BIN FAN
Part I. Pharmaceutical Analysis of the Prodrug Metronidazole Benzoate
Part II. Bioanalysis of Anti-HIV Drugs in Human Serum Using HPLC, CE, LC-MS-MS
(Under the direction of JAMES THOMAS STEWART)

The dissertation includes two parts: analytical methodology applied in formulation development and clinical healthcare. The focus of the first part was pharmaceutical analysis during new formulation development for the prodrug metronidazole benzoate. In the second part, bioanalytical techniques (HPLC, CE, and LC-MS-MS) were applied in the monitoring of anti HIV drugs in combination therapies for HIV infection.

A brief background about the interdisciplinary application of analytical methodology was introduced. Antiretroviral drugs approved by the FDA and triple cocktail therapies were discussed as well as the prodrug metronidazole benzoate.

In Chapters 1 and 2, two new dosage forms, an oil-in-water emulsion and a lozenge, were developed. A sensitive, stability-indicating HPLC assay was also developed and validated for the stability, degradation and dissolution studies of the prodrug metronidazole benzoate in these formulations. Waters Millennium^{32} PDA software and photodiode-array (PDA) detection were applied to determine the degradation products of metronidazole benzoate and their purity through library matching procedures and peak purity processing.

In Chapters 3 and 4, ion-pair chromatography (IPC) was applied to separate and quantitate the anti-HIV drugs zidovudine/lamivudine/nevirapine and zidovudine/ zalcitabine/nevirapine simultaneously in human plasma. Octane sulfonic acid sodium salt was chosen as the ion pair reagent.

In Chapters 5 and 6, gradient chromatography methods utilizing tandem mass spectrometry (LC-MS-MS) were developed and validated for the simultaneous measurement of the anti-HIV drugs lamivudine/stavudine /efavirenz and didanosine/stavudine/ritonavir in human serum. An ionization polarity switching technique was employed in the separation of lamivudine/stavudine /efavirenz. The effect of ion suppression from the matrix was studied.

In Chapters 7 and 8, capillary zone electrophoresis (CZE) was employed for the simultaneous determination of the anti-HIV drugs lamivudine/didanosine/saquinavir and lamivudine/didanosine/nevirapine in human serum. The effects of run buffer type, buffer concentration, and pH on the separation were investigated.

In Chapters 9 and 10, micellar electrokinetic chromatography (MEKC) methods were described in human serum for simultaneous determination of the anti-HIV drugs zidovudine/didanosine/nevirapine, zidovudine/didanosine/ritonavir, stavudine/ didanosine/saquinavir and stavudine/didanosine/efavirenz. Sodium dodecylsulfate (SDS) was used as the surfactant.

INDEX WORD: Pharmaceutical Analysis, Bioanalysis, Antiretroviral, HPLC, CZE, MEKC, LC-MS-MS, Photodiode-Array Detection, Prodrug, Human Serum, Ionization Polarity Switching, Ion Suppression.

PART I. PHARMACEUTICAL ANALYSIS OF THE PRODRUG METRONIDAZOLE BENZOATE

PART II. BIOANALYSIS OF ANTI-HIV DRUGS IN HUMAN SERUM USING HPLC, CE, LC-MS-MS

by

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DEDICATION

To my dear wife minmin and my parents for their love and support.

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I have enjoyed my time at UGA as well as the College of Pharmacy since I came to Georgia four years ago. However, the time has passed by so quickly and my educational journey at UGA is approaching an end with a Ph.D. degree. Many thanks are coming from the bottom of my heart to many people.

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INTRODUCTION AND LITERATURE REVIEW

During the drug development process, key decisions are based upon data obtained from analytical methods. Well-validated, specific, and sensitive analytical procedures are required to ensure accurate and reliable data at each stage of the development process [1]. It is important to realize that the results of any studies for a new drug depend on the sensitivity of the analytical methods employed. If the analytical methods do not provide accurate data for the drug, the results and derived conclusions will be questionable. Analytical methods are an essential component, and are used in a variety of laboratory settings during all phases of the drug development process, from the initial preclinical phase to the final clinical trials.

1. Interdiscipline Use of Analytical Methodology

During drug development, a high priority item is to establish sensitive and robust analytical procedures. Fundamentally, the role of the laboratory analyst in the development process is to provide data to establish the identity, potency, purity, and overall quality of the drug substance and the formulated drug product. Analytical methods used in development activities are typically used in specialized laboratories beyond the central analytical development group [2]. Figure 1 displays typical recipients or end-users of the validated methods. Throughout clinical, pharmacokinetic, toxicology,

regulatory, chemical, and pharmaceutical development groups, analytical methodology serves to support development activities in a variety of roles and functional areas.

Depending on the stage of development, analytical methods are standard screening tools at the start of the development process, which over time are gradually updated to validate methods for new drug applications (NDA) and or international (European) registration file (IRF) submissions. The uses and requirements of analytical methodology during various stages of the drug development process include: 1. Formulation development, 2. Pharmacokinetic and clinical studies.

Analytical testing is a fundamental component of the pharmaceutical formulation development effort. Pharmaceuticals are rarely administered into the body as the active pharmaceutical ingredient alone. Instead, they are formulated with inactive ingredients or excipients into an effective and convenient dosage form. Even before development activities can proceed, the active ingredient must be thoroughly characterized. Then, formulation efforts can begin to improve on characteristics intrinsic to the active ingredient. The susceptibility of the active ingredient and the degree of improvement rendered in the formulation must be accurately measured by suitable methodology on qualified instrumentation. Stability tests provide evidence on how the quality of an active ingredient or drug product varies with time under the influence of a variety of environmental factors and enables recommended storage conditions and shelf lives to be established [3,4]. Dissolution tests are designed to monitor the rate at which solid, semisolid, suspensions and other dosage forms release the active drug substance(s) into a uniform liquid medium at a controlled temperature under standardized conditions at the

liquid-solid interface. The detection of the active ingredient in the dissolution medium can be accomplished by many varied analytical techniques [5].

Bioanalysis is the application of analytical techniques to determine drug concentration in biological samples, mostly plasma, serum, or urine samples [6]. Bioanalysis plays a key role in drug development from discovery to drug approval, which enables pharmacokinetics to be a guiding force in nonclinical and clinical development of drugs and biologics. Application of bioanalytical methods and pharmacokinetics has led to a shorter drug development timeline and rational drug therapies. The plasma concentrations can be used to assess compliance and to adjust the dose for concomitant use of interacting drugs. Monitoring of blood drug concentrations during therapies has significantly reduced drug toxicity and improved treatment outcomes and, hence, patients' quality of life [7,8]. Therapeutic drug monitoring has been made possible by the development of highly selective and sensitive analytical techniques for a quick determination of plasma levels.

2. Analytical Techniques and Instrumentation

The rapid growth and advancement of the pharmaceutical sciences have led to a critical demand for fast and accurate analytical methods. Chromatographic and electrophoretic methods are two of the most popular analytical techniques. Separation techniques such as high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and gas chromatography (GC) play an important role in drug discovery and development [9,10]. Mass spectrometry has become more and more popular, especially with the development of new ionization techniques. Over the past

decade, high-performance liquid chromatography-mass spectrometry (HPLC-MS) has become an important routine technique in analytical laboratories, particularly in the pharmaceutical and biotechnology industries.

High-Performance Liquid Chromatography (HPLC)

Chromatography is the most frequently used analytical technique in pharmaceutical analysis. It is a physical separation method in which the components to be separated are selectively distributed between two immiscible phases: a mobile phase flowing through a stationary phase bed. The technique is classified according to the physical state of the mobile phase: gas chromatography (GC), liquid chromatography (LC), or supercritical fluid chromatography (SFC).

High-performance liquid chromatography (HPLC) is the most popular chromatographic technique in the pharmaceutical laboratory [11]. Reverse phase is the most popular mode of HPLC because of its versatility and ability to handle polar analytes. Many compounds of pharmaceutical interest are polar and ionogenic, which have often presented special problems in chromatographic separation. Ion pair chromatography (IPC) is a preferred HPLC technique that takes advantage of a charged analyte. IPC can be performed with existing reversed-phase columns and equipment. Once the ion pair reagent has been added to the mobile phase, it is absorbed to the stationary phase of the column because of its hydrophobic alkyl group. Once the analyte is introduced, the analyte complexes with the oppositely charged ion pair reagent, forming a neutral complex, which results in retention of the analyte.

HPLC with gradient elution is a powerful tool for achieving successful separation of a wide variety of complex samples. In gradient elution, the composition of the mobile

phase changes during the separation. This technique has been applied to improve resolution and shorten retention time.

Mass Spectrometry

Mass spectrometry is a universal spectrometric technique for separating molecular ions according to their mass-to-charge ratio (m/z). It is extremely selective, sensitive, and has the potential to yield information about chemical structure of the analyte. A mass spectrometer consists of five parts: sample introduction, ionization source, mass analyzer, ion detection and data handling [12]. The ionization of analytes can be performed in a number of ways [13,14], including electron impact (EI), chemical ionization (CI), plasma desorption (PD), field desorption (FD), fast atom bombardment (FAB), matrix-assisted laser desorption-ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Five major types of mass analyzers are current available [15]: magnetic sector, quadrupole, ion trap, Fourier-transform ion-cyclotron resonance and time-of-flight.

Electrospray ionization (ESI) is the technique most amenable to coupling with chromatographic inlets such as HPLC [16]. It is capable of producing intact ions, with one or more charges, from remarkably large, complex and fragile parent species. The process of electrospray ionization includes both the nebulization of a liquid into an aerosol of highly charged droplets and the ionization of solvated analyte species after desolvation of the charged droplets (see Figure 2). The electrospray process is initiated by applying a high electrical potential of several kV to the effluent, which flows through the tip of a hollow needle. A cone-shaped meniscus is produced from which a spray of highly charged droplets emerges, with subsequent evaporation of the droplets resulting in the

formation of ions which can be measured through the use of a variety of detection systems such as a quadrupole. In ESI, ions may exist in solution as protonated molecules or adducts in the positive-ion mode or deprotonated molecules in the negative-ion mode.

Quadrupole mass analyzer is the most commonly applied mass analyzer [17]. It uses a combination of direct current (DC) and radio frequency (RF) field as a mass filter. A quadrupole consists of four cylindrical rods in parallel. Opposite rods are connected in pairs electrically, and attached to RF and DC sources. Ions are introduced into the quadrupole field by means of a low accelerating potential. The ions start to oscillate in a plane perpendicular to the rod length as they traverse through the quadrupole filter. At a particular magnitude and frequency of the electric field produced by the quadrupole rods, ions of a selected mass-to-charge ratio are passed through to the detector. Those ions with other mass-to-charge ratios strike the rods at some point and are not passed.

A triple quadrupole mass spectrometer is an extension of the single quadrupole. It is the most versatile and the most widely used tandem mass spectrometer. The application of tandem mass spectrometry is to provide further information of a more specific nature about a sample by generating and mass analyzing fragment ions from the sample-related ions created in the ionization source [18,19]. A diagram of a typical triple quadrupole mass analyzer can be seen in Figure 3. In the triple quadrupole mass spectrometer, the first and third quadrupoles (MS1 and MS2) are operated for mass analysis. The second quadrupole is used as the collision cell in the RF-only mode. In a typical MS-MS experiment, a precursor ion with a particular m/z value is selected in MS1, then is passed into the collision cell for collision with an inert gas (usually argon or xenon) to induce fragmentation. The fragment ions produced are passed to MS2 for detection. Hyphenated

with LC, MS-MS has also used in the quantitation of complex mixtures due to high sensitivity, selectivity and throughout. It is this selective nature that has made triple quadrupole mass spectrometry very popular detectors for LC-MS-MS methodologies.

Multiple reaction monitoring (MRM) is the most efficient scan mode in tandem mass spectrometry. It is highly specific and sensitive due to the great reduction in background chemical noise and hence a significant improvement in signal-to-noise ratio [20]. Before MRM mode can be used for quantitation, flow injection is need to study collision-induced dissociation patterns, identify the most intense fragment ion, and obtain the optimized collision energy and other instrumental parameters. The optimized parameters are used in MRM for quantitation. In the MRM mode, MS1 is set to transmit only the parent mass of interest and the parent ions are fragmented in the collision cell. In MS2, only selected fragment ions (from flow injection experiments) will be transmitted. The high selectivity of MRM is due to both quadrupoles monitoring single ions. No time is wasted collecting data over parts of the mass range where there is no relevant information. Quantitative results can be obtained for two or more components as long as they do not have exactly the same retention time from the LC separation, same m/z values, and produce exactly the same fragment ions.

Liquid Chromatograph-Mass Spectrometry (LC-MS)

The combination of liquid chromatography with mass spectrometry (LC-MS) offers the advantage of both LC as a high-resolution separation technique and MS as a powerful and sensitive detection and identification technique. The ability to monitor the mass attributed to the elution of a species in liquid chromatography allows for identification of the eluted peak according to its mass number [21,22]. The most popular

tool for LC-MS (and LC-MS-MS) is electrospray ionization (ESI) mass spectrometry coupled with a triple quadrupole mass spectrometer. The most important application area of LC-MS is in the pharmaceutical field, where LC-MS is involved in almost every step of drug development, testing and formulation. Because of its specificity and sensitivity, LC-MS, especially in combination with MS-MS, has rapidly become the technique of choice in quantitative bioanalysis.

Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) is a powerful new separation technique that is well suited to the analysis of small molecules, especially pharmaceutical compounds. There has been a substantial increase in the number of capillary electrophoresis (CE) applications in the pharmaceutical industry over the last several years [23,24]. CE represents an attractive alternative to HPLC, and is considered to be a significant complementary separation technique to liquid chromatography and gas chromatography. This technique appears well suited for specific tasks, such as the analysis of complex biological matrices, monitoring of drugs and assaying binding capacity to proteins. The advantages of CE include higher resolution, reduced analysis time, and lower operation cost than high-performance liquid chromatography (HPLC) or conventional gel electrophoresis [25,26]. Minimum sample consumption can be quite beneficial for bioanalytical applications especially in the *in vivo* monitoring of biological fluids.

A schematic diagram of a typical CE system is shown in Figure 4. The basic components of a capillary electrophoresis system are a high-voltage power supply, two buffer reservoirs, a fused-silica capillary and a detector. Several different modes of capillary electrophoresis separation can be performed using a standard CE instrument. In

general, it can be considered as the electrophoretic separation of a number of analytes inside a capillary. The most often used modes in CE are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF) and capillary isotachoelectrophoresis (CITP).

Capillary zone electrophoresis (CZE) is the most commonly used technique in CE. Many charged compounds can be separated rapidly and easily. The selectivity in CZE is based on the differences in electrophoretic mobilities of individual analytes, which depends on the charge-to-size ratio in the electrophoretic buffer contained in the narrow bore (25-100 μm) capillary. CZE is a very effective means of separation of ionic species, both anions and cations, but it can not discriminate between neutral molecules.

Micellar electrokinetic chromatography (MEKC) provides a way to resolve neutral molecules as well as charged molecules, and is the only CE technique that can be used to separate neutral analytes [27]. The principal separation mechanism is based on analytes partitioning between a micellar phase and a solution phase. Micelles are formed in the run buffer when ionic surfactants are added above their critical micelle concentration (CMC). The micelles, which have hydrophobic interiors and ionic exteriors, serve as a pseudostationary phase. Neutral molecules distribute themselves between the buffer and micelles based on the hydrophobicity, and the amount of time spent in the micelles provides a separation.

3. Pharmaceutical Analysis of the Prodrug Metronidazole Benzoate

Drug substances are seldom administrated alone, but rather as part of a formulation with one or more nonmedical agents that serve varied and specialized pharmaceutical functions. Formulation development is a balance of improving the inherent properties of the active ingredient by enhancing stability, improving solubility, simplifying manufacture, and improving bioavailability without imparting negative characteristics or designing too complex a formulation. The majority of applications of HPLC in pharmaceutical analysis involve quantitative determination of drugs in formulations. The potential interferences in analysis of a formulation are excipients and degradation products of the formulated drugs. Analyses of formulations are not quite as simple, but compared to analysis of drugs in biological fluids or elucidation of complex drug degradation pathways, they present fewer difficulties.

Taste masking is a common used technique in pharmaceutical formulation to avoid the unpleasant taste of some drugs. The positive or negative sensation is produced when the drug molecule interacts with the taste receptor, which requires the drug to be sufficiently dissolved in saliva. A taste problem can be masked by lowering the aqueous solubility with prodrugs in addition to the use of flavors or sweeteners.

As a drug in pure form with an extremely bitter taste, metronidazole has restricted application in veterinary medicine. Metronidazole benzoate (MB), an ester prodrug of metronidazole has been used as a suitable alternative because of its low aqueous solubility [28]. As a prodrug, metronidazole benzoate hydrolyzes very quickly to release therapeutic doses of metronidazole. No unhydrolyzed metronidazole benzoate was found in the serum [29].

With its poor aqueous solubility, metronidazole benzoate runs the risk of serious problems for formulation development and dissolution. Incomplete dissolution of MB in the gastrointestinal tract may result in poor absorption. Until the present, there have been no commercial formulations of MB available except for suspension dosage forms [30].

My first goal was to formulate two new MB dosage forms, a glycerinated gelatin candy-based lozenge and an oil-in-water emulsion. The stability and *in vitro* dissolution of MB in these formulations were studied. The *in vivo* bioavailability and bioequivalence were also planned for study in cats in order to find correlations between *in vitro* and *in vivo* studies. A sensitive, stability-indicating HPLC assay was developed and validated. Waters Millennium^{32} PDA software and photodiode-array (PDA) detection were applied to determine the degradation products of MB and their purity through library matching procedures and peak purity processing. Unfortunately, *in vivo* bioavailability and bioequivalence could not performed because technique problem.

HPLC assays have been reported for the determination of MB in suspension and liquid preparations [30-32]. However, few methods were stability-indicating. No method was available to simultaneously identify MB degradation products and to check for peak purity as well as measure MB concentrations. The assay developed in this lab also had broad linearity, and lower limit of detection than other reported methods.

4. Bioanalysis in AIDS Clinical Healthcare and Pharmacokinetic Studies

Bioanalytical methods and pharmacokinetics have wide application in drug therapies as well as drug development. Bioanalytical disciplines have contributed immensely to the field of routine therapeutic drug monitoring by providing rationale and

tools, especially in the management of antiretroviral drug therapies. As dose decisions are often made on the basis of analytical results, it is pertinent that sensitive, reproducible, and thoroughly validated analytical methods are used for drug monitoring applications. Therapeutic drug monitoring is useful in clinical practice for the routine measurement of drugs in blood (plasma or serum) or other biological fluids as an aid to improving drug therapy. There is growing evidence that therapeutic drug monitoring can ensure that HIV-infected patients have adequate blood levels or that their plasma or serum concentrations are not in the toxic range [33,34]. An optimal therapeutic effect without toxicity could be achieved in a variety of patients by monitoring the drug plasma or serum concentration versus dose data. The plasma or serum concentrations could also be used to assess compliance and to adjust the dose for concomitant use of interacting drugs.

Human immunodeficiency virus (HIV), the causative agent of the debilitating disease acquired immunodeficiency syndrome (AIDS) has accounted for millions of death worldwide. Antiretroviral therapy for HIV infection has achieved unprecedented advances since the availability of antiretroviral drugs has increased to 18 approved drugs in a very short period of time. The FDA approved anti-HIV drugs represent three novel classes including: nucleoside analog reverse transcriptase inhibitors (NRTIs), non-nucleoside analog reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) (see Figure 5).

Triple cocktail treatments, also known as highly active antiretroviral therapy (HAART) have proven the most effective approach to treat HIV disease [35-37]. This therapy is a three-drug regimen with at least two NRTIs and one highly active PIs or one NNRTIs. The key to its success lies in the drug combination's ability to disrupt HIV at

different stages in its replication. Reverse transcriptase inhibitors inhibit the reverse transcriptase enzyme of HIV, which is crucial in an early stage of HIV duplication [38]. NRTIs, also known as nucleoside analogues, are the cornerstone of the majority of the antiretroviral regimen. They are taken up by target cells and phosphorylated to triphosphate metabolites by cellular enzymes to produce active drugs [39], which competitively inhibit HIV reverse transcriptase. These drugs are incorporated into the DNA of the provirus, and result in an incomplete strand of DNA provirus and termination of the virus life cycle. NNRTIs inhibit HIV reverse transcriptase through a very different mechanism. By noncompetitive binding to the enzyme [40], these drugs disrupt the catalytic site and therefore block the RNA-dependent DNA polymerase activity [41]. Protease inhibitors block the activity of the HIV protease enzyme at the crucial step of protein cleavage near the end of the HIV replication process, and result in the production of virus particles with immature morphology that are unable to initiate new rounds of infection [42]. The sites of action of these drugs are shown in Figure 6. The differing mechanisms of action of these three classes of anti-HIV drugs suggested that they might complement each other's antiviral activity, as has been shown in clinical trials. The aggressive combination therapy not only increases antiviral suppression, but also prevents drug resistance and optimizes drug exposure.

Highly selective bioanalytical methodology is required for multiple antiretroviral drugs monitoring to achieve the optimum therapeutic effect and to minimize adverse reactions, especially in the triple cocktail combination therapies. The assay methods are the basis for the evaluation of clinical efficacy of these drugs.

Almost all anti-HIV drugs are non-volatile compounds containing several polar functional groups, a situation not well suited to analysis by gas chromatography (GC). Because of their polarity, derivatization is essential prior to their analysis by GC. The analysis of these compounds by liquid chromatography is generally preferred. Additionally, the high polarity of these antiviral drugs largely precludes the use of normal-phase system for their determination in biological fluids. The most commonly employed methods are reversed-phase systems. Liquid chromatography with detection by ultraviolet (UV) absorption is the method of choice for the routine determination of nucleosides in biological fluids. Fluorescence and mass spectrometry are other options in liquid chromatography for more specialized applications. The intense absorption of UV light arises from the heterocyclic ring systems of the nucleoside. Fluorescence has not been as widely used as UV absorbance for the detection of anti-HIV drugs in biological samples because the purine and pyrimidine ring systems are only significantly fluorescent in their protonated forms.

Analytical methods for analysis of the newly approved anti-HIV agents have been reported. Numerous analytical methods, such as high performance liquid chromatography (HPLC) with UV detection [43-45], mass spectrometry (MS) detection [46-48], capillary electrophoresis (CE) [49,50] and immunoassay [51,52] have been described to quantify these individual drugs in biological media. However, no methods have been reported for the simultaneous determination of antiretroviral drugs in triple combination therapies. Anti-HIV drugs used in cocktail combination therapy belong to three different classes, whose physiochemical properties are different and it is difficult to separate them under isocratic HPLC conditions.

My second goal was to apply bioanalytical techniques in drug monitoring of antiretroviral therapy for HIV-1 infection. Various bioanalytical methods have been developed and validated to monitor and manage anti-HIV drugs in triple cocktail combination therapies. An ion pair chromatography method offered a reliable solution to separate different kinds of anti-HIV drugs under isocratic conditions. The ion pair reagent was added to alter the chromatographic selectivity of the ionized drugs for the retention of hydrophilic NRTI drugs. Chromatography with gradient elution presented another efficient solution to separation. To avoid baseline drift with UV detection, mass spectrometry was coupled to gradient chromatography. The LC-MS-MS method developed has the advantage of high selective and sensitive sepecially with multiple reaction monitoring detection mode. As a complementary separation technique to chromatography, capillary electrophoresis overcomes many of the drawbacks of chromatography. The advantages are high efficiency and low sample size. The CE methods developed included capillary zone electrophoresis (CZE) for charged anti-HIV drugs and micellar electrokinetic chromatography (MEKC) for both charged and uncharged anti-HIV drugs.

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Figure 1. "Stakeholders" in analytical testing during drug development

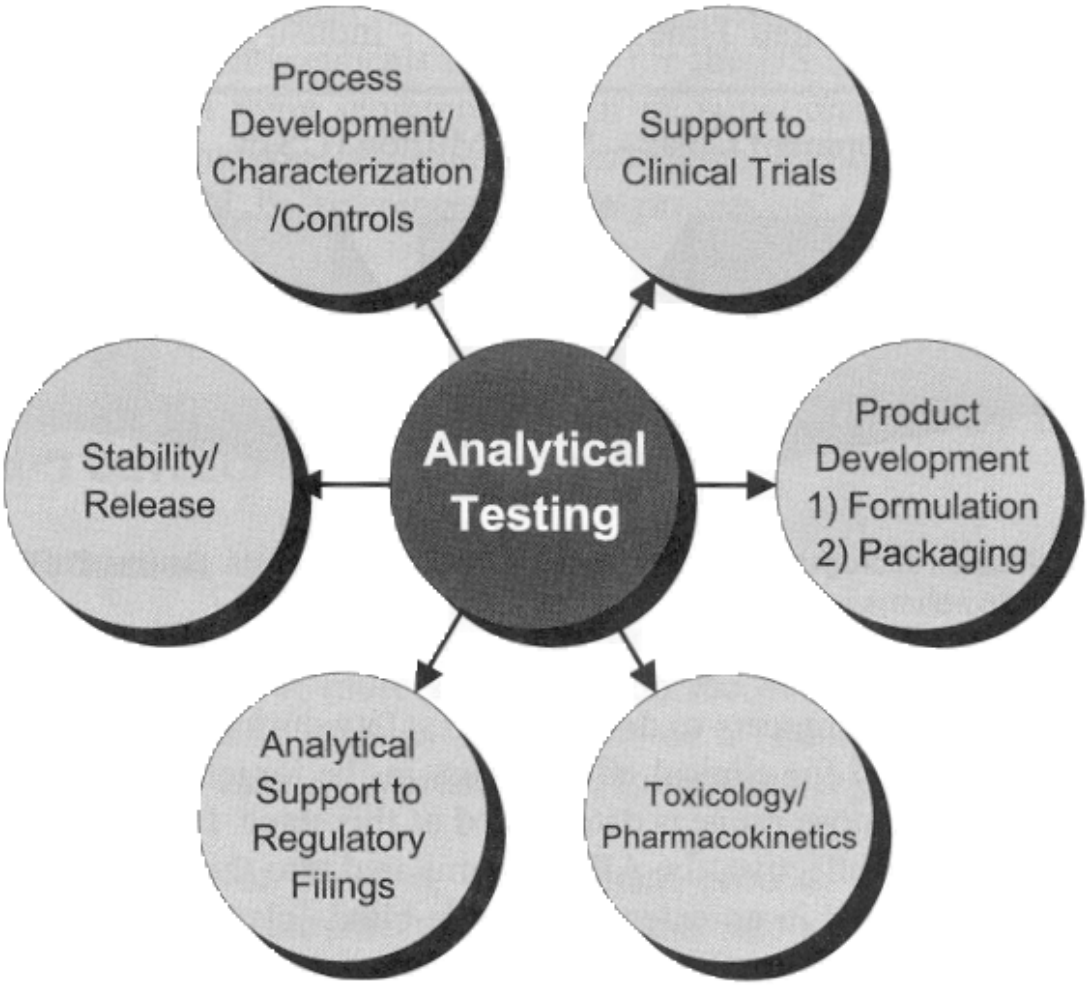

Process Development/ Characterization /Controls
Support to Clinical Trials
Stability/ Release
Analytical Testing
Product Development 1) Formulation 2) Packaging
Analytical Support to Regulatory Filings
Toxicology/ Pharmacokinetics

Figure 2. Schematic of the major process in electrospray ionization

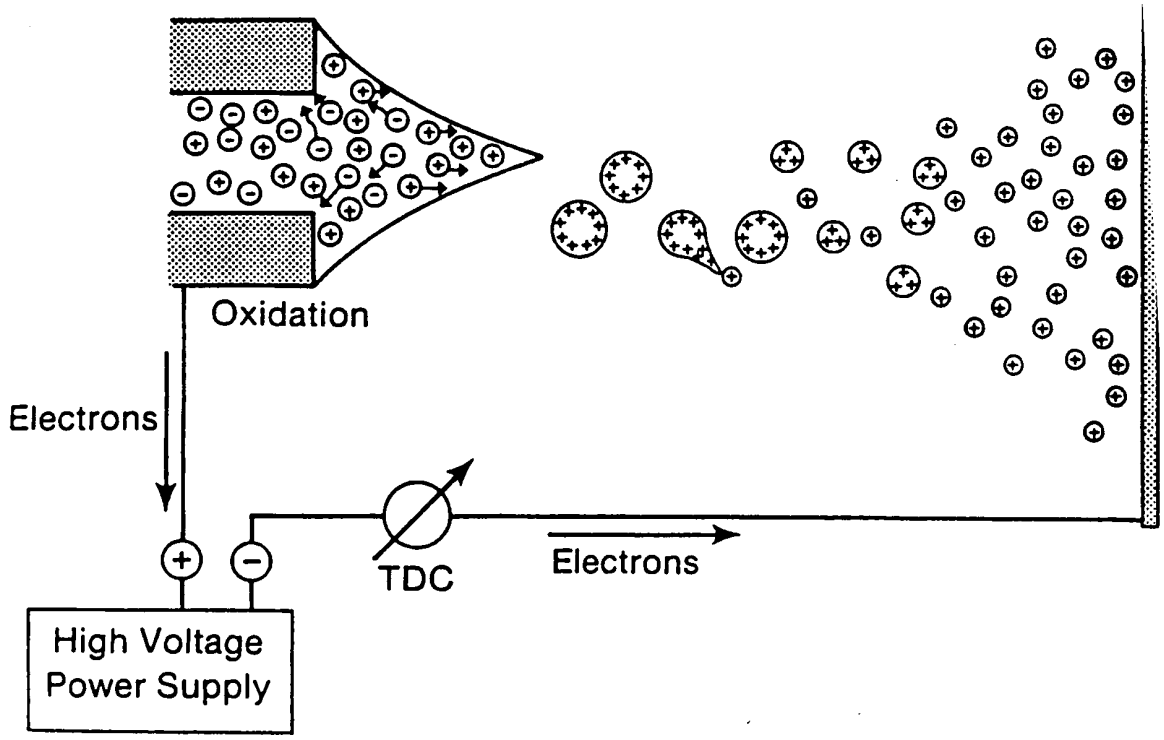

Oxidation
Electrons
TDC
Electrons
High Voltage
Power Supply

Figure 3. Diagram of a triple quadrupole mass spectrometry

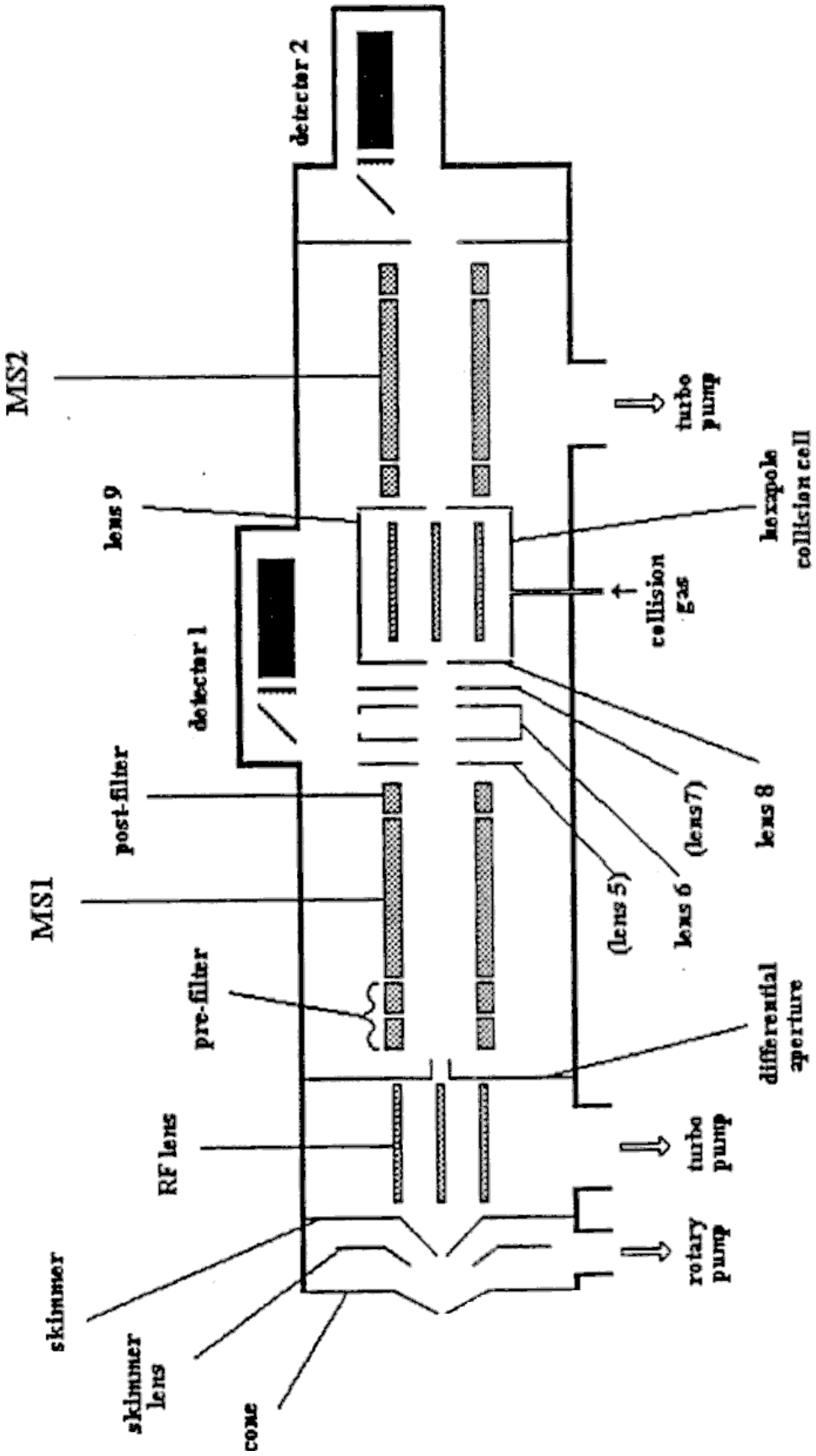

MS2
MS1
detector 2
detector 1
lens 9
post-filter
pre-filter
RF lens
skimmer
skimmer lens
cone
turbo pump
turbo pump
rotary pump
collision gas
hexapole collision cell
(lens 5)
lens 6
(lens 7)
lens 8
differential aperture

Figure 4. Basic schematic of a capillary electrophoresis (CE) instrument

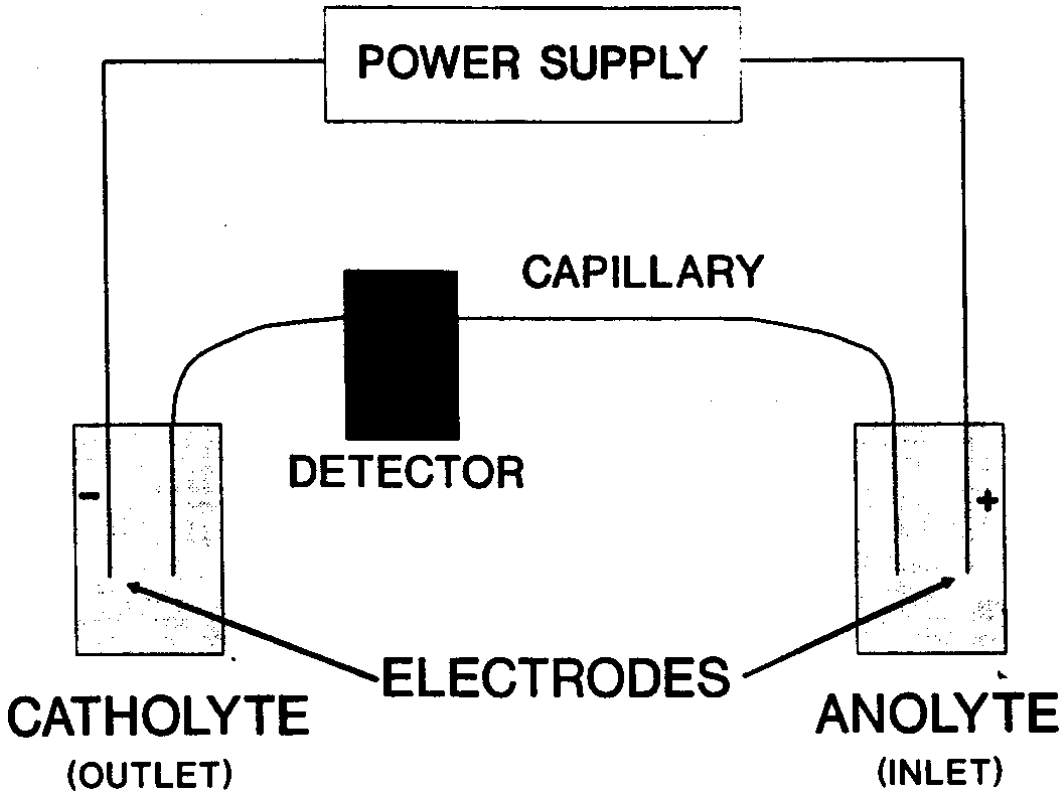

POWER SUPPLY
CAPILLARY
DETECTOR
-
+
ELECTRODES
CATHOLYTE
(OUTLET)
ANOLYTE
(INLET)

Figure 5. List of FDA approved anti-HIV drugs

Nucleoside Analogs

Zidovudine; AZT; Azidothymidine; Retrovir(R)

Didanosine; Dideoxyinosine; ddI; Videx(R)

Zalcitabine; Dideoxycytidine; ddC; Hivid(R)

Lamivudine; 3TC; Epivir(R)

Stavudine; 2',3'-Didehydro-3'-deoxythymidine; D4T; Zerit(R)

Abacavir Succinate; 1592U89 Succinate; Ziagen(R) ABC

Combivir(R); Lamivudine & Zidovudine; (-)-3TC & AZT

Trizivir(R); Abacavir & Lamivudine & Zidovudine; ABC & (-)-3TC & AZT

Non-Nucleoside Reverse Transcriptase Inhibitors; NNRTI's)

Nevirapine; BI-RG-587; Viramune(R)

Delavirdine; BHAP; U-90152; Rescriptor(R)

Efavirenz; DMP-266; Sustiva(R)

Protease Inhibitors

Saquinavir; Ro 31-8959; Fortovase(R); Invirase(R)

Indinavir; MK639; L-735,524; Crixivan(R)

Ritonavir; ABT-538; Norvir(R)

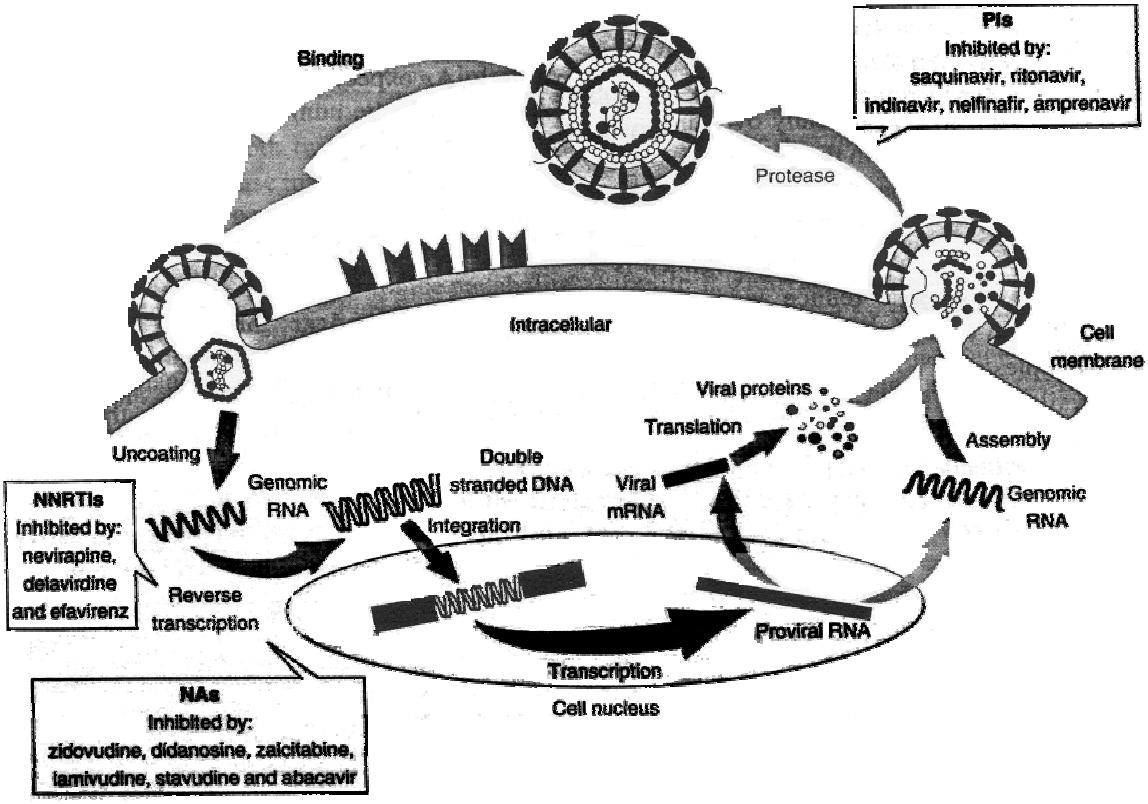
Nelfinavir; Viracept(R); AG-1343

Amprenavir; Agenerase(R); VX-478; 141W94

Lopinavir; ABT-378; Aluviran(R); Component of Kaletra

Kaletra(R); Lopinavir & Ritonavir; ABT-378 & ABT-538; Aluviran(R) & Norvir(R)

Figure 6. Sites of action of anti-HIV drugs. Nas = nucleoside analogues (nucleoside reverse transcriptase inhibitors); NNRTIs = non-nucleoside reverse transcriptase inhibitors, PIs = protease inhibitors


Binding
PIs
Inhibited by:
saquinavir, ritonavir,
indinavir, nelfinafir, amprenavir
Protease
Intracellular
Cell
membrane
Viral proteins
Translation
Assembly
Uncoating
Genomic
RNA
Double
stranded DNA
Viral
mRNA
Genomic
RNA
NNRTIs
Inhibited by:
nevirapine,
delavirdine
and efavirenz
Integration
Reverse
transcription
Proviral RNA
Transcription
Cell nucleus
NAs
Inhibited by:
zidovudine, didanosine, zalcitabine,
lamivudine, stavudine and abacavir

PART I

PHARMACEUTICAL ANALYSIS OF THE PRODRUG METRONIDAZOLE BENZOATE

CHAPTER 1

STABILITY AND DISSOLUTION OF LOZENGE AND EMULSION FORMULATIONS OF METRONIDAZOLE BENZOATE (MB)[1]

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[1]Fan, Bin. James T. Stewart and Catherine A. White. 2001. International Journal of Pharmaceutical Compounding. 5(2):153-156.

ABSTRACT

Chicken flavored glycerinated gelatin candy-based lozenges and oil-in-water emulsions of metronidazole benzoate (MB) were prepared for veterinary use. Their stability and dissolution were investigated using an HPLC assay. The separation and quantitation of MB were achieved on a Phenomenex IB-SIL 5 C_8 column (250 x 4.6 mm, id.) at ambient temperature with a 55:45 v/v pH 7.0 phosphate buffer-acetonitrile mobile phase at a flow rate of 1.0 ml/min. Tinidazole was used as the internal standard. A MB sample from the lozenge and emulsion was prepared for assay by dissolving one lozenge or 1 g of the emulsion in 50:50 v/v methanol-water solution, and filtered through a 0.2 μm membrane filter prior to assay. The chromatogram was monitored with UV detection at 230 nm. The HPLC separation of MB was achieved in less than 10 min with sensitivity in the 10 ng/ml range. The method is capable of separating the MB degradation products metronidazole and benzoic acid and showed linearity for MB in the 0.01-100 μg/ml ranges. Accuracy and precision were <1.0 % and <0.54 %, respectively. The limit of quantitation was 10 ng/ml, and the limit of detection was 0.01 ng/ml based on a signal to noise ratio of 3 and a 20 μl injection. The recoveries of MB from lozenge and emulsion were 98 ± 4 % and 99 ± 2 %, respectively. Stability testing of the new formulations was performed at both ambient temperature and 4 °C. Dissolution testing used the USP paddle method at 37 º C and 100 rpm in simulated gastric fluid without pepsin. Both formulations were stable at ambient temperature and 4 °C. MB is completely released from the lozenge and emulsion formulations based on dissolution $T_{50\%}$ values of 4.2 and 24 minutes, and $T_{90\%}$ values of 12.8 and 83 minutes, respectively.

INTRODUCTION

Metronidazole is widely used in the treatment of a number of protozoal parasitic diseases as well as in the prevention and treatment of bacterial infection (1,2). As an extremely bitter substance, it has restricted applications, especially in veterinary medicine. Therefore, metronidazole benzoate (MB), an ester prodrug, was developed as an alternative (3). It is practically tasteless because of its low aqueous solubility. Since the interaction of a drug with taste receptors requires the drug to be sufficiently soluble in saliva, lowering the aqueous solubility can help to mask the taste of MB.

Its sparingly aqueous solubility runs the risk of a serious problem. Incomplete dissolution of MB in the gastrointestinal tract may result in poor absorption. Until the present, there have been no commercial formulations of MB available except for suspension dosage forms (4). Extemporaneously prepared dosage forms, a glycerinated gelatin candy-based lozenge and an oil-in-water emulsion, were developed for this study. A rapid and sensitive stability-indicating HPLC assay was developed and validated. The stability and *in vitro* dissolution of the lozenge and emulsion were investigated.

MATERIALS AND METHODS

Materials

Metronidazole benzoate powder and chicken flavor were obtained from Professional Compounding Center of America, Inc. (9901 South Wilcrest, Houston, Texas 77099). Tinidazole and polyoxyethylene sorbitan mono-oleate (Tween 80) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Absolute methanol

and acetonitrile (J. T. Baker, phillipsburg, NJ.USA) were HPLC grade and water was purified by a cartridge system (Continental Water System, Roswell, GA, USA).

Partition Coefficient Determination

Following the shake-flask procedure (5), metronidazole benzoate was dissolved in pre-saturated octanol and mixed with buffered water (pH 7.0). After agitation of the two phases by a mechanical shaker for 48 hours and centrifugation for 20 minutes at 3000 rpm, each phase was analyzed for solute and the partition coefficient was calculated.

Formulation of the Chewable Lozenge

The lozenge formulation was compounded according to Secundum Artem methods (6). The glycerinated gelatin base was prepared with 21.6 ml water, 155 ml glycerin and 0.44 g methylparaben. The mixture was stirred and heated in a boiling water bath for 5 minutes. Then 43.4 g gelatin was slowly added until it was thoroughly dispersed and free of lumps. After that, 800 mg bentonite, 900 mg aspartame, 990 mg citric acid anhydrate, 720 mg acacia powder and 2.4 g MB were triturated together, added to the gelatin base and thoroughly mixed until evenly dispersed. Then 16 drops of chicken flavor was added and continuously mixed. Finally the mixture was poured into pediatric chewable lozenge molds and allowed to cool at 4 °C overnight.

Formulation of the Emulsion

The emulsion formulation was compounded according to the English Method (Wet Gum Method) (7). MB was dissolved in olive oil with heat, if necessary. Tween 80 was added as the emulsifier. Purified water was added and the mixture was emulsified using a Brinkmann homogenizer. The ratio of oil: water: emulsifer was 3:2:1 v/v/v. Chicken flavor (16 drops) was added to the final preparation.

Chromatographic Conditions

The HPLC system consisted of a Waters 515 HPLC pump (Milford, MA); Waters 996 photodiode array detector (Milford, MA) and a Rheodyne 20 µl injection loop (Rohnert Park, CA). The separation and quantitation were achieved on a Phenomenex IB-SIL 5 C_8 column (250 x 4.6 mm, id.) (Torrance, CA) at ambient temperature with 55:45, v/v phosphate buffer (pH 7.0)-acetonitrile mobile phase at a flow rate of 1.0 ml/min. The chromatogram was monitored with UV detection at 230 nm. Tinidazole was used as the internal standard.

Preparation of the Standard Solutions

A stock internal standard solution was prepared by dissolving 1.22 mg tinidazole in 5 ml methanol to obtain a final concentration of 244 µg/ml.

A stock standard solution of metronidazole benzoate was prepared in methanol to obtain a final concentration of 247 µg/ml.

Preparation of the Sample Solutions for Assay

The sample solution of the lozenge was prepared by dissolving one lozenge (about 1.24 g) containing about 200 mg metronidazole benzoate in 100 ml 50:50 v/v methanol-water solution with sonication for 20 minutes.

The sample solution of the emulsion was prepared by dissolving accurately weighed amounts of emulsion (equivalent to about 1 g) in 100 ml 50:50 v/v methanol-water solution with sonication for 20 minutes.

Analysis Procedure of MB in Samples

A typical calibration curve consisting of 0.247, 2.47 and 24.7 µg/ml MB was prepared and linear regression analysis of MB concentration vs area ratio of D/IS gave

slope, intercept and coefficient of determination (r^2) of 0.3865, 0.0118 and 0.9999 (n=6). Each sample solution of lozenge or emulsion (100 μl) was mixed with 40 μl of stock internal standard solution, diluted to 1 ml with 50:50 methanol-water v/v and filtered through a 0.2 μm membrane filter. The diluted sample was directly injected into the HPLC and chromatographed under the conditions described above.

Stability Testing

Each lozenge or emulsion formulation was stored in amber glass prescription bottles. For each formulation, one bottle was stored at ambient temperature and the other one was stored at 4 °C. Samples were withdrawn on days 0, 1, 3, 5, 7, 10 and 14 after preparation, and analyzed in triplicate by HPLC.

***In Vitro* Dissolution Testing**

Following the USP paddle method (8), each lozenge or emulsion dosage form was added into 900 ml of simulated gastric fluid without pepsin (pH 1.2) at 37 ± 1 °C and stirred at 100 rpm. A regenerated cellular membrane with molecular weight cut-off 12,000-14,000 was used as a dialysis membrane for the emulsion dissolution. Samples (1ml) were removed at 5, 10, 15, 30, 45, 60, 90, 120 and 150 minutes and buffered with 1ml of 1M pH 7.0 phosphate buffer. After mixing with stock internal standard solution (40 μl), the mixture was filtered and analyzed by HPLC.

RESULTS AND DISCUSSION

Formulation Development

The formulation of a new chemical entity with a expected therapeutic benefit requires that the drug be formulated into a delivery form that is chemical stable, and free from taste and odor problems, particularly if used for pediatric or veterinary use.

Metronidazole benzoate has a poor aqueous solubility, approximately 0.1 mg/ml at 25 °C (4). It is a challenge for formulation development. That is the reason why until now only suspensions of MB are available. The partition coefficient of metronidazole benzoate is 2.05, indicating that the drug is lipophilic, and easier to dissolve in oil than water.

Lozenges are solid dosage forms usually containing a medicinal agent and a flavoring substance. They are an excellent way of administering drug products as the taste of the drug often can be masked very effectively, especially for pediatric or veterinary use. The glycerinated gelatin base helps to deliver metronidazole benzoate for gastrointestinal absorption and systemic use.

An oil-in-water emulsion is also a convenient means of orally administering water insoluble drugs, especially a poorly tasting drug. Metronidazole benzoate was incorporated into an oil internal phase, and an external aqueous phase was flavored to enhance bioavailability.

Development of Chromatographic Separation

The HPLC assay of metronidazole benzoate was achieved in less than 10 min with sensitivity in the 10 ng/ml range (Figure 1). The method is stability indicating and will separate the degradation products metronidazole and benzoic acid. The linearity of

metronidazole benzoate is in the 0.01-100 μg/ml range. Accuracy and precision were <1.0 % and <0.54 %, respectively. The limit of quantitation was 10 ng/ml, and the limit of detection was 0.01 ng/ml based on a signal to noise ratio of 3 and a 20 μl injection. The absolute recoveries of MB from lozenge and emulsion were 98 ± 4 % and 99 ± 2 %, respectively.

Different HPLC reverse phase columns, such as $C_{18,}$ C_8 and phenyl, were studied to compare peak shapes and retention times of metronidazole benzoate and its degradation products. The desired retention times and peak shapes were obtained on the C_8 column. To confirm that the degradation products would not interfere with the assay, metronidazole benzoate was stressed for 15 minutes at ambient temperature with both 0.025 M hydrochloric acid and 0.005 M sodium hydroxide. The UV spectra and retention times of the degradation products were identical with metronidazole and benzoic acid standards using Waters Millennium software (version 3.2) to process library matching and peak purity identification.

Stability testing

A drug is considered stable as long as the drug remaining is equal or greater than 90 % of its initial concentration (9). MB was found to be stable in both lozenge and emulsion formulations at ambient temperature and 4 °C for up to 14 days (Table1). It was also found that metronidazole benzoate is stable when incubated in simulated gastric fluid with/without pepsin and in simulated intestinal fluid with/without pancreatin at 37 ° C. Other published data (10) had indicated that metronidazole benzoate was stable in suspension formulations.

In vitro dissolution testing

Metronidazole benzoate is rapidly released from lozenge and emulsion formulations with $T_{50\%}$ values of 4.2 and 24 minutes, and $T_{90\%}$ values of 12.8 and 83 minutes, respectively.

There were no initial time delays in the dissolution studies. The release of metronidazole benzoate from the lozenge was non-disintegration dissolution. When percent-dissolved values were plotted on the probability scale as a function of time values on the logarithmic scale, a single linear trend was observed, showing that the dissolution data followed a log normal distribution (Figure 2 and 3). The dissolution of both lozenge and emulsion formulations followed first-order kinetics.

Emulsion dissolution was studied under sink conditions using dialysis in which a wire was attached to the dialysis membrane containing the emulsion. The choice of the membrane was important in that it must have short equilibrium time and adequate physical strength to retain solid particles. The membrane was hydrolyzed in water the day before the start of the experiment. The rate at which metronidazole benzoate appeared on the distal surface of the dialysis membrane was not a function of the dialysis rate, but the dissolution rate; thus dissolution was the rate-limiting step. The dissolution rate is dependent on the solute diffusion coefficient, solubility and density of the suspended solid as well as the area of the membrane. A drug in the oil-in water emulsion must diffuse through the oil globules and then pass across the oil/water interface before it can dissolve. That is the reason MB displays a longer dissolution time in emulsion versus lozenge formulation.

ACKNOWLEGMENTS

The authors thank Drs J.C. Price and J.W. Beach of the College of Pharmacy, University of Georgia for their advice.

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8. The United States Pharmacopeia 24 / The National Formulary 19, Rockville, MD, the United States Pharmacopeial Convention, Inc.

9. Trissel LA. Avoiding common flaws in stability and compatibility studies of injectable drugs. American Journal of Hospital Pharmacy, 1983; 40:1159-60 (Editorial).
10. Alestig K, Freij L, Arnold E. Adsorption and extraction of metronidazole after administration of metronidazole benzoate mixture. Scandinavian Journal of Infectious Disease, 1980; 12:149-152.

Table 1. Stability of Metronidazole benzoate (MB) in lozenge and emulsion at ambient temperature and 4 °C.

	% of Initial Concentration Remaining[a]				
Lozenge Storage Time (days)	Ambient	4 °C	Emulsion Storage Time (days)	Ambient	4 °C
1	99.5±0.6[b]	98.3±1.0	1	97.9±1.0	98.1±1.3
3	101.6±0.1	102.6±1.3	3	101.6±0.5	100.1±0.4
5	99.6±0.7	99.0±1.0	5	100.4±0.3	101.7±0.8
7	99.7±0.2	99.0±0.3	7	99.8±0.7	97.8±0.2
10	99.9±0.2	99.4±0.2	10	101.6±0.3	98.2±0.1
14	102.1±0.5	100.2±0.4	14	96.5±0.4	100.2±0.3

a. Actual mean ± standard deviation of initial concentration of MB at time of preparation was 157.4 ± 1.4 mg/ml for the lozenge and 8.34 ± 0.01mg/ml for the emulsion.

b. n=3

Figure 1. Chromatograms of benzoic acid (a), metronidazole (b), internal standard tinidazole (c) and metronidazole benzoate(d).

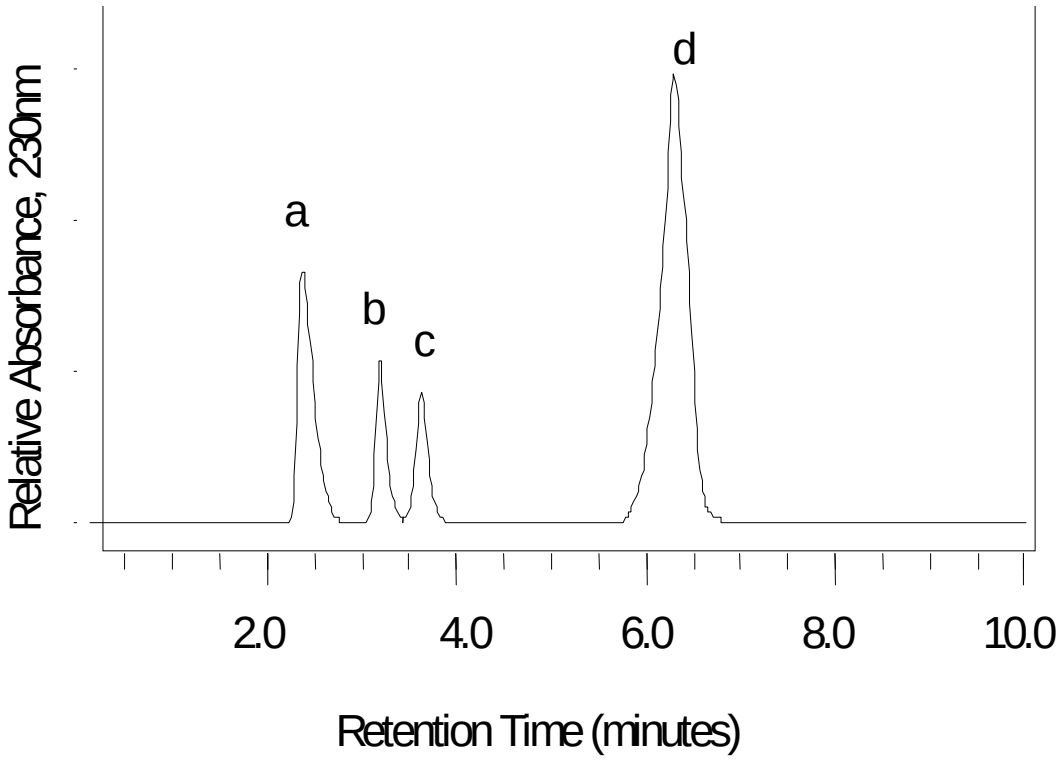

Relative Absorbance, 230nm
a
b
c
d
2.0
4.0
6.0
8.0
10.0
Retention Time (minutes)

Figure 2. Plot of percent dissolved versus time in minutes for lozenge (▲) and emulsion (◆) formulations in simulated gastric fluid without pepsin.

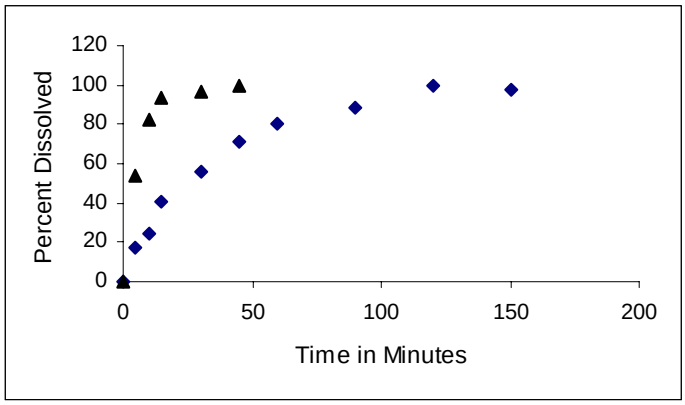
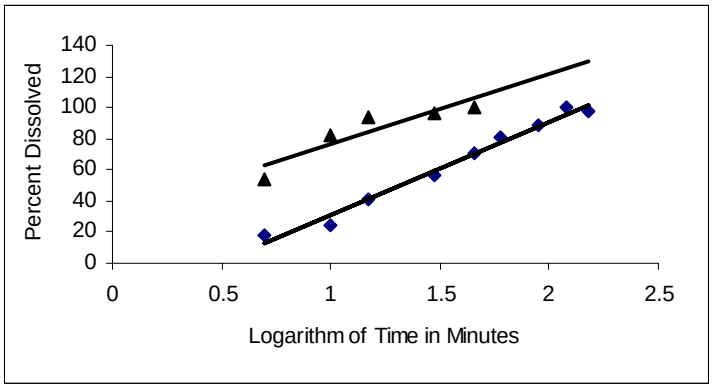

120
100
80
60
40
20
0
Percent Dissolved
0
50
100
150
200
Time in Minutes

Figure 3. Plot of percent dissolved versus logarithm time in minutes for lozenge (▲) and emulsion (◆) formulations in simulated gastric fluid without pepsin.


140
120
100
80
60
40
20
0
Percent Dissolved
0
0.5
1
1.5
2
2.5
Logarithm of Time in Minutes

CHAPTER 2

ANALYSIS OF DEGRADATION PRODUCTS OF METRONIDAZOLE BENZOATE USING HPLC/PDA[1]

[1]Fan, Bin. and James T. Stewart. To be submitted to Drug Development and Industral Pharmacy, 2001.

Abstract

Stress degradation of the prodrug, metronidazole benzoate (MB) was studied with high-performance liquid chromatography coupled with photodiode-array detection (HPLC-PDA). Waters Millennium^{32} PDA software was applied to determine the degradation products of MB and their purity through library matching procedures and peak purity processing. MB is stable when exposed to heat, oxidation or UV irradiation. However, MB degrades rapidly under strong acidic and basic conditions. The HPLC separation and PDA detection allow for both maximum sensitivity and compound identification.

Keyword: Metronidazole Benzoate; HPLC-PDA; Stress Degradation; Library Matching; Peak Purity Processing

Introduction

Metronidazole, 1-(2-hydroxyethyl)-2-methy-5-nitroimidazole, is widely used in veterinary medicine against both anaerobic bacteria and protozoal parasites [1,2]. As a drug in its pure form, it has an extremely bitter taste. The tasteless benzoate ester prodrug was developed as a suitable alternative [3]. As an ideal prodrug, metronidazole benzoate should be stable in solution or formulation, but completely and rapidly hydrolyzed within the human body to release therapeutic doses of metronidazole [4]. Stability and degradation studies for MB are important in terms of physicochemical properties necessary for this prodrug, and for formulation development across the entire spectrum of drug development activities.

A common practice in the pharmaceutical industry is to evaluate a number of formulations for critical attributes such as stability. Stability tests provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors. These tests establish recommended storage conditions and shelf lives. There is no universally accepted procedure for evaluating stability of R&D formulations. However, stress and accelerated testing are useful. High-performance liquid chromatography with UV detection was used as a stability-indicating assay to monitor MB [5]. Photodiode array (PDA) detection was used to collect data at several wavelengths simultaneously during an analysis as well as obtain spectral information of each compound. A variety of tasks are performed such as peak purity (comparing UV spectra at various points along the peak), compound confirmation (adding spectral information to the retention time), and reprocessing any single wavelength as a chromatogram. The purity of the chromatographic peaks eluting from the

HPLC column can be determined with PDA. Because an entire UV spectrum is acquired across the width of a peak, the purity of the peak can be checked for variations of the shape of the absorption spectrum across the chromatographic peak. The purpose of this paper is to demonstrate the usefulness of HPLC with PDA detection for the identification and spectral confirmation of MB and its degradation products.

Experimental

Reagents and Chemicals

Metronidazole benzoate powder was obtained from the Professional Compounding Center of America, Inc. (South Wilcrest, Houston, Texas 77099). Metronidazole and benzoic acid were purchased from Sigma Chemical Company (St. Louis, MO 63178). HPLC grade methanol and acetonitrile, 3% hydrogen peroxide, sodium hydroxide and concentrated hydrochloric acid were obtained from J. T. Baker (Phillipsburg, NJ). Water was purified by a cartridge system (Continental Water System, Roswell, GA 30076).

Preparation of Standard Solutions

A stock standard solution of metronidazole benzoate was prepared in absolute methanol to obtain a final concentration of 247 µg/ml. Solutions of 0.025 M hydrochloric acid and 0.005 M sodium hydroxide were prepared for use in the degradation studies.

Stress Degradation Conditions

For base degradation studies, 50 µl of MB stock solution was added to 1 ml of 0.005 M sodium hydroxide and then stored at ambient temperature for 15 min. The solution was neutralized with 1 ml of 1 M phosphate buffer (pH 7.0) prior to analysis.

For acid degradation studies, 50 µl of MB stock solution was added to 1 ml of 0.025 M hydrochloric acid and stored at ambient temperature for 15 min. The solution was neutralized with 1 ml of 1 M phosphate buffer (pH 7.0) prior to assay.

For oxidative degradation studies, 50 µl of MB stock solution was added to 1 ml of 3% hydrogen peroxide solution. The solution was stored at ambient temperature for up to 24 h.

For UV irradiation studies, 50 µl of MB stock solution was diluted with 1 ml deionized water and stored inside a cabinet equipped with a 254 nm UV lamp at ambient temperature for up to 24 h.

For heat degradation studies, 50 µl of MB stock solution was diluted with 1 ml deionized water and heated at 90 °C in an oven for up to 24 h.

Instrumentation and Chromatographic Conditions

The HPLC/PDA system consisted of a Waters 515 HPLC pump (Milford, MA), a Waters 996 Photodiode Array detector (Milford, MA) and a Rheodyne Model 7125 manual injector with 20 µl loop (Cotati, CA). Separations were achieved on a Phenomenex IB-SIL 5 C_8 column (250 x 4.6 mm, id., 5 µm particle size, Torrance, CA) at ambient temperature with a 55:45, v/v phosphate buffer (pH 7.0)-acetonitrile mobile

phase. The flow rate was 1.0 ml/min. Data acquisition was performed using Waters Millennium[32] PDA software (Milford, MA) operating on a PC.

Library Matching and Peak Purity Processing

A photodiode-array detector with Millennium[32] PDA software was used to perform library matching and peak purity processing. Library matching is a procedure used to create a library and to identify peaks by comparing spectra from unknown peaks to spectra from standards in the library. A library of known spectra is created before performing the library matching. The first step is to add the spectra of standard metronidazole, benzoic acid, and MB to the library. Then a PDA processing method for library matching was built using the Processing Method Wizard tool. Millennium[32] software Integrate function was used to compute the Match Angle and Threshold Angles for library matching. The Millennium[32] PDA software sets Thresholds Criteria to Noise plus Solvent with Solvent Angle set to one degree. Finally, the calculated results were viewed by Match Plot.

Millennium[32] PDA software was used to develop a PDA processing method for peak purity. Peak purity was calculated to determine if a peak is spectrally homogeneous. Spectral heterogeneity may indicate the presence of a coeluting peak. A Max Plot chromatogram, which plots the maximum spectral absorbance measured at each time point, was used to derive chromatograms of all peaks in the sample. The Processing Method Wizard tool was used to develop a processing method to assess peak purity. The Max Plot chromatogram was integrated and peak purity was calculated using spectral

absorbances. The Purity plot displayed the chromatographic peak and plotted the Purity Angle and Purity Threshold across the peak.

Results and Discussion

The chemical structures of benzoic acid, metronidazole and metronidazole benzoate (MB) are shown in Figure 1. MB is highly stable when exposed to heat, oxidation or UV irradiation. However, under strong acidic and basic conditions, MB degraded rapidly. Using a stability-indicating HPLC method, MB was separated from its degradation products as demonstrated in Figure 2. The total analysis time was less than 8 min.

Because a photodiode array detector (PDA) was coupled with HPLC, spectra or chromatograms can be extracted from all of the wavelengths collected, at each time point throughout the run. Usually, the concentrations of degradation products are rather low compared to the parent compound. With PDA detection, a maxplot, which provides a chromatogram of the maximum absorbance, can be generated at maximum sensitivity for further identification of the compounds.

UV spectra of each degradation product were obtained with PDA detection. Figure 3 shows a Match Plot spectrum of standard and degradation products. Using these spectra, library matching and peak homogeneity were performed using various algorithms in the Waters Millennium software [4,5].

The spectra of degradation products were compared to standards in the library and a match angle was calculated. Library Match displayed the data file spectrum overlaid with any possible matches to the library spectra. The Match Angle should be less than the

Match Threshold to indicate a good match. The lower the Match Angle, the higher the degree of match. The results of library matching are demonstrated in Figure 3 and Table 1. All of the Match Angles for peaks 2, 3 and 4 were less than the Match Threshold, which was considered a good match. Even for the small, partially separated peak 2, the spectral library matching was very effective.

The HPLC retention times were also used to aid in peak identification. The retention times for peaks 2, 3 and 4 were 2.4, 3.4 and 6.3 min while the retention times for standard benzoic acid, metronidazole and MB were 2.4, 3.3 and 6.3 min. Concerning the results of spectra library matching, it was confirmed that peak 2 was benzoic acid, peak 3 was metronidazole, and peak 4 was MB.

The purity of each peak in the degradation studies was evaluated by comparing the spectrum from the peak apex to all spectra across the peak. Any differences between the spectra are reported as the Purity Angle. The value is compared to a Purity Threshold, which is the measurement based on the baseline spectrum obtained from the system during the run. If the Purity Angle is less than the Purity Threshold, the peak is spectrally homogeneous. Coelution of two or more spectrally distinct compounds can produce a spectrally heterogeneous peak. The results are reported in Figure 4 and Table 2. For peak 3 and 4, Purity Angles were less than the Purity Threshold, therefore the peaks were considered spectrally pure. In the center of the peak 2, Purity Angles were larger than the Purity Threshold, and peak 2 was not a spectrally homogenous peak. This may have occurred because the concentration of peak 2 was too low to be detected.

Conclusions

Metronidazole benzoate is highly stable to heat, oxidation or UV irradiation. However, under strong acidic and basic conditions, MB degraded rapidly to metronidazole and benzoic aid. A photodiode-array detector coupled with Waters Millennium[32] PDA software were shown to be effective for the determination of the MB degradation products and peak purity checking.

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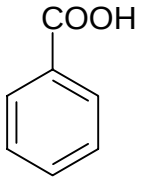
Table 1. Library Matching for MB degradation products

Peak Number	Match Angle	Match Threshold	Spectrum Name in Library	Match Flag	Match Ideal
2	4.622	5.553	Benzoic Acid	No	Yes
3	0.597	1.231	Metronidazole	No	Yes
4	0.284	1.050	MB	No	Yes

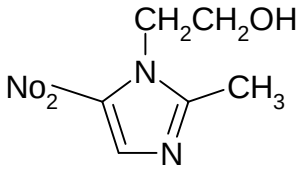
Table 2. Peak Purity Processing for MB degradation products

Peak Number	Purity Angle	Purity Threshold	Purity Flag	Purity Ideal
2	5.985	3.994	Yes	No
3	0.495	0.655	No	Yes
4	0.049	0.261	No	Yes

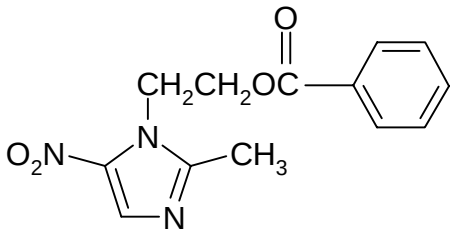
Figure 1. Structures of benzoic acid, metronidazole and metronidazole benzoate



Benzoic Acid



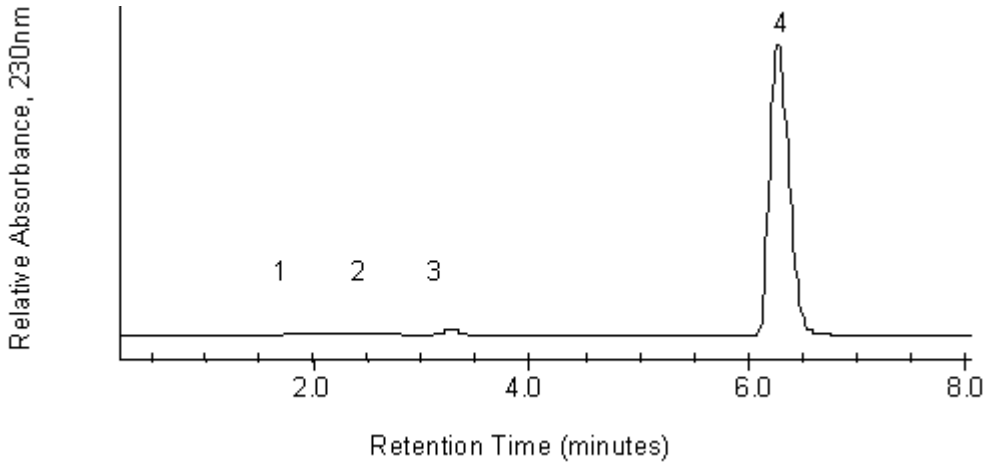
Metronidazole



Metronidazole Benzoate

Figure 2. Chromatograms of I. metronidazole benzoate degradation studies: peak 1 solvent front, peak 2 and 3 degradation products, and peak 4 metronidazole benzoate II. Standards: (a) benzoic acid, (b) metronidazole, (c) internal standard tinidazole and (d) metronidazole benzoate

I.


Relative Absorbance, 230nm
1
2
3
4
2.0
4.0
6.0
8.0
Retention Time (minutes)

II.

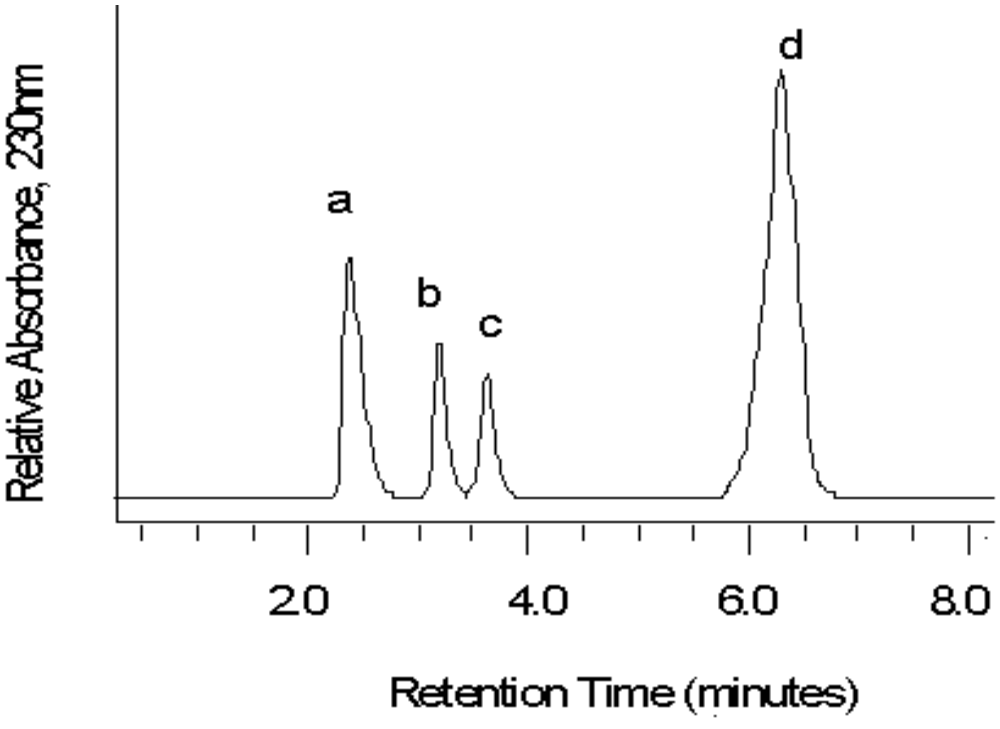
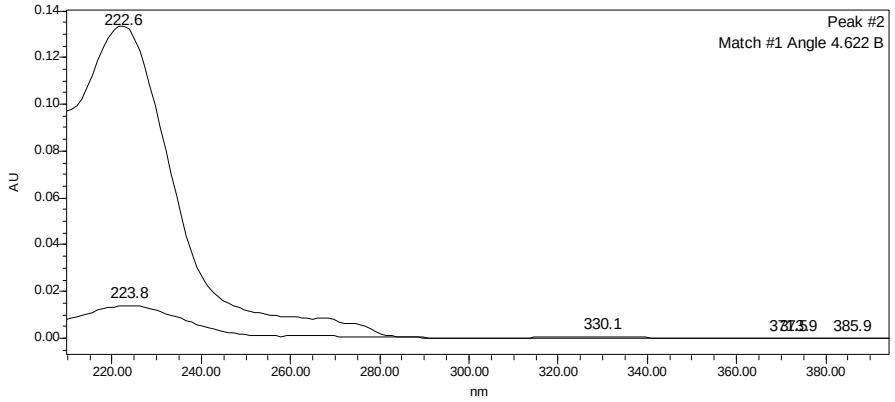
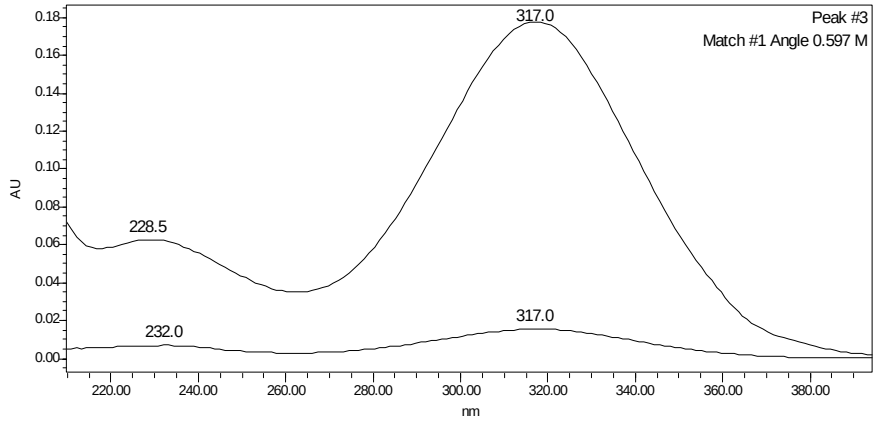

Relative Absorbance, 230nm
a
b
c
d
2.0
4.0
6.0
8.0
Retention Time (minutes)

Figure 3. Library match plots for metronidazole benzoate and each degradation product


Peak #2
Match #1 Angle 4.622 B
222.6
223.8
330.1
385.9
AU
nm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Match #1 Angle 0.597 M
317.0
228.5
232.0
317.0
AU
nm

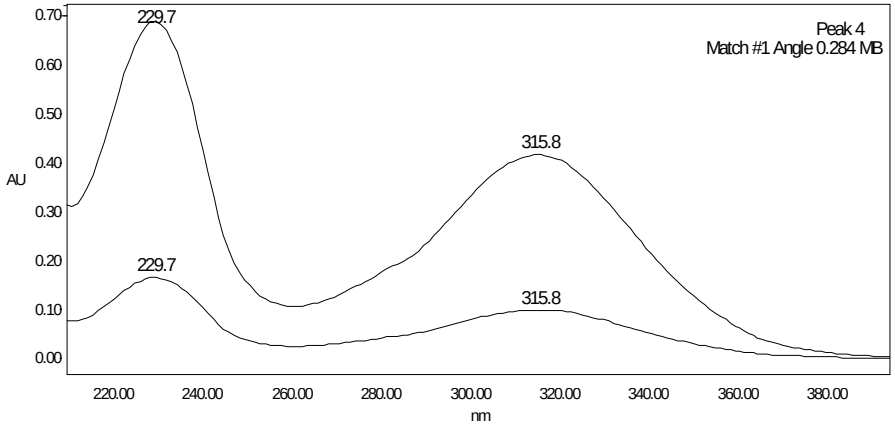
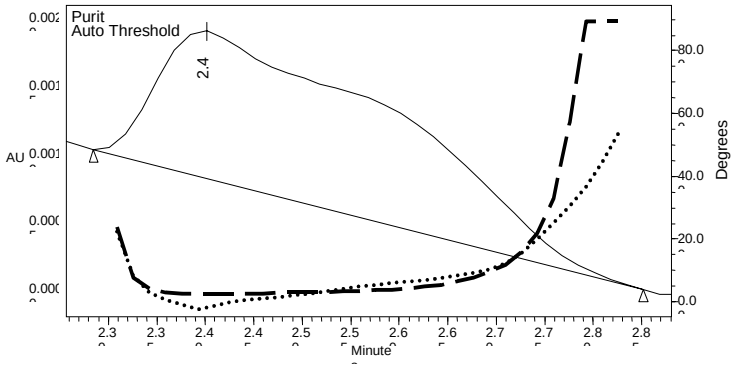
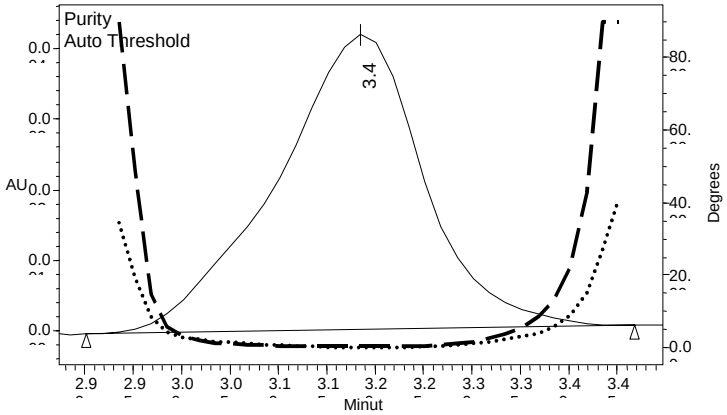
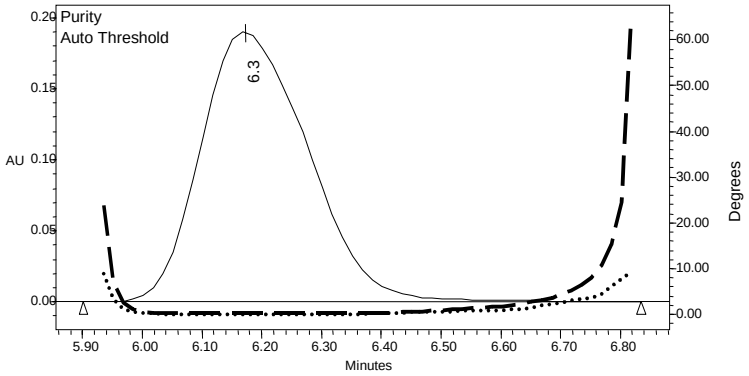

Peak 4
Match #1 Angle 0.284 MB
229.7
315.8
229.7
315.8
AU
nm

Figure 4. Purity plots of each peak of metronidazole benzoate and its degradation products (····· Purity, — — Auto Threshold)


Purit
Auto Threshold
2.4
AU
Degrees
Minute


Purity
Auto Threshold
3.4
AU
Degrees
Minut


Purity
Auto Threshold
6.3
AU
Degrees
Minutes

PART II

BIOANALYSIS OF ANTI-HIV DRUGS IN HUMAN SERUM USING HPLC, CE, LC-MS-MS

CHAPTER 3

DETERMINATION OF ZIDOVUDINE/LAMIVUDINE/NEVIRAPINE IN HUMAN PLASMA USING ION-PAIR HPLC[1]

[1]Fan, Bin. and James T. Stewart. Submitted to Journal of Pharmaceutical and Biomedical Analysis, 2001.

Abstract

A new high-performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of zidovudine(AZT)/lamivudine(3TC)/nevirapine in human plasma. Plasma samples were treated using a solid-phase extraction procedure. The compounds were separated using a mobile phase of 20 mM sodium phosphate buffer (containing 8 mM 1-octane sulfonic acid sodium salt) - acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid on an octylsilane column (150 x 3.9 mm i.d.) with UV detection at 265 nm. Aprobarbital was chosen as the internal standard (IS). The method was validated over the range of 57.6-2880 ng/ml for AZT, 59.0-17650 ng/ml for 3TC and 53.2-13300 ng/ml for nevirapine. The method was shown to be accurate, with intra-day and inter-day accuracy from 0.1 to 11% and precise, with intra-day and inter-day precision from 0.4 to 14%. Extraction recoveries of the analytes and I.S. from plasma were higher than 92%. The assay should be suitable for use in pharmacokinetic studies and routine plasma monitoring of this triple-drug therapy in AIDS patients.

Keywords: Zidovudine; Lamivudine; Nevirapine; Aprobarbital; Solid-phase extraction; Ion pair; Human plasma

1. Introduction

Over the last couple of years, combination therapy has proven to be the most effective approach to treat HIV disease [1-3]. The preferred options for patients who have not been previously treated include a three-drug regimen with at least two nucleoside reverse transcriptase inhibitors (NRTI) such as AZT and 3TC and a non-nucleoside reverse transcriptase inhibitor (NNRTI), such as nevirapine [4]. The profound and sustained viral suppression achievable with combinations such as indinavir (IDV), lamivudine (3TC) and zidovudine (AZT) has resulted in a dramatic shift in HIV treatment paradigms [2,5]. There are several combinations of NRTI to choose from in the initial three-drug regimen and, until very recently, the primary treatment combinations were based on AZT [4]. AZT and 3TC are front line therapies for the treatment of HIV infection and recently have become available in a combination tablet dosage form (Cimbivir^{TM}) [6].

Numerous analytical methods, such as high performance liquid chromatography (HPLC) with UV detection [7,8], mass spectrometry detection [6] and immunoassay [9,10], have been reported for either AZT or 3TC. However, no methods have been reported for the simultaneous determination of an AZT, 3TC and nevirapine mixture in human plasma. We have developed and validated an isocratic ion pair high-performance liquid chromatography (HPLC) assay with UV detection for the simultaneous determination of AZT, 3TC and nevirapine in human plasma. The method is potentially suitable for drug monitoring and determination of pharmacokinetic profiles.

2. Material and Methods

2.1 Chemicals and reagents

Zidovudine (AZT), aprobarbital (internal standard) and 1-octanesulfonic acid sodium salt were purchased from Sigma Chemical Company (St. Louis, MO 63178). Nevirapine was kindly provided by Abbott Laboratories (North Chicago, IL 60064). Lamivudine (3TC) was provided by Dr. Chung K. Chu (University of Georgia, Athens, GA, USA). Monobasic sodium phosphate, phosphoric acid, HPLC grade methanol and acetonitrile were from J.T. Baker Inc. (Phillipsburg, NJ 08865). Water was purified by a cartridge system (Continental Water System, Roswell, GA, USA).

2.2 Instrumentation

The high performance liquid chromatographic system consisted of a Beckman model 110 B pump (Fullerton, CA), an Alcott model 738 autosampler (Alcott Chromatography Inc., Norcross, GA), a Lamdba-Max Model 481 LC spectrophotometer (Waters, Milford, MA), and a HP 3394A integrator (Hewlett Packard, Avondale, PA). Separation was performed on a Waters Nova-Pak C_8 column (150 x 3.9 mm i.d., 5 µm particle size, Waters, Millford, MA) protected by an Applied Biosystems RP-8 guard column (Foster City, CA).

2.3 Chromatographic conditions

The chromatographic analysis was performed at ambient temperature with isocratic elution. The mobile phase consisted of 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid sodium salt) - acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid. The pump was set at a flow rate of 1.0 ml/min. A sample volume of 20 µl was injected in triplicate onto the HPLC column. The chromatogram was monitored with UV detection at 265 nm.

2.4 Preparation of standard stock solutions

Standard stock solutions of zidovudine (AZT), lamivudine (3TC), nevirapine and the internal standard (aprobarbital) were prepared by dissolving appropriate amounts of each drug in absolute methanol to obtain final drug concentrations of 288, 295, 266 and 1430 µg/ml, respectively. Working solutions were prepared by further diluting these stock solutions with 20 mM sodium phosphate buffer solution.

2.5 Sample preparation procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted standard solutions and 50 µl of the internal standard solution were added to blank plasma to a final volume of 1 ml. Extraction cartridges (Waters Oasis^{TM} HLB 1cc 30 mg Extraction Cartridge) were placed on a vacuum elution

manifold (VAC-ELUT^{TM}, Varian Sample Preparation Products, Harbor City, CA 90710) and rinsed with 1 ml of methanol followed by 1 ml of purified water. Care was taken that the cartridges did not run dry. One ml of the spiked plasma samples was then loaded onto the SPE cartridges and the vacuum applied. The cartridges were then washed with 1 ml 25 mM ammonium acetate buffer (pH 7.0) followed by vacuum suction for 1 min. One ml of water-acetonitrile (70:30, v/v) was used to elute the analytes and 20 μl was injected into the HPLC system.

2.6 Limit of detection (LOD) and limit of quantification (LOQ)

Plasma samples were spiked with decreasing concentrations of the analytes and 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 that could be measured with a signal-to-noise ratio of 10.

2.7 Linearity of method

Calibration plots for the analytes in plasma were prepared by spiking drug-free plasma with standard stock solutions to yield concentrations of 57.6-2880 ng/ml (57.6, 144, 288, 1440 and 2880 ng/ml) for AZT, 59-17650 ng/ml (59, 147.5, 295, 2950 and 17650 ng/ml) for 3TC and 53.2-13300 ng/ml (53.2, 133, 266, 2660 and 13300 ng/ml) for nevirapine. Triplicate injections of each concentration were performed. Calibration curves were constructed using ratios of the observed analyte peak area to internal

standard versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficient data which were then used to calculate analyte concentration in each sample.

2.8 Precision and accuracy of method

The intra-day accuracy and precision of the assay in plasma were determined by assaying two quality control samples in triplicate at low and high concentrations for each compound (144 ng/ml and 288 ng/ml for AZT, 295 ng/ml and 2950 ng/ml for 3TC, 133 ng/ml and 2660 ng/ml for nevirapine) within the same day (n=6). The inter-day accuracy and precision of the samples were analyzed on three different days (n=18). Accuracy was calculated by comparing concentration of spiked samples to the known concentrations. Precision was reported as percent relative standard deviation (%RSD).

2.9 Recovery of analytes from plasma

The recoveries of each drug and internal standard from plasma were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solutions at the same concentration. Both low and high concentrations for each compound were checked (144 and 288 ng/ml for AZT, 295 and 2950 ng/ml for 3TC, 133 and 2660 ng/ml for nevirapine). The concentration of the internal standard aprobarbital was 71.5 μg/ml.

3. Results and Discussion

Because of different physiochemical properties among AZT, 3TC and nevirapine, it was difficult to separate them simultaneously under isocratic conditions. The chemical structures of AZT, 3TC, nevirapine and aprobarbital (internal standard) are shown in Figure 1. A series of HPLC columns from silica to C_{18} were investigated, but none of them gave satisfactory chromatographic separations. With polar columns like silica, 3TC and AZT were eluted near the solvent front, and nevirapine was eluted at about 5 min even at a low percent organic solvent in the mobile phase. With nonpolar columns such as $C_{18,}$ 3TC and AZT were baseline separated at a low persent organic solvent in the mobile phase, but nevirapine did not elute within 30 min. Thus a reverse phase ion pair HPLC method was developed to alter the chromatographic selectivity of 3TC and nevirapine, and separate them from AZT under isocratic conditions. This ion pair method offered a reliable solution to the chromatography of analytes that were difficult to separate. It would also be a good technique for separating a mixture of 3TC and AZT.

In order to study an ion pair method for separating 3TC and nevirapine from AZT, factors such as organic solvent, choice of ion pair reagent and pH of mobile phase were investigated to manipulate chromatographic retention. Acetonitrile was chosen over methanol as organic modifier in the mobile phase because of its solvent strength. Negatively charged sulfonate ions (SO_3^-) with different lengths of alkyl chain, such as 1-pentanesulfonic acid, 1-hexanesulfonic acid, 1-heptanesulfonic acid, 1-octanesulfonic acid and lauryl sulfate were investigated as a suitable ion pair reagent. The ion pairs formed can enhance selectivity because of hydrogen bonding capacity and also offer

electrostatic interaction with analytes of opposite charges. With the addition of the ion pair reagent into the mobile phase and the use of an octylsilane column, the retention times of 3TC and nevirapine increased as predicted. The order of elution switched from 3TC, AZT and nevirapine to AZT, 3TC and nevirapine. The hydrophobicity of the ion pair was important to the optimizing of the separation; the higher the hydrophobicity of the ion pair, the larger the retention factors of 3TC and nevirapine. Concentrations from 0 to 50 mM of 1-octanesulfonic acid sodium salt were investigated to optimize the separation. It was found that 1-octanesulfonic acid sodium salt added to the mobile phase achieved suitable retention times for all three analytes with baseline resolution. The retention of 3TC increased and the retention of AZT slightly decreased as the concentration of the ion pair reagent was increased. Above 50 mM ion pair concentration, analyte retention decreased because micelles of the ion pair molecules began to be formed, and their formation led to competing partition processes. Eight mM 1-octanesulfonic acid was the optimum ion pair concentration that gave the best separation of AZT, 3TC and nevirapine.

The chromatographic selectivity of 3TC and AZT under these ion-pair conditions exhibited a profound pH dependency. As the pH of the phosphate buffer increased from 2.5 to 4.5, elution time and resolution initially increased and then decreased as buffer pH increased. The best separation for 3TC and nevirapine from AZT as ion pairs was achieved at pH 3.2.

Baseline separations of AZT, 3TC and nevirapine were achieved with retention times of 3.1, 6.1 and 15.0 min, respectively. The internal standard aprobarbital gave a retention time of 11.0 min. Aprobarbital was chosen because it is structurally similar to

NRTI drugs and gave good recoveries from plasma using the SPE method. Figure 2 I and II show the chromatograms obtained from drug-free human plasma and a spiked plasma sample, respectively. All the drugs showed good separation from endogenous plasma interferences. The only significant plasma peak present in both blank and sample eluted at 8.2-8.4 min and did not interfere with the separations.

During development of the solid-phase extraction method, a series of different extraction cartridges and discs were investigated, such as C_{18}, C_8, phenyl, Oasis^{TM} cartridges and C_{18}, C_{18}AR, and mixed-mode Discs. The recoveries using discs were less than 30% for AZT and 3TC. Cartridges gave much higher recoveries, particular for C_{18} and Oasis^{TM}. Oasis^{TM} provided the highest recoveries of all of these drugs as well as a much cleaner sample. Twenty-five mM ammonium acetate (pH 7.0) was used to wash the cartridges after loading spiked plasma to help retain the highly hydrophilic analytes AZT and 3TC. Higher recoveries were observed using ammonium acetate than water washes. Because there was no organic solvent in the wash solution, not all of the endogenous interferences could be removed. An endogenous non-interfering plasma component was observed around 8 min. A 30% acetonitrile solution was strong enough to elute all of the analytes including nevirapine and leave most of the highly hydrophobic plasma interferences on the SPE cartridges. Absolute plasma recoveries greater than 92% were obtained for all three analytes and internal standard. The detailed data is listed in Table 2. The recovery of the internal standard from plasma was 96%.

The calibration curves showed good linearity in the range of 57.6-2880 ng/ml for AZT, 59.0-17650 ng/ml for 3TC and 53.2-13300 ng/ml for nevirapine. The correlation coefficients (r^2) of calibration curves of each drug were higher than 0.999 as determined

by least-squares analysis. LOD and LOQ data are shown in Table 1. The LOD for AZT, 3TC and nevirapine were 28.8, 14.8 and 13.3 ng/ml, respectively. The LOQ for AZT, 3TC and nevirapine were 57.6, 59.0 and 53.2 ng/ml, respectively. The results from the validation of the method in human plasma are listed in Table 2. The method proved to be accurate (relative error at high and low concentration from 0.1 to 10.7% for intra-day and from 0.2 to 9.7 % for inter-day) and precise (intra-day precision ranged from 0.4 to 5.4% and inter-day precision ranged from 0.4 to 13.8%).

4. Conclusions

A sensitive, specific and validated HPLC isocratic assay was developed for the simultaneous analysis of AZT/3TC/nevirapine in human plasma. The HPLC method should be useful for monitoring plasma drug concentrations, and for pharmacokinetic studies in HIV-infected patients.

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Table 1. Range of calibration curves, limits of detection (LOD) and limits of quantification (LOQ) of AZT, 3TC and nevirapine in spiked human plasma

Drug	Range of calibration curves (ng/ml)	Limit of detection (LOD) (ng/ml)[a]	Limit of quantification (LOQ) (ng/ml)[b]
AZT	57.6—2880	28.8	57.6
3TC	59.0—17650	14.8	59.0
Nevirapine	53.2—13300	13.3	53.2

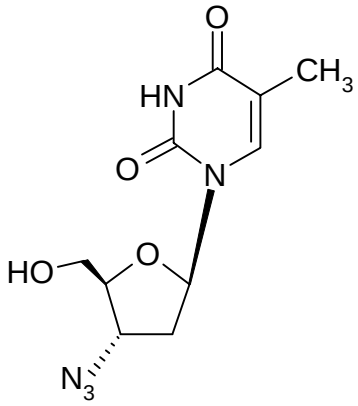
a S/N=3
b S/N=10

Table 2. Inter-day and intra-day accuracy, precision and recovery for the analysis of AZT, 3TC and nevirapine in human plasma

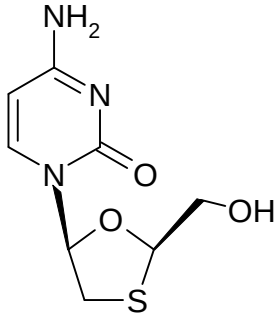
	Concentration	Precision (%)		Accuracy (%)		Plasma Recovery[c]
	(ng/ml)	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	(%)
AZT	288	5.4	6.2	2.6	5.4	102.3±3.3
	144	3.5	13.8	3.8	9.7	103.0±2.6
3TC	2950	4.0	0.4	2.2	0.2	97.9±1.9
	295	4.8	2.7	5.4	6.9	100.6±4.1
Nevirapine	2660	0.4	4.1	0.1	2.0	91.8±2.1
	133	4.5	12.1	10.7	4.2	106.3±3.8

a. Based on n = 3
b. Based on n = 9
c. Mean ± SD based on n = 6

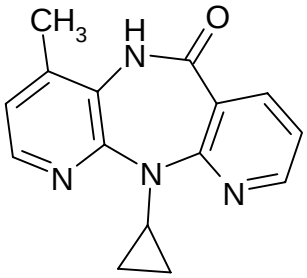
Figure 1. The chemical structures of analytes



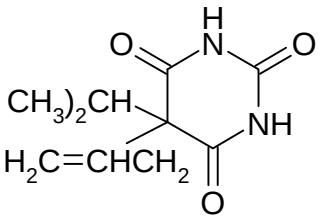
Zidovudine (AZT)



Lamivudine (3TC)

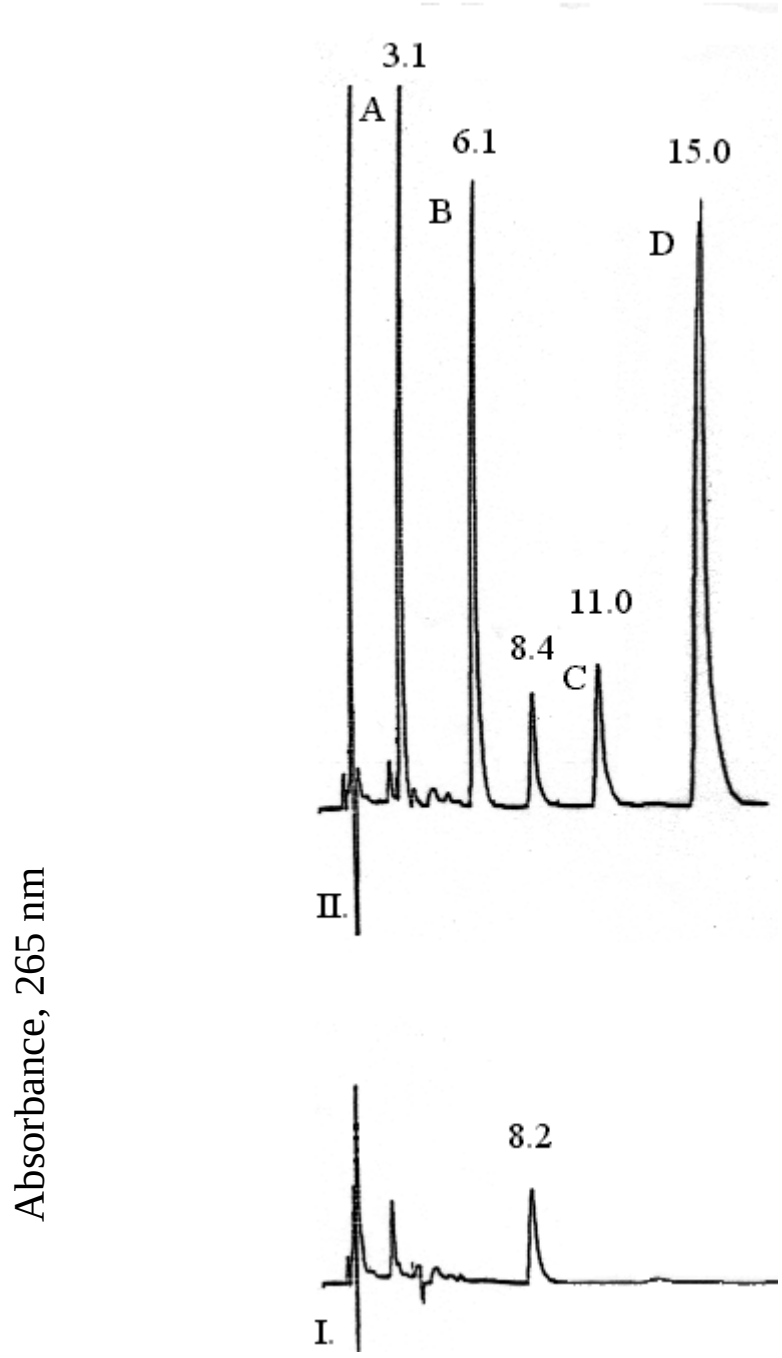


Nevirapine



Aprobarbital

Figure 2. Chromatogram of I. blank human plasma and II. human plasma spiked with (A) AZT, (B) 3TC, (C) internal standard and (D) nevirapine. The peak at 8.2-8.4 min is an unknown plasma component


3.1
A
6.1
B
15.0
D
11.0
8.4
C
II.
8.2
I.
Absorbance, 265 nm
Retention Time, min

CHAPTER 4

DETERMINATION OF ZIDOVUDINE/ZALCITABINE/NEVIRAPINE IN HUMAN PLASMA BY ION-PAIR HPLC[1]

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[1]Fan, Bin. and James T. Stewart. Accepted by Journal of Liquid Chromatography & Related Technologies. Reprint by courtesy of Marcel Dekker Inc. from the forthcoming Marcel Dekker Inc. publication, 2001.

ABSTRACT

A novel high-performance liquid chromatography (HPLC) assay was developed for the simultaneous determination of zidovudine(AZT)/zalcitabine(ddC)/nevirapine in human plasma. Plasma samples were treated using a solid-phase extraction procedure. The analytes were separated using a mobile phase containing 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid, sodium salt) - acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid on an octylsilane column (150 x 3.9 mm I.D.) with UV detection at 265 nm. Aprobarbital was chosen as internal standard. The method was validated over the range of 57.6-2880 ng/ml for AZT, 20.2-2020 ng/ml for ddC and 53.2-13300 ng/ml for nevirapine. Intra-day and inter-day accuracy were less than 10.7% and intra-day and inter-day precision were less than 13.7%. Extraction recoveries of all analytes from plasma were higher than 88.5%. The assay should be applicable for pharmacokinetic studies and routine monitoring of these drugs in plasma.

INTRODUCTION

Over the past several years, the use of multidrug therapy has greatly enhanced the success of acquired immunodeficiency syndrome (AIDS) treatment (1-3). Combination therapy of two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) is one of the preferred treatment options (4,5). Nevirapine was the first NNRTI, which is notorious for rapidly eliciting resistance due to mutations of the amino acids surrounding the NNRTI binding site (6). In association with two NRTI, nevirapine significantly reduces the viral load and increases CD4 cell counts, particularly in treatment-naive patients (7,8). There are several combinations of NRTI to choose from in the initial three-drug regimen and, until very recently, the primary treatment combinations were based on AZT. The combination of AZT with zalcitabine (ddC) is a well-proven, highly effective regimen (5).

Therapeutic monitoring of these drugs is recommended in order to avoid or delay resistance from the virus, to avoid the usual underestimated non-adherence and to manage drug interactions. Analytical methods have been described to quantify individual drugs in biological media (9-11), but few methods have been reported for combined anti-HIV drugs. We have developed and validated a high-performance liquid chromatography (HPLC) assay with UV detection for the simultaneous determination of AZT, ddC and nevirapine in human plasma. Because this combination includes drugs from both NRTI and NNRTI categories, they have quite different physiochemical characteristics, such as polarity and solubility, and their assay was a challenge for method development. The

method could also be applicable for drug monitoring and determination of pharmacokinetic profiles for this drug combination.

EXPERIMENTAL

Chemicals

Zidovudine (AZT), zalcitabine (ddC), aprobarbital (internal standard) and 1-octanesulfonic acid sodium salt were purchased from Sigma Chemical Company (St. Louis, MO 63178). Nevirapine was kindly provided by Abbott Laboratories(North Chicago, IL 60064). Monobasic sodium phosphate, phosphoric acid and HPLC grade methanol and acetonitrile were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). Water was purified by a cartridge system (Continental Water System, Roswell, GA, USA). Drug free human plasma was obtained from Bioreclamation Inc., Lot # BRH01495 (Hicksville, NY 11801).

Instrumentation

The high performance liquid chromatographic system was equipped with a Beckman model 110 B pump (Fullerton, CA), an Alcott 738 model autosampler (Norcross, GA), a Lambda-Max Model 481 LC spectrophotometer (Waters, Milford, MA), and a HP 3394A integrator (Hewlett Packard, Avondale, PA). Separation was performed on a Waters Nova-Pak C_8 column (150 x 3.9 mm I.D., 5 µm particle size,

Waters, Milford, MA) protected by an Applied Biosystems RP-8 guard column (Foster City, CA).

Chromatographic Conditions

The chromatographic analysis was performed at ambient temperature with isocratic elution. The mobile phase consisted of 20 mM sodium phosphate buffer (containing 8 mM 1-octanesulfonic acid sodium salt) - acetonitrile (86:14, v/v) with pH adjusted to 3.2 with phosphoric acid. The pump was set at a flow rate of 1.0 ml/min. A sample volume of 20 µl was injected in triplicate into the HPLC system. The UV detector was operated at a wavelength of 265 nm.

Preparation of Standard Stock Solutions

Stock solutions of zidovudine (AZT), zalcitabine (ddC), nevirapine and the internal standard (aprobarbital) were prepared by dissolving appropriate amounts of each drug in absolute methanol to obtain final drug concentrations of 288, 202, 266 and 1430 µg/ml, respectively. Working solutions were prepared by further diluting these stock solutions with 20 mM sodium phosphate buffer solution.

Sample Preparation Procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted stock solutions and 50 µl of the internal standard solution were added to blank plasma to obtain a final volume of 1 ml. Extraction cartridges (Waters Oasis^{TM} HLB 1cc 30 mg Extraction Cartridges) were placed on a vacuum elution manifold (VAC-ELUT^{TM}, Varian Sample Preparation Products, Harbor City, CA 90710) and rinsed with 1 ml of absolute methanol followed by 1 ml of purified water. Care was taken such that the cartridges did not run dry. One ml of each spiked plasma sample was then loaded onto a SPE cartridge and a vacuum applied. The cartridges were then washed with 1 ml 25 mM ammonium acetate buffer (pH 7.0) followed by vacuum suction for 1 min. Then, 1 ml of water-acetonitrile (70:30, v/v) was used to elute the analytes, followed by a 20 µl injection into the liquid chromatograph.

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

Plasma samples were spiked with decreasing concentrations of each analyte and analyzed. The limit of detection (LOD) was defined by the concentration that yields a signal-to-noise ratio of 3. The limit of quantification (LOQ) was calculated to be the lowest concentration that could be measured with a signal to noise ratio of 10.

Linearity of Method

Calibration plots for the analytes in human plasma were prepared by adding standard stock solutions to drug-free plasma yielding concentrations of 57.6-2880 ng/ml (57.6, 144, 288, 1440 and 2880 ng/ml) for AZT, 20.2-2020 ng/ml (20.2, 40.4, 101, 202 and 2020 ng/ml) for ddC and 53.2-13300 ng/ml (53.2, 133, 266, 2660 and 13300 ng/ml) for nevirapine. Triplicate injections of each concentration were performed. Calibration curves were constructed using ratios of the observed analyte peak area to internal standard versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficient which were then used to calculate analyte concentration in each spiked sample.

Precision and Accuracy of Method

The intra-day accuracy and precision of the assay in human plasma were determined by assaying two quality control samples in triplicate at low and high concentrations for each compound (144 and 288 ng/ml for AZT, 40.4 and 202 ng/ml for ddC, 133 and 2660 ng/ml for nevirapine) within the same day (n=6). The inter-day accuracy and precision for the samples were analyzed on three different days (n=18). Accuracy was reported as percent error. Precision was reported as percent relative standard deviation (%RSD).

Recovery of Analytes from Plasma

The recoveries of each drug and internal standard from plasma were determined by comparing the peak area of each compound after extraction with the respective non-extracted standard solution at the same concentration. Both low and high concentrations for each compound were checked, (144 and 288 ng/ml for AZT, 40.4 and 202 ng/ml for ddC, 133 and 2660 ng/ml for nevirapine). The concentration of the aprobarbital internal standard in the samples was 71.5 μg/ml.

RESULTS AND DISCUSSION

Because of different physiochemical properties among these drugs, it was difficult to separate them under isocratic HPLC conditions. A series of HPLC columns were investigated from silica to C_{18}, but none of them gave a satisfactory chromatographic separation. With a highly polar column such as silica, ddC and AZT eluted near the solvent front, and nevirapine eluted at about 5 min even at a low percent organic solvent in the mobile phase. With a nonpolar column such as C_{18}, ddC and AZT were baseline separated at a low percent organic solvent in the mobile phase, but nevirapine would not elute within 30 min. Thus, a reverse phase ion pair HPLC method was developed to alter the chromatographic selectivity of ddC and nevirapine, and separate them from AZT under isocratic conditions. This ion pair method offered a reliable solution for the chromatography of analytes that would be difficult to separate by other means.

Acetonitrile was chosen over methanol as the organic modifier in the mobile phase because of its solvent strength. The chemical structures of AZT, ddC, nevirapine and aprobarbital (internal standard) are shown in Figure 1. Under acidic mobile phase conditions, ddC and nevirapine are ionized, and AZT is unionized. Negatively charged sulfonate ions (SO_3^-) with different lengths of alkyl chain, such as 1-pentanesulfonic acid, 1-hexanesulfonic acid, 1-heptanesulfonic acid, 1-octanesulfonic acid and lauryl sulfate were investigated for use as the ion pair reagent. After the addition of the ion pair reagent into the acidic mobile phase and the use of an octylsilane column, the retention times of ddC and nevirapine increased as predicted by ion pair theory. The order of elution switched from ddC, AZT and nevirapine to AZT, ddC and nevirapine. The hydrophobicity of the ion pair reagent was important to the optimization of the separation, since the higher the hydrophobicity of the ion pair, the larger the retention factors of ddC and nevirapine. Concentrations from 0 to 50 mM of 1-octanesulfonic acid sodium salt were investigated to optimize the separation of ddC and nevirapine from AZT. The octanesulfonic acid compound achieved suitable retention times for all three analytes on the octylsilane column. The retention of ddC increased while the retention for AZT slightly decreased as the concentration of the ion pair reagent increased. Above 50 mM ion pair reagent, the retention of all analytes decreased because micelles of the ion pair molecules began to be formed and this led to competing partition processes. Eight mM 1-octanesulfonic acid sodium salt gave the best separation for all analytes. The chromatographic selectivity of ddC and AZT exhibited a pH dependency as the pH of the phosphate buffer was varied from 2.5 to 4.5. Elution times and resolution of ddC and

AZT increased and then decreased as buffer pH increased. The best separation of ddC and nevirapine from AZT was achieved at pH 3.2.

Baseline separations of AZT, ddC and nivirapine were achieved with retention times of 3.1, 5.2 and 15.0 min, respectively. The internal standard, aprobarbital, gave a retention time of 11.0 min. Aprobarbital was chosen because of its structural similarity to the NRTI drugs and its good recovery from plasma using the SPE method. Figures 2 I and II show typical chromatograms obtained from drug-free human plasma and a spiked plasma sample, respectively. The drugs show a good separation from endogenous plasma interferences. The peak present in both blank and spiked plasma, eluting around 8.2-8.5 min, is an unknown endogenous component of plasma.

During the development of the solid-phase extraction method, a series of different extraction cartridges and discs were investigated, such as C_{18}, C_8, phenyl, Oasis^{TM} cartridges and C_{18}, C_{18}AR, and mixed-mode Discs. The plasma recoveries using discs were less than 30% for AZT and ddC. Cartridges gave much higher recoveries, particular the C_{18} and Oasis^{TM}. Oasis^{TM} provided the highest recoveries of the drugs as well as relatively cleaner assay samples. Twenty-five mM ammonium acetate (pH 7.0) was used to wash cartridges after loading spiked plasma to help retain the hydrophilic analytes AZT and ddC. Higher recoveries were observed using ammonium acetate than water washes. Because no organic solvent was present in the wash solution, it was not strong enough to clean up all the endogenous interferences. The eluent including 30% acetonitrile was strong enough to elute the analytes and leave most of the highly hydrophobic interferences of plasma in the SPE cartridges. Absolute recoveries greater

than 88.5% were obtained for all three analytes. The detailed data is listed in Table 1. The recovery of the internal standard was 96.2%.

The calibration curves showed good linearity in the range of 57.6-2880 ng/ml for AZT, 20.2-2020 ng/ml for ddC and 53.2-13300 ng/ml for nevirapine. The correlation coefficients (r^2) of calibration curves of each drug were higher than 0.999 as determined by least-squares analysis. LOD and LOQ data are shown in Table 2. The LOD for AZT, ddC and nevirapine were 28.8, 20.2 and 13.3 ng/ml, respectively. The LOQ for AZT, ddC and nevirapine were 57.6, 20.2 and 53.2 ng/ml, respectively. The results from the validation of the method in human plasma are listed in Table 1. The method proved to be accurate (percent error at high and low concentration varied from 0.1 to 10.7% for intra-day and 1.7 to 9.7 % for inter-day) and precise (intra-day precision ranged from 0.4 to 5.4% and inter-day precision ranged from 4.1 to 13.7%).

CONCLUSION

A sensitive and specific HPLC isocratic ion pair assay was developed and validated for the simultaneous analysis of AZT/ddC/nevirapine in plasma. The HPLC method could be used for monitoring drug concentrations in human plasma, and for pharmacokinetic studies in HIV-infected patients.

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Table 1. Inter-Day and Intra-Day Accuracy, Precision and Recovery for the Analysis of AZT, ddC and Nevirapine in Human Plasma

	Concentration	Precision (%)		Accuracy (%)		Plasma Recovery[c]
	(ng/ml)	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	(%)
AZT	288	5.4	6.2	2.6	5.4	102.3±3.3
	144	3.5	13.8	3.8	9.7	103.0±2.6
ddC	202	1.1	4.2	3.9	1.7	88.5±3.8
	40.4	7.0	9.1	6.2	1.8	91.1±4.3
Nevirapine	2660	0.4	4.1	0.1	2.0	91.8±2.1
	133	4.5	12.1	10.7	4.2	106.3±3.8

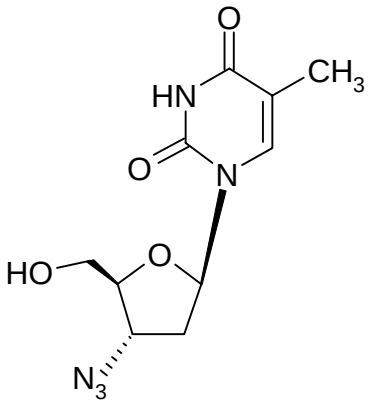
a. Based on n = 3
b. Based on n = 9
c. Mean ± SD based on n = 6

Table 2. Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantification (LOQ) of AZT, ddC And Nevirapine in Spiked Human Plasma

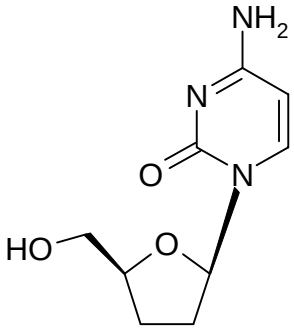
Drug	Range of calibration curves (ng/ml)	Limit of detection (LOD) (ng/ml)[a]	Limit of quantification (LOQ) (ng/ml)[b]
AZT	57.6—2880	28.8	57.6
ddC	20.2—2020	20.2	20.2
Nevirapine	53.2—13300	13.3	53.2

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

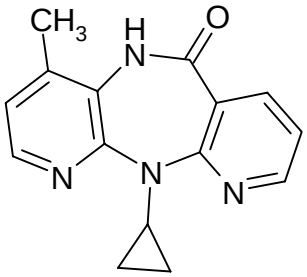
Figure 1. The chemical structures of analytes



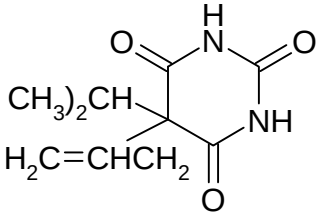
Zidovudine (AZT)



zalcitabine (ddC)

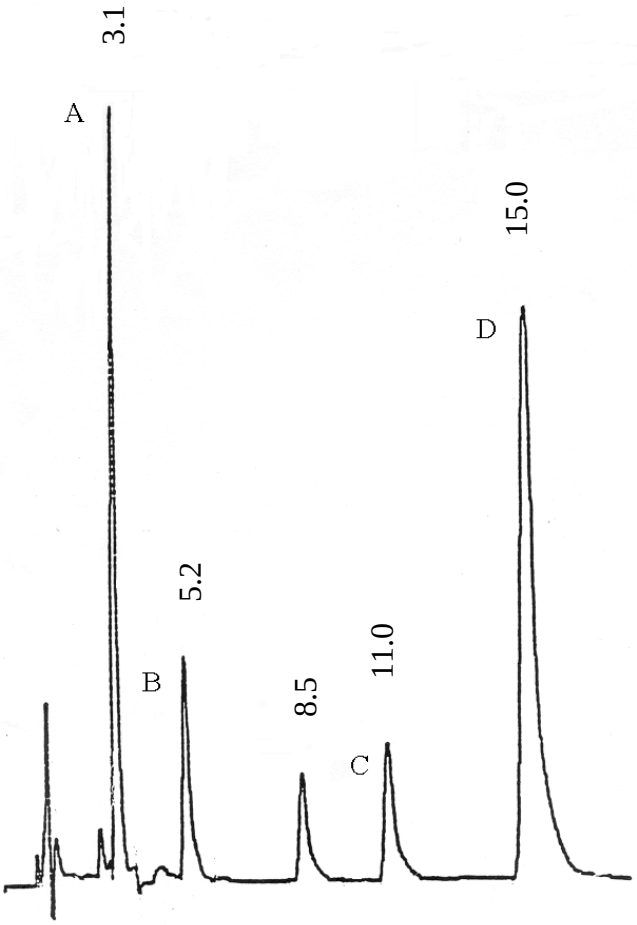


Nevirapine



Aprobarbital

Figure 2. Chromatogram of I. blank human plasma and II. human plasma spiked with (A) AZT, (B) ddC, (C) internal standard and (D) nevirapine. The peak at 8.2-8.5 min is an unknown plasma component.



I.

II.

Absorbance, 265 nm

Retention Time, min

CHAPTER 5

DETERMINATION OF LAMIVUDINE/STAVUDINE/EFAVIRENZ IN HUMAN SERUM USING LIQUID CHROMATOGRAPHY/ELECTROSPRAY TANDEM MASS SPECTROMETRY WITH IONIZATION POLARITY SWITCH[1]

[1]Fan, Bin. Michael G. Bartlett and James T. Stewart. Submitted to Biomedical Chromatography, 2001.

Abstract

A high-performance liquid chromatography/tandem mass spectrometry (LC-MS-MS) method with ionization polarity switch was developed and validated in human serum for the determination of a lamivudine (3TC)/stavudine (d4T)/efavirenz combination HIV therapy. A gradient mobile phase consisting of acetonitrile and 20 mM ammonium acetate buffer with pH adjusted to 4.5 using glacial acetic acid was utilized to separate these compounds on a hexylsilane column (150 x 2.0 mm i.d.). The precursor and major product ions of the analytes were monitored on a triple quadrupole mass spectrometer in the multiple reaction monitoring (MRM) mode. The effect of ion suppression from human serum was studied and no interference with the analysis was noted. The method was validated over the range of 1.1-540 ng/ml for 3TC, 12.5-6228 ng/ml for d4T and 1.0-519 ng/ml for efavirenz. The method was shown to be accurate, with intra-day and inter-day accuracy less than 14.0% and precise, with intra-day and inter-day precision less than 13.1%. The extraction recoveries of all analytes were higher than 90%.

Introduction

The availability of new potent antiretroviral therapies has dramatically changed the management of patients infected with the human immunodeficiency virus (HIV). Combination therapies have proven to be the most effective approach for the treatment of HIV [1-3], not only to increase antiviral suppression, but also to prevent drug resistance and optimize drug exposure. The International AIDS Society-USA (IAS) and the Department of Health and Human Services (HHS) antiretroviral guidelines recommend the use of a three-drug regimen with at least two nucleoside reverse transcriptase inhibitors (NRTI) and a highly active protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) [1, 4]. NRTIs are the cornerstone of the majority of antiretroviral regimens. There are several NRTI combinations to choose from in the initial three-drug regimen and, until very recently, the primary treatment combinations were largely based on AZT [5]. An alternative is to base the NRTI combination on d4T, which is easily tolerated and penetrates the central nervous system. The combination of lamivudine (3TC) and stavudine (d4T) is safe and effective, and it reduces the viral load in the cerebral spinal fluid as confirmed by clinical trials [6-8]. The current trend, widely utilized in clinical practice, is to substitute d4T for AZT in combination regimens using AZT and 3TC when patients experience intolerance and also as the initial use of the well tolerated combination in a potent suppressive regimen [9]. Efavirenz is a new NNRTI. It appears to be particularly useful since it is potent, extremely well tolerated, and has a long half-life [10].

Therapeutic monitoring of anti-HIV drugs is recommended in order to avoid or delay resistance from the virus, to avoid the usual underestimated non-adherence and to

manage drug interactions. Analytical methods have been reported to quantify individual HIV drugs in biological media [11-13]. However, no methods have been reported for the simultaneous determination of important antiretroviral combinations in human serum. We have developed and validated a gradient high-performance liquid chromatography (HPLC) method utilizing triple quadrupole mass spectrometry (MS) detection for the simultaneous determination of 3TC, d4T and efavirenz in human serum. Because this combination includes drugs from NRTI and NNRTI categories, they have quite different physiochemical characteristics, such as polarity and solubility. The method described herein should also be suitable for drug monitoring and determination of pharmacokinetic profiles.

Experimental

Chemicals and reagents

Stavudine (d4T) and aprobarbital (internal standard) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Efavirenz was kindly provided by DuPont Pharmaceuticals Company (Wilmington, DE 19805). Lamivudine (3TC) was provided by Dr. Chung K. Chu (University of Georgia, Athens, GA, USA). Ammonium acetate, glacial acetic acid, HPLC grade methanol and acetonitrile were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). Deionized water was purified by a cartridge system (Continental Water System, Rosewell, CA 30076). Oasis HLB solid phase cartridges were obtained from Waters Corporation (Milford, MA 01757). Drug free human serum was obtained from Bioreclamation Inc. (Hicksville, NJ 11801).

Instrumentation and LC-MS-MS conditions

The HPLC system consisted of an Agilent Technologies 1100 HPLC system equipped with a vacuum degasser, quaternary pump and an autosampler (Palo Alto, CA, USA). The column utilized was a Phenomenex Sphereclone hexylsilane (150 x 2.0 mm i.d., 3 µm particle size, Torrance, CA, USA) protected by an ADVCART^{TM} Advantage 100 octadecylsilane guard cartridges (15 x 3.2 mm, 5 µm, SCI-CON OF FLORIDA, Winter Park, FL 32789).

A gradient elution method was applied at ambient temperature using (A) 20 mM ammonium acetate buffer with pH adjusted to 4.5 with glacial acetic acid and (B) acetonitrile. The gradient conditions were as follow: From 0 min, 93% A and 7% B, flow rate 0.2 ml/min; ramp over 1 min to 10% A and 90% B, flow rate 0.3 ml/min; hold for 6 min; ramp over 1 min to 93% A and 7% B, flow rate 0.2 ml/min; Hold for 10 min to reequilibrate the system. A sample volume of 10 µl was injected in triplicate onto the column.

Positive and negative ion electrospray MS-MS was performed on a Micromass Quattro II triple quadrupole mass spectrometer (Beverly, MA, USA) interfaced to the 1100 HPLC system using a megaflow electrospray probe. High purity nitrogen was used as ESI nebulizing gas and drying gas. MS control and spectral processing was carried out using Masslynx software, version 2.22 (Micromass, Beverly, MA, USA). The abundant precursor ion of each analyte produced by positive and negative electrospray ionization (ESI) was selected for fragmentation in the collision cell containing argon gas (99.999% purity) maintained at approximately 1.2 x 10^{-4} Torr. The precursor and collision-induced fragment ions were monitored by the post-collision quadrupole analyzer, 0-5.3 min for

3TC and d4T, and 5.3-9.0 min for aprobarbital and efavirenz. A summary of the cone voltages, collision energies, and precursor and product ions of the analytes is presented in Table 1. The source temperature and needle voltage were set at 150 °C and 3.0 kV, respectively.

Preparation of standards

Stock solutions of lamivudine (3TC), stavudine (d4T), efavirenz and the internal standard (aprobarbital) were prepared by dissolving appropriate amounts of drug in absolute methanol to obtain final drug concentrations of 540, 1038, 173 and 1600 µg/ml, respectively. Working solutions were prepared by further diluting these stock solutions in an ammonium acetate buffer solution. Calibration plots for the analytes in serum were prepared by spiking drug-free serum with the standard stock solution to yield concentrations of 1.1-540 ng/ml (1.1, 5.4, 10.8, 54, 270 and 540 ng/ml) for 3TC, 12.5-6228 ng/ml (12.5, 62.8, 124.6, 622.8, 3114 and 6228 ng/ml) for d4T and 1.0-519 ng/ml (1.0, 5.2, 10.4, 51.9, 260, and 519 ng/ml) for efavirenz.

Sample preparation procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted solutions and 10 µl of a 1600 µg/ml internal standard aprobarbital solution were added to blank serum to a final volume of 1 ml. Solid phase extraction (SPE) was used to extract anti-HIV drugs and the internal standard from human serum. Extraction cartridges (Waters Oasis^{TM} HLB 1cc 30 mg) were placed on a vacuum elution manifold (VAC-ELUT^{TM}, Varian Sample Preparation Products, Harbor

City, CA 90710) and rinsed with 1 ml of methanol followed by 1 ml of purified water. Care was taken that the cartridges did not run dry. One milliliter of the spiked serum samples was loaded onto the cartridges and drawn by applying vacuum. The cartridges were then washed with 1 ml of 20 mM ammonium acetate buffer (pH 7.0) followed by vacuum suction for 1 min. One milliliter of methanol was used to elute the adsorbed analytes, which were then concentrated in a vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY, USA). Extracts were reconstituted in 50 µl of mobile phase and injected into the LC-MS-MS system.

Ion suppression testing

A postcolumn infusion of the individual analytes was performed during injections of extracted blank serum samples using the same LC-MS-MS conditions described previously [15]. Lamivudine (3TC), stavudine (d4T), efavirenz and the internal standard (aprobarbital) solutions were prepared by diluting the stock solutions in ammonium acetate buffer to obtain final drug concentrations of 5.40, 10.38, 17.30 and 16.00 µg/ml, respectively. Each analyte was infused individually through a zero dead volume tee at a flow rate 30 µl/min. Eluate from the HPLC column combined with the infused analytes and entered the mass spectrometer through the electrospray interface.

Limit of detection (LOD) and limit of quantification (LOQ)

Serum samples were spiked with decreasing concentrations of the analytes. The limit of detection (LOD) was defined by the concentration of analyte that yields a signal-

to-noise ratio of 3. The limit of quantification (LOQ) was considered to be the lowest concentration that could be measured with a signal-to-noise ratio of 10.

Accuracy, precision, linearity and recovery

Accuracy was calculated by comparing the concentration of spiked samples to each nominal concentration. Precision was reported as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay in serum were determined by assaying three quality control samples at low, medium and high concentrations of each compound (28, 110 and 540 ng/ml for 3TC, 310, 1245 and 5000 ng/ml for d4T, 20, 75 and 400 ng/ml for efavirenz) in three analytical runs within the same day. The inter-day accuracy and precision of samples were analyzed on three different days. Calibration curves were constructed using ratios of the observed analyte peak area to internal standard versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficients, which were then used to determine the concentration of each analyte in the quality control samples. The recoveries of each drug and the internal standard were determined by comparing the peak area of each analyte after extraction with the respective non-extracted standard solutions at the same concentration.

Results and Discussion

3TC and d4T are highly hydrophilic compounds, and thus are different from the highly hydrophobic efavirenz. It was difficult to separate them simultaneously under isocratic conditions, even using ion pair chromatography. A series of HPLC columns

were investigated from silica to C_{18}, but none gave satisfactory chromatographic separations. With highly polar columns like silica, 3TC and d4T co-eluted at the solvent front. With a nonpolar column such as $C_{18,}$ efavirenz did not elute within 60 min. Thus, a gradient HPLC method was developed and validated to separate 3TC, d4T and efavirenz by changing the ratio of organic modifier to buffer and flow rate. It offered a reliable solution for the chromatography of these analytes that were difficult to analyze by other means.

The gradient elution profile was optimized to improve the spacing of peaks in the chromatogram while maintaining the required resolution and to approximate the ideal separation. It began with the selection of the concave gradient shape, which was preferred for the chromatographic separation of the early eluted polar analytes 3TC and d4T. The steepness of the gradient was then optimized in order to achieve the required resolution of the critical pair of analytes 3TC and d4T as well as shorten the retention time for all the chromatographed compounds. Finally, the initial ratio of acetonitrile in the mobile phase was conveniently adjusted with the aim of keeping the retention times as short as possible. Acetonitrile was chosen as the organic modifier in the mobile phase because of its solvent strength. Efavirenz was not eluted quickly when using methanol.

Initially, a two-step elution with the first isocratic step (gradient delay) followed by gradient elution in the second step was tried. A good separation of 3TC and d4T was achieved under near optimum isocratic conditions in the first step, while the retention of the more strongly retained efavirenz was accelerated in the second gradient step, but was still longer than 15 min. In gradient elution chromatography, a certain minimum gradient delay existed, which was determined by the inner volume of the instrumentation between

the point of mixing of the mobile phase components and the top of the column, usually 1-5 ml. This gradient delay was large for the 2.1 mm i.d. nanobore column used, and produced a longer retention time for efavirenz. When the gradient program started at the time of sample injection, a baseline separation of 3TC and d4T was achieved as well as a shorter retention time for efavirenz. In this situation, the first isocratic step (the gradient delay) was set by the inner volume of the instruments and the composition of the mobile phase was equal to the initial composition set by the gradient program.

In order to keep the separation time short, flow programming was combined with gradient elution to decrease the retention time of the last "over-resolved" analyte efavirenz. When the flow rate was increased from 0.2 to 0.3 ml/min, the retention time of efavirenz was shortened from 14 to 7 min, while column pressure increased from 150 to 210 bar.

The main problem encountered in gradient elution chromatography with UV detection is baseline drift, which may originate from different sources such as change in refractive index during gradient elution and differences in the absorption of light between components of the mobile phase. Mass spectrometry is extremely powerful in gradient elution chromatography especially with specifically selected m/z channels. A highly efficient narrow-bore column was chosen for coupling to the ion source of the quadrupole mass spectrometer using electrospray ionization (ESI) with low flow rate of the mobile phase.

The chemical structures of 3TC, d4T, efavirenz and aprobarbital (internal standard) are shown in Figure 1. Aprobarbital was chosen as internal standard because it has a similar structure to NRTI drugs and gave a good recovery from serum using the

SPE method. The positive ion electrospray ionization (ESI) mode was selected for 3TC and d4T because of improved sensitivity due to the presence of amino groups, which are easily protonated under the acidic mobile phase conditions (pH 4.5). For efavirenz, even during flow injection at a high concentration (10µg/ml), the signal of the protonated molecular ion was weak. However, the deprotonated molecular signal, $(M-H)^-$ was abundant during the negative mode. Similarly, aprobarbital was readily deprotonated due to its chemical structure.

Electrospray ionization has good ion current stability, which when coupled to the lower applied accelerating voltages in the quadrupole mass spectrometer make it possible to monitor both positive and negative ions in the same LC-MS-MS run. An HPLC-MS method involving a ionization polarity switch was first reported in 1997 by Barnes and co-workers for the analysis of pesticides [14]. The ionization polarity switch was operated during each acquisition to determine the presence of 8 compounds in the positive ion mode and 2 compounds in the negative ion mode. The instrument used by Wang et al switched between ion polarities every 0.05 s while maintaining a stable baseline [15]. In this study, positive and negative ion modes were initially monitored simultaneously using the same instrument as Wang et al. However, at low concentration levels, it was difficult to acquire enough data points to adequately define the peaks for d4T and aprobarbital. In order to improve the peak shape, the detection polarity was switched from positive to negative in the middle of the LC run (at 5.3 min) because aprobarbital and efavirenz have longer retention times on the C_6 column than 3TC and d4T.

For the NRTI drugs, 3TC and d4T, the precursor ions $[M+H]^+$ were formed as a result of the addition of a proton to form the positively charged molecular ion. For aprobarbital and efavirenz, $[M-H]^-$ ions were observed following loss of a proton. The base peaks in the CID mass spectra were m/z 112 for 3TC, m/z 127 for d4T, m/z 244 for efavirenz and m/z 166 for aprobarbital (Figure 2). The precursor and major product ions of the analytes were monitored in the multiple reaction monitoring mode (Table 1). In the case of 3TC, protonation of the nitrogen on the purine base initiates glycosidic bond cleavage and results in the loss of the sugar moiety via alpha cleavage to give the ion at m/z 112. For d4T, the fragmentation is similar to 3TC, because of its structural similarity, resulting in the formation of the ion at m/z 127 by an alpha cleavage of the glycosidic bond. Complex tandem mass spectra were generated from the parent ion of efavirenz ($[M-H]^-$ at m/z 314) with the formation of multiple fragment ions. Major fragment ions at m/z 250, 230, 69 and a base peak at m/z 244 were observed following the loss of functional groups attached to the C-4 position of the efavirenz ring system. The proposed fragmentation pathways for all analytes are illustrated in Figure 4.

Baseline separations of 3TC, d4T and efavirenz were achieved with retention times of 3.67, 4.28 and 6.86 min, respectively. The internal standard, aprobarbital, had a retention time of 5.59 min. Figure 3 shows the chromatograms obtained from drug-free human serum and a spiked serum sample. Since the precursor and major product ions of the compounds were monitored in the MRM mode, the method is highly selective and specific. There were no peaks from endogenous components observed from blank serum.

During development of the solid-phase extraction method, a series of different extraction cartridges were investigated, such as C_{18}, C_8, phenyl and Oasis^{TM} cartridges.

C_{18} and Oasis™ cartridges did not show any loss of analytes when loading spiked serum samples. The Oasis™ SPE cartridges provided the highest recoveries for all drugs analyzed in this study. Twenty millimolar ammonium acetate (pH 7.0) was used to wash the cartridges after loading spiked serum to help retain the highly hydrophilic analytes 3TC and d4T and thus obtain a clean sample. Methanol was strong enough to elute all of the analytes and was also easily evaporated. Absolute recoveries greater than 89.6% were obtained for all three analytes and the internal standard. The detailed data is listed in Table 2. The recovery for the internal standard was 96.2%.

As a part of the method development, the effect of ion suppression from the matrix on the quantitative LC-MS-MS analysis of the four analytes was studied. In related chromatograms for each analyte including the internal standard, ion suppression was observed around 2.0 min. A second region of ion suppression occurred around 5.0 min. No ion suppression was observed at the retention times of the analytes or the internal standard. The current explanation for ion suppression in electrospray ionization is that a combination of solution and gas-phase reactions involving analyte ions and other components from the biological sample result in the loss of signal from the analyte ion [16]. The matrix effect is especially dependent on the degree of sample cleanup, the degree of chromatographic separation, and the retention of analytes on the analytical column [17]. In this method, solid-phase extraction was used to isolate the samples and the analytes were retained in the column long enough to avoid ion suppression. Careful assessment of matrix effects constitutes an integral and important part of any quantitative LC-MS-MS assay validation procedure in biological fluids.

The calibration curves showed good linearity in the range of 1.1-540 ng/ml for 3TC, 12.5-6228 ng/ml for d4T and 1.0-519 ng/ml for efavirenz. The correlation coefficients (r^2) of calibration curves of each drug were higher than 0.99 as determined by least-squares analysis. LOD and LOQ data are shown in Table 3. The LOD for 3TC, d4T and efavirenz were 0. 5, 6.2 and 0.5 ng/ml, respectively. The LOQ for 3TC, d4T and efavirenz were 1.1, 12.5 and 1.0 ng/ml, respectively. The results from the validation of the method in human serum are listed in Table 2. The method proved to be accurate (relative error at high, medium and low concentration) less than 14.0% for inter-day and less than 12.7% for intra-day) and precise (inter-day precision less than12.8% and intra-day precision less than 13.1%).

Conclusion

Solid phase extraction and LC-ESI-MS-MS methods with ionization polarity swith provide a fast, sensitive and selective procedure for the simultaneous determination of a 3TC/d4T/efavirenz mixture in human serum. The analytes in this method were shown to be free from ion supprerssion effects generated by the matrix. This method is one of the first demonstrations of a quantitative LC-MS-MS assay employing ionization potarity switching and should be useful in the routine monitoring of serum drug concentrations and in pharmacokinetic studies of HIV-infected patients.

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novel non-nucleoside reverse transcriptase inhibitor, in human plasma using isocratic reversed-phase high-performance liquid chromatography with ultraviolet detection. J.Chromatogr. B. 1999; **734(1)**: 55-61.

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Table 1. Optimized ESI (+) and ESI (-) mass spectrometric conditions for multiple reaction monitoring (MRM)

Compound	Parent Ion (m/z)	Daughter Ions (m/z)	Collision Energy (eV)	Cone Voltage (V)
Lamivudine (3TC)	230	112	15	30
Stavudine (d4T)	225	127	10	15
Aprobarbital (IS)	209	166	22	30
Efavirenz	314	244	19	35

Table 2. Inter-day and intra-day accuracy, precision and recovery for the analysis of 3TC, d4T and efavirenz in human serum

	Concentration	Precision[a] (%)		Accuracy[a] (%error)		Recovery[b]
	(ng/ml)	Intra-day	Inter-day	Intra-day	Inter-day	(%)
3TC	500	2.4	7.4	2.1	3.3	97.9±1.9
	108	2.2	12.8	11.8	11.4	100.6±4.1
	30	0.2	6.4	3.1	9.8	91.9±3.5
d4T	5000	5.8	11.9	12.2	14.0	94.3±3.5
	1245	2.3	8.6	12.7	0.5	91.7±2.3
	310	3.9	2.7	2.3	3.4	89.6±1.7
Efavirenz	400	13.1	5.2	5.9	13.9	100.2±1.2
	75	4.3	3.8	4.8	11.3	93.0±2.9
	20	5.5	5.9	2.8	13.9	92.8±3.0

a. Based on n = 9

b. Mean ± SD based on n = 6

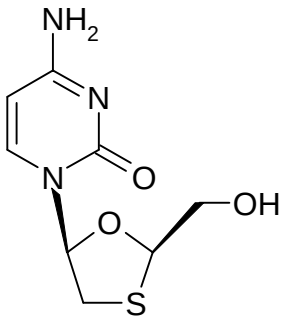
Table 3. Range of calibration curves, limits of detection (LOD) and limits of quantification (LOQ) of 3TC, d4T and efavirenz in spiked human serum

Drug	Range of calibration curves (ng/ml)	Limit of detection (LOD) (ng/ml)[a]	Limit of quantification (LOQ) (ng/ml)[b]
3TC	1.1—540	0.5	1.1
d4T	12.5—6228	6.2	12.5
Efavirenz	1.0—519	0.5	1.0

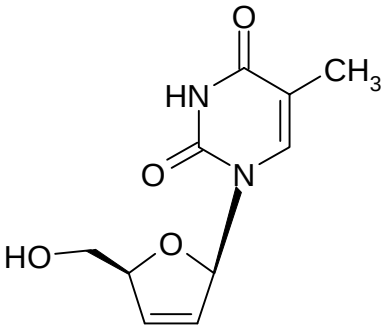
a. S/N=3

b. S/N=10

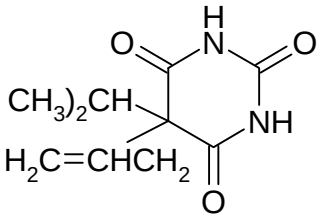
Figure 1. Chemical structures of 3TC, d4T, aprobarbital (I.S.) and efavirenz



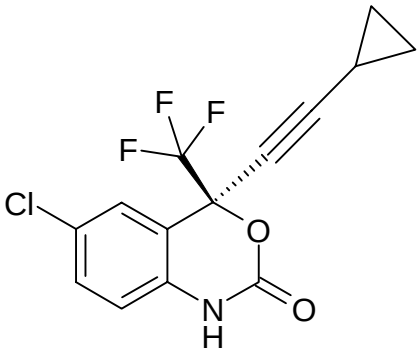
Lamivudine (3TC)



Stavudine (d4T)



Aprobarbital



Efavirenz

Figure 2. Mass spectra of efavirenz, aprobarbital (internal standard), d4T and 3TC

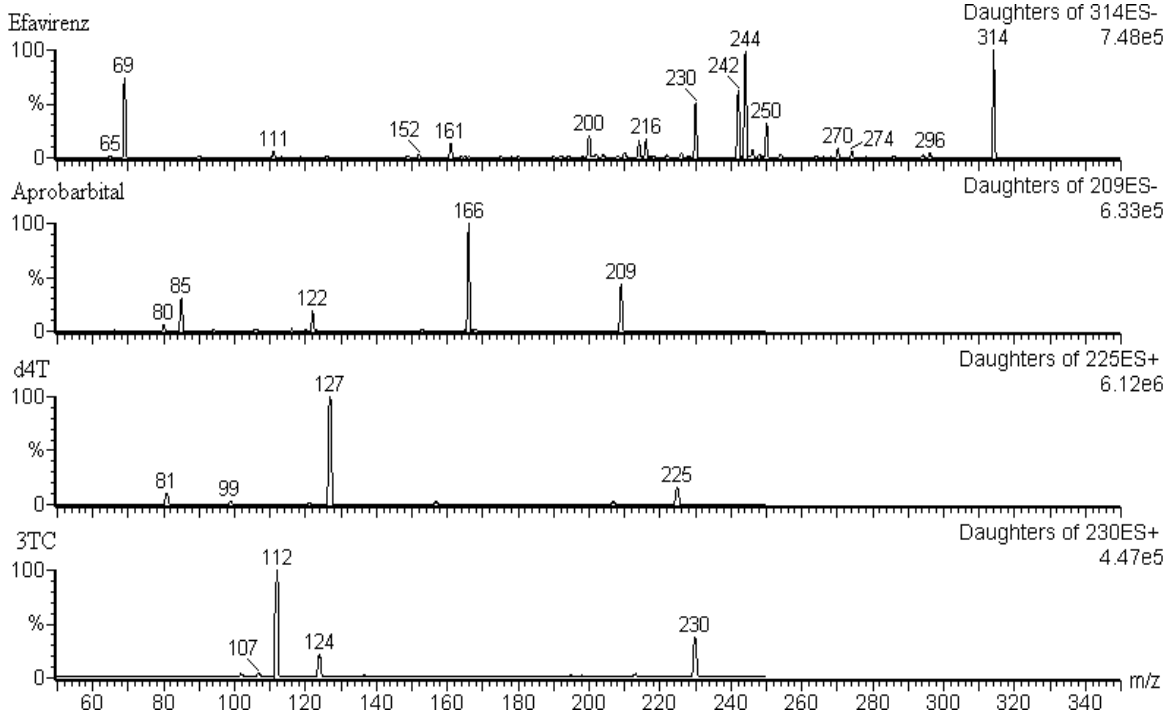
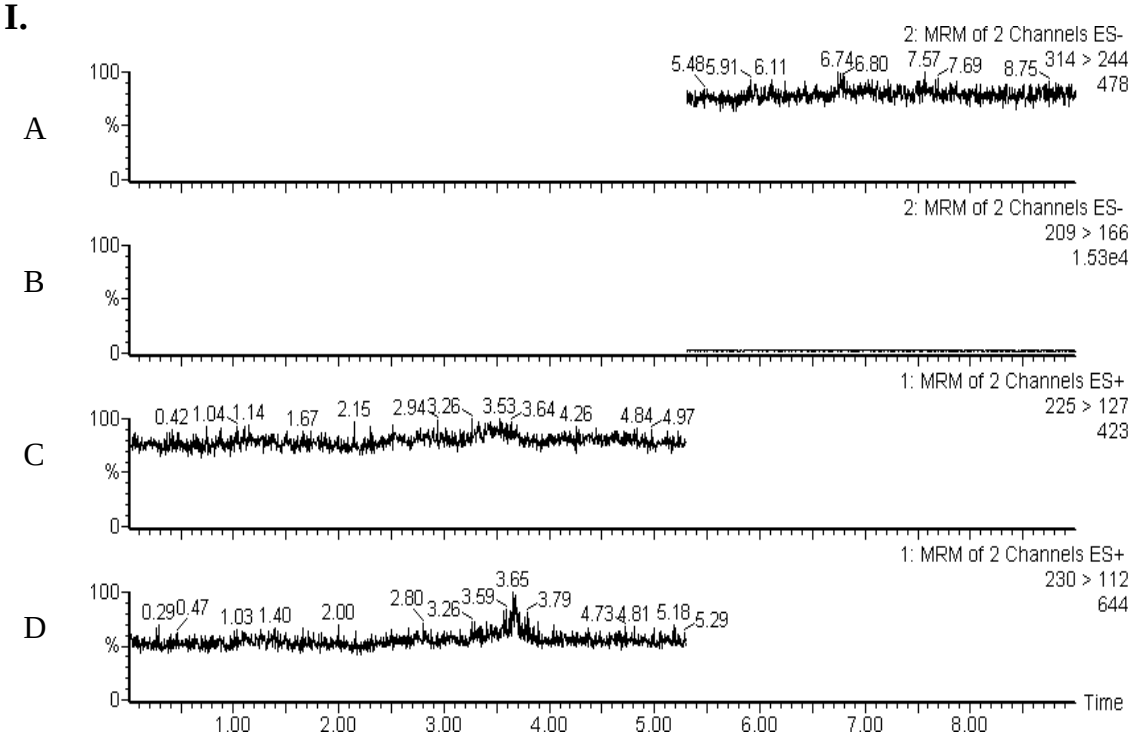

Efavirenz
Daughters of 314ES-
314
7.48e5
100
%
0
69
65
111
152
161
200
216
230
242
244
250
270
274
296
Aprobarbital
Daughters of 209ES-
6.33e5
166
209
85
80
122
d4T
Daughters of 225ES+
6.12e6
127
225
81
99
3TC
Daughters of 230ES+
4.47e5
112
230
107
124
m/z
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340

Figure 3. Chromatograms of I. blank human serum and II. human serum spiked with (A) efavirenz (B) internal standard, (C) d4T and (D) 3TC.

I.


A
2: MRM of 2 Channels ES-
314 > 244
478
5.48 5.91 6.11 6.74 6.80 7.57 7.69 8.75
B
2: MRM of 2 Channels ES-
209 > 166
1.53e4
C
1: MRM of 2 Channels ES+
225 > 127
423
0.42 1.04 1.14 1.67 2.15 2.94 3.26 3.53 3.64 4.26 4.84 4.97
D
1: MRM of 2 Channels ES+
230 > 112
644
0.29 0.47 1.03 1.40 2.00 2.80 3.26 3.59 3.65 3.79 4.73 4.81 5.18 5.29
100
%
0
Time
1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00

II.

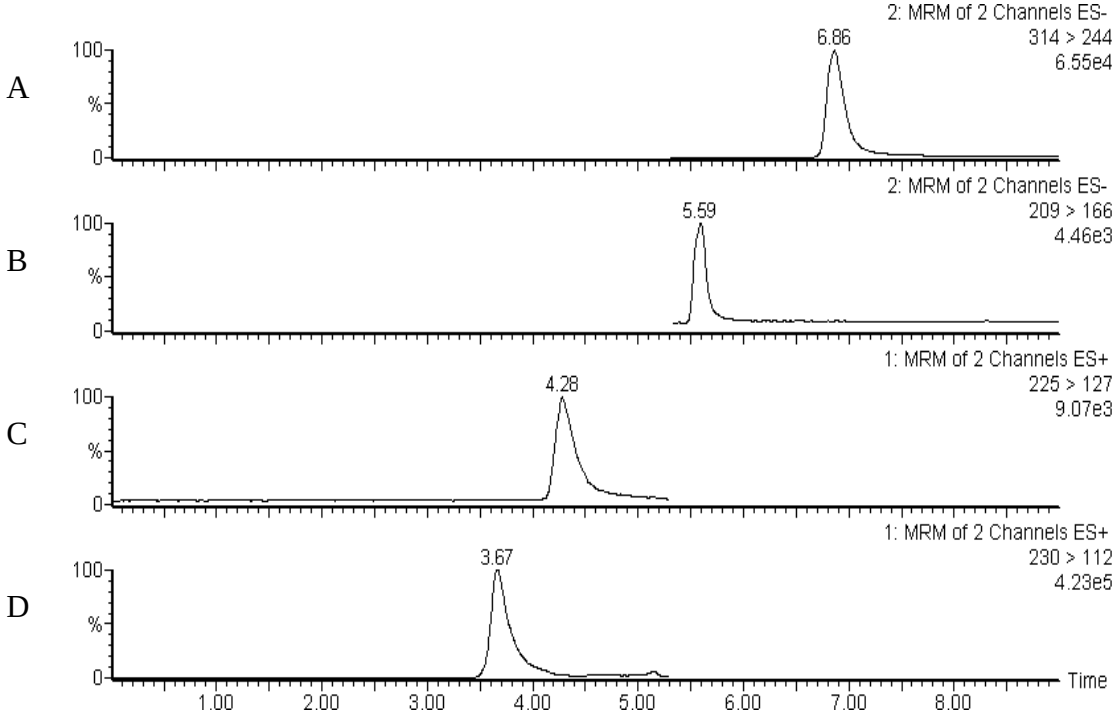
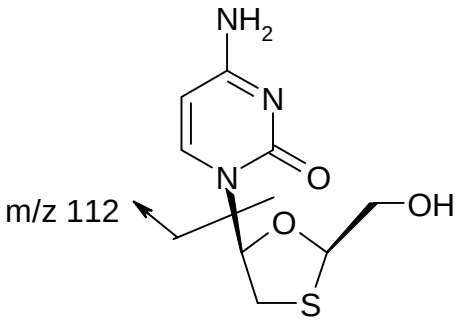
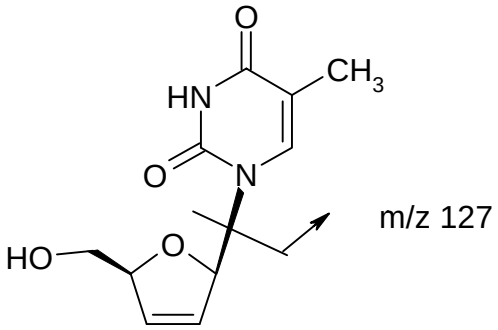

A
2: MRM of 2 Channels ES-
314 > 244
6.55e4
6.86
B
2: MRM of 2 Channels ES-
209 > 166
4.46e3
5.59
C
1: MRM of 2 Channels ES+
225 > 127
9.07e3
4.28
D
1: MRM of 2 Channels ES+
230 > 112
4.23e5
3.67
100
%
0
Time
1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00

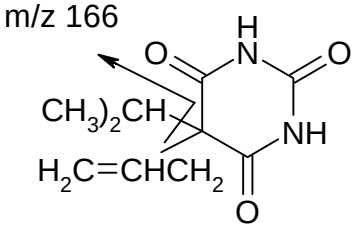
Figure 4. Proposed fragmentation pathways for 3TC, d4T, aprobarbital (I.S.), and efavirenz



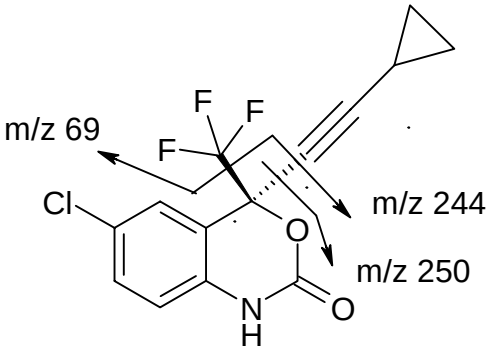
Lamivudine (3TC)



Stavudine (d4T)



Aprobarbital



Efavirenz

CHAPTER 6

DETERMINATION OF DIDANOSINE/STAVUDINE/RITONAVIR IN HUMAN SERUM USING SOLID PHASE EXTRACTION/LIQUID CHROMATOGRAPHY/ ELECTROSPRAY TANDEM MASS SPECTROMETRY[1]

[1]Fan, Bin. Michael G. Bartlett and James T. Stewart. To be submitted to Journal of Pharmaceutical and Biomediacal Analysis, 2001.

Abstract

A high-performance liquid chromatography (HPLC) method utilizing tandem mass spectrometric (MS-MS) detection was developed and validated in human serum for the simultaneous measurement of the didanosine (ddI)/stavudine (d4T)/ritonavir triple combination therapy for HIV. Solid phase extraction (SPE) was used to extract these anti-HIV drugs and the internal standard, nifedipine. The compounds were separated on a C_{18} column (150 x 2.1 mm i.d.) using a gradient consisting of acetonitrile and 10 mM ammonium acetate buffer with pH adjusted to 4.5 with acetic acid. The precursor and major product ions of the analytes were monitored on a triple quadrupole mass spectrometer with positive ion electrospray ionization (ESI) operated in the multiple reaction monitoring (MRM) mode. The effect of ion suppression from the matrix was studied and found not to interfere with the analysis. The method was validated over the range of 10.2-5100 ng/ml for ddI, 20.8-5190 ng/ml for d4T and 1.7-5760 ng/ml for ritonavir. The method was shown to be accurate, with intra-day and inter-day accuracy less than 12.9% and precise, with intra-day and inter-day precision less than 13.8%. Extraction recoveries of all analytes were greater than 85.0%. The assay should be suitable for use in pharmacokinetic studies and the routine plasma monitoring of this triple drug therapy.

Keywords: Didanosine; Stavudine; Ritonavir; Nifedipine; Solid-phase extraction; Tandem Mass Spectrometry; Electrospray Ionization; Multiple Reaction Monitoring; Human Serum

Introduction

The appearance in the clinic of two to three new antiretroviral agents yearly since 1995 has permitted unprecedented advances in HIV treatment. These combination therapies have proven to be the most effective approach for the treatment of HIV disease [1-3]. Combination therapies not only increase viral suppression, but also prevent drug resistance and optimize drug exposure. The preferred option from the International AIDS Society-USA (IAS) and the U.S. Department of Health and Human Services (HHS) is a three-drug regimen with at least two nucleoside reverse transcriptase inhibitors (NRTI) and a highly active protease inhibitor (PI) such as ritonavir [1, 4]. NRTIs are the cornerstone of most antiretroviral regimens. There are several combinations of NRTI to choose from in the initial three-drug regimen and, until very recently, the primary treatment combinations were based on AZT [5]. An alternative is to base the NRTI combination on d4T, which is easy to tolerate and penetrates the central nervous system. Didanosine (ddI) and stavudine (d4T), once thought to be contraindicated in combination because of their overlapping peripheral neuropathic toxicity, have proven to be well tolerated and effective [6-8]. These drugs are safe and effective in prolonging life, particularly when used in combination [9,10]. When used as part of a multidrug regimen with ritonavir, they delay the development of HIV resistance to ritonavir [5, 8].

Therapeutic monitoring of these drugs is recommended in order to avoid or delay drug resistance from the virus, to avoid the usual underestimated patient compliance and to manage drug interactions. Analytical methods have been described to quantify these individual drugs in biological media [11-13]; however, no methods have been reported for the simultaneous determination of this important antiretroviral triple combination. We

have developed and validated a gradient high-performance liquid chromatography (HPLC) method utilizing triple quadrupole mass spectrometry (MS) detection for the simultaneous determinations of ddI, d4T and ritonavir in human serum. Because this combination includes drugs from both NRTI and PI categories, they have quite different physiochemical characteristics, such as polarity and solubility. The method is also potentially suitable for drug monitoring and determination of pharmacokinetic profiles.

Material and Methods

Chemicals and reagents

Didanosine (ddI), stavudine (d4T) and nifedipine (internal standard) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Ritonavir was kindly provided by Abbott Laboratories (North Chicago, IL 60064). Ammonium acetate, acetic acid, HPLC grade methanol and acetonitrile were from J.T. Baker Inc. (Phillipsburg, NJ 08865). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Oasis HLB solid phase cartridges were obtained from Waters Corporation (Milford, MA 01757). Drug free human serum was obtained from Bioreclamation Inc., Lot # BRH01495 (Hicksville, NY 11801).

Instrumentation and LC-MS-MS conditions

The HPLC system consisted of an Agilent Technologies 1100 HPLC system equipped with a vacuum degassing module, quaternary pump and an autosampler (Palo Alto, CA, USA). The column utilized was a Supelcosil LC-18DB (150 x 2.1 mm i.d., 3

μm particle size, Supelco, Inc. Bellefonte, PA, USA) protected by an ADVCART™ Advantage 100 C_{18} guard cartridge (15 x 3.2, 5 μm, SCI-CON, Winter Park, FL 32789).

A gradient condition was applied at ambient temperature with (A) 10 mM ammonium acetate buffer with pH adjusted to 4.5 with acetic acid and (B) acetonitrile. The gradient conditions were as follows: from 0 min, 92% A and 8% B, flow rate 0.2 ml/min; ramp over 1 min to 10% A and 90% B, flow rate 0.3 ml/min; hold for 6 min; ramp over 1 min to 92% A and 8% B, flow rate 0.2 ml/min; hold for 10 min to reequilibrate the system.

Positive ion electrospray MS-MS was performed on a Micromass Quattro II triple quadrupole mass spectrometer (Beverly, MA, USA) interfaced to the 1100 HPLC system using a megaflow electrospray probe. High purity nitrogen was used as the ESI nebulizing and drying gas. MS control and spectral processing was carried out using Masslynx software, version 2.22 (Micromass, Beverly, MA, USA). The positively charged molecules of each analyte were selected and focused into the collision cell containing argon gas (99.999% purity) maintained at a pressure of approximately 1.2 x 10^{-3} Torr. The precursor and collision-induced fragment ions were monitored by a post-collision quadrupole analyzer. A summary of the cone voltages, collision energies, and precursor and product ions of the analytes are presented in Table 1. The source temperature and needle voltage were set at 150 °C and 3.0 kV, respectively.

Preparation of standard stock solutions

Stock solutions of didanosine (ddI), stavudine (d4T), ritonavir and the internal standard (nifedipine) were prepared by dissolving appropriate amounts of each drug in

absolute methanol to obtain final drug concentrations of 1020, 1038, 1152 and 592 µg/ml, respectively. Working solutions were prepared by further diluting these stock solutions in ammonium acetate buffer solution. Calibration plots for the analytes in serum were prepared by spiking drug-free serum with standard stock solution to yield concentrations of 10.2-5100 ng/ml (10.2, 25.5, 102, 510, 1020, 2550 and 5100 ng/ml) for ddI, 20.8-5190 ng/ml (20.8, 51.9, 103.8, 519, 2595 and 5190 ng/ml) for d4T and 1.7-5760 ng/ml (1.7, 8.6, 17.3, 34.5, 172.8, 864, and 5760 ng/ml) for ritonavir. A sample volume of 10 µl was injected in triplicate into the LC-MS-MS system.

Sample preparation procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various diluted solutions and 30 µl of a 2.96 µg/ml internal standard solution were added to blank serum to a final volume of 1 ml. Extraction cartridges (Waters Oasis^{TM} HLB 1cc 30 mg) were placed on a vacuum elution manifold (VAC-ELUT^{TM}, Varian Sample Preparation Products, Harbor City, CA 90710) and rinsed with 1 ml of methanol followed by 1 ml of deionized water. Care was taken that the cartridges did not run dry. One ml of the spiked serum samples was loaded onto the cartridges and drawn by applying a vacuum. The cartridges were then washed with 1 ml 99:1 v/v 25 mM ammonium acetate buffer (pH 7.0)/acetonitrile followed by vacuum suction for 1 min. One ml of methanol was used to elute the adsorbed analytes, which were then concentrated in a Model 110A vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY, USA) and reconstituted in 50 µl of mobile phase prior to injection into the LC-MS-MS system.

Ionization suppression

A postcolumn infusion of the individual analytes was performed during injections of extracted blank serum sample using the same LC-MS-MS condition described previously [15]. The analytes didanosine (ddI), stavudine (d4T), ritonavir and the internal standard (nifedipine) solution were prepared by diluting the stock solutions in ammonium acetate buffer to final drug concentrations of 10.20, 10.38, 11.52 and 17.30 µg/ml, respectively. Each analyte including the internal standard was infused individually using another Agilent Technologies 1100 HPLC system through a zero dead volume tee at flow rate of 30 µl/min. Effluent from the HPLC column combined with the infused analytes and entered mass spectrometer through the electrospray interface.

Limit of detection (LOD) and limit of quantification (LOQ)

Serum samples were spiked with decreasing concentrations of the analytes. The limit of detection (LOD) was defined by the concentration that yields a signal-to-noise ratio of 3. The limit of quantification (LOQ) was considered to be the lowest concentration that could be measured with a signal-to-noise ratio of 10.

Calculations of accuracy, precision, linearity and recovery

Accuracy was calculated by comparing the concentration of spiked samples to the nominal concentration of each analytes. Precision was reported as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay in plasma were determined by assaying three quality control samples at low, medium and high

concentrations for each compound (50, 250 and 1500 ng/ml for ddI, 200, 1250 and 4000 ng/ml for d4T, 75, 250 and 1250 ng/ml for ritonavir) in three analytical runs within the same day. The inter-day accuracy and precision of samples were analyzed on three different days. Triplicate injections of each analyte concentration were performed. The concentration of each quality control sample was determined from the slopes of standard curves of the peak area ratio of compound to internal standard against concentration used for the calibration curve. Calibration curves were constructed using ratios of the observed analyte peak area to internal standard versus concentration of analyte. Linear regression analysis of the data gave slope, intercept and correlation coefficient data, which were then used to calculate analyte concentration in each sample.

The recoveries of each drug and internal standard in serum were determined by comparing the peak area of each analyte after SPE extraction with the respective non-extracted standard solution at the same concentration. Low, medium and high concentrations for each compound were checked (50, 250 and 1500 ng/ml for ddI, 200, 1250 and 4000 ng/ml for d4T, 75, 250 and 1250 ng/ml for ritonavir).

Results and Discussion

Both ddI and d4T are very similar in chemical structure as well as physiochemical properties, but they are completely different from ritonavir (see Figure 1). Thus, it was difficult to separate the analytes simultaneously under isocratic conditions, even using ion-pairing reagents. A series of HPLC columns were investigated from silica to C_{18}, but none provided satisfactory chromatographic separations. In a polar column like silica, ddI and d4T eluted near the solvent front, and were not well separated from each other. In

a nonpolar column such as $C_{18,}$ ddI and d4T were baseline separated, but ritonavir did not elute within 90 min.

This antiretroviral mixture was then chromatographically separated by a newly developed and validated gradient HPLC method, which was able to separate this chemically diverse combination. The gradient profile was optimized in order to improve the spacing of peaks in the chromatogram while maintaining the required resolution. It began with the selection of the concave gradient shape, which was preferred for better chromatographic separation of the early eluting polar analytes ddI and d4T. Then the steepness of the gradient was optimized in order to achieve the required resolution of the critical pair of analytes ddI and d4T, as well as providing a short retention time for all chromatographed compounds. Finally, the initial ratio of acetonitrile in the mobile phase was adjusted with the aim of keeping the time of separation as short as possible. Acetonitrile was chosen as organic solvent in the mobile phase because of its solvent strength. Ritonavir did not elute quickly when using methanol in the mobile phase.

Initially a two-step elution, with an isocratic step (gradient delay) followed by gradient elution in the second step was tried. Good separation of ddI and d4T was achieved under isocratic conditions in the first step, while the retention of the more strongly retained ritonavir was accelerated in the second gradient step, but was still longer than 15 min. In practical operation with gradient elution chromatography, a certain minimum gradient delay exists, which is determined by the inner volume of the connecting tubing between the point of mixing of the mobile phase components and the top of the column (usually 1-5 ml). This gradient delay was relatively large to the 2.1 mm i.d. narrowbore column used, and thus gave longer retention times. When the gradient

program started at the time of sample injection, baseline separation of ddI and d4T was still achieved at a much shorter retention time. Thus, the first isocratic step (gradient delay) was set by the inner volume of the HPLC connecting tubing and the composition of the mobile phase was equal to the initial composition set by the gradient program.

In order to keep the separation time as short as possible, flow programming was combined with gradient elution to decrease the retention time of the "over-resolved" late eluting analyte ritonavir. When the flow rate was increased from 0.2 ml/min to 0.3 ml/min, the retention time of ritonavir was shortened from 13 min to 7 min, while the column pressure increased from 160 to 200 bar.

The main problem in gradient elution liquid chromatography with UV detection is baseline drift, which may originate from different sources such as changes in the refractive index during gradient elution and differences in the absorption of light between components of the mobile phase. Mass spectrometry is extremely powerful when using gradient elution chromatography with specifically selected m/z channels. When developing the method, a highly efficient narrowbore column was chosen for coupling to the ion source of a quadrupole mass spectrometer using electrospray ionization (ESI) with low flow-rates of the mobile phase.

For electrospray ionization, the positive ion mode was selected because of improved sensitivity due to the presence of amine groups, which were easily protonated under acidic conditions (pH 4.5). Nifedipine, a calcium channel inhibitor, was chosen as the internal standard because it would also be positively charged, giving a strong signal in the mass spectrometer as well as good recovery from serum using the SPE method, and was unlikely to be co-administered to patients with an HIV combination therapy.

For each compound, the precursor ion $[M+H]^+$, was formed as a result of the addition of a proton to form the positively charged molecular ion. The product ion base peak from the CID mass spectra were m/z 137 for ddI, m/z 127 for d4T, m/z 296 for ritonavir and m/z 315 for nifedipine (see Figure 2). The precursor and major product ions of the analytes were monitored in the multiple reaction mode (Table 1). In the case of ddI, protonation of N-9 on the purine base initiates glycosidic bond cleavage resulting in the loss of the sugar moiety via alpha cleavage to give the ion at m/z 137. For d4T, the fragmentation is similar to ddI because of their structural similarity. The ion at m/z 127 was formed by an alpha cleavage in the glycosidic bond following protonation at N-1. Protonated ritonavir generated a complex tandem mass spectrum with the formation of multiple fragment ions. The major fragment ions at m/z 426, 268, 197, 171 and the base peak at m/z 296 were observed by cleavage along the carbamide backbone. The proposed fragmentation pathways are summarized in Figure 4. The internal standard, nifedipine is a derivative of 1,4-dihydropyridine (DHP), and fragments to form the ion at m/z 315 following elimination of methanol.

Baseline separations of ddI, d4T and ritonavir were achieved with retention times of 3.4, 4.0 and 7.0 min, respectively. The internal standard, nifedipine gave a retention time of 6.5 min. Figure 3 shows the chromatograms obtained from drug-free human serum and a spiked serum sample. Since the precursor and major product ions of the compounds were monitored using MRM, the method is highly selective and specific. There are no other peaks from endogenous serum interferences, and there is only a low amount of random electronic noise.

During development of the solid-phase extraction method, a series of different extraction cartridges were investigated, such as C_{18}, C_8, phenyl, Oasis^{TM} cartridges. The C_{18} and Oasis^{TM} Cartridges did not show sample breakthrough when loading the spiked serum samples. The Oasis^{TM} cartridge provided the highest overall recoveries of these analytes. Twenty-five mM ammonium acetate (pH 7.0) was used to wash the cartridges after loading spiked serum to help retain the highly hydrophilic analytes ddI and d4T. Acetonitrile (1%) was added to the wash solution to remove endogenous interferences from serum and to provide a cleaner sample. Methanol was strong enough to elute all of the analytes and was easily evaporated. Absolute recoveries greater than 85% were obtained for all three analytes and the internal standard. The detailed recovery data is provided in Table 2.

In order to investigate the matrix effect in quantitative LC-MS-MS analysis of biological samples, such as in human serum, determination of ion suppression was performed. In the related chromatograms for each analyte including the internal standard, ion suppression was observed around 2.0 min. A second region of ion suppression occurred around 5.4 min. No ion suppression was shown at the retention times for the analytes or the internal standard. The current explanation for ion suppression in electrospray ionization is that gas-phase reactions involving analyte ions and other components from the biological sample result in the loss of signal from the analyte ion [14]. The matrix effect is especially dependent on the degree of sample cleanup and the degree of chromatographic separation and retention of the analytes on the analytical column [15]. In this method, solid-phase extraction was used to isolate the samples and the analytes were held in the column long enough to avoid ion suppression.

The calibration curves showed good linearity in the concentration range of 10.2-5100 ng/ml for ddI, 20.8-20760 ng/ml for d4T and 1.7-5760 ng/ml for ritonavir. The correlation coefficients (r^2) of calibration curves of each drug were higher than 0.99 as determined by least-squares analysis. LOD and LOQ data are shown in Table 3. The LOD for ddI, d4T and ritonavir were 1.0, 4.2 and 0.3 ng/ml, respectively. The LOQ for ddI, d4T and ritonavir were 10.2, 20.8 and 1.7 ng/ml, respectively. The results from the validation of the method in human serum are listed in Table 2. The method proved to be accurate (relative error at high and low concentration from 5.8 to 11.9% for intra-day and from 1.1 to 12.9 % for inter-day) and precise (intra-day precision ranged from 1.6 to 6.6% and inter-day precision ranged from 1.0 to 13.8%).

Conclusion

A solid phase extraction and electrospray LC-MS-MS method provided a fast, sensitive and selective procedure for the simultaneous determination of ddI/d4T/ritonavir in human serum. The HPLC method should be suitable for monitoring serum drug concentrations, and for pharmacokinetic studies in HIV-infected patients.

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Table 1. Optimized ESI (+) mass spectrometric conditions for multiple reaction monitoring (MRM)

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (eV)	Cone Voltage (V)
Didanosine (ddI)	237	137	10	21
Stavudine (d4T)	225	127	10	15
Nifedipine (IS)	347	315	15	25
Ritonavir	721	296	20	50

Table 2. Inter-day and intra-day accuracy, precision and recovery for the analysis of ddI, d4T and ritonavir in human serum

	Concentration	Precision[a] (%)		Accuracy[a] (%error)		Plasma Recovery[b]
	(ng/ml)	Intra-day	Inter-day	Intra-day	Inter-day	(%)
ddI	1500	1.7	4.8	11.0	5.0	93.6±2.1
	250	5.8	6.3	5.8	11.8	94.0±2.3
	50	6.6	13.8	8.9	1.1	85.0 ±1.6
d4T	4000	1.9	1.0	9.2	5.1	92.3±3.3
	1250	2.1	6.9	8.1	1.4	91.7±2.1
	200	1.6	1.4	11.9	5.1	88.0±1.5
Ritonavir	1250	2.8	9.5	8.6	11.6	108.7±6.3
	250	4.4	11.3	10.3	12.9	102.8±4.4
	75	6.5	13.6	8.1	1.7	95.0±1.6

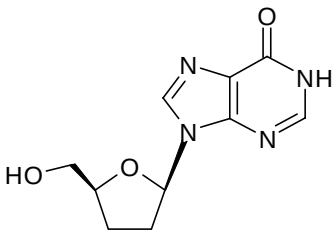
a. Based on n = 9
b. Mean ± SD based on n = 6

Table 3. Range of calibration curves, limits of detection (LOD) and limits of quantification (LOQ) of ddI, d4T and ritonavir in spiked human serum

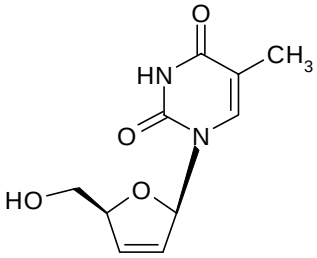
Drug	Range of calibration curves (ng/ml)	Limit of detection (LOD) (ng/ml)[a]	Limit of quantification (LOQ) (ng/ml)[b]
ddI	10.2—5100	1.0	10.2
d4T	20.8—5190	4.2	20.8
Ritonavir	1.7—5760	0.3	1.7

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

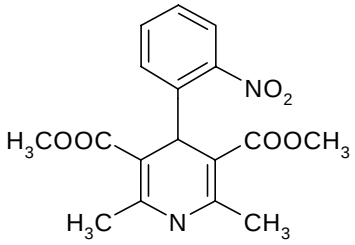
Figure 1. Chemical structures of the analytes studied



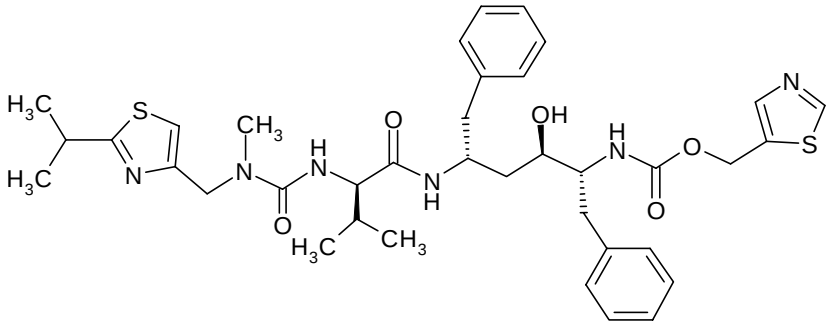
Didanosine (ddI)



Stavudine (d4T)



Nifedipine



Ritonavir

Figure 2. Mass spectra of ddI, d4T, internal standard nifedipine and ritonavir

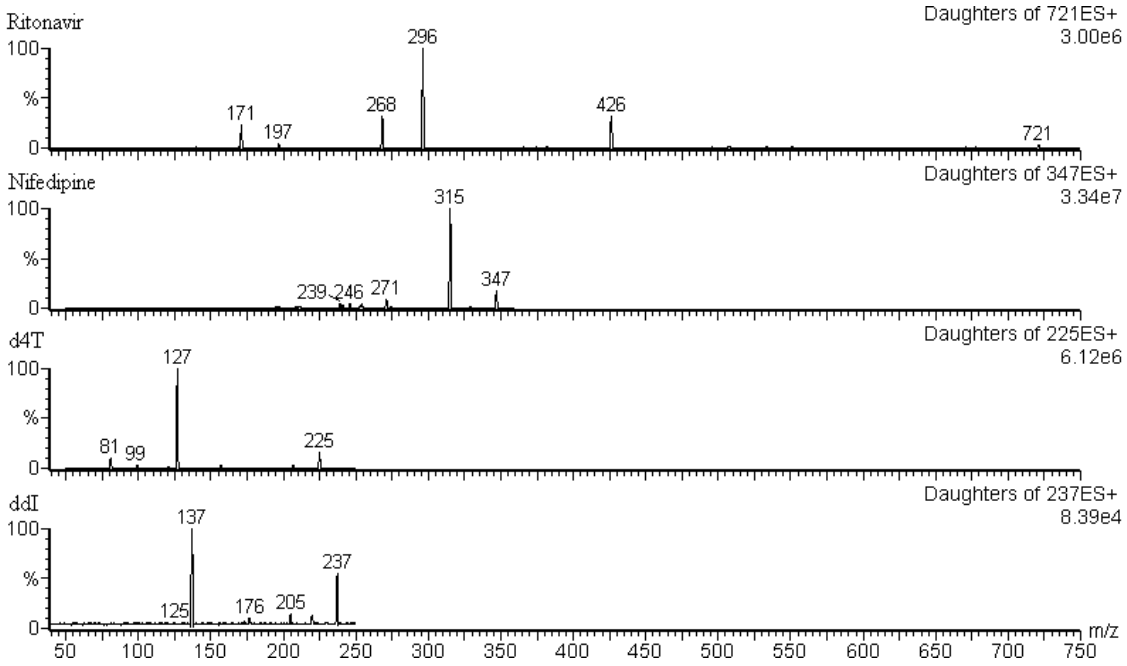
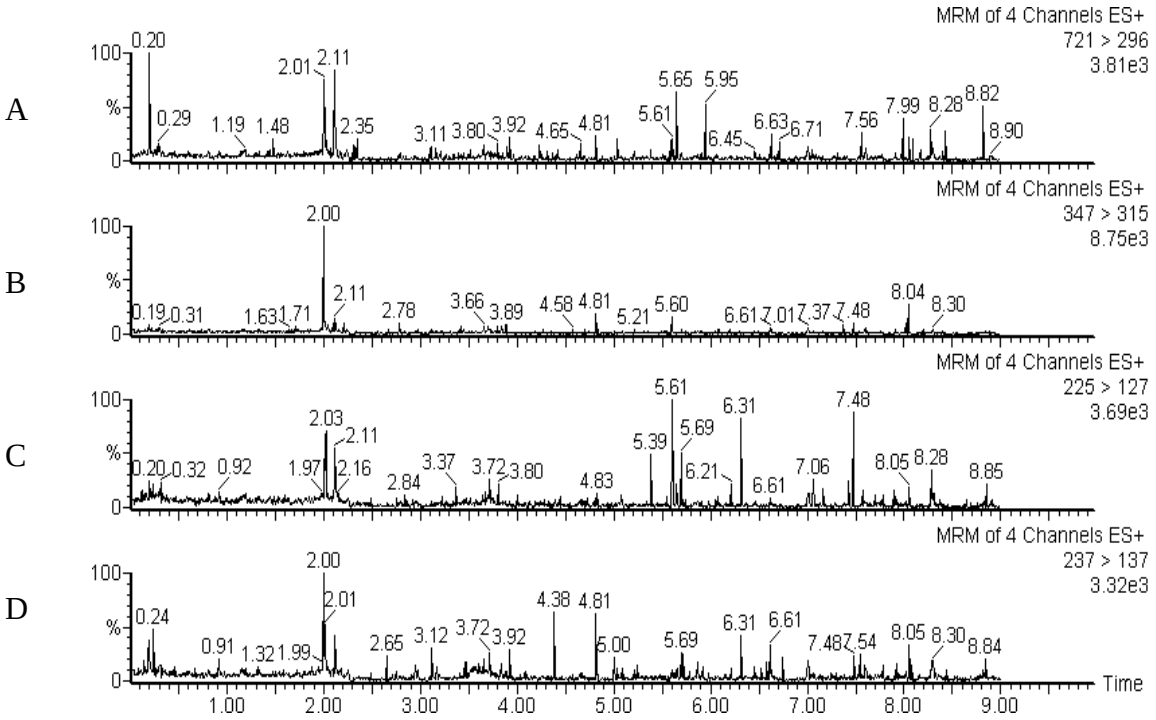

Ritonavir
Daughters of 721ES+
3.00e6
100
%
0
296
171
197
268
426
721
Nifedipine
Daughters of 347ES+
3.34e7
315
239
246
271
347
d4T
Daughters of 225ES+
6.12e6
127
81
99
225
ddI
Daughters of 237ES+
8.39e4
137
237
125
176
205
m/z
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750

Figure 3. Chromatograms of I. blank human serum and II. human serum spiked with (A) ritonavir (B) internal standard, (C) d4T and (D) ddI

I.


A
MRM of 4 Channels ES+
721 > 296
3.81e3
B
MRM of 4 Channels ES+
347 > 315
8.75e3
C
MRM of 4 Channels ES+
225 > 127
3.69e3
D
MRM of 4 Channels ES+
237 > 137
3.32e3
Time

II.

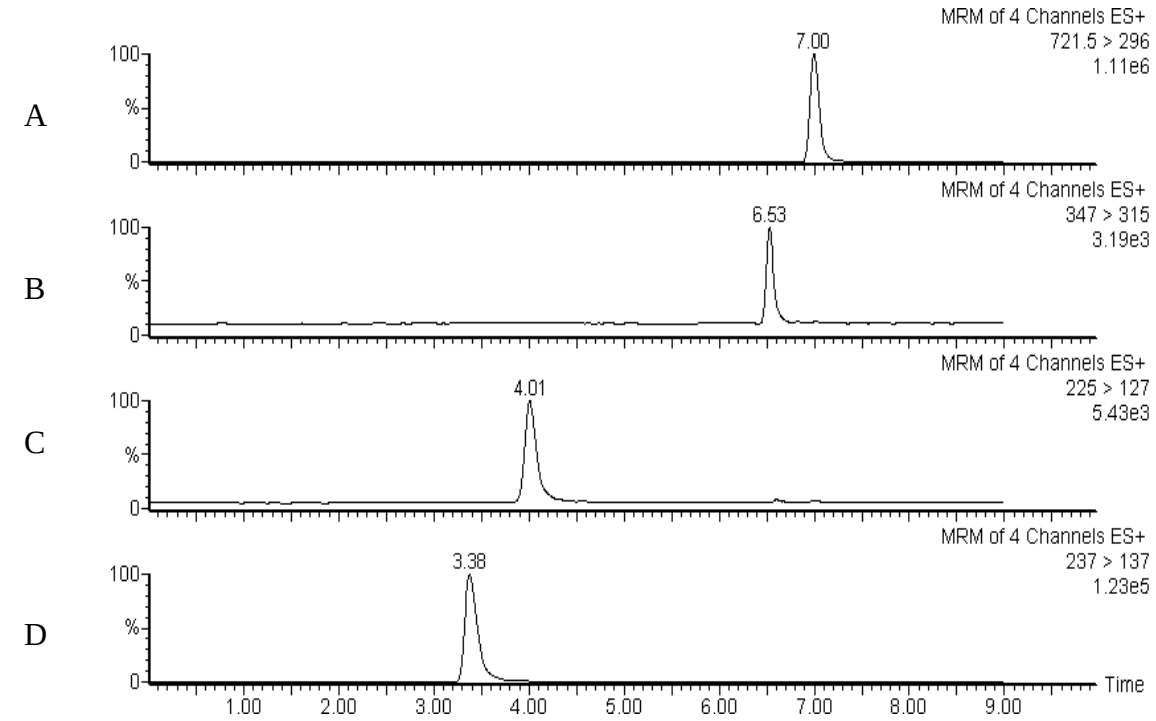
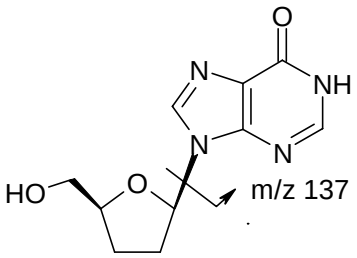
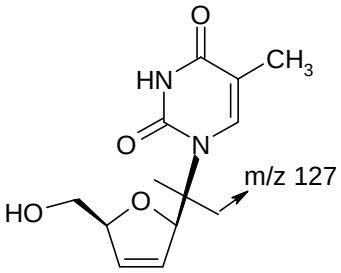

A
MRM of 4 Channels ES+
721.5 > 296
1.11e6
B
MRM of 4 Channels ES+
347 > 315
3.19e3
C
MRM of 4 Channels ES+
225 > 127
5.43e3
D
MRM of 4 Channels ES+
237 > 137
1.23e5
Time

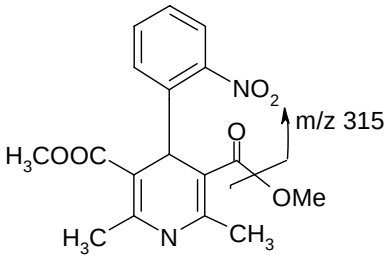
Figure 4. Proposed fragmentation pathway for ddI, d4T, ritonavir and nifedipine (I.S.)



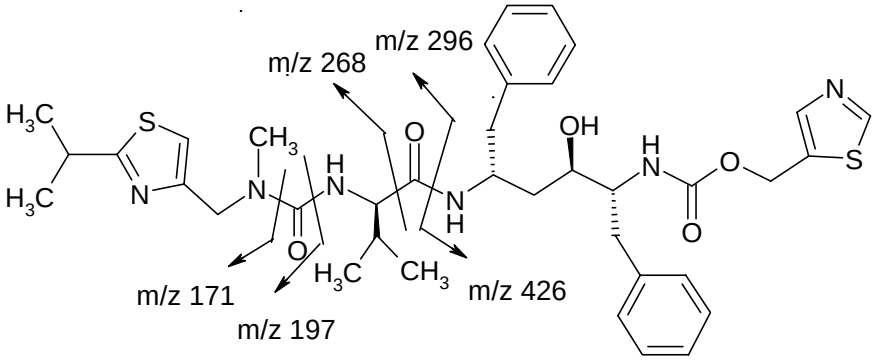
Didanosine (ddI)



Stavudine (d4T)



Nifedipine



Ritonavir

CHAPTER 7

DETERMINATION OF LAMIVUDINE/DIDANOSINE/SAQUINAVIR IN HUMAN SERUM USING CAPILLARY ZONE ELECTROPHORESIS[1]

[1]Fan, Bin. and James T. Stewart. Accepted by Journal of Liquid Chromatography & Related Technologies. Reprint by courtesy of Marcel Dekker Inc. from the forthcoming Marcel Dekker Inc. publication, 2001

ABSTRACT

The anti-HIV drug mixture of lamivudine (3TC), didanosine (ddI) and saquinavir was separated and quantitated in human serum with capillary zone electrophoresis. Serum samples were treated using a solid-phase extraction procedure. The effects of various factors such as run buffer type, buffer concentration, and pH on the separation were investigated. The optimized resolution was achieved with a run buffer containing 100 nM N, N-dimethyloctylamine in 80 mM phosphate buffer (pH 2.5). An uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica capillary operated at 30 °C was used in the analysis with UV detection at 210 nm. Diltiazem was chosen as an internal standard. All analytes were separated within 10 min with a voltage of +20 kV and a current around 30 μA. The method was validated over the range of 0.4-37.8 μg/ml for 3TC, 1.4-34 μg/ml for ddI and 0.5-24.4 μg/ml for saquinavir. Intra-day and inter-day accuracy was less than 13.7% and intra-day and inter-day precision was less than 13.3%. Extraction recoveries of all analytes from plasma were higher than 79%. The assay should be applicable for pharmacokinetic studies and routine monitoring of these drugs in serum.

Keywords: Lamivudine; Didanosine; Saquinavir; Capillary Zone Electrophoresis (CZE); Human Serum

INTRODUCTION

The availability of new and potent drugs and progress in understanding the pathogenesis of HIV-1 infection has led to the establishment of new treatment paradigms. The use of multidrug therapy has greatly enhanced the success of AIDS treatment [1-3]. Highly active antiretroviral therapy (HAART) and the use of aggressive combination antiretroviral regimens consisting of reverse transcriptase inhibitor and protease inhibitors, have become the standard of care [4,5]. These combination regimens achieved near-complete suppression of HIV-RNA concentrations and lead to considerable improvements in life expectancy of infected individuals [6-8].

The concentrations of antiretroviral drugs in human serum have become a useful parameter in the clinical management of HIV disease. The serum levels of these drugs seem to be connected with virologic efficacy [9-11]. It is important to have a simple and routine assay method for monitoring these drug levels in order to prevent the emergence of drug resistance and to identify problems with compliance [12].

Reported methods for the determination of antiretroviral drugs in patients sera have largely been based on HPLC methodology [13-15]. HPLC has the disadvantages of requiring rather large sample volumes, high cost of consumable supplies, complicated system operation and maintenance, and the technique generates substantial quantities of hazardous organic solvents with high disposal costs. The technique of capillary zone electrophoresis (CZE) overcomes many of the drawbacks of HPLC and has emerged in recent years as a proven clinical tool for the pursuit of pharmacological studies [16,17].

CZE will allow the simultaneous measurement of several drugs, has low operating and consumable costs, and uses primarily aqueous run buffers.

The CZE method described herein was developed and validated to evaluate serum concentrations of 3TC, ddI and saquinavir in a single assay run. The assay employs a solid-phase extraction protocol and a common phosphate run buffer and could be useful for drug monitoring, determination of pharmacokinetic profiles and evaluation of drug-drug interactions.

EXPERIMENTAL

Chemicals and Reagents

Didanosine (ddI) and the internal standard diltiazem were purchased from Sigma Chemical Company (St. Louis, MO 63178). Lamivudine (3TC) was kindly provided by Dr. Chung K. Chu (The University of Georgia, Athens, GA, USA). Saquinavir was provided by Roche Pharmaceuticals (Hertfordshire, UK). Monobasic sodium phosphate, concentrated phosphoric acid, sodium hydroxide and HPLC grade methanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). N, N-Dimethyloctylamine was purchased from Aldrich Chemical Company, Inc (Milwaukee, WI 53233). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Waters Oasis^{TM} HLB 1cc 30 mg cartridges were purchased from Waters Corp (Milford, MA. USA). Drug free human serum was obtained from Bioreclamation Inc (East Meadow, NJ 11554).

Instrumentation

The CZE experiments were performed on an Applied Biosystems 270A electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA, USA). Separations were carried out in an uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The capillary was thermostated at 30 °C with air coolant. The detection window was created by stripping the polyimide coating of the capillary in length of 5 mm. The capillary was conditioned with 1N sodium hydroxide for 1 hour followed by 30 min of deionized water before each day's run. Before each run, the capillary was also rinsed with 0.1 N sodium hydroxide for 3 min and run buffer for 3 min. The applied voltage was +20 kV for the separation and the detection wavelength was set at 210 nm. All the samples were injected by applying a 5 in. Hg vacuum to the outlet of the capillary for 3 s.

Preparation of Standard Stock Solution

Stock solutions of lamivudine (3TC), didanosine (ddI), saquinavir and diltiazem (internal standard) were prepared in deionized water to give concentrations of 1020, 540, 488 and 820 μg/ml, respectively. Working solutions were prepared by further diluting these stock solutions with deionized water. Calibration plots for the analytes in serum were prepared by spiking drug-free serum with standard stock solution to yield concentrations of 0.4-37.8 μg/ml (0.4, 1.8, 7.6, 18.0 and 37.8 μg/ml) for 3TC, 1.4- 34.0

μg/ml (1.4, 4.1, 6.8, 10.2 and 34.0 μg/ml) for ddI and 0.5-24.4 μg/ml (0.5, 2.4, 6.1, 12.2 and 24.4 μg/ml) for saquinavir. Samples were stored at 4 °C until use.

Sample Preparation Procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various solutions and 10 μl of a 820 μg/ml internal standard solution were added to drug-free serum to obtain a final volume of 1 ml. Waters Oasis^{TM} HLB 1cc cartridges were conditioned with 1 ml of methanol followed by 1 ml deionized water. One milliliter of the spiked serum samples was loaded onto the cartridges and drawn through by applying a vacuum. The cartridges were then washed with 1 ml methanol-water (10:90, v/v). One milliliter methanol was used to elute the adsorbed analytes. The eluting solvent was evaporated and reconstituted in 1ml of deionized water. Three second hydrodynamic injections of samples were made at the anodic end of the capillary.

Assay Validation

The method accuracy was obtained by comparing the concentrations calculated from the calibration curves versus concentrations added. Precision was calculated as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay were determined by assaying three quality control samples at low, medium and high concentrations for each compound (1.4, 7.2 and 15.1 μg/ml for 3TC; 3.4, 8.7 and 13.6 μg/ml for ddI; 1.0, 5.0 and 10.0 μg/ml for saquinavir) in three analytical runs within

the same day. The inter-day accuracy and precision samples were analyzed on three different days. Triplicate injections of each analyte concentration were performed. The absolute recoveries of each drug and internal standard clean-up procedure were obtained by comparing the extracted serum analytes to unextracted stock solutions.

RESULTS AND DISCUSSION

Two methods were evaluated for sample preparation of spiked human serum prior to analysis by CZE. Protein precipitation using acetonitrile resulted in a number of endogenous peaks, which interfered with the compounds analyzed. The recoveries for all analytes using protein precipitation were lower (< 40%) compared to the solid-phase extraction method (>79%). Solid-phase extraction using a series of different extraction cartridges, such as C_{18}, C_8, Oasis^{TM} cartridges, was investigated. Oasis^{TM} cartridges gave the highest recoveries of the drugs as well as cleaner assay samples.

The effects of phosphate run buffer concentration (20-100 mM) and pH (2-9) on the separation were investigated. Besides run current, buffer concentrations affect buffering capacity and EOF. Generally, the higher the buffer concentration, the higher the current, and the greater the buffering capacity. This prevents buffer depletion and improves assay reproducibility. Higher run buffer concentration also decreased the effect of charges on the capillary wall, and consequently reduced EOF. Long migration times and sharper peaks for the analytes were obtained with higher run buffer concentrations. At pH< 3.0, the EOF was relatively small and peak efficiency was high. EOF increased with increasing pH, and the migration times decreased but with an adverse impact on

peak efficiency. The optimized resolution of the mixture was achieved with 80 mM phosphate run buffer with the pH adjusted to 2.5 with concentrated phosphoric acid.

Another reason to use a high concentration run buffer is to apply the sample stacking technique to increase sensitivity. Deionized water was used to dissolve and dilute the analytes. Thus, the electrical conductance of the sample zone was lower than that of the run buffer. An overall applied voltage of +20 kV was held constant, and a higher electrical field was developed across the sample zone. The samples migrated faster until they reached the boundary of the sample zone, where they were stacked at the interface with the run buffer. This approach made it possible to load a larger volume of sample to improve the limits of detection of the analytes.

Our initial studies indicated that an asymmetric, split peak was observed for ddI in the electropherogram. Adsorption onto or interaction with the capillary wall could possibly be the main cause. A variety of additives to the run buffer, such as diethylamine, triethylamine and N, N-dimethyloctylamine were investigated to coat the capillary wall in order to reduce wall interactions. N, N-dimethyloctylamine (100 nM) added to the phosphate run buffer eliminated the adsorption effect and a sharp and symmetric peak was obtained for ddI.

Baseline separation of 3TC, ddI and saquinavir was achieved with retention times of 4.4, 10.8 and 6.9 min, respectively. The internal standard, diltiazem gave a migration time of 5.6 min. The structures of these compounds are shown in Figure 1. Figure 2 shows electropherograms of blank and spiked serum samples. The calibration curves showed good linearity in the concentration ranges of 0.4-37.8 μg/ml for 3TC, 0.5-24.4 μg/ml for saquinavir and 1.4-34.0 μg/ml for ddI. The regression coefficients (r^2) of

calibration curves of each drug were higher than 0.99. LOD and LOQ data are shown in Table 1. The results from the validation of the method in human serum are shown in Table 2.

CONCLUSION

A solid phase extraction procedure coupled with a capillary zone electrophoresis method provided a fast, sensitive and selective procedure for the simultaneous determination of a 3TC/ddI/saquinavir mixture in human serum.

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Table 1. Range of Calibration Curves, Limits of Detection (LOD) and Limits of Quantification (LOQ) of 3TC, ddI and Saquinavir in Spiked Human Serum

Drug	Range of calibration curves (μg/ml)	Limit of detection (LOD) (μg/ml)[a]	Limit of quantification (LOQ) (μg/ml)[b]
3TC	0.4—37.8	0.2	0.4
ddI	1.4—34.0	0.9	1.4
Saquinavir	0.5—24.4	0.2	0.5

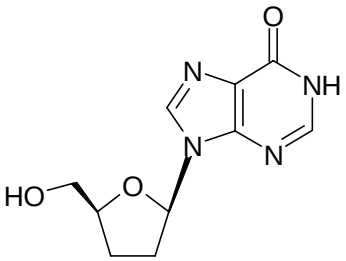
a. S/N=3
b. S/N=10

Table 2. Inter-Day and Intra-Day Accuracy, Precision and Recovery for the Analysis of 3TC, ddI and Saquinavir in Human Serum

	Concentration	Precision[a] (%)		Accuracy[a] (%)		Serum Recovery[b]
	(μg/ml)	Intra-day	Inter-day	Intra-day	Inter-day	(%)
3TC	15.1	0.5	1.7	8.1	6.6	91.9±3.5
	7.2	0.7	0.9	13.7	13.2	96.4±4.7
	1.4	13.3	7.7	4.3	7.1	88.3 ±1.5
ddI	13.6	9.7	2.6	3.4	1.8	82.3±3.1
	8.7	1.1	8.1	14.2	9.8	85.7±2.3
	3.4	7.1	7.6	13.7	8.8	78.5±1.6
Saquinavir	10.0	0.2	11.7	0.5	7.7	96.7±3.6
	5.0	10.6	8.3	3.0	8.0	85.6±2.4
	1.0	4.6	5.7	7.7	2.5	93.2±4.1

a. Based on n = 9
b. Mean ± SD based on n = 6

Figure 1. The chemical structures of analytes



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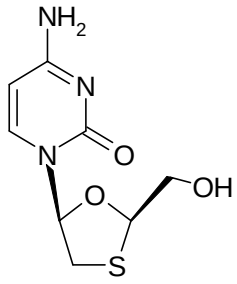

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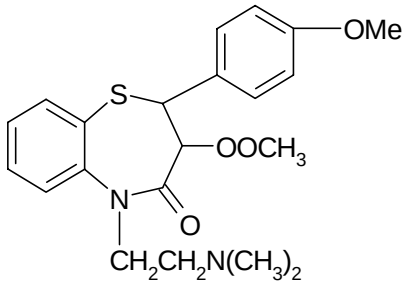
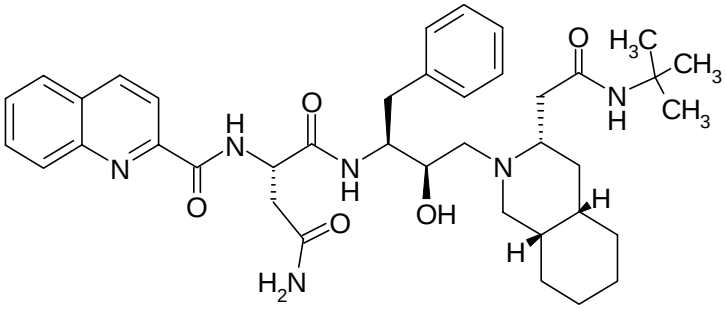


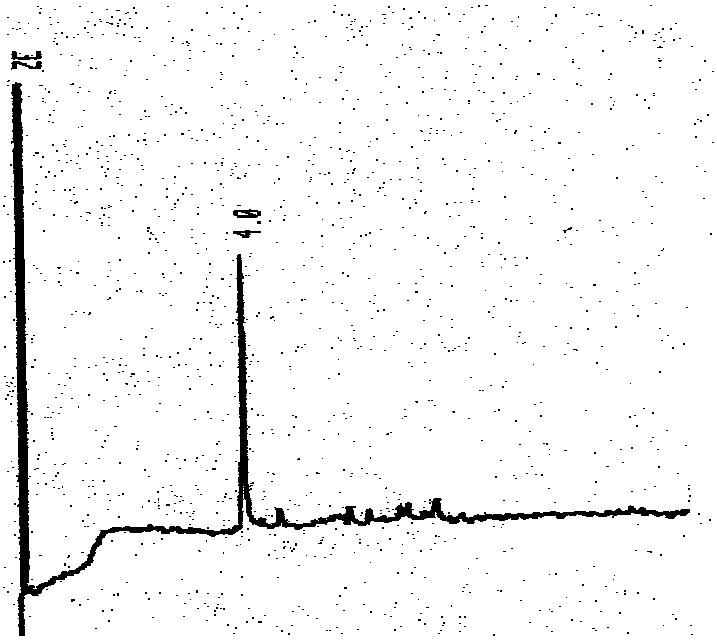
Diltiazem



Saquinavir

Figure 2. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 3TC (18.0 μg/ml), (B)) internal standard (C) saquinavir (12.2 μg/ml)and (D) ddI (10.2 μg/ml). The peak at 4.0 min is an unknown serum component

I.



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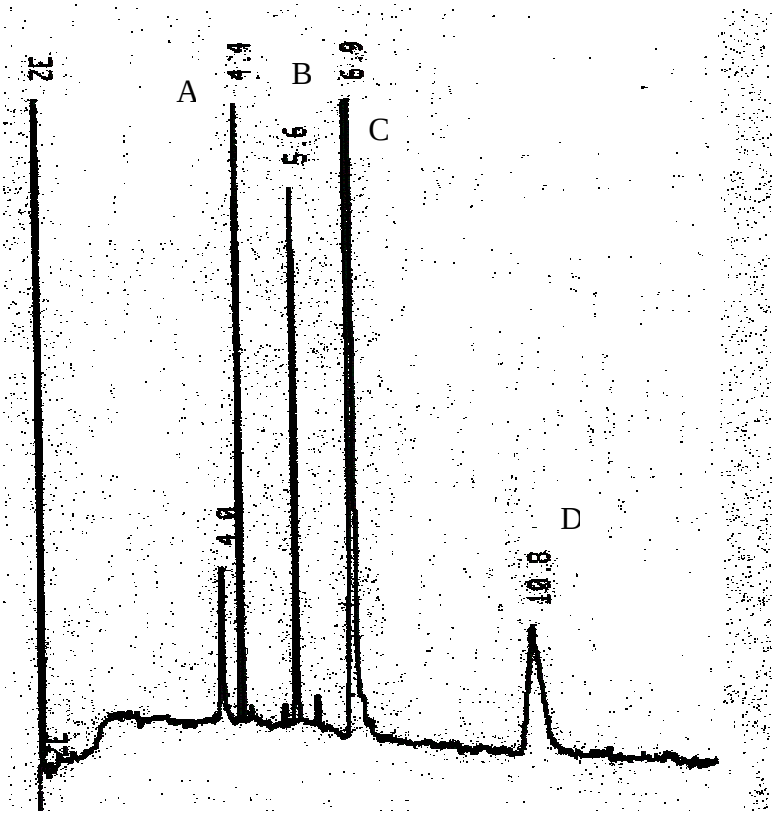

Absorbance, 210 nm

Migration Time (min)

CHAPTER 8

DETERMINATION OF LAMIVUDINE/DIDANOSINE/NEVIRAPINE IN HUMAN SERUM USING CAPILLARY ZONE ELECTROPHORESIS[1]

[1]Fan, Bin. and James T. Stewart. Submitted to Journal of Capillary Electrophoresis, 2001.

Abstract

A combination of the anti-HIV drugs lamivudine (3TC), didanosine (ddI) and nevirapine were separated and quantitated in human serum with capillary zone electrophoresis (CZE). The effects of various factors such as run buffer concentration and pH on the separation were investigated. The optimized resolution was achieved with a run buffer containing 100 nM N, N-Dimethyloctylamine in 80 mM phosphate buffer (pH 2.5). Diltiazem was chosen as the internal standard. All analytes were separated within 10 min at 30 °C with a voltage of + 20 kV and UV detection at 210 nm.

Keywords: Lamivudine; Didanosine; Nevirapine; Capillary Zone Electrophoresis (CZE), Human Serum

Introduction

In a very short period of time, the availability of antiretroviral drugs has increased from zidovudine (AZT), the first drug approved by the US Food and Drug Administration, up to 18 drugs now. The large number of available agents give a clinician the ability to tailor a therapeutic regimen to a particular patient. The use of multidrug therapy has become the rule rather than the exception in the treatment of patients with human immunodeficiency virus (HIV) infection [1-3]. This has been propelled by the need to delay the development of drug resistance and minimize potential dose-limiting side effects.

Clinical trials evaluating different combinations of these drugs have resulted in the generation of some basic guides for their appropriate use. Some very promising combination regimens contain at least two nucleoside reverse transcriptase inhibitors (NRTI) such as 3TC and ddI and one non- nucleoside reverse transcriptase inhibitor (NNRTI) such as nevirapine [1].

The concentrations of antiretroviral drugs in human serum have become a useful parameter in the clinical management of HIV disease. The serum levels of these drugs seem to be correlated with virologic efficacy [4-6]. It is important to have a simple and routine method for monitoring drug levels to prevent the emergence of drug resistance and to identify problems with compliance [7]. Reported methods for determination of antiretroviral drugs in patient serum have been based on HPLC methodology [8-10]. HPLC has the disadvantages of requiring rather large sample volumes, high cost of consumable supplies and the technique generates substantial quantities of hazardous organic solvent with high disposal costs. The technique of capillary zone electrophoresis

(CZE) overcomes many of the drawbacks of HPLC and has been emerging in recent years as a proven clinical tool for the pursuit of pharmacological studies [11,12]. CZE allows simultaneous measurement of several drugs, has low operating and consumable costs, and uses primarily aqueous run buffers.

The CZE method herein was developed and validated to determine serum concentrations of 3TC, ddI and nevirapine in a single run. This assay employs a solid-phase extraction protocol and a common run buffer. It should be useful for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug-drug interactions.

Experimental

Chemical and reagents

Didanosine (ddI) and the internal standard diltiazem were purchased from Sigma Chemical Company (St. Louis, MO 63178). Lamivudine (3TC) was kindly provided by Dr. Chung K. Chu (The University of Georgia, Athens, GA, USA). Nevirapine was provided by Abbott Laboratories (North Chicago, IL 60064). Monobasic sodium phosphate, concentrated phosphoric acid, sodium hydroxide and HPLC grade methanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). N, N-Dimethyloctylamine was purchased from Aldrich Chemical Company, Inc (Milwaukee, WI 53233). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Water Oasis^{TM} HLB 1cc 30 mg cartridges were purchased from Waters Corp (Milford, MA. USA). Drug free human serum was obtained from Bioreclamation Inc (East Meadow, NY 11554).

Instrumentation

CZE separations were performed on an ABI Model 270A electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA, USA) and an uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The applied voltage was + 20 kV in the reverse polarity mode. The capillary temperature was maintained at 30 °C with air coolant. The detection window was created by stripping the polyimide coating of the capillary in a length of 5 mm. Each day prior to use, the capillary was conditioned with 1 N sodium hydroxide for 1 hour followed by 30 min of deionized water. Before each run, the capillary was also rinsed with 0.1 N sodium hydroxide for 3 min and run buffer for 3 min. Samples were introduced into the CE capillary by pressure injection for 3 s. All analytes were monitored at a wavelength of 210 nm.

Preparation of standard stock solution

Stock solutions of lamivudine (3TC), didanosine (ddI), nevirapine and diltiazem (internal standard) were prepared in deionized water to give concentrations of 1020, 540, 912 and 820 μg/ml, respectively. Working solutions were prepared by further diluting these stock solutions with deionized water. Calibration plots for the analytes in serum were prepared by spiking drug-free serum with standard stock solution to yield concentrations of 0.4-37.8 μg/ml (0.4, 1.8, 7.6, 18.0 and 37.8 μg/ml) for 3TC, 1.4- 34.0 μg/ml (1.4, 4.1, 6.8, 10.2 and 34.0 μg/ml) for ddI and 0.3-30.4 μg/ml (0.3, 0.9, 3.0, 9.1 and 30.4 μg/ml) for nevirapine. Samples were stored at 4 °C until use.

Sample preparation procedure

To prepare calibration standards and quality control samples, appropriate quantities of the various solutions and 10 μl of a 820 μg/ml internal standard solution were added to drug-free serum to a final volume of 1ml. Waters Oasis^{TM} HLB 1cc cartridges were conditioned with 1 ml methanol followed by 1 ml deionized water. One ml of the spiked serum samples was loaded onto the cartridges and drawn by applying a vacuum. The cartridges were then washed with 1 ml methanol-water (10:90, v/v) and 1 ml methanol was used to elute the adsorbed analytes. The eluting solvent was evaporated and reconstituted in 1ml deionized water. 3s hydrodynamic injections of samples were made at the anodic end of the capillary.

Assay Validation

The method accuracy was obtained by comparing the concentrations calculated from the calibration curves versus concentrations added. Precision was calculated as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay were determined by assaying three quality control samples at low, medium and high concentrations for each compound (1.4, 7.2 and 15.1 μg/ml for 3TC; 3.4, 8.7 and 13.6 μg/ml for ddI; 1.0, 4.6 and 10.2 μg/ml for nevirapine) in three analytical runs within the same day. The inter-day accuracy and precision samples were also analyzed on three different days. Triplicate injections of each analyte concentration were performed.

Results and Discussion

Two methods were evaluated for the sample preparation of spiked human serum prior to analysis by CZE. Protein precipitation using acetonitrile resulted in a few endogenous peaks, which interfered with the compounds analyzed. The recoveries for all analytes by this method were much lower compared to the solid-phase extraction. Solid-phase extraction (SPE) using a series of different extraction cartridges, such as C_{18}, C_8 and Oasis^{TM} cartridges, was investigated. Significant loss of 3TC was observed in the C_8 cartridges during the loading step. Oasis^{TM} cartridges provided the highest recoveries of the drugs as well as relatively cleaner assay samples when a cartridge wash solution containing 10% methanol was used.

The run buffer plays a central role in capillary zone electrophoresis (CZE). In CZE, analytes migration velocity, separation, column efficiency, and the peak shape are sensitive to changes in buffer characteristics. In particular, the pH is of crucial importance, creating the need for stringent buffer control. The effects of commonly used phosphate run buffers and pH on the separation were investigated. Besides running current, buffer concentrations affect buffering capacity and EOF. Generally, the higher the buffer concentration, the higher the electrical current, and the better is the buffering capacity. This will prevent buffer depletion and improve assay reproducibility. Also, higher buffer concentrations will decrease the effect of charges on the capillary wall, and consequently reduce the EOF. Longer migration times and sharper peaks for the analytes were obtained with higher buffer concentrations. The effects of buffer pH on the separation were studied from pH 2 to 9. The separation of the drug mixture was optimized by changing selectivity through various pHs. At buffer pH< 3.0, the EOF is

relatively small and peak efficiency is high. EOF increases with increasing pH, and the migration times decrease but with an adverse impact on peak efficiency. The optimized resolution was achieved with 80 mM phosphate run buffer with the pH adjusted to 2.5 with concentrated phosphoric acid.

Another reason for a high concentration run buffer is to use the sample stacking technique to increase sensitivity. Deionized water was used to dissolve and dilute analytes. Thus, the electrical conductance of the sample zone is lower than that of the run buffer. An overall applied voltage of 20 kV was held constant, and a higher electrical field developed across the sample zone. The samples migrated faster until they reach the boundary of the sample zone, where they stack at the interface with the run buffer. This approach makes it possible to load a large volume of sample to improve the limits of detection.

An asymmetric and split peak was observed for ddI possibly due to adsorption onto or interaction with the capillary wall. A variety of amines, such as diethylamine, triethylamine and N, N-Dimethyloctylamine were investigated to coat the capillary wall in order to reduce wall interactions. Amines readily adsorb to silica and modify the surface properties of the sorbent. When an amine is applied at high concentrations, the surface properties of silica are completely masked. N, N-Dimethyloctylamine (100 nM) added to the phosphate run buffer eliminated the adsorption, and a sharp and symmetric peak for ddI was obtained.

Baseline separation of 3TC, ddI and nevirapine was achieved with migration times of 4.4, 9.6 and 6.5 min, respectively. The internal standard, diltiazem, migrated at 5.5 min. The structures of these analytes are shown in Figure 1. Figure 2 shows the

electropherograms of blank and spiked serum samples. The limits of detection for 3TC, nevirapine and ddI were 0.2, 0.9 and 0.2 μg/ml, respectively. Calibration curves were obtained over the range of 0.4-37.8 μg/ml for 3TC, 0.3-30.4 μg/ml for nevirapine and 1.4-34.0 μg/ml for ddI. The regression coefficients (r^2) of calibration curves of each drugs were higher than 0.99 (see Table 1). The quantitative aspects of the method were examined and the results are shown in Table 2.

Conclusion

The separation and quantitation of the anti-HIV drug mixture 3TC, ddI and nevirapine in human serum was achieved by CZE using a 80 mM phosphate run buffer containing 100 nM N, N-Dimethyloctylamine (pH 2.5). The method is fast, sensitive and selective for this three drug mixture.

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Table 1. Range of calibration curves, limits of detection (LOD) and limits of quantitation (LOQ) of 3TC,ddI and nevirapine in spiked human serum

Drug	Range of calibration curves (μg/ml)	Limit of detection (LOD) (μg/ml)[a]	Limit of quantitation (LOQ) (μg/ml)[b]
3TC	0.4—37.8	0.2	0.4
ddI	1.4—34.0	0.9	1.4
Nevirapine	0.3—30.4	0.1	0.3

a S/N=3

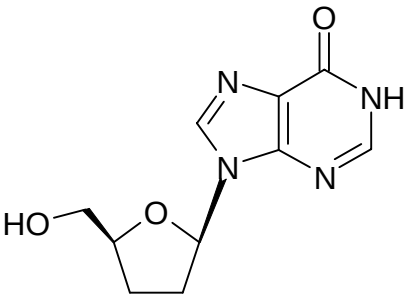
b S/N=10

Table 2. Inter-day and intra-day accuracy and precision for the analysis of 3TC, ddI and nevirapine in human serum

	Concentration	Precision[a] (%)		Accuracy[a] (%)	
	(μg/ml)	Intra-day	Inter-day	Intra-day	Inter-day
3TC	15.1	0.5	1.7	8.1	6.6
	7.2	0.7	0.9	13.7	13.2
	1.4	13.3	7.7	4.3	7.1
ddI	13.6	9.7	2.6	3.4	1.8
	8.7	1.1	8.1	14.2	9.8
	3.4	7.1	7.6	13.7	8.8
Nevirapine	10.2	1.4	0.6	12.9	13.2
	4.6	4.2	12.8	4.1	2.2
	1.0	7.4	7.4	3.5	5.0

a Based on n = 9

Figure 1. The chemical structures of analytes



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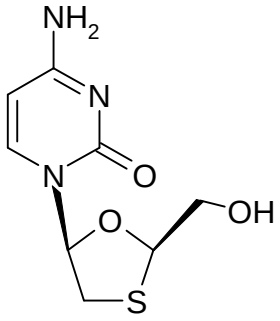

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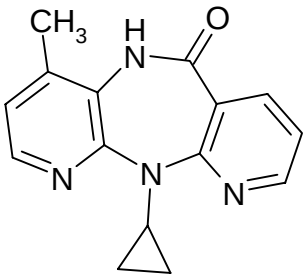
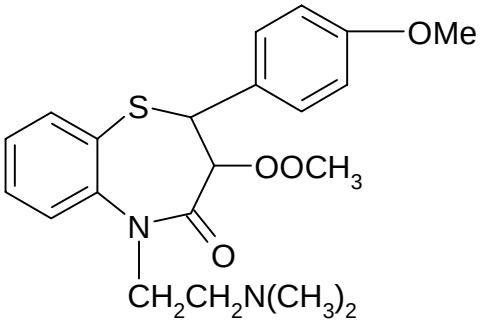


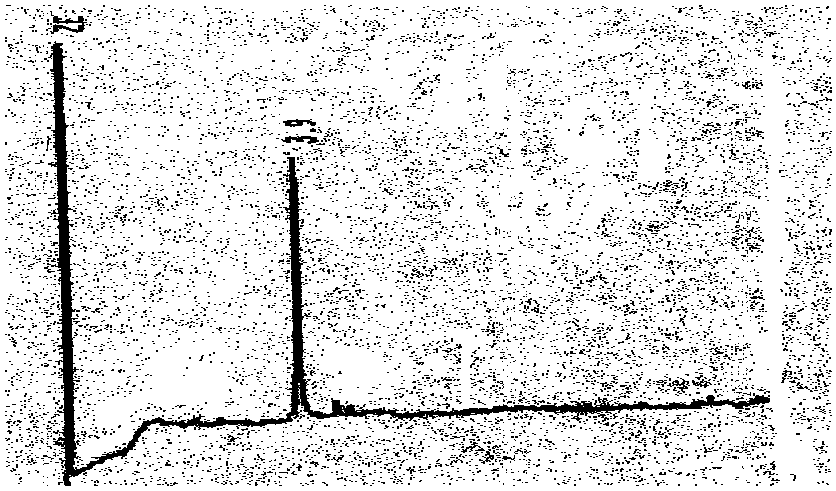
Nevirapine



Diltiazem

Figure 2. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 3TC (18.0 μg/ml), (B)) internal standard (C) nevirapine (9.1 μg/ml) and (D) ddI (10.2 μg/ml)

I.



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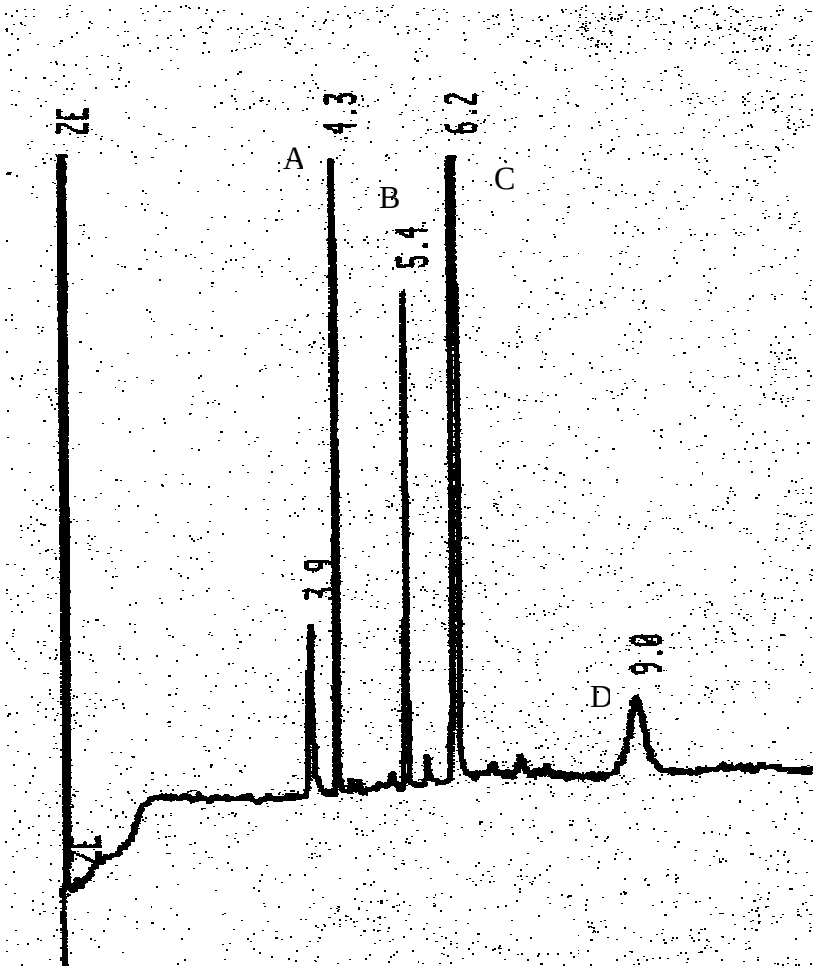

Absorbance, 210 nm

Migration Time (min)

CHAPTER 9

DETERMINATIONS OF ZIDOVUDINE/DIDANOSINE/NEVIRAPINE AND ZIDOVUDINE/DIDANOSINE/RITONAVIR IN HUMAN SERUM BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)[1]

[1]Fan, Bin. and James T. Stewart. To be submitted to Journal of Pharmaceutical and Biomedical Analysis, 2001.

Abstract

Micellar electrokinetic chromatography (MEKC) methods were developed and validated to separate and quantitate anti-HIV drug mixtures containing zidovudine(AZT)/ didanosine(ddI)/nevirapine (mixture A) and zidovudine(AZT)/didanosine (ddI)/ritonavir (mixture B) in human serum. Serum samples were prepared using a solid-phase extraction procedure. The effects of various factors such as buffer type, buffer and surfactant concentrations, and pH on the separations were investigated. The optimized resolution was achieved with a run buffer containing 18 mM sodium dodecylsulfate (SDS) in 15 mM phosphate and borate buffer (pH 9.0). An uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica capillary operated at 30 °C was used in the analysis with UV detection at 210 nm. Aprobarbital was chosen as the internal standard. All analytes were separated within 14 min with a voltage of +15 kV and a current around 30 μA. The methods were validated over the range of 0.5-25.0 μg/ml for AZT, 0.8-18.5 μg/ml for ddI, 0.5-22.8 μg/ml for nevirapine in mixture A and the range of 0.5-25.0 μg/ml for AZT, 0.8-18.5 μg/ml for ddI, 1.2-28.8 μg/ml for ritonavir in mixture B. Intra-day and inter-day accuracy was less than 12.4% and intra-day and inter-day precision was less than 13.9% for both mixtures. Extraction recoveries of all analytes from serum were higher than 75.9%. The assay should be applicable to pharmacokinetic studies and routine monitoring of these drugs in serum.

Keywords: Zidovudine; Didanosine; Nevirapine; Ritonavir; Micellar Electrokinetic Chromatography (MEKC), Human Serum

Introduction

The current standard of health care for patients with HIV disease is a triple therapy regimen, usually consisting of 2 nucleoside analogues in combination with a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor [1-3]. Current therapeutic guidelines are based on the use of Zidovudine (AZT), the first nucleoside analogue approved by FDA, as first-line therapy, and most combination therapy in clinical trials includes AZT [4]. Another nucleoside analogue, Didanosine (ddI), is a good candidate for combination anti-retroviral therapy along with Zidovudine, because of its synergistic anti-HIV effect in vitro and different patterns of adverse effects [5,6]. As more anti-HIV drugs are approved for use, various drug combinations are being used. Nevirapine, the first non-nucleoside reverse transcriptase inhibitor, and ritonavir, a protease inhibitor, are also included in other therapeutic options along with AZT.

The separation and determination of antiviral drugs are needed to study their pharmacokinetics in biological fluids as well as to monitor antiretroviral therapy in the treatment of HIV infection. Determination of antiretroviral drugs has been achieved using high-performance liquid chromatography [7-9]. Recently, capillary electrophoresis (CE) has been shown to be a powerful alternative to HPLC [10,11]. Since CE is generally faster, more efficient, requires less sample and solvent waste is negligible. Micellar electrokinetic chromatography (MEKC), a mode of CE, has been used for the separation of neutral molecules, which can not be analyzed by conventional capillary zone electrophoresis (CZE).

In these studies, MEKC assays to determine AZT/ddI/nevirapine (mixture A) and AZT/ddI/ritonavir (mixture B) in human serum were developed and validated. The assays

employ solid-phase extraction and a common CE run buffer, and should be useful for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug-drug interactions.

Experimental

Chemical and Reagents

Zidovudine (AZT), Didanosine (ddI), aprobarbital (internal standard), dibasic sodium phosphate and sodium dodecylsulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Ritonavir and nevirapine were kindly provided by Abbott Laboratories (North Chicago, IL 60064). Concentrated phosphoric acid, sodium hydroxide and HPLC grade methanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). Sodium tetraborate was purchased from Fisher Scientific, Inc (Fair Lawn, NJ 07410). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Water Oasis^{TM} HLB 1cc 30 mg cartridges were purchased from Waters Corp (Milford, MA. USA). Drug free human serum was obtained from Biological Specialty (Colmar, PA, USA).

Instrumentation

MEKC was performed with an Applied Biosystems 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA, USA). Separations were carried out at 30 °C with an uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica

capillary (Polymicro Technologies, Phoenix, AZ, USA). The capillary was conditioned with 1N sodium hydroxide for 1 hour followed by 30 min of the run buffer before each day's run. Before each run, the capillary was rinsed with 0.1 N sodium hydroxide for 3 min and run buffer for 3 min. The applied voltage was optimized at +15 kV for the separation and the detection wavelength was set at 210 nm. The detection window was created by stripping the polyimide coating of the capillary in a length of 5 mm. The sample was injected by applying a 5 in. Hg vacuum to the outlet of the capillary for 3 s.

Preparation of Standard Solutions

Stock solutions of zidovudine (AZT), didanosine (ddI), nevirapine, ritonavir and aprobarbital (internal standard) were prepared in deionized water to give concentrations of 998, 413, 912, 1152 and 1600 μg/ml, respectively. Standards were prepared by spiking drug-free serum with these stock solutions, and 10 μl of the stock internal standard solution was added to drug-free serum to a final volume of 1ml. The concentrations of calibration standards in mixture A were 0.5-25.0 μg/ml (0.5, 2.0, 5.0, 10.0 and 25.0 μg/ml) for AZT, 0.8-18.5 μg/ml (0.8, 1.9, 4.1, 8.3, and 18.5 μg/ml) for ddI, 0.2-22.8 μg/ml (0.2, 0.5, 1.8, 9.1 and 22.8 μg/ml) for nevirapine. The concentrations in mixture B were 0.5-25.0 μg/ml (0.5, 2.0, 5.0, 10.0 and 25.0 μg/ml) for AZT, 0.8-18.5 μg/ml (0.8, 1.9, 4.1, 8.3, and 18.5 μg/ml) for ddI, 1.2-28.8 μg/ml (1.2, 2.3, 5.8, 11.5, and 28.8 μg/ml) for ritonavir. Samples were stored at 4 °C.

Sample Preparation Procedure

Waters Oasis^{TM} HLB 1cc cartridges were conditioned with 1 ml methanol followed by 1 ml deionized water. One ml of the spiked serum samples was loaded onto the cartridges and drawn by applying a vacuum. The cartridges were then washed with 1 ml methanol-water (10:90, v/v). 1 ml methanol was used to elute the adsorbed analytes. The eluting solvent was evaporated to dryness and reconstituted in 1 ml deionized water. Three second hydrodynamic injections of samples were made at the anodic end of the capillary.

Assay Validation

The method accuracy was obtained by comparing the concentrations calculated from the calibration curves versus concentrations added. Precision was calculated as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay were determined by assaying three quality control samples at low, medium and high concentrations for each compound in mixture A (1.0, 5.0 and 20.0 μg/ml for AZT, 1.6, 8.0 and 16.0 μg/ml for ddI, 1.0, 4.5 and 18.0 μg/ml for nevirapine); and in mixture B (1.0, 5.0 and 20.0 μg/ml for AZT, 1.6, 8.0 and 16.0 μg/ml for ddI, 2.0, 10.0 and 25.0 μg/ml for ritonavir) in three analytical runs within the same day. The inter-day accuracy and precision samples were analyzed on three different days. Three sets of samples at each analyte concentration were performed. The absolute recoveries of each drug and internal standard were obtained by comparing the extracted serum analytes to unextracted stock solutions.

Result and Discussion

Two procedures were evaluated for preparation of analytical samples from spiked human serum prior to analysis by MEKC. Protein precipitation using acetonitrile resulted in large numbers of endogenous peaks, which interfered with the compounds being analyzed. Solid-phase extraction using a series of different extraction cartridges, such as C_{18}, C_8, Oasis^{TM} cartridges was investigated. Significant loss of AZT (> 20%) was observed in the C_8 cartridges during the loading step. Oasis^{TM} gave the highest recoveries of the drugs as well as cleaner assay samples when a cartridge wash solution containing 10% methanol was used.

AZT can not be determined by capillary zone electrophoresis (CZE) since it is uncharged. It can be assayed by MEKC that is a hybrid of reverse-phase liquid chromatography (RPLC) and capillary zone electrophoresis (CZE). The MEKC separation process incorporates hydrophobic and polar interactions, a partitioning mechanism, and electromigration. Because of its special mechanism for separation, MEKC can be used for the separation and quantitation of AZT in the presence of other charged anti-HIV drugs.

With MEKC, the effects of buffer type and concentration, surfactant concentration and pH on the separation were investigated. A sodium phosphate-sodium borate buffer provided sharp, symmetric peaks, and gave good reproducibility in migration times and buffer capacity over a broad pH range. Besides running current, buffer concentrations affected buffering capacity and electroosmosis flow (EOF). Generally, the higher the buffer concentration, the higher the electrical current, and the greater the buffering capacity. This prevented buffer depletion and improved assay

reproducibility. Longer migration times and sharper peaks for the analytes were obtained with higher buffer concentrations. With a 15 mM buffer concentration, the best separation of the analytes in both mixture A and B was obtained with migration times <14 min. The pH of the buffer was an important factor to manipulate selectivity of analytes except for AZT. Typically a pH between 7 and 9 is employed to assure proper MEKC flow characteristics. The effects of buffer pH on the separation were studied from pH 6.5 to 10.5. Electroosmotic flow had an important effect on resolution and analysis time in MEKC separation. At pH< 6.5, the EOF is relatively small, and it was difficult to get symmetric and reproducible peaks. EOF increases with increasing pH, and migration times decrease but with an adverse impact on peak efficiency. pH 9.0 was found to be the optimum pH for both mixture A and B since the best separations were achieved

Surfactant type and concentration are also important variables that influence the electrophoretic migration in MEKC. Sodium dodecyl sulfate (SDS) was selected because it permitted the separation of low to moderate hydrophobic compounds and provides selectivity similar to that of reverse-phase LC. With a low critical micellar concentration (CMC), SDS reduces the conductivity of the buffer and joule heating. The primary role of the surfactant concentration is to adjust the retention factor within the optimum range to achieve better resolution. The concentration of SDS was optimized at 18 mM for both mixture A and B.

The MEKC separations of mixture A and B were performed in an untreated fused-silica capillary. The capillary exhibited rapid electroosmosis flow, and resulted in a short elution range. The implementation of MEKC often entails washing the capillary with sodium hydroxide solution. The frequency of rinsing the capillary and the solutions

used for rinsing had the greatest effect on migration reproducibility. In addition, the migration behavior of solutes that interact with micelles is not repeatable unless the proper rinse protocol is applied. A correlation between inconsistent migration behavior and fluctuation in electric current was observed, which might indicate the existence of nonequilibrium conditions between the run buffer and the capillary wall. The washing procedure used decreased the deleterious effects of capillary "aging," which resulted in changes in sample-wall interactions and electroosmotic flow.

The structures of these compounds in mixtures A and B are shown in Figure 1. In mixture A, baseline separation of the AZT, ddI and nevirapine mixture was achieved with migration times of 3.7, 4.0 and 6.0 min, respectively. The internal standard, aprobarbital, gave a migration time of 5.1 min. Figure 2 shows the electropherograms of blank serum and spiked serum samples in mixture A. In mixture B, AZT, ddI and ritonavir mixture was baseline separated with migration times of 3.7, 4.0 and 13.5 min, respectively. The migration time for the internal standard was 5.1 min. Figure 3 shows the electropherograms of blank serum and spiked samples in mixture B. The calibration curves for mixture A showed good linearity in the concentration range of 0.5-25.0 µg/ml for AZT, 0.8-18.5 µg/ml for ddI, 0.5-22.4 µg/ml for nevirapine. The calibration curves for mixture B showed good linearity in the concentration range of 0.5-25.0 µg/ml for AZT, 0.8-18.5 µg/ml for ddI, and 1.2-28.8 µg/ml for ritonavir. The regression coefficients (r^2) of calibration curves of each drug were higher than 0.99. LOD and LOQ data are shown in Table1. The methods proved to be accurate and precise. Extraction recoveries of all analytes from serum were higher than 75.9%. The results from method validation in human serum are listed in Table 2.

Conclusions

Solid phase extraction (SPE) and micellar electrokinetic chromatography (MEKC) methods provided a fast, sensitive and selective procedure for the simultaneous determination of AZT/ddI/nevirapine (mixture A) and AZT/ddI/ritonavir (mixture B) in human serum.

Reference

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Table 1. Range of calibration curves, limits of detection (LOD) and limits of quantification (LOQ) of AZT, ddI, nevirapine (mixture A) and AZT, ddI, ritonavir (mixture B) in spiked human serum

Drug	Range of calibration curves (µg/ml)	Limits of detection (LOD) (µg/ml)[a]	Limits of quantification (LOQ) (µg/ml)[b]
AZT	0.5—25.0	0.2	0.5
ddI	0.8—18.5	0.4	0.8
Nevirapine[c]	0.2—22.8	0.1	0.2
Ritonavir[d]	1.2—28.8	0.6	1.2

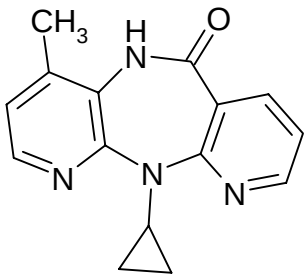
a S/N=3
b S/N=10
c Present in mixture A along with AZT and ddI
d Present in mixture B along with AZT and ddI

Table 2. Inter-day and intra-day accuracy, precision and recovery for the analysis of AZT, ddI, nevirapine (mixture A) and AZT, ddI, ritonavir (mixture B) in human serum

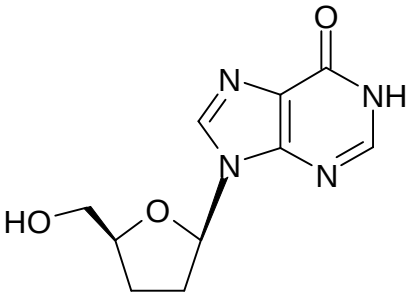
	Concentration	Precision (%)		Accuracy (%)		Plasma Recovery[c]
	(μg/ml)	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	(%)
AZT	20.0	4.4	2.1	3.2	1.5	95.2±1.7
	5.0	9.5	5.7	3.1	0.2	79.3±3.6
	1.0	9.8	2.0	8.2	5.8	76.1±5.4
ddI	16.0	13.9	12.3	2.3	0.8	80.8 ±3.0
	8.0	12.6	7.2	10.8	0.5	77.9 ±1.2
	1.6	10.8	10.7	9.4	7.1	75.9±5.2
Nevirapine[d]	18.0	3.1	0.4	0.7	0.4	102.1±2.3
	4.5	6.8	4.8	11.2	10.4	95.3±5.5
	1.0	5.1	7.3	4.4	11.9	101.1±2.0
Ritonavire[e]	25.0	5.4	8.5	3.7	6.2	94.4±1.8
	10.0	7.5	6.2	12.4	8.3	92.3±1.1
	2.0	11.4	10.6	11.3	10.1	92.4±7.1

a Based on n = 3
b Based on n = 9
c Mean ± SD based on n = 9
d Present in mixture A along with AZT and ddI
e Present in mixture B along with AZT and ddI

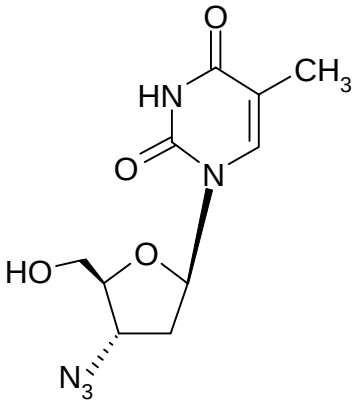
Figure 1. The chemical structures of analytes in mixtures A and B



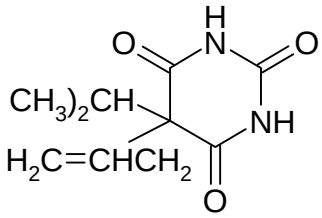
Nevirapine



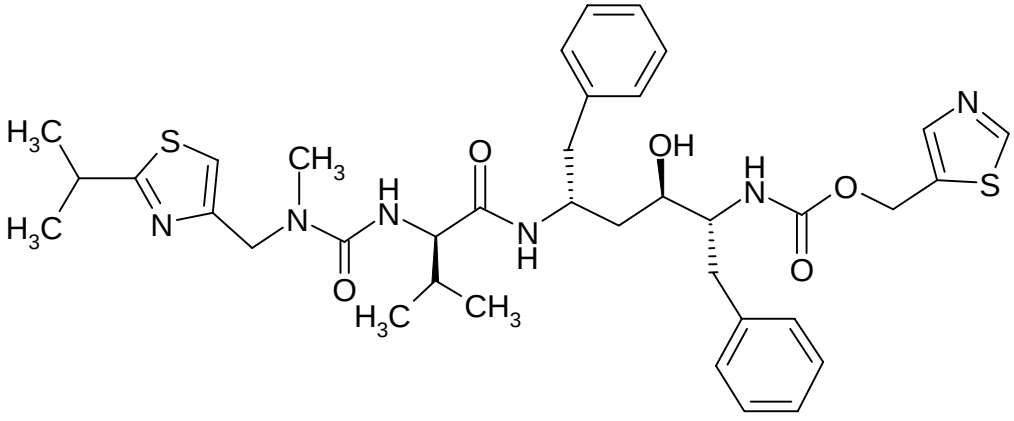
Didanosine (ddI)



Zidovudine (AZT)



Aprobarbital



Ritonavir

Figure 2. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 10.0 µg/ml AZT, (B) 8.0 µg/ml ddI (C) 16.0 µg/ml internal standard and (D) 9.1 µg/ml nevirapine in mixture A

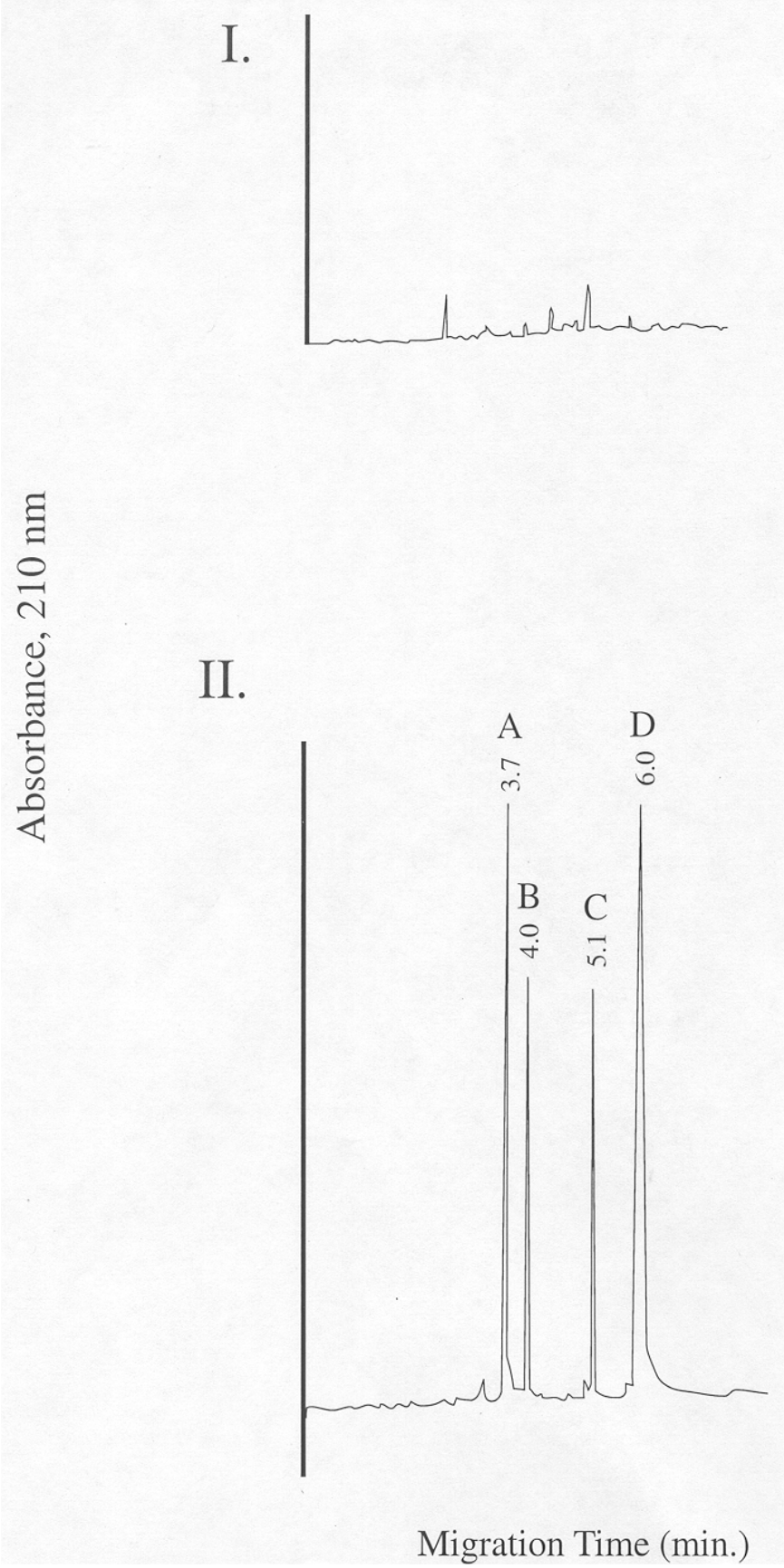
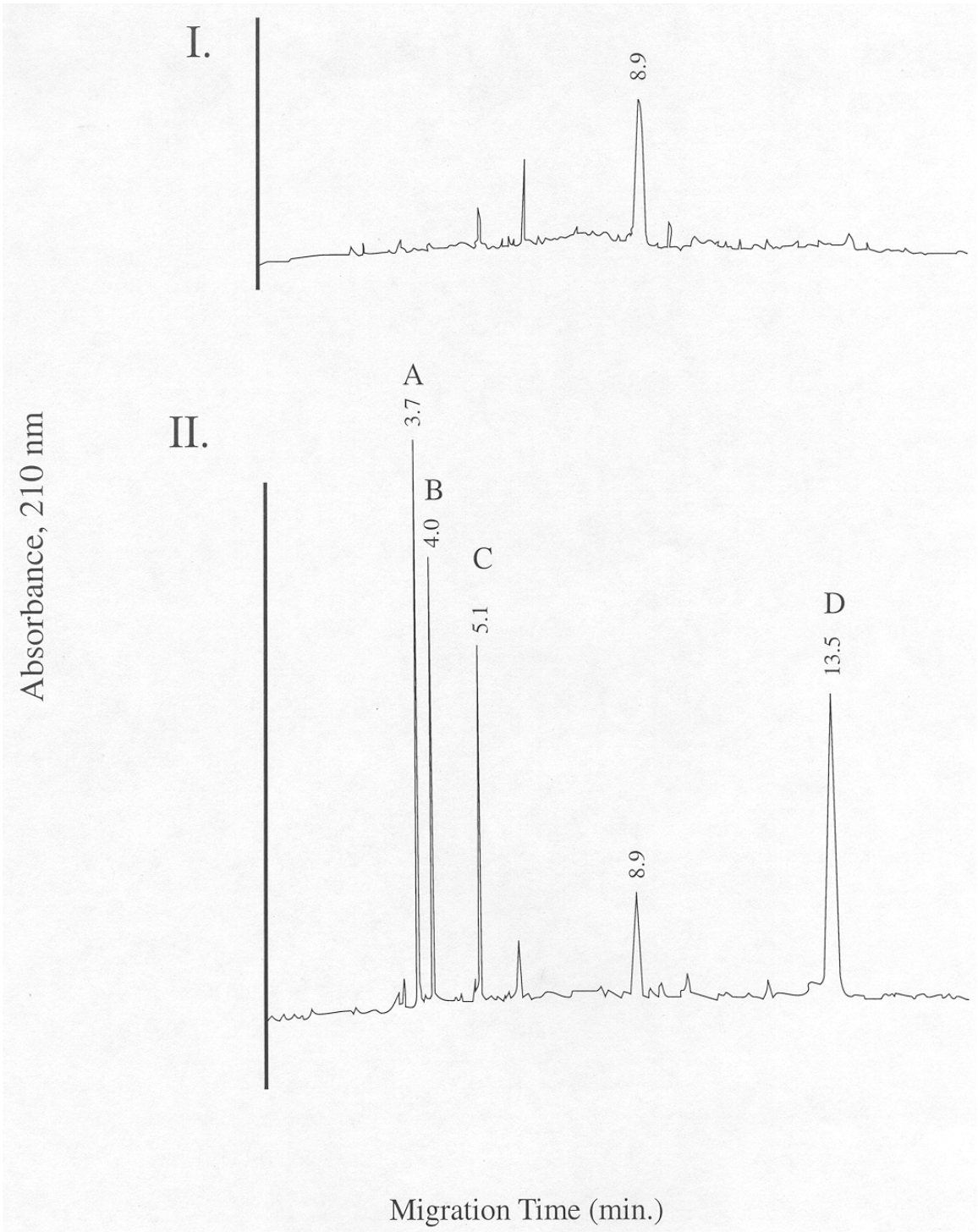

I.
II.
A
3.7
B
4.0
C
5.1
D
6.0
Absorbance, 210 nm
Migration Time (min.)

Figure 3. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 10.0 µg/ml AZT, (B) 8.3 µg/ml ddI (C) 16.0 µg/ml internal standard and (D) 11.5 µg/ml ritonavir in mixture B. The peak at 8.9 min is an unknown serum component.


I.
8.9
II.
A
3.7
B
4.0
C
5.1
8.9
D
13.5
Absorbance, 210 nm
Migration Time (min.)

CHAPTER 10

DETERMINATION OF STAVUDINE/DIDANOSINE/SAQUINAVIR AND STAVUDINE/DIDANOSINE/EFAVIRENZ IN HUMAN SERUM BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)[1]

[1] Fan, Bin. and James T. Stewart. To be submitted to Journal of Liquid Chromatography and Related Technologies, 2001.

Abstract

Anti-HIV drug mixtures A and B containing stavudine(d4T)/didanosine(ddI)/ saquinavir and stavudine(d4T)/didanosine(ddI)/efavirenz, respectively were separated and quantitated in human serum using micellar electrokinetic chromatography (MEKC). Serum samples were treated using a solid-phase extraction procedure. The effects of various factors such as buffer type, buffer and surfactant concentration, and pH on the separation of the analytes were investigated. The optimized resolution of both mixture was achieved with a run buffer containing 18 mM sodium dodecylsulfate (SDS) in 15 mM phosphate and borate buffer (pH 9.0). An uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica capillary operated at 30 °C was used in the analysis with UV detection at 210 nm. Aprobarbital was chosen as the internal standard. All analytes were separated within 15 min with a voltage of +15 kV and a current around 30 μA. The methods were validated over the range of 0.7-35.3 μg/ml for d4T, 0.8-18.5 μg/ml for ddI, 0.5-12.2 μg/ml for saquinavir in mixture A and 0.7-35.3 μg/ml for d4T, 0.8-18.5 μg/ml for ddI, 0.6-31.9 μg/ml for efavirenz in mixture B. Intra-day and inter-day accuracy were less than 13.7% and intra-day and inter-day precision were less than 14.5% for both mixture. Extraction recoveries of all analytes from serum were higher than 77.3%. The assay should be applicable for pharmacokinetic studies and routine monitoring of these drugs in serum.

Keywords: Stavudine; Didanosine; Saquinavir; Efavirenz; Micellar Electrokinetic Chromatography MEKC, Human Serum

Introduction

Today, an estimated 30.6 million people are living with HIV infections or AIDS [1]. Early in the epidemic, survival rates of less than 1 year were observed. However, the situation has changed significantly over the past 2 to 3 years following a number of advances, including the availability of newer antiretroviral agents and the demonstration that combination drug therapy is more effective than monotherapy [2,3]. Highly active antiretroviral therapy (HAART) and the use of aggressive combination antiretroviral regimens consisting of reverse transcriptase inhibitors and protease inhibitors, have become the standard of patient care [4,5]. The rationale for combining one or more anti-HIV agents is to provide more complete viral suppression, to limit the emergence of drug resistance during chronic viral replication and to provide more effective antiretroviral treatment even when mixtures of drug-resistant and drug-sensitive strains are present [6,7].

Separation and quantitative analysis of antiviral drugs are needed to study their pharmacokinetics in biological fluids as well as to monitor antiretroviral therapy in the treatment of HIV infection. Reported methods for the determination of antiretroviral drugs in patient sera have been based on HPLC methodology [8-10]. Recently, capillary electrophoresis (CE) has also been shown to be a powerful alternative to HPLC [11,12]. Conventional capillary zone electrophoresis (CZE) is most commonly used, but it fails to resolve uncharged anti-HIV drugs, such as AZT and d4T. Micellar electrokinetic chromatography (MEKC) is a mode of CE that is capable of separating uncharged compounds [13,14]. It is viewed as a chromatographic technique in which migrating

charged micelles act as a pseudostationary phase. MEKC offers analyte partitioning between micelles formed by a surfactant in the run buffer such that mixture of charged and uncharged anti-HIV drugs can be satisfactorily separated.

The aim of this study was to develop and validate a MEKC method for the determination of serum concentrations of d4T/ddI/saquinavir (mixture A) and d4T/ddI/efavirenz (mixture B). These assays employ a solid-phase extraction protocol and could be useful for drug monitoring, determination of pharmacokinetic profiles, and evaluation of drug-drug interactions.

Experimental

Chemical and reagents

Stavudine (d4T), didanosine (ddI), aprobarbital (internal standard), dibasic sodium phosphate and sodium dodecylsulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO 63178). Saquinavir was kindly provided by Roche Pharmaceuticals (Hertfordshire, UK). Efavirenz was provided by DuPont Pharmaceuticals Company (Wilmington, DE 19805). Concentrated phosphoric acid, sodium hydroxide and HPLC grade methanol were obtained from J.T. Baker Inc. (Phillipsburg, NJ 08865). Sodium tetraborate was purchased from Fisher Scientific, Inc (Fair Lawn, NJ 07410). Deionized water was purified by a cartridge system (Continental Water System, Roswell, GA 30076). Water Oasis^{TM} HLB 1cc 30 mg cartridges were purchased from Waters Corp (Milford, MA. USA). Drug free serum was obtained from Biological Specialty (Colmar, PA, USA).

Instrumentation

MEKC was performed with an Applied Biosystems 270A capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) equipped with a HP3395 integrator (Hewlett-Packard, Avondale, PA, USA). Separation was carried out on an uncoated 52 cm (effective length 30 cm) x 50 μm ID fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA). The capillary temperature was maintained at 30 °C with air coolant. The capillary was conditioned with 1N sodium hydroxide for 1 hour followed by 30 min of the run buffer before each day's run. Before each run, the capillary was rinsed with 0.1 N sodium hydroxide for 3 min and run buffer for 3 min. The applied voltage was +15 kV for the separation and the detection wavelength was 210 nm. The detection window was created by stripping the polyimide coating of the capillary in a length of 5 mm. The sample was injected by applying a 5 in. Hg vacuum to the outlet of the capillary for 3 s.

Preparation of standard solutions

Stock solutions of stavudine (d4T), didanosine (ddI), saquinavir and aprobarbital (internal standard) were prepared in deionized water to give concentrations of 1414, 826, 912 and 1600 μg/ml in mixture A, respectively. Stock solutions of stavudine (d4T), didanosine (ddI), efavirenz and aprobarbital (internal standard) were prepared in deionized water to give concentrations of 1414, 826, 1275 and 1600 μg/ml in mixture B, respectively. Calibration standards were prepared by diluting these stock solutions with drug-free serum, and 10 μl of the stock aprobarbital internal standard solution was added to obtain a final volume of 1ml. The concentrations in mixture A were 0.7-35.3 μg/ml

(0.7, 2.8, 7.1, 14.1 and 35.3 μg/ml) for d4T, 0.8-18.5 μg/ml (0.8, 1.9, 4.1, 8.3 and 18.5 μg/ml) for ddI, 0.5-12.2 μg/ml (0.5, 2.4, 4.9, 9.8 and 12.2 μg/ml) for saquinavir. The concentrations in mixture B were 0.7-35.3 μg/ml (0.7, 2.8, 7.1, 14.1 and 35.3 μg/ml) for d4T, 0.8-18.5 μg/ml (0.8, 1.9, 4.1, 8.3 and 18.5 μg/ml) for ddI, 0.6-31.9 μg/ml (0.6, 2.6, 6.4, 12.8 and 31.9 μg/ml) for efavirenz. Samples were stored at 4 °C.

Assay Validation

The method accuracy was obtained by comparing the concentrations calculated from the calibration curves versus concentrations added. Precision was calculated as percent relative standard deviation (%RSD). The intra-day accuracy and precision of the assay were determined by assaying quality control samples at low, medium and high concentrations for d4T (1.4, 7.0 and 28.0 μg/ml), ddI (1.6, 8.0 and 16.0 μg/ml), saquinavir (1.0, 5.0 and 10.0 μg/ml) in mixture A in three analytical run within the same day; and for d4T (1.4, 7.0 and 28.0 μg/ml), ddI (1.6, 8.0 and 16.0 μg/ml), efavirenz (1.2, 7.5 and 27.5 μg/ml) in mixture B in three analytical runs within the same day. The inter-day accuracy and precision samples were analyzed on three different days. Three sets of samples at each analyte concentration were performed. The absolute recoveries of each drug and internal standard were obtained by comparing the extracted serum samples to unextracted stock solutions.

Sample preparation procedure

Waters Oasis^{TM} HLB 1cc cartridges were conditioned with 1 ml methanol followed by 1 ml deionized water. One ml of the spiked serum samples was loaded onto

the cartridges and drawn by applying a vacuum. The cartridges were then washed with 1 ml methanol-water (10:90, v/v). 1 ml methanol was used to elute the adsorbed analytes. The eluting solvent was evaporated to dryness and reconstituted in 1ml deionized water. 3s hydrodynamic injections of samples were made at the anodic end of the capillary.

Result and Discussion

Two precedures were evaluated for preparation of analytical samples of spiked analytes in human serum prior to analysis by MEKC. Protein precipitation using acetonitrile resulted in large numbers of endogenous peaks, which interfered with the compounds being analyzed. The recoveries for all analytes were much lower compared to the SPE method. Solid-phase extraction using a series of different extraction cartridges was investigated, such as C_{18}, C_8, and Oasis^{TM} cartridges. Larger than 20% loss of d4T was observed with the C_8 cartridges during the loading step. Oasis^{TM} gave the highest recoveries of the analytes as well as cleaner assay samples using a cartridge wash solution containing 10% methanol.

In MEKC, the choice of run buffer composition was crucial. The effects of buffer type, concentration and pH on the separation were investigated. A sodium phosphate-sodium borate run buffer provided sharp, symmetric peaks, and also gave good reproducibility in migration times and buffer capacity over a large pH range. Besides running current, buffer concentrations affected buffering capacity and electroosmosis flow (EOF). Generally, the higher the buffer concentration, the higher the electrical current, and the greater the buffering capacity. This will prevent buffer depletion and improve assay reproducibility. Longer migration times and sharper peaks for the analytes

were obtained with higher buffer concentrations. When the run buffer concentration was optimized to 15 mM, the best separations of both mixtures A and B were achieved with a migration time < 15 min. The pH of the buffer was an important factor to manipulate selectivity of the ionizable analytes except d4T. Typically a pH between 7 and 9 was employed to assure proper MEKC flow characteristics. The effects of buffer pH on the separations were studied from pH 6.5 to 10.5. Electroosmotic flow had an important effect on resolution and analysis time in the MEKC separations. At pH< 6.5, the EOF was relatively small, and it was difficult to get symmetric and reproducible peaks. EOF increased with increasing pH and the migration times decreased but with an adverse impact on peak efficiency. pH 9.0 was found to be the optimum pH for both mixtures A and B since the best separations of the analyte mixtures were achieved.

Surfactant type and concentration also influenced the electrophoretic migration in MEKC. Sodium dodecyl sulfate (SDS) in MEKC served two functions: it reduced the capillary surface interactions and it formed charged micelles. At a low critical micellar concentration (CMC), SDS reduced the conductivity of the buffer and joule heating. The primary role of the surfactant concentration was to adjust the retention factor within the optimum range to achieve better resolution of the analytes. The concentration of SDS was set at 18 mM for both mixtures A and B.

The MEKC separations of mixture A and B were performed using an untreated fused-silica capillary. The capillary exhibited rapid electroosmosis flow, and consequently, resulted in a short elution range. The implementation of MEKC entailed washing the capillary with sodium hydroxide solution. The frequency of rinsing the capillary and the wash solutions had the greatest effect on migration reproducibility. In

addition, the migration behavior of the analytes that interacted with micelles was not repeatable unless the proper rinse protocol was applied. A correlation between inconsistent migration behavior and fluctuation in electric current was observed, which might indicate the existence of nonequilibrium conditions between the run buffer and the capillary wall. The washing procedures used decreased the deleterious effects of capillary "aging," which can result in changes in sample-wall interactions and electroosmotic flow.

Figure 1 lists the chemical structures of the analytes in the mixtures A and B. In mixture A, d4T, ddI and saquinavir were baseline separated with migration times of 3.4, 4.0 and 14.1 min, respectively. The migration time for the internal standard aprobarbital was 5.2 min. Figure 2 shows the electropherograms of blank serum and spiked serum samples for mixture A. In mixture B, baseline separation of the d4T, ddI and efavirenz mixture was achieved with migration times of 3.4, 4.0 and 13.1 min, respectively. The internal standard aprobarbital gave a migration time of 5.1 min in this mixture. Figure 3 shows the electropherograms of blank serum and spiked serum samples with mixture B. The calibration curves for mixture A showed good linearity in the concentration range of 0.7-35.3 µg/ml for d4T, 0.8-18.5 µg/ml for ddI, and 0.5-12.2 µg/ml for saquinavir. The calibration curves for mixture B showed good linearity in the concentration range of 0.7-35.3 µg/ml for d4T, 0.8-18.5 µg/ml for ddI and 0.6-31.9 µg/ml for efavirenz. The regression coefficients (r^2) of calibration curves of each drug were higher than 0.99. LOD and LOQ data are shown in Table1. The methods proved to be accurate and precise. Extraction recoveries of all analytes from serum were higher than 77.3%. The data from validation of the methods in human serum are listed in Table 2.

Conclusion

The MEKC separation and quantitation of anti-HIV drug mixtures containing d4T/ ddI/saquinavir (mixture A) and d4T/ ddI/efavirenz (mixture B) using solid phase extraction provided fast, sensitive and selective procedures for these mixtures in human serum.

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Table 1. Range of calibration curves, limits of detection (LOD) and limits of quantification (LOQ) of d4T, ddI, saquinavir (mixture A) and d4T, ddI, efavirenz (mixture B) in spiked human serum

Drug	Range of calibration curves (µg/ml)	Limits of detection (LOD) (µg/ml)[a]	Limits of quantification (LOQ) (µg/ml)[b]
d4T	0.7—35.3	0.3	0.7
ddI	0.8—18.5	0.4	0.8
Saquinavir[c]	0.5—12.2	0.3	0.5
Efavirenz[d]	0.6—31.9	0.3	0.6

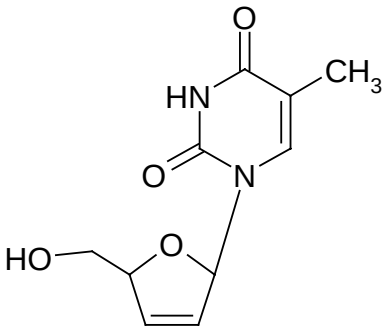
a S/N=3
b S/N=10
c Present in mixture A with d4T and ddI
d Present in mixture B with d4T and ddI

Table 2. Inter-day and intra-day accuracy, precision and recovery for the analysis of d4T, ddI, saquinavir (mixture A) and d4T, ddI, efavirenz (mixture B) in human serum

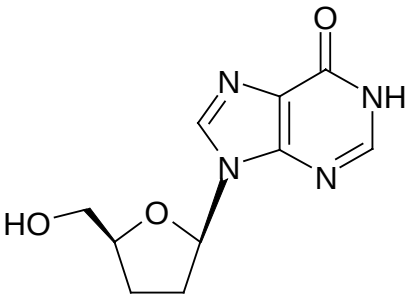
	Concentration	Precision (%)		Accuracy (%)		Plasma Recovery[c]
	(μg/ml)	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	(%)
d4T	28.0	1.5	1.8	3.7	1.7	95.4±3.0
	7.0	3.6	8.2	3.2	10.6	88.3±4.5
	1.4	7.8	1.5	11.4	6.4	84.6 ±4.8
ddI	16.0	4.8	10.8	4.2	12.4	83.1 ±5.1
	8.0	10.2	5.7	4.7	9.7	77.3 ±3.3
	1.6	12.2	14.5	9.8	2.8	82.0 ±6.5
Saquinavir[d]	10.0	1.7	4.7	6.4	5.6	99.1±2.2
	5.0	4.1	13.9	2.8	11.0	100.7±1.2
	1.0	8.2	12.8	7.2	9.8	95.6±4.4
Efavirenz[e]	27.5	2.1	6.0	5.2	5.7	103.2±2.8
	7.5	6.6	6.1	8.1	11.8	86.0±1.9
	1.2	5.0	7.0	13.7	8.6	89.7±2.0

a Based on n = 3
b Based on n = 9
c Mean ± SD based on n = 9
d Present in mixture A with d4T and ddI
e Present in mixture B with d4T and ddI

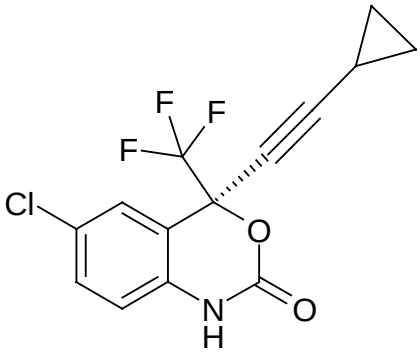
Figure 1. The chemical structures of analytes in Mixtures A and B



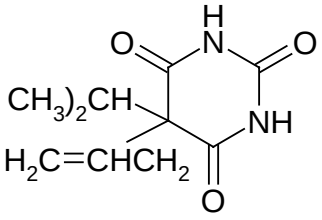
Stavudine (d4T)



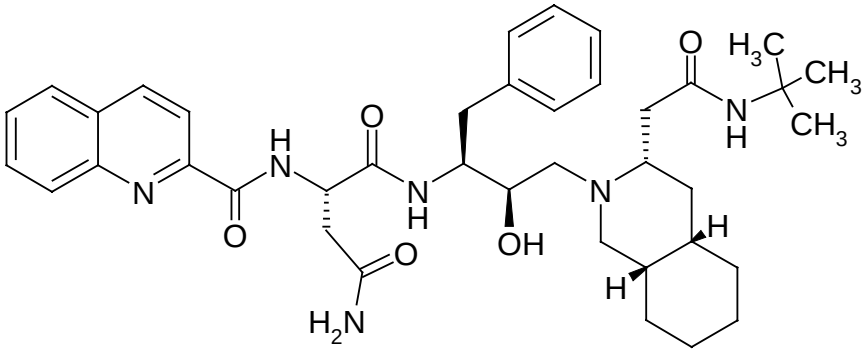
Didanosine (ddI)



Efavirenz



Aprobarbital



Saquinavir

Figure 2. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 14.1 µg/ml d4T, (B) 8.0 µg/ml ddI, (C) 16.0 µg/ml internal standard and (D) 9.8 µg/ml saquinavir in mixture A. The peak at 9.1 min is an unknown serum component.

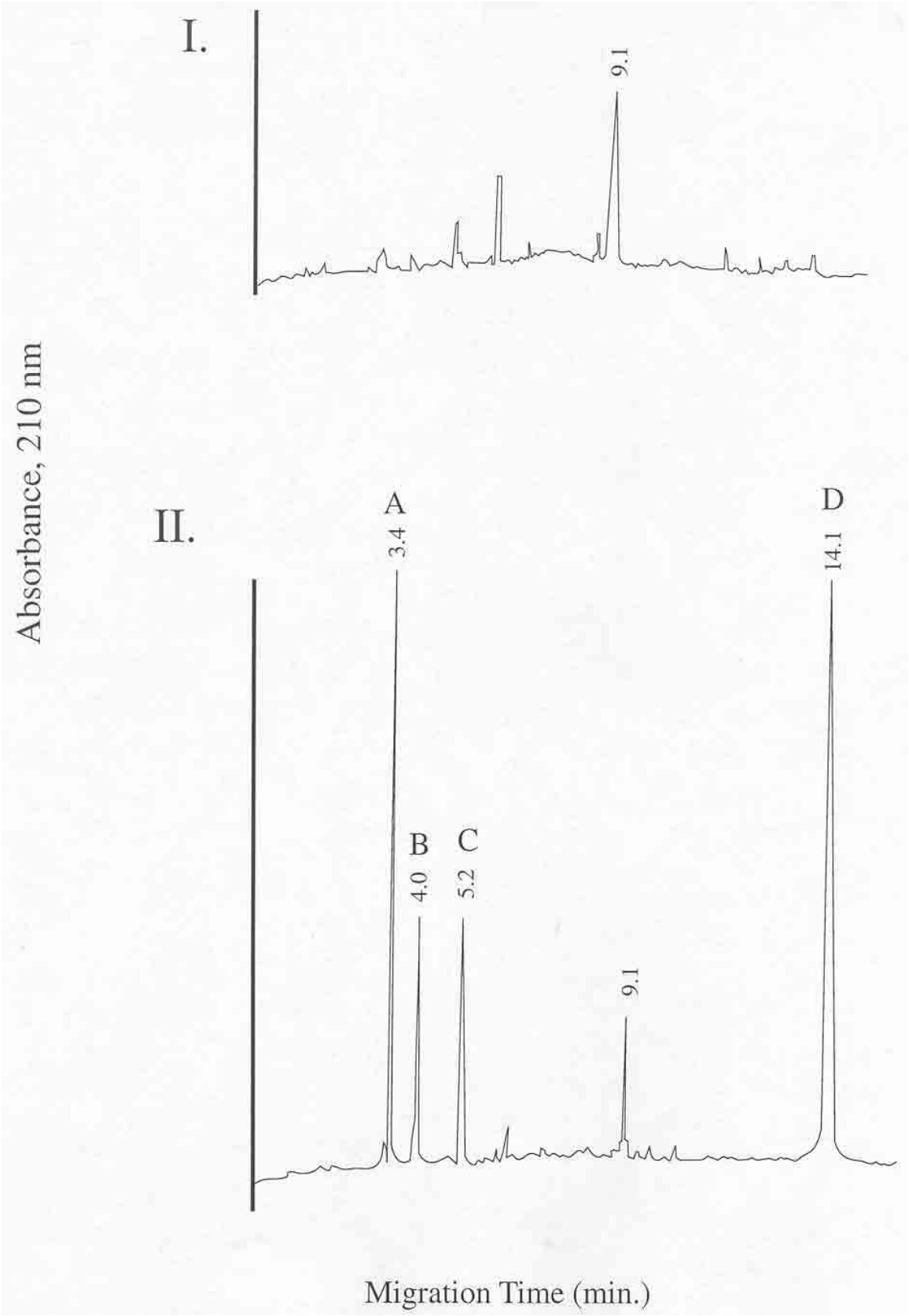
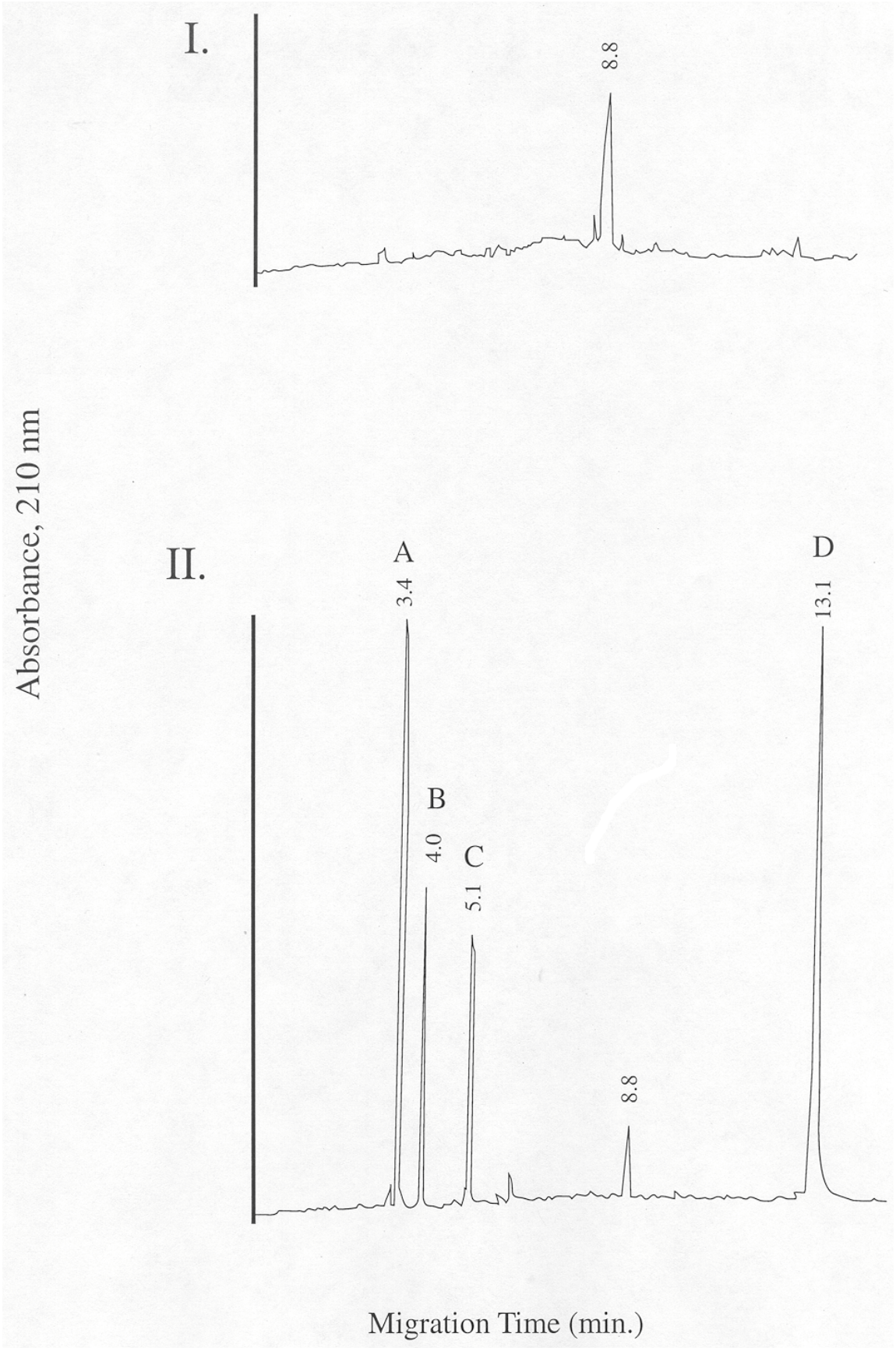

I.
9.1
II.
A
3.4
B
4.0
C
5.2
9.1
D
14.1
Absorbance, 210 nm
Migration Time (min.)

Figure 3. Representative electropherograms of I. blank human serum and II. human serum spiked with (A) 14.1 µg/ml d4T, (B) 8.3 µg/ml ddI, (C) 16.0 µg/ml internal standard and (D) 12.8 µg/ml efavirenz in mixture B. The peak at 8.8 min is an unknown serum component.


I.
8.8
II.
A
3.4
B
4.0
C
5.1
8.8
D
13.1
Absorbance, 210 nm
Migration Time (min.)

CONCLUSION

In this dissertation, pharmaceutical analysis and bioanalysis were discussed in term of formulation development and combination anti-HIV drug therapeutic monitoring.

Part I concerned pharmaceutical analysis during formulation development for the prodrug metronidazole benzoate. Two new dosage forms, an oil-in-water emulsion and a chicken-flavored glycerinated gelatin candy-based lozenge, were prepared for veterinary use. The HPLC assay developed was sensitive and stability-indicating. Waters Millennium^{32} PDA software with photodiode-array detection was effective for the determination of metronidazole benzoate degradation products and peak purity checking. Metronidazole benzoate was not only stable in both formulations at ambient temperature and 4ºC, but was also released rapidly and completely.

Part II concerned the bioanalysis in anti HIV drug monitoring. Bioanalytical techniques (HPLC, CE, and LC-MS-MS) were employed and various methods were developed and validated. An ion pair chromatography method with octanesulfonic acid sodium salt as the ion pair reagent offered a reliable solution to separate and quantitate some of the anti-HIV drugs under isocratic conditions. These drugs were zidovudine, lamivudine, zidovudine and nevirapine. Gradient chromatography utilizing tandem mass spectrometry (LC-MS-MS) provided a fast, sensitive and selective procedure. The anti-HIV drugs separated and quantitated were lamivudine, stavudine, didanosine, efavirenz and ritonavir. Capillary electrophoresis methods including capillary zone electrophoresis and micellar electrokinetic chromatography were employed as suitable alternatives for the simultaneous determination of anti-HIV drugs in human serum. These drugs were

zidovudine, lamivudine, didanosine, stavudine, saquinavir, ritonavir, efavirenz and nevirapine.

APPENDICES

APPENDIX 1

COMPUTER SIMULATION MODEL FOR VITAMIN EFFECTS ON HOMOCYSTEINE METABOLISM[1]

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[1]Fan, Bin. Nandita Bose, James L. Hargrove and Diane K. Hartle. 1999. Health Sciences Simulation, San Francisco: the Society for Computer Simulation International, pp170-174.

ABSTRACT

We have developed a STELLA computer simulation model for the metabolic regulation of homocysteine (HCY) plasma levels using kinetic data from the human medical literature. The model illustrates key regulatory steps in the metabolic interrelationships among the following sulfur-containing amino acids: methionine, homocysteine, and cysteine. Both instantaneous and steady state plasma levels of HCY are predicted by the model for various degrees of substrate loading in either normal or abnormal metabolic states. The simulation model demonstrates that the rate of transsulfuration controls the ultimate rate of HCY disposition. Moreover, the model predicts that S-adenosylmethionine (SAM) levels determine the degree to which folate supplementation lowers plasma HCY. An increase in SAM activates transsulfuration while it inhibits remethylation of HCY. The result is that lower SAM levels insure an adequate rate of remethylation to continuously supply methyl donors and higher SAM levels promote irreversible disposition of HCY to prevent unnecessary cycling of high levels of HCY in the methionine cycle. Altogether, the effect of SAM is to produce a metabolic "switch" that allows adequate HCY levels to supply the methionine cycle, but prevents the buildup of this toxic amino acid. In this way, levels of HCY in plasma can normally be regulated in the low μmolar range with varying intakes of methionine. Finally, the model predicts that folate will lower normal fasting plasma HCY levels only when the SAM "switch" is operating.

KEY WORDS: Methionine, homocysteine, S-adenosylmethionine, atherogenesis, transsulfuration, remethylation

INTRODUCTION

Homocysteine (HCY) is an intermediate sulfhydryl amino acid formed during the metabolism of methionine to cysteine. Current epidemiological research indicates that HCY is an independent risk factor for coronary artery disease (Malinow 1996 and Boers 1997) and contributes to the rate of progression of atherosclerosis (Mayer and Jacobsen 1996). A dose-response relationship appears to exist between plasma HCY concentration and the severity of cardiovascular disease (Stampfer and Malinow 1995). Multiple studies indicate that HCY is toxic to vascular endothelial cells (see Boers 1997 and Harpel 1996 for recent reviews). Endothelial cell toxicity or injury is generally considered to be a mechanism accelerating the rate of development of atherosclerosis.

HCY is metabolized by entering either a transsulfuration pathway or one of the remethylation pathways synthesizing methionine (Finkelstein and Martin 1986; Finkelstein 1990). Plasma HCY levels generally reflects the status of methionine metabolism.

The metabolic fate of HCY is regulated by the relative activities of cystathionine beta-synthase (CBS), methionine synthase complex, and methylene-tetrahydrofolate reductase (MTHFR). The dietary vitamins B_6 (pyridoxal phosphate), folic acid and B_{12} (cobalamin) serve as precursors of the requisite cofactors for HCY metabolism, pyridoxal-5-phosphate, methylcobalamin and methylene tetrahydrofolate, respectively (Ueland et al. 1992). Plasma HCY levels is markedly elevated in deficiencies of these vitamins. Supplementation with folic acid and vitamins B_6 and B_{12} not only reduces plasma HCY concentration but also ameliorates endothelial dysfunction caused by HCY.

MODEL DEVELOPMENT AND SIMULATION

MODEL DEVELOPMENT

Fig. 1 shows the metabolic scheme used for the simulation model. There are six compartments in this model, including three cycles: plasma methionine equilibrated with tissue methionine, HCY plasma levels equilibrated with tissue HCY levels and tissue methionine metabolism to tissue HCY. HCY accumulates when methionine is activated by ATP forming SAM, which donates a methyl group to multiple endogenous acceptors, in turn forming S-adenosylhomocysteine. The latter intermediate is then hydrolyzed forming HCY. The formation of SAM is the rate-limiting step in the conversion of methionine to HCY. The model therefore omits the non-rate-limiting SAM to S-adenosyl-homocysteine flux. HCY pools are depleted through remethylation and transsulfuration metabolic outflow pathways.

The model was constructed with two levels of operation. Initial values are entered on the control panel (using input devices called sliders) to provide the loading dosage, enzyme efficiencies, and vitamin dosages. Any combination of parameters may be used to simulate various metabolic states. An important feature of the model is that the SAM compartment acts as a metabolic "switch" that reciprocally activates the transsulfuration pathway while inhibiting the remethylation pathways as SAM accumulates after an increase in methionine intake.

MODEL OPERATION

After adjusting the metabolic parameters of the model to achieve a steady-state fasting level of HCY in the normal range (9 μM), the effects of separate and combined

vitamin supplementation (Vitamin $B_{12,}$ 1 mg/d; Vitamin $B_{6,}$ 10 mg/d; folic acid, 1 mg/d) were carried out to observe effects on the fasting levels of HCY under normal conditions.

RESULTS

Sensitivity of parameters controlling plasma levels of HCY

The parameters used to construct this model were from kinetic values published in the human clinical literature (Young 1991 and Hiranatsu 1994). In order to test which parameters in the model were most responsible for maintaining steady-state HCY levels, we arbitrarily doubled each parameter individually to test its relative influence on regulation of fasting plasma HCY levels. The model predicts that four of the parameters exert most control over the pathway (Table 1). Dietary intake rates and the transsulfuration rate (K_{ts}) are sensitive kinetic parameters (Table 1). According to the model, SAM controls the output via the TS pathway. Since this is the highest capacity metabolic outflow pathway, it plays a crucial role in disposition of plasma HCY at methionine intakes that increase SAM. However, the cycle from tissue HCY to plasma HCY is an equally sensitive part of the model.

Vitamin effects on fasting plasma HCY levels

Vitamin supplements significantly lower fasting plasma HCY levels. Given separately, folic acid (5mg/d) caused a 52% decrease in fasting plasma HCY levels (from 9 μM to 4.33 μM). Given separately, vitamin B_6 (10 mg/d), decreased plasma HCY level from 9 μM to 7.7 μM. Vitamin B_{12} (1 mg/d), produced no significant change in normal fasting plasma HCY. Given as a combined supplement, the vitamins decreased plasma HCY from 9 μM to 3.78 μM (Fig. 2).

The effect of SAM on the regulation of plasma HCY level

SAM affects plasma HCY level through activating the transsulfuration pathway and inhibiting remethylation. After adding a SAM compartment to simulate these effects, folic acid significantly lowered the plasma HCY level (Table 2).

DISCUSSION

Factors controlling disposition of HCY

HCY is a toxic amino acid intermediate that is tightly regulated to prevent plasma levels from accumulating above the low (10-15) μmolar range. Although HCY can be metabolized by both remethylation and transsulfuration pathways, remethylation leads to recycling via methionine synthesis while transsulfuration produces cystathionine. This step is irreversible under most physiological conditions. HCY is then committed to the cysteine synthetic pathway and effectively removed from the methionine metabolic cycle. Cysteine metabolism leads to permanent elimination. When we tested the sensitivity of HCY plasma levels to relative changes in the kinetics of the disposition pathways we verified that the transsulfuration pathway exerts more control over disposition of HCY than the remethylation pathways. An additional mechanism which regulates plasma HCY level is the flux between tissue and plasma. HCY export from tissues is very efficient and plasma HCY concentrations are most sensitive to this rate. This export mechanism maintains low intracellular HCY levels and prevents toxicity to tissue cells at the expense of increasing plasma HCY levels and potentially causing hyperhomocysteinemia and homocystinuria (Christensen et al. 1991; Selhub and Miller 1992).

Effect of SAM on the regulation of plasma HCY level

The model predicts that the responsiveness of plasma HCY levels to folic acid supplementation depends on the concentration of SAM, which inhibits remethylation but stimulates transsulfuration. This result is consistent with clinical results (Brattstrom et al. 1988). The HCY metabolic system is regulated in several ways. In situations of excess methionine intake, there is an abundance of SAM. In this situation the transsulfuration pathway is favored because of an activation of cystathionine beta-synthase and a concomitant inactivation of the remethylation pathways (Finkelstein 1986). Conversely, when methionine intake is low, the remethylation pathway is favored. This recycling of HCY insures that adequate methionine is available for protein synthesis and SAM synthesis. SAM is the major methyl donor molecule in intermediary metabolism (Finkelstein 1990). SAM also acts as a "switch" to regulate the rate of HCY remethylation and elimination. The behavior of the model supports the hypothesis that folic acid would have little effect on plasma HCY in the absence of SAM's regulatory effects on transsulfuration and remethylation. (Selhub and Miller 1992; Mason and Miller 1992).

ACKNOWLEDGMENTS

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Figure 1. Diagrammatic representation of key steps in methionine metabolism showing the compartments and the modeled flux and cycling among sulfur-containing amino acid compartments. Each reaction follows first order kinetics except the step from SAM to tissue homocysteine. SAM, S-adenosylmethionine; K_{out}, the rate of methionine transfer from tissue to plasma; K_{in}, the rate of methionine transfer from plasma to tissue; K_{mat}, the rate of methionine adenosyltransferase; K_{sdt}, S- adenosylmethionine-dependent transmethylation; $K_{outflow}$, the rate of homocysteine transfer from tissue to plasma; K_{inflow}, the rate of homocysteine transfer from plasma to tissue. K_{rm}, the rate of remethylation; K_{ts}, the rate of transsulfuration. K_m, the rate of cysteine metabolism

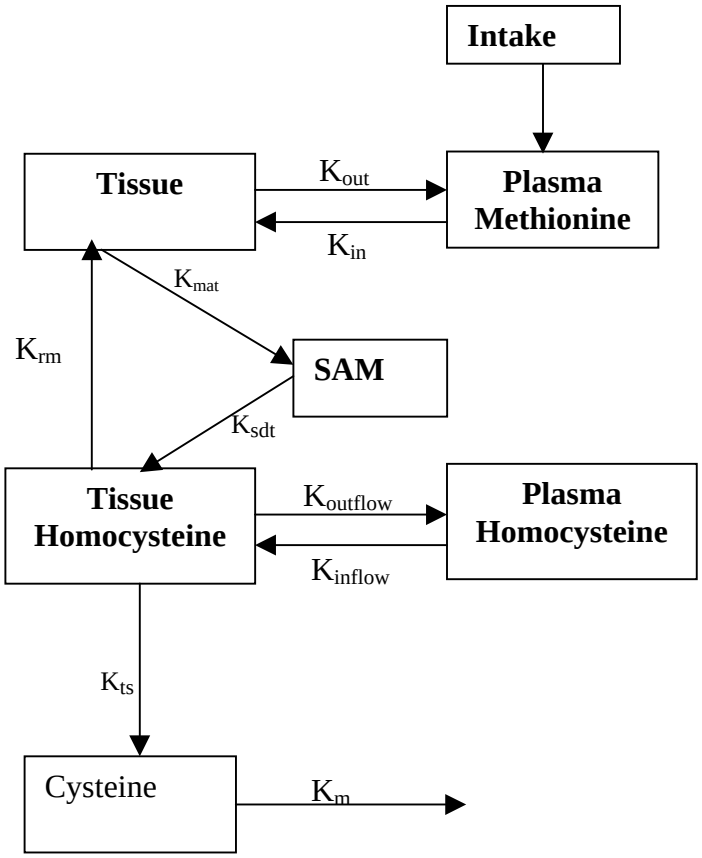
Intake
Tissue
Plasma Methionine
K_{out}
K_{in}
K_{mat}
K_{rm}
SAM
K_{sdt}
Tissue Homocysteine
Plasma Homocysteine
$K_{outflow}$
K_{inflow}
K_{ts}
Cysteine
K_m

Figure 2. Fasting plasma HCY concentrations before and after vitamin supplementation. 1. Before vitamin supplementation, 2. Vitamin B_{12} supplementation, 3. Folic acid supplementation, 4.Vitamin B_6 supplementation, 5. Combined Vitamin B_{12}, folic acid and Vitamin B_6 supplementation

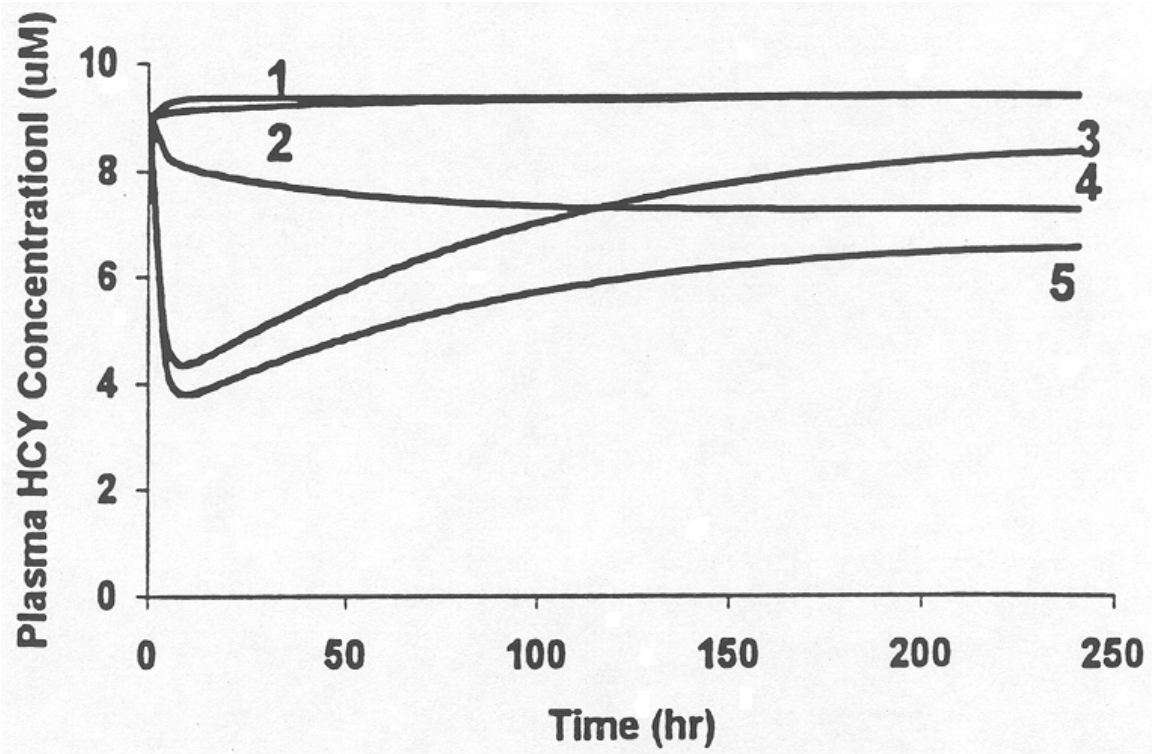
Plasma HCY Concentrationl (uM)
10
8
6
4
2
0
0
50
100
150
200
250
Time (hr)
1
2
3
4
5

Table 1. Sensitivity of fasting plasma HCY levels with an arbitrary increase in each kinetic parameter

Parameter*	Change in Fasting HCY Plasma Level
K_{mat}	+5.2%
K_{ts}	-39.1%
K_{rm}	-17.5%
K_{m}	No change
$K_{outflow}$	+97.6%
K_{inflow}	-46.6%
K_{in}	+3.8%
K_{out}	-2.5%
Methionine intake	+85.5%

*See the legend of Fig. 1 for parameter abbreviations.

Table 2. Effects of folic acid on plasma HCY level and relative rates of the transsulfuration (TS) and remethylation (RM) pathways with and without SAM regulatory switch

	HCY	RM	TS
Plus SAM switch	52%	70.90%	53.70%
Minus SAM switch	17%	52.60%	17.40%

APPENDIX 2

INTRAVENOUS REGIONAL ANESTHESIA IN CATS: LIDOCAINE PLASMA CONCENTRATIONS AND CARDIOVASCULAR EFFECTS[1]

[1]Kushner, Lynne I. Bin Fan and Frances S. Shofer. Submitted to Veterinary Anaesthesia and Analgesia, 2001.

Abstract

Objective To determine if intravenous regional anesthesia (IVRA) can be used in cats without resulting in excessive plasma lidocaine concentrations or adverse cardiovascular effects.
Study design Prospective, blinded crossover study.
Animals Seven healthy male young adult cats.
Methods At 2.3% end-tidal (ET) isoflurane concentration, lidocaine (L) 3mg/kg (1%) or saline (S) was injected in a distal cephalic catheter after application of 2 tourniquets to that forelimb which remained in place for 20 min. Heart and respiratory rates, arterial blood pressures and ECG were recorded every 5 min during tourniquet application and for 20 min following tourniquet removal. Lidocaine plasma concentrations were measured 5 min after injection and 0.5, 1, 2, 4, 8, and 20 min after tourniquet removal. ET isoflurane concentrations were reduced to 1.5-2.0 % to illicit a response to toe pinch (RTP) in the contralateral leg. The study was repeated similarly in the contralateral leg and RTP was graded for 40 min. Response was also illicited in the leg previously injected, the differences between the 2 scores determined and those differences were compared between the L and S groups. The data were analyzed using ANOVA in repeated measures and physiologic variables were performed on differences from baseline. Significance was set at a $p < 0.005$ using the Bonferonni method for multiple comparisons.
Results There were no significant differences in physiologic parameters at either isoflurane concentration. Differences in RTP were significantly larger in the lidocaine group. The highest mean lidocaine concentrations were measured 0.5 min after tourniquet removal after both injections and were 2.79 ± 1.05 and 3.10 ± 1.11 $\mu g\ ml^{-1}$. The highest individual plasma concentration was 6.46 $\mu g\ ml^{-1}$.
Conclusion No adverse hemodynamic effects were evident after IVRA lidocaine in any cat. The lidocaine dosage studied inhibited a RTP until 20 min after tourniquet removal. Lidocaine concentrations varied and were measurable prior to tourniquet removal.
Clinical relevance- IVRA may be a suitable technique for cats undergoing surgery of the distal limbs.

(Key words: IV regional anesthesia (IVRA); lidocaine; cats; analgesia).

Introduction

Intravenous regional anesthesia (IVRA) is a local anesthetic technique introduced by Bier in 1908 and modified by Holmes (Holmes 1963) for surgery on distal extremities in humans. Still practiced in human medicine, IVRA is used in ruminants and other domestic species to perform digit amputations and other surgeries involving the distal limb (Bogan and Weaver 1978; Kupper 1977). The technique involves placement of a tourniquet on the limb prior to injection of a local anesthetic in a vein distal to the tourniquet. The local anesthetic diffuses into the tissues producing local anesthesia in the affected area. The safety and efficacy is related to interruption of blood flow to the occluded limb thus preventing the local anesthetic from entering the circulation. Lidocaine is often but not exclusively used for IVRA.

Many anesthetic and analgesic protocols, from general anesthesia to injectable techniques have been employed for onychectomy in cats (Lin et al 1993; Ko et al 1993; Winkler et al 1997; Ringwood and Smith 2000). In veterinary teaching hospitals, it is this author's experience that the duration of the procedure coupled with the need for adequate analgesia necessitates general anesthesia or increased dosage rates of injectable agents. The former requires additional equipment and time; the latter may result in prolonged recoveries. Both techniques may result in significant cardiopulmonary depression. As an adjunct to light anesthesia, IVRA may provide optimum analgesia during surgery which could result in less post-operative pain.

Although IVRA appears to be well suited for surgery such as onchyectomy, to this author's knowledge, its usage has not been explored in cats. Safe dosage of lidocaine for feline clinical patients, in particular for IVRA, has not been defined. Although IVRA

is considered to be a simple, safe and reliable technique (Hartmannsgruber et al 1999; Kupper 1977), the potential exists for adverse reactions from excessive plasma anesthetic concentrations due to high dosage rates, rapid tourniquet removal or leakage of local anesthetic underneath the tourniquet.

The objectives of this study were to determine if bilateral IVR administration of 3 mg/kg 1% lidocaine can be administered to cats without producing excessive lidocaine plasma concentrations and deleterious cardiovascular and respiratory effects. In addition, effectiveness of dosage and technique was assessed by response to toe pinch and the duration of effect was determined. It is hypothesized that healthy cats can tolerate moderate lidocaine concentrations with no adverse affects.

Material and Methods

Animals

This study was reviewed and approved by the Animal Care and Use Committee at Mississippi State University College of Veterinary Medicine. Seven young adult male cats weighing 3.96 ± 0.63 kg were studied. Cats were determined healthy by physical exam and hematologic and blood chemistry testing. A crossover design was used and cats were randomly assigned to either drug A or B (lidocaine or saline) for the first treatment. The cat was studied again using the other treatment after a period of 7- 11 days and the investigator was blinded to treatment.

Anesthesia and instrumentation

Cats were placed in a plexiglass chamber and induced with 4% isoflurane and oxygen until intubation could be accomplished using a cuffed endotracheal tube.

Anesthesia was maintained using a non-rebreathing system (Jackson Reese, Owens-Brigham Medical Co, Newland NC) with an oxygen flow rate of 300 ml kg^{-1} min^{-1}. Anesthesia was delivered by a precision vaporizer (Vapor 19.1, Drager, Telford, PA) at an anesthetic concentration which prevented movement and autonomic responses to instrumentation. Breathing was spontaneous and respiratory rate was measured by counting chest excursions over 30 second intervals. End-tidal carbon dioxide ($ETC0_2$) and anesthetic concentrations, sampled from a catheter with its distal end proximal to the carina, were measured by an infrared analyzer (Ohmeda RGM; Ohmeda Anesthesia Systems; Madison, WI) that was calibrated prior to each study using the appropriate calibration gas (Anesthesia calibration gas; Ohmeda Anesthesia Systems; Madison, WI). Cats were placed on a warm water circulating heating pad to maintain body temperature. Rectal body temperature was measured with a digital thermometer.

A 20 ga 32mm IV catheter (Surflo; Terumo Medical Corp; Elkton, MD) was placed in the medial saphenous vein for administration of a balanced electrolyte solution (Lactated Ringers solution; McGraw Inc, Irvine, CA) at 5 ml kg^{-1} hr^{-1}. The femoral artery of the contralateral leg was surgically exposed at the femoral triangle using aseptic technique for placement of a 22 ga 25mm IV catheter (Surflo, Terumo Medical Corp; Elkton, MD) for measurement of arterial blood pressures. The catheter was attached to a pressure transducer via non-compliant tubing filled with heparinised saline. Blood pressure was measured using the pressure transducer (Gould-Stratham, Oxnard, CA) that was calibrated to a mercury manometer prior to each experiment The midline of the sternum was used as the reference point for transducer position. Blood pressure and lead II ECG were continuously displayed on a monitor (Physio control VSM 1 vital signs

monitor; Physio Control Corp.; Redmond, WA) and recorded at the specified time intervals. Heart rate was counted by cardiac auscultation over 15 second intervals. Arterial blood (0.5 ml) was anaerobically removed for pH, bicarbonate and blood gas analysis (Chiron Diagnostics 248 pH blood gas analyzer, Bayer Corp Norwood, MA) placed in an ice bath and analyzed within 30 min.

A 22 ga 25mm IVcatheter was placed in the cephalic vein with the tip of the catheter positioned proximal to the carpus and pointing distally for injection of lidocaine or saline. After instrumentation cats were kept in lateral recumbency and maintained at 2.3 % end-tidal isoflurane concentration for the first part of the experiment, which is approximately 1.5 MAC (Steffey 1977).

Experimental design

This experiment was designed to study the cardiovascular effects of IVR injection of lidocaine. To determine the adequacy of dosage and technique anesthetic concentration was reduced prior to the second injection in order to assess response to toe pinch. Lidocaine concentrations were measured at specified intervals after both injections. An overview of the experimental design is diagramed in figure 1.

Injection of first leg-2.3% isoflurane

After stabilization at 2.3% isoflurane for 15 min, baseline measurements of heart rate (HR) respiratory rate (RR) end-tidal $C0_2$ ($ETC0_2$), systolic, (SAP) diastolic (DAP), mean (MAP) arterial pressures, lead II electrocardiogram (ECG) and rectal temperature were recorded. Arterial blood (0.5 ml) was anaerobically removed for pH, bicarbonate and blood gas analysis. After baseline recordings, an inflatable blood pressure cuff (neonatal #2, Critikon, Tampa, Fla) placed proximal to the cephalic catheter was attached

to an aneroid manometer (Mabis-Signature Aneroid Manometer; Baumanometer; Clearwater, Fl) inflated and maintained at 100 mm Hg greater than the systolic pressure. A second tourniquet, 6.25 mm rubber tubing, was tied above the elbow. After 5 min of tourniquet placement, HR, RR, $ETC0_2$, SAP, DAP, MAP, and ECG were recorded.

Saline 0.9% and lidocaine 2% were previously placed in sterile injection vials by the pharmacist and labeled A or B. For the first study in each cat, the choice of treatment A or B was made at random by coin toss. The alternate drug was administered in the second study 7-10 days later. The volume of the drug to be injected (A or B) was equivalent to 3 mg kg^{-1} of 2% lidocaine (Lidocaine HCL, Burns Veterinary Supply, Rockville Centre, NY) which was diluted with saline to a concentration of 1%. The average volume of the injectate was 1.20 ml and was injected in the cephalic catheter over 4 sec. After injection, HR, RR, $ETC0_2$, SAP, DAP, MAP and ECG were recorded every 5 min for 20 min. After 20 min, the cuff was deflated and the second tourniquet was removed and HR, RR, $ETC0_2$, SAP, DAP, MAP and ECG were recorded every 5 min for twenty minutes. Rectal temperature was recorded and arterial blood was removed for blood gas analysis 10 min after cuff removal.

After the final readings and blood samples were collected a response to toe pinch using a mosquito hemostat was illicited in the contralateral leg after the anesthetic concentration was reduced in 0.25% decrements. All cats responded at anesthetic end-tidal concentrations of 1.5 – 2.0%.

Injection of the second leg- 1.5-2 % isoflurane

After 20 min of stabilization at that anesthetic concentration, tourniquets were applied to the contralateral leg and the same drug was injected in a similar manner as was

done in the first leg. Response to toe pinch was determined in the following manner. The second toe of the injected leg was squeezed for 2 seconds with a 12.7 cm Halsted mosquito hemostat at the second ratchet and if no response, the third toe was squeezed. After each response, a similar response was then elicited and scored in the opposite previously injected (control) foot. Response was graded as 0 (no response), 1 (slight movement), or 2 (marked flexion or withdrawal) and was recorded every 5 min for 20 min after drug injection and every 5 min for 20 min after tourniquet removal. The difference between the scores of both feet was determined and those differences were statistically compared between drugs A and B. All other physiologic indices were recorded similarly as was done after injection of the first foot.

Lidocaine concentrations

Two milliliters of blood was removed from the arterial catheter for lidocaine plasma concentrations 5 min after injection (while tourniquet was in place) and 0.5, 1, 2, 4, 8, and 20 min after tourniquet removal. Lidocaine concentrations were not sampled prior to tourniquet removal after injection of the second leg. Blood was placed into EDTA vacutainers and centrifuged within 1 hr. The plasma was separated, placed into clean plastic tubes and frozen at –45 ºC. until assayed for lidocaine concentrations at a later date.

The method used to assay the lidocaine was a modification of the technique previously reported (Chen et al. 1992). The technique used was a reversed phase high performance liquid chromatography (HPLC) with ultraviolet detection at 210 nm. Recovery was 105±1% and the limit of detection for the HPLC method was 20 ng ml^{-1}.

Statistical analysis

An analysis of variance in repeated measures was performed where the 2 repeated measures were treatment and time. To account for possible carryover effects of treatment, an order factor was included in the model. To adjust for differences in baseline in the physiological data, baseline values were subtracted out and analyses were performed on differences from baseline. To adjust for multiple tests, the p-value was set at $p<0.005$ by the Bonferroni method. All analyses were performed using SAS statistical software.

Results

One cat was dropped after the first study for issues unrelated to the experiment. Because lidocaine was the drug randomly chosen for the first experiment plasma lidocaine concentrations from this cat are included in the plasma concentration analyses. This cat was not included in the statistical analyses of the cardiovascular data and response to toe pinch.

The total amount of blood removed in each cat was 28 milliliters, which is approximately 12% of the cat's blood volume. Healthy adult anesthetized animals can tolerate blood loss of 10% of the blood volume without treatment (Wagner and Dunlop 1993). All cats in this study received crystalloid fluids and blood loss was identical in both groups.

Physiologic variables

There were no statistically significant differences between the groups in any of the physiologic parameters at either anesthetic concentration. Heart rate and arterial blood

pressures were generally higher at the lower anesthetic concentration (Table 1). There were no dysrhythmias or abnormalities in ECG waveforms.

Lidocaine plasma concentrations

Mean lidocaine plasma concentrations (± SD) and ranges (Cmax and Cmin) for each time period are reported in Table 2.

After the first lidocaine injection, the highest mean plasma concentration was 2.79 ± 1.05 μg ml $^{-1}$, measured 0.5 min after tourniquet removal (range 1.07 to 4.56 μg ml $^{-1}$). Five minutes after injection of the first leg (before tourniquet removal), the highest individual plasma concentration was 4.42 μg ml $^{-1}$ whereas in two cats there were no measurable concentrations. The highest individual plasma concentration was 6.46 μg ml $^{-1}$ 20 min after tourniquet removal. Due to loss of the arterial catheter in that cat immediately prior to injection of the second leg, no further lidocaine concentrations were determined. After the second injection, the highest mean plasma concentration was 3.10 ± 1.11 μg ml $^{-1}$, measured 0.5 min after tourniquet removal (range 2.23 to 5.01 μg ml $^{-1}$). Twenty minutes after tourniquet removal all cats had measurable concentrations ranging from 0.77 to 1.58 μg ml $^{-1}$.

Plasma concentrations after injection of both legs were compared for times 1, 4, 8, and 20 min (Table 3). There were no significant differences in lidocaine plasma concentrations at those time periods. The mean time interval between injection of the first leg to injection of the second leg was 58.7 ± 7.8 min.

Response to toe pinch

Toe pinch score difference between the saline injected leg and the previously injected (control) leg was negligible ie) response to toe pinch occurred in all instances

resulting in similar scores. There were large differences in scores between the lidocaine injected leg and the previously injected (control) leg. When those differences were compared, statistically significant group differences were found at all time points up to 40 min (20 min after tourniquet removal; Fig 2).

Discussion

Local anesthetic techniques are not commonly employed in cats although some local anesthetic techniques have been described (Hall and Taylor 1994; Ringwood and Smith 2000). This author is unaware of any clinical reports of IVRA in cats. In humans, IVRA has been a popular anesthetic method for hand surgery, is considered effective, safe and technically easy to perform (Coleman 1999). The dosage and volume used in this study was extrapolated from the IVR lidocaine dosage and volume suggested for dogs, which was 2.5-5 mg kg^{-1} and 0.5-1.0 ml kg^{-1} of 1% lidocaine (Skarda 1996; Kupper 1977). However, the technique is not without potential for mild to life threatening complications attributed to local anesthetic toxicity

The CNS and cardiovascular toxic effects of excessive lidocaine plasma concentrations are well described (Berde and Strichartz 2000; Scott 1975; Chadwick 1985). The dosage used in this study is below the reported convulsive dosages of 11 .7 ± 4.6 mg kg^{-1} (Chadwick 1985) and 22.0 ± 4.4 mg kg^{-1} (DeJong et al 1982) determined in cats. Dosages that produced cardiovascular collapse were 2.5-4 times greater than the CNS toxic dosage but differences in study design would account for differences in plasma levels and toxic dosages. Corresponding lidocaine plasma concentrations during

seizure varied between those studies and ranged from 19.6 ± 5.5 μg ml^{-1} (DeJong et al 1982) to greater than 100μg ml^{-1} (Chadwick 1985).

In the present study, there were no significant differences in any of the physiologic variables between the groups at either anesthetic concentration. Significant differences may have been found if a larger group was studied. In the lidocaine group at 2.3% there was a tendency for HR to be lower after tourniquet removal but the decrease was not of clinical concern.

Heart rate and blood pressure in the present study can be compared to those reported after IV bolus administration of 3 mg kg^{-1} 4% lidocaine to N_20-0_2 anesthetized cats (Rosenbaum et al 1978). Only slight decreases in HR and arterial blood pressure were reported. In a different study, dosages of 2, 5, and 10 mg kg^{-1} IV lidocaine were administered to N_20-0_2 and N_20-0_2-halothane anesthetized cats (Nishikawa et al 1990). A brief but significant decrease in MAP occurred after 5 and 10 mg kg^{-1} bolus of IV lidocaine. The decrease in heart rate was significant only with the highest dosage. However, when halothane was added, the decreases were significantly greater. It was concluded that the cardiovascular depressant effects of halothane counterbalanced lidocaine's indirect stimulatory action mediated by the autonomic nervous system. Therefore background anesthetic activity may influence the cardiovascular responses to IV administered lidocaine.

The anesthetic concentration, although reduced in the second part of the experiment, may mask any CNS signs of toxicity in the cats of this report. Subjective signs of toxicity in people such as dizziness is reported to occur when plasma concentrations approach 5 μg ml^{-1} while cardiovascular instability is associated with

concentrations approaching 10 μg ml $^{-1}$ (Hartmannsgruber et al 1999; Merrifield and Carter 1965). However, lack of correlation between the incidence of subject symptoms and arterial plasma levels have been demonstrated in people during IVRA (Tucker and Boas 1971; Chan et al 1999). In a pharmacokinetic study of lidocaine in cats, sedative effects without overt signs of toxicity where described after IV administration of 2.2 mg kg $^{-1}$ and peak mean plasma concentrations were 5.8 μg ml $^{-1}$ (Brock and Webb 1988).

The highest plasma concentration in this study, 6.46 μg ml $^{-1}$was measured 20 min after tourniquet removal in one cat. Although a concentration of this magnitude after 20 min is difficult to explain smaller secondary peaks in concentration did occur in other cats as has been described in people after IVRA (Hargrove et al 1966; Thorn-Alquist 1969; Cotev and Robin 1966). This has been attributed to movement or exercise of the limb, or reactive hyperemia after reperfusion producing a washout of drug from the tissues (Thorn-Alquist 1969; Cotev and Robin 1966). Plasma concentrations at other time periods were high in this cat contributing to the variable ranges in concentrations among the cats (Table 2). Plasma lidocaine concentrations in human volunteers were also variable after 3 mg ml $^{-1}$of 1% IVR lidocaine (Tucker and Boas 1971). Unfortunately, the arterial catheter was lost in this cat prior to injection of the second leg so no subsequent plasma concentrations were measured. Consequently arterial blood pressure could not be measured although heart rate remained stable. Subjectively there was no suggestion of cardiovascular instability in this cat after the second lidocaine injection.

The relatively high plasma concentrations of lidocaine measured in the majority of cats prior to tourniquet removal in the present study despite the application of 2 tourniquets was unexpected, although recovery of small amounts of lidocaine would not

have been surprising. Despite adequate and sustained tourniquet pressures leakage of local anesthetic into the circulation have been reported and investigated in human patients (Coleman et al 1999; Hoffmann et al 1995; Davies and Walford 1986; Rosenberg et al 1983). Leaks were quantified by measuring the radioactivity remaining in the limb after injection of a radiolabeled substance in human volunteers (Coleman 1999). Leakage (mean ± SD) under an upper arm tourniquet was 10% ± 20; however one individual demonstrated a 76% leakage. Because this individual was hypertensive, unrecorded surges in systolic pressure may have led to inadequate tourniquet pressures. In another study, radiographic contrast media was injected in volunteers and radiographs of the limb revealed contrast filling the proximal side of the cuff within seconds of injection despite occluded arterial flow and high cuff pressures (Rosenberg et al 1983). The high pressure distal to the cuff from venous injection was determined to be the main cause of contrast leakage in that study.

Recommended measures to avoid leakage include exsanguination of the limb, injection at a distal vein, slow rate of injection and maintenance of tourniquet pressures to at least 100 mmHg in excess of systolic pressure (Hoffmann et al 1995). In addition, adequate cuff width to > 20% of the diameter of the limb has been recommended to avoid leakage under the cuff (Grice et al 1986). A narrow cuff may not provide effective tourniquet pressure and in these instances a cuff pressure of 300 mmHg is recommended. In the present study, injections were made as distal as possible (proximal to the carpus), cuff pressures were maintained to at least 100 mm Hg above systolic with an additional rubber tourniquet placed at the distal humerus. However, the cuff width was not > 20% of the width of the limb, the limb was not exsanguinated and the injection time was not > 4

sec. However, the technique was applied consistently to all cats and the wide variation of concentrations measured prior to tourniquet release is difficult to explain.

Because onychectomy is performed on two legs, lidocaine concentrations after 2 injections would be additive potentially resulting in excessive plasma concentrations. Paired lidocaine concentrations at all time periods in all cats were not available either due to study design (no 5min post injection of second leg) or to loss of sample. No samples were taken at 5 min after injection (prior to tourniquet release) in the second leg because it would be unclear if the amount reflected leakage under the cuff or residual concentrations from previous injection. Therefore, lidocaine concentrations from injection in both legs were compared at 1, 4, 8 and 20 min in 6 cats. There were no significant differences between the concentrations at these time periods (Table 3). The time interval (mean ± SD) between lidocaine injections was 58.7 ± 7.8 min. The tourniquet time of 20 min in this study was used to approximate the surgical time required for onychectomy in the veterinary teaching hospital. Peak plasma concentrations after IVRA are inversely related to tourniquet time (Tucker, Boas 1971). Therefore, one must be aware of the potential of excessive plasma concentrations if surgical and tourniquet times are reduced. In such cases the use of lower dosages may be warranted.

The dosage and technique used in the present study was effective as evidence to the large difference in toe pinch score in the lidocaine group compared to the saline group (Fig 2). The duration of apparent analgesia was consistent and sensory responses returned by 40 min (20 min after tourniquet removal) in all cats. Although the local analgesic effects of IVR lidocaine is short lived after tourniquet removal, central effects of lidocaine may contribute to intraoperative sedation and analgesia and perhaps residual

postoperative analgesia. Systemically administered lidocaine has been effective for some but not all pain states. Lidocaine infusions to human patients during cholecystectomy and continued for 24 hours postoperatively resulted in significantly lower postoperative pain scores and opioid requirements than in the control group. Whole blood lidocaine concentrations ranged between 1-2 $\mu g\ ml^{-1}$ in those patients (Cassuto et al 1985). However, other studies have found no or only minor analgesic effects on acute pain from IV lidocaine (Dirks et al 2000). Further study is needed to determine if any preemptive analgesia from both the local and systemic effects of lidocaine result in any postoperative benefit.

In summary, IVRA with a dosage of 3 mg kg^{-1} 1% lidocaine did not produce deleterious cardiovascular effects in six healthy isoflurane anesthetized cats. Arterial lidocaine plasma concentrations were variable among the cats; concentrations were in the ranges where human patients describe subjective symptoms of dizziness, but below the ranges where cardiovascular instability is reported to occur. Lidocaine concentrations were measurable prior to tourniquet release in 4 cats demonstrating significant leakage under the tourniquet. The dosage studied produced a consistent lack of response to noxious stimuli until 20 minutes after tourniquet release. IVRA may be a useful adjunct to general or injectable anesthesia for surgical procedures such as onychectomy in cats. Careful attention to dosage and tourniquet application time is important to minimize possible local anesthetic toxicity.

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Table 1. Cardiovascular and respiratory variables (mean ± SD) after IV regional injection of lidocaine (L) or saline (S) at 2.3% (high) and 1.5-2.0% (low) isoflurane concentrations

Variable	Isoflurane	Drug	Time (min)								
			0	5	10	15	20	25	30	35	40
HR (min^{-1})	High	S	131 ±17	131 ±18	129 ± 17	129 ±16	127 ± 15	126 ±17	130 ±19	133 ±17	135 ± 16
		L	124 ±13	122 ±13	122 ± 13	123 ±14	122 ± 13	118 ±12	118 ±14	119 ±14	119 ± 14
	Low	S	149 ±15	155 ±23	153 ± 25	162 ±23	164 ± 3	161 ±29	157 ±25	157 ±24	159 ± 19
		L	138 ±24	131 ±18	143 ± 30	143 ±31	145 ± 45	146 ±37	146 ±37	145± 34	152 ± 36
SAP (mmHg)	High	S	93 ±23	94 ±16	89 ± 18	93 ±20	88 ± 15	92 ± 15	89 ±19	96 ± 22	92 ± 19
		L	91 ±14	92 ±17	92 ± 16	90 ±13	93 ± 15	92 ± 16	91 ±15	89 ± 14	89 ± 16
	Low	S	121 ± 15	113 ±22	119 ± 21	125 ±24	123± 22	113 ±17	113 ±13	109 ±15	116 ± 21
		L	132 ± 17	110 ±29	125 ± 36	121 ±46	126 ± 48	118 ±35	113 ±33	118 ±30	121 ±32
DAP (mmHg)	High	S	58 ± 20	59 ± 14	55 ± 15	59 ± 18	57 ± 19	56 ± 12	55 ±17	62 ± 20	55 ± 14
		L	55 ± 8	57 ± 13	57 ± 11	56 ± 8	58 ± 11	55 ± 13	54 ±11	54 ± 12	57 ± 15
	Low	S	81 ± 12	74 ± 15	79 ± 16	81 ± 17	80 ± 14	71 ± 13	72 ± 12	70 ± 13	74 ± 13
		L	93 ± 17	73 ± 26	86 ± 30	83 ± 39	87 ± 40	81 ± 30	76 ± 30	82 ± 26	84 ± 27
MAP (mmHg)	High	S	73 ± 23	75 ± 17	70 ± 18	73 ± 20	70 ± 18	70 ± 14	69 ±19	77 ± 22	71 ± 17
		L	70 ± 11	72 ± 16	71 ± 14	70 ± 11	72 ± 13	71 ± 15	69 ±14	68 ± 14	69 ± 16
	Low	S	99 ± 15	93 ± 20	98 ± 19	102 ±21	100 ± 18	89 ± 15	90 ± 12	87 ± 13	93 ± 16
		L	112 ± 17	90 ± 29	105 ± 34	101 ±44	105 ± 45	98 ± 34	93 ± 33	92 ± 26	102 ± 31
RR (min^{-1})	High	S	25 ±11	28 ± 15	25 ± 7	25 ± 6	25 ± 8	26 ± 10	27 ±12	28 ± 9	29 ± 9
		L	21 ± 6	22 ± 7	22 ± 7	22 ± 6	22 ± 6	22 ± 6	23 ± 7	24 ± 7	23 ± 6
	Low	S	32 ± 14	29 ± 9	34 ± 13	38 ± 13	38 ± 12	38 ± 10	38 ± 9	39 ± 13	38 ±11
		L	33 ± 14	33 ± 12	34 ± 15	32 ± 14	33 ± 16	34 ± 14	34 ± 13	33 ± 14	32 ± 10
$ETC0_2$ (mmHg)	High	S	39 ± 4	39 ± 5	40± 5	39 ± 4	39 ± 5	39 ± 5	40 ± 5	39 ± 5	39± 6
		L	38 ± 5	40 ± 7	39 ± 5	41 ± 8	41 ± 6	40 ± 4	40 ± 2	41 ± 4	42 ± 4
	Low	S	37 ± 2	38 ± 4	36 ± 2	36 ± 3	36 ± 4	34 ± 3	36 ± 4	36 ± 5	36 ± 4
		L	36 ± 8	37 ± 8	38 ± 10	40 ± 10	37 ± 7	37 ± 7	38 ± 7	40 ± 10	37 ± 7

Table 2. Mean arterial lidocaine plasma concentrations and the maximal (Cmax) and minimum (Cmin) concentrations after IV regional injection of lidocaine in 7 isoflurane anesthetized cats

Leg 1	Time (min)	Mean ± SD	Cmax ($\mu g\ ml^{-1}$)	Cmin ($\mu g\ ml^{-1}$)
Tourniquet on	5	2.28 ± 1.83	4.42	0
Tourniquet off	0.5	2.79 ± 1.05	4.56 *	1.07
	1	2.70 ± 0.95	3.79 *	0.90
	2	2.68 ± 1.21	4.26	1.11
	4	2.60 ± 1.12	3.96 *	0.63
	8	2.32 ± 1.17	4.26 *	0.26
	20	2.0 ± 1.59	6.46 *	0
Leg 2 §				
Tourniquet off	0.5	3.10 ± 1.11	5.01	2.23
	1	2.78 ± 1.10	4.82	1.85
	2	2.45 ± 0.51	3.13	1.73
	4	1.95 ± 0.56	2.46	0.87
	8	2.07 ± 0.50	3.03	1.73
	20	1.29 ± 0.28	1.58	0.77

§ n = 6;
* denotes same cat.

Table 3. Comparison of arterial lidocaine plasma concentrations after IV regional injection of lidocaine in both legs at 1, 4, 8, and 20 min after tourniquet removal

Time (min)	Leg 1 (μg ml $^{-1}$)	Leg 2 (μg ml $^{-1}$)	P value
1	2.398	2.782	0.252
4	2.380	1.948	0.199
8	1.668	2.068	0.232
20	0.832	1.286	0.177
Mean	1.819	2.021	0.328

Pooled SEM 0.227

Figure 1. Time line (min) representing one study time. 0, baseline; T, tourniquet; open arrow, injection; closed arrow, lidocaine concentrations

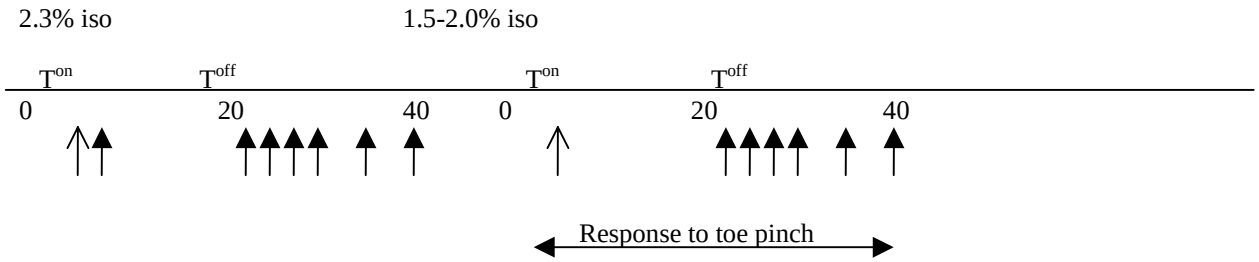
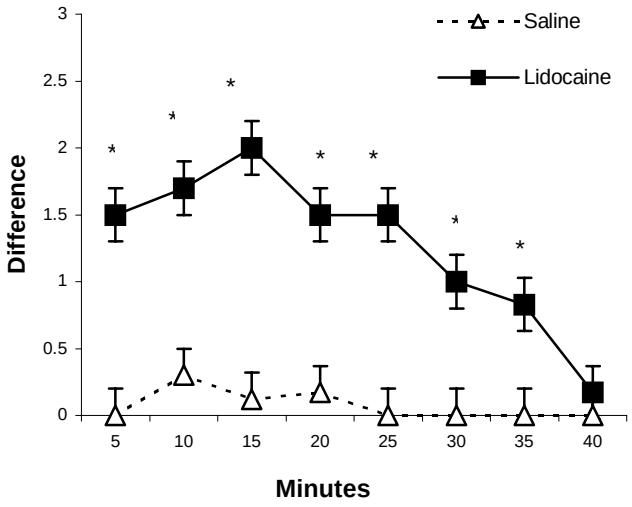

2.3% iso
1.5-2.0% iso
Ton
Toff
Ton
Toff
0
20
40
0
20
40
Response to toe pinch

Figure 2. Toe pinch response differences between IVR injected leg (saline or lidocaine) and the previously injected (control) leg. * significantly different ($p < 0.005$) between groups

*


Saline
Lidocaine
Difference
Minutes
3
2.5
2
1.5
1
0.5
0
5
10
15
20
25
30
35
40
*