PART I. DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR THE ANALYSIS OF SELECTED PHARMACEUTICALS IN INTRAVENOUS FLUID MIXTURES

PART II. DEVELOPMENT AND VALIDATION OF BIOANALYTICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR THE ANALYSIS OF SELECTED PHARMACEUTICALS

by

WILLIAM VINCENT CAUFIELD

(Under the direction of James T. Stewart, PhD)

ABSTRACT

This dissertation is composed of two sections. The first section is concerned with the development and validation of stability indicating high performance liquid chromatography (HPLC) methods for the analysis of selected pharmaceuticals in intravenous fluid mixtures. The second section focuses on the development and validation of bioanalytical HPLC methods for the determination of selected pharmaceuticals in human plasma. Part I contains two chapters. In Chapter 2 four stability indicating HPLC methods were developed for the assay of the carbapenem antibiotic meropenem in combination with dopamine, aminophylline, metoclopramide or ranitidine in intravenous fluid mixtures. In Chapter 3 five stability indicating HPLC methods were developed for the assay of the protease inhibitor zidovudine (AZT) in combination with ceftazidime, chlordiazepoxide, dobutamine, lorazepam or ranitidine in intravenous fluid mixtures. UV detection was used for all of the separations. Accelerated stability studies were carried out on each drug by exposure to several different stressors for different time periods. The degraded drugs were then analyzed using the developed methods with a photodiode-array (PDA) detector and Waters Millennium32 PDA software to verify that degradation products did not interfere with the quantitation of each drug. In chapter 4 solid phase extraction and HPLC with UV detection was used to determine AZT and levofloxacin in human plasma. In chapter 5 high speed HPLC methods utilizing solid phase extraction and a new monolithic silica column were developed for the determination of drugs of abuse in human plasma.

INDEX WORDS: Antiretroviral, Bioanalysis, HPLC, Human plasma, Ion-pair chromatography, Monolithic silica, Photodiode-array detection, Stability indicating, Therapeutic drug monitoring.

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DEDICATION

This dissertation is dedicated to my beloved wife, Margaret. Her love, friendship, support, humor, patience, and confidence were the glue that held me together during these six years. This work is as much hers as it is mine and it stands as a testament to the power of teamwork, perseverance and love.

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It Takes A Village to Raise a Child.
Ancient African proverb

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

There are many steps that a chemical entity takes on the long journey to becoming an approved and profitable pharmaceutical and many of these steps require some type of analytical data. In the early stage of drug discovery, synthetic chemists simply want to know, in the simplest terms, "what is this green goo that I have made?" As part of the preclinical studies, a new drug candidate must be examined with respect to its chemistry, physical properties, animal pharmacokinetics, pharmacology, metabolism, and toxicity. In this phase, the stability characteristics of the bulk drug substance are determined. The data provided must demonstrate that the bulk drug is stable under the duration and conditions used in the toxicity studies. The stability of the proposed formulation that will be used in the clinical pharmacological studies must also be assessed. If the drug candidate seems promising, it will be scaled up to produce kilogram quantities. One of the purposes of this scale-up is to generate enough material to conduct more extensive testing. Each new batch will require testing for identity, purity, and physical properties. The next stage, the development of a dosage form and formulation, will require the analysis of both active and inactive ingredients. In addition, the stability of the formulation that will be used in the clinical trials must be studied. At this point, it is also necessary to determine if the drug and the excipients used in the final formulation are compatible. Prior to submitting a new drug application (NDA) to the Food and Drug Administration (FDA), it is necessary to demonstrate that the drug is stable in the proposed packaging (1).

It should be evident from this brief tour through the drug development process that analytical chemists are heavily relied upon to provide accurate, precise, and sensitive data to many scientific disciplines. It should also be apparent that stability testing plays a key role in almost every stage of drug development. But why is stability testing necessary? The primary purpose of stability testing is to demonstrate that a drug will maintain a portion of its potency over the duration of its normal shelf life. Federal regulations require drug manufacturers to demonstrate that their product will maintain its purported potency, purity, quality, and identity

throughout the shelf life of the drug (2). Most regulatory authorities would consider a drug with less than 90% of its original potency as unfit for human consumption (3).

In the hospital, pharmacists are frequently asked to prepare formulations that are not commercially available. These formulations are often mixed in parenteral or dialysis solutions as well as topical and oral preparations. It is necessary to assess the chemical stability of these formulations and to determine the shelf life (4). As in the industrial setting, the adsorption of the drug and the excipients to the product container must be investigated.

In the first half of the past decade, there were a limited number of analyses that addressed the determination of a product's stability. Qualitative assessments were made by visual and olfactory analyses, i.e., "Did a precipitate form?" or "Does it smell different?" Quantitative tests used color producing chemical reactions and colorimeters to measure stability. In the late 1940's, spectrophotometers became available but their utility was limited because degradation products often have the same chromophore as the parent compound (5). More specific analyses became possible with the advent of thin layer chromatography (TLC). In TLC, a thin coating of silica gel or alumina is applied to a glass plate and dried. The sample is usually spotted at one end of the plate and then the plate is placed upright in a covered tank containing a small amount of mobile phase. The sample moves up the plate by capillary attraction and the mixture is separated by an adsorption process. The separated components may be detected under a UV light or a color change may be induced by spraying the plate with a reagent (6). However, despite many attempts at quantitating TLC, it remains a semi-quantitative analytical method. The need for a truly quantitative analytical technique led to the use of High Performance Liquid Chromatography (HPLC) in stability assessments. In HPLC, sample components are separated based on non-covalent interactions with a mobile and a stationary phase. The different modes of HPLC include reversed-phase (RP-HPLC), normal-phase (NP-HPLC), and ion-exchange (IEC), with RP-HPLC being the most commonly used (7). In RP-HPLC, retention is the result of the partitioning of the sample components between a nonpolar stationary phase such as octadecyl

(C18) and a polar mobile phase. Sample retention can also occur by a combination of hydrophobic interactions and normal phase interactions with un-protected silanol groups on the stationary phase. Ion pair chromatography (IPC) is a variation of RP-HPLC that is often used when the analyte of interest is a highly hydrophilic weak acid or base. In this method, a hydrophobic ion pairing reagent containing a counter ion is added to the mobile phase. The ion pair reagent then partitions into the stationary phase because of its hydrophobic alkyl group. A charged sample ion will then exchange with the counter ion, resulting in retention by an ion exchange process. (8).

Retention of sample components in HPLC can be manipulated by altering the mobile phase conditions. Isocratic separations occur with a constant mobile phase composition throughout the analytical run. In gradient elution, the composition of the mobile phase is changed, usually in a linear fashion, over the course of the run. The retention of highly lipophilic compounds can be dramatically decreased by the judicious application of a mobile phase gradient. The result is that analytical run times can be decreased, resolution enhanced, and sensitivity improved due to the decrease in band broadening.

The use of organic solvents in HPLC is becoming increasingly problematic due to the initial purchase costs as well as the increasing cost of disposal. One technique that has been developed to deal with this problem involves distillation and purification of less expensive technical or reagent grade solvents. Although the initial cost of the solvent is decreased, it is time consuming and does not address the problem of waste disposal. It is also possible to purify the waste from the HPLC, but again, this can be a difficult and laborious task due to the complexity of most chromatographic wastes. Commercially available mobile phase recycling systems can be used when a single component is to be assayed. However, many samples contain low levels of impurities that can decrease the efficiency of the recycling system and lead to poor sensitivity and linearity. The obvious solution to this problem is to decrease solvent consumption and thus the amount of generated organic waste. The use of a narrow bore column (2.1 mm

internal diameter or less) can reduce mobile phase consumption by a factor of 3-4 (assuming a flow rate of 1-2 mL min^{-1}) (9). Since column flow is proportional to internal diameter the same linear velocities and retention times can be preserved by using a narrow bore column operated at a reduced flow rate.

The ability to separate complex mixtures by HPLC is meaningless if there is no way to see, and ultimately quantitate, the resulting separation. Today, there are a variety of detectors that can be used, including ultraviolet absorption (UV), fluorencence, refractive index, and mass spectrometers. The most popular HPLC detectors for drug analysis and the assessment of product stability are UV, diode array detectors (DAD) (10-11) and mass spectrometers. The reason for the popularity of UV detectors is that it is both durable and inexpensive (7). UV detectors measure the loss in intensity of ultraviolet or visible light as it passes through the solution exiting the HPLC column. A typical variable wavelength detector will consist of a continuous light source, such as a deuterium lamp, a monochrometer, a flow cell, either a photodiode or a photomultiplier to measure the light exiting the flow cell, and an amplifier circuit to provide an output in voltage proportional to the measured absorbance (12). The amount of monochromatic light that is absorbed by the sample in the flow cell is linearly proportional to the concentration of the sample and is represented by Beer's Law:

$$A = \varepsilon bc$$

Where A is absorbance, ε is the amount of light a particular analyte will absorb (molar absorbtivity), b is the path length of the cell in centimeters, and c is the sample concentration in moles/liter. However, Beer's law is not without limit. As sample concentration increases, a shielding effect of all the molecules in solution impairs the interaction between photons and molecules and Beer's law becomes non-linear.

The development of diode array detectors in the early 1980s represented a significant advance in HPLC detection because it allowed the continual acquisition of absorbance data across

the UV/visible spectrum (13). The chief difference between a variable wavelength UV/visible and DAD detector is the type of optics used. Conventional UV/visible detectors utilize forward optics where the flow cell is placed after the dispersion grating. DAD detectors utilize reverse optics in which polychromatic light is allowed to pass through the sample first, before being directed into a diffraction grating where it is dispersed into its component wavelengths. The dispersed light is then focused onto an array of diodes that generate electrical signals that are proportionate to the intensity of the impinging light. The number of elements in the array determines the wavelength resolution and sensitivity of the detector. The amount of data generated by a DAD detector necessitates the use of a computer to process the data as well as control the detector itself. The ability to generate a full UV spectrum for each peak in a chromatogram makes the DAD detector very useful in choosing an optimal wavelength in HPLC method development. One of the major reasons DAD detectors are purchased is to conduct peak purity assessments (13). The issue of peak purity has plagued chromatographers ever since Tswett described the technique in the early 20^{th} century (14). The problem was that, aside from using an alternate analytical technique to analyze a mixture, there was no way to know for sure if a decomposition product was coeluting with the parent drug. The situation improved considerably with the development of the DAD detector. The underlying assumption for peak purity assessment is that for any pure peak, spectra taken at any time during peak elution should have exactly the same profile (13). If an impurity is present, the spectrum from the parent component will be distorted. This assumption is based on the premise that the UV spectrum of the parent component and the decomposition product are different enough to make a measurable change in the spectrum. This is not always the case in stability testing where the parent drug and the degradant may have the same or similar chromophore. Therefore, DAD peak purity assessments are a valuable tool in the development of stability indicating HPLC methods, but the results are not definitive.

The development of a stability indicating HPLC method involves two essential stages. In the first stage the parent drug is degraded thermally, chemically (acid, base, oxidation), and

photolytically (exposed to strong UV light). It is usually not advisable to stress the parent drug beyond 10 – 30% by assay comparison against non-degraded drug. The objective is to induce a small amount of degradation but not so much that secondary degradation products (i.e., degradants of degradants) are generated because they generally are not observed in stability studies (14). If the method is stability indicating the degradation peaks will be baseline resolved from the parent peak. If this is not the case, the method is then modified, if possible, to improve the separation. The next step in this stage is to assess the purity of the parent peak. If the parent peak is found to be co-eluting with a degradant the method must again be modified to obtain the best possible separation. It is not usually necessary to have baseline resolution of all the degradants but the parent drug must be baseline resolved. The second stage of the method development process requires that the method be validated with respect to linearity (concentration-detector response relationship), accuracy, precision, selectivity, and limits of detection and quantitation. The purpose of the validation is to demonstrate that the method is scientifically sound and that it has been systematically evaluated to meet the requirements of the intended application (4).

In Chapter 2 of this dissertation, isocratic, stability indicating HPLC assays were developed for the simultaneous analysis of meropenem in combination with dopamine, aminophylline, metoclopramide, and ranitidine in intravenous fluid mixtures. Meropenem is a new carbapenem antibiotic with a broad spectrum of activity in-vitro. It is active against gram-positive and gram-negative organisms and is highly effective in the treatment of infections caused by many clinically relevant aerobic, nutritionally fastidious and anaerobic bacterial species (15). Meropenem is administered by I.V. infusion or by I.V. bolus injection, often in conjunction with one or more other injectable drugs. It has been analyzed by HPLC with UV detection in serum, plasma and urine (16-19). Meropenem has also been studied with respect to its compatibility with commonly used injectable drugs by examination of I.V. admixtures for precipitation, gas production and color change (20). A stability indicating HPLC method was developed for the

analysis of meropenem and ofloxacin in 5% dextrose in water USP and 0.9% sodium chloride USP using UV detection at 270 nm (21). However, there was a need for the development of stability indicating HPLC methods that could be used to evaluate the compatibility of meropenem with other injectable drugs. Aminophylline, dopamine, metoclopramide and ranitidine are compounds that can be administered in conjunction with meropenem. There were no stability indicating methods available to assay each of these compounds with meropenem reported in the literature.

Aminophylline is a methylxanthine, which is formed when theophylline complexes with ethylenediamine. It can be used to reverse narcosis in anesthetized patients as well as in the treatment of asthma (22). Aminophylline/theophylline has typically been analyzed by reversed phase HPLC with UV detection (23-25). Dopamine, a monoamine neurotransmitter, is used in the treatment of shock. (26). HPLC assay methods for the analysis of dopamine using electrochemical (ECD) (27-28), fluorescence (29) and combined ultraviolet absorbency-electrochemical detection (UV-ECD) (30) have been reported. The methods utilized aqueous – methanol mobile phases and ODS columns. Metoclopramide is a member of the benzamide class of antiemetic agents and can be administered during cancer chemotherapy as well as in pregnancy (31). Assay methods for metoclopramide include ultraviolet (32) spectroscopy, HPLC-UV (33-34), and gas chromatography – mass spectrometry (GC-MS) (35). Ranitidine is an H_2-receptor antagonist used to inhibit gastric acid secretion in the treatment of peptic ulcer (36). Assay methods for ranitidine include HPLC with UV detection (37-38), HPLC with post-column fluorescence derivatization (39), high performance thin-layer chromatography (HPTLC) (40), TLC (41), supercritical fluid chromatography (SFC) (42) and capillary electrophoresis (CE) (43). Several of the HPLC methods utilized aqueous – acetonitrile mobile phases in the separations.

Three of the separations were carried out using a polar endcapped narrow bore column (2.0 mm i.d., 3µm particle size, and 120Å pore size) specifically designed for use in analyses that utilize mostly aqueous mobile phases. The column is manufactured with a monomeric bonding of

ODS and is endcapped with a hydrophyllic reagent, which allows the stationary phase to be wetted with polar solvents. The stationary phase design gives longer retention of polar compounds and greater resistance to acid hydrolysis. A satisfactory separation of meropenem and dopamine was not achieved on the polar endcapped column but the mixture was successfully separated on a conventional ODS narrow-bore column (2.1 mm i.d., 5μm particle size, Varian Associates Inc., Harbor City, CA 90710). All mixtures were separated with aqueous-acetonitrile eluents within 20 minutes with sensitivities in the ng mL^{-1} range. Forced degradation studies were conducted on each drug individually to determine if the methods were stability indicating.

In Chapter 3 stability indicating HPLC methods were developed for the simultaneous analysis of zidovudine in combination with ceftazidime, chlordiazepoxide, dobutamine, lorazepam, or ranitidine in intravenous fluid mixtures.

Zidovudine (3'-azido-3'-deoxythymidine; also referred to as AZT) is a nucleoside reverse transcriptase inhibitor with antiviral activity against HIV-1, HIV-2, human T lymphotrophic virus and other retroviruses. When taken up by target cells, zidovudine is phosphorylated to a triphosphate metabolite by cellular enzymes to produce the active drug (44).

Zidovudine is administered either orally as a capsule or syrup or by I.V. infusion, often in conjunction with one or more other injectable drugs. It has been analyzed by HPLC with UV detection in the three dosage forms (45), serum (46-47), plasma (48-49), and urine (49-50). Radioimmunoassay methods have been used to quantitate zidovudine in plasma, urine, and cerebrospinal fluid (51-52). HPLC and radioimmunoassay were used to determine zidovudine and its three phosphorylated metabolites, zidovudine mono-, di- and triphosphate, in peripheral blood mononuclear cells (53). Zidovudine has also been studied with respect to its stability in I.V. admixtures by HPLC analysis with 266 nm UV detection and by visual inspection for precipitation, gas production, turbidity, and color change (54). However, there continues to be a need for the development of stability indicating HPLC methods that can be used to evaluate the compatibility of zidovudine with other injectable drugs. Ceftazidime, chlordiazepoxide,

dobutamine, lorazepam and ranitidine are compounds that can be administered in conjunction with zidovudine. There are no published stability indicating methods available to assay each of these compounds with zidovudine.

Ceftazidime is a semi-synthetic cephalosporin, broad spectrum, beta-lactam antibiotic with activity against many gram negative pseudomonas and enterobacteriaceae microorganisms. It is used in the treatment of lower respiratory infections and urinary tract infections (55). Ceftazidime has been analyzed by reversed-phase HPLC with UV (56-57) and electrochemical detection (58). HPLC and micellar capillary electrophoresis with UV detection have been used to investigate the stability of aqueous reconstituted ceftazidime injection vials (59). An enzyme linked immunosorbent assay (ELISA) with fluorescence detection has been used for the determination of ceftazidime in urine, serum and airborne material (60). Chlordiazepoxide and lorazepam belong to the benzodiazepine class of drugs and are used in the treatment of anxiety disorders and in anesthetic premedication. In addition, chlordiazepoxide may be used in the management of alcohol withdrawal (61). Reversed-phase HPLC with UV (62-63) and electrochemical detection (64) have been used to assay chlordiazepoxide. An assay for chlordiazepoxide has also been developed using gas chromatography - negative-ion chemical-ionization mass spectrometry (65). Assay methods for lorazepam include HPLC-UV (66-67), micellar electrokinetic chromatography (68), gas chromatography – tandem mass spectrometry (69) and immunoassay (70-71). Dobutamine, a synthetic sympathomimetic catecholamine, is used primarily in the treatment of severe congestive heart failure (72). HPLC assay methods for the analysis of dobutamine using UV (73), electrochemical (ECD)(74-75), and fluorescence (76) detection have been reported. Ranitidine is an H_2-receptor antagonist used to inhibit gastric acid secretion in the treatment of peptic ulcer (77). Assay methods for ranitidine include HPLC with UV detection (78-79), HPLC with post-column fluorescence derivatization (80), high performance thin-layer chromatography (HPTLC) (81), TLC (45), supercritical fluid chromatography (SFC) (82) and capillary electrophoresis (CE) (83).

All of the separations were carried out using an amide hexadecylsilane (C16) column. The column is manufactured with a polar amide functionality located between the C3 and C4 members of the C18 alkyl chain. The amide functionality is able to take part in polar interactions between the phase and the analytes, resulting in a column with hydrophobic selectivity more similar to an octylsilane (C8) phase than a traditional octadecylsilane (C18) phase. All mixtures were separated with aqueous-acetonitrile eluents within 20 minutes.

Up to this point, the focus of this dissertation has been on the development of stability indicating methods for stability testing in parenteral fluids. There is no denying that these methods play a key role in the drug development process. Equally as important are the bioanalytical methods that are used to quantitate drug levels for studies in pharmacology, toxicology, Phase I and II clinical trials, metabolism, pharmacokinetics, formulation development and pharmacodynamics. Beyond the drug development process there is a need for effective bioanalytical methods in forensic toxicology, clinical medicine, routine workplace drug testing and therapeutic drug monitoring (84). Drug analysis in a complex matrix like serum or plasma is one of the most challenging tasks for an analytical chemist. Knowledge of the drug molecule and possible matrix compounds as well as the analytical measurement technique all need to be considered when developing a bioanalytical method (85). The primary difference between stability indicating and bioanalytical HPLC method development is in the sample preparation. Proteinacious samples such as whole blood, plasma, and urine will cause “plugging” of most HPLC columns if the drug is not first separated from the matrix. There are a number of techniques that have been used to accomplish this, including protein precipitation, ultrafiltration, liquid-liquid extraction (LLE), and solid phase extraction (SPE). Protein precipitation can be accomplished by the addition of an acid or alkali precipitant with a salt, but these techniques can have low recoveries and should not be used if the analyte is acid labile. Water miscible organic solvents (such as acetonitrile) can also be used to precipitate proteins but can also have poor recoveries and late eluting peaks. Ultrafiltration can be accomplished by forcing the sample

(usually by centrifugation) through a size selective semi-permeable membrane. If the analyte is protein bound it is necessary to displace it from the binding sites prior to filtration. The advantages of this technique are that it is amenable to very small sample volumes and there is no dilution of the sample. LLE depends on the partitioning of the analyte between two immiscible liquids, but this method is slow, labor intensive, difficult to automate, and can suffer from poor recoveries (86). Solid phase extraction can be thought of as HPLC on a miniature scale since the same principles that drive HPLC also apply to SPE. However, SPE is not efficient enough to separate individual analytes as in HPLC. The solid phase in SPE typically consists of silica particles with a chemically bonded organic phase with, C8 and C18 bonded phases being the most popular, versatile and useful. (87). The particles are usually packed into a small narrow cartridge or tube through which the sample is passed. A SPE method usually consists of four steps: conditioning, adsorption, washing and elution. Flow through the cartridge is driven by vacuum or pressure. Some advantages of SPE verses LLE include: 1. It uses much less organic solvents. 2. Extractions by SPE are more efficient than LLE extractions. 3. It is possible to achieve much higher concentration factors with SPE than LLE. 4. It is faster and requires less manipulation than LLE.

The requirement for fast analytical methods has become a huge issue in the pharmaceutical industry as analytical laboratories come under ever increasing pressure to provide analyses more quickly and at lower cost. The number of samples that chromatographers have been asked to analyze has increased and the time it takes to run them is required to decrease. Improved automation of instrumentation has resulted in a significant increase in sample throughput but has also focused attention on the next analytical bottleneck: the HPLC column. Due to the inherent nature of particulate columns, the maximum mobile phase flow rate is dictated by the resultant backpressure. Excessive backpressure will cause excessive wear on the solvent delivery system and can damage the column. The result of this limitation is that HPLC run times can take up to 30 minutes.

In the past, efforts to decrease analysis times have focused on the use of short columns with particles that are smaller than the standard 5μm. These columns offer good efficiency with higher flow rates but also have a tendency to "plug" and backpressures tend to be high (8). Recently, columns made of a single piece of monolithic silica were introduced as an alternative to particle-based columns. These columns possess a biporous structure consisting of larger macropores (2 μm) that permit high flow rates with low backpressure and smaller mesopores (13 nm) that provide a high surface area for high efficiency (88). Therefore, it is possible to perform analyses with high linear flow velocity but without significantly reduced separation efficiency. The utility of monolithic silica columns for high throughput bioanalysis in a drug discovery environment has been demonstrated (89). The columns have also been used to analyze metabolites (90) and natural products (91).

Chapter 4 of this dissertation describes the development and validation of an HPLC method that is both rapid and sensitive for the determination of AZT and levofloxacin in human plasma. For sample pre-treatment, the method utilized solid phase extraction that does not require an evaporation step. Elution was performed isocratically on a conventional 150 x 4.6 mm C18 column with 266 nm UV detection.

At the end of 2001, there had been an estimated 3 million deaths worldwide due to human immunodeficiency virus (HIV) infections and 40 million people are currently living with HIV (92). Although several drugs have been developed to combat this epidemic, zidovudine (3'-azido-3'-deoxythymidine; AZT) continues to be one of the first-line therapeutic agents in treating HIV. AZT is a nucleoside reverse transcriptase inhibitor with antiviral activity against HIV-1, HIV-2, human T lymphotrophic virus and other retroviruses. It is metabolized to a triphosphate metabolite by cellular enzymes to produce the active drug (44). Side effects associated with AZT therapy include gastrointestinal intolerance, bone marrow toxicity and myelosuppression (93). Dose-related toxicities can be reduced and patient outcomes can be improved when a specific

concentration range of AZT is maintained (94). HIV patients might benefit from a pharmacokinetic approach to AZT therapy (95). Therapeutic drug monitoring of antiviral drugs such as AZT is necessary to avoid or delay resistance from the virus, to monitor compliance, and to monitor drug-drug and drug-food interactions.

Many human immunodeficiency virus (HIV)-infected patients develop secondary bacterial infections because of their compromised immune systems. Coinfection with mycobacterium species, especially Mycobacterium avium complex (MAC) and mycobacterium tuberculosis is often treated with multiple antibacterial agents, including levofloxacin (96-98). Levofloxacin is a chiral fluorinated carboxyquinolone and is the L-isomer of the racemate ofloxacin. It is a broad-spectrum antibacterial agent with activity against a wide range of gram-positive, gram-negative and anaerobic bacteria. Levofloxacin has been found in vitro to be generally twice as active as ofloxacin against many of these organisms (99). The bactericidal activity of levofloxacin is maximized when the ratios of peak plasma drug concentrations (C_{max}) : minimum inhibitory concentrations (MIC) or area under the concentration-time curve (AUC) : MIC exceed certain threshold levels (100). Therefore, therapeutic drug monitoring of levofloxacin plasma levels would make it possible to administer the correct dose of the drug to the patient at the appropriate interval. Monitoring
of the Cmax to MIC ratio is particularly important in patients at risk for malabsorption, such as those infected with HIV (101).

Analytical methods have been described to quantify AZT and Levofloxacin separately in biological media (102-105), but no methods have been reported for the simultaneous determination of AZT and levofloxacin in human plasma.

Chapter 5 of this dissertation describes the development and validation of two separate HPLC methods that are both rapid and sensitive for determining cocaine and three of its metabolites in human plasma as well as five commonly abused opium alkaloids in human plasma.

For sample pre-treatment, the methods utilized mixed mode solid phase extraction. Elution was performed by binary mobile phase gradients with 231 and 208 nm UV detection, respectively.

In 1998, there were estimated to be 1.8 million Americans age 12 and older who were chronic cocaine users. Although this represents a decrease from the 5.7 million users in 1985, the abuse of this addictive stimulant has become a persistent problem in this country (106). The euphoria associated with cocaine use is caused by an inhibition of neuronal reuptake of biogenic amines in the central nervous system (CNS). In addition to its addictive properties, cocaine has been shown to be toxic to both the CNS and the cardiovascular system (107-108). Benzoylecgonine, one of the major metabolites, is formed by ester hydrolysis of cocaine. The human plasma elimination half lives of cocaine and benzoylecgonine are 30 to 90 min and 7.5 h, respectively(109). Therefore, benzoylecgonine is often the analyte of choice for detecting cocaine use. Norcocaine is formed by N-demethylation of cocaine. Cocaethylene is a neurologically active compound that is formed when cocaine is coadministered with ethanol. It provides the same degree of euphoria as cocaine but for longer periods of time and with more toxicity than cocaine alone. Co-administration of cocaine and ethanol is the most common two-drug combination that results in drug-related death(106).

In addition to the abuse of cocaine, an estimated 4 million people age 12 and over used prescription drugs for non-medical purposes in 1999 (110). The most commonly abused prescription drugs are opiates, CNS depressants and stimulants. Opium alkaloids are very potent analgesics that bind to opiate receptors in the brain, spinal cord and gastrointestinal tract and block the transmission of pain signals. Commonly abused opiates include heroin, morphine, hydromorphone, codeine, oxycodone, and hydrocodone. Heroin and codeine are both metabolized, in part, to morphine, although at different rates. Therefore, a ratio of the two drugs is often used to determine if the morphine was due to the consumption of heroin or morphine itself. Morphine has an elimination half-life of 1.7 h and is largely metabolized to glucuronide conjugates. Hydromorphone is a synthetic derivative of morphine that has an elimination half-life

of 2.5 h and is 7-10 times more potent than morphine. Oxycodone is a derivative of codeine with an elimination half-life and potency that are similar to morphine. Hydrocodone is very similar to codeine and is converted in the body to hydromorphone (111).

The analysis for the possible presence of drugs of abuse is usually a two-stage process in which an initial screening test is followed by a confirmation test. This approach requires that the test methods be fast and inexpensive. Currently the preferred first stage screening method utilizes some form of enzyme immunoassay (112). Samples that test positive are usually analyzed by GC/MS due to its high sensitivity and selectivity but the necessity of sample derivatization and the cost of the technique itself restrict its applicability(113-114). High performance liquid chromatography (HPLC) is able to separate both lipophilic and hydrophilic analytes without any chemical treatment. This technique is slowly gaining acceptance as a confirmatory method for the analysis of drugs of abuse. However, as the workload in toxicology and forensic laboratories increases the need for faster HPLC methods has become an important issue.

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PART I

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR THE ANALYSIS OF SELECTED PHARMACEUTICALS IN INTRAVENOUS FLUID MIXTURES

CHAPTER 2

HPLC SEPARATIONS OF MEROPENEM AND SELECTED PHARMACEUTICALS USING A POLAR ENDCAPPED OCTADECYLSILANE NARROW BORE COLUMN[1]

[1] Caufield, W.V. and Stewart, J.T. *Chromatographia* **2000,** *51, No. 5/6*, 307-313. Reprinted with permission of publisher

CHAPTER 2

ABSTRACT

Stability indicating high performance liquid chromatography methods have been developed for the assay of meropenem in combination with either dopamine (A), aminophylline (B), metoclopramide (C) or ranitidine (D) in intravenous fluid mixtures.

Separations B, C and D were performed on a polar endcapped ODS column (150 x 2 mm) with aqueous, pH 3.0 - acetonitrile (89:11, 88:12, and 92:8) eluent and detection at 270, 290, 317 nm respectively. Meropenem was linear over the concentration ranges 126.88-507.50, 131.25-525, and 131.25-525 μg mL^{-1}. Aminophylline, metoclopramide and ranitidine were linear over the concentration ranges 13-52, 37.5-150, and 25-100 μg mL^{-1}. Separation A was performed on a conventional ODS column (150 x 2.1 mm) with aqueous, pH 3.0 - acetonitrile (85:15) eluent and detection at 280 nm. Meropenem and dopamine were linear in the 61.25-245 and 10-40 μg mL^{-1} ranges, respectively. Accuracy and precision for all methods were 0.20-3.30% and 0.10-1.58%, respectively.

Accelerated stability studies have been carried out on each drug by exposure to acid, base, H_2O_2, and heat for different time periods.

INTRODUCTION

Meropenem is a new carbapenem antibiotic with a broad spectrum of activity in-vitro. It is active against gram-positive and gram-negative organisms and is highly effective in the treatment of infections caused by many clinically relevant aerobic, nutritionally fastidious and anaerobic bacterial species [1].

Meropenem is administered by I.V. infusion or by I.V. bolus injection, often in conjunction with one or more other injectable drugs. It has been analyzed by HPLC with UV detection in serum, plasma and urine [2-5]. A carbon dioxide adduct of meropenem has also been

determined by proton and carbon-13 NMR coupled with flow injection mass spectrometry [6]. Meropenem has also been studied with respect to its compatibility with commonly used injectable drugs by examination of I.V. admixtures for precipitation, gas production and color change [7]. A stability indicating HPLC method was developed for the analysis of meropenem and ofloxacin in 5% dextrose in water injection USP and 0.9% sodium chloride injection USP using UV detection at 270 nm [8]. However, there continues to be a need for the development of stability indicating HPLC methods, which can be used to evaluate the compatibility of meropenem with other injectable drugs. Aminophylline, dopamine, metoclopramide and ranitidine are compounds, which can be administered in conjunction with meropenem. To date, there are no stability indicating methods available to assay each of these compounds with meropenem.

Aminophylline is a methylxanthine, which is formed when theophylline complexes with ethylenediamine. It can be used to reverse narcosis in anesthetized patients as well as in the treatment of asthma [9]. Aminophylline/theophylline has typically been analyzed by reversed phase HPLC with UV detection [10-12]. Dopamine, a monoamine central neurotransmitter, is used in the treatment of some types of shock, including cardiogenic and septic shock [13]. HPLC assay methods for the analysis of dopamine using electrochemical (ECD) [14-15], fluorescence [16] and combined ultraviolet absorbency-electrochemical detection (UV-ECD) [17] have been reported. These methods utilized aqueous – methanol mobile phases and ODS columns. Metoclopramide is a member of the benzamide class of antiemetic agents and can be administered during cancer chemotherapy as well as in pregnancy [18]. Assay methods for metoclopramide include ultraviolet [19] and fluorescence spectroscopy [20], HPLC-UV [21-22], gas–liquid chromatography (GLC) [23] and gas chromatography – mass spectrometry (GC-MS) [24]. The HPLC-UV methods utilized aqueous – acetonitrile mobile phases in the separations. Ranitidine is an H_2-receptor antagonist used to inhibit gastric acid secretion in the treatment of peptic ulcer [25]. Assay methods for ranitidine include HPLC with UV detection [26-27], HPLC with post-column fluorescence derivatization [28], high performance thin-layer chromatography

(HPTLC) [29], TLC [30], supercritical fluid chromatography (SFC) [31] and capillary electrophoresis (CE) [32]. Several of the HPLC methods utilized aqueous – acetonitrile mobile phases in the separations.

The use of organic solvents in liquid chromatography is becoming increasingly problematic as the initial purchase costs as well as the cost of disposal continues to rise. One technique that has been developed to deal with this problem involves distillation and purification of less expensive technical or reagent grade solvents. While this does decrease the initial cost of the solvent, it is also time consuming and does not address the problem of waste disposal. It is also possible to purify the waste from the HPLC, but again, this can be a difficult and laborious task due to the complexity of most chromatographic waste streams. Commercially available mobile phase recycling systems can also be used when a single component is to be assayed. However, many samples contain low levels of impurities that tend to decrease the efficiency of the recycling system and lead to poor sensitivity and linearity. The obvious solution to this problem is to decrease the solvent consumption and thus the amount of generated organic waste. The use of a narrow bore column (2.1 mm internal diameter or less) can reduce mobile phase consumption by a factor of 3-4 (assuming a flow rate of 1-2 mL min^{-1}) verses a traditional 3.9 mm ID column [33]. Since column flow is proportional to internal diameter the same linear velocities and retention times can be preserved by using a narrow bore column operated at a reduced flow rate.

In this paper, isocratic, stability indicating HPLC assays are presented for the simultaneous analysis of meropenem and dopamine (Mixture A), meropenem and aminophylline (Mixture B), meropenem and metoclopramide (Mixture C), and meropenem and ranitidine (Mixture D) in intravenous fluid mixtures. Due to the highly aqueous nature of intravenous fluid mixtures, three of the separations were carried out using a polar endcapped narrow bore column (2.0 mm i.d., 3μm particle size, and 120Å pore size, YMC-ODS-AQ, YMC INC., Wilmington, NC 28403) specifically designed for use in analyses which utilize mostly aqueous mobile phases.

The column is manufactured with a monomeric bonding of ODS and is endcapped with a hydrophyllic reagent, which allows the stationary phase to be wetted with polar solvents. The stationary phase design gives longer retention of polar compounds and greater resistance to acid hydrolysis. A satisfactory separation of meropenem and dopamine was not achieved on the polar endcapped column but the mixture was successfully separated on a conventional ODS narrow-bore column (2.1 mm i.d., 5μm particle size, Varian Associates Inc., Harbor City, CA 90710). All mixtures were separated with aqueous-acetonitrile eluents within 20 minutes with sensitivities in the ng mL^{-1} range.

EXPERIMENTAL

Reagents and Chemicals

The structural formulae of the compounds studied are shown in Figure 2.1. Meropenem was obtained from Zeneca Pharmaceuticals, Inc. (Wilmington, DE, USA). Aminophylline, metoclopramide and triethylamine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dopamine and ranitidine were obtained from the United States Pharmacopeial Convention Inc. (Rockville, MD, USA). Acetonitrile (Fisher Scientific, Norcross, GA, USA) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, GA, USA). Glacial acetic acid and Na_2-EDTA dihydrate (Fisher Scientific, Norcross, GA, USA) were ACS reagent grade.

Instrumentation

The chromatographic separations were performed on a Model 1090 HPLC system (Hewlett Packard Co., Palo Alto, CA, USA). This system included a pump, an autosampler equipped with a 25μL loop and a Model 117 variable wavelength UV detector (Gilson, Middleton, WI, USA). 0.005” ID tubing was used before and after the column and was kept at a minimum length. Peak-Pro (Beckman Coulter, Allendale, NJ, USA) chromatography software was used for integration. A Waters 996 photodiode array detector with Millenium-32 chromatography software was used to confirm the purity of the analyte peaks from forced

degradation. Separation of Mixture A was achieved on a 15-cm ODS narrow-bore column. The mobile phase consisted of 85:15 *v/v* aqueous, pH 3.0 (adjusted with glacial acetic acid) – acetonitrile and the detector was set to 280 nm. Separation of Mixtures B, C, and D were achieved on a 15-cm polar endcapped ODS narrow-bore column. The mobile phase for mixture B consisted of 89:11 *v/v* aqueous, pH 3.0 (adjusted with glacial acetic acid) –acetonitrile and the detector was set to 270 nm. The mobile phase for mixture C consisted of 88:12 *v/v* aqueous 10 mM triethylamine, pH 3.0 (adjusted with glacial acetic acid) –acetonitrile and the detector was set to 290 nm. The mobile phase for mixture D consisted of 92:8 *v/v* aqueous, pH 3.0 (adjusted with glacial acetic acid) –acetonitrile and the detector was set to 317 nm. The mobile phases were filtered through 0.45 μm nylon-66 filter (MSI, Westborough, MA, USA) and degassed by sparging with helium prior to use. The flow rate was 0.2 mL min^{-1} for all four mixtures.

Preparation of Solutions

All stock solutions were prepared fresh daily and were protected from light. Dilutions of Mixture A were made using a 85:15 *v/v* 0.9 mM Na_2-EDTA-acetonitrile diluent. Dilutions of the remaining mixtures were made with an aqueous-acetonitrile diluent matching the mobile phase composition. A combined stock solution was prepared for meropenem and dopamine at concentrations of 245 and 40 μg mL^{-1}, respectively. Standard solutions of the analytes for linear regression were made by dilution of the respective stock solutions. These standard solutions, along with 2:8 and 4:8 dilutions, gave solutions containing 61.25 and 122.5 μg mL^{-1} meropenem, while dopamine was diluted to 10 and 20 μg mL^{-1}.

For the three remaining drugs, combined standard solutions were prepared which consisted of meropenem and either aminophylline, metoclopramide or ranitidine at concentrations of 500, 52, 150 and 100 μg mL^{-1}, respectively. Standard solutions of the analytes for linear regression were made by dilution of the respective stock solutions. In addition, 2:8 and 4:8

dilutions were prepared giving solutions containing 125 and 250 μg mL^{-1} meropenem, 13 and 26 μg mL^{-1} aminophylline, 37.5 and 75 μg mL^{-1} metoclopramide and 25 and 50 μg mL^{-1} ranitidine.

Three point calibration curves were constructed for each analyte using the analytical conditions established for each mixture. Additional dilutions (3:8 and 6:8) of the stock solutions were prepared in diluent to serve as spiked samples for each analyte to determine accuracy and precision. Quantitation was based on linear regression analysis of analyte peak area versus analyte concentration in μg mL^{-1}.

To show that the methods are stability indicating it was necessary to subject the analytes to extreme conditions to cause them to degrade. Since each analyte possessed different degrees of stability it was necessary to utilize different concentrations of HCl, NaOH, and H_2O_2 as well as different temperatures to force the degradation. The details of these degradation studies can be found in Tables III and IV. In each case, 1 mg (or 2 mg with acid and base degradation) were dissolved in the appropriate degradation solution and allowed to stand from 1 minute to 10 hours, according to stability testing procedures discussed by Weiser [34]. Samples degraded with peroxide were injected directly into the HPLC. Most acid and base degraded samples were first neutralized with equal volumes and concentrations of either acid or base prior to injection into the HPLC system. 6 N HCl stressed samples were neutralized with an equal volume of 5 M sodium acetate while 3 N NaOH stressed samples were neutralized with an equal volume of glacial acetic acid.

RESULTS AND DISCUSSION

The purpose of this study was to develop stability indicating methods for the analysis of meropenem in combination with aminophylline, dopamine, metoclopramide or ranitidine in intravenous fluid mixtures. A search of the literature revealed no other HPLC methods for the analysis of meropenem with any of the other four compounds. In addition, narrow bore columns

were used in all four methods in an attempt to decrease the quantity of solvents used and the waste generated in the course of the analyses.

Initial method development efforts centered on the use of the 15-cm polar endcapped ODS narrow bore column. Mobile phase conditions developed by Al-Meshal [3] were utilized for the first chromatographic runs with a flow rate of 0.2 mL min^{-1}. and an injection volume of 5µL. Since meropenem was found to elute at the solvent front under those conditions, the concentration of acetonitrile was decreased to cause meropenem to elute after the solvent front and also to obtain the best separation of meropenem and each of the four drugs being studied. It was determined that 8, 11, and 12% acetonitrile provided the optimal separations for ranitidine, aminophylline and metoclopramide, respectively. The mobile phase for the separation of Mixture C was further modified by the addition of 10 mM triethylamine to the aqueous phase. This was done to minimize tailing of metoclopramide, a basic drug. Dopamine eluted at the solvent front in all mobile phase conditions except 100% water (pH adjusted to 3.0 with glacial acetic acid). However, meropenem had an excessively long retention time in 100% water. Therefore, the polar endcapped column did not yield an optimal separation for Mixture A and a conventional ODS narrow bore column was substituted. It was determined that 15% acetonitrile provided the optimal separation. Since catechols such as dopamine are subject to oxidative breakdown, 0.9 mM Na_2-EDTA was added to the Mixture A diluent to improve the time stability in accuracy, precision and linearity studies. The detection wavelength was optimized for each drug mixture and wavelengths of 280, 270, 290 and 317 nm were found to yield the best sensitivity for dopamine, aminophylline, metoclopramide and ranitidine, respectively. Limits of detection were 123, 92, 97, and 68 ng mL^{-1}, for dopamine, aminophylline, metoclopramide and ranitidine, respectively. Limits of detection for meropenem were 167, 150, 75, and 131 ng mL^{-1}, for Mixture A, B, C and D, respectively. All limits of detection were based on a S/N of 3. The analytical figures of merit for each method are shown in Table 2.1.

The HPLC method for Mixture A showed concentration verses absorbance linearity for meropenem and dopamine in the 61.25-245 and 10-40 μg mL^{-1} ranges, respectively at 280 nm. The HPLC method for Mixture B showed concentration verses absorbance linearity for meropenem and aminophylline in the 126.88-507.50 and 13-52 μg mL^{-1} ranges, respectively at 270 nm. The HPLC method for Mixture C showed concentration verses absorbance linearity for meropenem and metoclopramide in the 131.25-525 and 37.5-150 μg mL^{-1} ranges, respectively at 290 nm. The HPLC method for Mixture D showed concentration verses absorbance linearity for meropenem and ranitidine in the 131.25-525 and 25-100 μg mL^{-1} ranges, respectively at 317 nm. Correlation coefficients for the four mixtures were in a range of 0.9996 – 1.0 (n=6).

Accuracy and precision of the methods were evaluated using spiked samples containing each analyte. The results shown in Table 2.2 indicate that the procedures give acceptable accuracy and precision for each analyte.

Intra-day variabilities for meropenem and dopamine (Mixture A) expressed as % RSD were 0.26 and 1.04% (n=15), respectively. Inter-day variabilities were calculated from assays run on three consecutive days. The %RSD was 0.33% for meropenem and 3.00% for dopamine. Intra-day variabilities for meropenem and aminophylline (Mixture B) were 0.71 and 0.69% (n=15), respectively. Inter-day variabilities were 0.46% for meropenem and 0.38% for aminophylline. Intra-day variabilities for meropenem and metoclopramide (Mixture C) were 2.12 and 0.45% (n=15), respectively. Inter-day variabilities were 0.47% for meropenem and 0.60% for metoclopramide. Intra-day variabilities for meropenem and ranitidine (Mixture D) were 0.63 and 0.64% (n=15), respectively. Inter-day variabilities were 0.93% for meropenem and 0.94% for ranitidine.

A photodiode array detector was used to verify that none of the degradation products of the analytes in either Mixture A, B, C, or D interfered with the quantitation of each drug. Each mixture was analyzed under their respective analytical conditions. The results of these studies are

summarized in Tables 2.3 and 2.4. Chromatograms of the separations of each of the four mixtures following forced degradation are shown in Figures 2.2, 2.3, 2.4 and 2.5.

In summary, a polar endcapped narrow-bore (2.0 mm I.D.) ODS column with aqueous (pH 3 with acetic acid) – acetonitrile eluents was shown to be suitable for the separation of meropenem and aminophylline mixtures (B), meropenem and metoclopramide mixtures (C), and meropenem and ranitidine mixtures (D) in intravenous fluid mixtures. Meropenem and dopamine mixtures (A) were successfully separated on a conventional narrow-bore (2.1 mm I.D.) ODS column with aqueous (pH 3 with acetic acid) – acetonitrile eluent. The methods had run times that were ≤ 20 min. with reduced solvent usage and the columns showed good efficiencies for all analytes. The methods were found to be free from interferences from degradants and are suitable for the investigation of the chemical stability of the analytes in each of the mixtures.

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TABLE 2.1: Analytical Figures of Merit for Mixtures A-D

Analyte	k	N^a	*Tailing Factor*[b]	R_s	α
Mixture A					
Meropenem	3.37	2759	1.62	6.24	2.04
Dopamine	1.65	2351	1.54		
Mixture B					
Meropenem	0.81	1905	1.40	3.89	1.78
Aminophylline	1.44	3959	1.33		
Mixture C					
Meropenem	0.64	1550	1.38	19.77	7.63
Metoclopramide	4.88	7915	1.43		
Mixture D					
Meropenem	2.20	3929	1.46	5.57	2.20
Ranitidine	1.00	1247	1.98		

[a] Calculated as 5.54 $(t_R/W_{0.5})^2$
[b] Calculated at 5% peak height

TABLE 2.2: Accuracy and Precision Using Spiked Drug Samples

Mixture	Analyte	Concn Added [a] μg mL^{-1}	Concn Found μg mL^{-1}	Percent Error	RSD (%)
A	Meropenem	91.88	91.23 ± 0.24	0.70	0.27
		183.75	183.34 ± 0.33	0.22	0.18
	Dopamine	15.00	15.12 ± 0.07	0.76	0.43
		30.00	30.13 ± 0.13	0.42	0.42
B	Meropenem	190.31	184.00 ± 0.35	3.30	0.19
		380.63	373.01 ± 0.89	2.00	0.24
	Aminophylline	19.50	18.86 ± 0.05	3.30	0.27
		39.00	38.23 ± 0.12	2.00	0.31
C	Meropenem	196.88	193.74 ± 1.36	1.59	0.78
		393.75	385.01 ± 5.76	2.22	1.58
	Metoclopramide	56.25	55.89 ± 0.19	0.64	0.35
		112.50	111.14 ± 0.45	1.21	0.40
D	Meropenem	196.88	197.21 ± 0.19	0.20	0.10
		393.75	389.33 ± 1.54	1.12	0.40
	Ranitidine	37.50	37.60 ± 0.06	0.30	0.16
		75.00	74.85 ± 0.25	0.20	0.33

[a] mean ± standard deviation based on n=3

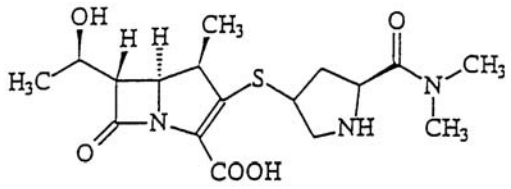
TABLE 2.3: Stability Indicating Nature of Assays

Drug	Treatment	Duration	%Δ in Peak Area	t_R of Parent Peak (min)	t_R of Degradent Peak (min)
Mixture A					
Meropenem	0.1 N HCl	15 min	- 29 %	9.8	2.0, 2.6
	0.01 N NaOH	1 min	- 24 %	9.7	2.2, 2.6, 3.1
	0.03% H_2O_2	2 h	- 12 %	9.3	2.1, 2.6, 4.2
	60° C	4 h	- 14 %	9.4	2.2
Dopamine	0.1 N HCl	2 h	- 11 %	5.5	2.0, 2.5
	0.0001 N NaOH	2 h	- 14 %	5.6	2.6
	3 % H_2O_2	1 h	- 10 %	5.8	5.4
	60° C	1.5 h	- 12 %	5.7	2.6
Mixture B					
Meropenem	0.1 N HCl	15 min	- 23.3 %	4.4	2.3, 2.9
	0.01 N NaOH	1 min	- 33.0 %	4.4	2.9
	0.03% H_2O_2	2 h	- 10.9 %	4.3	2.4, 3.0, 3.3
	60° C	4 h	- 13.6 %	4.3	2.9, 3.3
Aminophylline	6 N HCl, 95° C	5 days	0 %	6.0	N/A
	1 N NaOH, 60° C	3 days	- 18.5 %	5.9	2.3, 2.5
	3 % H_2O_2	3 days	- 14.6 %	5.9	2.3, 2.5
	95° C	10 days	0 %	5.9	N/A

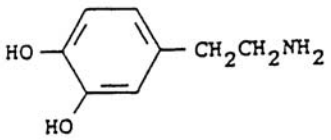
TABLE 2.4: Stability Indicating Nature of Assays

Drug	Treatment	Duration	%Δ in Peak Area	t_R of Parent Peak (min)	t_R of Degradent Peak (min)
Mixture C					
Meropenem	0.1 N HCl	15 min	- 36.6 %	3.6	2.7
	0.01 N NaOH	1 min	- 31.0 %	3.6	2.7
	0.03% H_2O_2	4 h	- 10.1 %	3.6	2.7, 2.9
	60° C	4 h	- 12.7 %	3.6	2.8, 5.5
Metoclopramide	6 N HCl, 60° C	1 day	-32.0 %	11.9	8.8, 19.6
	3 N NaOH, 60° C	2 days	- 10.2 %	11.5	19.5
	3 % H_2O_2	4 days	-10.6 %	11.2	2.4, 6.4, 8.1, 14.4, 19.5
	95° C	9 days	0 %	12.1	N/A
Mixture D					
Meropenem	0.1 N HCl	15 min	- 30.2 %	7.7	2.4, 3.6
	0.01 N NaOH	2 min	- 41.9 %	7.9	3.7
	0.03% H_2O_2	1.75 h	- 18.6 %	7.7	3.2, 3.7, 4.3
	60° C	4 h	- 13.6 %	7.7	3.7
Ranitidine	0.1 N HCl	6 days	-13.0 %	4.8	2.4, 2.7
	0.1 N NaOH	17 h	- 20.4 %	4.7	2.4
	0.3 % H_2O_2	30 min	- 10.0 %	4.8	2.4, 3.0
	80° C	6 days	- 15.0 %	4.6	2.4

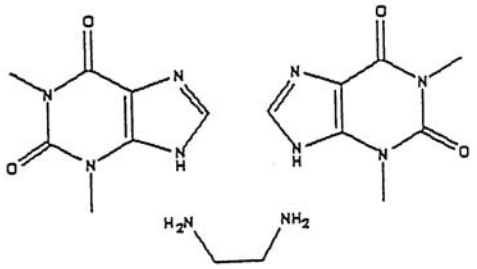
Figure 2.1: Chemical structures of the analytes



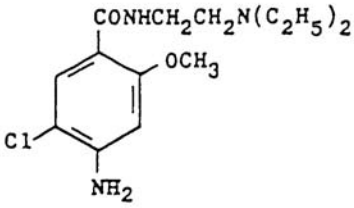
Meropenem



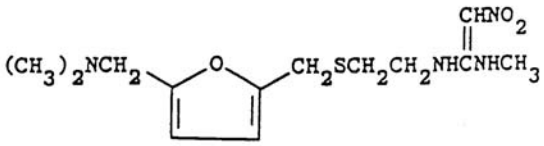
Dopamine



Aminophylline



Metoclopramide



Ranitidine

Figure 2.2: Chromatograms from accelerated degradation studies of dopamine and meropenem. Top, dopamine in diluent at 60°C for 90 min. Bottom, meropenem in 0.01 N NaOH for 3 min. Separations took place on a Rainin Microsorb ODS (150 x 2.1 mm i.d., 5 μm particle size) column using a mobile phase of 15:85 v/v acetonitrile – aqueous acetic acid pH 3, a flow rate of 0.2 mL/min and a detection wavelength of 280 nm. Injection volumes were 5 μL. Unlabeled peaks are unknown degradation products. The dopamine chromatogram is offset from the meropenem chromatogram with an angle of elevation of 10 degrees.

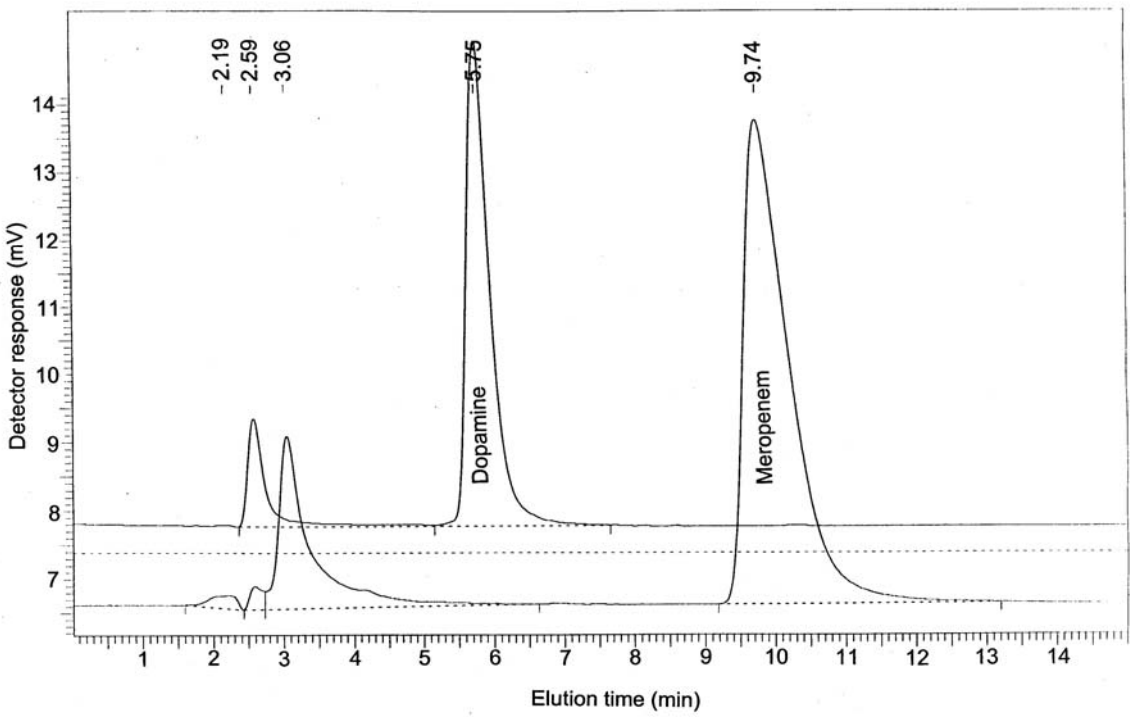

2.19
2.59
3.06
5.75
9.74
Dopamine
Meropenem
Detector response (mV)
Elution time (min)

Figure 2.3: Chromatograms from accelerated degradation studies of aminophylline and meropenem. Top, aminophylline in 3% H_2O_2 for 3 days. Bottom, meropenem in 0.1 N HCl for 30 min. Separations took place on a YMC ODS-AQ (150 x 2.0 mm i.d., 3 μm particle size) column using a mobile phase of 11:89 v/v acetonitrile – aqueous acetic acid pH 3, a flow rate of 0.2 mL/min and a detection wavelength of 270 nm. Injection volumes were 5 μL. Unlabeled peaks are unknown degradation products. The aminophylline chromatogram is offset from the meropenem chromatogram with an angle of elevation of 10 degrees

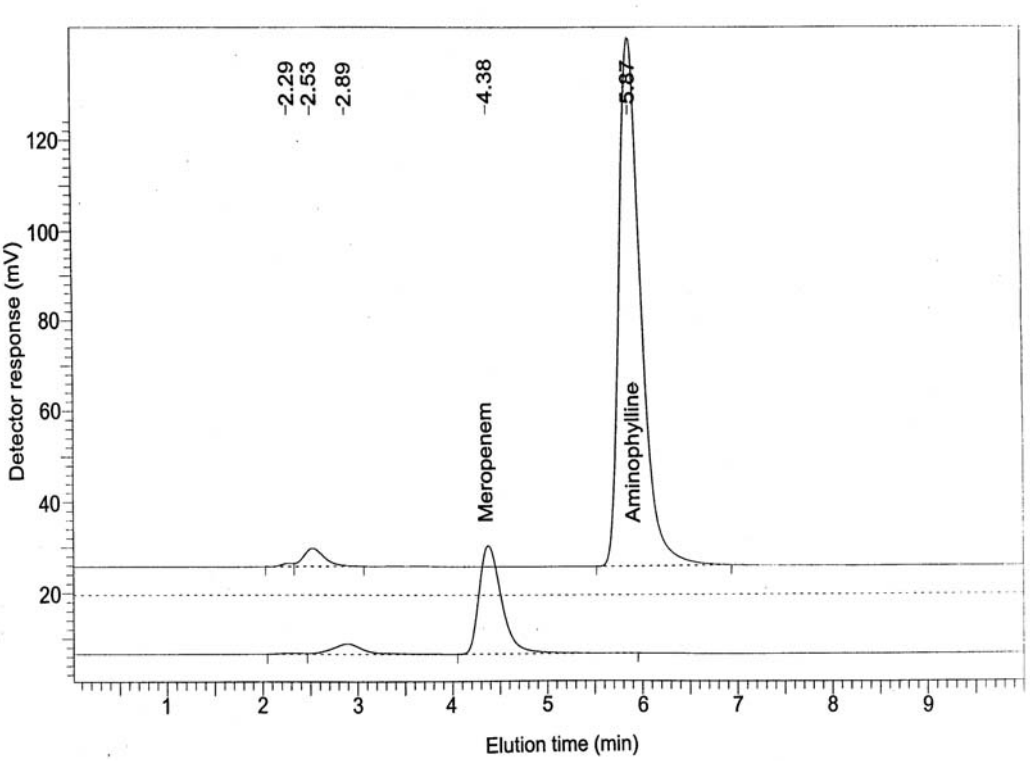

2.29
2.53
2.89
4.38
5.87
Meropenem
Aminophylline
Detector response (mV)
120
100
80
60
40
20
1
2
3
4
5
6
7
8
9
Elution time (min)

Figure 2.4: Chromatograms from accelerated degradation studies of ranitidine and meropenem. Top, ranitidine in 0.3% H_2O_2 for 30 min. Bottom, meropenem in 0.01 N NaOH for 3 min. Separations took place on a YMC ODS-AQ (150 x 2.0 mm i.d., 3 μm particle size) column using a mobile phase of 8:92 v/v acetonitrile – aqueous acetic acid pH 3, a flow rate of 0.2 mL/min and a detection wavelength of 317 nm. Injection volumes were 5 μL. Unlabeled peaks are unknown degradation products. The ranitidine chromatogram is offset from the meropenem chromatogram with an angle of elevation of 10 degrees.

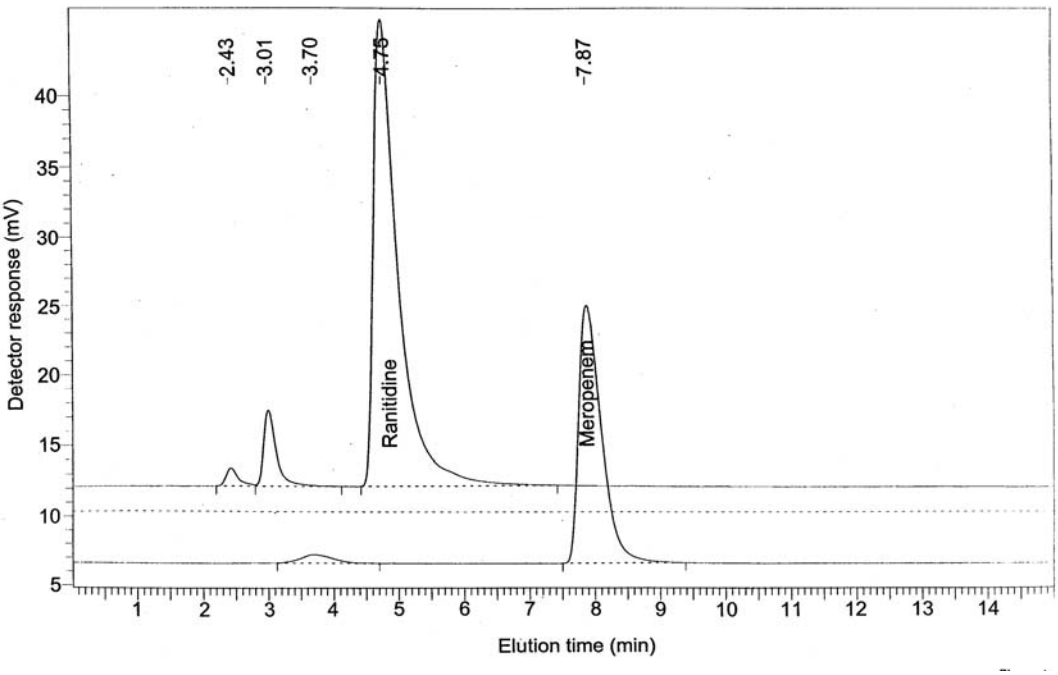
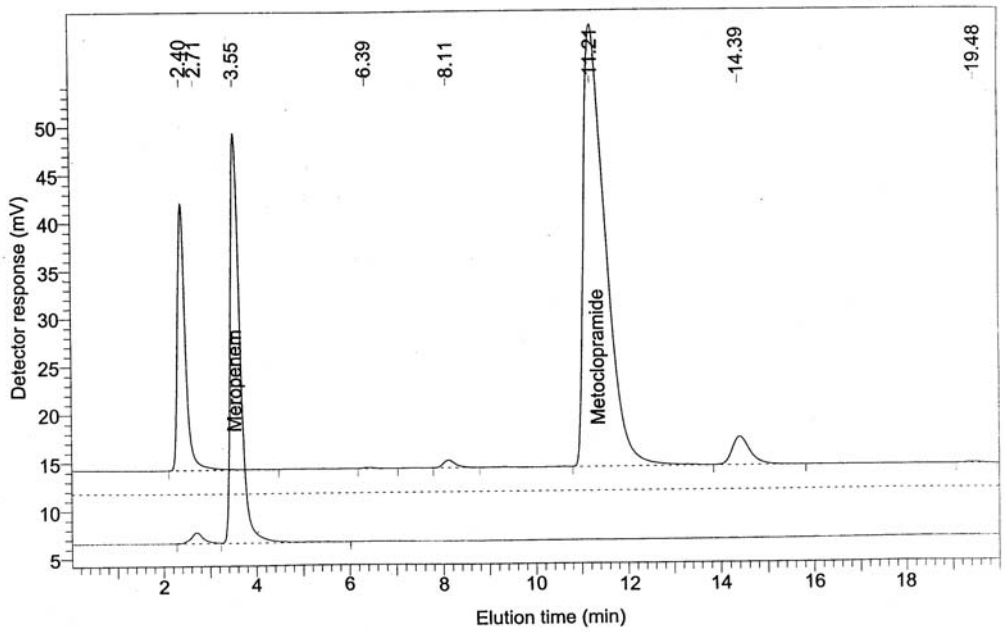

2.43
3.01
3.70
4.76
7.87
Ranitidine
Meropenem
Detector response (mV)
Elution time (min)

Figure 2.5: Chromatograms from accelerated degradation studies of metoclopramide and meropenem. Top, metoclopramide in 3.0% H_2O_2 for 4 days. Bottom, meropenem in 0.01 N NaOH for 2 min. Separations took place on a YMC ODS-AQ (150 x 2.0 mm i.d., 3 μm particle size) column using a mobile phase of 12:88 v/v acetonitrile – aqueous 0.01 M triethylamine pH 3 (adjusted with acetic acid), a flow rate of 0.2 mL/min and a detection wavelength of 290 nm. Injection volumes were 5 μL. Unlabeled peaks are unknown degradation products. The metoclopramide chromatogram is offset from the meropenem chromatogram with an angle of elevation of 10 degrees.


2.40
2.71
3.55
6.39
8.11
11.21
14.39
19.48
Metoprolam
Metoclopramide
Detector response (mV)
Elution time (min)

CHAPTER 3

HPLC SEPARATIONS OF ZIDOVUDINE AND SELECTED PHARMACEUTICALS USING A HEXADECYLSILANE AMIDE COLUMN[2]

[2] Caufield, W.V. and Stewart, J.T. *Chromatographia* **2001,** *54, No. 9/10*, 561-568. Reprinted with permission of publisher

CHAPTER 3

ABSTRACT

Stability indicating high performance liquid chromatography methods have been developed for the assay of zidovudine in combination with either ceftazidime (A), chlordiazepoxide (B), dobutamine (C), lorazepam (D) or ranitidine (E) in intravenous fluid mixtures.

All separations were performed on an amide hexadecylsilane column (250 x 4.6 mm) with 25 mM phosphate buffer, pH 3.0 - acetonitrile eluent. Isocratic methods were developed using 16, 20, 16, and 12 percent *v/v* acetonitrile for separations A, B, C, and E, respectively. A gradient method (18 – 60 percent v/v acetonitrile) was developed for separation D. UV detection at 280 nm was used for separation A, while detection at 265 nm was used for the remaining separations. Zidovudine was linear over the concentration ranges 5-200, 52.5-210, 52.5-210, 52.5-210, and 50-200 μg mL^{-1}. Ceftazidime, chlordiazepoxide, dobutamine, lorazepam and ranitidine were linear over the concentration ranges 54.9-219.7, 250-1000, 50-200, 25-100, and 24.9-99.6 μg mL^{-1}. Accuracy and precision for all methods were 0.05-1.09% and 0.02-1.06%, respectively.

Accelerated stability studies have been carried out on each drug by exposure to acid, base, H_2O_2, heat and 254 nm light for different time periods.

INTRODUCTION

Zidovudine (3'-azido-3'-deoxythymidine; also referred to as AZT) is a nucleoside reverse transcriptase inhibitor with antiviral activity against HIV-1, HIV-2, human T lymphotrophic virus and other retroviruses. When it is taken up by target cells, zidovudine is phosphorylated to a triphosphate metabolite by cellular enzymes to produce the active drug [1].

Zidovudine is administered either orally as a capsule or syrup or by I.V. infusion, often in conjunction with one or more other injectable drugs. It has been analyzed by HPLC with UV

detection in the three dosage forms [2], serum [3-4], plasma [5-6], and urine [6-7]. Radioimmunoassay methods have been used to quantitate zidovudine in plasma, urine, and cerebrospinal fluid [8-9]. HPLC and radioimmunoassay were used to determine zidovudine and its three phosphorylated metabolites, zidovudine mono-, di- and triphosphate, in peripheral blood mononuclear cells [10]. Zidovudine has also been studied with respect to its stability in I.V. admixtures by HPLC analysis with 266 nm UV detection and by visual inspection for precipitation, gas production, turbidity, and color change [11]. However, there continues to be a need for the development of stability indicating HPLC methods, which can be used to evaluate the compatibility of zidovudine with other injectable drugs. Ceftazidime, chlordiazepoxide, dobutamine, lorazepam and ranitidine are compounds that can be administered in conjunction with zidovudine. To date, there are no stability indicating methods available to assay each of these compounds with zidovudine.

Ceftazidime is a semi synthetic cephalosporin, broad spectrum, beta lactam antibiotic with activity against many gram negative pseudomonas and enterobacteriaceae microorganisms. It is used in the treatment of lower respiratory infections and urinary tract infections [12]. Ceftazidime has been analyzed by reversed-phase HPLC with UV [13-14] and electrochemical detection [15]. HPLC and micellar capillary electrophoresis with UV detection have been used to investigate the stability of aqueous reconstituted ceftazidime injection vials [16]. An enzyme linked immunosorbent assay (ELISA) with fluorescence detection has been used for the determination of ceftazidime in urine, serum and airborne material [17]. Chlordiazepoxide and lorazepam belong to the benzodiazepine class of drugs and are used in the treatment of anxiety disorders and in anesthetic premedication. In addition, chlordiazepoxide may be used in the management of alcohol withdrawal [18]. Reversed-phase HPLC with UV [19-20] and electrochemical detection [21] has been used to determine chlordiazepoxide. An assay for chlordiazepoxide has also been developed using gas chromatography - negative-ion chemical-ionization mass spectrometry [22]. Assay methods for lorazepam include HPLC-UV [23-24],

micellar electrokinetic chromatography [25], gas chromatography – tandem mass spectrometry [26] and immunoassay [27-28]. Dobutamine, a synthetic sympathomimetic catecholamine, is used primarily in the treatment of severe congestive heart failure [29]. HPLC assay methods for the analysis of dobutamine using UV [30], electrochemical (ECD) [31-32], and fluorescence [33] detection have been reported. Ranitidine is an H_2-receptor antagonist used to inhibit gastric acid secretion in the treatment of peptic ulcer [34]. Assay methods for ranitidine include HPLC with UV detection [35-36], HPLC with post-column fluorescence derivatization [37], high performance thin-layer chromatography (HPTLC) [38], TLC [2], supercritical fluid chromatography (SFC) [39] and capillary electrophoresis (CE) [40]. A stability indicating HPLC-UV method for the assay of ranitidine and meropenem has also been developed [41].

In this paper stability indicating HPLC assays are presented for the simultaneous analysis of zidovudine and ceftazidime (Mixture A), zidovudine and chlordiazepoxide (Mixture B), zidovudine and dobutamine (Mixture C), zidovudine and lorazepam (Mixture D), and zidovudine and ranitidine (Mixture E) in intravenous fluid mixtures. All of the separations were carried out using an amide hexadecylsilane (C16) column. The column is manufactured with a polar amide functionality located between the C3 and C4 members of the C18 alkyl chain. The amide functionality is able to take part in polar interactions between the phase and the analytes, resulting in a column with hydrophobic selectivity more similar to an octylsilane (C8) phase than a traditional octadecylsilane (C18) phase. All mixtures were separated with aqueous-acetonitrile eluents within 20 minutes.

EXPERIMENTAL

Reagents and Chemicals

The structural formulae of the compounds studied are shown in Figure 3.1. Zidovudine and dobutamine hydrochloride were obtained from the United States Pharmacopeial Convention Inc. (Rockville, MD, USA). Lorazepam was obtained from Sigma Chemical Co. (St Louis, MO, USA). Ceftazidime and ranitidine were obtained from GlaxoSmithKline (Research Triangle Park,

NC, USA). Chlordiazepoxide was obtained from Hoffman-La Roche Inc. (Nutley, NJ, USA). Acetonitrile (Fisher Scientific, Norcross, GA, USA) was HPLC grade and water was purified by a cartridge system (Continental Water Systems, Roswell, GA, USA). Phosphoric acid and sodium phosphate monobasic monohydrate (Fisher Scientific, Norcross, GA, USA) were ACS reagent grade. 0.9% Sodium Chloride Injection USP was obtained from Baxter Healthcare Corporation (Deerfield, IL, USA).

Instrumentation

The chromatographic separations were performed on a Model 1090 HPLC system (Hewlett Packard Co., Palo Alto, CA, USA). This system included a pump, an autosampler equipped with a 25µL loop and a Model 117 variable wavelength UV detector (Gilson, Middleton, WI, USA). 0.010" ID tubing was used before and after the column and was kept at a minimum length. Turbochrom (Perkin Elmer, Norwalk, CT, USA) chromatography software was used for data integration. A Model 996 photodiode array detector with Millenium-32 chromatography software (Waters, Milford, MA, USA) was used to confirm the purity of the analyte peaks from forced degradation. Separation of Mixtures A –E was achieved on a 250 mm x 4.6 mm I.D. amide C16 column (5µm particle size, and 180Å pore size, Discovery RP-AmideC16, Supelco, Bellafonte, PA 16823). Detection of separations A, B, D, and E was at 265 nm whereas detection of separation C was at 280 nm. The mobile phase for mixtures A and C consisted of 84:16 *v/v* 25 mM sodium phosphate monobasic monohydrate, pH 3.0 (adjusted with 0.1 M phosphoric acid) – acetonitrile. The mobile phase for mixture B consisted of 80:20 *v/v* 25 mM sodium phosphate monobasic monohydrate, pH 3.0 (adjusted with 0.1 M phosphoric acid) – acetonitrile. A binary gradient was utilized in the separation of Mixture D. Mobile phase A for Mixture D consisted of 82:18 *v/v* 25 mM sodium phosphate monobasic monohydrate, pH 3.0 (adjusted with 0.1 M phosphoric acid) – acetonitrile. Mobile phase B for Mixture D consisted of 40:60 *v/v* 25 mM sodium phosphate monobasic monohydrate, pH 3.0 (adjusted with 0.1 M phosphoric acid) – acetonitrile. The gradient program was as follows: 0 – 4 minutes, 100%

mobile phase A; 4 – 14 minutes, linear change from 100% mobile phase A to 100% mobile phase B; 14 – 15 minutes linear change from 100% mobile phase B to 100% mobile phase A; 15 – 20 minutes, equilibration at 100% mobile phase A. The mobile phase for Mixture E consisted of 88:12 *v/v* 25 mM sodium phosphate monobasic monohydrate, pH 3.0 (adjusted with 0.1 M phosphoric acid) – acetonitrile. The mobile phases were filtered through 0.22 μm nylon-66 filter (MSI, Westborough, MA, USA) and degassed by sparging with helium prior to use. The flow rate was 1.0 mL min^{-1} for all five mixtures.

Preparation of Solutions

All stock solutions were prepared fresh daily and were protected from light. Dilutions of all mixtures were made with an aqueous-acetonitrile diluent matching the mobile phase composition. A combined stock solution was prepared for zidovudine and ceftazidime at concentrations of 20 and 220 μg mL^{-1}, respectively. Standard solutions of the analytes for linear regression were made by dilution of the stock solution. This standard solution, along with 2:8 and 4:8 dilutions, gave solutions containing 10 and 5 μg mL^{-1} zidovudine, while ceftazidime was diluted to 110 and 55 μg mL^{-1}.

For Mixtures B, C and D, combined standard solutions were prepared which consisted of zidovudine and either chlordiazepoxide, dobutamine, or lorazepam at concentrations of 210, 1000, 200 and 100 μg mL^{-1}, respectively. Standard solutions of the analytes for linear regression were made by dilution of the respective stock solutions. In addition, 2:8 and 4:8 dilutions were prepared giving solutions containing 105 and 52.5 μg mL^{-1} zidovudine, 500 and 250 μg mL^{-1} chlordiazepoxide, 100 and 50 μg mL^{-1} dobutamine and 50 and 25 μg mL^{-1} lorazepam.

A combined stock solution was prepared for zidovudine and ranitidine at concentrations of 200 and 100 μg mL^{-1}, respectively. Standard solutions of the analytes for linear regression were made by dilution of the stock solution. This standard solution, along with 2:8 and 4:8

dilutions, gave solutions containing 100 and 50 μg mL^{-1} zidovudine, while ranitidine was diluted to 50 and 25 μg mL^{-1}.

Three point calibration curves were constructed for each analyte using the analytical conditions established for each mixture. Additional dilutions (3:8 and 6:8) of the stock solutions were prepared in diluent to serve as spiked samples for each analyte to determine accuracy and precision. Quantitation was based on linear regression analysis of analyte peak area versus analyte concentration in μg mL^{-1}.

Mixtures A – E were also prepared in 0.9% NaCl intravenous solutions. Zidovudine solutions were prepared at 1 mg mL^{-1} and ceftazidime, chlordiazepoxide, dobutamine, lorazepam and ranitidine solutions were prepared at 10, 5, 1, 1, and 0.3 mg mL^{-1}, respectively. For mixtures B - E, one mL of each intravenous solution was diluted to volume in a 10 mL volumetric flask using diluent. For mixture A, one mL of each intravenous solution was diluted to volume in a 100 mL volumetric flask using diluent.

To show that the methods are stability indicating it was necessary to subject the analytes to extreme conditions to cause them to degrade. Since each analyte possessed different degrees of stability, it was necessary to utilize different concentrations of HCl, NaOH, and H_2O_2 as well as different temperatures to force the degradation. In each case, 1 mg (or 2 mg with acid, base and H_2O_2 degradation) were dissolved in the appropriate degradation solution and allowed to stand from 1 minute to 7 days, according to stability testing procedures discussed by Weiser [42]. Samples degraded with peroxide were first neutralized with an equal volume and concentration of sodium sulfite solution (pH adjusted to 7 with 18 M sulfuric acid) prior to injecting into the HPLC system. Most acid and base degraded samples were first neutralized with equal volumes and concentrations of either acid or base prior to injection into the HPLC system. 6 M HCl stressed samples were neutralized with an equal volume of 5 M sodium acetate while 6 M NaOH stressed samples were neutralized with an equal volume of concentrated acetic acid.

RESULTS AND DISCUSSION

The purpose of this study was to develop stability-indicating methods for the analysis of zidovudine in combination with ceftazidime, chlordiazepoxide, dobutamine, lorazepam, or ranitidine in intravenous fluid mixtures. A search of the literature revealed no other stability indicating HPLC methods for the analysis of zidovudine with any of the other five compounds.

Zidovudine is typically administered intravenously at a concentration of 2 mg ml^{-1} and is diluted to a concentration of 1 mg ml^{-1} when it is mixed one to one with a second drug. Ceftazidime, chlordiazepoxide, dobutamine, lorazepam, and ranitidine are typically administered intravenously at concentrations of 20, 10, 2, 2, and 0.6 mg ml^{-1} and are diluted to concentrations of 10, 5, 1, 1, and 0.3 mg ml^{-1} when they are mixed one to one with zidovudine.

Initial method development efforts centered on the use of the compendial method for the separation of zidovudine, zidovudine related compound B, and zidovudine related compound C [2]. The flow rate was 1.0 mL min^{-1} and the injection volume was 10 µL for all developed methods. One of each of the five drugs was combined with zidovudine and the zidovudine related compounds and the percent acetonitrile was adjusted to obtain baseline resolution between zidovudine and zidovudine related compound B. This was necessary because zidovudine related compound B exists as an impurity in the zidovudine reference standard. If the impurity is not well resolved from the parent drug, the peak purity assessment of zidovudine will indicate that the peak is heterogeneous. The separation was then optimized with respect to the resolution between the drug that was being paired with zidovudine and all other related compounds. It was determined that 16, 20, 16, and 12% acetonitrile provided the optimal separations for ceftazidime, chlordiazepoxide, dobutamine, and ranitidine, respectively. Due to the extreme difference in polarities of zidovudine and lorazepam, an isocratic method with a reasonably short run time could not be developed. Instead, gradient conditions were used to separate the mixture with a 20-minute run time (including equilibration time). A wavelength of 265 nm was found to yield acceptable sensitivity for mixtures A, B, D, and E. Mixture C required detection at 280 nm for

good sensitivity of dobutamine. The analytical figures of merit for each method are shown in Table 3.1.

The HPLC method for Mixture A showed concentration verses absorbance linearity for zidovudine and ceftazidime in the 5-200 and 55-220 μg mL^{-1} ranges, respectively at 265 nm. The HPLC method for Mixture B showed concentration verses absorbance linearity for zidovudine and chlordiazepoxide in the 52.5-210 and 250-1000 μg mL^{-1} ranges, respectively at 265 nm. The HPLC method for Mixture C showed concentration verses absorbance linearity for zidovudine and dobutamine in the 52.5-210 and 50-200 μg mL^{-1} ranges, respectively at 280 nm. The HPLC method for Mixture D showed concentration verses absorbance linearity for zidovudine and lorazepam in the 52.5-210 and 25-100 μg mL^{-1} ranges, respectively at 265 nm. The HPLC method for Mixture E showed concentration verses absorbance linearity for zidovudine and ranitidine in the 50-200 and 25-100 μg mL^{-1} ranges, respectively at 265 nm. Correlation coefficients for the five mixtures were in a range of 0.9999 – 1.0 (n=6).

Accuracy and precision of the methods were evaluated using spiked samples containing each analyte. The results shown in Table 3.2 indicate that the procedures give acceptable accuracy and precision for each analyte.

Intra-day variabilities for zidovudine and ceftazidime (Mixture A) expressed as % RSD were 0.20 and 0.43% (n=15), respectively. Inter-day variabilities were calculated from assays run on three consecutive days. The %RSD was 0.19% for zidovudine and 1.80% for ceftazidime. Intra-day variabilities for zidovudine and chlordiazepoxide (Mixture B) were 0.17 and 0.08% (n=15), respectively. Inter-day variabilities were 0.86% for zidovudine and 0.25% for chlordiazepoxide. Intra-day variabilities for zidovudine and dobutamine (Mixture C) were 0.09 and 0.43% (n=15), respectively. Inter-day variabilities were 0.31% for zidovudine and 0.52% for dobutamine. Intra-day variabilities for zidovudine and lorazepam (Mixture D) were 0.15 and 1.37% (n=15), respectively. Inter-day variabilities were 0.46% for zidovudine and 1.59% for

lorazepam. Intra-day variabilities for zidovudine and ranitidine (Mixture D) were 0.13 and 0.19% (n=15), respectively. Inter-day variabilities were 0.30% for zidovudine and 0.28% for ranitidine.

A photodiode array detector was used to verify that none of the degradation products of the analytes in either Mixture A, B, C, D, or E interfered with the quantitation of each drug. Each mixture was analyzed under their respective analytical conditions. The results of these studies are summarized in Tables 3.3, 3.4, and 3.5. Chromatograms of the separations of each of the five mixtures following forced degradation are shown in Figures 3.2 – 3.6.

The mixtures were also prepared in 0.9% NaCl intravenous solutions, diluted in diluent and analyzed under their respective analytical conditions. Example chromatograms for Mixtures A and E are shown in figures 3.7 – 3.8.

In summary, an amide C16 column with 25 mM phosphate buffer (pH 3 with phosphoric acid) – acetonitrile eluents was shown to be suitable for the separation of zidovudine in combination with ceftazidime (Mixture A), chlordiazepoxide (Mixture B), dobutamine (Mixture C), lorazepam (Mixture D) or ranitidine (Mixture E) in intravenous fluid mixtures. The methods had run times that were ≤ 20 min. and the column showed good efficiencies for all analytes. The methods were found to be free from interferences from degradants and are suitable for the investigation of the chemical stability of the analytes in each of the mixtures.

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TABLE 3.1: Analytical Figures of Merit for Mixtures A-E

Analyte	*k*	N^a	*Tailing Factor*[b]	R_s	α		
Mixture A							
Zidovudine	1.39	13512	1.10				
						13.62	3.77
Ceftazidime	0.37	6550	1.25				
Mixture B							
Zidovudine	0.96	7036	1.12				
						17.45	3.85
Chlordiazepoxide	3.70	7279	1.55				
Mixture C							
Zidovudine	1.42	13412	1.10				
						11.28	1.89
Dobutamine	2.69	10959	1.37				
Mixture D							
Zidovudine	1.10	N/A	1.16				
						45.22	3.91
Lorazepam	4.31	N/A	1.17				
Mixture E							
Zidovudine	2.46	12490	1.14				
						21.17	5.21
Ranitidine	0.47	8497	1.18				

[a] Calculated as 16 $(t_R/W)^2$. Calculations of theoretical plates in gradient separations do not yield meaningful data.
[b] Calculated at 5% peak height

TABLE 3.2: Accuracy and Precision Using Spiked Drug Samples

Mixture	Analyte	Concn Added [a] $\mu g\ mL^{-1}$	Concn Found $\mu g\ mL^{-1}$	Percent Error	RSD (%)
A	Zidovudine	7.50	7.49 ± 0.02	0.23	0.26
		15.00	15.02 ± 0.02	0.16	0.16
	Ceftazidime	82.50	82.60 ± 0.08	0.12	0.10
		165.00	165.11 ± 0.20	0.10	0.12
B	Zidovudine	78.75	79.26 ± 0.10	0.64	0.13
		157.50	158.82 ± 1.68	1.09	1.06
	Chlordiazepoxide	375.00	377.00 ± 0.85	0.53	0.23
		750.00	754.26 ± 0.74	0.57	0.10
C	Zidovudine	78.75	78.56 ± 0.11	0.24	0.13
		157.5	157.97 ± 0.08	0.30	0.05
	Dobutamine	75.00	75.00 ± 0.06	0.06	0.09
		150.00	150.64 ± 0.19	0.43	0.12
D	Zidovudine	78.75	78.57 ± 0.06	0.23	0.08
		157.50	157.72 ± 0.50	0.21	0.32
	Lorazepam	37.50	37.27 ± 0.17	0.62	0.46
		75.00	74.79 ± 0.16	0.27	0.21
E	Zidovudine	75.00	75.21 ± 0.08	0.27	0.11
		150.00	150.42 ± 0.21	0.28	0.14
	Ranitidine	37.50	37.42 ± 0.05	0.22	0.15
		75.00	75.04 ± 0.02	0.05	0.02

[a] mean ± standard deviation based on n=3.

TABLE 3.3: Stability Indicating Nature of Assays

Drug	Treatment	Duration	%Decrease in Peak Area	t_R of Parent Peak (min)	t_R of Degradent Peak (min)
Mixture A					
Zidovudine	6 M HCl	4 days	10.3	7.6	3.1, 3.5, 3.8, 5.8, 6.7, 7.3
	6 M NaOH	4 days	21.8	7.5	3.1, 3.5, 3.8
	0.3% H_2O_2	2 days	10.7	7.5	3.0, 3.4, 3.7
	90° C	7 days	9.7	7.4	3.7
	254 nm	5.5 h	12.3	7.4	3.1, 3.2, 3.4, 3.7, 3.9, 6.6
Ceftazidime	1.0 M HCl	1 day	20.7	4.2	3.2, 3.9, 4.9, 8.3
	0.001 M NaOH	5 days	13.8	4.2	3.5, 5.7, 6.0
	0.03 % H_2O_2	2 days	20.8	4.3	3.2, 3.5, 4.0, 5.4, 9.4
	60° C	2 days	10.6	4.2	3.1, 3.4, 3.9, 4.8, 5.3, 6.7
	254 nm	4 h	21.9	4.2	3.1, 3.7, 3.9, 5.2
Mixture B					
Zidovudine	6 M HCl	4 days	11.4	6.3	3.5, 5.0, 5.8
	6 M NaOH	4 days	20.3	6.2	3.3, 3.9
	0.3% H_2O_2	2 days	15.5	6.3	3.1, 3.3, 3.6, 3.9, 4.3
	90° C	7 days	11.3	6.2	3.7
	254 nm	5.5 h	11.9	6.2	3.2, 3.5, 3.7, 4.0, 4.5, 5.7
Chlordiazepoxide	1 M HCl	2 days	27.5	14.7	3.0
	1 M NaOH	5 days	27.3	14.2	2.4, 3.1
	0.3 % H_2O_2	2 days	15.5	12.1	3.5, 13.0
	60° C	4 h	20.5	14.3	
	254 nm	30 min	22.1	14.7	

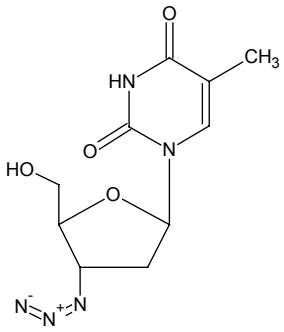
TABLE 3.4: Stability Indicating Nature of Assays

Drug	Treatment	Duration	%Decrease in Peak Area	t_R of Parent Peak (min)	t_R of Degradent Peak (min)
Mixture C					
Zidovudine	6 M HCl	4 days	8.3	7.5	3.0, 3.4, 3.7, 6.6, 7.2
	6 M NaOH	4 days	21.0	7.4	3.0, 3.4, 3.8
	0.3% H_2O_2	2 days	11.1	7.4	3.0, 3.4
	90° C	7 days	10.0	7.4	3.7
	254 nm	5.5 h	12.1	7.4	3.0, 3.4, 3.7, 3.9, 6.5
Dobutamine	6 M HCl	3 h	35.5	10.8	3.3, 3.8
	0.001 M NaOH	15 min.	18.0	11.1	
	0.03 % H_2O_2	20 h	12.4	10.8	3.4
	60° C	1 h	12.1	10.2	
	254 nm	1.75 h	10.8	10.2	
Mixture D					
Zidovudine	6 M HCl	4 days	13.2	6.5	3.0, 3.4, 3.7, 5.1, 5.8, 6.1
	6 M NaOH	4 days	21.1	6.4	3.1, 3.3, 3.7
	0.3 % H_2O_2	2 days	12.5	6.4	3.0, 3.3, 3.9, 4.3
	90° C	7 days	11.0	6.4	3.5, 3.6
	254 nm	5.5 h	12.4	6.4	3.0, 3.2, 3.6
Lorazepam	0.1 M HCl	2 days	24.3	11.1	11.5, 12.0, 12.9
	0.1 M NaOH	18 h	27.1	11.1	11.5, 12.5, 13.4
	3.0 % H_2O_2	2 days	13.0	10.9	3.4, 3.9, 10.3, 12.3
	60° C	1.5 h	10.1	11.0	11.4
	254 nm	19 h	15.0	11.0	11.5, 11.9

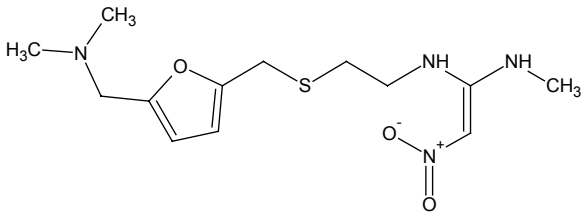
TABLE 3.5: Stability Indicating Nature of Assays

Drug	Treatment	Duration	%Decrease in Peak Area	t_R of Parent Peak (min)	t_R of Degradent Peak (min)
Mixture E					
Zidovudine	6 M HCl	4 days	8.9	10.5	3.1, 3.5, 3.9, 7.4, 9.1
	6 M NaOH	4 days	21.0	10.4	3.2, 3.2, 3.5, 3.9
	0.3 % H_2O_2	2 days	13.6	10.4	3.0, 3.5, 3.8, 5.7
	90° C	7 days	11.7	10.5	3.9
	254 nm	5.5 h	12.4	10.4	3.2, 3.7, 3.9, 4.1
Ranitidine	1 M HCl	4 days	15.9	4.7	3.6, 4.2
	1 M NaOH	20 h	55.6	4.9	3.1, 6.9
	0.3 % H_2O_2	30 min	15.8	4.9	3.1, 3.3, 3.8, 4.4
	80° C	3 days	14.4	4.9	3.3
	254 nm	2.5 h	12.1	4.8	3.7, 4.4

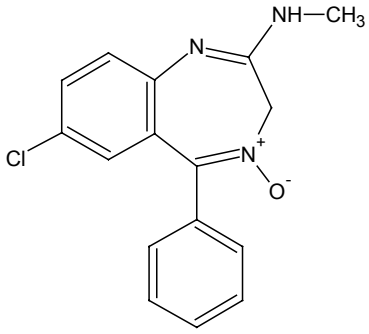
Figure 3.1: Chemical structures of the analytes



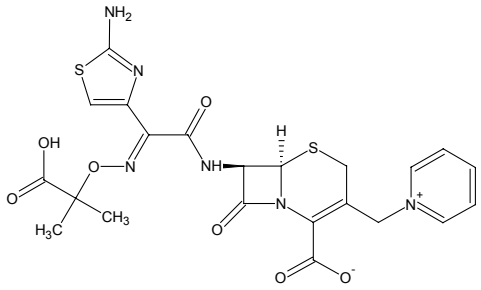
Zidovudine



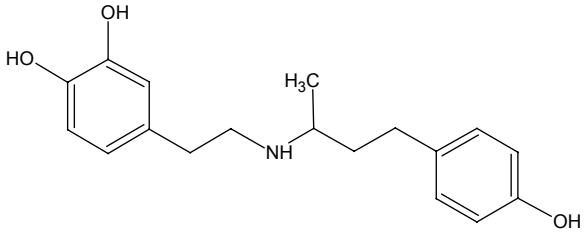
Ranitidine



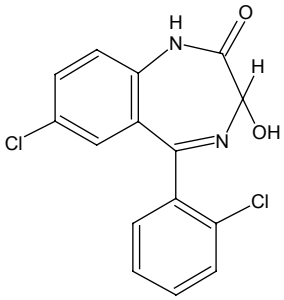
Chlordiazepoxide



Ceftazidime



Dobutamine



Lorazepam

Figure 3.2: Chromatograms from accelerated degradation studies of ceftazidime and zidovudine. Top, ceftazidime in 0.03% H_2O_2 for 2 days. Bottom, zidovudine following exposure to 254-nm uv light for 5.5 hours. Unlabeled peaks are unknown degradation products. The ceftazidime chromatogram is offset from the zidovudine chromatogram with an angle of elevation of 10 degrees.

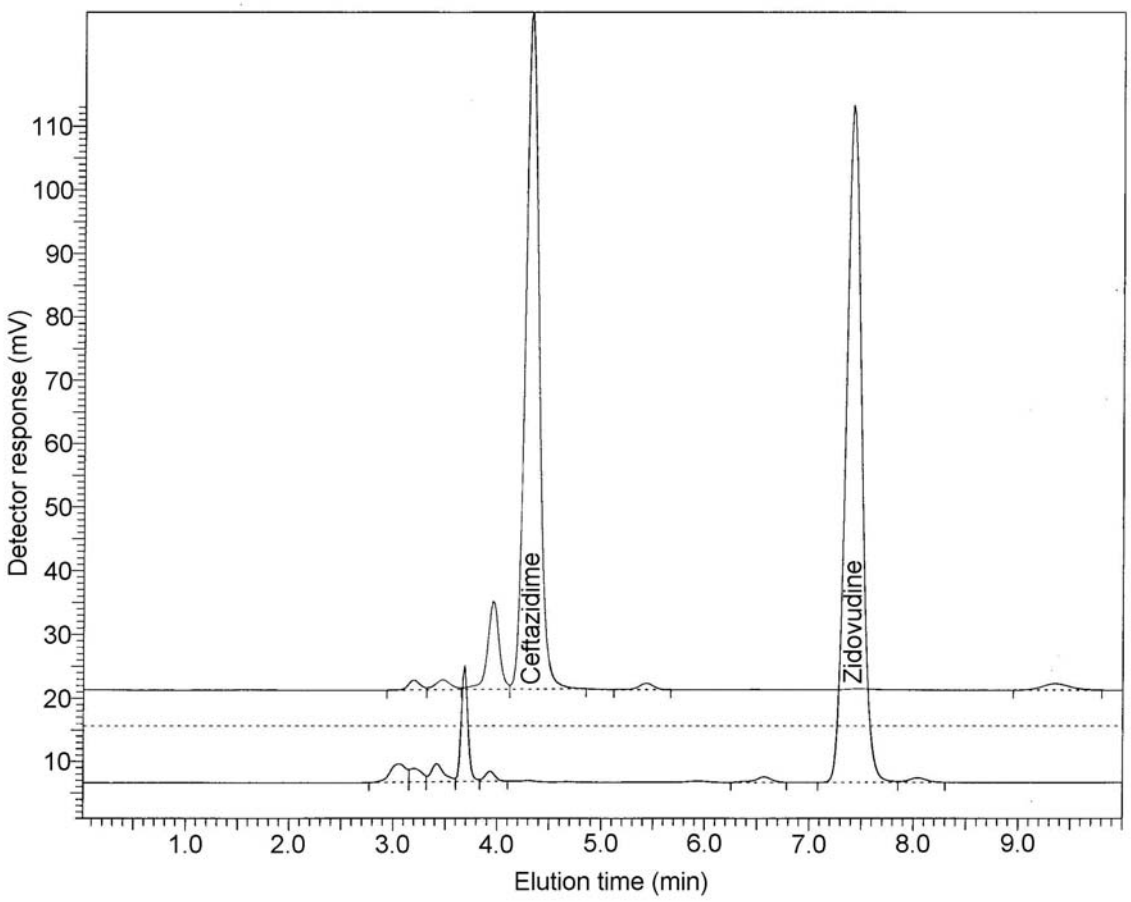

Detector response (mV)
110
100
90
80
70
60
50
40
30
20
10
Ceftazidime
Zidovudine
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Elution time (min)

Figure 3.3: Chromatograms from accelerated degradation studies of chlordiazepoxide and zidovudine. Top, chlordiazepoxide in 1 M NaOH for 5 days. Bottom, zidovudine in 0.3% H_2O_2 for 2 days. Unlabeled peaks are unknown degradation products. The chlordiazepoxide chromatogram is offset from the zidovudine chromatogram with an angle of elevation of 10 degrees.

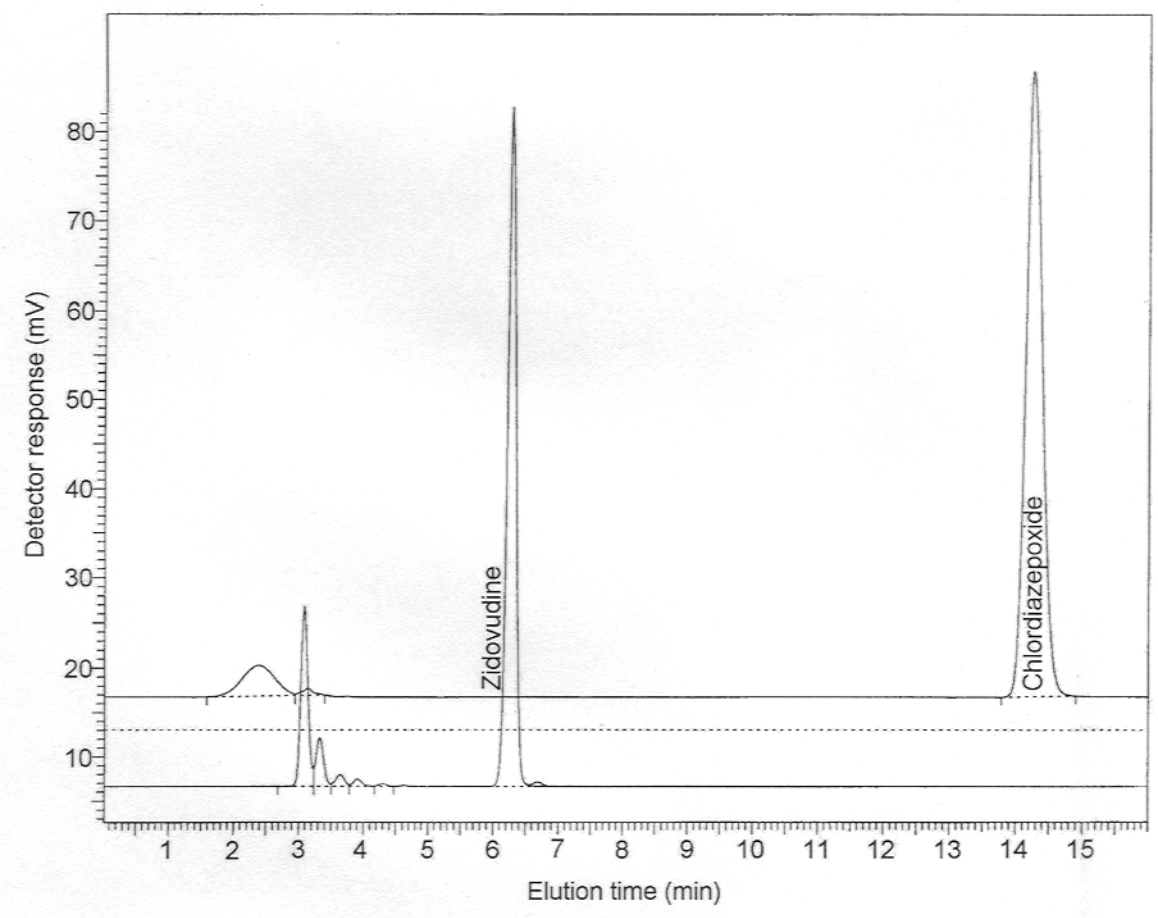

Detector response (mV)
80
70
60
50
40
30
20
10
Zidovudine
Chlordiazepoxide
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Elution time (min)

Figure 3.4: Chromatograms from accelerated degradation studies of dobutamine and zidovudine. Top, dobutamine in 6 M HCl for 3 hours. Bottom, zidovudine following exposure to 254-nm uv light for 5.5 hours. Unlabeled peaks are unknown degradation products. The dobutamine chromatogram is offset from the zidovudine chromatogram with an angle of elevation of 10 degrees.

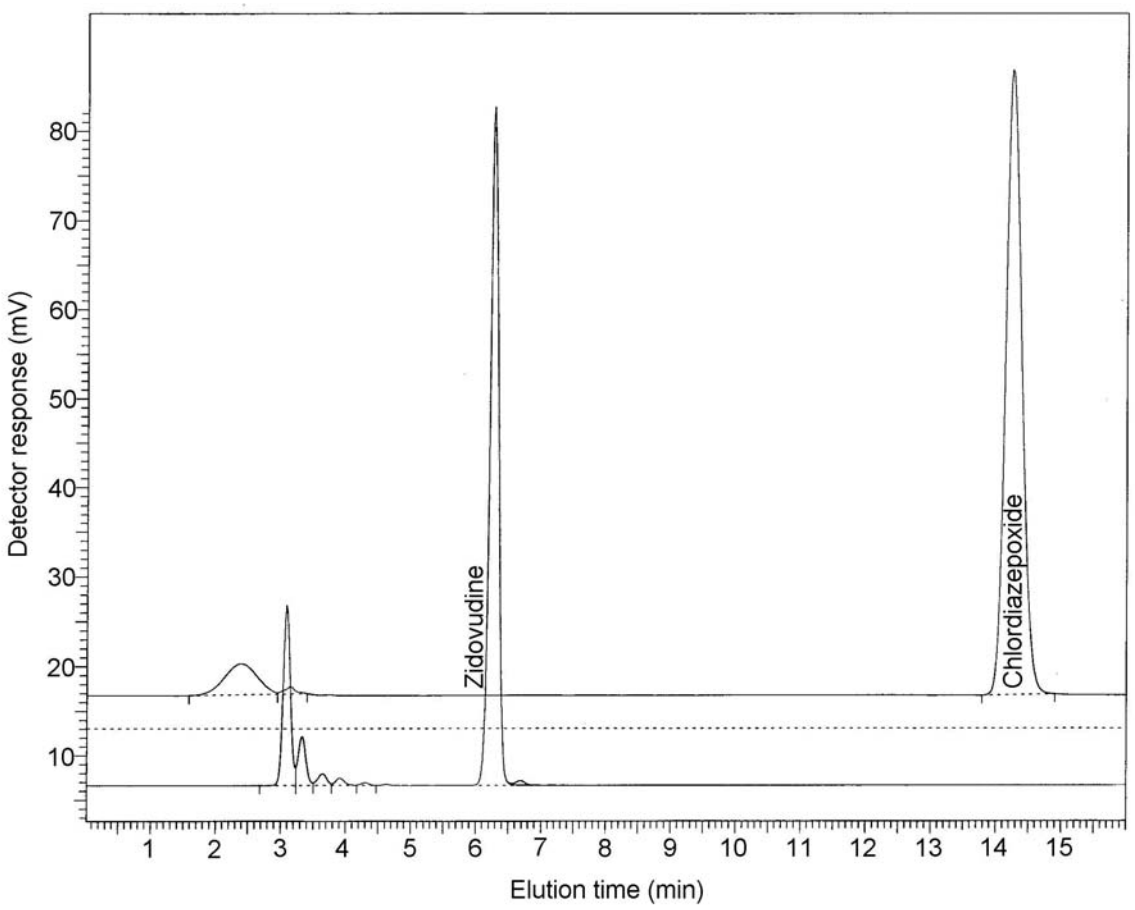

Detector response (mV)
80
70
60
50
40
30
20
10
Zidovudine
Chlordiazepoxide
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Elution time (min)

Figure 3.5: Chromatograms from accelerated degradation studies of lorazepam and zidovudine. Top, lorazepam in 0.1 M NaOH for 18 hours. Bottom, zidovudine in 6 M HCl for 4 days. Unlabeled peaks are unknown degradation products. The lorazepam chromatogram is offset from the zidovudine chromatogram with an angle of elevation of 10 degrees.

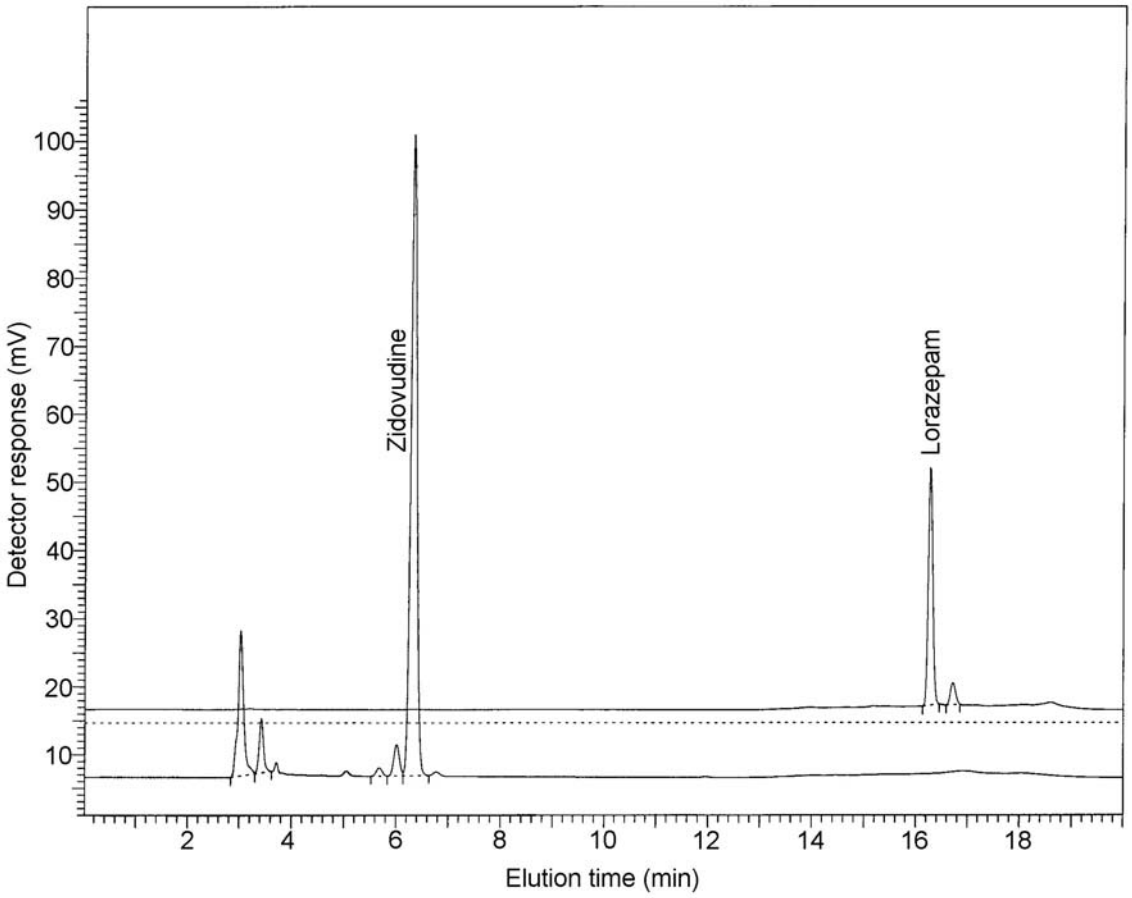

Detector response (mV)
100
90
80
70
60
50
40
30
20
10
Zidovudine
Lorazepam
2
4
6
8
10
12
14
16
18
Elution time (min)

Figure 3.6: Chromatograms from accelerated degradation studies of ranitidine and zidovudine.Top, ranitidine in 1 M HCl for 4 days. Bottom, zidovudine in 6 M HCl for 4 days. Unlabeled peaks are unknown degradation products. The ranitidine chromatogram is offset from the zidovudine chromatogram with an angle of elevation of 10 degrees.

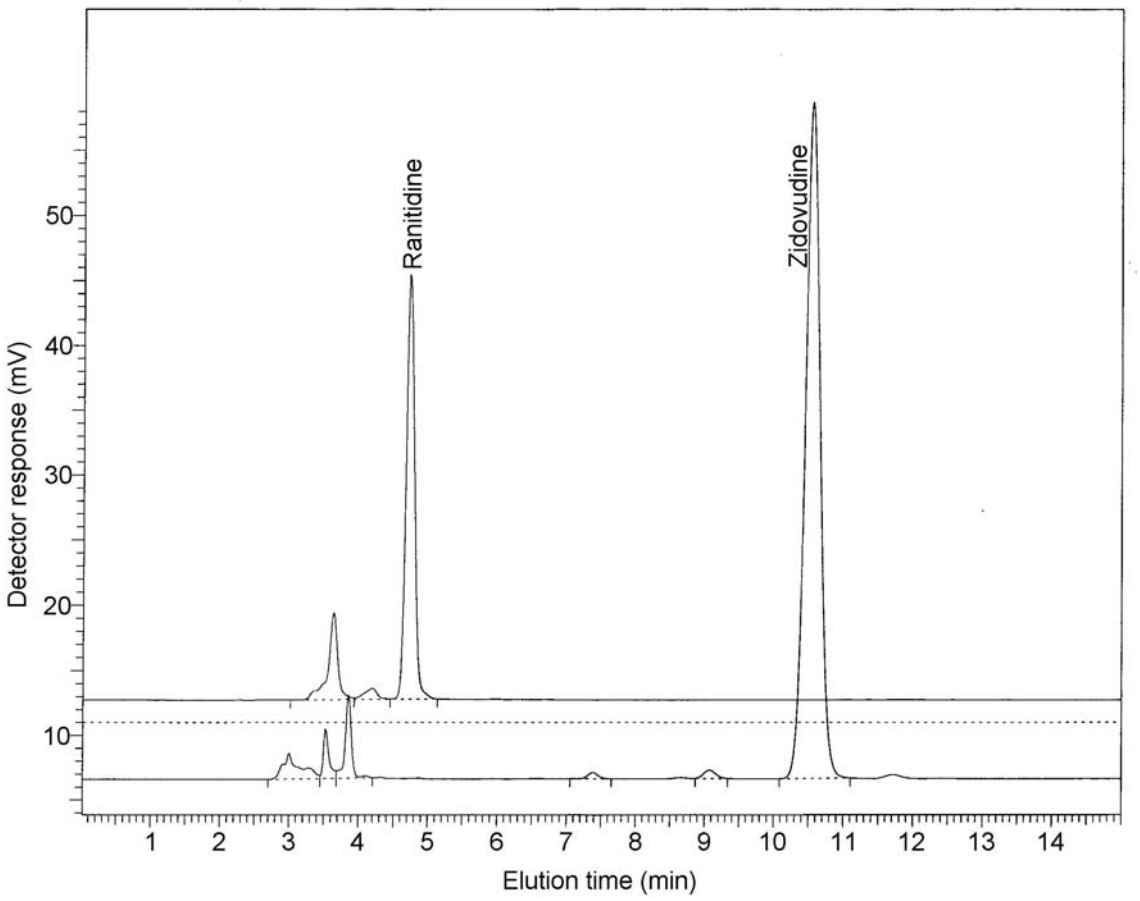

Ranitidine
Zidovudine
Detector response (mV)
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Elution time (min)

Figure 3.7: Chromatogram of Mixture A, prepared in 0.9% NaCl intravenous fluid. Concentrations of ceftazidime and zidovudine were determined to be 1.00 and 1.101 mg mL^{-1}, respectively.

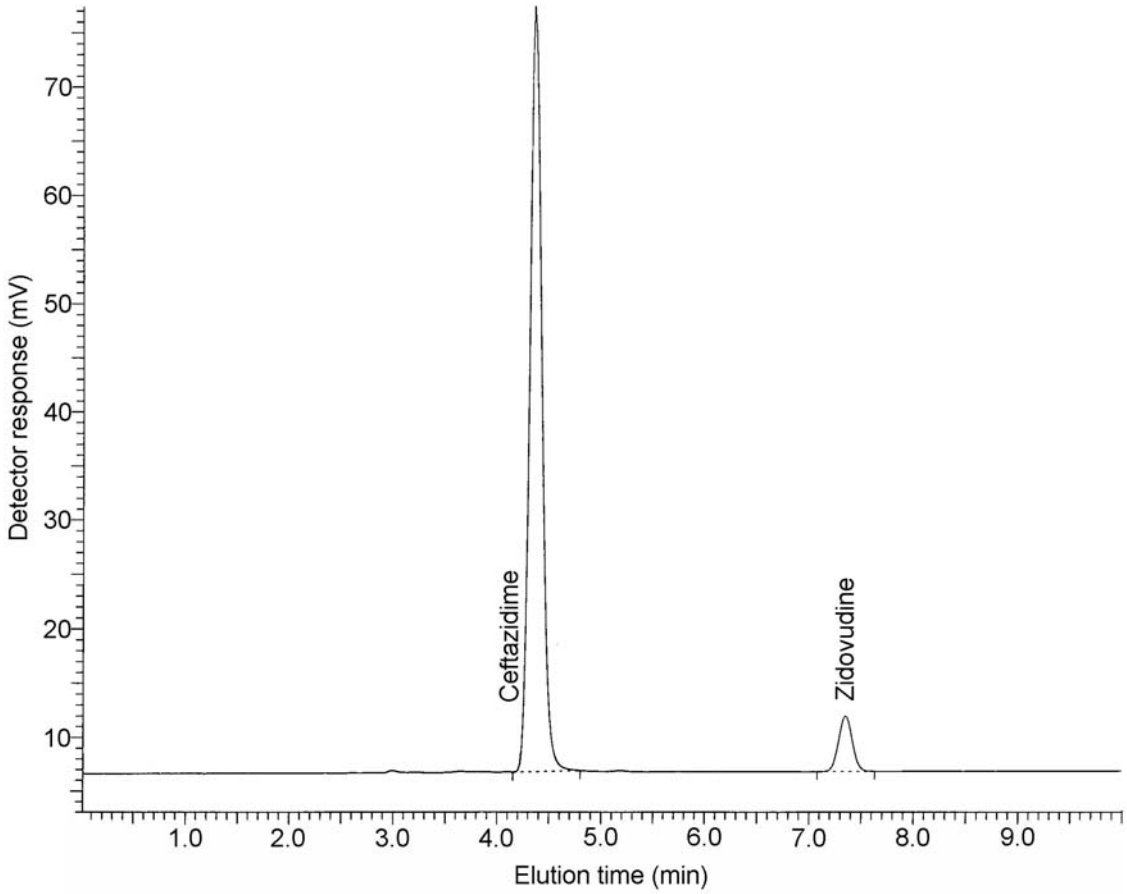
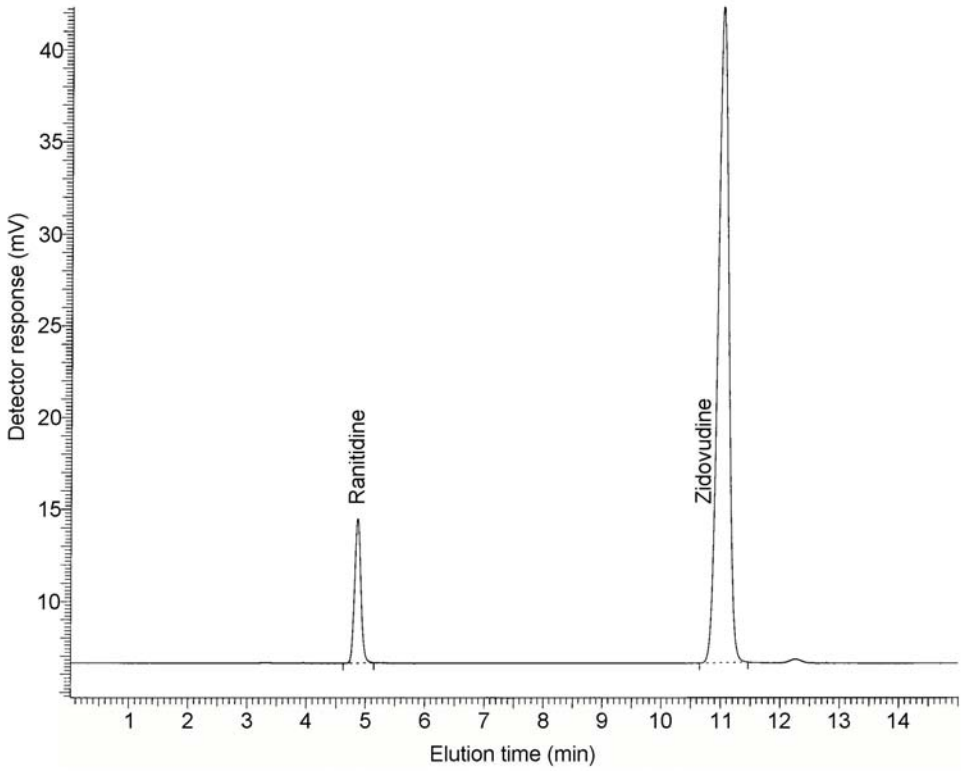

Detector response (mV)
70
60
50
40
30
20
10
Ceftazidime
Zidovudine
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Elution time (min)

Figure 3.8: Chromatogram of Mixture E, prepared in 0.9% NaCl intravenous fluid. Concentrations of ranitidine and zidovudine were determined to be 0.33 and 0.996 mg mL^{-1}, respectively.


Detector response (mV)
40
35
30
25
20
15
10
Ranitidine
Zidovudine
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Elution time (min)

PART II

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR THE ANALYSIS OF SELECTED PHARMACEUTICALS

CHAPTER 4

DETERMINATION OF ZIDOVUDINE AND LEVOFLOXACIN IN HUMAN PLASMA BY REVERSED PHASE HPLC AND SOLID PHASE EXTRACTION[3]

[3] Caufield, W.V. and Stewart, J.T. Accepted by Journal of Liquid Chromatography and Related Technologies. Reprinted here with permission of publisher.

CHAPTER 4

ABSTRACT

A new high-performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of zidovudine (AZT) and levofloxacin in human plasma. Plasma samples were treated with a solid-phase extraction procedure. The compounds were separated using a mobile phase of 86:14 *v/v* 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4) – acetonitrile on an octadecylsilane column (150 x 4.6 mm i.d.) with UV detection at 266 nm. Ciprofloxacin was used as the internal standard (IS). The method was validated over the range of 26.3-2600 ng/mL for AZT, and 51.2-5069 ng/mL for levofloxacin. The method proved to be accurate (percent bias for all calibration samples varied from –6.2 to 5.6%) and precise (within-run precision ranged from 0.9 to 9.7% and between-run precision ranged from 1.3 to 7.5%). The mean absolute recoveries were 94.1% for AZT, 91.2% for levofloxacin, and 84.7% for the internal standard. The assay should be suitable for use in pharmacokinetic studies and routine plasma monitoring of these drugs in HIV infected patients.

INTRODUCTION

At the end of 2001, there had been an estimated 3 million deaths worldwide due to human immunodeficiency virus (HIV) infections and 40 million people were currently living with HIV (1). Although several drugs have been developed to combat this epidemic, zidovudine (3'-azido-3'-deoxythymidine; AZT) continues to be one of the first-line therapeutic agents in treating HIV. AZT is a nucleoside reverse transcriptase inhibitor with antiviral activity against HIV-1, HIV-2, human T lymphotrophic virus and other retroviruses. It is anabolized intracellularly to a triphosphate metabolite by cellular enzymes to produce the active drug (2). Side effects that have been associated with AZT therapy include gastrointestinal intolerance, bone marrow toxicity and myelosuppression (3). Long-term exposure to AZT has also been shown to result in the development of AZT resistant strains of HIV-1 (4). It has been demonstrated that dose-related toxicities can be reduced and patient outcomes can be improved when a specific concentration

range of AZT is maintained (5). It has also been suggested that HIV patients might benefit from a pharmacokinetic approach to AZT therapy (6). Therapeutic drug monitoring of antiviral drugs such as AZT is also necessary to avoid or delay resistance from the virus, to monitor adherence, and to monitor drug-drug and drug-food interactions.

Many human immunodeficiency virus (HIV)-infected patients develop secondary bacterial infections because of their compromised immune systems. Coinfection with mycobacterium species, especially Mycobacterium avium complex (MAC) and mycobacterium tuberculosis is often treated with multiple antibacterial agents, including levofloxacin (7-9). Levofloxacin is a chiral fluorinated carboxyquinolone and is the L-isomer of the racemate ofloxacin. It is a broad-spectrum antibacterial agent with activity against a wide range of gram-positive, gram-negative and anaerobic bacteria. Levofloxacin has been found in vitro to be generally twice as active as ofloxacin against many of these organisms (10). The bactericidal activity of levofloxacin is maximized when the ratios of peak plasma drug concentrations (C_{max}) : minimum inhibitory concentrations (MIC) or area under the concentration-time curve (AUC) : MIC exceed certain threshold levels (11). Therefore, therapeutic drug monitoring of levofloxacin plasma levels would make it possible to administer the correct dose of the drug to the patient at the appropriate interval. Monitoring of the Cmax to MIC ratio is particularly important in patients at risk for malabsorption, such as those infected with HIV (12).

Analytical methods have been described to quantify the individual drugs in biological media (13-16), but no methods have been reported for the simultaneous determination of AZT and levofloxacin in human plasma. This paper describes the development and validation of an assay that is both rapid and sensitive for determining AZT and levofloxacin in human plasma. For sample pre-treatment, the method utilizes solid phase extraction that does not require an evaporation step. Elution is performed isocratically with 266nm UV detection.

EXPERIMENTAL

Chemicals

Zidovudine (AZT) was obtained from the United States Pharmacopeial Convention Inc. (Rockville, MD, USA). Levofloxacin was kindly provided by R. W. Johnson Pharmaceutical Research Institute (Spring House, PA 19477). Ciprofloxacin Hydrochloride was purchased from Serologicals Proteins Inc. (Kankakee, IL 60901). Monobasic sodium phosphate, phosphoric acid, HPLC grade methanol and acetonitrile were from J.T. Baker Inc. (Phillipsburg, NJ 08865). Trifluoroacetic acid was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201). Water was purified by a cartridge system (Continental Water System, Roswell, GA, USA). Drug free human plasma was obtained from Bioreclamation Inc. (Hicksville, NY 11801).

Instrumentation

The chromatographic separations were performed on a Model 1090 HPLC system (Hewlett Packard Co., Palo Alto, CA, USA). This system included a pump, an autosampler equipped with a 250 µL loop and a Model 117 variable wavelength UV detector (Gilson, Middleton, WI, USA). 0.010" ID tubing was used before and after the column and was kept at a minimum length. Turbochrom (Perkin Elmer, Norwalk, CT, USA) chromatography software was used for data integration. Separations were performed on a 150 mm x 4.6 mm I.D. octadecylsilane (C18) column (5µm particle size, and 100Å pore size, Luna, Phenomenex, Torrance, CA 90501).

Chromatographic Conditions

The mobile phase consisted of 86:14 *v/v* 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4) – acetonitrile. The mobile phase was filtered through a 0.22 µm nylon-66 filter (MSI, Westborough, MA, USA) and degassed in an ultrasonic bath for 15 min before use. The HPLC pump flow rate was 1.5 mL/min and all analyses were

conducted at ambient temperature. The injection volume was 50 µL and the UV detector was operated at 266 nm.

Preparation of Standard Stock Solutions

Standard stock solutions of zidovudine (AZT) and levofloxacin were prepared by dissolving appropriate amounts of each drug in pooled human plasma to obtain final drug concentrations of 503 and 980 µg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with pooled human plasma. The internal standard (ciprofloxacin) stock solution was prepared by dissolving an appropriate amount of the drug in methanol to obtain a final concentration of 540 µg/mL. A working internal standard solution was prepared by further diluting this stock solution with 25 mM sodium phosphate buffer solution to yield a concentration of 540 ng/100 µL.

Sample Preparation Procedure

Calibration standards and quality control samples were prepared by making appropriate dilutions of the working standard solution with pooled human plasma. Solid phase extraction cartridges (Varian Inc., Bond Elut C18, 1cc 100 mg) were placed on a vacuum elution manifold (Alltech, Deerfield, IL 60015) and rinsed with 1 mL of methanol followed by 1 mL of purified water and 1 mL of 25 mM sodium phosphate monobasic monohydrate containing 0.1% trifluoroacetic acid (pH 2.4). Care was taken that the cartridges did not run dry. 1.1 mL of each standard or sample was transferred to a 1.5 mL polypropylene microcentrifuge tube and centrifuged at 13,000 rpm for 15 min. Following centrifugation, 1 mL of each standard or sample was transferred to a 1.5 mL polypropylene microcentrifuge tube and mixed with 100 µL of internal standard. The entire spiked plasma samples were then transferred to the SPE cartridges. The microcentifuge tubes were then rinsed with 250 uL of 25 mM sodium phosphate monobasic monohydrate containing 0.1% trifluoroacetic acid (pH 2.4) and the rinses were transferred to the cartridge. Vacuum was then applied to obtain a flow through the cartridges of 1-2 mL/min. The cartridges were then washed with two 1-mL aliquots of 95:5 *v/v* 25 mM sodium phosphate

monobasic monohydrate containing 0.1% trifluoroacetic acid (pH 2.4) –methanol followed by vacuum suction for one min. The analytes were eluted from the cartridges with two 250 uL aliquots of 80:20 *v/v* 25 mM sodium phosphate monobasic monohydrate containing 0.1% trifluoroacetic acid (pH 2.4) – acetonitrile followed by vacuum suction for one min. Extracts were collected directly into 1.5 mL autosampler vials, vortex mixed and 50 uL was then injected onto the liquid chromatograph.

Specificity

The specificity of the assay was checked by analyzing four independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analytes. The specificity was also assessed for other compounds that could reasonable be expected to be present in the plasma of HIV infected patients.

Linearity

Calibration plots for the analytes in plasma were prepared by diluting stock solutions with pooled human plasma to yield concentrations of 26.3-2600 ng/mL (26.3, 138, 261, 1325 and 2600 ng/mL) for AZT and 51.2-5069 ng/mL (51.2, 268, 509, 2582 and 5068 ng/mL) for levofloxacin. Calibration standards at each concentration were extracted and analyzed in triplicate. Calibration curves were constructed using ratios of the observed analyte peak area to internal standard versus nominal concentrations of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficient data. From this data, a first order polynomial model was selected for each analyte. To confirm that the chosen linear model was correct, a statistical lack of fit test was performed.

Benchtop stability

The stability of the processed sample in the sample compartment of the HPLC was also assessed. Prepared samples at two concentrations (80.3 and 2062 ng/mL for AZT, 156 and 4019 ng/mL for levofloxacin) from day 1 of the precision and accuracy assessment were pooled and

injected every two hr. over a 24-hr period. During this time the samples were protected from light at ambient temperature. The peak area ratio of analyte to internal standard was plotted verses time and was used for least squares regression analysis. The regression data was used to determine if a significant change in analyte concentration occurred over the course of the 24-hr period. For the purposes of this validation, a change in concentration was considered significant if it exceeded 10%.

Precision and Accuracy

The within-run and between-run accuracy and precision of the assay in plasma were determined by assaying four quality control samples in triplicate over a period of three days. The concentrations represented the entire range of the calibration curves. The lowest level was at the expected LOQ for each analyte (26.3 ng/mL for AZT and 51.2 ng/mL for levofloxacin). The second level was at three times the LOQ (80.3 ng/mL for AZT and 156 ng/mL for levofloxacin) and the third level was at the mid-point of the calibration curves (261 ng/mL for AZT and 509 ng/mL for levofloxacin). The fourth level was at 80 percent of the upper boundary of the calibration curves (2062 ng/mL for AZT and 4019 ng/mL for levofloxacin). Calibration curves were prepared and analyzed daily and linear models were used to determine concentrations in the quality control samples. The nine measured concentrations per concentration level (triplicates from three runs) were subjected to analysis of variance (ANOVA) to estimate the within-run and between run precision. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Precision was reported as percent relative standard deviation (%RSD).

Limit of Detection and Limit of Quantification

Decreasing concentrations of the analytes were prepared by diluting stock solutions with pooled human plasma, and then analyzed. The limit of detection (LOD) was defined as the concentration that yields a signal-to-noise ratio of 3. The limit of quantification (LOQ) was

calculated to be the lowest analyte concentration in plasma that could be measured with a between run relative standard deviation (RSD) of <20% and an accuracy between 80 and 120%.

Recovery

The absolute recoveries of AZT and levofloxacin from plasma were assessed at two concentrations (80.3 and 2062 ng/mL for AZT, and 156 and 4019 ng/mL for levofloxacin). The recovery of the internal standard from plasma was assessed at the working concentration of 540 ng/mL. For each level three samples were extracted and analyzed in triplicate. Three replicates of each concentration, prepared in the eluent, were directly injected. The assay absolute recovery for each compound at each concentration was computed using the following equation: absolute recovery = (peak area in extract)/(mean peak area direct injection) x 100.

Freezing and thawing stability

The stability of plasma samples after three freeze-thaw cycles was also examined. The two concentrations used in the benchtop stability study were assayed in triplicate over a period of three days. After each analysis, the samples were re-frozen until the next day. After three freeze-thaw cycles, the results were compared to the initial fresh unfrozen samples from the accuracy and precision assessment. The unpaired t-test (two tailed) was used to determine if the means from each level were significantly ($\alpha = 0.05$) different. An f-test was also used to determine if the variances were significantly different.

RESULTS AND DISCUSSION

The chemical structures for AZT, levofloxacin and the internal standard ciprofloxacin are shown in Figure 4.1. The goals in developing this method were low ng/mL sensitivity, a run time of less than ten min and a simple extraction method that could be easily automated. The large difference in lipophilicity between AZT and levofloxacin posed the greatest challenge in the development of the separation. The more hydrophilic AZT tended to elute with endogenous substances in the plasma extract whereas levofloxacin and the internal standard tended to elute much later in the run. Initially, a series of reversed phase columns were investigated including

C8, C16-amide, halogen specific C18 and conventional C18. A gradient separation was not desirable due to the additional time required for the column to re-equilibrate to the initial conditions. Ultimately, a 150 x 4.6 mm conventional C18 column and an isocratic run were selected with a mobile phase of 86:14 *v/v* 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4) – acetonitrile. Trifluoroacetic acid was added to lower the mobile phase pH and to minimize the retention of endogenous sample components. These conditions were found to give good selectivity and sensitivity in a 10 min run.

The primary objectives in the development of the extraction method were to minimize interfering endogenous sample components while at the same time providing high recoveries of the analytes. Liquid-liquid extraction and several protein precipitation techniques were evaluated but were not as effective as solid phase extraction (SPE) in the removal of endogenous sample components. During development of the solid-phase extraction method, a series of different extraction cartridges were investigated, such as C_{18}, C_8, phenyl, Oasis™ , and Absolute™ cartridges. The 1 cc Bond Elut C18 cartridge was found to give the highest recoveries while at the same time removing endogenous interferences. 95:5 *v/v* 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4) – methanol was used to wash the cartridges after loading spiked plasma to help retain the hydrophilic analytes. Cleaner extracts were observed using 5% methanol-buffer washes than buffer washes alone. An 80:20 *v/v* 25 mM sodium phosphate monobasic monohydrate and 0.1% trifluoroacetic acid (pH 2.4) – acetonitrile solution was strong enough to elute all of the analytes including the quinolones and leave most of the highly hydrophobic plasma interferences on the SPE cartridges. Good recoveries were obtained after the addition of 1 mL of spiked plasma by elution with only 0.5 mL of the SPE eluent. This resulted in a two-fold sample concentration and avoided the necessity of a long extract evaporation step.

Specificity

AZT and levofloxacin were well separated under the HPLC conditions applied. Retention times were 4.9 min. for AZT and 8.0 min. for levofloxacin. The internal standard (ciprofloxacin) was well resolved from levofloxacin with a retention time of 9.4 min. No interferences were observed in drug free human plasma samples. Figures 4.2 and 4.3 show chromatograms of a blank plasma sample and a calibration sample, respectively. Since AZT is often prescribed with other antiviral agents, the specificity was assessed with regard to several HIV drugs as well as drugs that may be used to treat opportunistic infections. Several non-prescription drugs were also evaluated as potential interferences. Table 4.1 shows the retention factors (k) of these drugs in order of ascending k value. As can be seen, none of the evaluated drugs interfere with AZT or levofloxacin. However, guafenesin was found to have a k very close to that of the internal standard. Therefore, this method would not be suitable for individuals who have recently used guafenesin.

Linearity

The calibration curves showed good linearity in the range of 26.3-2600 ng/mL for AZT and 51.2-5069 ng/mL for levofloxacin. The correlation coefficients (r) of calibration curves of each drug were higher than 0.996 as determined by least squares analysis. The test for lack of fit (α=0.05) indicated that the linear models are appropriate for establishing a relationship between the concentration and the response. No significant lack of fit was observed.

Benchtop stability

The benchtop stability assessment showed a relatively small change in the concentrations of both drugs with the greatest change being less than 5%. This indicates that an autosampler can be loaded with enough samples to span a 24 hr time period with very little change in sample composition between the beginning and the end of the run. The results of the benchtop stability experiments are presented in Table 4.2.

Precision and Accuracy

A summary of the accuracy and precision results is given in Table 4.3. The method

proved to be accurate (percent bias for all calibration samples varied from –6.2 to 5.6%) and precise (within-run precision ranged from 0.9 to 9.7% and between-run precision ranged from 1.3 to 7.5%). The acceptance criteria (within-run and between run %RSD's of <15% and an accuracy between 85 and 115%) were met in all cases.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD, as defined in the Experimental section, was 8.8 ng/mL for AZT and 25.0 ng/mL for levofloxacin. The lowest concentration of each calibration graph was 26.3 ng/mL for AZT and 51.2 ng/mL for levofloxacin, which was therefore the LOQ. LOD and LOQ data are shown in Table 4.4. Accuracy and precision data for the LOQ were also acceptable and are reported in Table 4.3.

Recovery

The results of the recovery experiments were satisfactory. The mean absolute recoveries were 94.1% for AZT, 91.2% for levofloxacin, and 84.7% for the internal standard.

Freezing and Thawing Stability

The mean of the measured concentrations after three freezing and thawing cycles were not significantly different from the data obtained in the precision and accuracy assessment. Also, the variances of the freezing and thawing data were not significantly different from equivalent levels of the precision and accuracy data. No significant deterioration was observed after three freezing and thawing cycles.

CONCLUSION

A method has been developed and validated for the determination of zidovudine (AZT) and levofloxacin in human plasma. The method combines a solid phase extraction procedure with a fast and sensitive isocratic reversed phase HPLC analysis with UV detection. The method is suitable for monitoring drug concentrations in human plasma and for pharmacokinetic studies.

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TABLE 4.1: HPLC Retention Data for Compounds Evaluated as Possible Interferents

Compound	Retention Factor, k
zalcitabine	0.25
lamivudine	0.38
theophylline	1.13
acetaminophen	1.22
caffeine	1.72
pseudoephedrine	1.92
zidovudine (AZT)	3.61
trimethoprim	4.57
levofloxacin	6.66
guafenesin	7.89
ciprofloxacin	7.96
acetylsalacylic acid	>9.38
ibuprofen	>9.38
indinavir	>9.38
naproxen	>9.38
nevirapine	>9.38
pyrimethamine	>9.38
saquinavir	>9.38
sulfamethoxazole	>9.38

TABLE 4.2: Benchtop Stability (24 hr) of AZT and Levofloxacin at Low and High Concentrations.

Drug	Nominal Concentration (ng/mL)	Area Ratio at t = 0 (ng/mL)	Area Ratio at t = 24 hr (ng/mL)	Concentration Change (%)
AZT	80.3	0.1066	0.1016	-4.8
	2062	2.350	2.292	-2.5
Levofloxacin	156	0.1102	0.1100	-0.2
	4019	2.728	2.654	-2.7

TABLE 4.3: Within-Run and Between-Run Accuracy and Precision for the Analysis of AZT and Levofloxacin in Human Plasma (n = 9)

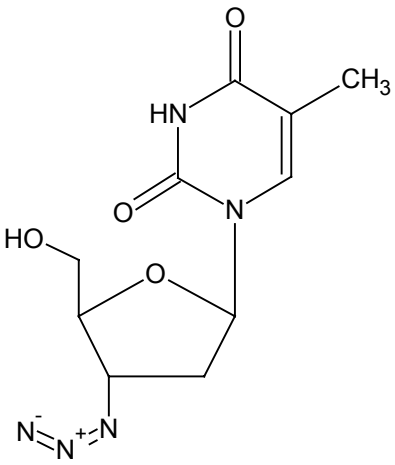
	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)	Within-run RSD (%)	Between-run RSD (%)
AZT	26.3	24.6	-6.2	7.5	7.5
	80.3	75.5	-6.0	6.6	7.1
	261	256	-1.9	2.5	1.3
	2062	2072	0.5	0.9	2.1
Levofloxacin	51.2	50.6	-1.2	9.7	3.1
	156	165	5.6	4.3	5.6
	509	520	2.2	2.0	5.3
	4019	4055	0.9	0.9	5.5

TABLE 4.4: Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantitation (LOQ) of AZT and Levofloxacin in Spiked Human Plasma.

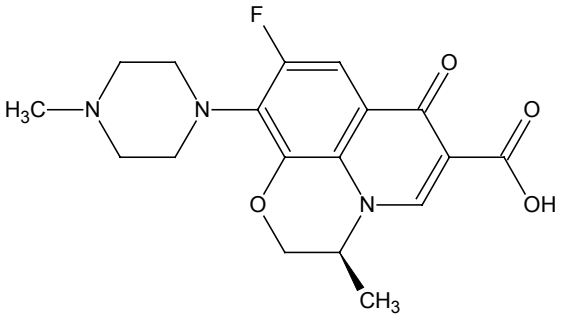
Drug	Range of calibration curves (ng/mL)	Limit of detection (LOD) (ng/mL)[a]	Limit of quantitation (LOQ) (ng/mL)[b]
AZT	26.3—2600	8.8	26.3
Levofloxacin	51.2—5069	25.0	51.2

a S/N=3
b S/N=10

Figure 4.1: The chemical structures of the analytes.



Zidovudine



Levofloxacin

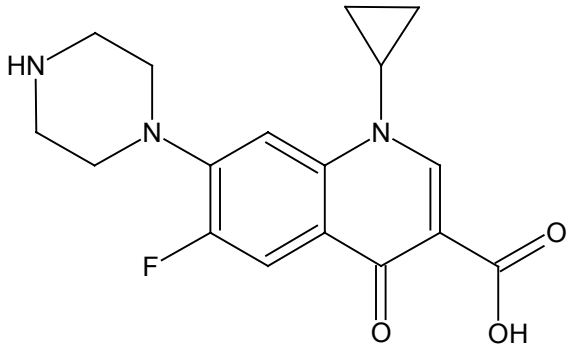


Figure 4.2: Chromatogram of blank pooled human plasma.

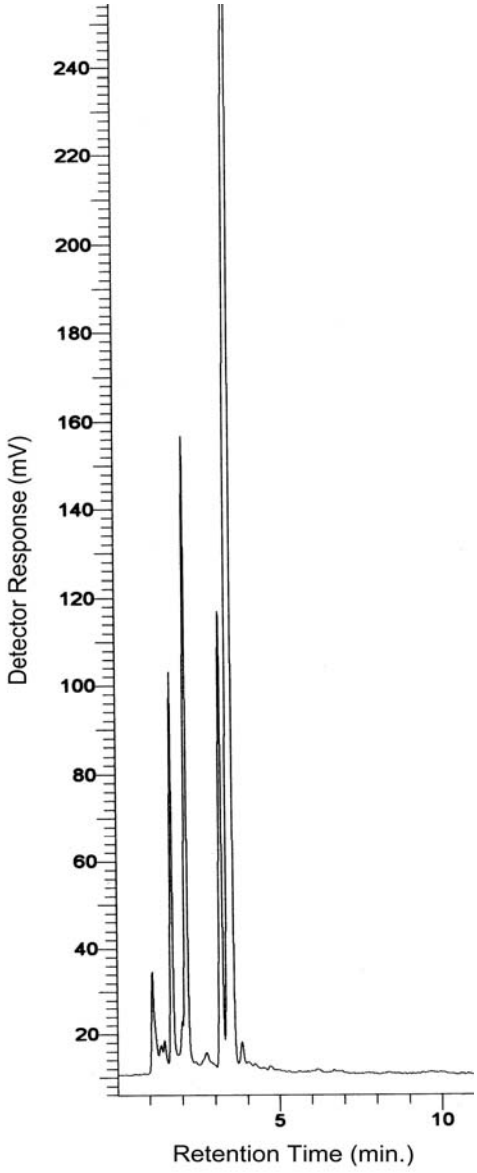
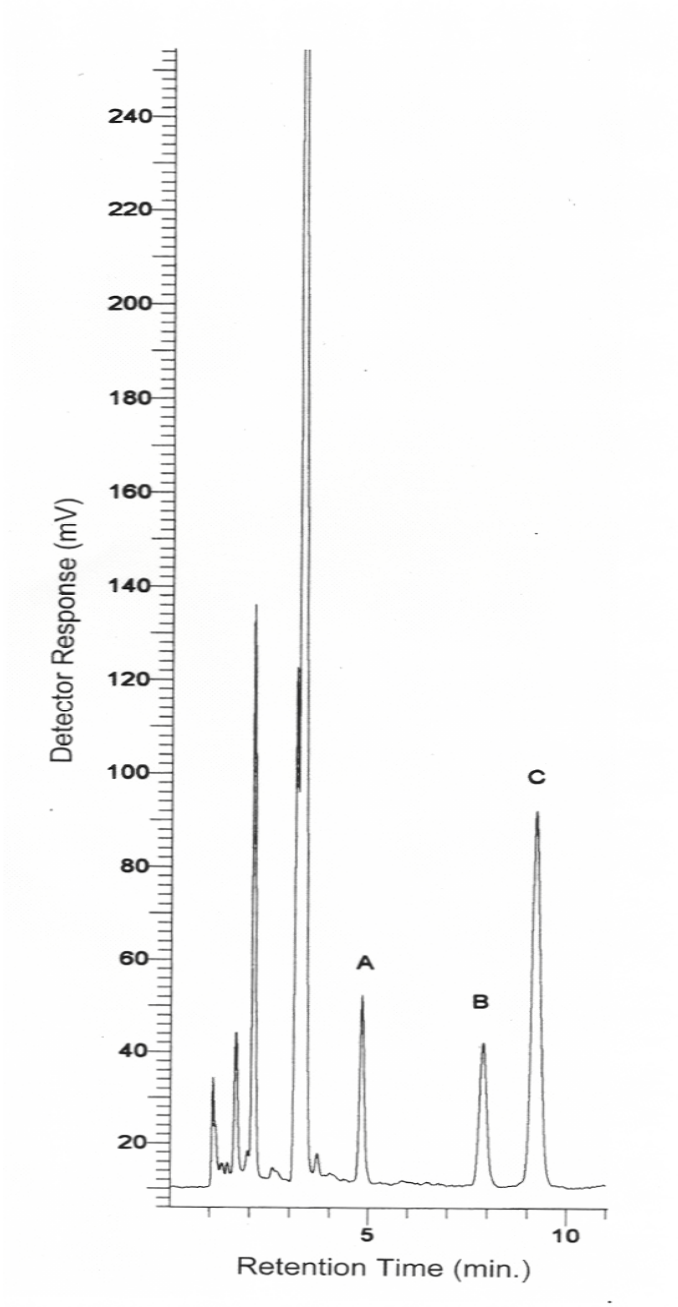

Detector Response (mV)
240
220
200
180
160
140
120
100
80
60
40
20
5
10
Retention Time (min.)

Figure 4.3: Chromatogram of pooled human plasma spiked with (A) 261 ng/mL AZT, (B) 509 ng/mL levofloxacin and (C) 540 ng/mL internal standard (ciprofloxacin).


Detector Response (mV)
240
220
200
180
160
140
120
100
80
60
40
20
A
B
C
5
10
Retention Time (min.)

CHAPTER 5

RAPID DETERMINATION OF SELECTED DRUGS OF ABUSE IN HUMAN PLASMA USING A MONOLITHIC SILICA HPLC COLUMN AND SOLID PHASE EXTRACTION[4]

[4]Caufield, W.V. and Stewart, J.T. To be submitted to Journal of Liquid Chromatography and Related Technologies.

CHAPTER 5

ABSTRACT

Two new high-performance liquid chromatography (HPLC) assays were developed which utilized a 100 x 4.6 mm ID monolithic silica column and binary mobile phase gradients for the simultaneous determination of selected drugs of abuse in human plasma. Both methods used gradients consisting of 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate– acetonitrile (pH 2.9) and mixed mode solid phase extraction procedures. In the first method, cocaine (COC) and its metabolites benzoylecgonine (BE), norcocaine (NC), and cocaethylene (CE) were separated with a pump flow rate of 5.0 mL/min in a total run time of five minutes. All analyses were conducted at ambient temperature. The injection volume was 100 µL and the UV detector was operated at 231 nm. The method was validated over the range of 50-5000 ng/mL for BE, COC and CE and 25-2500 ng/mL for NC. The method proved to be accurate (percent bias for all calibration samples varied from –4.5 to 8.5%) and precise (within-run precision ranged from 1.5 to 12.8% and between-run precision ranged from 0.4 to 12.7%). The mean absolute recoveries were 93.5, 95.7, 105.4, and 98.8% for BE, COC, NC and CE, respectively. In the second method, morphine (MO), hydromorphone (HM), tolazoline (ISTD), codeine (CO), oxycodone (OC), and hydrocodone (HC) were separated with a pump flow rate of 8.0 mL/min in a total run time of two minutes. All analyses were conducted at 30°C temperature. The injection volume was 100 µL and the UV detector was operated at 208 nm. The method was validated over the range of 50-5000 ng/mL for the opiates studied. The method proved to be accurate (percent bias for all calibration samples varied from –8.4 to 2.0%) and precise (within-run precision ranged from 1.7 to 16.9% and between-run precision ranged from 0.4 to 14.8%). The mean absolute recoveries were 95.6, 104, 103, 97.9, and 105% for MO, HM, CO, OC, and HC, respectively. The recovery for the internal standard was 99.6%. The assays should be suitable for use in routine determination of the selected drugs of abuse in human plasma.

INTRODUCTION

In 1998, there were estimated to be 1.8 million Americans age 12 and older who were chronic cocaine users. Although this represents a decrease from the 5.7 million users in 1985, the abuse of this addictive stimulant has become a persistent problem in the USA (1). The euphoria that is associated with cocaine (COC) use is caused by an inhibition of neuronal reuptake of biogenic amines in the central nervous system (CNS). In addition to its addictive properties, cocaine has been shown to be toxic to both the CNS and the cardiovascular system (2-3). Benzoylecgonine (BE), one of the major metabolites, is formed by de-esterification of cocaine. The human plasma elimination half lives of cocaine and benzoylecgonine are 30 to 90 min and 7.5 h, respectively (4). Therefore, benzoylecgonine is often the analyte of choice for detecting cocaine use. Norcocaine (NC), the only pharmacologically active metabolite, is formed by N-demethylation of cocaine. Cocaethylene (CE) is a neurologically active compound that is formed when COC is coadministered with ethanol. It provides the same degree of euphoria as COC but for longer periods of time and with more toxicity than COC alone (5). Co-administration of COC and ethanol is the most common two-drug combination that results in drug-related death(1).

In addition to the abuse of COC, an estimated 4 million people age 12 and over used prescription drugs for non-medical purposes in 1999 (6). The most commonly abused prescription drugs are opiates, CNS depressants and stimulants. Opium alkaloids are very potent analgesics that bind to opiate receptors in the brain, spinal cord and gastrointestinal tract and block the transmission of pain signals. Commonly abused opiates include heroin, morphine, hydromorphone, codeine, oxycodone, and hydrocodone. Heroin and codeine are both metabolized, in part, to morphine, although at different rates. Therefore, a ratio of the two drugs is often used to determine if the morphine found was due to the consumption of heroin or morphine itself. Morphine has an elimination half-life of 1.7 h and is largely metabolized to glucuronide conjugates. Hydromorphone is a synthetic derivative of morphine that has an elimination half-life of 2.5 h and is 7-10 times more potent than morphine. Oxycodone is a

derivative of codeine with an elimination half-life and potency similar to morphine. Hydrocodone is very similar to codeine and is converted in humans to hydromorphone (7).

The analysis for the possible presence of drugs of abuse is usually a two-stage process in which an initial screening test is followed by a confirmation test. This approach requires that the test methods be fast and inexpensive. Currently the preferred first stage screening method utilizes an enzyme immunoassay(8). Samples that test positive are further analyzed by GC/MS due to its high sensitivity and selectivity but the necessity of sample derivatization and the cost of the equipment itself restrict its applicability(9-10). High performance liquid chromatography (HPLC) can be used for the direct analysis of a wide spectrum of compounds and is not dependent on solute volatility or polarity. This technique is slowly gaining acceptance as a confirmatory method for the analysis of drugs of abuse. However, as the workload in toxicology and forensic laboratories increases, the need for faster HPLC methods has become an important issue. In the past, efforts to decrease analysis times have focused on the use of short columns with particles that are smaller than the standard 5μm. These columns offer good efficiency with higher flow rates but also have a tendency to "plug" and backpressures tend to be high(11). Recently, columns made of a single piece of monolithic silica were introduced as an alternative to particle-based columns. These columns possess a biporous structure consisting of larger macropores (2 μm) that permit high flow rates with low backpressure and smaller mesopores (13 nm) that provide a high surface area for high efficiency(12). Therefore, it is possible to perform analyses with high linear flow velocity but without significantly reduced separation efficiency. The utility of monolithic silica columns for high throughput bioanalysis in a drug discovery environment has been demonstrated(13). The columns have also been used to analyze metabolites(14) and natural products(15).

This paper describes the development and validation of two separate HPLC methods that are both rapid and sensitive for determining cocaine and three of its metabolites in human plasma

as well as five commonly abused opium alkaloids in human plasma. For sample pre-treatment, the methods utilize mixed mode solid phase extraction. Elution is performed by binary mobile phase gradients with 231 and 208 nm UV detection, respectively.

EXPERIMENTAL

Chemicals

Benzoylecgonine (BE), benzoylnorecgonine hydrochloride (BN), cocaine hydrochloride (COC), and cocaethylene fumarate (CE) were obtained from the National Institute of Drug Abuse (Bethesda, MD). Norcocaine (NC) and tolazoline hydrochloride were obtained from Sigma Chemical Co. (St Louis, MO, USA). Morphine sulfate pentahydrate (MO), hydromorphone hydrochloride (HM), codeine phosphate hemihydrate (CO), Oxycodone hydrochloride (OC), and hydrocodone bitartrate (HC) were obtained from the United States Pharmacopeial Convention Inc. (Rockville, MD, USA). Monobasic sodium phosphate, phosphoric acid, pentanesulfonic acid monohydrate sodium salt, HPLC grade methanol and acetonitrile were from J.T. Baker Inc. (Phillipsburg, NJ 08865). Trifluoroacetic acid was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI 53201). Water was purified by a cartridge system (Continental Water System, Roswell, GA, USA). Drug free human plasma was obtained from Bioreclamation Inc. (Hicksville, NY 11801).

Instrumentation

The chromatographic separation of COC and the selected metabolites was performed on a Model 1090 HPLC system (Hewlett Packard Co., Palo Alto, CA, USA). This system included a pump, an autosampler equipped with a 250 μL loop and a Model 117 variable wavelength UV detector (Gilson, Middleton, WI, USA). 0.010" ID tubing was used before and after the column and was kept at a minimum length. Turbochrom (Perkin Elmer, Norwalk, CT, USA) chromatography software was used for data integration. Separations were performed on a reversed phase monolithic silica column (Chromolith Performance RP-18e, 100 mm x 4.6 mm I.D., Merck KgaA, Darmstadt, Germany).

The chromatographic separation of the selected opiates was performed on an HPLC system that consisted of two Model 515 pumps and a Model 996 photodiode array detector with Millenium-32 chromatography software (Waters, Milford, MA, USA). Samples were injected in triplicate with a Model 728 autosampler (Alcott, Norcross, Georgia, USA) with a 100 µL loop. The monolithic column that was used on the Hewlett Packard system above was also used for the separation of the selected opiates.

Chromatographic Conditions for Analysis of COC and Selected Metabolites

COC and the selected metabolites were separated using a binary mobile phase gradient. Mobile Phase A consisted of 86:14 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate– acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Mobile Phase B consisted of 76:24 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate (pH 2.9) – acetonitrile. The gradient program was as follows: 0 – 2.7 min, linear change from 100% Mobile Phase A to 100% Mobile Phase B; 2.7 – 3.5 min, linear change from 100% Mobile Phase B to 100% Mobile Phase A; 3.5 – 5 minutes, equilibration at 100% Mobile Phase A. The HPLC pump flow rate was 5.0 mL/min and all analyses were conducted at ambient temperature. The injection volume was 100 µL and the UV detector was operated at 231 nm.

Chromatographic Conditions for Analysis of Selected Opiates

The five opiate drugs and internal standard were also separated using a binary mobile phase gradient. Mobile Phase A consisted of 95:5 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate– acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Mobile Phase B consisted of 80:20 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate (pH = 2.9) – acetonitrile. The gradient program was as follows: 0 – 1.4 min, linear change from 60% Mobile Phase A to 60% Mobile Phase B; 1.4 – 1.5 min, linear change from 60% Mobile Phase B to 60%

Mobile Phase A; 1.5 – 2 minutes, equilibration at 60% Mobile Phase A. The HPLC pump flow rate was 8.0 mL/min and the column was thermostated at 30º C. The injection volume was 100 µL and the diode array detector was operated at 208 nm. All mobile phase was filtered through a 0.45 µm nylon-66 filter (MSI, Westborough, MA, USA) and degassed in an ultrasonic bath for 15 min before use.

Preparation of Standard Stock Solutions

Standard stock solutions of COC, BE, NC and CE were prepared by dissolving appropriate amounts of each drug in deionized water to obtain final drug concentrations of 196, 90, 115, 51 and 102 µg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with pooled human plasma. The pooled human plasma was supplemented with sodium fluoride (NaF) to a final concentration of 0.064 M. to inhibit esterases thereby preventing hydrolysis of COC and NC.

Standard stock solutions of MO, HM, CO, OC, and HC were prepared by dissolving appropriate amounts of each drug in deionized water to obtain final drug concentrations of 5.30, 5.13, 5.36, 5.30 and 5.28 mg/mL, respectively. Working solutions were prepared by further diluting these stock solutions with pooled human plasma. The internal standard (tolazoline hydrochloride) stock solution was prepared by dissolving an appropriate amount of the drug in deionized water to obtain a final concentration of 208 µg/mL. A working internal standard solution was prepared by further diluting this stock solution with deionized water to yield a concentration of 2491 ng/750 µL.

Sample Preparation Procedure

Calibration standards and quality control samples were prepared by making appropriate dilutions of the working standard solution with pooled human plasma. The extraction methods used in these studies were based on the generally accepted mixed mode solid phase extraction protocols (16).

Extraction of COC and metabolites

Solid phase extraction (SPE) cartridges (Varian Inc., Bond Elut Certify, 3cc 130 mg) were placed on a vacuum elution manifold (Alltech, Deerfield, IL 60015) and rinsed with 3 mL of methanol followed by 3 mL of 100 mM sodium phosphate dibasic (pH 6.0). Care was taken that the cartridges did not run dry. 1.1 mL of each standard or sample was transferred to a 1.5 mL polypropylene microcentrifuge tube and centrifuged at 13,000 rpm for 15 min. Following centrifugation, 1 mL of each standard or sample was transferred to a 13 mm test tube and mixed with 3 mL of 100 mM sodium phosphate dibasic (pH 6.0). The entire spiked plasma samples were then transferred to the SPE cartridges. Vacuum was then applied to obtain a flow through the cartridges of 1-2 mL/min. The cartridges were then washed with 6 mL deionized water and 3 mL 1 M acetic acid. The cartridges were then dried under vacuum for 5 min. This was followed by a final rinse with 6 mL of methanol. The analytes were eluted from the cartridges with two 1 mL aliquots of methylene chloride/2-propanol/ammonium hydroxide (78:20:2 *v/v*). Extracts were dried under a stream of nitrogen in a 40º C water bath. The extracts were then reconstituted in 500 µL of Mobile Phase A, vortex mixed and 100 uL was injected onto the liquid chromatograph.

Extraction of Selected Opiates

SPE cartridges (Varian Inc., Bond Elut Certify, 3 cc 130 mg) were placed on a vacuum elution manifold (Alltech, Deerfield, IL 60015) and rinsed with 2 mL of methanol followed by 2 mL of 100 mM sodium phosphate dibasic (pH 8.5). Care was taken that the cartridges did not run dry. Each standard or sample (1.1 mL) was transferred to a 1.5 mL polypropylene microcentrifuge tube and centrifuged at 13,000 rpm for 15 min. Following centrifugation, 1 mL of each standard or sample was transferred to a 13mm test tube and mixed with 750 µL of internal standard and 2 mL of 100 mM sodium phosphate dibasic (pH 8.5). The entire spiked plasma samples were then transferred to the SPE cartridges. Vacuum was then applied to obtain a flow through the cartridges of 1-2 mL/min. The cartridges were then washed with 2 mL deionized water, 2 mL of 0.1 M sodium acetate buffer (pH 4.0) and 2 mL of methanol. The cartridges were

then dried under vacuum for 5 min. The analytes were eluted from the cartridges with two 1 mL aliquots of methanol/ammonium hydroxide (98:2 *v/v*). Extracts were dried using a vacuum centrifuge (SC110A SpeedVac Plus and RVT400 refrigerated vapor trap, Savant Inc., Farmingdale, NY). The extracts were then reconstituted in 750 µL of Mobile Phase A, vortex mixed and 100 uL was injected onto the liquid chromatograph.

Specificity

The specificity of the assay was checked by analyzing four independent blank human plasma samples. The chromatograms of these blank plasma samples were compared with chromatograms obtained by analyzing human plasma samples spiked with the analytes.

Linearity

Calibration plots for the analytes in plasma were prepared by diluting stock solutions with pooled human plasma to yield five concentrations over a range of 50-5000 ng/mL. For NC the range of the calibration standards was 25-2500 ng/mL. Calibration standards at each concentration were extracted and analyzed in triplicate. Calibration curves of COC and the selected metabolites were constructed using the observed analyte peak area versus nominal concentrations of the analytes. Calibration curves of the selected opiates were constructed using ratios of the observed analyte peak height to internal standard versus nominal concentrations of analyte. Weighted (W = 1/x) least squares linear regression analysis of the data gave slope, intercept and correlation coefficient data. From this data, a first order polynomial model was selected for each analyte.

Precision and Accuracy

The within-run and between-run accuracy and precision of the assays in plasma were determined by assaying four quality control samples in triplicate over a period of three days. The concentrations represented the entire range of the calibration curves. The lowest level was at the expected LOQ for each analyte (49.9, 44.6, 44.9, and 50.1 ng/mL for BE, COC, NC and CE and 103, 53.2, 53.2, 53.2, and 53.2 ng/mL for MO, HM, CO, OC, and HC respectively). The second level was within three times the LOQ (125, 112, 62.7, and 125 ng/mL for BE, COC, NC and CE and 153, 152, 152, 152, and 153 ng/mL for MO, HM, CO, OC, and HC respectively) and the third level was at the mid-point of the calibration curves (2501, 2233, 1252, and 2501 ng/mL for BE, COC, NC and CE and 2519, 2519, 2517, 2519, and 2519 ng/mL for MO, HM, CO, OC, and HC respectively). The fourth level was at 80 percent of the upper boundary of the calibration curves (4001, 3752, 2003, and 4000 ng/mL for BE, COC, NC and CE and 3978, 3978, 3976, 3977, and 3978 ng/mL for MO, HM, CO, OC, and HC respectively). Calibration curves were prepared and analyzed daily and linear models were used to determine concentrations in the quality control samples. The nine measured concentrations per concentration level (triplicates from three runs) were subjected to analysis of variance (ANOVA) to estimate the within-run and between run precision. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards. Precision was reported as percent relative standard deviation (% RSD).

Limit of Detection and Limit of Quantification

Decreasing concentrations of the analytes were prepared by diluting stock solutions with pooled human plasma, and then analyzed. The limit of detection (LOD) was defined as the concentration that yields a signal-to-noise ratio of 3. The limit of quantification (LOQ) was calculated to be the lowest analyte concentration in plasma that could be measured with a between run relative standard deviation (RSD) of <20% and an accuracy between 80 and 120%.

Recovery

The absolute recoveries of the analytes from plasma were assessed at two concentrations (within three times the LOQ and 80 percent of the upper boundary of the calibration curves). The recovery of the internal standard from plasma was assessed at the working concentration of 2491 ng/750 μL. For each level three samples were extracted and analyzed in triplicate. Three replicates of each concentration, prepared in the eluent, were directly injected. The assay absolute recovery for each compound at each concentration was computed using the following equation: absolute recovery = (peak area of extract)/(mean peak area of direct injection) x 100.

RESULTS AND DISCUSSION

Assay of COC and Selected Metabolites

The chemical structures for COC and the selected metabolites are shown in Figure 5.1. The goals in developing this method were low ng/mL sensitivity, a run time of less than five min and a simple extraction method that could be easily automated. Initial experiments utilized isocratic conditions with a 25 mM phosphate buffer (pH 2.9) – acetonitrile mobile phase. However, under these conditions it was not possible to achieve baseline separation of BE from the structurally similar benzoylnorecgonine (BN). The next approach was to improve the resolution by the addition of an ion pair reagent to the aqueous phase. Under acidic conditions, the basic amine group of COC and its metabolites is protonated and will therefore react with an alkylsulfonate in a cation exchange process. Several alkylsulfonates, including octane-, heptane-, and pentane-sulfonic acid sodium salt were tried with the latter giving the shortest run time with baseline resolution between BE and BN. The large difference in lipophilicity between BE, COC, and CE posed the greatest challenge in the development of the separation. The more hydrophilic BE tended to elute with endogenous substances in the plasma extract whereas COC and CE tended to elute much later in the run. Ordinarily, a gradient separation would not be desirable due to the additional time required for a particle based reversed phase column to re-equilibrate to the initial conditions. This is particularly so in the case of ion pair separations where gradient elution is usually not recommended. However, this was not an issue with the monolithic silica column

used in these studies. Due to the high degree of porosity of the monolithic column it was possible to re-equilibrate the column in 1.5 min at a flow rate of 5 mL/min. At this flow the system pressure was only 120 bar. Ultimately, it was possible to separate COC and the selected metabolites using a binary mobile phase gradient consisting of 86:14 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate– acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Trifluoroacetic acid was added to lower the mobile phase pH and to minimize the retention of endogenous sample components. The initial conditions were followed by a linear increase in the acetonitrile concentration to 24% *v/v* in 3.5 min. These conditions were found to give good selectivity and sensitivity in a 5 min run.

The solid phase extraction method utilized in these studies has been used extensively in the analysis of COC and metabolites in biological fluids (16-18). The method utilizes a copolymeric phase combining a C8 and a strong cation exchange phase to achieve a mixed mode separation mechanism. The 3 cc, 130 mg Bond Elut Certify cartridge was found to give high recoveries for COC and the metabolites in this study while at the same time removing endogenous interferences. The method sensitivity was improved by a factor of two by extracting 1 mL of plasma and reconstituting in 0.5 mL of mobile phase.

Specificity

The analytical figures of merit for this method are shown in Table 5.1. BE, COC, NC and CE were well separated under the HPLC conditions applied. Retention times were 0.9, 2.4, 2.6, and 3.1 min. for BE, COC, NC and CE, respectively. No interferences were observed in drug free human plasma samples. Figures 5.2 and 5.3 show chromatograms of a blank plasma sample and a calibration sample, respectively.

Linearity

The calibration curves showed good linearity in the range of 50-5000 ng/mL for BE, COC, and CE and 25-2500 ng/mL for NC. The correlation coefficients (r) of calibration curves of

each drug were higher than 0.99 as determined by least squares analysis.

Precision and Accuracy

A summary of the accuracy and precision results is given in Table 5.2. The method proved to be accurate (percent bias for all calibration samples varied from –4.5 to 8.5%) and \ precise (within-run precision ranged from 1.5 to 12.8% and between-run precision ranged from 0.4 to 12.7%). The acceptance criteria (within-run and between run %RSD's of <15% and an accuracy between 85 and 115%) were met in all cases.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD, as defined in the Experimental section, were 11.6, 16.9, 18.5, and 22.0 ng/mL for BE, COC, NC and CE, respectively. The LOQ of each calibration graph was 49.9, 44.6, 25.1, and 50.1 ng/mL . LOD and LOQ data are shown in Table 5.3. Accuracy and precision data for the LOQ were also acceptable and are reported in Table 5.2.

Recovery

The results of the recovery experiments were acceptable. The mean absolute recoveries were 93.5, 95.7, 105.4, and 98.8% for BE, COC, NC and CE, respectively.

Assay of Selected Opiates

The chemical structures for the selected opiates are shown in Figure 5.4. As in the previous method, the goals in developing this method were low ng/mL sensitivity, a run time of less than five min and a simple extraction method that could be easily automated. Since the opiates also contain basic tertiary amines, the initial experiments utilized the method parameters developed for the separation of COC and the selected metabolites (see above). However, under these conditions it was not possible to achieve baseline separation of MO from the structurally similar HM. However, by increasing the percentage of the aqueous phase to 89% v/v the two peaks were well resolved. The gradient conditions and flow rate were then modified to obtain good resolution of the other opiates in the shortest possible run time. Experiments were also conducted to evaluate the utility of replacing the mobile phase gradient with a flow gradient.

Although good resolution was achieved for all components, band broadening of the later eluting peaks resulted in decreased sensitivity. The final method used a binary gradient in which Mobile Phase A contained 95:5 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate– acetonitrile. The buffer pH was adjusted to 2.9 with trifluoroacetic acid prior to mixing with the acetonitrile. Mobile phase B contained 80:20 *v/v* 25 mM pentanesulfonic acid and 25 mM sodium phosphate monobasic monohydrate– acetonitrile. The gradient program was as follows: 0 – 1.4 min, linear change from 60% Mobile Phase A to 60% Mobile Phase B; 1.4 – 1.5 min, linear change from 60% Mobile Phase B to 60% Mobile Phase A; 1.5 – 2 minutes, equilibration at 60% Mobile Phase A. The flow rate throughout the gradient was 8 mL/min. At this flow the system pressure was 190 bar. These conditions were found to give good selectivity and sensitivity in a 2 min run.

As with the COC and metabolites method, a well-established solid phase extraction method was selected for the extraction of the opiates from plasma (16). The method again utilized the mixed mode Certify cartridge and yielded high recoveries for all of the opiates tested while at the same time removing endogenous interferences. The method sensitivity was improved by a factor of 1.3 by extracting 1 mL of plasma and reconstituting in 0.75 mL of mobile phase.

Specificity

The analytical figures of merit for this method are shown in Table 5.4. MO, HM, ISTD, CO, OC, and HC were well separated under the HPLC conditions applied. Retention times were 0.4, 0.5, 0.7, 0.8, 1.1, and 1.3 min. for MO, HM, ISTD, CO, OC, and HC, respectively. No interferences were observed in drug free human plasma samples. Figures 5.5 and 5.6 show chromatograms of a blank plasma sample and a calibration sample, respectively.

Linearity

The calibration curves showed good linearity in the range of 50-5000 ng/mL for all of the opiates studied. The correlation coefficients (r) of calibration curves of each drug were higher than 0.997 as determined by least squares analysis.

Precision and Accuracy

A summary of the accuracy and precision results is given in Table 5.5. The method proved to be accurate (percent bias for all calibration samples varied from –8.4 to 2.0%) and precise (within-run precision ranged from 1.7 to 16.9% and between-run precision ranged from 0.4 to 14.8%). The acceptance criteria (within-run and between run %RSD's of <15% and an accuracy between 85 and 115%) were met in all cases.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LODs, as defined in the Experimental section, were 38.6, 12.0, 8.7, 20.9, and 25.2 ng/mL for MO, HM, ISTD, CO, OC, and HC, respectively. The LOQ was 103 ng/mL for MO and 53.2 ng/mL for all of the other opiates studied. LOD and LOQ data are shown in Table 5.6. Accuracy and precision data for the LOQ were also acceptable and are reported in Table 5.5.

Recovery

The results of the recovery experiments were satisfactory. The mean absolute recoveries were 95.6, 104, 103, 97.9, and 105% for MO, HM, ISTD, CO, OC, and HC, respectively. The recovery for the internal standard was 99.6%.

CONCLUSION

High-speed methods have been developed and validated for the determination of cocaine and selected metabolites and five common opiates in human plasma. The methods utilize a new monolithic silica column technology, efficient solid phase extraction procedures and fast and sensitive gradient reversed phase HPLC analyses with UV detection. The methods are suitable for use in routine determinations of the selected drugs of abuse in human plasma.

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TABLE 5.1: Analytical Figures of Merit for Cocaine and Selected Metabolites

Analyte	k	*Tailing Factor*[a]	R_s	α
Benzoylecgonine	1.5	1.1	N/A	N/A
Cocaine	5.9	1.0	22.0	3.9
Norcocaine	6.5	1.0	2.6	1.1
Cocaethylene	8.0	1.0	6.7	1.2

[a] Calculated at 5% peak height

TABLE 5.2.: Within-Run and Between-Run Accuracy and Precision for the Analysis of Cocaine and Metabolites in Human Plasma (n = 15)

	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)	Within-run RSD (%)	Between-run RSD (%)	Recovery (%)
BE	49.9	50.1	0.4	4.1	1.1	N/A
	125	119.3	-4.5	5.5	1.7	89.5
	2501	2599	3.9	2.3	0.8	97.0
	4001	3983	-0.5	2.6	1.7	93.9
COC	44.6	44.4	-0.5	7.7	1.7	N/A
	112	120.3	7.9	7.4	6.7	93.2
	2233	2230	-0.2	3.3	1.1	95.2
	3752	3749	5.0	1.8	0.4	98.6
NC	25.1	25.4	1.1	12.8	5.3	N/A
	62.7	64.8	3.6	9.8	10.1	109.5
	1252	1224	-2.2	1.9	2.4	101.8
	2003	2173	8.5	2.8	2.5	105.0
CE	50.1	50.0	-0.1	10.0	1.3	N/A
	125	130	3.8	5.5	12.7	96.6
	2501	2494	-0.3	3.1	1.4	98.7
	4000	4137	3.4	1.5	2.5	101.1

TABLE 5.3: Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantitation (LOQ) of Cocaine and Metabolites in Spiked Human Plasma.

Drug	Range of calibration curves (ng/mL)	Limit of detection (LOD) (ng/mL)[a]	Limit of quantitation (LOQ) (ng/mL)
BE	49.9-4999	11.6	49.9
COC	44.6-4475	16.9	44.6
NC	44.9-4471	18.5	44.9
CE	50.1-4983	22.0	50.1

a S/N=3

TABLE 5.4: Analytical Figures of Merit for Selected Opiates

Analyte	*K*	*Tailing Factor*[a]	R_s	α
Morphine	1.3	1.0	N/A	N/A
Hydromorphone	2.0	1.1	1.6	1.5
INT STD	2.9	1.0	1.4	1.5
Codeine	3.6	1.0	2.8	1.2
Oxycodone	5.2	1.1	2.8	1.4
Hydrocodone	6.2	1.0	1.8	1.2

[a] Calculated at 5% peak height

TABLE 5.5: Within-Run and Between-Run Accuracy and Precision for the Analysis of Selected Opiates in Human Plasma (n = 15)

	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Bias (%)	Within-run RSD (%)	Between-run RSD (%)	Recovery (%)
MO	103	103	0.0	12.1	1.9	N/A
	153	145	-5.0	6.3	4.7	87.4
	2519	2502	-0.7	2.1	2.4	99.3
	3978	3962	-0.4	1.9	2.4	100
HM	53.2	52.5	-1.4	16.9	5.2	N/A
	152	146	-4.4	7.6	2.7	108
	2519	2491	-1.1	3.0	1.6	101
	3978	3936	-1.0	3.0	2.6	103
CO	53.2	53.4	0.4	12.4	0.7	N/A
	152	140	-8.4	5.3	14.8	112
	2517	2496	-0.9	1.7	2.0	97.9
	3976	3908	-1.7	2.0	1.3	100
OC	53.2	53.7	0.9	13.2	3.0	N/A
	152	151	-1.0	5.0	2.6	98.6
	2519	2487	-1.3	2.5	1.6	96.4
	3977	3891	-2.2	2.5	3.3	98.8
HC	53.2	54.3	2.0	10.6	1.5	N/A
	153	147	-4.4	2.6	4.9	113
	2519	2459	-2.4	2.5	0.4	100
	3978	3831	-3.7	2.4	1.5	103

TABLE 5.6: Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantitation (LOQ) of Selected Opiates in Spiked Human Plasma.

Drug	Range of calibration curves (ng/mL)	Limit of detection (LOD) (ng/mL)[a]	Limit of quantitation (LOQ) (ng/mL)
MO	103 – 5002	38.6	103
HM	53.2 – 5002	12.0	53.2
CO	53.2 – 4999	8.7	53.2
OC	53.2 – 5001	20.9	53.2
HC	53.2 – 5003	25.2	53.2

a S/N=3

Figure 5.1: The chemical structures of cocaine and the selected metabolites.

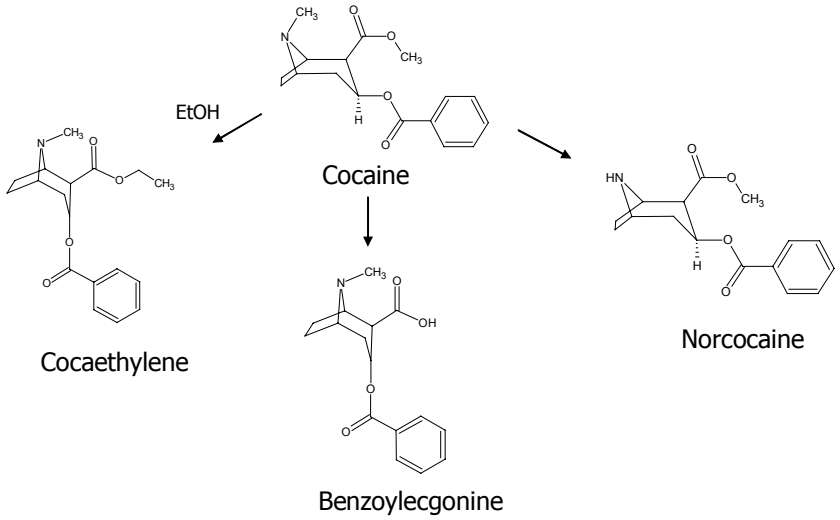

EtOH
Cocaine
Cocaethylene
Benzoylecgonine
Norcocaine

Figure 5.2: Chromatogram of blank pooled human plasma using the method for the analysis of cocaine and the selected metabolites.

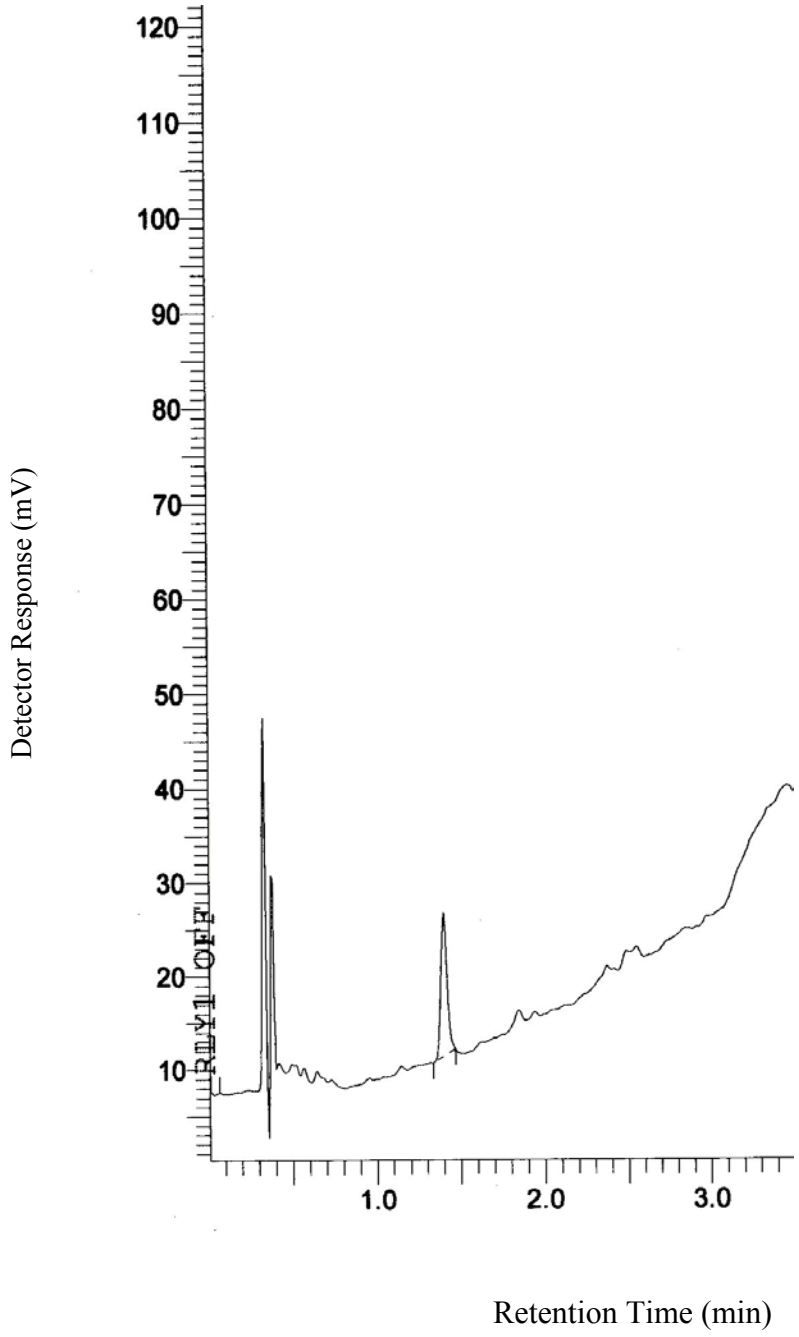

Detector Response (mV)
120
110
100
90
80
70
60
50
40
30
20
10
1.0
2.0
3.0
Retention Time (min)

Figure 5.3: Chromatogram of pooled human plasma spiked with 2500 ng/mL benzoylecgonine (BE), benzoylnorecgonine (BN), cocaine (COC), cocaethylene (CE), and 1250 ng/mL norcocaine

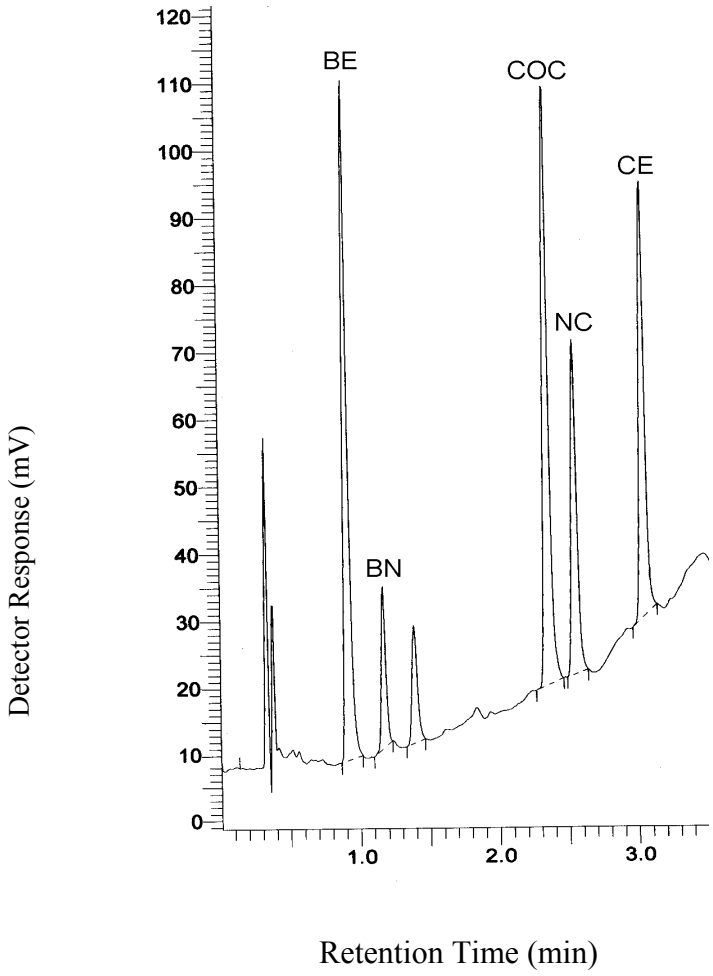
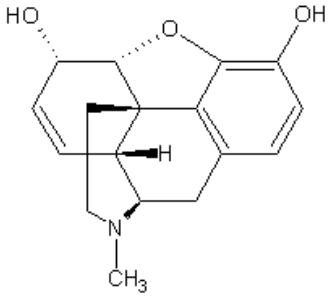
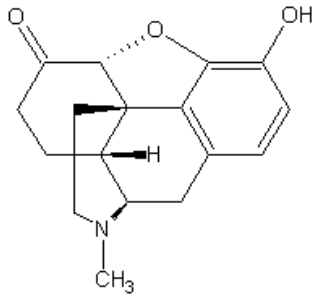

Detector Response (mV)
120
110
100
90
80
70
60
50
40
30
20
10
0
BE
COC
CE
NC
BN
1.0
2.0
3.0
Retention Time (min)

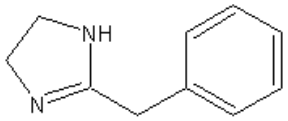
Figure 5.4: The chemical structures of the selected opiates.



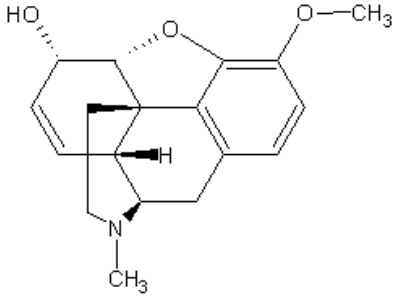
Morphine



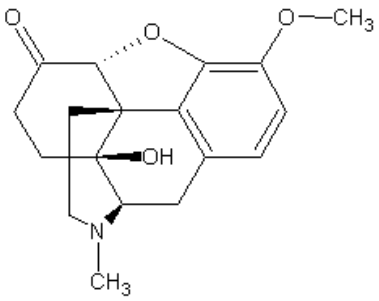
Hydromorphone



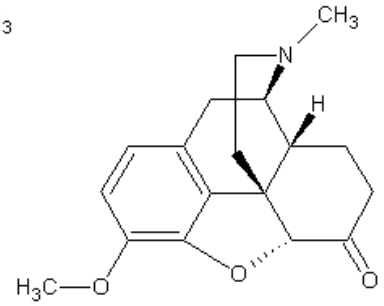
Tolazoline



Codeine



Oxycodone



Hydrocodone

Figure 5.5: Chromatogram of blank pooled human plasma using the method for the analysis of the selected opiates.

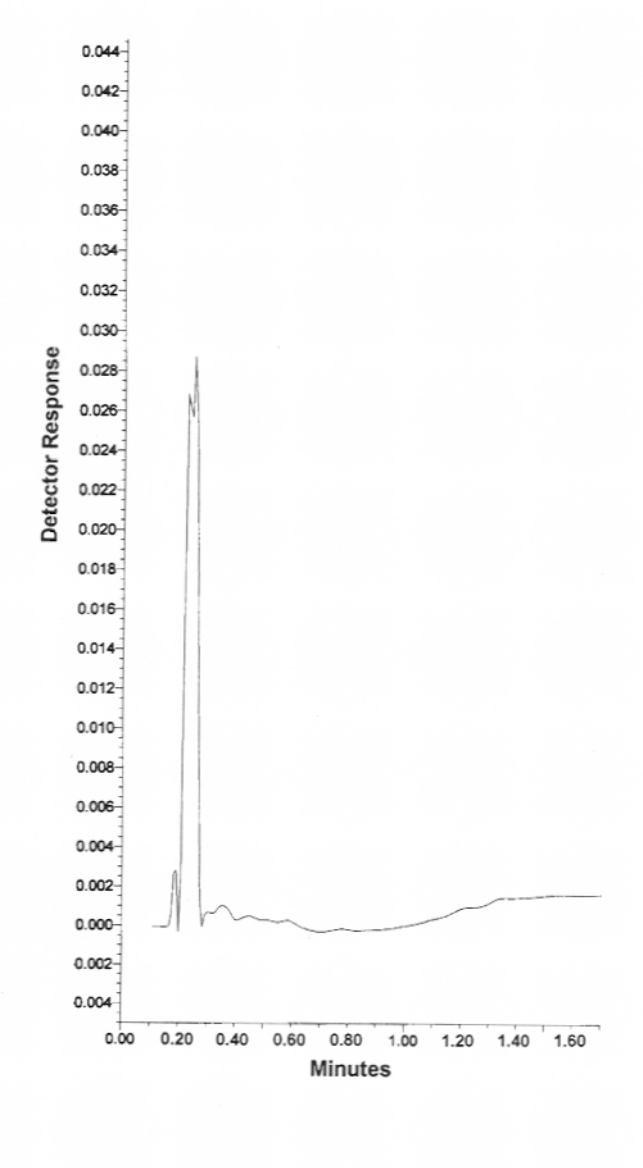
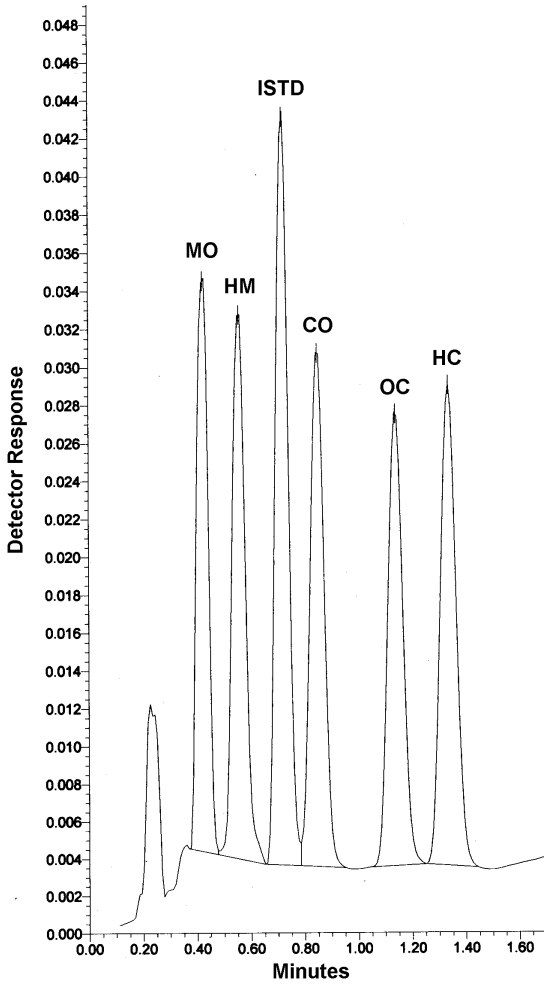

Detector Response
0.044
0.042
0.040
0.038
0.036
0.034
0.032
0.030
0.028
0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0.002
0.004
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
Minutes

Figure 5.6: Chromatogram of pooled human plasma spiked with 600 ng/mL morphine (MO), hydromorphone (HM), tolazoline (ISTD), codeine (CO), oxycodone (OC), andhydrocodone (HC).


ISTD
MO
HM
CO
OC
HC
Detector Response
Minutes
0.000
0.002
0.004
0.006
0.008
0.010
0.012
0.014
0.016
0.018
0.020
0.022
0.024
0.026
0.028
0.030
0.032
0.034
0.036
0.038
0.040
0.042
0.044
0.046
0.048
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60

CHAPTER 6

CONCLUSIONS

CONCLUSIONS

In Chapters 2 and 3, stability indicating HPLC methods were developed and validated for the determination of selected pharmaceuticals in parenteral solutions. Accelerated stability studies were conducted on the individual drugs and the purity of the chromatographic peaks was determined with a photodiode array detector and Waters Millennium data analysis software.

In Chapter 2 a narrow bore (2.0 mm I.D.), polar end-capped octadecylsilane (ODS) column was used to develop isocratic separations with aqueous (pH 3 with acetic acid) – acetonitrile eluents in the development of stability indicating methods for the determination of meropenem in combination with aminophylline, metoclopramide or ranitidine in intravenous fluid mixtures. Meropenem and dopamine mixtures were successfully separated on a conventional narrow-bore (2.1 mm I.D.) ODS column with aqueous (pH 3 with acetic acid) – acetonitrile eluent. The methods had run times that were ≤ 20 min. with reduced solvent usage and the columns showed good efficiencies for all analytes. The methods were found to be free from interferences from degradants and are suitable for the investigation of the chemical stability of the analytes in each of the mixtures.

In Chapter 3, an amide C16 column with 25 mM phosphate buffer (pH 3 with phosphoric acid) – acetonitrile eluents was shown to be suitable for the determination of zidovudine in combination with ceftazidime, chlordiazepoxide, dobutamine, lorazepam or ranitidine in intravenous fluid mixtures. The methods had run times that were ≤ 20 min. and the column showed good efficiencies for all analytes. The methods were found to be free from interferences from degradants and are suitable for the investigation of the chemical stability of the analytes in each of the mixtures.

In Chapters 4 and 5 bioanalytical HPLC and solid phase extraction methods were developed for the analysis of pharmaceuticals in human plasma. Each of the methods was validated with respect to linearity, accuracy, precision and limits of detection and quantitation.

In Chapter 4, a method was developed and validated for the determination of zidovudine (AZT) and levofloxacin in human plasma. The method combined a solid phase extraction procedure with a fast and sensitive isocratic reversed phase HPLC analysis with UV detection. The method would be suitable for monitoring drug concentrations in human plasma and for pharmacokinetic studies.

In Chapter 5, high-speed HPLC methods were developed and validated for the determination of cocaine and selected metabolites and five common opiates in human plasma. The methods utilized a new monolithic silica column technology, efficient solid phase extraction procedures and fast and sensitive gradient reversed phase HPLC analyses with UV detection. The analytical run times were 5 and 2 minutes for the cocaine/metabolites and opiates methods, respectively. The methods would be suitable for use in routine determinations of the selected drugs of abuse in human plasma.