I. TRACE LEVEL DETERMINATION OF TRICHLOROETHYLENE FROM LIVER, LUNG, AND KIDNEY TISSUES BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

II. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS AND COMPARATIVE PHARMACOKINETICS OF ACYCLOVIR AND ACYCLOVIR/ZIDOVUDINE THERAPIES IN THE PREGNANT RAT

by

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(Under the direction of Michael G. Bartlett)

ABSTRACT

This dissertation is divided into two parts entitled **I. Trace Level Determination of Trichloroethylene from Liver, Lung, and Kidney Tissues by Gas Chromatography/Mass Spectrometry** and **II. High Performance Liquid Chromatographic Analysis and Comparative Pharmacokinetics of Acyclovir and Acyclovir/Zidovudine Therapies in the Pregnant Rat**. The chapters contained therein describe techniques of analytical chemistry as well as some pharmacokinetic analysis and toxicology studies. The introduction to this document should help the reader understand not only why specific subject matters are being examined, but also why analytical chemistry plays such a vital role in the scientific process.

Part I focuses on the method development aimed at lowering the limits of detection for the common drinking water contaminant, trichloroethylene (TCE). The ability to quantitate trace levels of this chemical in biological matrices will enable toxicologists to develop more environmentally relevant models of the risk associated with TCE exposure. Chapter 1 presents the validated method used for quantitating TCE from drinking water from which the tissue methods were derived. Chapter 2 describes the final method and validation for quantitating low levels of TCE from target organs.

Part II describes the analytical and pharmacokinetic studies conducted to examine the placental transfer of the anti-herpes drug acyclovir (ACV). This study also incorporated the use of the anti-HIV compound zidovudine (AZT) in a comparative

pharmacokinetic analysis between ACV or AZT mono-therapies and a therapy involving a combination of the two. Chapters 3-5 outline the various analytical methods used to help quantitate acyclovir (and zidovudine) in a variety of biological matrices. Chapter 6 presents the pharmacokinetic analysis of both the ACV and AZT mono-therapies and the results obtained from a study of the co-administration of ACV and AZT.

INDEX WORDS: Trichloroethylene, TCE, Acyclovir, Zidovudine, AZT, Anti-virals,Gas Chromatography, GC/MS, Liquid Chromatography, LC/MS

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INTRODUCTION

Prologue

> *Analytical chemistry, or the art of recognizing different substances and determining their constituents, takes a prominent position among the applications of science, since the questions which it enables us to answer arise wherever chemical processes are employed for scientific or technical purposes. Its supreme importance has caused it to be assiduously cultivated from a very early period in the history of chemistry, and its records comprise a large part of the quantitative work which is spread over the whole domain of science.*
>
> -Wilhelm Ostwald, 1894
> *Foundations of Analytical Chemistry*

Ostwald's words, first written in his analytical chemistry textbook over a century ago are no less relevant today. [1]. The supportive and integral role that analytical chemistry plays in other sciences will always exist. For example, pharmacokinetists and toxicologists call on analytical chemists to analyze drugs from bizarre biological matrices like fingernails, meconium, sweat, and breast milk. Environmental chemists and risk assessors need analytical chemists to quantitate ultra-low levels of chemicals in the environment. Synthetic chemists depend on the analytical chemist to determine the identity of the trace impurity present in their product that is contaminating their entire synthesis. Forensic scientists rely on analytical techniques to identify the accelerant used in an arson case or the drugs on board in an over-dose case. Billions of dollars are on the line every day waiting for the results of quality control tests conducted by analytical

chemists in the pharmaceutical, cosmetic, chemical, and food industries. Are the questions facing analytical chemists getting harder, or are technological advances in the field making it easier to approach more difficult problems? One thing is certain – as with all facets of science, even an infinite number of research hours and dollars could never begin to answer all the questions that are posed in the field of analytical chemistry.

Some of the most commonly used techniques in the biological field of analytical chemistry (bioanalysis) evolved from relatively new technologies. Extraction methods such as solid-phase extraction (SPE) and solid-phase microextraction (SPME) enable purification and/or concentration of analytes such that minute concentrations of drugs or environmental compounds can be quantitated in complex biological matrices. Separation techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) enable the resolution of numerous analytes out of everything from air to brain tissue. When these separation techniques are coupled with the detection capabilities of mass spectrometry (MS), even analytes that could not be resolved on a million HPLC or GC columns can be quantitated at parts-per-billion (ppb) or parts-per-trillion (ppt) levels.

Unfortunately, these technologies often contribute to misconceptions concerning the capabilities of analytical chemists. On the one hand, some scientists are so mystifiesd by analytical instrumentation that the technology is often perceived as a magic box capable of answering any question. Personal experience in one particular industrial setting led to hearing the frequent comment, “Just do mass spec on it!” when a problem arose. While there is no need to underestimate the capabilities of mass spectrometry, few that understand this technique feel that it can provide fortune cookie answers to all

analytical problems. On the flip side, some feel that all it takes to be an analytical chemist is the capability to operate a syringe. The truth is that while there is no magic involved in analytical chemistry, there is a great deal of background knowledge needed to make a good analytical chemist. A strong foundation in all fields of chemistry (organic, physical, biochemistry, inorganic) as well as a firm understanding of the principles of chromatography, a working knowledge of human and animal physiology, and a reasonable set of expectations of the capabilities of instrumentation contribute to building capable and successful analytical chemists. Also, because of the supportive role many analytical chemists play in other fields, a basic understanding of these fields (i.e. toxicology, pharmacology, environmental sciences, pharmacokinetics, forensics) is also necessary to effectively contribute to answering some difficult questions.

The Extraction

Many times, the most difficult part of a method development, especially a bioanalytical method development, is the extraction. The sample preparation is usually the most time – consuming step and is the source of the majority of precision and accuracy problems in an overall analysis [2]. The goals of an extraction include removing interferences, converting the sample into a medium that is suitable for an analytical technique, and concentrating the analytes to maximize sensitivity. There is also added pressure to use sample analysis techniques that can be easily automated and that are "earth friendly" in that they require the consumption of a minimal amount of organic solvents. For these two final reasons, the techniques of solid-phase extraction (SPE) and solid-phase microextraction (SPME) will be discussed in further detail.

Solid-Phase Extraction (SPE)

SPE is basically a miniature liquid chromatography system. An SPE cartridge resembles the barrel of a syringe. The bottom of the barrel can contain a variety of packing materials that correspond to the column packings available for HPLC. As in HPLC, the most common packings for SPE are derivatized silica (C_8, C_{18}, etc.) for a reversed phase separation. The particle size of the packing is larger than HPLC packing and is often irregular in size and shape, thus keeping the cost of SPE to ~$1 per cartridge [3]. For an extraction, the packing is initially conditioned with an organic solvent and secondly with a solution that will maximize the retention of the analytes onto the packing. The liquid sample (plasma, serum, tissue homogenate) is loaded and washed with a solution or solvent that will remove interferences without removing the analyte molecules (wash solutions are often aqueous with a small percentage of organic) [3]. Finally, the analyte(s) are eluted with an organic solvent. This is often followed by evaporation of the organic and reconstitution in a small volume for chromatographic analysis. SPE is theoretically simple, it can be automated, produces minimal organic waste, and effectively removes interferences and particulates from the most complex of biological matrices [3].

Solid-Phase Microextraction (SPME)

SPME is the process of using a fiber coated with a gas chromatography stationary phase to concentrate sample analytes and deliver them into a GC injection port. Essentially, a SPME fiber is a GC column turned “inside out” therefore the principles

governing GC apply to SPME as well. Since its introduction in 1987, SPME has experienced rapid growth and acceptance in the analytical world [4]. The concepts behind SPME are simple: volatile analytes contained in the matrix (that can be solid, liquid, or gas) are adsorbed onto the SPME fiber and subsequently desorbed into the GC injection port [5]. The SPME method can be optimized by changing the length of adsorption/desorption time, the temperature of the injection port or sample, or the degree of agitation/stirring [5,6]. SPME is theoretically simple, easily automated, and completely solvent free. It has found an important niche in environmental analysis of volatile organics, but can be applied to other fields where the analysis of volatiles is necessary (i.e. toxicology, forensics, food science, etc.) [7-16].

The Separation

Quantitating drugs and environmental compounds requires an efficient and reproducible separation technique. Separation science has progressed tremendously since Tswett's 1906 work with the column chromatography separation of chlorophyll and xanthophyll plant pigments [17]. Two of the most commonly used separation techniques in the environmental, pharmaceutical, and industrial worlds are gas chromatography (GC) and high performance liquid chromatography (HPLC).

Gas Chromatography (GC)

The concept of gas chromatography (gas-liquid chromatography) was elucidated in 1941 by Martin and Synge, but it took more than a decade later (James and Martin, 1952) for the applicable technique to be introduced [17-20]. For this type of separation

technique, the stationary phase is a liquid bonded to an inert solid (usually silica) and the mobile phase is a gas (usually He, Ar, N_2, H, or CO_2) [17]. The gas selection depends on the type of detector being used. Most GC columns are long capillaries made of fused silica coated with an immobilized liquid such as polydimethyl siloxane, phenyl-polydimethyl siloxane, or polyethylene glycol [17]. Suitable stationary phases for GC are thermally stable, chemically inert, and have low volatility. Analytes are separated based on their polarity and volatility and are carried through the capillary by the carrier gas to the detector. Flame ionization (FID), electron capture (ECD), and thermal conductivity (TCD) are three common types of detectors for gas chromatography. An FID measures the current resulting from the pyrolysis of the organic analytes in a hydrogen/air flame. Detection using a TCD involves sensing changes in the thermal conductivity of the carrier gas (usually N_2) when analyte molecules are present. The utility of the ECD depends on the electron affinity of the analyte molecules where detection is based on gas-phase electron capture reactions [20,21]. Because many environmental compounds are highly halogenated, the ECD has been heavily used for trace environmental analyses [21]. The mass spectrometer, a much more selective and sometimes more sensitive detector for GC, will be discussed more extensively later.

High Performance Liquid Chromatography (HPLC)

Although various manifestations of liquid chromatography have existed for decades, the term HPLC was not coined until the 1960's. The addition of the words "high performance" to the name of this technique indicated the evolution of new technology for drastically minimizing particle size and column length and thus increasing

the number of theoretical plates [17,20]. HPLC uses a liquid mobile phase (a combination of buffers, water, and solvents) to move analytes through a column filled with a solid stationary phase (derivatized silica). Differences in polarities often account for the differences in retention for HPLC separations [3]. Column type, flow rate, column temperature, and mobile phase composition are some of the parameters used to optimize HPLC separations [3]. Ultraviolet detection is the most common type of detection used in HPLC. It requires the presence of UV absorbing chromophores in the molecule of interest, but can be used in a large number of compounds and is readily available [3]. As with GC, the coupling of the HPLC separation to a mass spectrometer for detection can provide qualitative information and sensitivity that the UV detector lacks.

The *Magic* of Mass Spectrometry

Hyphenated techniques such as GC – MS and LC – MS have begun to dominate both industrial and academic applications in bioanalysis. Although a mass spectrometer can be a stand-alone spectroscopic technique, it is more commonly used in conjunction with chromatography. The history of mass spectrometry is almost as old as chromatography beginning in 1907 with J.J. Thompson's production of a mass spectroscope [22,23]. The modern manifestation of mass spectrometry is sometimes deemed as "magical" because only a few picomoles of an analyte are required to provide structural and molecular weight information [23].

A block diagram of a mass spectrometer always includes at least the following three components: ionization source, mass analyzer, and detector. The second two

components are useless without an effective way to transfer the pure analyte molecules to the gas phase (ionization). Ionization techniques can be described as "hard" or "soft" depending on the intensity of the energy delivered during the ionization process and thus the resulting degree of fragmentation [23]. Electron ionization (EI) is the most common type of hard ionization where analyte molecules in the gas phase are bombarded with electrons from a filament (70eV). A positive charge is left on the analyte molecule when one of its electrons is removed. This results in the formation of the molecular ion, denoted $M^{+\bullet}$, which may further fragment in order to dissipate the excess energy absorbed during the ionization process [23]. EI and chemical ionization (CI) are the most commonly used ionization techniques in the interface between GC and MS. Chemical ionization is a soft ionization technique that requires the use of a reagent gas to produce reagent ions that collide with analyte molecules to promote ionization. EI and CI sources look very similar except that the CI source has much more narrow slits in order to promote sufficient collisions between the analyte molecules and the reagent ions [23].

Electrospray (ESI), another soft ionization technique, is commonly used to interface HPLC with a mass spectrometer. Malcolm Dole performed some of the early development for electrospray in the late 1960's [24]. Twenty years later, John Fenn's group elaborated on Dole's ideas and applied electrospray to introduce a sample into the mass spectrometer [25]. Electrospray uses an electric field to ionize analyte molecules and spray them in very fine droplets from a capillary. The electric field exists because of the high voltage (2-5kV) applied to the capillary needle relative to a counter electrode [23]. The presence of a nebulizing gas around the capillary and the occurrence of redox chemistry at the ESI interface contributes to ionization and droplet formation [23]. ESI is

the softest of the ionization techniques, thus producing few fragments. It provides a way to produce ions from non-volatile sources, hence its compatibility with the types of compounds usually separated by HPLC. Also, the fact that electrospray produces multiply charged ions gives it a flexible mass-to-charge (m/z) range, compatible with most mass analyzers [23].

As with mass spectrometer sources, there are several different types of mass analyzers. The single or triple quadrupole mass analyzer is most commonly coupled with HPLC. The word quadrupole is indicative of the four rods that are connected to radiofrequency (RF) and direct current (DC) voltage sources to serve as a "mass filter." According to quadrupole theory, the hyperbolic field created by this geometric arrangement of the rods facilitates the ability for ions to move through the filter [26-28]. By changing the magnitude of the RF amplitude and the DC voltages, the quadrupole filter is scanned [23]. Values corresponding to mathematically stable ion trajectories are values that are bounded solutions to the Mathieu equation [27,28]. Triple quadrupole systems have three sets of quadrupole rods in sequence, often designated Q_1, q_2, and Q_3. Q_1 and Q_3 serve as mass filters as described for a single quadrupole, and q_2 acts as a gas-filled collision cell, operating with only RF voltage applied to it. Typically Q_1 is used to select the parent or precurser ion, q_2 aids in the collisionally induced fragmentation, and Q_3 facilitates the characterization of fragment or daughter ions [23]. Quadrupole mass analyzers are advantageous because of their low cost (relative to other mass analyzers) and their ability to tolerate the high pressures associated with ESI and HPLC. Quadrupoles are found coupled to GCs as well as HPLCs.

A magnetic sector mass analyzer is often coupled to an EI or CI source and a gas chromatograph. In a sector instrument, a magnetic field is used to differentiate discrete ions from the total ion beam [23]. The ions accelerate through an electric field (usually with an accelerating voltage of 8000V) and gain kinetic energy. This accelerating voltage and the strength of the magnetic field ultimately determine the radius of the circular path of which the ions will travel. The m/z value can then be calculated if this radius is known [23]. Sector instruments are notorious for their electronic instability, but can be very sensitive when working with trace concentrations of analytes.

Preface to Part I

Risk assessment became an organized activity for federal agencies in the 1970's and has been an influence on environmental policy ever since. Risk assessment is defined as "the systematic scientific characterization of potential adverse health effects resulting from human exposure to hazardous agents or situations" [29]. A full assessment must not only include an evaluation of quantitative dose-response data, but must also incorporate qualitative information on the reliability of the available data and an idea of the amount of estimation or uncertainty involved in making the assessment [29]. The primary objectives of a risk assessment can be divided into four categories. First, an estimation of risk versus benefits must be made, especially for substances that are known to be useful but can potentially harm human life. Secondly, acceptable levels of risk are set for cases of pollutant or contaminants. Also, regulatory priorities must be outlined as a result of the assessment so government agencies and manufacturers can maintain a

balance of compliance. Finally, the "residual risks" must be estimated so the risk reduction process can continue for as long as the risk is present [29].

Several risk assessments have been conducted for trichloroethylene (TCE) over the past two decades. A review of twenty-nine of these TCE risk assessments found that the data sets were often incomplete and indicated biased data selection [30]. Although both epidemiological studies and animal experiments have indicated TCE carcinogenesis in several tissue sites (including the liver, kidneys, and lungs), not all risk assessors come to the same conclusions concerning the carcinogenic risks associated with TCE [30]. Differences in metabolism, morphology, extrapolating human risks from animal data, and human susceptibility to "peroxisome proliferation" may explain the differences in opinion [31-33]. The different approaches to TCE risk assessment use of linear versus non-linear mdoels have also sparked debate [34]. The variety of ways that TCE is classified also contributes to the confusion surrounding its carcinogenic nature. The International Agency of Research on Cancer (IARC) classifies TCE into Group 2A which indicates a "probable carcinogen to humans" and the U.S. Department of Health and Human Services classifies TCE as "reasonably anticipated to be a human carcinogen" [34,35]. However, the American Conference of Government Industrial Hygenists (ACGIH) places TCE into Group A5 which defines it as "not suspected as a human carcinogen" [34].

The pressing question in risk assessment now is whether or not environmentally relevant concentrations of chemicals like TCE pose a real threat to human health. In order to collect quantitative information on internal TCE levels that are associated with trace-level exposure, analytical techniques for biological samples must be improved.

They must also be reliable and reproducible enough that the technique itself has a known and acceptable variability and is not a source of uncertainty in the risk assessment process. The practice of establishing governmental policy based simply on the current limits of detection may not be an effective way to protect the public or the environment. High-resolution mass spectrometry can be used to quantitate trace levels of environmental contaminants in everything from water to mammalian tissues. Such techniques will be necessary to collect all the data needed for low-level risk assessment.

Preface to Part II

The ancient Egyptians revered the human placenta as a home to the external soul. Ceremonial processions were even formed to present "royal placentas" to the pharaoh in the belief that it would bring health to the kingdom [36,37]. Many carnivores consume the placentas of their offspring so as to not waste its valuable nutrients [38]. The study of placental transfer helps scientists understand the mechanisms by which the fetus is exposed to much needed minerals, vitamins, and gasses as well as potentially harmful environmental contaminants and drugs of abuse. Toxicity to the fetus is usually the main concern for long-term administration of drugs during pregnancy because it is assumed that the drug will cross the placenta to some extent [39]. The extent of placental transfer and the rate of this transfer becomes an issue when the drug is given more acutely in late pregnancy [39]. The exchange of compounds across the placenta can occur by passive diffusion, facilitated diffusion, or active transport [40]. Blood flow at the site of exchange, pressure and concentration gradients, the thickness of the membranes, and the surface area available for exchange will affect the mechanism and extent of placental

transfer [39-41]. A specific compound's ability to cross the placenta depends on its molecular size, lipid solubility, protein binding, and degree of ionization [36,40,41]. Late pregnancy is associated with a reduction in the thickness of the membrane barrier between the maternal and fetal circulation, thus resulting in a higher permeability for the transfer of compounds across the placenta [36]. For this reason, studies targeting drugs that are commonly administered in late pregnancy or during labor must be done in animals that are nearing the end of their gestation.

The use of anti-viral drugs has been on the rise since the approval of zidovudine (AZT) for use in pregnant women. Acyclovir, an anti-herpes simplex compound, has been increasingly used in pregnancy as evidence of its safety and efficacy accumulates. Animal studies indicate that acyclovir is not a carcinogen, mutagen, or teratogen, and a collection of registered pregnancies during which acyclovir was used indicates no detrimental effects on the fetus [42,43]. As the number of genital herpes cases increases, the number of female users (of reproductive age) of acyclovir has increased to account for more than 50% of the totality of the drug's use [42]. Studies with the dually perfused isolated human placenta cotyledon model show that acyclovir crosses the placenta by means of passive diffusion, but because of its physiochemical properties and similarity to endogenous nucleotides, some suspect that movement of acyclovir across the placenta may be facilitated by a number of transporters [42,44,45]. Since acyclovir is highly polar, it will inherently cross the placenta more slowly than other anti-virals [36]. Because of its ability to prevent the possible manifestation of a life-threatening disseminated herpes-simplex infection in the neonate, acyclovir will continue to be used in pregnancy. An understanding of the extent and the mechanism of placental transport

of this and other anti-virals may contribute to the development of more effective therapies for the prevention of *in utero* and *intra partum* transmission of viruses.

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PART I

CHAPTER 1

A VALIDATED GC-MS ASSAY FOR THE QUANTITATION OF TRICHLOROETHYLENE (TCE) FROM DRINKING WATER[1]

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Abstract

Trichloroethylene (TCE) is a common ground and surface water contaminent found in the United States. A validated GC-MS assay for the quantitation of trichloroethylene (TCE) in drinking water is presented here. The limit of quantitation, 5 ng/mL, is lower than current validated methods for the analysis of TCE from water. This assay requires a small sample volume, has simple sample preparation, fast run time, high recovery, and reproducible and accurate results.

Introduction

Trichloroethylene (TCE) is a common industrial solvent that has been used for over 100 years as a metal degreaser, anesthetic, chemical intermediate, and dry cleaning agent (1,2). The presence of TCE in the environment can be attributed to industrial discharge of the chemical to and leaching from hazardous waste sites (1). As a result of its widespread and long-term use, TCE can be found in groundwater at more than 50% of the hazardous waste sites on the United States Environmental Protection Agency's National Priorities List (1). A 1989 survey indicated that TCE could be found in more then 34% of municipal drinking water supplies in the United States (3). Concentrations found in U.S. water supplies vary from levels below the EPA's acceptable limit (5 ppb) to levels up to 239 ppb and 267 ppb in contaminated sites of Tuscon, AZ and Woborn, MS respectively (1,4,5). TCE is also one of the chemicals found to be prevalent in blood samples from the general population, detectable in 10% of the samples taken in the Third National Health and Nutrition Examination (NHANES III) conducted by the U.S. Centers

for Disease Control (6). Exposure to TCE has been linked to CNS depression, cardiac arrythmias, and some cancers (1,7,8).

The U.S. EPA currently uses a GC-ECD method for the analysis of TCE from drinking water (9). This method requires a liquid-liquid extraction with methyl-tert-butyl ether (MTBE); however, the EPA recognizes the potential for this solvent to be contaminated with TCE. As a result, multiple distillations of the MTBE may be required prior to analysis, thus delaying analysis of highly volatile samples. Other groups report very low limits of quantitation for TCE from water, but provide no information on assay validation (10,11). Large samples sizes (up to one liter) are also required by some methods to attain reported limits of detection (11).

Experimental

Materials

Analytical grade TCE was purchased from Aldrich (Milwaukee, WI, USA). Anhydrous diethyl ether was purchased from J.T. Baker (Phillipsburg, NJ, USA). Perfluorokerosene was obtained from Sigma (St. Louis, MO, USA). The deionized water was generated from a Continental Deionized Water System (Natick, MA, USA).

Instrumentation

A Hewlett Packard (Agilent) 5890 Series II gas chromatograph (Palo Alto, CA, USA) interfaced with a Micromass AutoSpec Magnetic Sector with an electron ionization source (Manchester, UK) was used for all GC-MS experiments. The resolution of the

mass spectrometer was kept at 1500 and the electron energy at 70 eV. A LEAP Technologies CTC-A200S Autosampler (Carrboro, NC, USA) with an SGE gas-tight syringe (Victoria, Australia) was used for sample introduction. A DB-5ms capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) from J & W Scientific (Palo Alto, CA, USA) was used for all chromatographic separations. The GC temperature program was isothermal for 4 minutes at 35°C with TCE eluting at ~3.5 minutes. The injector was kept at 100°C. Each sample injection volume was 2 μL.

Procedure

A stock solution of 10 μg/mL TCE was prepared in deionized water. From the stock solution, dilutions of 1 μg/mL, 600 ng/mL, 400 ng/mL, 200 ng/mL, 100 ng/mL, 60 ng/mL, 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, and 1 ng/mL were made in deionized water. Dilutions of 750 ng/mL, 75 ng/mL, and 7.5 ng/mL were also made for use in the assessment of assay precision and accuracy. A new set of stock and standard solutions were made on each day of validation.

Samples were prepared by adding 200 μL diethyl ether to 200 μL of water sample into a conical bottomed glass vial. Samples were capped and vortexed for 15 seconds using a Scientific Industries Vortex Genie 2 (Bohemia, NY, USA). Once phase separation had occurred, the ether layer was transferred to an autosampler vial and analyzed.

The mass spectrometer was calibrated daily using perfluorokerosene (PFK). The SIR Voltage experiment (equivalent to Selected Ion Monitoring or SIM) was used in the

quantitation of TCE. The PFK peak of m/z 130.99202 was used as a lock mass for the monitoring of the TCE molecular ion, m/z 129.9144.

The assay was validated over three different days. A ten-point calibration curve was generated on each day with the following calibration points: 1 μg/mL, 600 ng/mL, 400 ng/mL, 200 ng/mL, 100 ng/mL, 60 ng/mL, 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL. Blanks of the deionized water and a 1 ng/mL limit of detection (LOD) sample was run on each day of validation. The LOD was determined by a 3:1 signal to noise ratio. Five replicate samples of 750 ng/mL, 75 ng/mL, and 7.5 ng/mL were prepared each day to test precision and accuracy. Each calibration and validation sample was injected in duplicate. Precision was expressed in terms of relative standard deviation: % RSD = 100 * (st.dev./mean). Accuracy (% Error) was expressed as the percent difference between the theoretical concentrations and the experimental concentrations of the replicate samples in each validation set. Microsoft Excel was used to generate linear regression equations for the calibration curves and to calculate % RSD and % Error for each validation set.

Results and Discussion

Sample chromatograms for a blank water extract and a water extract at the limit of quantitation (5 ng/mL TCE) are shown in Figure 1.1 The absence of interfering matrix peaks is attributed to the use of the SIR Voltage experiment for monitoring TCE. An external calibration technique is used because addition of a second analyte to the experiment would ultimately lower the sensitivity of the assay. The possibility of using deuterated (d^1) TCE was investigated, but the increase in resolution required to resolve

d^1-TCE from the PFK calibrant peak at m/z 130.99202 would have drastically lowered the sensitivity.

Several chemicals were tested for possible liquid-liquid extraction solvents including MTBE, chloroform, n-hexane, toluene, isooctane, ethyl acetate, and petroleum ether. The highest recoveries of TCE were obtained with MTBE and diethyl ether extraction; however, MTBE was not ultimately chosen for the extraction solvent because of the high frequency of TCE contamination of different MTBE batches. As with MTBE, many other commercially available solvents also had the consistent problem of high TCE background levels. In some cases, diethyl ether batches contained trace quantities of TCE, but due to the wide range between diethyl ether and TCE boiling points, the TCE could be removed with a single simple distillation.

Over the three days of validation, the assay demonstrated % RSD and % Error < 15%. This data is shown in Table 1.1. Recovery was evaluated at the 100 ng/mL level by comparing 5 water extracts with ether standards of the same concentration. The recovery for the assay was 96.9 $\pm$ 3%, which was higher than that reported by the U.S. EPA (7).

Conclusions

This assay is a fast, simple, and reproducible way to measure a wide range of TCE concentrations in drinking water. The recovery for diethyl ether liquid-liquid extraction is high, and the use of this solvent minimizes the concern for TCE contamination. Unlike other methods for measuring TCE from water, this assay has been validated over three days demonstrating a % RSD < 13% and % Error < 15%.

Acknowledgements

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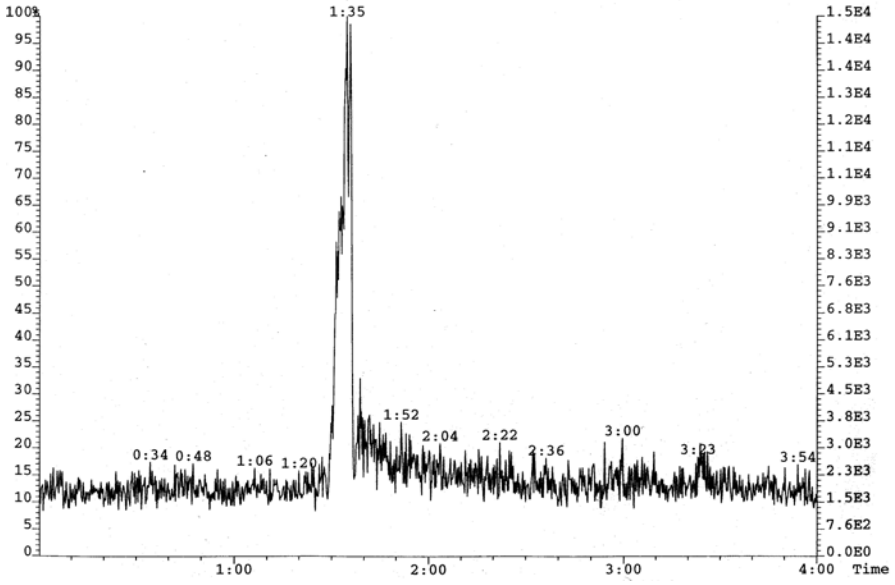
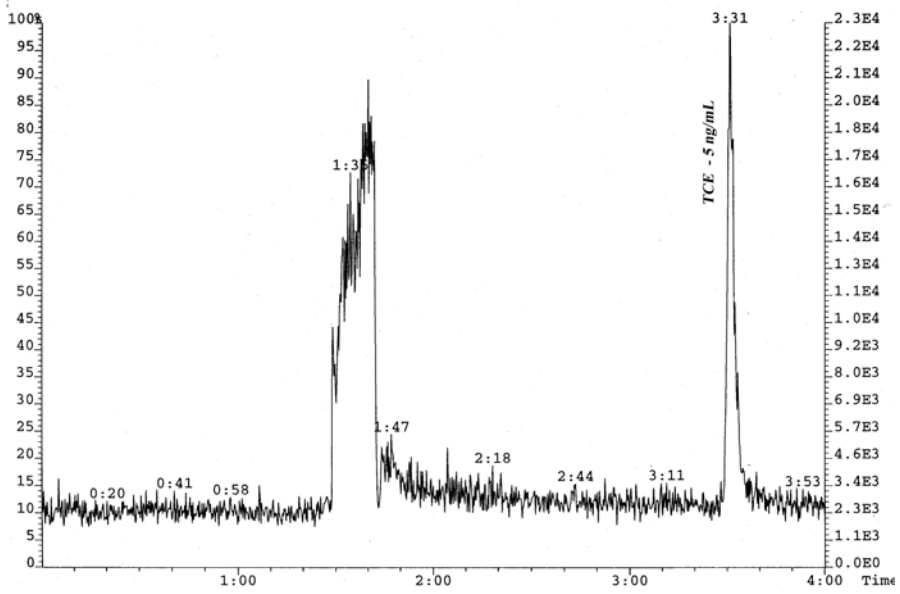
Figure 1.1

(a)



(a) Blank water extract

(b)



(b) Spiked water extract at the 5 ng/mL level

Table 1.1

The precision (% RSD) and accuracy (% Error) of TCE quantitation from water over 3 days (n = 30 for each validation concentration)

Concentration TCE added (ng/mL)	Concentration TCE found (ng/mL) average $\pm$ st. dev.	% RSD	% Error
7.5	7.92 $\pm$ 0.59	7.46	7.73
75	72.8 $\pm$ 7.8	10.7	8.52
750	648.2 $\pm$ 81.1	12.5	14.7

CHAPTER 2

TRACE LEVEL DETERMINATION OF TRICHLOROETHYLENE FROM LIVER, LUNG, AND KIDNEY TISSUES BY GAS CHROMATOGRAPHY/MAGNETIC SECTOR MASS SPECTROMETRY[1]

[1]Brown, S.D., Muralidhara, S., Bruckner, J.V., and M.G. Bartlett. Submitted to *The Journal of Chromatography B*, 05/02

Abstract

Trichloroethylene (TCE) is a common industrial chemical that has been heavily used as a metal degreaser and a solvent for the past 100 years. As a result of the extensive use and production of this compound, it has become prevalent in the environment, appearing at over 50% of the hazardous waste sites on the U.S. EPA's National Priorities List (NPL). TCE exposure has been linked to neurological dysfunction as well as to several types of cancer in animals. This paper describes the development and validation of a gas chromatography/mass spectrometry (GC/MS) method for the quantitation of trace levels of TCE in its target tissues (i.e. liver, kidney, and lungs). The limit of quantitation (5 ng/mL) is substantially lower than currently published methods for the analysis of TCE in tissues. The % RSD and % Error for the assay falls within the acceptable range (<15% for middle and high QC points and <20% for low QC points), and the recovery is high from all tissues (>79%).

Introduction

Trichloroethylene is most commonly used in industrial settings as a general-purpose solvent for lipophilic compounds and to remove grease from machinery. Known by the trade names of Vitran® and Triclene®, TCE also has many applications in household products, dry cleaning, taxidermy, and as a chemical intermediate [1,2]. Environmental releases of TCE are most commonly associated with vapor degreasing operations, but can also be linked to waste and water treatment facilities and landfills [2]. TCE contamination of ground and surface waters is a result of industrial discharge or leaching from hazardous waste sites [1]. According to the Third National Health and

Nutrition Examination (NHANES III), an estimated 10% of the U.S. population has detectable levels of TCE in their blood [3]. Pharmacokinetic models relating environmental concentrations of TCE to body burdens suggest that the prevalence of TCE in the general population is a result of multiple exposure routes including water ingestion, inhalation, dermal absorption, and ingestion of TCE-contaminated food [4].

The main health risk associated with mild acute TCE exposure is central nervous system (CNS) depression. At vapor levels higher than 100 ppm, CNS effects such as sleepiness, headache, and dizziness can occur [1,5]. Coma, cardiac arrythmias, and even death are associated with very high acute TCE exposures [1]. Chronic rodent studies and epidemiological evidence suggests that chronic, high-level TCE exposures may cause liver, kidney, and lung cancer. There is more limited epidemiological evidence of increased incidences of non-Hodgkin's lymphoma, cervical cancer, testicular cancer, and multiple myeloma in humans [1,6]. Although TCE is a known carcinogen in rats and mice, it has been officially classified by the National Toxicology Program (NTP) and by the International Agency for Research on Cancer (IARC) as a "probable carcinogen in humans" because of the limited epidemiological data to support TCE as a cause of cancer in humans [6,8,9]. Although the subject is controversial, a number of leading authorities feel that environmentally-relevant concentrations of TCE are not likely to be a significant cancer risk [7,8,10].

Several analytical methods exist for the quantitation of TCE in water. The EPA has a GC-ECD method for determination of TCE and several other halogenated hydrocarbons that uses liquid-liquid extraction sample preparation [11]. The recommended extraction solvent for this method is methyl-t-butyl ether (MTBE), but this

solvent is frequently contaminated with traces of TCE. Karp (1994) describes a method with a TCE detection limit of 1 ng/mL in water, but there is no mention of validation or the type of instrument that was used [12]. Zoccolillo and Rellori report quantitating TCE at levels below 1 ng/L, but their method is not validated and requires a sample of at least one liter. This far exceeds volumes of sample that can be secured for a bioanalytical assay [13]. Purge-and-trap instrumentation has also been utilized to analyze trace levels of TCE and similar compounds, but these procedures involve time-consuming methods [14,15].

Quantitation of drugs or chemicals in a biological matrix is much more difficult than analysis in water. Chen et al. describe a GC-ECD method that is useful for analyzing TCE in several tissues including liver, kidney, and lungs. They indicate a limit of detection of 50 ng/mL, which is expressed as 1 ng on-column [16]. Muralidhara and Bruckner report a rapid assay for the measurement of TCE and its metabolites from blood [17]. Their LOQ is 50 ng/mL but it lacks complete validation data.

The ability to monitor the time-course of TCE in tissues is of particular importance to toxicologists and risk assessors. There is a limited amount of pharmacokinetic data generated from relatively high-level TCE exposures found in occupational settings. It has not been possible, however, to study the systemic uptake and disposition of trace levels of TCE typically encountered in environmental media (i.e., air and water). An assay that can accommodate the exposures at the lower end of the dose-response curve is needed to help provide more accurate information for cancer risk assessments. Recent papers on the development of physiologically-based pharmacokinetic (PBPK) models for TCE state that tissue concentration data for the

three primary target organs (i.e. liver, kidney, and lungs) would be necessary to develop and validate useful models [18,19]. The present paper describes an assay that may help meet the needs of the toxicologists and kineticists who struggle to obtain such data. This method has the potential for high throughput of samples with its simple extraction procedure and fast run-time. It is more sensitive than previously reported assays for quantitation of TCE from tissues and requires a very small sample size. Most importantly, this assay has been validated to measure TCE concentrations in three target tissues, thus guaranteeing precision and accuracy at environmentally-relevant exposure levels.

Experimental

Reagents and Chemicals

Analytical grade trichloroethylene (TCE) was purchased from Aldrich (Milwaukee, WI, USA). Reagent grade anhydrous diethyl ether was obtained from J.T. Baker (Phillipsburg, NJ, USA). The perfluorokerosene used as a calibrant for the mass spectrometer was purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA). The helium used as a carrier gas for the GC was purchased from National Welders (Charlotte, NC, USA). Alkamuls, the emulsifying agent used in preparing the doses for the animal study, was obtained from Rhone-Poulenc (Cranbury, NJ, USA).

Preparation of Stock and Standard Solutions

A stock solution of TCE was prepared in deionized water to yield a final concentration of 10 μg/mL TCE. Standard solutions for the calibration curve were prepared from the stock solution in the following concentrations: 1 μg/mL, 600 ng/mL, 400 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10ng/mL, 5ng/mL, and 1 ng/mL. Standards used to assess precision and accuracy were prepared in deionized water from the 10 μg/mL stock solution in concentrations of 750 ng/mL, 75 ng/mL, and 7.5 ng/mL. All stock and standard solutions were refrigerated at 4^{o}C during the day of use and were prepared fresh daily.

GC/MS System and Conditions

All GC experiments were conducted with the use of a Hewlett Packard (Agilent) 5890 Series II gas chromatograph (Palo Alto, CA, USA) interfaced with a Micromass AutoSpec Magnetic Sector Mass Spectrometer (Manchester, UK). The electron energy in the electron ionization source of the mass spectrometer was set at 70 eV. A resolution of 1500 was used. The mass spectrometer was calibrated daily using perfluorokerosene (PFK). All samples were injected using a LEAP Technologies CTC-A200S Autosampler (Carrboro, NC, USA). Chromatographic separations were achieved on a DB-5ms capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) from J &W Scientific (Agilent, Palo Alto, CA, USA). The temperature program for the GC was isothermal heating at 35^{o}C for four min. The injector temperature was set at a constant 100^{o}C. Helium was used as the carrier gas. The retention time for TCE was ~3.5 min.

Quantitation

TCE peaks (m/z 129.9144) were monitored using the SIR Voltage experiment in the Micromass OPUS software (equivalent to Selected Ion Monitoring or SIM) using a PFK peak of m/z 130.99202 as the lock mass. Concentrations of TCE in real samples were calculated using an external calibration curve prepared with spiked blank tissue homogenates. JMP® statistical software was used to generate linear regression equations for all calibration curves. Each curve contained the following points (n = 9): 1 μg/mL, 600 ng/mL, 400 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL, and 5 ng/mL.

Liquid-Liquid Extraction

All tissue samples were prepared using liquid-liquid extraction with anhydrous diethyl ether. Prior to extraction, tissues were homogenized with two volumes of deionized water (w/v) using a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). One hundred μL of tissue homogenate plus 200 μL of ether (or 100 μL blank tissue homogenate plus spike solution plus 200 μL ether) were combined in a glass tube for extraction and sealed with parafilm. Plastic tubes were found to adsorb TCE to some extent; therefore glass tubes were used consistently throughout the experiments. The tissue/ether mixture was vortexed for 10 s using a Scientific Industries Vortex Genie 2 (Bohemia, NY, USA). The samples were then centrifuged at 2200 g, 4^{o}C for 15 min in a Jouan CR422 refrigerated centrifuge (Winchester, VA, USA). The ether layers were immediately transferred to autosampler vials and analyzed. The samples were always

kept on ice during the physical transfer of sample vials due to the highly volatile nature of TCE.

Solvent Selection

During the method development stage of this project, several solvents were investigated as potential liquid-liquid extractants. Initially methyl-t-butyl ether (MTBE) was used according to the EPA Method 551.1 for drinking water analysis [10]. Upon observation of a high response for TCE from the "blank" solvent injections, we discovered that a majority of MTBE batches are highly contaminated with TCE. Multiple fractional distillations became necessary to prepare MTBE for use, and this was ultimately deemed unacceptable. A limited survey of solvents located in our laboratory showed that TCE contamination is not restricted to MTBE (see Table 2.1). Finally, diethyl ether was chosen as the best extraction solvent. Not only does it provide acceptable recovery, but it also can be purified by a single distillation.

Sampling

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, NC, USA) weighing an average 277 $\pm$ 11 g (n = 30) were used for a tissue disposition study. An emulsion of 0.55 mg/mL TCE was prepared by combining 15.2 μL pure TCE with 2.0 mL Alkamuls® and 38.0 mL of physiological saline. An appropriate volume of the emulsion, based on the weight of each rat, was administered to yield a final dose of 2.0 mg/kg. The animals were divided into six groups of five rats each. Members of each group were dosed orally using a curved gavage needle. Groups were sacrificed by

cervical dislocation at 2, 5, 10, 30, 60, and 120 min post dosing. The liver, kidney, and lungs were perfused *in situ* with cold saline to remove as much blood as possible. Each tissue specimen was weighed and homogenized with two volumes of cold deionized water. All samples were analyzed immediately.

Results and Discussion

Figure 2.1(a,b) shows a representative chromatogram of TCE at 5 ng/mL, the lowest point on the calibration curve (LOQ), extracted from a liver tissue homogenate (spiked with 5 ng/mL) and a chromatogram from a blank (liver) tissue extract. Because the experiments were done using the SIR Voltage function, no interfering matrix peaks can be seen. This also helps maximize sensitivity of the assay by eliminating the need to scan a large range of masses.

Calibration curves were produced during each day of validation and during the analysis of the samples from the animal study. Since the calibration curve encompassed such a wide range (5 ng/mL – 1 μg/mL), the points on the curves were weighted by a factor of "1/y" using JMP® Statistical Software to ensure that all points contributed equally to the slope of the regression line. The range of concentrations in the curve encompasses the range of concentrations present in the various tissues in a 2-hr period following administration of the 2 mg/kg oral bolus dose.

The limit of detection (LOD) for TCE in the tissue matrices was determined to be 1 ng/mL according to the 3:1 signal/noise ratio seen at this concentration. The assay was validated by analyzing five replicates of three different concentrations of TCE in spiked

tissue over a period of three days. The concentrations of 7.5 ng/mL, 75 ng/mL, and 750 ng/mL were chosen to represent low, middle, and high portions of the curve. The precision (%RSD) represents the reproduceability of the assay while the accuracy (% error) shows how well the assay can predict concentrations correctly. Table 2.2 summarizes the validation data that were collected. All % RSD and % error values were under fifteen percent for the middle and high QC points and below twenty percent for the lowest QC point for each day.

Recovery of TCE from the various tissues was measured by comparing the responses from spiked samples to the responses from ether standards. Five samples from each matrix homogenate were each spiked with 100 ng/mL TCE. The peak heights from each of these was compared to the peak heights of five ether standards. The recovery from lung and kidney was > 79% and the recovery from liver was > 87%. The results from this experiment are presented in Table 2.3.

The lung, liver, and kidney tissues that were collected from the test rats were extracted and analyzed as described above. The peak heights of the TCE peaks from the real samples were compared to the calibration curve to calculate concentrations of TCE in these target tissues. Figure 2.2 shows a concentration versus time profile of TCE in the three tissue matrices. The elimination phase in these tissues is rather lengthy compared to the distribution phase. Some time points in the latest group (120 min) approached the limit of quantitation for this assay. The profiles shown here are very similar to concentration-time profiles of TCE in blood reported previously [20].

Conclusions

A sensitive, efficient, and validated method for the extraction and analysis of TCE in liver, kidney, and lung tissues is described. This method yields acceptable recovery, precision, and accuracy over the calibration range of 5 ng/mL to 1 μg/mL. Liquid-liquid extraction is a quick, efficient way to minimize evaporation of the volatile TCE analyte during preparation of tissue samples for GC/MS analysis. The use of the SIR Voltage function in the data acquisition capabilities of the mass spectrometer enables the quantitation of trace levels of TCE due to the low noise level and the absence of interfering matrix peaks. The most sensitive assay for quantitating TCE from biological matrices is the purge-and-trap MS method used by the CDC [15]. By starting with a 5 mL blood sample, the CDC assay reaches an LOD of 5 pg/mL. The method reported in this manuscript begins with a much smaller sample volume and is still capable of reaching an LOD of 1 ng/mL. Although slightly less sensitive than the CDC method, this assay is capable of much higher throughput. This assay can effectively be applied to the quantitiation of trace levels of TCE in tissue samples.

Acknowledgements

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Table 2.1

Estimated trichloroethylene levels found in various solvent types.

Solvent type	Estimated TCE concentration (ng/mL)	Manufacturer
Acetonitrile	1.21	J.T. Baker
Acetonitrile	1.80	J.T. Baker
Acetonitrile	2.11	Fisher
Acetonitrile	1.97	Aldrich
Acetonitrile	1.46	Fisher
Methyl-t-butyl ether	730.6	Aldrich
Methyl-t-butyl ether	1.75	Aldrich
Diethyl ether	4.17	J.T. Baker
Diethyl ether	1.00	J.T. Baker
Diethyl ether	0.378	J.T. Baker
Heptane	3.39	E.M. Science
n-Hexane	2.38	J.T. Baker

Table 2.2

The precision (% RSD) and accuracy (% error) of TCE in rat liver, kidney, and lung tissue.

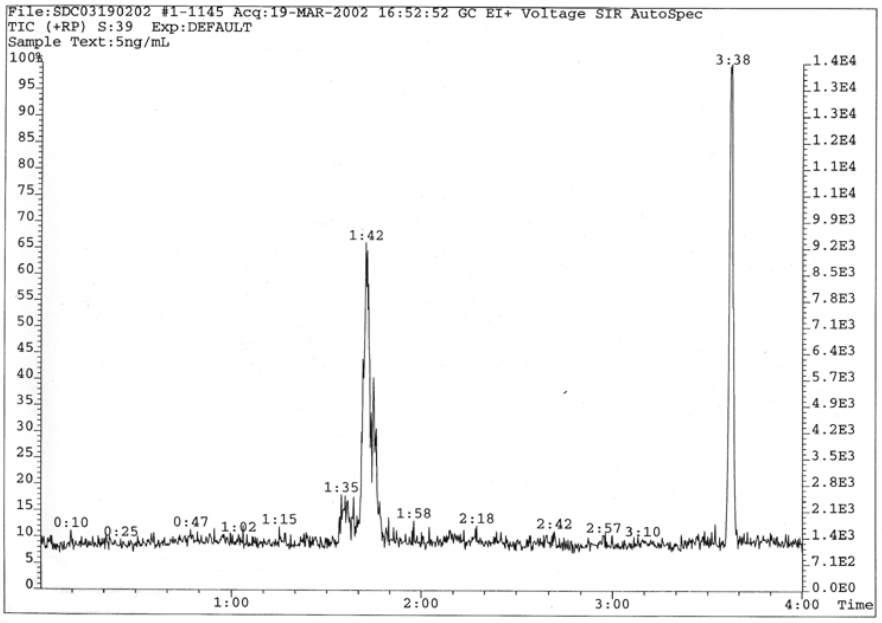
Liver Tissue Validation (n = 15)			
[ ] TCE added (ng/mL)	**[ ] TCE found (ng/mL)**	**% RSD**	**% Error**
7.5	7.93 ± 1.6	19.9	18.6
75	75.7 ± 8.7	11.4	9.83
750	766.2 ± 110	14.4	12.3
Lung Tissue Validation (n =15)			
[ ] TCE added (ng/mL)	**[ ] TCE found (ng/mL)**	**% RSD**	**% Error**
7.5	7.15 ± 0.87	9.27	12.8
75	74.5 ± 9.2	3.61	10.4
750	718.6 ± 99	9.48	11.9
Kidney Tissue Validation (n = 15)			
[ ] TCE added (ng/mL)	**[ ] TCE found (ng/mL)**	**% RSD**	**% Error**
7.5	7.26 ± 0.86	8.59	8.95
75	76.0 ± 8.9	8.67	11.0
750	715.7 ± 84	9.24	13.8

Table 2.3

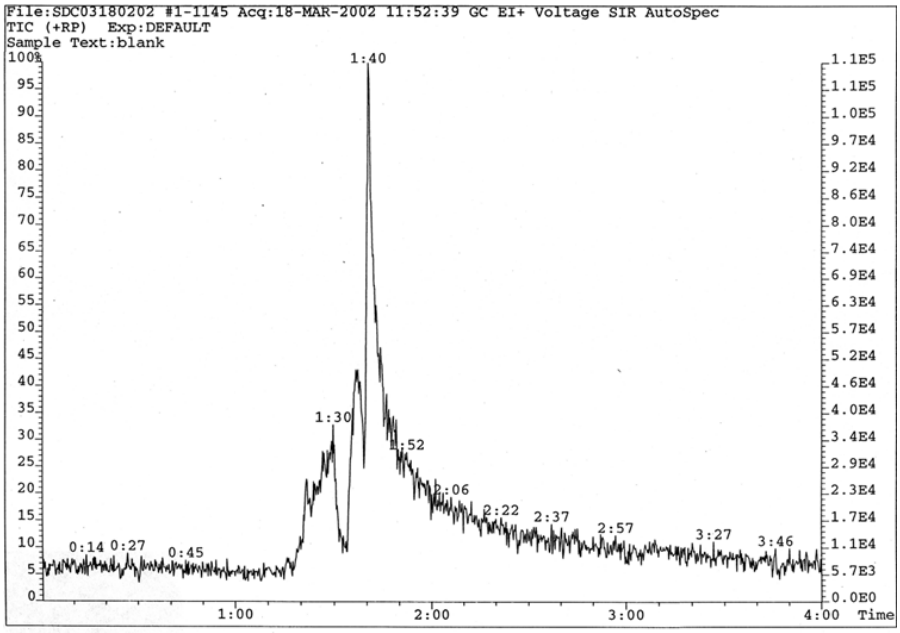
The % relative recovery (± standard deviation) of liver, kidney, and lung tissues spiked with 100 ng/mL TCE (n = 5 for each matrix) as compared to ether standards of 100 ng/mL

Liver	Kidney	Lung
87.23 ± 2.78	79.93 ± 14.2	79.20 ± 10.8

Figure 2.1

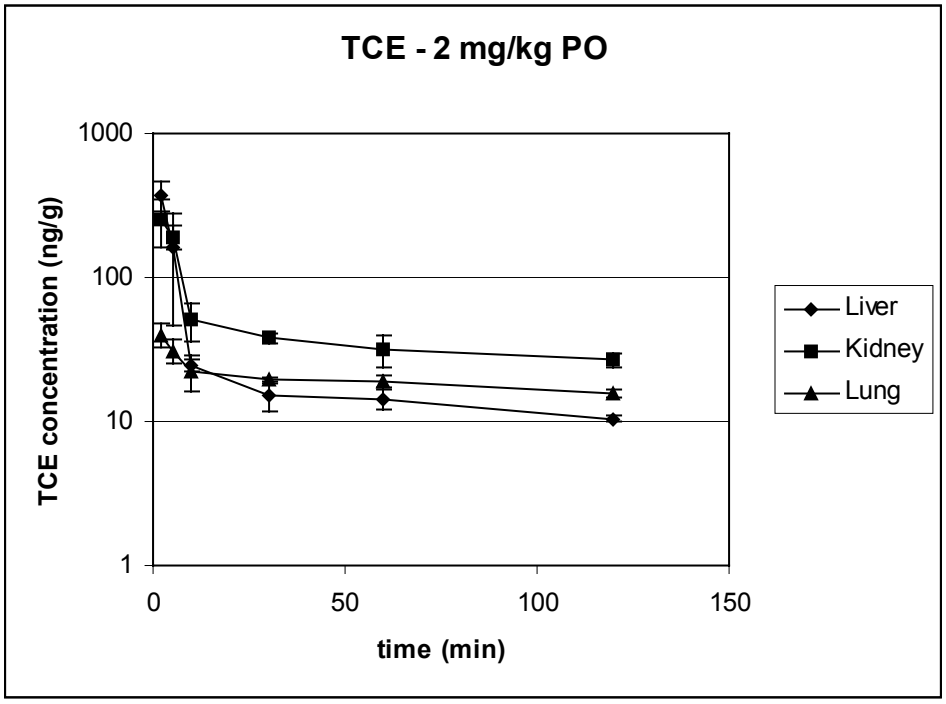


(a) Representative chromatogram of 5 ng/mL TCE from liver homogenate



(b) Representative chromatogram of blank liver extract

Figure 2.2



Concentration versus time profile of liver, kidney, and lung concentrations of TCE from rats dosed with 2 mg/kg oral TCE (mean concentration $\pm$ standard deviation, n = 5 for each time point)

PART II

CHAPTER 3

DETERMINATION OF ACYCLOVIR IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL AND PLACENTAL TISSUES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[1]

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[1]Brown, S.D., White, C.A., Chu, C.K., and M.G. Bartlett. 2002. *Journal of Chromatography B*. 772(2): 327-334.
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Abstract

Acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanosine, Zovirax®, ACV) is a synthetic purine nucleoside analog active against herpes simplex virus types 1 (HSV-1), 2 (HSV-2), and varicella zoster virus. Acyclovir has frequently been used in HSV-2 seropositive mothers to prevent prenatal transmission of herpesvirus to their unborn children. A fast and reproducible HPLC method for the determination of the highly polar acyclvoir in maternal rat plasma, amniotic fluid, placental tissue, and fetal tissue has been developed and validated. Plasma and amniotic fluid samples were prepared by protein precipitation using 2 M perchloric acid and syringe filtering. Tissue samples were homogenized in distilled water, centrifuged, and extracted using a C-18 solid phase extraction (SPE) method prior to analysis. Baseline resolution was achieved for acyclovir and the internal standard ganciclovir, an anti-viral of similar structure to acyclovir, using an Agilent Eclipse XDB C-8 column (150 x 2.1 mm, 5 μm). The mobile phase used for the plasma and amniotic fluid was 10 mM acetate/citrate buffer: 3.7 mM aqueous octanesulfonic acid (87.5:12.5 v/v) at a flow rate of 0.2 mL/min. The mobile phase used for the tissue samples was 30 mM acetate/citrate buffer with 5 mM octanesulfonic acid:acetonitrile (99:1 v/v). Both aqueous mobile phase portions were pH adjusted to 3.08. All separations were done using an Agilent 1100 Series HPLC system with ultra-violet (UV) detection of 254 nm. The assay was validated for each matrix over a range of 0.25 μg/mL – 100 μg/mL over three days using five replicates of three spiked concentrations. The relative standard deviation and percent error for each validation data set was <15% for middle and high QC points and <20% for all low QC points. All

calibration curves showed good linearity with an $R^2 > 0.99$. The extraction efficiency for recovery of acyclovir from all matrices was > 80%.

Introduction

Herpes Simplex Virus – 2 (HSV-2), also known as genital herpes, is one of the most common viral infections in humans. HSV-2 affects 20-25 million people in the United States, with approximately 500,000 new cases reported each year [1]. In adults of reproductive age, this accounts for a seroprevalence of HSV-2 of 16 – 22% [2]. HSV-2 is characterized by cycles of viral latency and subsequent reactivation that remain with the infected individual for the duration of his or her life [2,3]. Although there is no cure for genital herpes, several anti-viral compounds have been introduced which decrease the frequency of episodes of active lesions. Acyclovir, 9-[(2-hydroxyethoxy)-methyl]-guanosine, is the most widely used of these anti-virals either in its original form (Zovirax®) or as the pro-drug valacyclovir (Valtrex®) because it has been shown to be effective in the treatment of HSV-1, HSV-2, and varicella zoster virus [4]. It is widely tolerated in different populations and disease states, and has a high therapeutic index, possibly due to its highly selective biological activity [3,4].

Although acyclovir has not been officially approved for use in pregnancy, many obstetricians prescribe oral acyclovir for HSV-2 positive mothers to reduce the possibility of an episode immediately preceding delivery or to help prevent *in utero* transmission. Since 85% of neonatal herpes cases are acquired as a result of passage through an infected birth canal, most HSV-2 pregnant women undergo a cesarean section instead of

a vaginal delivery [2,5]. However, due to the numerous case studies reporting the successful use of acyclovir to suppress HSV-2 during pregnancy without evidence of toxicity to the newborn, many physicians feel the risks of cesarean delivery are much greater than those associated with the use of acyclovir [2, 5-9].

Although the safety and efficacy of acyclovir use during pregnancy has been demonstrated though case studies and the *Acyclovir in Pregnancy Registry*, little is known about the placental transfer of acyclovir [5-9]. Even at the clinical trial stage of acyclovir, placental and fetal drug distribution data is not obtained because pregnant women are excluded from clinical trials [10]. Some groups have attempted to characterize acyclovir transfer using the perfused human placenta model [11-12]. Although the results of these studies are interesting, they do not necessarily translate well to *in vivo* drug behavior. If human data from ACV dosed pregnant women was collected, the matrices gathered for analysis would be limited to maternal plasma, placenta, and possibly amniotic fluid, but a sample of fetal tissue could never be included. For this reason, an animal model that accurately represents the placental mechanisms of humans must be utilized. Previously, a pregnant rat model was developed and used in the study of the placental transfer of nucleoside analogs as well as a variety of other compounds [13-21]. This model is relevant because of the similar changes seen in the hemochordial placenta and the hemodynamic pregnancy for rats and humans [14,22]. The containment of each rat pup in an individual fetal sack and the large litter size also make it a useful model for serial sampling in pharmacokinetic studies.

Several HPLC methods exist for the quantitation of ACV from plasma, serum, and urine [23-35]. Some of these methods require more specialized equipment like

fluorimetric detection [30] or extremely large sample volumes [23,25,26,34]. Depending upon the internal standard chosen for the method, run time can also be lengthy [31]. Solid phase extraction (SPE) is commonly used as a sample clean-up technique, but may not always be necessary for relatively simple matrices [24,30,33]. Radioimmunoassays (RIA) and enzyme-linked immunosorbant assays can also be found for acyclovir [35,36,37]. While sensitive, these assays require specialized reagents and can be lengthy. This paper reports an efficient and reproducible HPLC-UV method that has been developed and validated for quantitating acyclovir from maternal plasma, amniotic fluid, fetal tissue, and placental tissue collected during a maternal – fetal drug transfer study. The assay reported here is the first to report quantitation of acyclovir from such complex tissue matrices. It requires small plasma sample volumes in order to maximize the number of pharmacokinetic time points that can be collected from the rat model. Sample preparation for the plasma and amniotic fluid samples is a simple protein precipitation, thus saving both time and money. This study utilized the pregnant rat model where all samples of four biological matrices were collected at various time-points to get a complete profile of the drug's distribution across the placenta.

Experimental

Reagents and Chemicals

Analytical standards of acyclovir and the internal standard, ganciclovir, were obtained from Sigma (St. Louis, MO, USA). Reagent grade citric acid was acquired from Sigma as well. Reagent grade ammonium acetate and reagent grade octanesulfonic acid were bought from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile and

methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sep-Pak Vac 1 cc C-18 cartridges were purchased from Waters (Milford, MA, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

Preparation of Stock and Standard Solutions

Appropriate amounts of ganciclovir and acyclovir were weighed and added to deionized water to yield final stock solution concentrations of 1.0 mg/mL. Acyclovir standard solutions were prepared with deionized water from the 1.0 mg/mL ACV stock to yield final concentrations of 750, 500, 100, 50, 25, 10, 5, 2.5, 1 μg/mL. A 100 μg/mL ganciclovir standard solution was prepared with deionized water from the 1.0 mg/mL GAN stock. Stock solutions were kept refrigerated when not in use and replaced on a bi-weekly basis. The stock solutions were assumed to be stable over a period of two weeks due to the low degree of variability (< 5% RSD) during that time. Fresh standard solutions were prepared for each day of analysis or validation.

Chromatographic System

The HPLC system consisted of Hewlett-Packard (Agilent) 1100 Series components including a quaternary pump, degasser, autosampler, and variable wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Agilent Eclipse XDB C-8 column (150 x 2.1 mm, 5 μm) (Palo Alto, CA, USA) with a Phenomenex Security Guard C-18 guard column (Torrance, CA, USA).

Chromatographic Conditions

The mobile phase used for the plasma and amniotic fluid matrices was 10 mM acetate/citrate buffer: 3.7 mM aqueous octanesulfonic acid (87.5:12.5 v/v) adjusted to pH 3.08 with phosphoric acid. The retention times under these conditions were ~8 min for GAN and ~11 min for ACV (see Figure II). The mobile phase used for the placental and fetal tissue samples was 30 mM acetate/citrate buffer with 5 mM octanesulfonic acid (pH 3.08) and acetonitrile (99:1 v/v). Under these conditions, GAN eluted at ~10 min and ACV eluted at ~12 min. A different mobile phase was required for the tissue matrices due to the greater number of endogenous peaks present that had to be separated from the analytes. All flow rates were kept at a constant 0.200 mL/min and the detection wavelength was fixed at 254 nm.

Calibration Curves

Blank plasma, amniotic fluid, placenta, and fetal tissue was collected from untreated anesthetized animals. The placenta and fetal tissues were minced and homogenized with two volumes of deionized water (w/v) using a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). Plasma calibration points were prepared by spiking 100 μL of plasma inside a 1.5 mL centrifuge tube with 10 μL of each acyclovir standard and 10 μL of the 100 μg/mL ganciclovir standard solution. Amniotic fluid calibration points were prepared by spiking 50 μL of fluid inside a 1.5 mL centrifuge tube with 5 μL of each acyclovir standard and 5 μL of the 100 μg/mL ganciclovir standard. Placental calibration samples were prepared using 200 μL of placental homogenate inside a 1.5 mL centrifuge tube spiked with 20 μL of each acyclovir standard

and 20 μL of the 100 μg/mL ganciclovir solution. Finally, fetal calibration standards were prepared using 300 μL of fetal homogenate inside a 1.5 mL centrifuge tube with 30 μL of each acyclovir standard and 30 μL of the 100 μg/mL ganciclovir standard solution. Ultimately, the calibration concentrations of acyclovir in each matrix would be as follows: 0.1, 0.5, 1, 5, 10, 50, 100 μg/mL with an internal standard concentration in each sample of 10 μg/mL. After each matrix was spiked, it was subject to further sample preparation before analysis.

Sample Preparation

Plasma and amniotic fluid samples were prepared with protein precipitation and filtration. After spiking, samples were vortexed briefly and 20 μL of 2 M perchloric acid (plasma) or 10 μL of 2 M perchloric acid (amniotic fluid) was added. The tubes were centrifuged for 10 min at 16,000 g using a Biofuge Pico Microcentrifuge (Heraeus Instruments, Hanau, Germany). After centrifuging, the supernatent was removed and filtered using either XPertek® syringe filters, 0.22 μm nylon filter (St.Louis, MO) or CoStar SpinX® centrifuge tube filters, 0.22 μm nylon filter (Corning, NY) and the pellet was discarded.

Placental and fetal tissue samples were prepared using solid phase extraction (SPE). The homogenates were vortexed briefly after spiking and were pH adjusted using the aqueous portion of the mobile phase (30 mM acetate citrate buffer with 5 mM octansulfonic acid, pH 3.08) by adding 300 μL of mobile phase to the fetal homogenates and 200 μL to the placental homogenates. The tubes were vortexed again and centrifuged for 10 min at 16,000 g. Supernatents were loaded onto Sep-Pak C-18 SPE

cartridges that had been preconditioned with 2 mL methanol followed by 2 mL of the aqueous portion of the tissue mobile phase. Samples were washed with 1 mL of deionized water and eluted into clean culture tubes with 3 mL methanol. The eluents were evaporated to dryness in a vacuum centrifuge (Model SC110A, Savant Instruments Inc., Holbrook, NY, USA) and the residues reconstituted in 100 μl of mobile phase. Reconstituted residues were then syringe filtered using 0.22 μm nylon syringe filters. An injection volume of 10 μL was used for all samples.

Sample Collection

The use of animals in this study was approved by the University of Georgia Animal Use and Care Committee. The rats were housed one animal per cage in the University if Georgia College of Pharmacy animal facility (AALAC accredited). The environment was controlled (20 – 22^{o}C, 14 hours of light per day) with daily feedings of standard chow pellets and water *ad libitum*.

Timed pregnant Sprauge-Dawley rats (Harlan, Indianapolis, IN) weighing an average of 333 g ($\pm$ 22 g) were anesthetized intramuscularly with ketamine:acepromazine (75:2.5 mg/kg) and dosed on day 19 of gestation. During anesthesia, animals were given atropine (0.5 mg/kg) subcutaneously. For dosing and blood sampling purposes, a cannula was surgically implanted in the right jugular vein. For sampling of the pups (amniotic fluid, placenta, and fetal tissues), a laparotamy was performed. The dose of acyclovir given to the rats was prepared as a 10 mg/mL solution of acyclovir in 0.1 M NaOH in physiological saline (pH 7.4). The rats were administered the IV bolus dose of acyclovir (60 mg/kg) via the jugular cannula followed by 1mL of phosphate buffered saline (pH

7.4) to rinse the cannula. Blood samples were collected at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, 480 min into heparinized tubes and centrifuged at 16,000 g for 10 min to enable plasma collection. Amniotic fluid, placenta, and fetus samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480 minutes. Placental and fetal tissue samples were homogenized in two volumes of deionized water. All samples were stored at –20^{o}C until analysis.

Results and Discussion

The structures for acyclovir and the internal standard used in this assay, ganciclovir, are shown in Figure 3.1. To achieve baseline resolution of ACV and GAN from each other as well as interfering matrix peaks, the levels of octanesulfonic acid and the concentrations of the buffer were altered until the desired separation was achieved. Figure 3.2 (a-d) shows chromatograms of each matrix spiked with ACV (2.5 μg/mL) and GAN (10 μg/mL). Other anti-virals including AZT (zidovudine), AZDU, 3TC (lamivudine), D4T (stavudine), DDI (didanosine), and DDC (zalcitabine) were run using this method to show that they did not have any interfering peaks.

The calibration curves for each day of validation and analysis showed acceptable linear response ($R^2 > 0.99$) through a range of 0.25 – 100 μg/mL. Microsoft Excel® or JMP® statistical software was used to generate linear regression equations for all calibration curves. Calibration curves for the different matrices are displayed in Table 3.1. The range of 0.25 – 100 μg/mL was sufficient for use in calculating ACV levels from samples taken from rats that were dosed with 60 mg/kg ACV. Concentrations in the

early plasma samples fell outside the range of the curve and had to be diluted prior to analysis.

The extraction efficiency for ACV and GAN from the various matrices is expressed in terms of relative recovery. Standard-spiked matrix samples at the 2.5 μg/mL level were extracted and analyzed (n = 5). An equal number of matrix blanks were extracted and spiked post-extraction. The peak areas of these two sample sets were compared showing high recoveries for both ACV and GAN for all matrices. Acyclovir recovery from maternal plasma, amniotic fluid, placenta, and fetus ranged from 82% to 90%. The relative recoveries for each individual matrix can be found in Table 3.2.

Assay precision and accuracy was calculated for each matrix over a range of three days. Blanks from each matrix were spiked with ACV and GAN to yield final concentrations corresponding with those in the calibration curve. Five replicates of blanks spiked with ACV concentration of 0.25 μg/mL (LOQ), 2.5 μg/mL, and 75 μg/mL were prepared for each validation day to test the precision (% Relative Standard Deviation, %RSD) and accuracy (%Error). According to the analytical method validation criteria set forth by the United States Food and Drug Administration, the assay precision and accuracy was within acceptable limits for each matrix over three days [38]. This validation data is compiled in Table 3.3. The intra-day and inter-day variability of the GAN response (peak area) was less than 10% for the course of the validation.

To demonstrate the utility of this assay, a pregnant rat was dosed with ACV at the level of 60 mg/kg. Maternal plasma, amniotic fluid, placenta, and fetal samples were collected, extracted, and analyzed as described above. A calibration curve from each matrix was prepared on the day of analysis to calculate the concentration of acyclovir

present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 10 μg/mL of the internal standard ganciclovir. The sample peak area ratios of ACV to GAN were used to calculate the concentration of ACV in each sample. Figure 3.3 shows the concentration – time profile of acyclovir in all four biological matrices of the pregnant rat. Figure 4 shows a closer look at the tissue profile of acyclovir in the rat pup. Using WinNonlin®, the ACV half-life was calculated to be 45.7 min. The volume of distribution at steady state of 1.1 L/kg, the total clearance of 1.03 mL/min, and the area under the curve of 14.7 μM·L were also calculated from maternal plasma. All values were in close agreement with previously reported literature values for acyclovir pharmacokinetics in rats [39,40]. Figure 3.4 shows that there is little evidence for extensive clearance of acyclovir from the amniotic fluid. This low clearance from the rat amniotic fluid may correlate with previously reported observations of acyclovir accumulation in human amniotic fluid [12].

Conclusions

Extracting and analyzing such highly polar compounds out of complex biological matrices poses some unique problems. The HPLC assay reported here combats these problems by combining the sample clean-up power of solid phase extraction with the prolonged retention ability acquired with an isocratic separation and an ion-pair mobile phase additive. This assay for the determination of acyclovir from plasma, amniotic fluid, placental homogenate, and fetal homogenate is sensitive, reproducable, and efficient. The extraction methods used yield high recoveries for acyclvoir, and the assay

shows good linearity, precision, and accuracy in the calibration range of 0.25 μg/mL - 100 μg/mL in all four complex biological matrices. The initial pharmacokinetic parameters generated from the maternal plasma showed good correlation with the reported literature values thus demonstrating the utility of this method for pharmacokinetic studies. Further pharmacokinetic investigations will be used to determine the efficiency of which acyclovir crosses the placental for the prevention of perinatal HSV.

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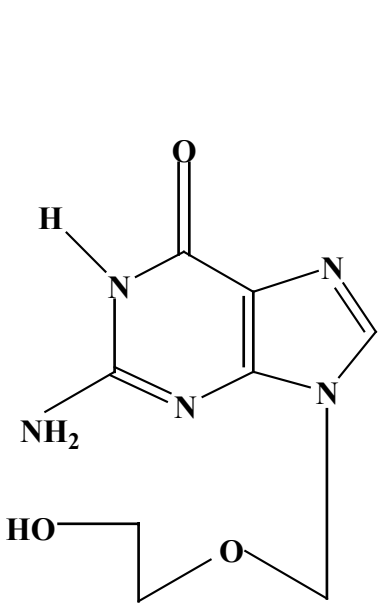
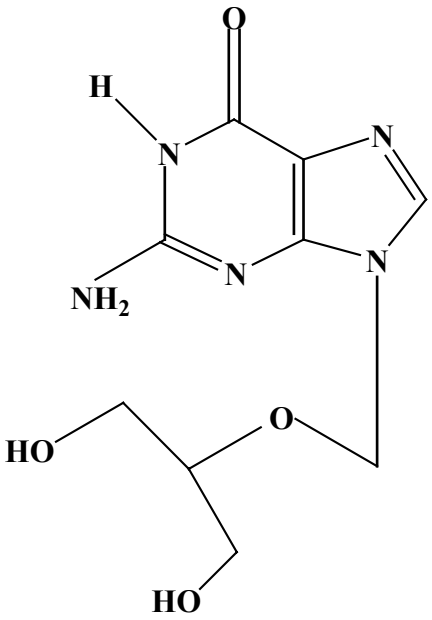
Figure 3.1



Acyclovir

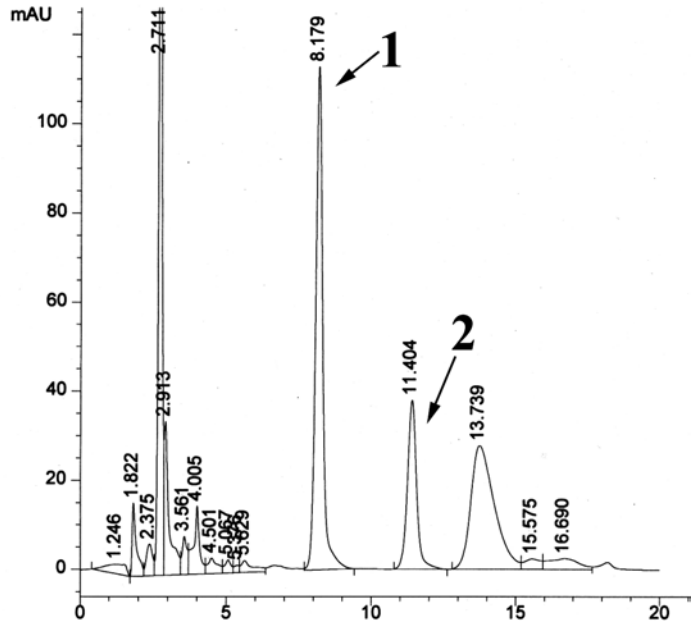


Ganciclovir

Structures of acyclovir and ganciclovir

Figure 3.2

(a)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8.179
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16.690
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80
100
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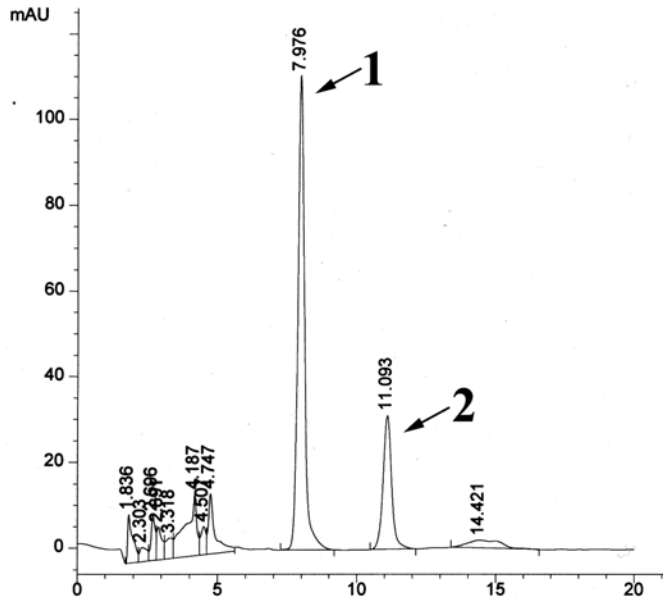
5
10
15
20

(b)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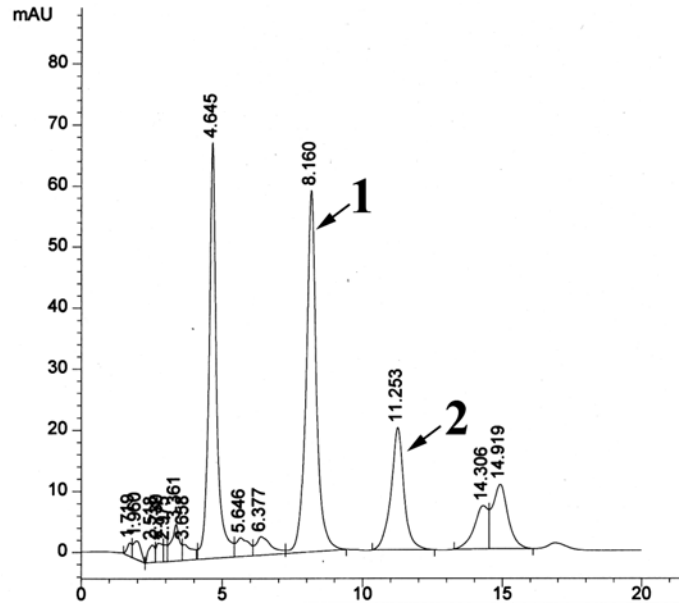
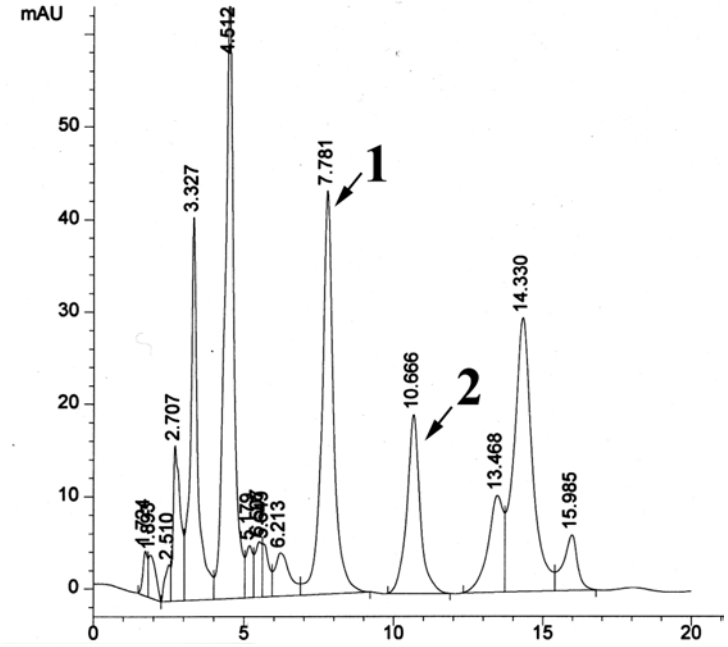
(c)

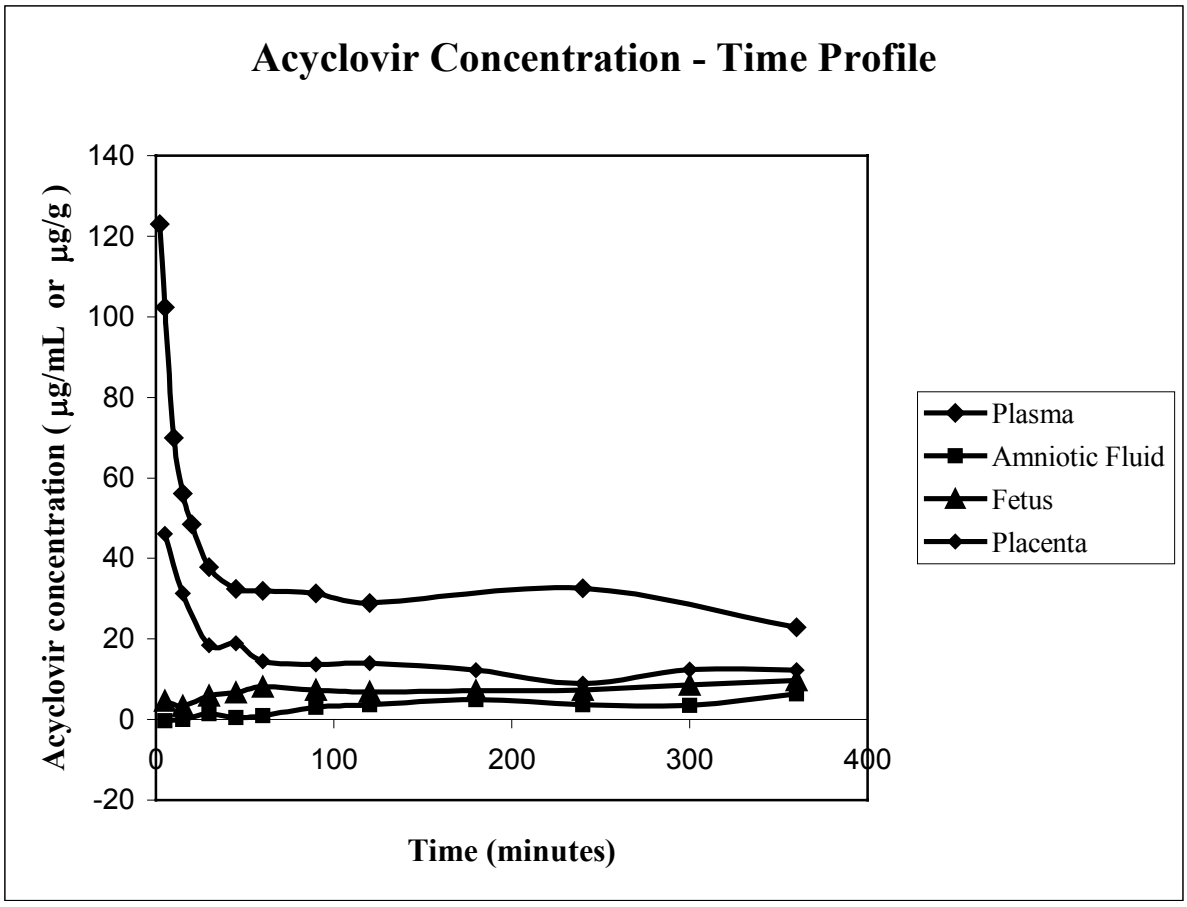


(d)



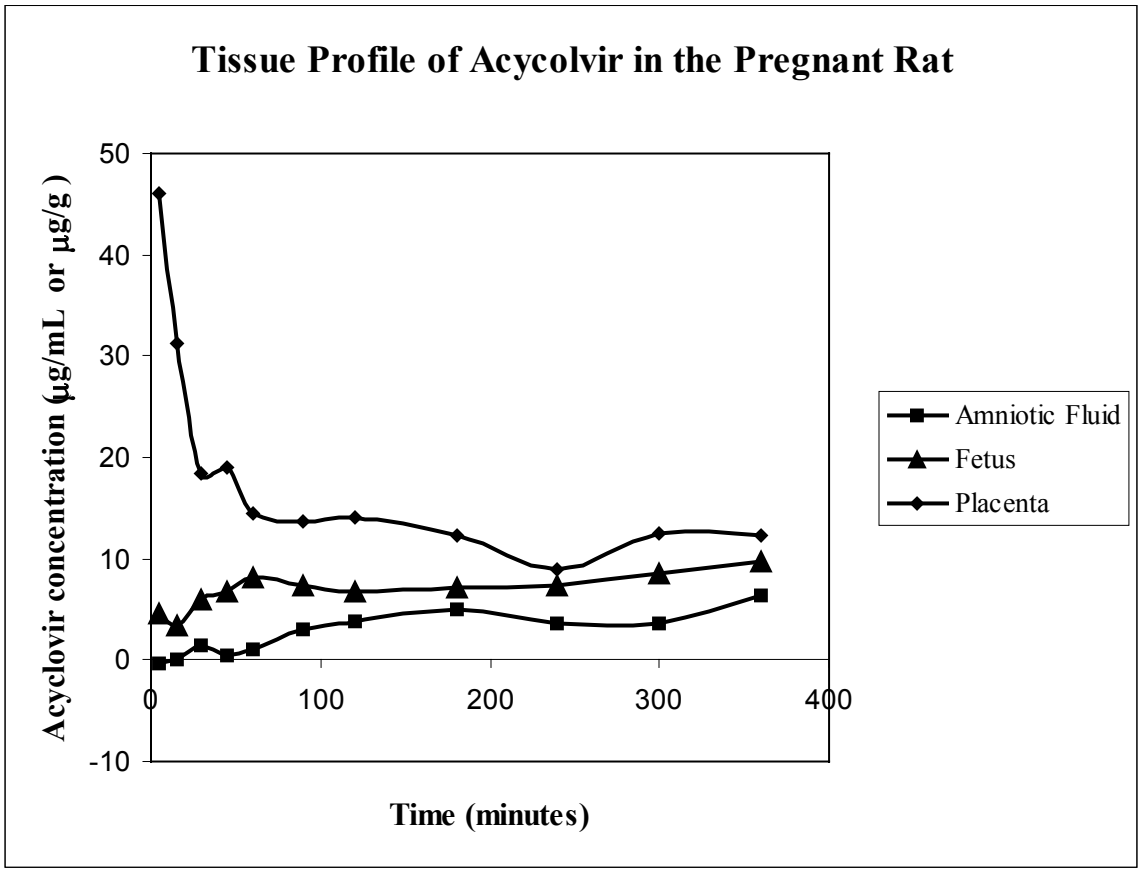
Chromatograms of (1) ganciclovir (~ 8 min retention time, 10 μg/mL) and (2) acyclovir (~ 11 min retention time, 2.5 μg/mL) spiked into (a) maternal plasma, (b) amniotic fluid, (c) placental homogenate, and (d) fetal homogenate

Figure 3.3



Concentration versus time curve of acyclovir in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate

Figure 3.4



Concentration – time profile of acyclovir accumulations in fetal tissue, placenta, and amniotic fluid

Table 3.1

Linear regression equations generated from validation data from each matrix; slope ± st.dev., intercept ± st.dev. and correlation coefficient ± st. dev. (n = 3 for each matrix)

Matrix	Slope	Intercept	R^2
Plasma	0.077 ± 0.009	0.047 ± 0.04	0.995 ± 0.004
Amniotic fluid	0.074 ± 0.011	0.084 ± 0.06	0.997 ± 0.002
Fetus	0.140 ± 0.021	0.0093 ± 0.009	0.997 ± 0.003
Placenta	0.170 ± 0.006	0.163 ± 0.05	0.998 ± 0.003

Table 3.2

The % relative recovery ± standard deviation (n = 5) of acyclovir and ganciclovir from maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate

	Maternal plasma	**Amniotic fluid**	**Placental homogenate**	**Fetal homogenate**
Acyclovir	87.11 ± 8.58	86.73 ± 4.63	90.02 ± 7.14	82.33 ± 12.3
Ganciclovir	75.83 ± 11.7	82.97 ± 5.16	69.27 ± 6.41	45.86 ± 8.39

Table 3.3

The precision (%Relative Standard Deviation) and accuracy (% error) (n = 15 at each spike concentration) of the HPLC-UV assay used to quantitate acyclovir in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate.

Concentration ACV added (μg/mL)	**Concentration ACV found (μg/mL)**	**% RSD**	**% Error**
Maternal Plasma			
0.25	0.269 ± 0.034	12.7	12.7
2.5	2.57 ± 0.273	10.6	9.70
75	70.3 ± 6.39	9.11	8.94
Amniotic Fluid			
0.25	0.260 ± 0.045	17.2	14.5
2.5	2.42 ± 0.343	14.2	12.2
75	71.3 ± 6.95	9.74	8.85
Placental Homogenate			
0.25	0.247 ± 0.030	12.1	10.1
2.5	2.38 ± 0.190	7.99	7.67
75	70.1 ± 5.32	7.58	9.07
Fetal Homogenate			
0.25	0.268 ± 0.0351	13.1	12.2
2.5	2.54 ± 0.321	12.6	11.4
75	71.3 ± 7.81	11.0	9.67

CHAPTER 4

HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY – ELECTROSPRAY MASS SPECTROMETRY DETERMINATION OF ACYCLOVIR IN PREGNANT RAT PLASMA AND TISSUES[1]

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[1]Brown, S.D., White, C.A., and M.G. Bartlett. Submitted to *Rapid Communications in Mass Spectrometry*, 06/02

Abstract

Reversed phase chromatography is the most common means of separation for small drug molecules. However, polar drugs may suffer from poor retention and peak shape in RP-HPLC. Hydrophilic interaction liquid chromatography (HILIC) provides a viable alternative to RP-HPLC and is an excellent way to separate polar compounds. This paper describes an HILIC-ESI-MS/MS assay for the determination of acyclovir from rat plasma, amniotic fluid, placental tissue, and fetal tissue. The isocratic separation utilizes an underivatized silica column with an acetonitrile: formate buffer mobile phase (80:20). The method is validated over a range of 50 ng/mL – 50 μg/mL with % Error and % RSD of < 15% over three days. All samples are prepared by acetonitrile protein precipitation, which yields high recovery (> 84% for acyclovir). This assay can be applied to the pharmacokinetic study of the placental transfer of acyclovir.

Introduction

Reversed-phase HPLC (RP-HPLC) is heavily relied upon in the pharmaceutical industry as a means of chromatographic separation because of its wide applicability to a variety of compounds. For very polar compounds, RP-HPLC may not be sufficient to provide the needed retention, resolution, and desired peak shape without the use of organic modifiers or ion-pair agents. While acceptable for use with UV detectors, high concentrations of these modifiers in the mobile phase can suppress ionization when HPLC is coupled with mass spectrometry [1]. Also, the highly aqueous nature of mobile phases used for the analysis of polar compounds in RP-HPLC may hinder ionization and desolvation and lead to shorter column lifetimes [2]. The use of a partially aqueous

mobile phase with a traditionally normal phase (NP) analytical column (i.e. silica or amino stationary phases) has been termed as hydrophilic interaction liquid chromatography (HILIC) [3]. This type of chromatography is becoming more widely accepted as an alternative to RP-HPLC for the analysis of polar drugs [4-7]. Since aqueous components are part of the mobile phase, interference from the aqueous nature of biological matrices is not a concern as it would be in a NP-HPLC environment [7]. Compounds elute as they would in NP-HPLC, but the presence of the aqueous portion of the mobile phase allows for further optimization options such as pH adjustment. The higher percentage of organic allowed in HILIC contributes to more efficient desolvation when the flow is introduced into the mass spectrometer and better peak shape, thus the potential for higher sensitivity. Silica columns used under partially aqueous conditions have been shown in to increase sensitivity of analyzing polar ionic compounds by up to eight-fold over RP columns [7].

Acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanine, ACV) and its structural analog, ganciclovir (9-[(2-hydroxy-1-(hydroxymethyl) ethoxy)-methyl]-guanine, GAN) are highly polar anti-viral drugs used to treat herpes simplex virus – 2 (HSV-2, genital herpes) and cytomegalovirus (CMV) respectively. ACV has been used for almost two decades to prevent vertical (mother-to-child) transmission of HSV-2 and has been shown to be safe and effective in doing so [8-13]. *In vivo* placental transfer of ACV has not been characterized. The LC-MS/MS assay presented here can be used to help characterize the placental transfer of this drug and is the first validated LC-MS/MS method reported for the quantitation of ACV. Other methods exist for the analysis of ACV from biological fluids, but involve less sensitive detectors such as UV or tedious

sample preparation techniques such as SPE [14-27]. Only one assay has currently been published for the analysis of ACV from complex tissue matrices such as those associated with pregnancy, but this method utilizes RP-HPLC, SPE, has a 15 min run-time, and has a higher LOQ than the method presented here [14].

Experimental

Reagents and Chemicals

Standards of ACV and GAN were purchased from Sigma (St. Louis, MO, USA). Reagent grade ammonium acetate was obtained from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile was from Fisher Scientific (Fair Lawn, NJ, USA). The formic acid used was reagent grade purchased from J.T.Baker (Phillipsburg, NJ, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

Supplies and Instrumentation

The HPLC column used was a Brownlee Spheri-5 Silica column (100 x 4.6 mm, 5 μm particle size) from Perkin Elmer (Norwalk, CT, USA) with a Spheri-10 RP-2 (30 x 4.6mm, 10 μm) guard column from the same manufacturer. The chromatographic system used consisted of a Hewlett-Packard (Agilent) 1100 Series quaternary pump, degasser, autosampler, and column thermostater (Palo Alto, CA, USA). The mass spectrometer was a Micromass Quattro-LC triple quadrupole instrument with an ESI Z-Spray® source (Manchester, UK). Tissues were homogenized using a Tekmar Model SDT-1810 tissue grinder (Cincinnati, OH, USA). All sample centrifugation was done in a Biofuge Pico

Microcentrifuge (Heraeus Instruments, Hanau, Germany). A Savant Model SC110A vacuum centrifuge (Holbrook, NY, USA) was used in the sample preparation. All rats used were purchased from Harlan (Indianapolis, IN, USA).

Method Development

Full-scan mass spectra (positive ion mode) were obtained for both ACV and GAN. Acyclovir and ganciclovir showed the protonated molecules, $[M + H]^+$, at m/z 226 and m/z 256 respectively. Protonated guanine (m/z 152) was the most abundant ion seen in the product ion mass spectra (Figure 4.1) for both compounds resulting from a neutral loss of 74 Da (ACV) and 104 Da (GAN). As a result of this experiment, the mass spectrometer was set up in multiple reaction monitoring mode (MRM) to monitor the transitions from the precursor ions (m/z 226 for ACV and m/z 256 for GAN) to the product ions (m/z 152 for ACV and GAN). Blanks of each matrix were examined under these conditions and no peaks were detected in either channel.

Several columns were investigated for the separation of ACV and GAN including the Aglient (Palo Alto, CA) Zorbax C-8 (2.1 x 150 mm, 5 μm) and the Phenomenex (Torrance, CA) Luna NH_2 (2.1 x 150 mm, 5 μm). Both ammonium formate (pH 3) and ammonium acetate (pH 6.5) buffers were investigated for mobile phase components. Various levels of acetonitrile, methanol, and methylene chloride were considered as possible mobile phase components. The use of triethylamine (TEA) was also studied as a mobile phase additive in concentrations of 0.1 – 1% in an attempt to improve peak shape and retention.

A previously developed SPE method has been demonstrated for sample preparation of placental and fetal tissues [14]. Protein precipitation using 2 M perchloric acid was assessed for a possible sample preparation for plasma and amniotic fluid. Acetonitrile precipitation was ultimately used for the sample preparation because it is inexpensive, fast, and simple and provides comparable recovery to SPE. As opposed to aqueous perchloric acid, acetonitrile can be easily evaporated after precipitation, allowing for reconstitution in any solvent/buffer and concentration of sample analytes. Volumes of ACN equal to three to ten times the volume of the sample were tested to determine the optimum amount of ACN to add to each sample. Buffer, mobile phase, ACN, and deionized water were all considered as possibilities for reconstituting the sample after evaporation.

Sample Preparation

Calibration curves were prepared by spiking blank plasma and tissues (amniotic fluid, placenta, and fetus) obtained from a non-dosed pregnant rat. All samples were prepared by protein precipitation using 50 μL of plasma or tissue homogenate (or 20 μL of amniotic fluid) and adding three volumes (150 μL for plasma, placenta, and fetus; 60 μL for amniotic fluid) of ice-cold acetonitrile. Samples were vortexed for 10 sec and centrifuged at 16,000 g for 10 min. The supernatant was removed and evaporated to dryness under vacuum. Samples were reconstituted in mobile phase (50 μL for plasma, placenta, and fetus; 20 μL for amniotic fluid) and 10 μL of the reconstituted sample was injected.

Liquid Chromatographic and Mass Spectrometric Conditions

All chromatographic separations were done using the Brownlee silica column (100 x 4.6 mm, 5 μm particle size). The mobile phase consisted of 80:20, acetonitrile:10 mM ammonium formate buffer, pH 3). Separations were isocratic at 0.700 mL/min. The column thermostat was set at 30^{o}C. The autosampler needle was rinsed with methanol between injections.

All mass spectrometric measurements were performed using a Micromass Quattro LC triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in positive ion mode. The cone voltage was set at 40 V and the capillary voltage at 2.80 kV. The source temperature was kept at 80^{o}C and the desolvation temperature at 120^{o}C. For quantitation of acyclovir and the internal standard ganciclovir, the transitions m/z 226 (Q1) => m/z 152 (Q3) (acyclovir) and m/z 256 (Q1) => m/z 152 (Q3) (ganciclovir) were monitored using the MRM experiment.

Ionization Suppression Study

Regions of ionization suppression were determined using a post-column infusion of a 10 μg/mL solution of ACV and GAN while injection of a "blank" of each matrix [28]. This experiment was repeated three times with three different blanks of each of the four matrices.

Validation and Recovery Study

Calibration and quality control standards of ACV were prepared in mobile phase with the following concentrations: 500 μg/mL, 250 μg/mL, 125 μg/mL, 50 μg/mL, 25

μg/mL, 15 μg/mL, 5 μg/mL, 1.5 μg/mL, and 0.5 μg/mL. A stock solution of GAN was prepared in mobile phase with a final concentration of 10 μg/mL. Volumes of each calibration or QC solution was spiked into blank matrix to yield final concentrations of 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 5 μg/mL, 2.5 μg/mL, 1.5 μg/mL, 500 ng/mL, 150 ng/mL, and 50 ng/mL.

Quantitiation was performed using the peak area ratios between ACV and the internal standard, GAN. GAN concentrations were kept at the 10 μg/mL level in all samples. A calibration curve including the points: 50 ng/mL, 250 ng/mL, 500 ng/mL, 2.5 μg/mL, 5 μg/mL, 12.5 μg/mL, 50 μg/mL ACV was generated in each matrix for each day of validation. Calibration curves for each day of validation (and for sample analysis) were weighted by a factor of "1/y" using JMP statistical software. The "1/y" weighting scheme was determined using a theoretical validation set of samples prepared in mobile phase. Five replicate samples of each of the following concentrations: 50 ng/mL, 150 ng/mL, 1.5 μg/mL, and 25 μg/mL were prepared on each day of validation to assess precision and accuracy. Precision was calculated as the relative standard deviation, % RSD = 100 (St.dev./Mean). To reflect the accuracy of the assay, the % Error was calculated as the percent difference between the theoretical concentrations and the experimentally determined concentrations of the replicate samples. All % Error and % RSD calculations were done in Microsoft Excel.

Recovery was expressed as the percent difference between samples spiked with ACV and GAN pre- and post-extraction. Five replicates of each matrix were spiked with 500 ng/mL ACV and 10 μg/mL GAN and extracted. These peak areas were compared to

five replicates of each matrix blank that were extracted and spiked with 500 ng/mL ACV and 10 μg/mL GAN after mobile phase reconstitution.

Results and Discussion

The silica column in HILIC mode was decided upon after considering a C-8 column (RP-LC) and an amino column (HILIC). The C-8 column produced extensive peak tailing for both compounds and failed to provide adequate retention of both compounds to remove them from the major regions of ion suppression. The amino column, although retaining both analytes well, showed very wide peaks (~1 min) for ACV. Addition of TEA to the aqueous portion of the mobile phase for both columns failed to correct the poor peak shape. Even a small percentage of this ion-pair agent also has the potential to contribute to ionization suppression [1]. Ammonium formate was chosen over ammonium acetate because its buffering capacity is better suited for pH 3, which is the optimum pH for the chromatographic analysis of ACV [14, 29]. Figure 4.2 shows the chromatograms for ACV (50 ng/mL) and GAN (10 μg/mL) from the four matrices. The optimum amount of ACN added for the sample preparation was determined to be three times the volume of the original sample. Addition of larger volumes did not improve recovery, but addition of a smaller portion yielded ~9% lower recoveries. The mobile phase was chosen as the reconstitution solvent because this produced the sharpest and most reproducible peaks.

Ionization suppression regions were found at 1.0 – 2.0 min for the plasma and 1.5 – 2.5 min for the amniotic fluid, fetus, and placenta (see Figure 4.3). The retention of

ACV at 3.25 min and GAN at 3.75 min was adequate to avoid the effects of these ion suppression areas.

Results from the recovery experiments can be seen in Table 4.1. Amniotic fluid showed the highest recovery of ACV (96%) while recovery from the fetal tissue was the lowest (85%). Table 4.2 shows the validation data over three days with n = 15 for each QC point. The % RSD ranged from 6.6 – 14.7 % while the % Error ranged from 5.4 – 12.8%. Calibration curves were deemed acceptable if $R^2 > 0.99$. Table 3 shows the slope and R^2 generated from the calibration curves used in the validation study. All validation data generated was in compliance with the suggested criteria for bioanalytical method validation [30].

Application of Method

To verify the utility of this assay for monitoring ACV concentrations in maternal plasma and other matrices associated with pregnancy, a pregnant rat was dosed with ACV. The use of this animal was approved by the University of Georgia Animal Use and Care Committee. The rat was housed at the University of Georgia College of Pharmacy animal facility (AALAC accredited) until the day of the experiment. The environment in the animal facility was controlled at 20 – 22^{o}C with 14 hr of light each day. Rats were fed standard chow pellets and allowed constant access to water. A timed-pregnant Sprague-Dawley rat (345 g) was used on day 19 of gestation for this study. An intramuscular dose of a ketamine-acepromazine combination was used for the anesthesia. To help prevent pulmonary edema, atropine was administered subcutaneously throughout the experiment. A jugular cannula was inserted to facilitate collection of maternal plasma

samples and to administer the intra-venous bolus dose of ACV (60 mg/kg). The dose was prepared in 0.1 M NaOH in physiological saline. A laporatomy was performed to allow for serial sampling of the rat pups. Maternal blood samples were collected at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min post-dose. Plasma was collected by centrifuging the blood samples at 16,000 g for 10 min. Amniotic fluid, placenta, and fetal samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min after the dose. Each tissue sample collection involved removing a single fetal sack, each containing the three tissues of interest. At the 480 time point, the final three fetal sacks were removed to assess the variability between tissue samples. Placental and fetal tissue samples were minced and homogenized in deionized water (1:2, w/v). All samples were stored at -20^{o}C prior to analysis.

On the day of rat sample analysis, calibrations curves were generated from each matrix (50 ng/mL – 50 μg/mL) and QC standards in each matrix were prepared to be analyzed at the end of the "real" sample set. Samples were prepared as indicated above. Because of the higher concentrations of drug found in the plasma, these samples were diluted by a factor of four prior to analysis. Before protein precipitation, each sample was spiked with the GAN stock solution to yield a final concentration of 10 μg/mL. The concentration versus time profile generated from the analysis of these samples is shown in Figure 4.4. The triplicate samples collected for each tissue at 480 min showed low % RSD among the samples (13.5% for amniotic fluid, 5.82% for fetus, and 9.95% for placenta). Using WinNonlin® pharmacokinetic software, the plasma data was fit to a two-compartment model with first order elimination and no lag-time. This model is consistent with reported literature models for ACV kinetics in the rat [31]. The volume

of distribution at steady-state was calculated to be 1.75 L/kg, also consistent with reported literature values [32]. As expected, the curves for maternal plasma and placenta mirror each other due to the "blood-rich" nature of this tissue. As previously reported, there seems to be an accumulation of ACV in the amniotic fluid as indicated by the higher lingering concentrations of ACV in this tissue at the later time-points [33]. Samples stored at –20^{o}C in the various matrices were re-analyzed over a period of one year. The responses generated from re-analysis of the samples after this time period indicated the same pharmacokinetic parameters and model to describe the distribution and elimination of acyclovir in the pregnant rat.

Conclusions

This method is the first validated LC-MS/MS method to be published for the quantitation of ACV from complex biological matrices such as plasma, amniotic fluid, fetal tissue, and placental tissue. Utilizing HILIC for the chromatographic separation helps aid in the retention, peak shape, and ionization of these highly polar compounds. The acetonitrile protein precipitation sample preparation is fast and simple, thus saving time and money over SPE and generating less waste than liquid-liquid extraction (LLE). The recovery associated with this sample preparation technique is high (85 – 96%) from the various matrices. The assay is accurate and reproducible yielding % RSD and % Error numbers of < 15% over three days of validation. The run time is short (4 min) allowing for high throughput potential needed for the analysis of large numbers of samples generated from pharmacokinetic studies.

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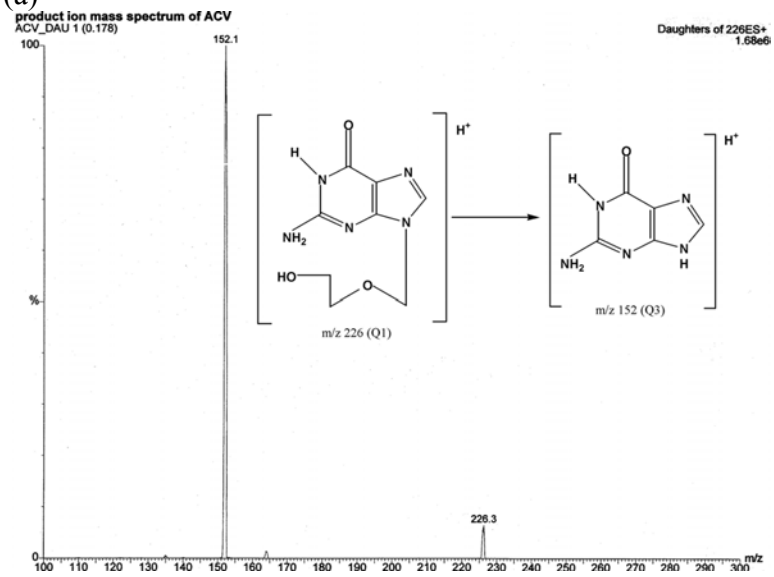
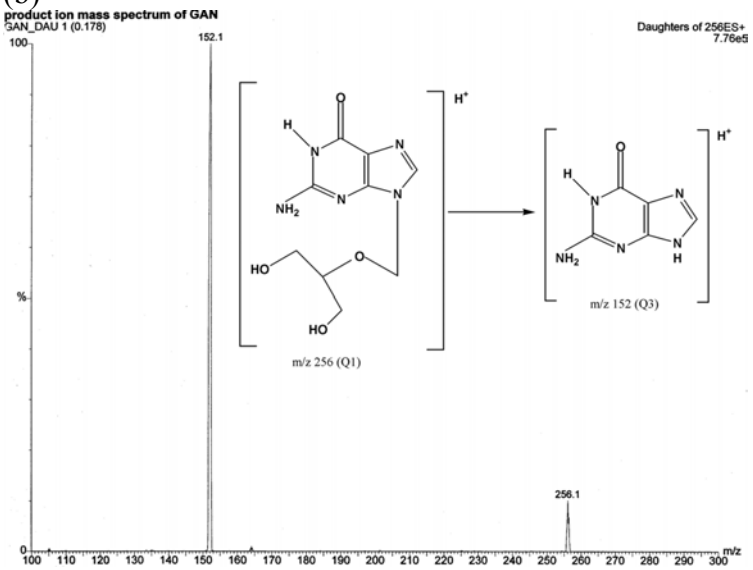
Figure 4.1

(a)



Product ion mass spectra of acyclovir

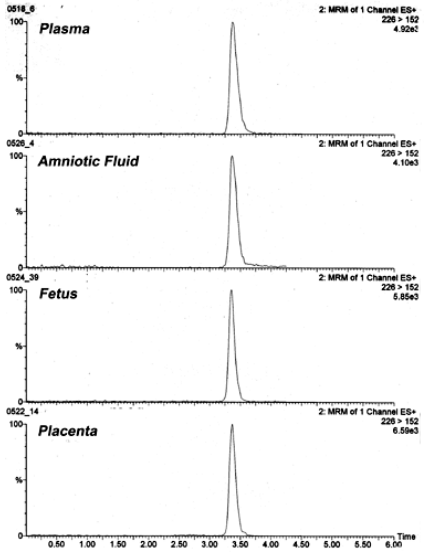
(b)



Product ion mass spectra of ganciclovir

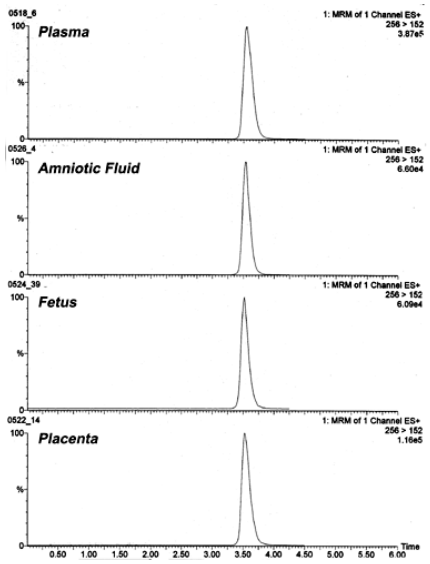
Figure 4.2

(a)



Chromatograms of ACV (50 ng/mL) generated from extracted plasma, amniotic fluid, fetal tissue, and placental tissue

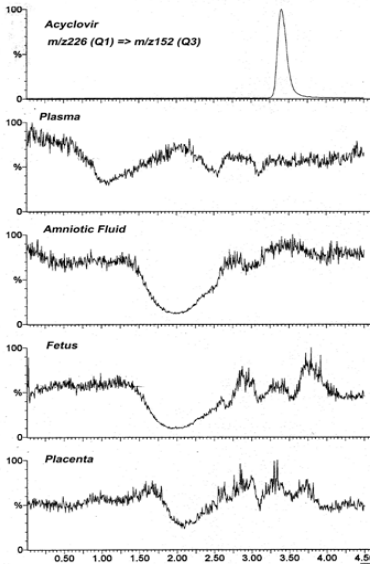
(b)



Chromatograms of GAN (10 μg/mL) generated from extracted plasma, amniotic fluid, fetal tissue, and placental tissue

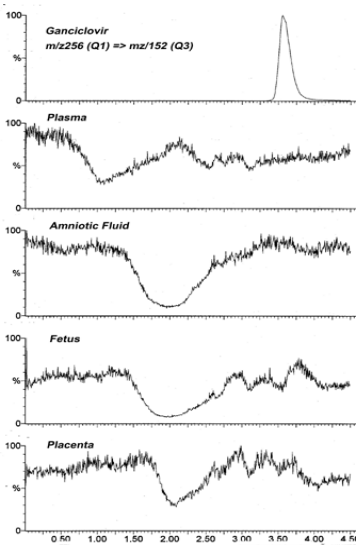
Figure 4.3

(a)



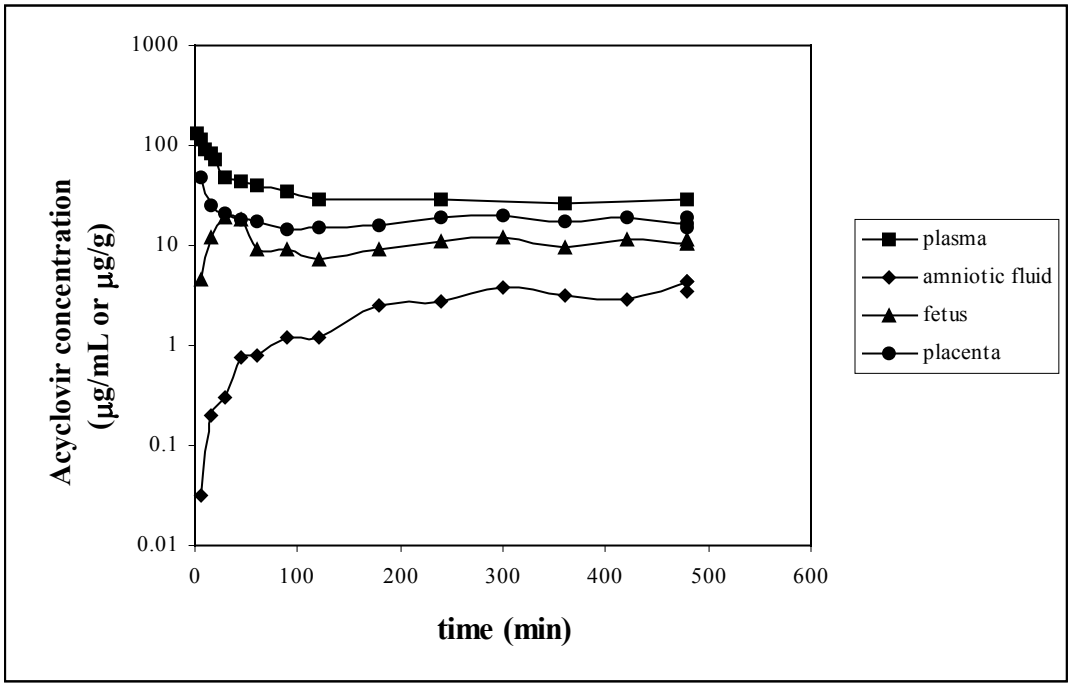
Regions of ionization suppression in maternal plasma, amniotic fluid, fetal tissue, and placental tissue for acyclovir

(b)



Regions of ionization suppression in maternal plasma, amniotic fluid, fetal tissue, and placental tissue for ganciclovir

Figure 4.4



The concentration versus time profile of ACV distribution in maternal plasma, amniotic fluid, placental tissue, and fetal tissue following a 60 mg/kg IV bolus dose.

Table 4.1

The % relative recovery ± standard deviation (n = 5) of ACV (500 ng/mL level) and GAN (10 μg/mL level) from plasma, amniotic fluid, placental homogenate, and fetal homogenate

	Acyclovir recovery	**Ganciclovir recovery**
Plasma	91.73 ± 2.68	87.64 ± 6.94
Amniotic fluid	95.91 ± 5.10	91.21 ± 2.38
Placental tissue	87.93 ± 5.90	87.50 ± 12.1
Fetal tissue	84.64 ± 12.1	80.45 ± 17.5

Table 4.2

The precision (% RSD) and accuracy (% Error) of the LC-MS/MS assay used to quantitate ACV in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate

Theoretical ACV concentration (μg/mL)	Experimental ACV concentration (μg/mL)	% RSD	% Error
Plasma (n = 15)			
0.05	0.050 ± 0.006	12.2	10.1
0.15	0.158 ± 0.015	9.35	9.41
1.5	1.48 ± 0.10	6.63	5.35
25	24.1 ± 2.1	8.52	7.33
Amniotic fluid (n = 15)			
0.05	0.052 ± 0.006	11.8	10.4
0.15	0.154 ± 0.018	11.7	10.4
1.5	1.58 ± 0.15	9.36	9.98
25	24.0 ± 2.3	9.65	9.09
Placental tissue (n = 15)			
0.05	0.049 ± 0.007	14.7	12.8
0.15	0.159 ± 0.015	9.14	9.54
1.5	1.59 ± 0.13	8.02	8.48
25	23.7 ± 1.6	6.69	6.66
Fetal tissue (n = 15)			
0.05	0.049 ± 0.005	10.8	8.56
0.15	0.148 ± 0.017	11.5	8.16
1.5	1.50 ± 0.14	9.06	8.12
25	24.0 ± 1.6	6.73	5.79

Table 4.3

Linear regression equations generated from validation data from each matrix; slope ± st.dev., intercept ± stdev., and correlation coefficient ± st. dev. (n = 3 for each matrix)

Matrix	Slope	Intercept	R^2
Plasma	0.103 ± 0.031	0.011 ± 0.006	0.994 ± 0.003
Amniotic fluid	0.309 ± 0.12	0.031 ± 0.005	0.993 ± 0.001
Fetus	0.327 ± 0.16	0.041 ± 0.016	0.995 ± 0.004
Placenta	0.316 ± 0.066	0.044 ± 0.009	0.991 ± 0.002

CHAPTER 5

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) DETERMINATION OF ACYCLOVIR AND ZIDOVUDINE IN MATERNAL PLASMA, AMNIOTIC FLUID, FETAL AND PLACENTAL TISSUES USING ULTRA-VIOLET (UV) DETECTION[1]

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[1]Brown, S.D., White, C.A., and M.G. Bartlett. Accepted by *The Journal of Liquid Chromatography & Related Technologies.*
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Abstract

A sensitive and reproducible HPLC assay has been developed and validated for the separation and analysis of acyclovir and zidovudine from plasma, amniotic fluid, placental homogenate, and fetal homogenate. Acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanosine, ACV) is the oldest and most widely used compound to treat episodes of genital herpes (herpes simplex virus-2, HSV-2). Zidovudine (3-azido-3'deoxythymidine, AZT) is the premier reverse transcriptase inhibitor released for the treatment of human immunodeficiency virus (HIV). Both of these drugs have been used in pregnant women to prevent the vertical (mother-to-child) transmission of their respective viruses. This gradient HLPC assay aids in the quantitation of these drugs from the matrices associated with pregnancy (maternal plasma, amniotic fluid, fetal tissue, and placenta). The mobile phase consists of 30mM acetate/citrate buffer (pH 3) and methanol. The plasma and amniotic fluid samples are prepared using a combination of protein precipitation and filtration while the more complex tissues are prepared with the use of solid-phase extraction (SPE). The method was validated in the calibration range of 0.1 – 100 μg/mL and showed precision (% RSD) and accuracy (% Error) of less than 15% for all matrices over three days. The assay was applied to a pharmacokinetic study involving the co-administration of ACV and AZT in the pregnant rat.

Introduction

Acyclovir has been used to treat the symptoms and to suppress recurrent episodes of genital herpes for almost two decades. Genital herpes (HSV-2) is not typically life threatening for adults, but it can be devastating for newborn infants. Newer statistics

indicate that genital herpes may affect up to 25-30% of reproductive age women making it a real concern for perinatal transmission of the disease [1,2]. Neonatal herpes can manifest itself in three forms, affecting either the skin, eyes, and mucous membranes, the central nervous system, or major organ systems (disseminated disease) [3,4]. All of these manifestations are associated with some degree of mortality with the disseminated infection being the most severe (20% survival rate) [3,4].

Zidovudine is the first reverse-transcriptase inhibitor released and is the only anti-viral approved for use in pregnant women. Pediatric AIDS is a leading cause of death in some developing nations, but the use of AZT, even if given only in the late stages of pregnancy, can reduce vertical HIV transmission by 51-68% [5]. Acyclovir and zidovudine have commonly been given in combination because of reports that ACV potentiates the *in vitro* activity of AZT [6,7]. Clinically, this drug combination has shown beneficial effects in patients with HIV, often by reducing the frequency of opportunistic infections in these patients [8,9]. Although extreme fatigue has been reported as a side effect of this combination, the benefits often outweigh the risks when attempting to treat both viruses simultaneously [10].

Mamede *et al.* conducted a study examining the effects of the ACV/AZT combination on maternal, fetal and placental weights [11]. This study found that only the ACV mono-therapy group exhibited a decrease in maternal body weight over the controls. AZT had previously been shown to have no effect on rat maternal body weights, and this finding was replicated in the Mamede study [11,12]. Mamede proposed that AZT elicits a “protective effect” against the ACV when they are administered in combination [11]. ACV also reduced the number of placentas and viable fetuses in the pregnant rats,

but the addition of AZT to the therapy seemed to attenuate the abortive effect of ACV [11].

This paper describes an HPLC assay used in the pharmacokinetic study of the ACV/AZT combination therapy in the pregnant rat. Because of the vast differences in the polarities of these two drugs, a gradient elution was used. Several assays exist for the quantitation of acyclovir [13-27] and zidovudine alone [28-36], but none have examined the seperation of this combination, especially in the complex biological matrices associated with pregnancy. This assay was used in conjugation with a previously developed assay for quantitating ACV for comparing the pharmacokinetic differences between ACV and AZT mono-therapies and the ACV/AZT combination therapy in pregnant rats.

Experimental

Reagents and Chemicals

Analytical standards of acyclovir and zidovudine were obtained from Sigma (St. Louis, MO, USA). AZDU (3'-azido-3'-deoxythymidine), one of the internal standards used, was synthesized as previously described [37]. Lamivudine (3TC), also an internal standard, was recrystallized from Epivir® tablets because of the lack of availability of a commercially available standard. Reagent grade citric acid was acquired from Sigma. Reagent grade ammonium acetate and reagent grade octanesulfonic acid were bought from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile and methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sep-Pak Vac 1cc C-18

cartridges were purchased from Waters (Milford, MA, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

Preparation of Stock and Standard Solutions

Stock solutions of ACV, AZT, AZDU, and 3TC were prepared in deionized water to yield final concentrations of 1.0 mg/mL drug. Acyclovir and zidovudine standard solutions were prepared with deionized water from the 1.0 mg/mL stocks to yield final concentrations of 750, 500, 100, 50, 25, 10, 5, 2.5, 1 μg/mL. 100 μg/mL standard solutions of AZDU and 3TC were prepared with deionized water from the 1.0 mg/mL stocks. Stock solutions were kept refrigerated when not in use and replaced on a bi-weekly basis. Fresh standard solutions were prepared for each day of analysis or validation.

Chromatographic System

The HPLC system consisted of Hewlett-Packard (Agilent) 1100 Series components including a quaternary pump, degasser, autosampler, and variable wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Agilent Eclipse XDB C-8 column (150 x 2.1 mm, 5 μm) (Palo Alto, CA, USA) with a Phenomenex Security Guard C-18 guard column (Torrance, CA, USA).

Chromatographic Conditions

The mobile phase used for the gradient was 30 mM acetate/citrate buffer (pH 3.1): methanol. The gradient tables used can be seen in Tables 5.1 and 5.2. The chromatograms for each matrix can be seen in Figure 5.2. The detection wavelength was fixed at 254 nm.

Calibration Curves

Blank plasma, amniotic fluid, placenta, and fetal tissue were collected from untreated anesthetized animals. The placenta and fetal tissues were minced and homogenized with two volumes of deionized water (w/v) using a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). Plasma calibration points were prepared by spiking 100 μL of plasma inside a 1.5 mL centrifuge tube with 10 μL of each ACV standard, 10 μL of each AZT standard, and 10 μL of the 250 μg/mL 3TC standard solution. Amniotic fluid calibration points were prepared by spiking 50 μL of fluid inside a 1.5 mL centrifuge tube with 5 μL of each ACV standard, 5 μL of each AZT standard, and 5 μL of the 250 μg/mL 3TC standard. Placental calibration samples were prepared using 150 μL of placental homogenate inside a 1.5 mL centrifuge tube spiked with 15 μL of each ACV standard, 15 μL of each AZT standard, and 15 μL of the 100 μg/mL AZDU solution. Fetal tissue calibration samples were prepared using 300 μL of fetal homogenate inside a 1.5 mL centrifuge tube with 30 μL of each ACV standard, 30 μL of each AZT standard, and 30 μL of the 100 μg/mL AZDU standard solution. Ultimately, the calibration concentrations of the analytes (ACV and AZT) in each matrix would be as follows: 0.1, 0.5, 1, 5, 10, 50, 100 μg/mL with an internal standard concentration in each

sample of 10 μg/mL (AZDU) or 25 μg/mL (3TC). After each matrix was spiked, it was subject to further sample preparation before analysis.

Sample Preparation

Plasma and amniotic fluid samples were prepared with protein precipitation and filtration. After spiking, samples were vortexed briefly and 20 μL of 2 M perchloric acid (plasma) or 10 μL of 2 M perchloric acid (amniotic fluid) was added. The tubes were centrifuged for 10 min at 16,000 g using a Biofuge Pico Microcentrifuge (Heraeus Instruments, Hanau, Germany). After centrifuging, the supernatent was removed and filtered using either XPertek® syringe filters, 0.22 μm nylon filter (St.Louis, MO) or CoStar SpinX® centrifuge tube filters, 0.22 μm nylon filter (Corning, NY) and the pellet was discarded.

Placental and fetal tissue samples were prepared using solid phase extraction (SPE). The homogenates were vortexed briefly after spiking and were pH adjusted using 30 mM acetate/citrate buffer with 5 mM octanesulfonic acid (pH 3.08) by adding 300 μL of mobile phase to the fetal homogenates and 150 μL to the placental homogenates. The tubes were vortexed again and centrifuged for 10 min at 16,000 g. Supernatents were loaded onto Sep-Pak C-18 SPE cartridges that had been preconditioned with 2 mL methanol followed by 2 mL of the acetate/citrate buffer with the ion-pair agent. The presence of the octanesulfonic acid helps the ACV stay retained to the cartridge long enough to facilitate clean-up of the sample. Samples were washed with 1 mL of deionized water/methanol (95:5 v/v) and eluted into clean culture tubes with 1 mL methanol. The eluents were evaporated to dryness in a vacuum centrifuge (Model

SC110A, Savant Instruments Inc., Holbrook, NY, USA) and the residues reconstituted in 100 μL (fetus) or 50 μL (placenta) of the aqueous component of the mobile phase. Reconstituted residues were then syringe filtered using 0.22 μm nylon syringe filters or filtered using the CoStar centrifuge filters (also 0.22 μm). An injection volume of 10 μL was used for all samples.

Sample Collection

The use of animals in this study was approved by the University of Georgia Animal Use and Care Committee, and was conducted in accordance with the Animal Welfare Act and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The rats were housed one animal per cage in the University of Georgia College of Pharmacy animal facility (AALAC accredited). The environment was controlled (20 – 22^{o}C, 14 hours of light per day) with daily feedings of standard chow pellets and water *ad libitum.*

Timed pregnant Sprauge-Dawley rats (Harlan, Indianapolis, IN) weighing an average of 331 $\pm$ 34 g were anesthetized intramuscularly with ketamine:acepromazine (75:2.5 mg/kg) and dosed on day 19 of gestation. During anesthesia, animals were given atropine (0.5 mg/kg) subcutaneously. For dosing and blood sampling purposes, a cannula was surgically implanted in the right jugular vein. For sampling of the pups (amniotic fluid, placenta, and fetal tissues), a laparotomy was performed. The dose (60 mg/kg ACV + 60 mg/kg AZT) was given to the rats prepared as a 10 mg/mL solution in 0.1 M NaOH in physiological saline (pH 7.4). The rats were administered the dose intra-venously via the jugular cannula followed by 1mL of phosphate buffered saline (pH 7.4) to rinse the

cannula. Blood samples were collected at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120,180, 240, 300, 360, 480 min into heparinized tubes and centrifuged at 16,000 g for 10 min to enable plasma collection. Amniotic fluid, placenta, and fetus samples were collected at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480 minutes. Placental and fetal tissue samples were homogenized in two volumes of deionized water. All samples were stored at –20^{o}C until analysis.

Results and Discussion

To achieve a timely separation between ACV and AZT, a gradient method had to be used. Lamivudine was initially chosen as the internal standard for this assay and was successfully used in the plasma and amniotic fluid samples. AZDU was chosen as the internal standard for the tissue samples because an endogenous peak in the tissue matrix interfered with the 3TC. Other anti-virals such as D4T (stavudine), DDI (didanosine), and DDC (zalcitabine) were run using the chromatographic conditions of this assay and were found to pose no interference with the analytes. Figure 5.2 shows chromatograms of ACV, AZT, and the internal standards separated in plasma, amniotic fluid, placental, and fetal homogenates.

The calibration curves for each day of validation and analysis showed acceptable linear response ($R^2 > 0.99$) through a range of 0.1 – 100 μg/mL. Microsoft Excel® or JMP® statistical software was used to generate linear regression equations for all calibration curves. The range of 0.1 – 100 μg/mL was sufficient for use in calculating ACV and AZT levels from samples taken from rats that were dosed with 60 mg/kg ACV

and 60 mg/kg AZT. Concentrations in the early plasma samples fell outside the range of the curve and had to be diluted prior to analysis.

The extraction efficiency for ACV and GAN from the various matrices is expressed in terms of relative recovery. Standard-spiked matrix samples at the 2.5 μg/mL level were extracted and analyzed (n = 5). An equal number of matrix blanks were extracted and spiked post-extraction. The peak areas of these two sample sets were compared showing high recoveries for ACV, AZT, AZDU, and 3TC. Acyclovir recovery from maternal plasma, amniotic fluid, placenta, and fetus ranged from 85% to 98%. AZT recoveries in each matrix ranged from 84% to 98%. The relative recoveries for each individual matrix can be found in Table 5.3.

Assay precision and accuracy was calculated for each matrix over a range of three days. Blanks from each matrix were spiked with ACV, AZT, and the appropriate internal standard to yield final concentrations corresponding with those in the calibration curve. Five replicates of blanks spiked with ACV and AZT concentrations of 0.25 μg/mL, 2.5 μg/mL, and 75 μg/mL were prepared for each validation day to test the precision (% Relative Standard Deviation, %RSD) and accuracy (%Error). This validation data is compiled in Tables 5.4 and 5.5.

To demonstrate the utility of this assay, a pregnant rat was dosed with ACV and AZT at the level of 60 mg/kg. Maternal plasma, amniotic fluid, placenta, and fetal samples were collected, extracted, and analyzed as described above. A calibration curve from each matrix was prepared on the day of analysis to calculate the concentrations of acyclovir and zidovudine present in the real samples. Before analysis, each sample collected from the dosed pregnant rat was spiked to yield a concentration of 10 μg/mL of

the appropriate internal standard (AZDU or 3TC). The sample peak area ratios of ACV and AZT to the internal standard were used to calculate the concentrations of ACV and AZT in each sample. Figures 3 and 4 show the concentration versus time profiles for acyclovir and zidovudine in all four biological matrices of the pregnant rat. The half-life ($t_{1/2}$) for AZT when given alone was calculated from the plasma data using WinNonlin pharmacokinetics software. The $t_{1/2}$ was found to be 0.76 ± 0.07 hr, which correlates well with previously reported studies of AZT in the pregnant rat [28]. However, this and other pharmacokinetic parameters for AZT change drastically when ACV is co-administered. Overall, maternal plasma exposure to AZT is prolonged when given with ACV, but the opposite is true for ACV. Both $t_{1/2}$ and area under the curve (AUC) of ACV is decreased when ACV and AZT are given in combination.

Conclusions

This assay has been validated for the separation and quantitation of acyclovir and zidovudine, two compounds very different in polarity. A balance had to be achieved between retaining the ACV (a highly polar drug) and eluting the AZT (a highly non-polar drug). This was done in four complex biological matrices using gradient elution and selective sample preparation techniques like solid-phase extraction. The method can be applied to the analysis of maternal plasma, amniotic fluid, placental tissue, and fetal tissue from rats that have been dosed with a combination of ACV and AZT. This assay can be used to analyze samples for the support of pharmacokinetic studies for comparing the placental transfer of ACV and AZT mono- and combination therapies.

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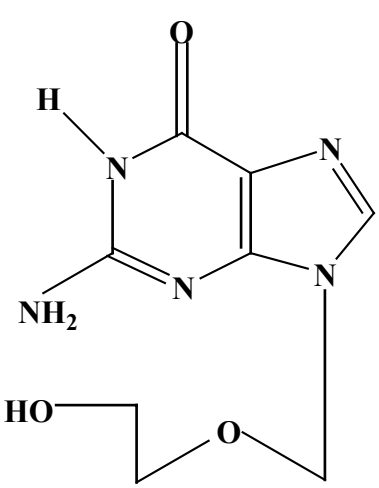
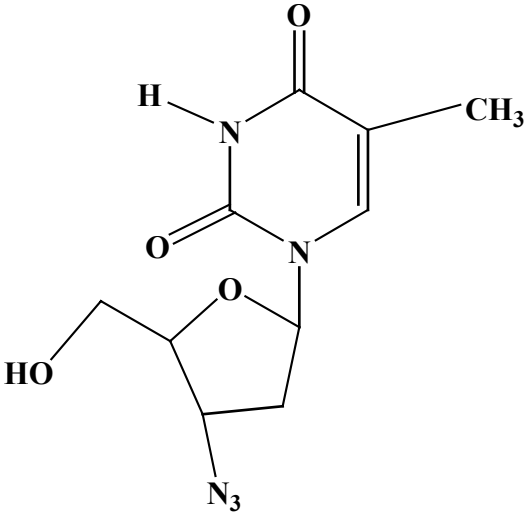
Figure 5.1



Acyclovir

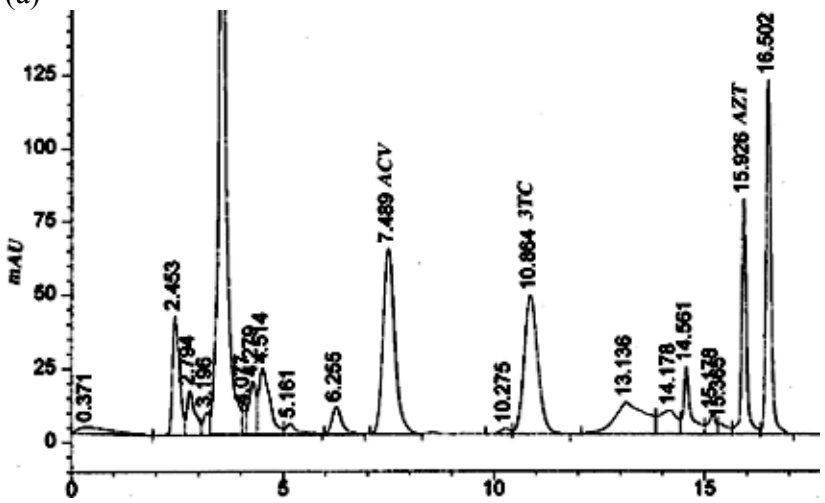


Zidovudine

Structures of acyclovir and zidovudine

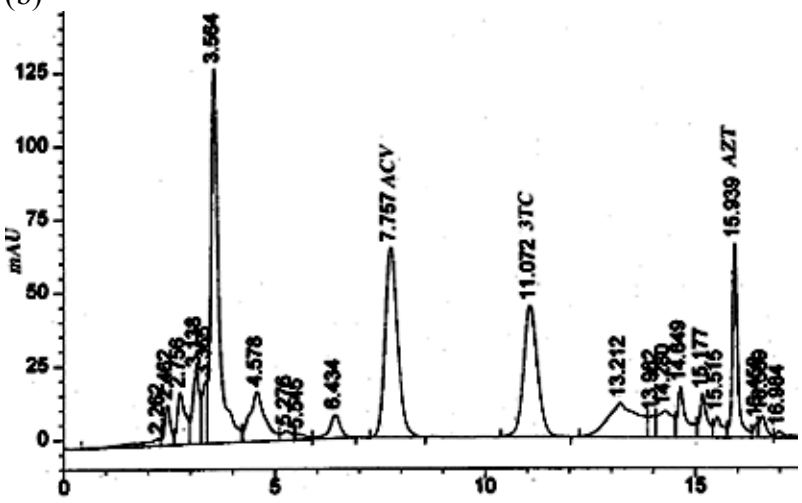
Figure 5.2

(a)



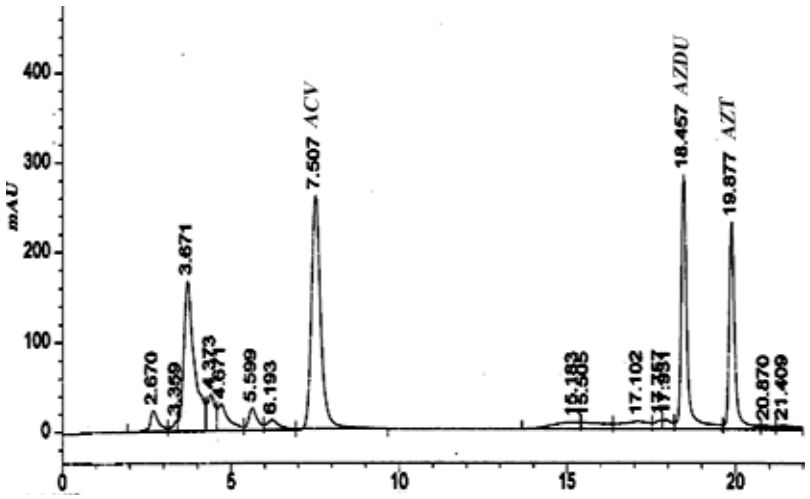
Chromatograms of ACV and AZT (10 μg/mL), and internal standard 3TC (25 μg/mL) in maternal plasma

(b)



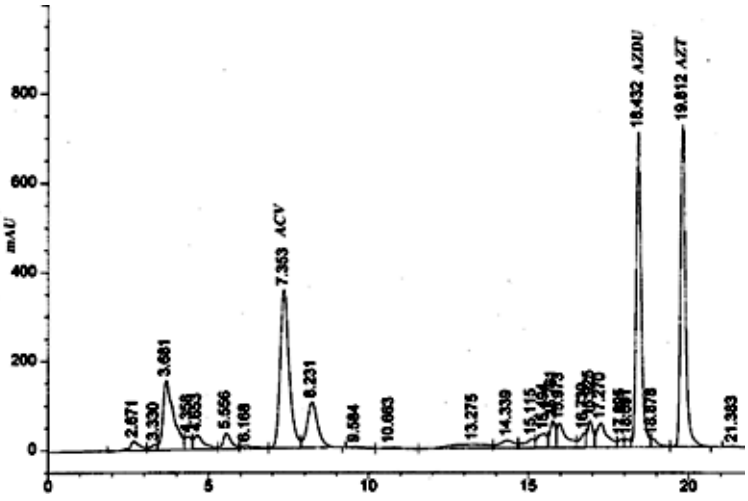
Chromatograms of ACV and AZT (10 μg/mL), and internal standard 3TC (25 μg/mL) in amniotic fluid

(c)



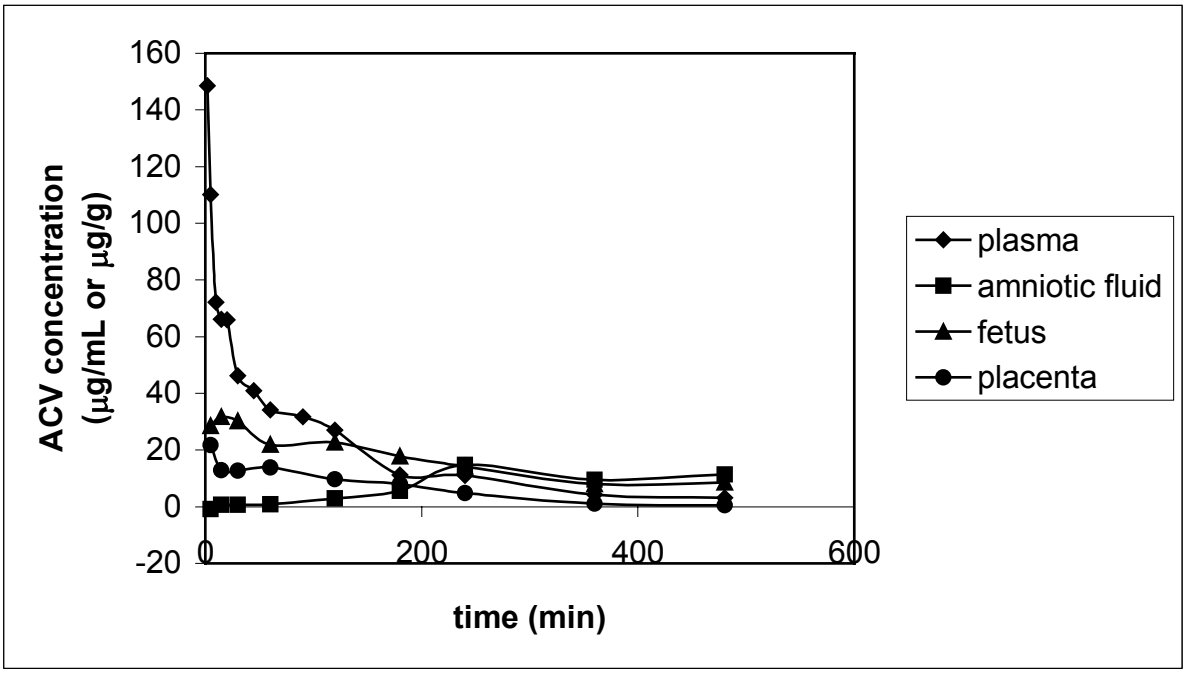
Chromatograms of ACV and AZT (10 μg/mL), and internal standard AZDU (10 μg/mL) in placental homogenate

(d)



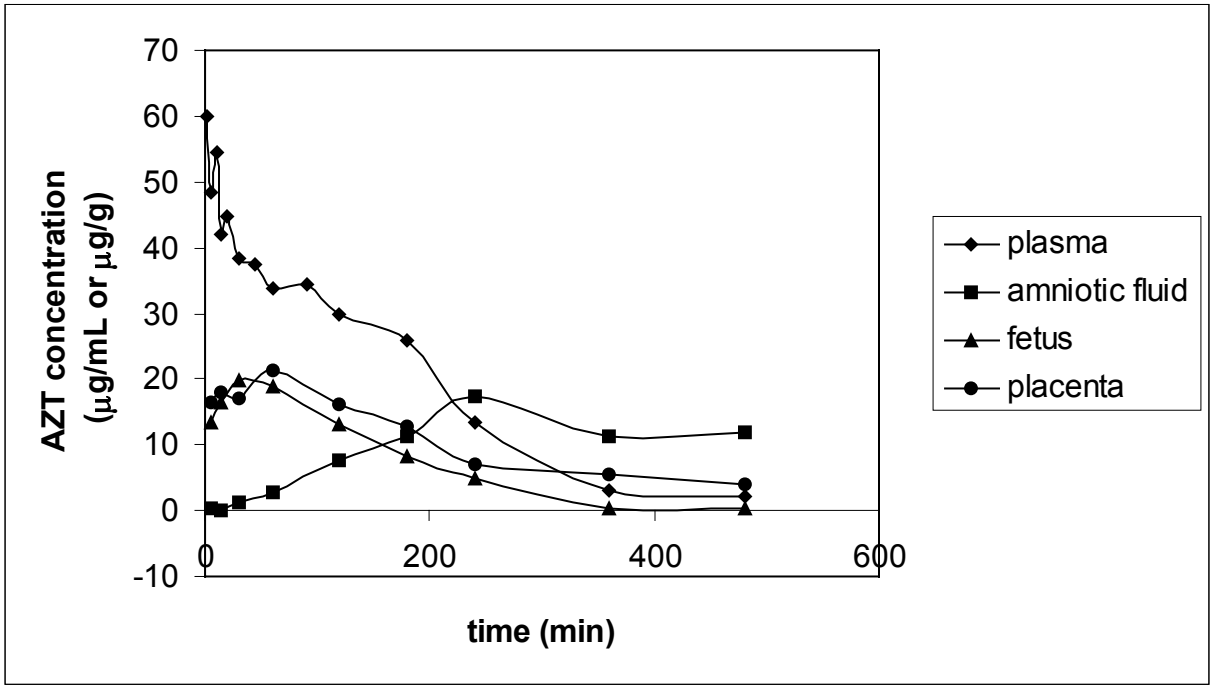
Chromatograms of ACV and AZT (10 μg/mL), and internal standard AZDU (10 μg/mL) in fetal homogenate

Figure 5.3



Concentration versus time profile of ACV in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate from a rat dosed with ACV/AZT combination-therapy (60 mg/kg of each drug)

Figure 5.4



Concentration versus time profile of AZT in maternal plasma, amniotic fluid, placental homogenate, and fetal homogenate from a rat dosed with ACV/AZT combination-therapy (60 mg/kg of each drug)

Table 5.1

Gradient used for plasma and amniotic fluid samples (A = 30 mM acetate/citrate buffer, pH 3.1, B = Methanol)

Time	% B	Flow rate (mL/min)
5	0	0.150
16	100	0.250
17	0	0.150

Table 5.2

Gradient used for placenta and fetal tissue samples (A = 30 mM acetate/citrate buffer, pH 3.1, B = Methanol)

Time	% B	Flow rate (mL/min)
5	0	0.150
20	100	0.150

Table 5.3

The relative recovery (± standard deviation, n = 5) of analytes ACV and AZT (2.5 μg/mL) and internal standards 3TC (25 μg/mL) and AZDU (10 μg/mL)

Matrix	ACV	AZT	3TC	AZDU
Plasma	89.93 ± 5.01	91.87 ± 8.12	92.94 ± 1.69	n/a
Amniotic fluid	97.65 ± 2.43	98.39 ± 1.16	96.48 ± 0.63	n/a
Placenta	86.54 ± 8.60	93.16 ± 3.93	n/a	79.94 ± 13.1
Fetal Tissue	84.87 ± 8.76	83.54 ± 4.13	n/a	83.14 ± 2.64

Table 5.4

The precision (% RSD) and accuracy (% Error) for ACV in maternal plasma, amniotic fluid, fetal tissue, and placental tissue over 3 days (n = 15 for each spike point)

Concentration added (μg/mL)	Concentration found (μg/mL)	% RSD	% Error
Maternal plasma			
0.25	0.240 ± 0.028	11.6	10.3
2.5	2.68 ± 0.23	8.50	11.1
75	72.9 ± 4.5	6.30	5.22
Amniotic fluid			
0.25	0.244 ± 0.023	9.59	7.73
2.5	2.61 ± 0.21	7.89	7.76
75	72.2 ± 5.3	7.34	6.21
Placenta			
0.25	0.239 ± 0.027	11.1	9.55
2.5	2.49 ± 0.14	5.75	4.37
75	75.1 ± 5.9	7.79	6.48
Fetal tissue			
0.25	0.243 ± 0.033	13.5	12.4
2.5	2.56 ± 0.22	8.76	7.90
75	75.8 ± 5.3	7.00	5.34

Table 5.5

The precision (% RSD) and accuracy (% Error) for AZT in maternal plasma, amniotic fluid, fetal tissue, and placental tissue over 3 days (n = 15 for each spike point)

Concentration added (μg/mL)	Concentration found (μg/mL)	% RSD	% Error
Maternal plasma			
0.25	0.240 ± 0.032	13.4	11.4
2.5	2.53 ± 0.21	8.14	6.23
75	70.5 ± 4.9	6.97	7.60
Amniotic fluid			
0.25	0.262 ± 0.025	9.46	9.05
2.5	2.67 ± 0.18	6.85	8.55
75	76.5 ± 5.4	7.00	6.19
Placenta			
0.25	0.231 ± 0.020	8.45	8.76
2.5	2.51 ± 0.19	7.47	5.63
75	73.4 ± 2.8	3.79	3.16
Fetal tissue			
0.25	0.254 ± 0.023	9.17	8.64
2.5	2.62 ± 0.15	5.68	6.87
75	77.2 ± 6.5	8.48	7.90

CHAPTER 6

COMPARATIVE PHARMACOKINETICS OF INTRAVENOUS ACYCLOVIR AND ACYCLVOIR/ZIDOVUDINE THERAPIES IN THE PREGNANT RAT[1]

[1] Brown, S.D., Bartlett, M.G., and C.A. White, To be submitted to *Antimicrobial Agents and Chemotherapy*

Abstract

The pharmacokinetics and placental transfer of acyclovir and zidovudine mono-therapies and acyclovir/zidovudine combination therapy were compared in the pregnant rat. Timed-pregnant Sprague Dawley rats were used for the study. Doses of 60 mg/kg of each drug in mono-and in combination therapy were given by IV bolus and samples of maternal plasma, amniotic fluid, fetal tissue, and placental tissue were collected over a period of eight hours post dose. Concentrations of each drug in the various matrices were measured by HPLC. All data was analyzed using WinNonlin. A one-compartment model with first-order elimination was used to fit the AZT plasma data from the combination therapy rats, but the plasma data from the other groups was fit to a two-compartment model. Tissue data was analyzed by non-compartmental analysis to generate AUC values. Implementation of the combination therapy altered the pharmacokinetics of each drug compared to their mono-therapy pharmacokinetics. The combination of these two drugs may potentiate fetal and placental exposures to each drug.

Introduction

Acyclovir (9-[(2-hydroxyethoxy)-methyl]-guanosine, ACV), an acyclic analog of the natural nucleoside 2'deoxyguanosine (Figure 6.1) is active against the members of the herpes group of DNA viruses [1,2]. For over two decades, acyclovir has been considered the first choice of treatment for herpes simplex virus (HSV-1 and HSV-2), but it has also been shown to effectively treat varicella zoster virus (VZV) and provide protection from cytomegalovirus (CMV) in immunosuppressed patients receiving transplants [3,4]. The

success of ACV in treating HSV has prompted the synthesis of several structural analogs, but none have shown to be as tolerable as and have shown to have such a high therapeutic index as ACV [5-7]. Zidovudine (3'-azido-3'-deoxythymidine, AZT) (Figure 1) is the premier reverse transcriptase inhibitor released for the treatment of human immunodeficiency virus (HIV). A therapy involving the combination of ACV and AZT is not uncommon to help suppress symptoms in patients who are both HIV-positive and HSV-2 positive. These drugs, both in mono-therapy and in combination, have been used to prevent vertical (mother-to-child) transmission of HVS-2 and HIV.

The *Acyclovir in Pregnancy Registry* has compiled a large amount of case study information regarding the relative safety and efficacy of acyclovir use in HSV-2 positive pregnant women [8]. Although a great deal is known about the pharmacokinetic properties of acyclovir, little work has been done to characterize the placental transfer of ACV *in vivo* because pregnant women are routinely excluded from clinical trials. Pharmacokinetic parameters may be altered during pregnancy due to the increase in body fat content, cardiac output, and total body water seen in pregnant women [9-11]. There may also be changes in plasma albumin concentration and protein binding affinities [12,13]. The perfused human placenta model has been used on occasion in attempts to characterize the placental transfer of acyclovir [14,15]. However, this type of model does not mimic the dynamic among fetus, amniotic fluid, and placenta that exists in the whole animal. Unlike ACV, AZT is approved by the FDA for use during pregnancy. To date, several groups have investigated the placental transfer of AZT mono-therapy [16-21]. Huang *et al.* developed a compartmental pharmacokinetic model that described AZT distribution in all matrices associated with pregnancy (maternal plasma, amniotic fluid,

placenta, and fetal tissue) [16]. The concensus concerning AZT behavior in pregnancy is that it readily crosses the placenta via passive diffusion [16-20].

The pregnant rat model has been used successfully in the study of the placental transfer of many compounds, including nucleoside analogs [16, 22-33]. The hemodynamic changes present in the pregnant rat are similar to those seen in a human pregnancy [23,34]. The pregnant rat model is also ideal for pharmacokinetic studies because of the short gestation time and the containment of each fetus, placenta, and amniotic fluid in individual fetal sacs that allows for concurrent serial sampling of the pups.

To date, the effect of pregnancy on the pharmacokinetic parameters of acyclovir have not been investigated. Although anti-viral combinations are often administered to pregnant women, the pharmacokinetic changes associated with each individual drug have not been studied under these circumstances. A study of the safety and efficacy of zidovudine with and without acyclovir found no changes in the efficacy of the drugs and no indication of renal dysfunction or hepatotoxicity associated with the drugs given in combination [35]. Cooper *et al.* also found that when ACV and AZT were given together, the HIV-induced cytopathic effect was increased 2-3 fold over AZT mono-therapy [35]. Mamede *et al.* showed that this drug combination did not lower the birth weights of rats, but rather the combination showed a protective effect against the low birth weights seen in acyclovir mono-therapy [36]. This study examines the pharmacokinetics of acyclovir mono-therapy and acyclovir/zidovudine combination therapy during pregnancy.

Materials and Methods

Reagents and Chemicals

Analytical standards of acyclovir, ganciclovir, and zidovudine were obtained from Sigma (St. Louis, MO, USA). AZDU (3'-azido-3'-deoxythymidine), one of the internal standards used, was synthesized as previously described [37]. Lamivudine (3-TC), also an internal standard, was recrystallized from Epivir® tablets. Reagent grade citric acid was acquired from Sigma. Reagent grade ammonium acetate and reagent grade octanesulfonic acid were bought from Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile and methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sep-Pak Vac 1cc C-18 cartridges were purchased from Waters (Milford, MA, USA). The deionized water used was generated from a Continental Deionized Water System (Natick, MA, USA).

Animal Study

The use of animals for this study was approved by the University of Georgia Animal Use and Care Committee, and conducted in accordance with the Animal Welfare Act and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The rats were housed one animal per cage in the UGA College of Pharmacy AALAC accredited animal facility. The living environment of the animals was controlled to 14 hr of light per day, constant temperature of 20 – 22^{o}C, daily feedings of standard chow, and water *ad libitum*.

Timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) with an average weight of 331 $\pm$ 35g were used for the study. The anesthesia (ketamine:acepromazine,

75:2.5 mg/kg IM) was given in conjunction with subcutaneous atropine (0.5 mg/kg). Prior to dosing, a laparotamy was performed to allow for sampling of the pups and a cannula was surgically implanted in the right jugular vein. IV bolus doses (60 mg/kg) of each therapy group were prepared in 0.1M NaOH in physiological saline (pH 7.4) and administered via the jugular cannula followed by 1 mL of physiological saline (pH 7.4) to rinse the cannula. Three dosing groups were used to complete the study: I. Acyclovir mono-therapy (60 mg/kg) (n = 6), II. Zidovudine mono-therapy (60 mg/kg) (n = 3), and III. ACV/AZT combination therapy (60 mg/kg each) (n = 7). Pups were harvested at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min. Blood samples of 200-250 μL were collected at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360, and 480 min into heparinized tubes and centrifuged for 10 min at 16,000 g using a Biofuge Pico Microcentrifuge (Heraeus Instruments, Hanau, Germany) to allow for collection of the plasma. Amniotic fluid samples were pulled from the fetal sacs and deposited into clean eppendorf tubes. Placental and fetal tissues were homogenized in two volumes of deionized water (w/v) using a Tekmar tissue grinder (model SDT-1810, Cincinnati, OH, USA). All samples were stored at –20^{o}C until analysis. Following the eight hr time-course of the experiment, animals were euthanized using phenobarbital (150 mg/kg).

HPLC Analysis –Acyclovir Mono-therapy

The plasma and amniotic fluid samples were prepared by acid protein precipitation by adding 10 μL (50 μL amniotic fluid sample) or 20 μL (100 μL plasma sample) of 2 M perchloric acid. Placental and fetal tissue homogenates were processed by solid phase extraction using Waters Sep-Pak Vac C18 SPE cartridges. The SPE

procedure included a conditioning step with methanol and the mobile phase followed by the sample load and a wash of the sample with deionized water, and finally an elution with methanol. The internal standard, ganciclovir was also spiked into each sample to yield a final ganciclovir concentration of 10 μg/mL in the sample. Calibration curves were generated using samples from spiked blank matrix to yield final calibration points of 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL.

The chromatographic system consisted of a Hewlett-Packard (Agilent) 1100 Series HPLC with a quaternary pump, degasser, autosampler, and variable wavelength UV detector (Palo Alto, CA, USA). Chromatographic separations were achieved using an Agilent Eclipse XDB C-8 column (150 x 2.1mm, 5μm) (Palo Alto, CA, USA) with a Phenomenex Security Guard C-18 guard column (Torrance, CA, USA).

The mobile phase used for the plasma and amniotic fluid matrices was a 10 mM acetate/citrate buffer: 3.7 mM aqueous octanesulfonic acid (87.5:12.5 v/v) adjusted to pH 3.08 with phosphoric acid. Under these conditions, GAN eluted at ~8 min and ACV eluted at ~11 min. The mobile phase used for the placental and fetal tissue samples was a 30mM acetate/citrate buffer with 5mM octanesulfonic acid (pH 3.08) and acetonitrile (99:1 v/v). Under these conditions, GAN eluted at ~10 minutes and ACV eluted at ~12 min. All flow rates were kept at a constant 0.200 mL/min, the injection volume used was 10 μL, and the detection wavelength was fixed at 254 nm. This method has been previously validated to show acceptable precision and accuracy for the quantitation of ACV in the range of 0.1 – 100 μg/mL [38].

HPLC Analysis – AZT Mono-therapy and ACV/AZT Combination Therapy

Sample preparation for plasma and all tissues is as described above. Lamivudine (3-TC) was spiked into each plasma and amniotic fluid sample (25 μg/mL) to serve as an internal standard. Because of chromatographic interference of endogenous peaks, 3-TC could not be used as an internal standard for the placental and fetal tissues and was replaced by AZDU. AZDU was spiked into each placenta and fetal tissue sample at a level of 10 μg/mL. Calibration curves were generated from using spiked blank matrix to yield final calibration points of 0.1, 0.5, 1, 5, 10, 50, and 100 μg/mL.

The HPLC system used in this assay is the same as described above. Because of the relative differences in the polarities of ACV and AZT, a gradient elution technique had to be utilized for timely analysis. The mobile phase consisted of a 30 mM acetate/citrate buffer at pH 3.08 (component A) and methanol (component B). Under these conditions, ACV eluted at 7.6 min, AZT at 15.9 min, and 3-TC at 10.9 min in the plasma and amniotic fluid. In the fetus and placental tissues, ACV eluted at 7.4 min, AZT at 19.8 min, and AZDU at 18.4 min. This assay was validated to ensure both precision and accuracy in accordance to the FDA guidelines for bioanalytical method validation [39]. The assay showed acceptable reproducibility (% RSD < 15%) and accuracy (% Error < 15%) over the calibration range of 0.1 – 100 μg/mL [40].

Data Analysis

Using Win Nonlin, the plasma data from all rats was subjected to compartmental analysis. A two-compartment IV-bolus model with first order elimination was used to fit the plasma data generated from AZT mono-therapy dosed rats and ACV for both mono-

and combination therapy animals. A one-compartment IV-bolus model with first order elimination was used to fit the AZT plasma data from the combination therapy animals. A "1/y" weighting scheme was used throughout the analysis. Amniotic fluid, fetus, and placenta was subjected to non-compartmental analysis. To express relative exposure to each matrix, the extrapolated area under the curve (AUC) values for the individual tissues were compared to the AUC values for the corresponding plasma data. The pharmacokinetic parameters generated for each dosing group and the relative exposure numbers were compared using the t-test ($\alpha = 0.05$) to detect statistically significant differences. All calculations were done using Microsoft Excel.

Results and Discussion

The fitted plasma concentration-time profiles for AZT and ACV are shown in Figure 6.2. The pharmacokinetic parameters generated from the compartmental analysis of the plasma data are presented in Table 6.1. Co-administration of ACV resulted in a 60% decrease in total clearance of AZT. Considering that renal excretion is the major route of elimination for both drugs, and that ACV and AZT are both transported by the organic anion transporter, this decrease in clearance is probably due to the inhibition of active tubular secretion in the kidney [7,30]. A significant increase in both half-life and AUC for AZT was also seen when ACV is co-administered. These differences result from the decrease in AZT clearance. No statistically significant changes can be noted in the volume of distribution or C_{max} of AZT when given in the combination therapy.

The decrease in the clearance of AZT in the combination therapy group is coupled with a 60% increase in ACV clearance. Also noted is a 50% decrease in the volume of

distribution of ACV when administered with AZT. It is unlikely that this can be attributed to a change in plasma protein binding, for ACV inherently has a low affinity for plasma protein binding sites (4.4 – 15.4% bound) [41]. An increase in uptake of ACV by the fetus in the combination therapy may help explain this change in volume. There is a three-fold increase in the amount of ACV (expressed as a percentage of dose) taken up into the fetal compartment when ACV is co-administered with AZT. The decrease in half-life of ACV in the combination therapy rats results from a decrease in volume of distribution and an increase in clearance.

Duplicate and triplicate pups were harvested in several animals throughout the time course to ensure that each fetal sac was "equal." No corrections were made for metabolic differences between male and females pups; however this was not of great concern considering that neither ACV nor AZT is extensively metabolized in the rat. Low coefficients of variation were observed among fetal sacs removed at the same time point (7.4% in fetal tissue, 6.8% in placenta, and 4.3% in amniotic fluid).

The concentration time profiles of the two drugs in amniotic fluid, placenta, and fetus are shown in Figures 6.3 (AZT) and 6.4 (ACV), and the pharmacokinetic parameters for these matrices generated from non-compartmental analysis are tabulated in Tables 6.2 through 6.4. In the rats receiving AZT alone or in combination with ACV, the initial rate of uptake in amniotic fluid and fetus is similar; however, the peak concentration (C_{max}), the time to peak (T_{max}), and the AUC are higher in these tissues with the animals receiving combination therapy. A similar pattern can be seen in the placental tissue of the AZT group; a longer time to peak, a higher peak AZT concentration, and a larger AUC is seen in the combination therapy group. For ACV, no

significant differences are noted in the placental concentration versus time profiles. However, both amniotic fluid and fetal exposures to ACV are much lower when ACV is given alone. An increase in the peak concentrations of ACV in the amniotic fluid (3-fold) and the fetus (2-fold) are demonstrated in the combination therapy animals. This is coupled with a shorter T_{max} in both of these tissues due to a faster rate of uptake of ACV for the combination therapy group. An increase in the overall exposure of the fetal compartment to ACV in the ACV/AZT dosed group is indicated by the 2-fold increase in AUC in both amniotic fluid and fetal tissue.

Relative exposure numbers are shown in Table 6.5. This table shows the ratios of extrapolated AUC values for each tissue versus plasma AUC. For AZT, decreases in exposure to all three tissues were seen in the presence of ACV (16 – 24% decrease). This decrease suggests saturation of uptake into the fetal compartment (placenta, amniotic fluid, and fetus). On the other hand, ACV showed a three-fold increase in drug exposure in amniotic fluid and fetal tissue with the combination therapy. No change in placental exposure was seen for ACV in the two therapy groups. Previous reports indicate that ACV accumulates in the amniotic fluid [42,43]. Although this may not be obvious from the concentration versus time profiles of ACV, the accumulation of ACV in the amniotic fluid is apparent from the prolonged half-life in this tissue (5.93 ± 3.9 hr).

Conclusions

The disposition of AZT and ACV in the pregnant rat was significantly altered when the two drugs were co-administered. The changes noted in the placenta and fetus, suggest that transporters, in addition to passive diffusion, play a role in the uptake of both

ACV and AZT in these tissues. Nucleoside and organic anion transporters are two that could possibly contribute to the placental transfer of these two compounds [44,45]. Interestingly, the uptake of ACV into the placenta was not affected by co-administration of AZT. However, the fetal and amniotic fluid ACV exposure was increased approximately three-fold. The increase in plasma clearance coupled with an increase in fetal uptake suggests up-regulation of a transport process when ACV and AZT are co-administered.

The driving force behind the "protective effect" of AZT against ACV proposed by Mamede *et al.* is not apparent from this study [36]. The exposures to fetus and amniotic fluid of ACV are increased dramatically in the presence of AZT. However, this is not of extreme concern considering that ACV has been shown to exhibit no detrimental effects on the fetus when used during pregnancy [8,42,43,46-48]. The theory that the ACV potentiates the actions of AZT when given in combination is supported by this data. The significant increase in half-life and AUC coupled with the 60% decrease in clearance, which resulted in higher AZT concentrations observed in both the mother and the fetus, could support the observation of an increased activity of AZT when given in this combination.

Both ACV and AZT are known to be safe and effective against protecting unborn children against their respective viruses. This combination of drugs would allow for a potentiated activity of AZT while increasing the exposure of the fetus to ACV. Although the pharmacokinetics of each drug is altered in the combinations, therapeutic plasma levels of each can be maintained when they are given together.

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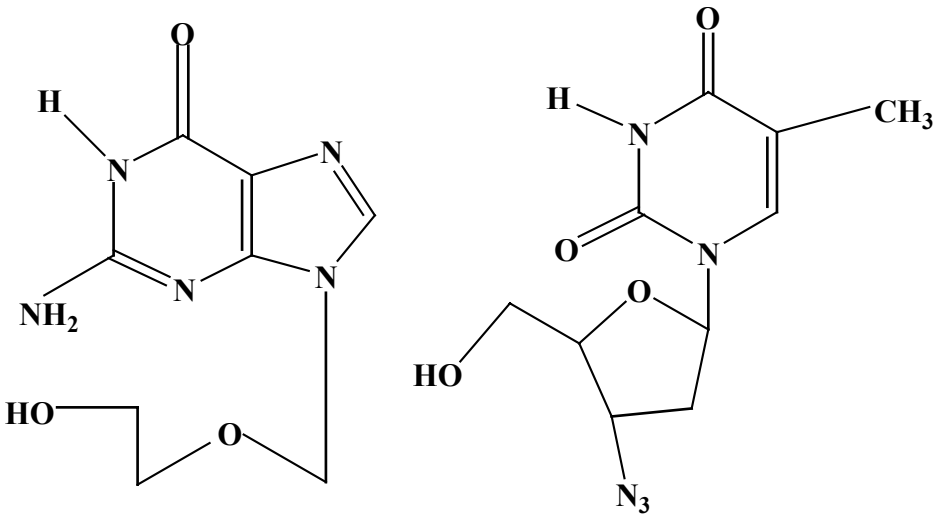
Figure 6.1

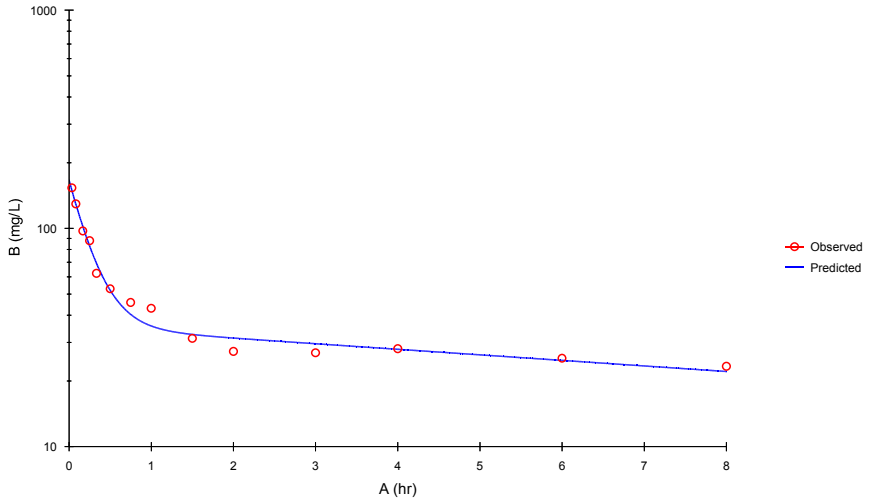


Acyclovir Zidovudine

Structures of acyclovir and zidovudine

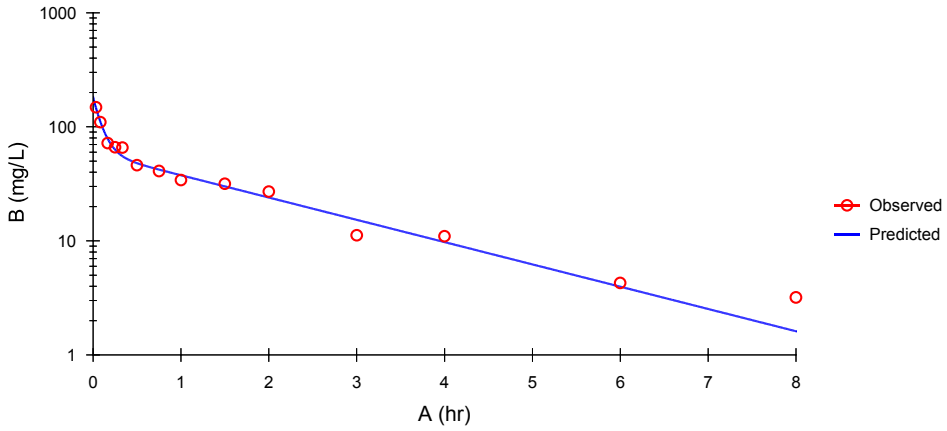
Figure 6.2

(a)



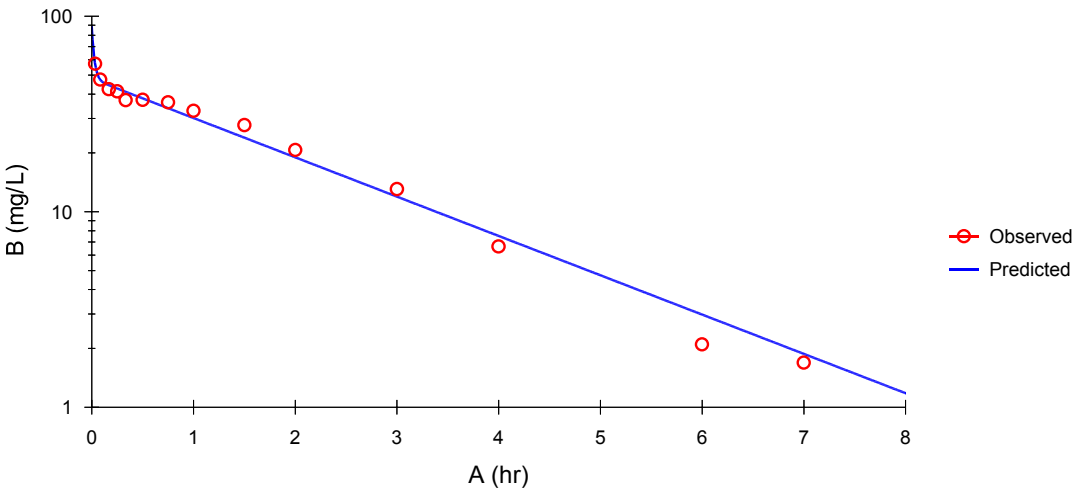
Fitted plasma concentration versus time profiles for ACV mono-therapy

(b)



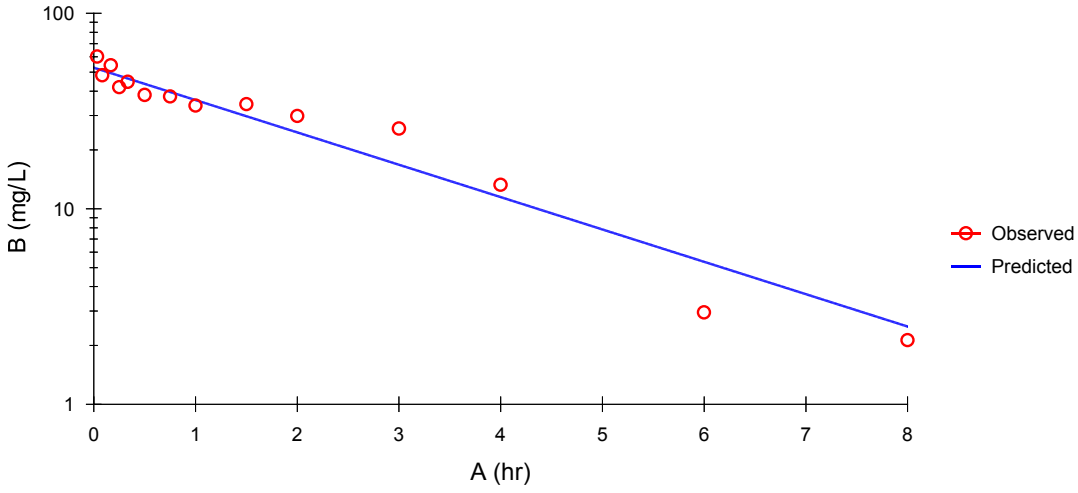
Fitted plasma concentration versus time profiles for ACV in the presence of AZT

(c)



Fitted plasma concentration versus time profiles for AZT mono-therapy

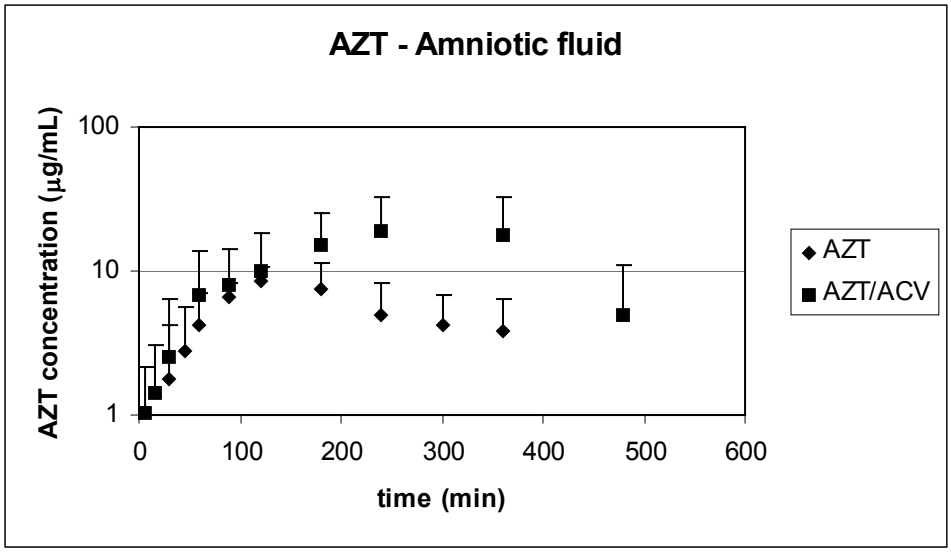
(d)



Fitted plasma concentration versus time profiles for AZT in the presence of ACV

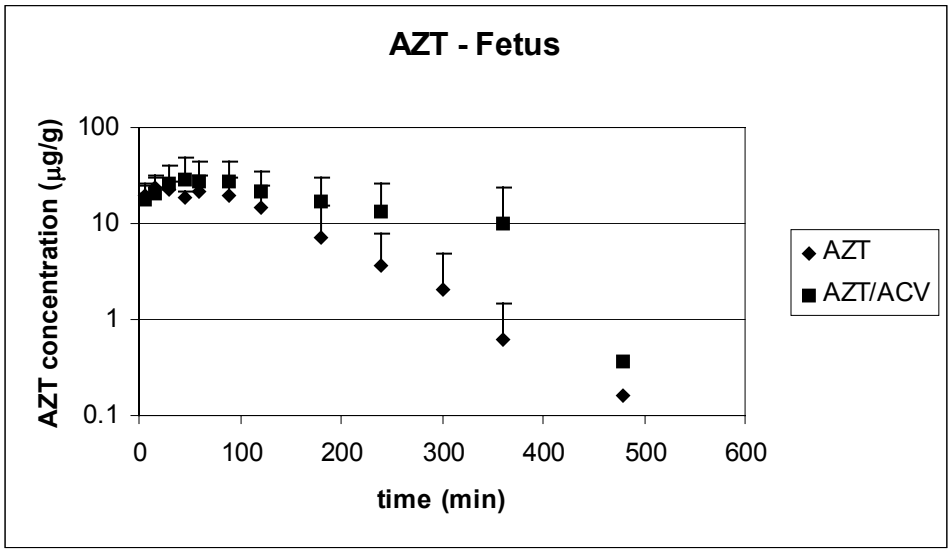
Figure 6.3

(a)



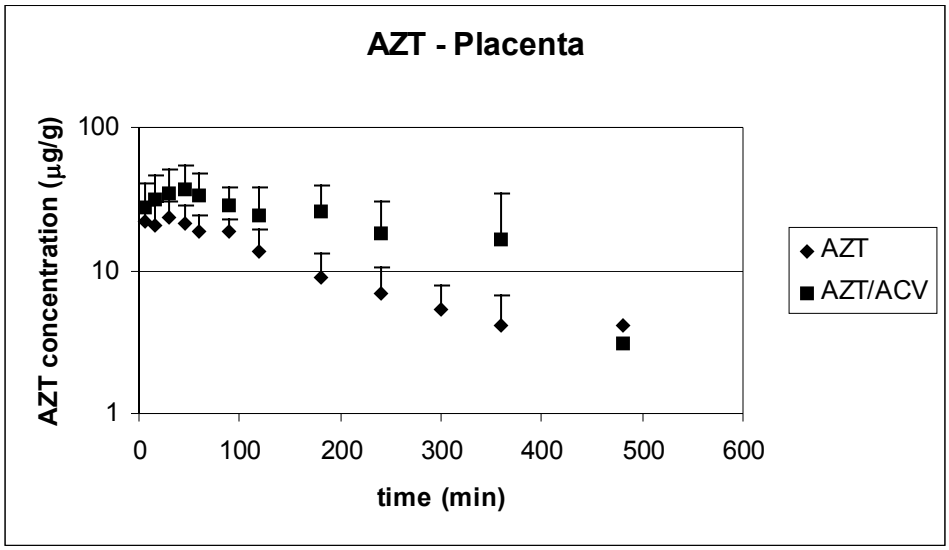
The concentration versus time profile of AZT mono-therapy and in combination with ACV from amniotic fluid

(b)



The concentration versus time profile of AZT mono-therapy and in combination with ACV from fetal tissue

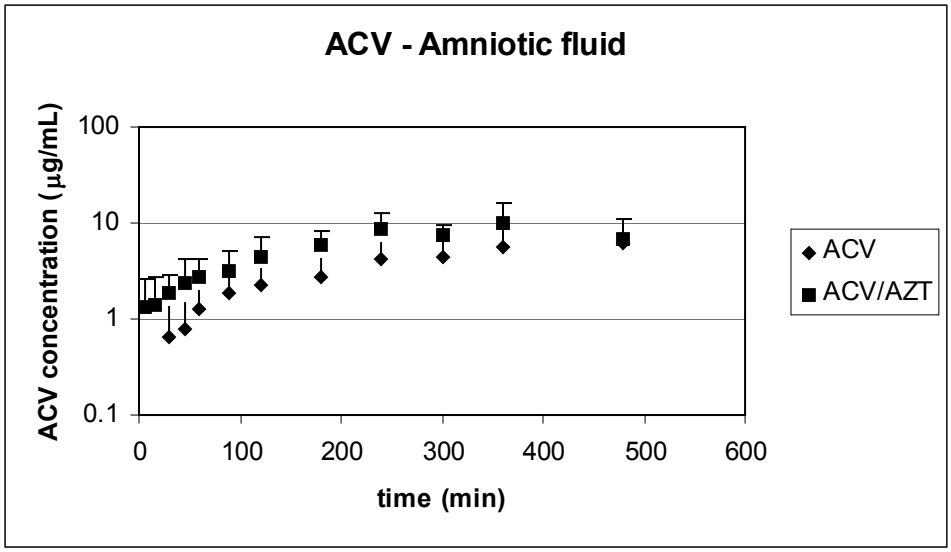
(c)



The concentration versus time profile of AZT mono-therapy and in combination with ACV from placental tissue

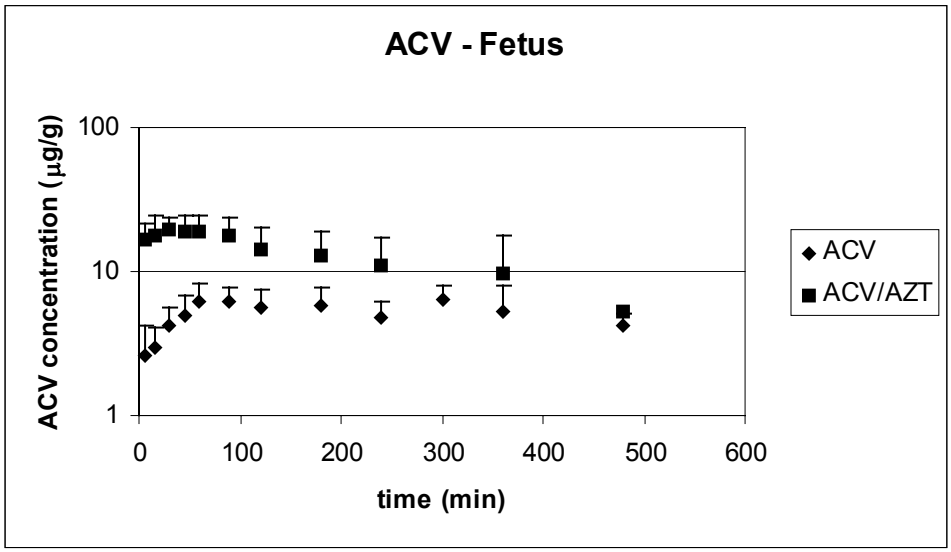
Figure 6.4

(a)



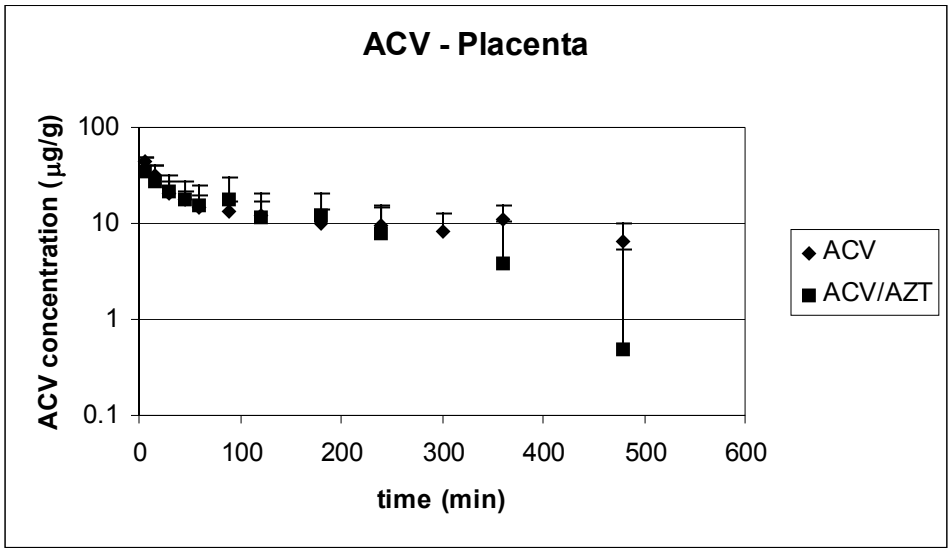
The concentration versus time profile of ACV mono-therapy and in combination with AZT from amniotic fluid

(b)



The concentration versus time profile of ACV mono-therapy and in combination with AZT from fetal tissue

(c)



The concentration versus time profile of ACV mono-therapy and in combination with AZT from placental tissue

Table 6.1

Pharmacokinetic parameters (mean ± standard deviation) generated from the compartmental analysis of plasma data collected from ACV mono-therapy, AZT mono-therapy and ACV – AZT combination therapy pregnant rats (60 mg/kg)

Parameter	**ACV**	**ACV/AZT**	**AZT**	**AZT/ACV**
Half-life (hr)	9.12 ± 1.1	2.48 ± 1.9	1.39 ± 0.3	2.69 ± 1.2
*AUC (hr*mg/L)*	467.4 ± 183	241.4 ± 165	80.1 ± 21	239.9 ± 130
*Clearance *L/hr-kg)*	0.14 ± 0.05	0.34 ± 0.2	0.78 ± 0.2	0.31 ± 0.2
V_{ss} *(L/kg)*	1.61 ± 0.26	0.80 ± 0.1	1.52 ± 0.42	1.02 ± 0.2
C_{max} *(mg/L)*	148.4 ± 88	196.7 ± 27	72.1 ± 14	59.9 ± 10

Table 6.2

Pharmacokinetic parameters for amniotic fluid generated using non-compartmental analysis

Parameter	ACV	ACV/AZT	AZT	AZT/ACV
C_{max} *(mg/L)*	4.99 $\pm$ 2.7	10.5 $\pm$ 4.4	8.57 $\pm$ 2.0	19.6 $\pm$ 13
T_{max} *(hr)*	6.20 $\pm$ 2.5	4.00 $\pm$ 1.2	2.33 $\pm$ 0.58	3.67 $\pm$ 0.52
AUC (hr-mg/L)	18.6 $\pm$ 9.3	39.6 $\pm$ 16	35.7 $\pm$ 18	74.1 $\pm$ 48

Table 6.3

Pharmacokinetic parameters for placenta generated using non-compartmental analysis

Parameter	ACV	ACV/AZT	AZT	AZT/ACV
C_{max} *(μg/g)*	34.6 ± 16	38.5 ± 19	24.2 ± 8.5	40.8 ± 17
T_{max} *(hr)*	0.26 ± 0.3	0.12 ± 0.07	0.36 ± 0.3	0.68 ± 0.5
AUC (hr-μg/g)	82.0 ± 42	81.9 ± 62	72.9 ± 23	139 ± 62

Table 6.4

Pharmacokinetic parameters for fetus generated using non-compartmental analysis

Parameter	ACV	ACV/AZT	AZT	AZT/ACV
C_{max} *(μg/g)*	7.89 ± 1.4	24.5 ± 4.7	28.9 ± 5.3	32.7 ± 15
T_{max} *(hr)*	2.50 ± 2.0	0.65 ± 0.3	0.50 ± 0.4	1.2 ± 0.5
AUC (hr-μg/g)	37.4 ± 10	88.1 ± 27	59.4 ± 32	108 ± 64

Table 6.5

Relative exposure ($AUC_{tissue}/AUC_{plasma}$) data (mean $\pm$ standard deviation) from amniotic fluid, fetal tissue, and placental tissue generated from the non-compartmental analysis of tissue data collected from ACV mono-therapy, AZT mono-therapy and ACV – AZT combination therapy pregnant rats (60 mg/kg)

Tissue	**ACV**	**ACV/AZT**	**AZT**	**AZT/ACV**
Fetus	0.20 $\pm$ 0.05	0.56 $\pm$ 0.2	0.72 $\pm$ 0.2	0.59 $\pm$ 0.2
Placenta	0.42 $\pm$ 0.1	0.43 $\pm$ 0.3	1.00 $\pm$ 0.5	0.76 $\pm$ 0.2
Amniotic fluid	0.091 $\pm$ 0.02	0.26 $\pm$ 0.1	0.48 $\pm$ 0.3	0.40 $\pm$ 0.2