced in both rats and humans .[12,13] The large litter size and individual fetal sacs allow for serial maternal blood, placental, fetal and amniotic fluid sampling, making it even more useful for pharmacokinetic studies. The pregnant rat model has been utilized in maternal-fetal drug transfer studies of a variety of compounds, including AZT.[10,11,13-19]

Here we report a rapid and efficient HPLC method that has been developed and validated for the determination of DDI concentrations in samples taken in a placental transport study of this widely used antiviral. DDI concentration levels were determined in maternal plasma, fetal, placental and amniotic fluid samples after solid phase extraction. Due to the chemical instability of the compound and limited sample volumes for several matrices, several factors had to be taken into consideration when developing the method. Therefore we see that all analysis takes place under low temperature and almost neutral pH conditions. In doing so, we are able to quantitate didanosine at relatively low levels with small sample volumes. Several methods for the quantitation of didanosine from various biological matrices have previously been reported.[20-23]

However, none show the validation data, small sample size and complexity of biological matrices as demonstrated here.

## EXPERIMENTAL

### Reagents and Chemicals

Didanosine (DDI) and the internal standard Stavudine (D4T), were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and optima water were purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA).

### Instrumentation and Supplies

All analyses were performed on an Agilent (Palo Alto, CA, USA) 1100 Series HPLC equipped with a quaternary pump and autosampler coupled to a Micromass (Beverly, MA, USA) Quattro II triple quadrupole mass spectrometer equipped with a Z-Spray® atmospheric pressure electrospray ionization (ESI) source. Chromatographic separations were achieved on a Nova-Pak phenyl analytical column (4 μm, 2.0 x 150 mm, Waters Corp., Milford, MA, USA) equipped with a Security-guard phenyl guard column (2.0 x 4.0 mm, Phenomenex, Torrance, CA, USA). Tissues were homogenized using a Polytron PCU-2 tissue grinder (Kinematica GmbH, Luzern, Switzerland). All sample centrifugation was conducted in a Biofuge Pico microcentrifuge (Heraeus Instruments, Hanau, Germany). All rats used were purchased from Charles River Laboratories (Wilmington, MA, USA).

**Preparation of Standards**

Individual DDI and D4T stock solutions were prepared in optima water to give a concentration of 1.0 mg/ml. Calibration standards of DDI were prepared by serial dilution in optima water to obtain concentrations of 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, 100.0 and 250.0 μg/ml. Validation standards of DDI at concentrations of 1.50, 12.5 and 225.0 μg/ml were prepared, as well. Stock solutions of D4T at concentrations of 37.5 and 25 μg/ml were prepared in optima water. All solutions were stored at -20$^{o}$C when not in use. Stock solutions were prepared weekly throughout the validation period and on each day of sample analysis. Calibration curves were prepared by spiking blank plasma, amniotic fluid and tissues obtained from a non-dosed pregnant rat. Blank placental and fetal tissue homogenates were prepared from untreated animals by homogenization with two volumes of optima water (w/v). Calibration samples were prepared on each day of analysis by spiking 135 μl of the tissue homogenate and 90 μl of plasma or amniotic fluid with appropriate volumes of DDI and D4T standard solutions to obtain DDI concentrations of 50 ng/ml - 25 μg/ml and 2.5 μg/ml D4T concentration.

**Sample Preparation**

In a 0.65 ml centrifuge tube 100 μl of plasma or amniotic fluid and 10 μl of D4T internal standard (25 μg/ml) solution were combined and vortexed. The samples were then loaded onto a Sep-Pak $C_{18}$ solid phase extraction cartridge preconditioned with 1 ml of methanol followed by 1 ml of 10 mM ammonium acetate buffer. Samples were washed with 1 ml of 5% methanol in buffer and eluted with 1 ml of methanol into clean plastic culture tubes. Eluents from the cartridge were then dried under a stream of

nitrogen and reconstituted in 50 μl of optima water. The reconstitute was transferred to injection vials where 20 μl of sample was injected.

Tissue samples were prepared in a similar fashion. In a 1.5 ml centrifuge tube 150 μl of fetus or placenta homogenate and 10 μl of D4T internal standard (37.5 μg/ml) were combined and vortexed. Following the addition of 350 μl of optima water and vortexing to mix, the tubes were centrifuged at 13 000 rpm for 5 minutes in the microcentrifuge. The supernatant was then loaded onto a Sep-Pak $C_{18}$ solid phase extraction cartridge preconditioned with 1 ml of methanol followed by 1 ml of 10 mM ammonium acetate buffer. Samples were washed with 1 ml of 5% methanol in buffer and eluted with 1 ml of methanol into clean plastic culture tubes. Eluents from the cartridge were then dried under a stream of nitrogen and reconstituted in 50 μl of optima water. The reconstitute was transferred to injection vials where 20 μl of sample was injected.

**Liquid Chromatography**

The mobile phase used was 10 mM ammonium acetate (pH 6.3):MeOH (40:60) operated under isocratic conditions at a flow rate of 0.15 ml/min at ambient temperature. Under the chromatographic conditions described, DDI and D4T eluted at approximately 2.9 and 3.1 minutes respectively. The mobile was held at near neutral pH in order to prevent the breakdown of didanosine to form hypoxanthine upon exposure.

**Mass Spectrometry**

The chemical structures for didanosine and the internal standard stavudine are shown in Figure 5.1. Full scan mass spectra were obtained operating in positive ion mode for didanosine and stavudine. Didanosine showed the protonated molecule, $[M+H]^+$, at *m/z* 237 and stavudine showed the protonated molecule, $[M+H]^+$, at *m/z* 225. Product ion mass spectra (Figure 5.2) were obtained for both compounds with the most abundant ions being protonated hypoxanthine, *m/z* 137, for didanosine and protonated thymine, *m/z* 127 for stavudine. Both product ions are the result of the loss of the sugar moiety to give the resulting purine or pyrimidine base. As a result of this experiment, the mass spectrometer was set up in multiple-reaction monitoring (MRM) mode to monitor the transitions from the precursor ions (*m/z* 237 for DDI and *m/z* 225 for D4T) to the product ions (*m/z* 137 for DDI and *m/z* 127 for D4T). Blanks of each matrix were examined under these conditions and no peaks from endogenous compounds were detected in either transition.

The ESI source of the Quattro II was operated in positive ion mode with nitrogen serving as the desolvation gas and high purity argon as the collision gas. The capillary voltage was held constant at 3.5 kV while the cone voltage was set at 40 V for DDI and at 35 V for D4T. The source temperature was set at 30$^{o}$C to prevent in source fragmentation and the desolvation temperature was held at 250$^{o}$C. For quantitation of didanosine and the internal standard stavudine, the transitions *m/z* 237 (Q1) → *m/z* 137 (Q3) (didanosine) and *m/z* 225 (Q1) → *m/z* 127 (Q3) (stavudine) were monitored using the MRM experiment.

**Ionization Suppression Study**

Regions of ionization suppression were determined using a post-column infusion of a 25 μg/ml solution of DDI and D4T while injecting a "blank" of each matrix.[24] This experiment was repeated 3 times with 3 different blanks of each of the four matrices.

**Validation and Recovery Study**

Calibration and validation samples were prepared as described above on each day of validation. Quantitation was performed using the peak area ratios between DDI and the internal standard, D4T. D4T concentrations were held constant at 2.5 μg/ml for all samples. A calibration curve including the points: 50 ng/ml, 100 ng/ml, 500 ng/ml, 1.0 μg/ml, 2.5 μg/ml, 5.0 μg/ml, 10.0 μg/ml and 25.0 μg/ml DDI was prepared in each matrix on each day of validation. Calibration curves for each day of validation (and of sample analysis) were weighted by a factor of 1/y using JMP IN statistical software.

Five replicate samples of each of the following concentrations: 50 ng/ml, 150 ng/ml, 1.25 μg/ml and 22.5 μg/ml were prepared on each validation day to assess precision and accuracy. Precision was calculated as the relative standard deviation (%RSD) and accuracy was reported as percent error. All precision and accuracy calculations were performed using Microsoft Excel.

Recovery was expressed as the percent difference between samples spiked with DDI and D4T pre- and post-extraction. Five replicates of each matrix were spiked with 1.25 μg/ml DDI and 2.5 μg/ml D4T and extracted. The peak areas of the extracted samples were compared with peak areas of five replicates of blank samples that had been

extracted and spiked with 1.25 μg/ml DDI and 2.5 μg/ml D4T following reconstitution in optima water.

## RESULTS AND DISCUSSION

Separation of DDI and D4T from the ion suppression region was explored using various ratios of buffer and organic modifier. Suitable resolution was achieved at a mobile phase consisting of 40% 10 mM ammonium acetate and 60% methanol. Figure 5.3 shows chromatograms of spiked DDI (50 ng/ml) and D4T (2.5 μg/ml) in each of the four matrices. The ionization expression region is present from 2.5 - 5.0 minutes for the four matrices (Figure 5.4). Although DDI and D4T elute at the beginning of the suppression region, area counts are high and reproducible at the LOQ (50 ng/ml) and allow for a LOD of 1 ng/ml.

The calibration curve showed a good linearity in the range of 0.05 - 25 μg/ml for the individual matrices. The range of concentrations encompasses the estimated range of post IV bolus dose (25 mg/kg) concentrations in these biological matrices.

The limits of detection (LOD) for DDI in the biological matrices were determined by analysis of standard-spiked blank samples gradually decreasing in concentration. The LOD was determined as a concentration at which the signal/noise ratio was 3 and were found to be 1 ng/ml for all four biological matrices.

To investigate the extraction efficiency of DDI and D4T from the various biological matrices (plasma, amniotic fluid, fetal and placental tissues), standard-spiked matrix samples were subjected to extraction and then analyzed. The resulting peak areas were compared to peak areas of blank samples that were spiked with equal amounts of

analyte and internal standard following extraction. The recoveries were 70% or greater for both compounds in all four matrices. The recoveries for both DDI and D4T in the individual matrices are shown in Table 5.1.

Within-run (n=5) and between-run (n=15) precision and accuracy were calculated from standard curves constructed from each of the four biological matrices studied. Precision and accuracy values for DDI were less than 15% for the low, medium and high validation points and less than 20% for the LOQ. These values fall in compliance with the suggested criteria for bioanalytical method development.[25] Within-run and between-run precision and accuracy data are shown in Table 5.2.

**Application of Method**

To verify the utility of the method for determining didanosine concentrations in the specified matrices in support of a fetal exposure study, a pregnant rat was dosed with DDI and samples were taken as specified for this particular pregnancy model. Animal use was approved by the University of Georgia's Institutional Animal Care and Use Committee (IACUC). The rat was housed at the University of Georgia College of Pharmacy animal facility (AALAC accredited) until the day of use.

A timed pregnant female Sprague-Dawley rat weighing 311 g was used. On day 19 of gestation the rat was anesthetized using a ketamine:acepromazine:xylazine (50:3.3:3.4 mg/kg) solution injected intramuscularly. A cannula was placed in the right jugular vein prior to shipment by Charles River Laboratories. This allows for IV administration of didanosine and for serial sampling of maternal blood. On the day of the surgery, a laparotamy was performed to allow concurrent serial sampling of fetus, placenta and amniotic fluid. The rat was administered an IV infusion dose of 15 mg/kg of

DDI dissolved in phosphate buffered saline over 10 minutes via the jugular cannula. Samples were collected at 5, 15, 30, 45, 60, 90, 120, 180 and 240 minutes post-dose and stored on ice until processed. Blood samples were placed in heparinized microcentrifuge tubes. Due to the instability of didanosine in whole blood,[8] the tubes were immediately centrifuged at 5 000 rpm for 10 minutes and the plasma was transferred to clean dry tubes for storage. Fetal and placental samples were collected and homogenized in 2 volumes of optima water. Amniotic fluid samples were placed in clean dry microcentrifuge tubes. All samples were stored at –20$^{o}$C until analysis.

On the day of sample analysis, calibration curves were generated and QC samples were prepared for each matrix as previously described. Prior to extraction, each collected sample was spiked with the appropriate amount of D4T to yield a final concentration of 2.5 μg/ml.

Figure 5.5 shows the concentration vs. time curve of the DDI in the pregnant rat in all four biological matrices. A DDI half-life of 36 min, a steady state volume of distribution of 1.51 L/kg and total clearance of 4.84 L/hr·kg were calculated by noncompartmental analysis using WinNonlin® pharmacokinetic software. Half-life and volume of distribution values were determined to be in agreement with literature values previously reported for humans.[26,27] Because differences in total body clearance values between species is a common occurrence, the difference in the values observed here is not surprising.

## CONCLUSION

This is the first validated method for the quantitation of didanosine from complex matrices such as amniotic fluid and fetal and placental tissue homogenates. Using low temperatures and nearly neutral pH conditions prevents the degradation of didanosine to hypoxanthine, affording the sensitivity required for our assay while maintaining a small sample volume. The solid phase extraction is effective at removing large amounts of endogenous compounds while still retaining a significant amount of didanosine and stavudine. Even though DDI and D4T recovery is not optimal, validation data falls well under the suggested regulations. The dose response results from the analysis of collected samples were comparable to literature data, further validating the reliability of this particular method for the determination of didanosine concentrations in biological samples. This method allows for a pharmacokinetic investigation for the determination of the maternal-fetal pharmacokinetics as well as placental/fetal uptake of DDI during pregnancy.

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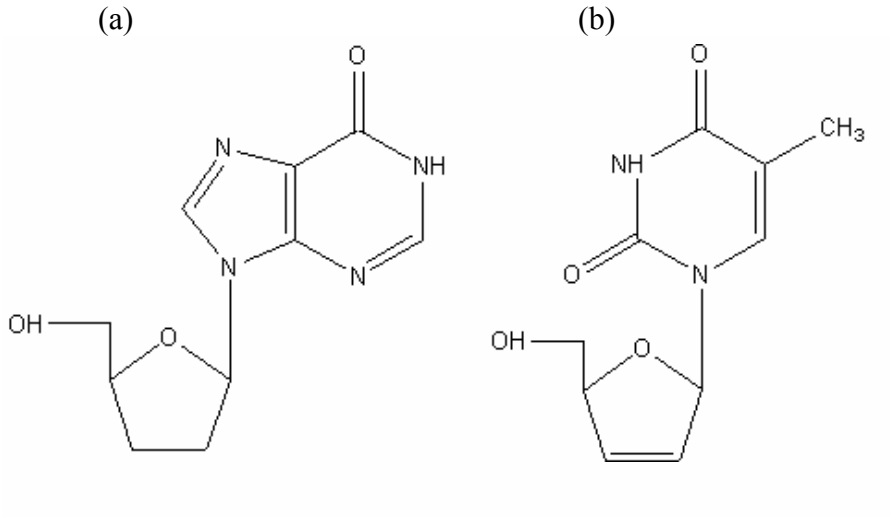


**Figure 5.1.** Molecular structures of (a) Didanosine and (b) Stavudine

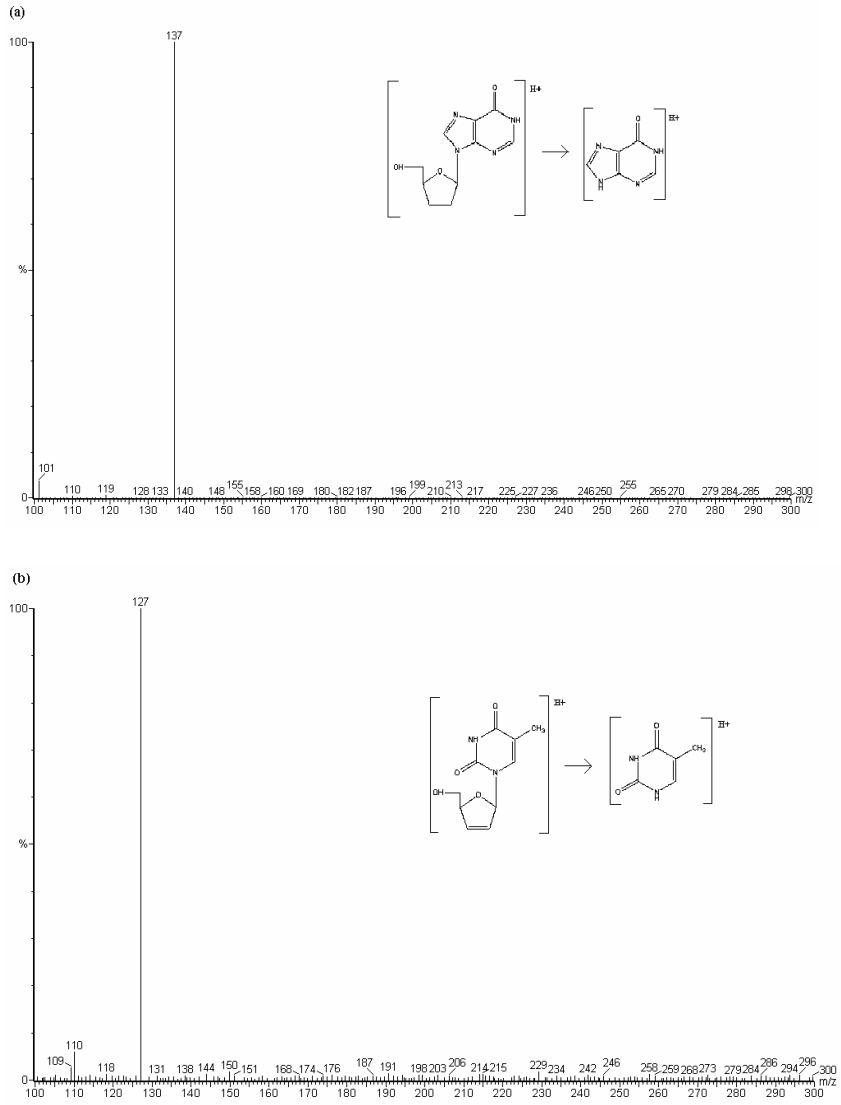


**Figure 5.2.** Product ion mass spectra of (a) Didanosine and (b) Stavudine

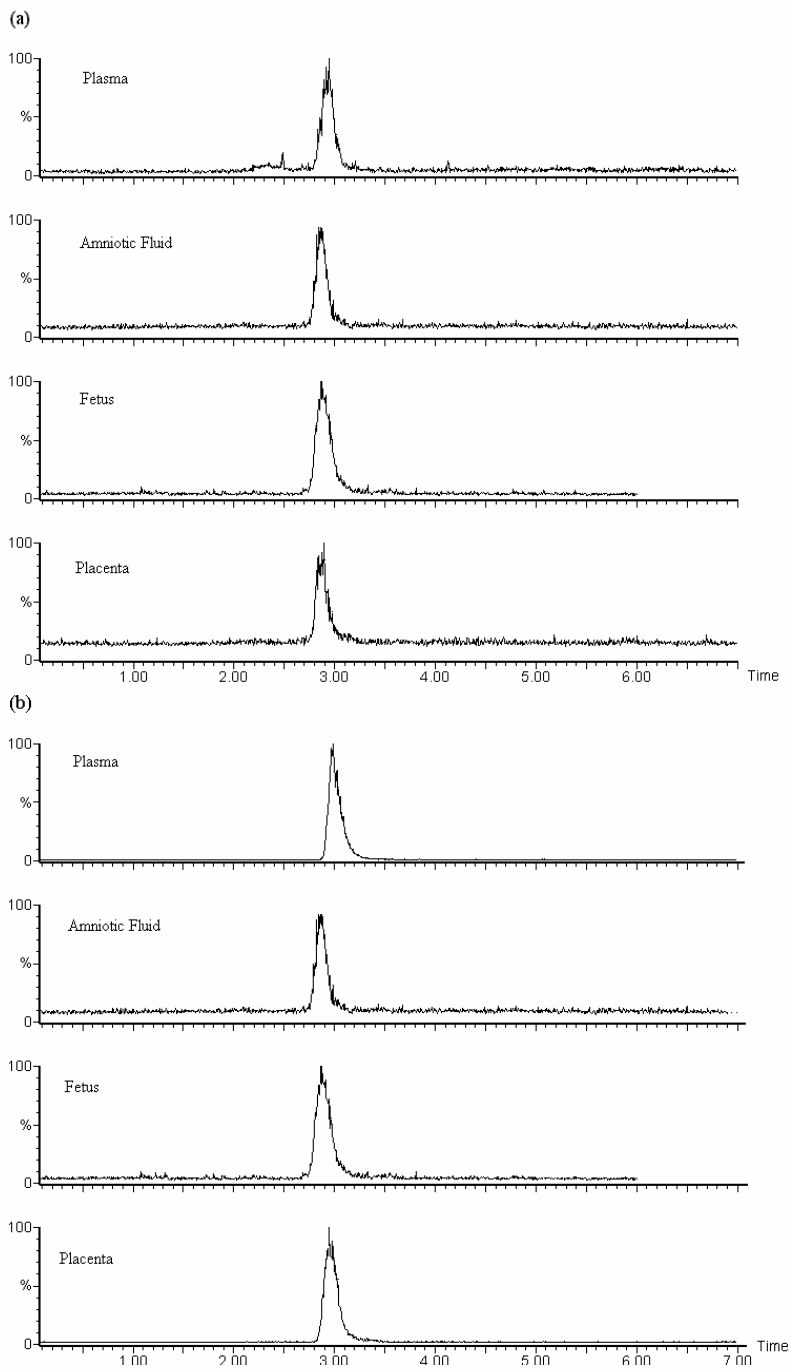


**Figure 5.3.** Chromatograms of (a) Didanosine (50 ng/ml) and (b) Stavudine (2.5 μg/ml) generated from extracted plasma, amniotic fluid, placental and fetal tissues

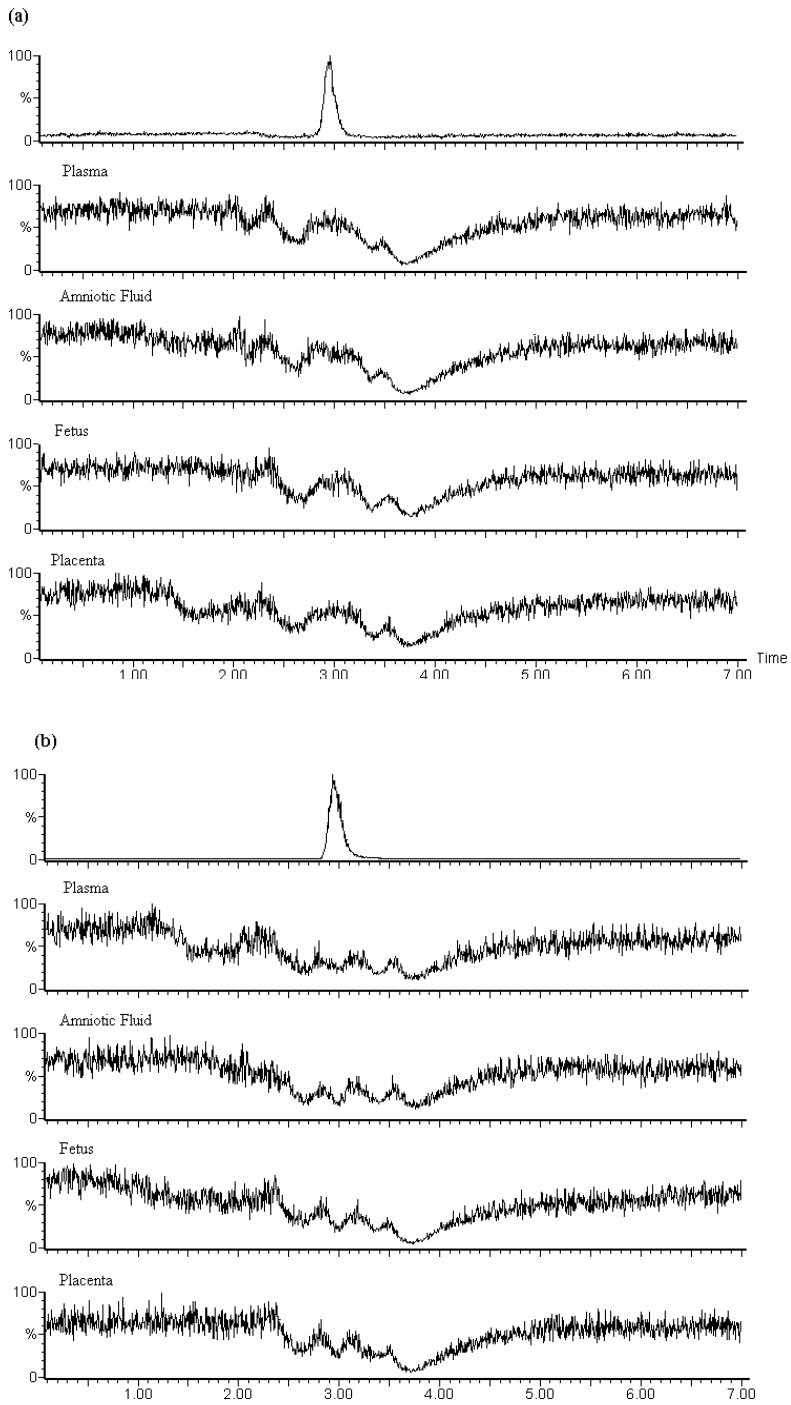


**Figure 5.4.** Regions of ionization suppression in maternal plasma, amniotic fluid, placental and fetal tissues for (a) didanosine and (b) stavudine

**Table 5.1.** Percent relative recovery ± standard deviation (n=5) of DDI (1.25 μg/ml) and D4T (2.5 μg/ml) from maternal plasma, amniotic fluid, placental and fetal tissues

| Analyte | % Relative Recovery (±SD) | | | |
|---|---|---|---|---|
| | **Maternal Plasma** | **Amniotic Fluid** | **Placenta** | **Fetus** |
| **DDI** | 86.4± 20.0 | 73.4 ± 13.6 | 70.0 ± 10.3 | 81.4 ± 3.5 |
| **D4T** | 73.9 ± 6.0 | 72.0 ± 8.9 | 70.9 ± 12.2 | 77.9 ± 16.8 |

**Table 5.2.** The within- and between-run precision (% RSD) and accuracy (% Error) of Didanosine in maternal plasma, amniotic fluid, placenta and fetus

| Theoretical Concentration | Within-run (n=5) | | | Between-run (n=15) | | |
|---|---|---|---|---|---|---|
| | Concentration Found (μg/ml) | RSD (%) | Error (%) | Concentration Found (μg/ml) | RSD (%) | Error (%) |
| **Maternal Plasma** | | | | | | |
| 0.05 μg/ml | 0.045 ± 0.005 | 10.17 | 12.29 | 0.047 ± 0.004 | 8.55 | 9.03 |
| 0.15 μg/ml | 0.148 ± 0.014 | 9.20 | 8.04 | 0.149 ± 0.014 | 9.49 | 7.60 |
| 1.25 μg/ml | 1.21 ± 0.04 | 3.52 | 3.99 | 1.21 ± 0.12 | 10.27 | 8.60 |
| 22.5 μg/ml | 22.80 ± 1.02 | 4.46 | 3.77 | 20.94 ± 2.70 | 12.91 | 10.29 |
| **Amniotic Fluid** | | | | | | |
| 0.05 μg/ml | 0.050 ± 0.003 | 6.31 | 8.58 | 0.050 ± 0.007 | 14.14 | 9.65 |
| 0.15 μg/ml | 0.131 ± 0.010 | 7.28 | 12.56 | 0.149 ± 0.020 | 13.75 | 11.33 |
| 1.25 μg/ml | 1.17 ± 0.07 | 5.87 | 6.08 | 1.22 ± 0.07 | 5.49 | 4.44 |
| 22.5 μg/ml | 21.65 ± 0.84 | 3.88 | 4.30 | 21.30 ± 1.73 | 8.11 | 7.55 |
| **Placenta** | | | | | | |
| 0.05 μg/ml | 0.053 ± 0.008 | 14.46 | 10.65 | 0.053 ± 0.008 | 15.40 | 13.60 |
| 0.15 μg/ml | 0.155 ± 0.013 | 8.62 | 7.24 | 0.143 ± 0.019 | 13.65 | 9.68 |
| 1.25 μg/ml | 1.28 ± 0.17 | 12.94 | 11.37 | 1.26 ± 0.18 | 14.61 | 12.96 |
| 22.5 μg/ml | 20.50 ± 1.66 | 8.11 | 9.56 | 19.97 ± 2.06 | 10.29 | 11.22 |
| **Fetus** | | | | | | |
| 0.05 μg/ml | 0.051 ± 0.006 | 11.42 | 8.92 | 0.048 ± 0.006 | 12.22 | 9.97 |
| 0.15 μg/ml | 0.166 ± 0.017 | 10.30 | 12.64 | 0.162 ± 0.018 | 10.88 | 9.49 |
| 1.25 μg/ml | 1.27 ± 0.16 | 12.59 | 10.63 | 1.26 ± 0.15 | 11.86 | 9.63 |
| 22.5 μg/ml | 23.07 ± 0.94 | 4.09 | 3.73 | 22.51 ± 2.12 | 9.43 | 6.95 |

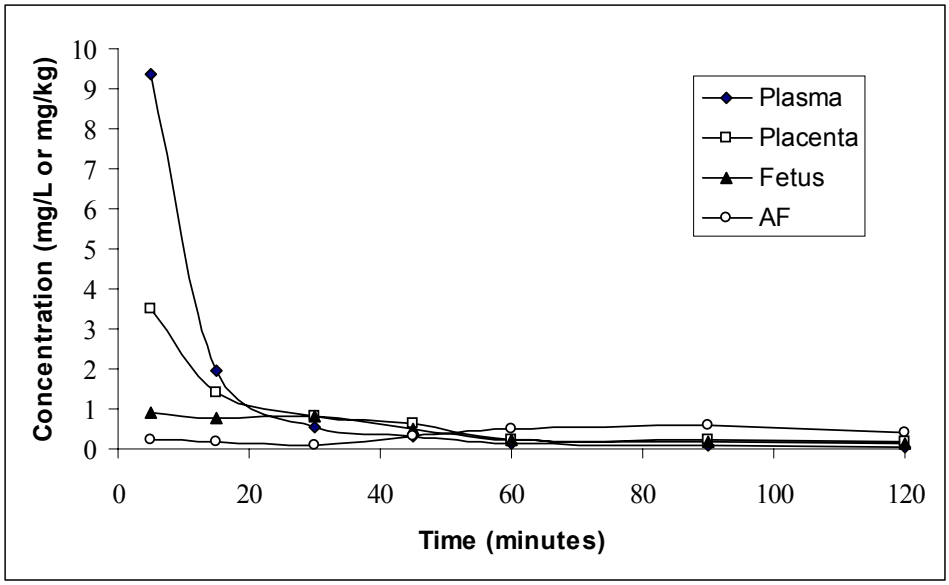


**Figure 5.5.** Concentration versus time profile of Didanosine in maternal plasma, amniotic fluid, placenta and fetus