UC781: BETA-CYCLODEXTRIN COMPLEXATION AND FORMULATION AS AN ANTI-HIV MICROBICIDE

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ABBREVIATION

ABC	Area Between Curves
ACN	Acetonitrile
AZT	Zidovudine, Retrovir
βCD	beta-cyclodextrin
BIV	Bovine immunodeficiency-like virus
CA	Capsid
CA	Cellulose acetate 1,2-benzenedicarboxylate
CCR5	Chemokine (C-C motif) receptor 5
CD4	Cluster of differentiation type 4
CDs	Cyclodextrins
CXCR4	CXC chemokine Receptor, co-receptor for HIV infection
CD or CDs	Cyclodextrins
DCs or DC	Dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule Grabbing Nonintegrin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
vDNA	viral DNA
EtOH	Ethyl Alcohol
ER	Endoplasmatic reticulum
FIV	Feline Immunodeficiency Virus
FTIR	Fourier Transform Infrared Spectroscopy

HAART	Highly active antiretroviral therapy
HEC	Hydroxyethylcellulose
HPβCD	Hydroxypropyl -beta-Cyclodextrin
HPLC	High Performance Liquid Chromatography
HPMC	Hydroxypropyl Methylcellulose
HTLV-III	Human T-lymphotropic Virus-III
IN	Integrase
LTR	Long-Terminal-Repeats
LVA	Lymphadenopathy-associated virus
MA	Matrix
MβCD	Methyl -beta-Cyclodextrin
MC	Methylcellulose
MDT	Mean Dissolution Time
MED	Multiple Exposure Device
MeOH	Methyl Alcohol
MMWR	Morbidity and Mortality Weekly Report
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
N9	Nonoxynol 9
NMR	Nuclear Magnetic Resonance
NNRTI	Non-Nucleotide Reverse Transcriptase Inhibitor
NC	Nucleocapsid
NRTI	Nucleotide Reverse Transcriptase Inhibitor
SIV	Simian Immunodeficiency Virus
PBS	Phosphate Buffer
PCP	Pneumocystis Carinii Pneumonia
PEP	Post-exposure prophylaxis
PrEP	Pre-exposure prophylaxis
PR	Protease
PVA	Polyvinyl Alcohol
PVP K-30	Polyvinylpyrrolidone K30
PVP K-90	Polyvinylpyrrolidone K90

RNA	Ribonucleic Acid
ROESY	Rotating frame Overhause Effect Spectroscopy
RT	Reverse Transcriptase
UV	Ultraviolet

UC781: BETA-CYCLODEXTRIN COMPLEXATION AND FORMULATION AS AN ANTI-HIV MICROBICIDE

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ABSTRACT

Background: UC781, a tight-binding non-nucleotide reverse transcriptase inhibitor (NNRTI) of HIV-1, is a thiocarboxanilide that has been identified as a potential microbicide agent. UC781 prevents HIV-1 infection by potently inhibiting HIV-1 replication (EC_{50}≈8nM) with a broad therapeutic index (>62,000). However, its extremely poor water solubility leads to a great challenge for its formulation development. A β-cyclodextrin (βCD) based drug delivery system was developed for UC781 to overcome this issue.

Method: The complex of UC781: βCD was assessed with UV, FTIR, DSC, and NMR. An HPLC method was used to investigate the thermodynamic behavior of the UC781 complex.

Complexation of UC781 with either hydroxypropyl β-Cyclodextrin (HPβCD) or methyl β-Cyclodextrin (MβCD) was optimized by evaluation of four processing methods (autoclave, lyophilization, shaking, and kneading), incorporation of four water-soluble polymers (HPMC, HEC, PVA, and PVP K30), and utilization of three buffering systems (pH 7.0, 9.0 and 11.0).

Finally, three formulations—methylcellulose (MC) gel, hydroxyethylcellulose (HEC) gel, and polyvinyl alcohol (PVA) film—were developed for UC781. The physical properties, toxicity, and anti-HIV activity of UC781 containing formulations were evaluated with *in vitro* and *ex vivo* models.

Results: Complexation of UC781 with CDs was confirmed and characterized with UV, FTIR, DSC, and NMR. UC781's complexation was found to be an enthalpy driven process. The

solubility of UC781 was increased from almost none to 35 μg/ml in 15% HPβCD and 180 μg/ml in 15% MβCD solutions after optimization.

Complexation technique significantly improved the release of UC781 from all three formulations. The complexation of UC781 with HPβCD or MβCD greatly increased the osmolality and decreased the viscosity of MC and HEC gel; shortened the disintegration time of PVA film; and reduced IC_{50} for UC781 in all three formulations. No observed toxicity was found in all complexed UC781 containing formulations.

Conclusion: βCD complexation technique provided an effective method to overcome the aqueous solubility challenge for UC781. UC781 complexation can be used as a safe and effective drug delivery system for UC781. Of the formulations tested, PVA film with complexed UC781 provided the most promising option for microbicide product development.

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PREFACE

I would like to express a wealth of gratitude to my advisor, Dr. Lisa Cencia Rohan. Your work ethic and dedication to science are unparalleled, and the delicate balance that you keep between career and family is most commendable. Thank you for your patience and guidance throughout my time here. Without your generous help, I could not have done the most difficult projects in my thesis.

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1.0 INTRODUCTION OF HIV INFECTION AND PREVENTION STRATEGIES

1.1 HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 AND AIDS

1.1.1 History of HIV and AIDS Discovery

A report on Kaposi's sarcoma in young homosexual men roused the beginning of early general awareness of acquired immunodeficiency syndrome (AIDS) (Hymes et al.,1981). On June 5, 1981, a report of five cases of Pneumocystis carinii pneumonia (PCP), a fungal infection of the lungs, among previously healthy young men in Los Angeles was published by Morbidity and Mortality Weekly Report (MMWR) (Angeles,1981). This report was commonly referred to as the "First Report on AIDS" in the USA (CDC,2001).

On April 22, 1984, Dr. James O. Mason, head of the Federal Centers for Disease Control, believed a lymphadenopathy-associated virus (LAV) discovered in France was the cause of AIDS (Altman,1984). One day later, Margaret Heckler from United States Health and Human Services announced that AIDS is caused by Human T-lymphotropic Virus-III (HTLV-III), which had been isolated by Dr. Robert Gallo of the National Cancer Institute(AVERT,1984). LAV and HTLV-III were quickly proven to be the same virus: Human Immunodeficiency Virus (HIV) (Marx,1986; Ratner et al.,1985). In 1986, both viruses were given the same name, HIV-1. Ten

years later, another member of the HIV family, HIV-2 was first reported in Egypt (Hassan et al.,1996). These two viruses are different in both origination and clinical behavour. HIV-1 is thought to have originated from Chimpanzees with higher virulence and transmittability (Gao et al.,1999) which accounts for the epidemic of HIV transmission. In contrast, HIV-2 which originated from the Sooty Mangabey with less virulence and transmittability and is primarily found in West Africa (Gao et al.,1992; Hirsch et al.,1989).

1.1.2 The AIDS Epidemic and Pathogenesis

AIDS is now a pandemic (Kallings,2008). The prevalence of AIDS is one of the most serious challenges of infectious disease to public health all over the world. In 2007, AIDS killed 2.1 million people worldwide, including 330,000 children, and led to 33.2 million people living with the disease (UNAIDS.,2007). An estimated 2.5 million persons were newly infected in 2007, including 420,000 children (UNAIDS.,2007). Every day, over 6,800 persons become infected with HIV and over 5,700 persons die from AIDS, mostly due to inadequate access to HIV prevention and treatment services.

AIDS is defined as a clinical condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections. HIV infects several cell types: $CD4^+$ T cells, macrophages, and dendritic cells (DCs)(Miceli and Parnes,1993; Mohan,2005). This infection directly and indirectly destroys $CD4^+$ T cells (Alimonti et al.,2003). As $CD4^+$ T cells are required for the proper functioning of the immune system, AIDS happens when no sufficient $CD4^+$ T cells can be maintained for normal immune response. Clinically, CD4 count is used to assess immune status, susceptibility to opportunistic infections, and for defining AIDS (CD4 <200).

Due to the loss of $CD4^+$ cells by HIV infection, the major health issues faced by AIDS patients are certain opportunistic infections. These infections or cancers include the following diseases: Pneumocystis pneumonia (PCP), a lung infection; Kaposi's sarcoma (KS), a skin cancer; Cytomegalovirus (CMV), an infection that usually affects the eyes; and Candida, a fungal infection that can cause thrush (a white film in mouth) or infections in the throat or vagina *et al* (Casabona et al.,1993; Stein et al.,1992). These infections do not normally develop in individuals with healthy immune systems and are caused by bacteria, viruses, fungi and parasites (Holmes et al.,2003; Holmes et al.,2006). AIDS related death is highly related to the latest CD4 cells count (Margolis,2005). A lower CD4 cell counts lead to a greater risk for particular AIDS conditions due to the weakened immune function, such as PCP *et al* (Kumar and Krieger,1998). As a consequence, the leading cause of death in AIDS patients is infection without effective treatment, with 30% of these infections being bacteria, and 25% being other opportunistic organisms (Stein et al.,1992).

1.1.3 Molecular Biology of HIV-1

1.1.3.1 Virion Classification and Tropism of HIV

HIV is a member of the genus Lentivirus belonging to the family of Retroviridae, which have genes composed of ribonucleic acid (RNA) molecules. Generally, retrovirus converts its RNA into a DNA intermediate with its reverse transcriptase. The newly formed viral DNA is then permanently integrated the host cell chromosome DNA (Tang et al.,1999).

Like HIV-1 cause immunodeficiency syndrome in human, other members of this family cause an immunodeficiency syndrome in mammals, such as monkeys (Daniel et al.,1985), cats (Yamamoto et al.,1986), and cattle (Wang et al.,1999). These viruses share many similar

physical and biochemical properties with HIV-1 and HIV-2. Therefore, they were designated as simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and bovine immunodeficiency-like virus (BIV), respectively.

Two species of HIV have thus far been found that infect humans: HIV-1 and HIV-2 (Gao et al.,1999). HIV-1, HIV-2, and SIV comprise the subgenus "primate lentiviruses." The genomic organization of these viruses is also very similar (Reeves and Doms,2002). HIV-2 shares approximately 60% nucleotide sequence similarity with HIV-1 (Figure 1-1).

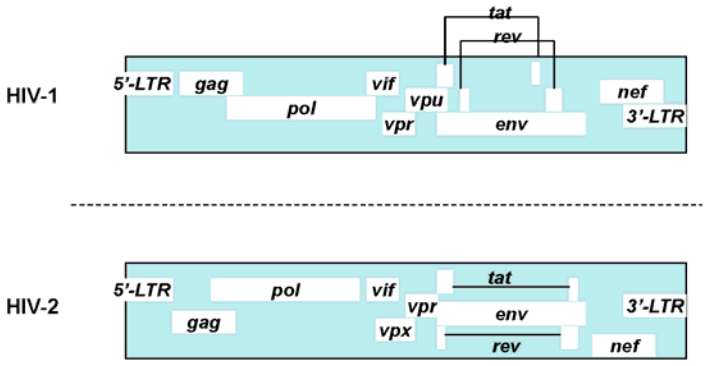


Figure 1-1. Genes laid out in HIV RNA.
Schemetic presentation shows genes of HIV-1 and HIV-2 genes on their RNA. These genes code out proteins with similar function with approximately 60% nucleotide sequence similarity between HIV-1 and HIV-2.

This diagram *is based on a map of the HIV-1, HIV-2, and SIV genomes, available at* hiv-web.lanl.gov/content/immunology/pdf/2000/intro/GenomeMaps.pdf

CD4 protein is the main receptor on host cells for HIV to recognize and bind to during HIV entry (Moore,1990; Sattentau et al.,1989). The affinity for CD4 of HIV-1 gp120 is 25 times higher than that for HIV-2 gp120 (Sattentau and Moore,1993). This may help to explain the lower virulence of HIV-2 than of HIV-1.

Furthermore, HIV-1 shows specificity of entering into different target cell types in transmission, which are classified as T-cell-tropic (T-Tropic) virus (also called syncytium-inducing (SI) strains) and Macrophage-tropic (M-Tropic) virus (called nonsyncytium-inducing

(NSI) strains). Syncytium is defined as a large cell-like structure formed by the joining together of two or more cells (National Cancer Institute). It was reported that reported that patients infected with syncytium-inducing phenotype HIV-1 strains were more likely to progress to the AIDS than patients with non-syncytium-inducing strains (Koot et al.,1993). This HIV-1 tropism phenomenon suggests the existence of co-receptors in HIV-1 infection. Two co-receptors were identified as involved in the HIV-1 infection *in vivo*- CCR5 and CXCR4 (Berger et al.,1999; Choe et al.,1996; Dragic et al.,1996).

The M-Tropic virus utilizes the β-chemokine receptors CCR5 (sometimes the CCR3 co-receptor (Choe et al.,1996)), which are associated with mucosal and intravenous transmission of HIV infection. On the other hand, T-Tropic virus utilizes the α-chemokine receptor, CXCR4 (Power et al.,1995), as a co-receptor (Berger et al.,1999; Moore,1997). HIV that use only the CCR5 receptor are termed R5 virus, those that use only CXCR4 are termed X4 virus, and those that use both, X4R5 virus. In summary, both M-Tropic viruses and T-Tropic viruses bind to the CD4 receptor; however, they utilize different co-receptors in order to enter the host cells.

1.1.3.2 Virion Structure and Genomic Organization of HIV

The HIV virus particle is roughly spherical in shape with a diameter from 110 to 128 nm in its mature form and 132 to 146 nm in its immature form (Gentile et al.,1994). Typically, HIV-1 has a high-density core encircled with an envelope in the transmission electron micrograph (Kumar et al.,2008; Salahuddin et al.,2007). The structure of the HIV particle is shown in Figure 1-2. .

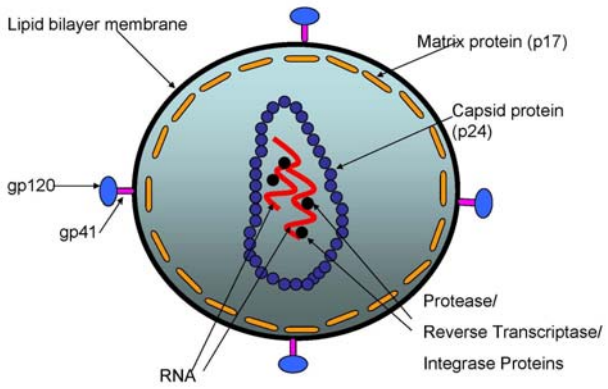


Figure 1-2. Structure of HIV Virus
HIV particles compose of outer coat and core. The outer coat contains gp120 and gp41 proteins complex as spike to recognize host cells, biomembrane as envolope, and matrix protein. The core of HIV composes of two single strand of RNA and enzymes surrounded by capsid protein.

The outer coating of the virus, known as the viral envelope, is composed of two layers of phospholipids, which come from the membrane of host cells when newly formed virus bud from the cell. 72 copies (on average) of the complex HIV proteins are embedded in the viral envelope (National Institute of Allergy and Infectious Diseases,2004). This complex, also called "spikes", is composed of three glycoprotein 120 (gp120) as cap and three glycoprotein 41 (gp 41) as stem. A layer of a matrix protein composed of the viral protein p17 lines the inner surface of the membrane of HIV to keep the integrity of the virus (Wang et al.,1999). 2,000 copies of the viral protein p24 form a conical capsid (Folkers,1996) of HIV which contains the HIV gene in the core.

The core of HIV contains two copies of positive single-stranded RNA, reverse transcriptase, integrase, protease, and NucleoCapsid (p7) (National Institute of Allergy and Infectious Diseases,2004).

The HIV-1 genome codes for the viruses' nine genes: *gag, pol, env*, *tat, rev, nef, vif, vpr*, and *vpu*. The virus RNA is flanked by two long-terminal-repeats (LTRs or LTR) as shown in

Figure 1-3. Three of these genes, *gag, pol,* and *env*, contain information needed to produce structural proteins for new virus particles. The *gag* codes structural proteins MA (matrix), CA (capsid), NC (nucleocapsid), p6; The *pol* enzymes PR (protease), RT (reverse transcriptase), and IN (integrase). Six regulatory genes, *tat, rev, nef, vif, vpr,* and *vpu*, contain the information necessary for HIV replication (HIV Databases). The gene regulatory proteins are coded by *tat* and *rev*; and the accessory proteins are coded by *nef, vif, vpr,* and *vpu*. The LTR acts as a switch to control the production of new viruses and can be triggered by proteins from either HIV or the host cell (Reed-Inderbitzina and Maury,2003). The 5' LTR contains the promoter sequence that controls viral expression, while the 3'-LTR is involved in polyadenylation.

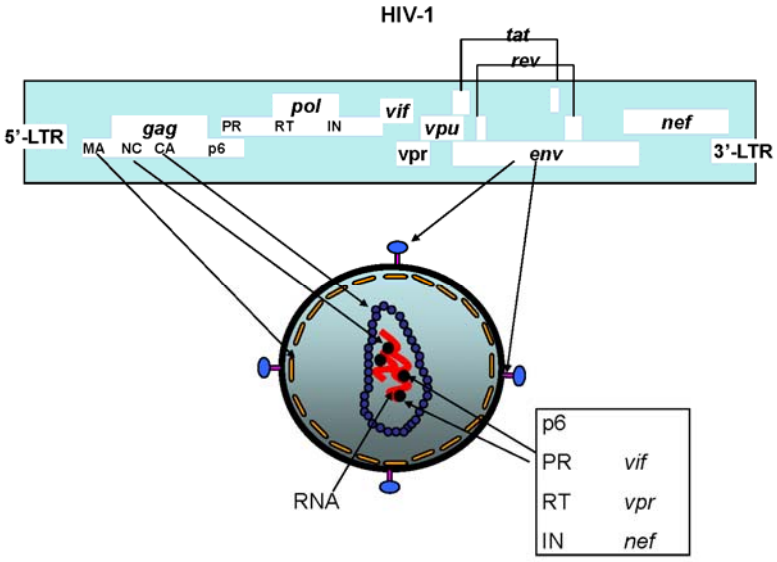


Figure 1-3. HIV-1 Organization of the HIV-1 genome and virion

The genes of HIV and their corresponding products were shown Figure 1-3. Three genes, *gag, pol,* and *env*, code for structural proteins for HIV particles. Six regulatory genes, *tat, rev, nef, vif, vpr,* and *vpu*, contain the proteins necessary for HIV replication.

1.1.3.3 Life Cycle of HIV

Generally, HIV infection is recognized as having four steps (Figure 1-4): **1 Virus entry (attachment and fusion)**: HIV uses its membrane proteins to find and bind to CD4 receptors on target cells and transfer its genome into host cells; **2 Reverse transcription and integration of virus genes**: viral gene on the HIV RNA is converted into viral DNA and integrated into the host cells' DNA; **3 Transcription/translation**: after the integration of virus DNA into the host cells' genome DNA, the components of the HIV begin to be manufactured from target cells; **4 Virus particle release**: The HIV virus components are assembled and released in a "bud" form to become a new born HIV virus.

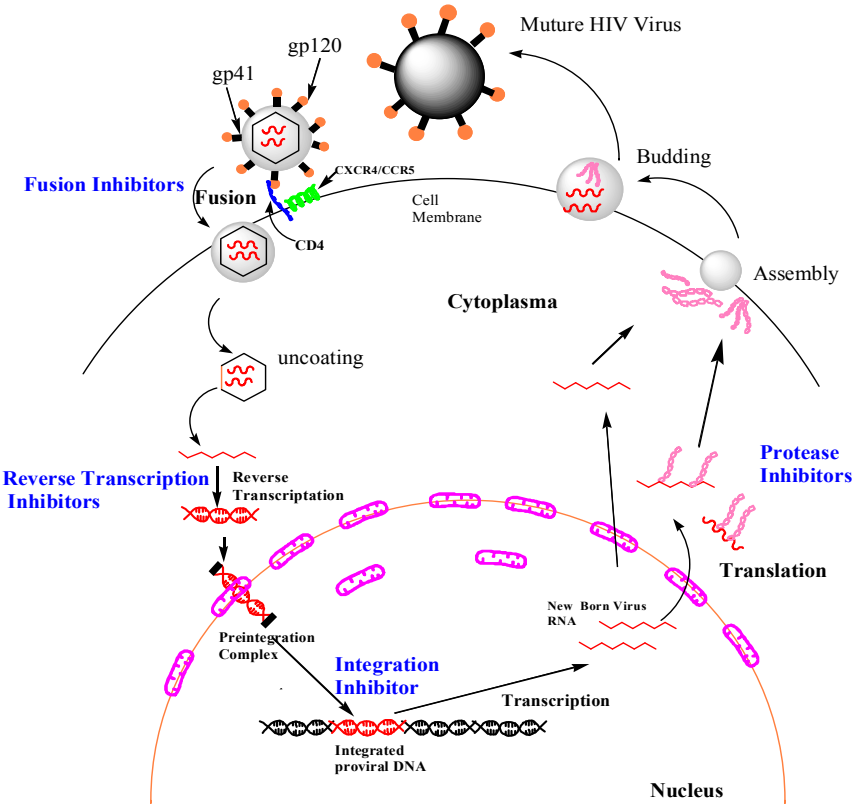


Figure 1-4. HIV life cycle and anti-HIV drug target

The HIV life cycle is shown in the figure above. The figure illustrates the different stages of infection: attachment and fusion; reverse transcription and integration; replication; budding; and release.

1 Virus entry : In order to infect its target cells, HIV must transfer its genome into these host cells, crossing both the viral and cell bilayer membranes (Doms and Trono,2000). The virus entry events initiate from the contact and recognition between proteins from both virus membrane and cell membrane (Blobel et al.,1992; Hernandez et al.,1996). With the help of receptors and co-receptors for the host cell, a portal of entry is provided for virus penetration allowing membrane fusion to occur.

The process of HIV entry involves fusion of the viral envelope with the host cell membrane, which requires the specific interaction of the envelope "spike" from HIV with specific cell surface receptors-CD4. The spike is a complex comprised of three functional units of gp120 molecules as a cap and functional units of gp41 molecules as a stick inserted into the viral lipid membrane. The binding of gp120 to CD4 on the surface of the target cell induces a conformational change of gp120 leading to a further binding of co-receptors and the fusion process.

The binding of gp120 to CD4 by itself does not trigger membrane fusion (Ashorn et al.,1990; Clapham et al.,1991). Therefore, chemokine co-receptors are necessary for HIV-1 fusion. The conformation change of the V3 and V2 regions of gp120 after binding to CD4 enable it to interact with CCR5 or CXCR4, chemokine co-receptors that facilitate HIV entry into macrophage and T cells *in vivo,* respectively. This interaction triggers the fusion of HIV-1 to host cells by exposing the hidden gp41 to the target cell membrane through the formation of a triple-stranded coiled-coil (Pierson and Doms,2003), leading to fusion of the virus and cell membrane. The content of HIV-1 is then transfered into the host cell for replication.

In addition, both CD4, chemokine co-receptors for HIV-1 entry and budding process are found to be associated with the lipid rafts domains in the cell membrane (Kozak et al.,2002;

Popik et al.,2002). These cholesterol- and sphingolipid-enriched microdomains provide the platform for the interaction between HIV-1 and target cells. Decreasing cholesterol concentration in virion and target cell membrane will greatly decrease the infectivity of HIV-1. Therefore, the cholesterol-depleting modulators might be a viable strategy to prevent HIV-1 transmission at mucosal surfaces such as βCD.

2 Reverse Transcription and integration of virus genes: Once the viral capsid enters the cell, HIV-1 genome is quickly reverse-transcribed into a double stranded viral DNA intermediate (vDNA) by the reverse transcriptase (RT), which contains RNA- and DNA-templated polymerase and RNase H activities. This vDNA then forms a nucleoprotein complex called the pre-integration complex (PIC) and is transported to the nucleus by nuclear localization sequences (NLS) (Bouyac-Bertoia et al.,2001). After nuclear import, integrase (IN) recognizes and inserts the vDNA into the cellular DNA of the host cell in the nucleus. The vDNA remains permanently associated with the host cellular DNA for the lifetime of the cells. The understanding of infection steps is highly correlated with the prevention concept and methods of HIV-1 infection. Ideally, an effective prophylactic method should block the HIV infection before vDNA is integrated into the host cell genome.

3 Transcription/translation: The integrated vDNA serves as the template for the synthesis of newly generated HIV-1 mRNAs transcripts, which are transported out of the nucleus for translation in the cytoplasm, and then form the infectious virion particles. The polyproteins Gag and Gag-Pol for viral matrix and capsid are localized at the lipid raft domains of the cell membrane (Halwani et al.,2003; Leung et al.,2008). The Env precursor protein gp160 (gp120 and gp41) is synthesized by the endoplasmatic reticulum (ER) using Env mRNA as template (Fenouillet and Jones,1995).

4 Virus particles release: Once all components of HIV are assembled at the inner surface of the cell membrane, the viral particles begin to bud from the cell surface to form new born immature viral particles. These noninfectious particles are then released and undergo a maturation process involving processing of Gag and Gag-Pol by HIV protease and assembly of the core particle (Freed,1998). Protease digests the polyproteins into Matrix (p17), Capsid (CA), NucleoCapsid (NC), Protease (PR), Reverse Transcriptase (RT), and Integrase (IN). This mature HIV virion is now ready to start another replication cycle by infecting next host cell (Kohl et al.,1988).

1.1.3.4 Classification of Anti-HIV Drugs

Generally, most anti-HIV compounds are desigend to interfere with different stages of the HIV life cycle. Therefore, they can be classified according to the stages of the HIV life cycle. A number of anti-HIV drugs have been approved by the FDA for HIV treatment and are listed in Table 1-1.

Table 1-1. FDA approved Anti-HIV drugs

Entry and Fusion inhibitors	Reverse Transcriptase Inhibitors		Integrase Inhibitors	Protease Inhibitors
	NNRTIs	NRTIs		
Fuzeon	Intelence	Combivir	Raltegravir	Atazanavir
Selzentry (CCR-5 inhibitor)	Rescriptor	Emtriva	(Isentress)	Agenerase
	Sustiva	Epivir		Aptivus
	Viramune	Epzicom		Crixivan
		Retrovir		Invirase
		Trizivir		Kaletra
		Videx		Lexiva
		Truvada		Norvir
		Viread		Prezista
		Ziagen		Reyataz
		Zerit		Viracept

Table 1-1 shows FDA approved anti-HIV drugs listed by brand name. Drug information was obtained from aidsinfor.nih.gov.

Entry inhibitors (or fusion inhibitors): interfere with binding, fusion, and entry of HIV-1 into the host cell by interfering with HIV gp120 or blocking receptors or coreceptors on target cells surface such as CD4, CXCR4 or CCR5. Fuzeon and Selzentry are the two currently available drugs in this class.

Reverse transcriptase inhibitors: can be classified into two categories—nucleoside reverse transcriptase inhibitors (NRTIs), and non-nucleoside reverse transcriptase inhibitors (NNRTIs) according to the different mechanism their RT inhibition.

NRTIs inhibit reverse transcription by being incorporated into the newly synthesized viral DNA and preventing its further elongation. NRTIs are analogues of the naturally occurring deoxynucleotides necessary for synthesizing the viral DNA, and they compete with the natural deoxynucleotides for incorporation into the growing viral DNA chain. Due to their lack of a 3'-hydroxyl group on the deoxyribose moiety in their structure, NRTIs act as chain terminators for viral DNA synthesis. In that a 5'-3' phosphodiester bond with the next incoming deoxynucleotide needed to extend the DNA chain cannot be formed resulting in the halt of viral DNA synthesis. All NRTIs are classified as chain terminators and competitive substrate inhibitors.

NNRTIs inhibit reverse transcriptase directly by binding to the enzyme and interfering with its function. Instead of competing with natural deoxynucleotides as NRTIs, all NNRTIs bind tightly to the allosteric hydrophobic site with a common binding mode (Ren et al.,1995). This NNRTI binding pocket is located between two beta sheets of the p66 subunit of RT, approximately 10 Angstroms away from the polymerase active site of the RT (Ding et al.,1995). NNRTIs inhibit HIV-1 RT allosterically by displacing the catalytic aspartate residues relative to the polymerase binding site. NNRTIs are non-competitive (or mixed-type) inhibitors with respect to substrate and non-competitive or uncompetitive inhibitors with respect to template/primer.

Integrase inhibitors inhibit the enzymatic activity of HIV integrase, which is responsible for integration of viral DNA into the DNA of the infected cell.

Protease inhibitors (PIs) target viral assembly by inhibiting the activity of protease, an enzyme used by HIV to cleave nascent proteins for final assembly of new virions.

Other anti-HIV compounds can inhibit or interfere with the HIV life cycle at stages other than those mentioned above. With the development of understanding and research on HIV, more and more compounds are under investigation for the treatment of HIV infection focusing on new targets directed toward different viral or cellular components of the life cycle of HIV replication. Compounds in this category can inhibit or interfere with viral maturation, viral packaging, polyamine biosynthesis in host cells, or RNA interference (RNAi) to silence the RNA of HIV. A high occurrence of HIV resistance development with anti-HIV drugs and the adverse effects associated with early regimens of HAART led to increases in the failure rate associated with anti-HIV treatment (Recsky et al.,2004). However, newer combination drug strategies look promising. The development of these new anti-HIV molecules provides strategies toward new anti-HIV targets which may potentially improve the effectiveness and efficacy of AIDS therapy in the future.

1.1.4 Prevention and Treatment of HIV/AIDS

1.1.4.1 HIV Transmission and Its Prevention

HIV is transmitted through three main routes: sexual contact (anal or vaginal), exposure to infected body fluids or tissues (including the sharing of contaminated needles/syringes; blood transfusion), and from mother to fetus or child during pregnancy, childbirth, and breastfeeding (CDC,1999). Although HIV can also be found in the saliva, tears, and sweat of HIV positive

patients, no recorded cases of infection by contact with these secretions has been shown to result in transmission of HIV (CDC,1999).

Unprotected sexual contact is one of the primary modes of HIV infection worldwide (Johnson and Laga,1988). The utilization of male or female condoms can reduce the chances of infection with HIV. Consistent condom use results in 80% reduction in HIV incidence (Davis and Weller,1999; Weller and Davis,2001).

Injecting drug users (IDUs) have been among the high-risk groups affected by HIV and AIDS (23.8% (Gomma et al.,1993)). Syringe sharing has made HIV, as well as other blood-borne pathogens, spread rapidly through intravenous drug users' population. Harm reduction strategies such as needle-exchange programs are used in attempt to reduce the infections caused by drug abuse (Kerr et al.,2007; Rhodes et al.,2006).

Occupational HIV infection is mostly caused by exposure to HIV-infected blood via a percutaneous injury (i.e. from needles, blades, instruments, etc.). The average risk for HIV transmission after percutaneous exposure to HIV infected blood is not high, only about three per 1,000 injuries (Wilks et al.,2003), this is still a considerable concern for many health care workers. Precautions should be taken to reduce the exposure to HIV. These precautions include barriers such as gloves, masks, and protective goggles or shields, which prevent exposure of the skin or mucous membranes to blood-borne pathogens. Additionally, post-exposure prophylaxis (PEP) is used in cases in which there was a failure to avoid virus exposure. This post-exposure prophylaxis is an antiretroviral treatment administered directly after a highly significant exposure to HIV (CDC,2005; Hamlyn and Easterbrook,2007). However, the effectiveness of PEP is still under investigation. Another approach called pre-exposure prophylaxis (PrEP) is also under

clinical evaluation for efficacy as a female-controlled prevention method for women worldwide (CDC,2008a).

As for mother-to-child transmission of HIV (1% worldwide in 2007, (UNAIDS.,2007)), using anti-HIV drugs prophylaxis in late pregnancy with no breast-feeding can greatly reduce the infection of HIV from 27% to 9% in infants (Steel-Duncan et al.,2004). Currently, WHO recommends that long term breast-feeding of infants should be avoided by HIV positive mothers. (WHO and CDC,2008).

1.1.4.2 Treatment of HIV/AIDS with Highly Active Antiretroviral Agents

Due to the lack of vaccine or cure for AIDS, current antiviral treatments of HIV consist of a highly active antiretroviral therapy (HAART) in current clinical practice. HAART has been highly beneficial to many HIV-infected individuals since its introduction in 1996 when the protease inhibitor-based HAART initially became available (Melroe et al.,1999; Vandamme et al.,1998) .

The key in the HAART is to disrupt HIV at different stages in its replication. HAART is a combination (or "cocktail") of drugs belonging to at least two types, or "classes," of antiretroviral agents targeting different HIV replication stages. Typical regimens consist of two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI). It is reported that NNRTI-based HAART was more effective than PI-based HAART for virus suppression and was similar to PI-based HAART for clinical outcomes of death, disease progression, and withdrawals (Chou et al.,2006).

HAART is a very effective regimen for AIDS treatment and has saved thousand of lives since it was introduced in 1995 (Ho et al.,1995). HAART had been designed to treat AIDS by suppressing HIV replication and reconstituting the patient's immune system (Flint et al.,2006).

In the absence of HAART, the median survival time for patients after developing AIDS is only 9.2 months (Morgan et al.,2002). Fortunately, HAART therapy increases survival time by between 4 and 12 years (King et al.,2003; Tassie et al.,2002). Furthermore, with the application of powerful HAART therapy, the AIDS death rate in the U.S has been greatly decreased since 1995 for the first time as shown in Figure 1-5.

However, HAART treatment has significant drawbacks: it cannot completely suppress the HIV-1 replication and long-term therapy carries a risk of toxicity. Selection of (multi) drug-resistant viral variants or adverse effect from HAART drug components are the primary reason for failure of this regimen (Day,2003; Vandamme et al.,1998).

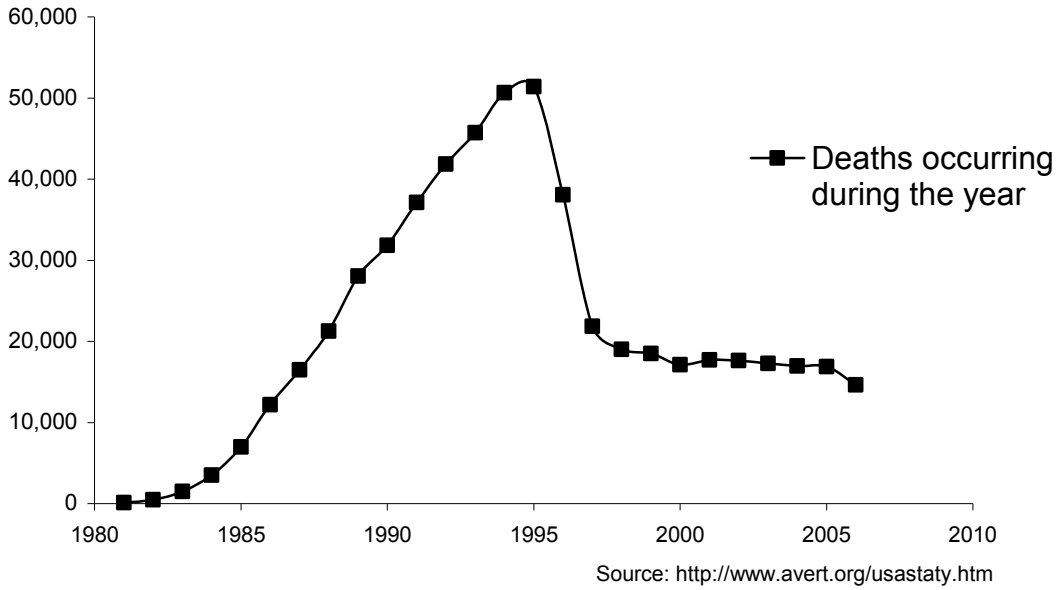


Figure 1-5. Yearly AIDS deaths in US since 1981

Figure 1-5 shows the yearly number of AIDS deaths in the United States since 1981. The death rate of AIDS patients increased yearly after HIV was discovered in 1981 until the clinical implementation of HAART in 1995. With the appearance of HAART the number of deaths dropped. The number of deaths of HIV patients in 1997 dropped to approximately half of that in 1995 and has been maintained at that level.

Due to the success of current therapies in HIV-positive individuals, these drugs have also been investigated as a means to prevent HIV infection. It was reported that male HIV positive patients treated with AZT were 50% less likely to transmit HIV to their female partners than were untreated patients, due to reduction in the viral load in biofluid (Musicco et al.,1994). In a study evaluating the potential use of HAART as a prevention strategy it was estimated that HAART could reduce the yearly rate of HIV incidence by as much as 50% in Canada (Montaner et al.,2006).

However, the effect of HAART on HIV prevention is still in dispute (Anema et al.,2008). There is no direct evidence to support the use of a combination of multi-drug regimen following occupational exposure to HIV (Young et al.,2007). Furthermore, cost is also a critical issue in applying HAART to prevent HIV transmission. For example, using generic Lamivudine (Epivir®, or 3TC) to avoid HIV infection, the cost will be $6,625 per infection avoided (Derdelinckx et al.,2006). Therefore, cost-effective methods must be developed for the prevention of HIV transmission, especially for developing countries. Microbicides may represent a safe, effective, and cost-saving method for HIV prevention under development.

1.2 MICROBICIDES: A FEMALE CONTROLLED METHOD OF PROTECTION AGAINST HIV INFECTION

1.2.1 HIV/AIDS and Women

HIV infection or AIDS has reached pandemic levels and poses one of the greatest challenges to global public health. Although HIV/AIDS was originally thought to be associated

with sexual practices of male homosexuals, women are more easily to be infected by HIV than men. The number of infected women has significantly increased since HIV/AIDS was first identified. At the end of 2007, UNAIDS estimated that out of the 33.0 million people worldwide living with HIV, nearly half are women (15.5 million) (UNAIDS.,2007). Young women age between 15 and 24 years old are at least three times more likely to be infected than young men in South Africa, Zambia, and Zimbabwe (Quinn and Overbaugh,2005).

Heterosexual transmission and drug abuse are the two most common routes of HIV infection in women. One of the first studies in Zaire revealed a strong relationship between HIV infection and heterosexual intercourse in the 80's (Piot et al.,1984). Heterosexual transmission accounts for the fastest growing risk group in the United States. AIDS cases attributed to heterosexual transmission increased from 3% of all cumulative AIDS cases among women from 1983 to 1984 to 64% from 1999 to 2002 (CDC,2004). More than 80% of newly diagnosed infections in the U.S are the result from heterosexual intercourse, and AIDS is the fifth leading cause of death for American women from ages 25 to 44, according to the newest CDC report (CDC,2008b)..

Women are more vulnerable to HIV infection than their male counterparts due to their anatomical and biological differences. These differences include greater area of mucous membrane exposure to virus during intercourse in women than in men, larger quantity of body fluids transferred from men to women, the higher viral content of male sexual fluids, and microtears in the cervix and vaginal epithelium that commonly occur during sexual intercourse. These properties facilitate HIV crossing the reproductive tract and enter the bloodstream.

The risk of male-to-female HIV transmission has been reported in several studies. Females were shown to be up to 20 times more likely to be infected than their male counterparts

in these studies (Nicolosi et al.,1994; Padian et al.,1991; Padian et al.,1997). This risk can also be greatly amplified with non-consensual sex, sex without condom use, and high-risk behaviors of their partners (Cameron et al.,1989; Fowler et al.,1997). Furthermore, women are 4 to 13 times more vulnerable to acquiring other sexually transmitted infections (STIs) (UNAIDS.,26 February - 9 March 2007). These STDs and some other genital tract diseases, such as HSV infection and ulcers, are also cofactors to enhance HIV infection in women (Corey et al.,2004). Moreover, social, cultural, economic, and legal discrimination or inequities also greatly affect the behavior of women in heterosexual transmission of HIV.

In addition to the direct effects from HIV infection, millions of women have been indirectly affected by the HIV/AIDS epidemic. To women, the impact of HIV/AIDS is not limited only to the disease itself. AIDS disproportionately affects women in many other ways. Pregnancy and childbearing may lead to mother-to-child transmission of HIV in HIV positive women. The responsibility of caring for AIDS patients and orphans in the family may affect women more strongly than men. AIDS also has a huge economic impact on HIV positive patients, especially women. The average cost of HAART therapy is $10,500 per HIV positive patient per annum (Chen et al.,2006). For the prevention of mother-to-child transmission of HIV, the treatment costs are about $104,502 for 100 HIV-positive pregnant women and their newborns (Mauskopf et al.,1996). These costs are not considering the impact of resistance to antiretroviral therapy, the effect of metabolic abnormalities, and toxicities. Therefore, the responses for solutions to the problem of HIV/AIDS are particularly urgent for the female population. Effective prophylactic methods must be developed for the prevention of HIV in sexual transmission for women.

1.2.2 Microbicides as an effective prevention method against HIV/AIDS transmission in Women

1.2.2.1 HIV-1 Invading the Female Genital Tract

The vagina has several innate factors which serve as a protective barrier to infection, such as its acidic pH (4.2), and peroxides produced by vaginal lactobacilli (Schwebke,1966). However, pre-exiting inflammatory conditions, such as sexually transmitted diseases, ulcers, or bacterial vaginosis; and menstrual cycle may greatly reduce the innate barrier function and thus increase the likeliness of HIV transmission (Martin et al.,1999).

For heterosexual transmission, HIV-1 has to cross the mucosal barrier of the genital tract before it can infect CD4+ T cells or macrophage cells. In women, the morphology of the epithelium changes from the squamous epithelium of the vagina and ectocervix to the single-layer columnar epithelium of the endocervix. It may be easier for HIV-1 to cross the single layer of endocervical epithelium in comparison with the multilayer squamous epithelium of the ectocervical and vaginal mucosa (Moss et al.,1991). However, with more than 15 times the surface area as compared with that of endocervix, the vaginal wall and ectocervix may provide potential access sites for HIV invasion, particularly when breaches or damages occur in the epithelial layer (Hladik and McElrath,2008). HIV-1 can invade mucosal barriers as either a cell-free or cell-associated virus. It is then available to infect local leukocytes or to be captured by Langerhans cells for further systemic infection as shown in Figure 1-6 (Bass,2004; Boggiano and Littman,2007).

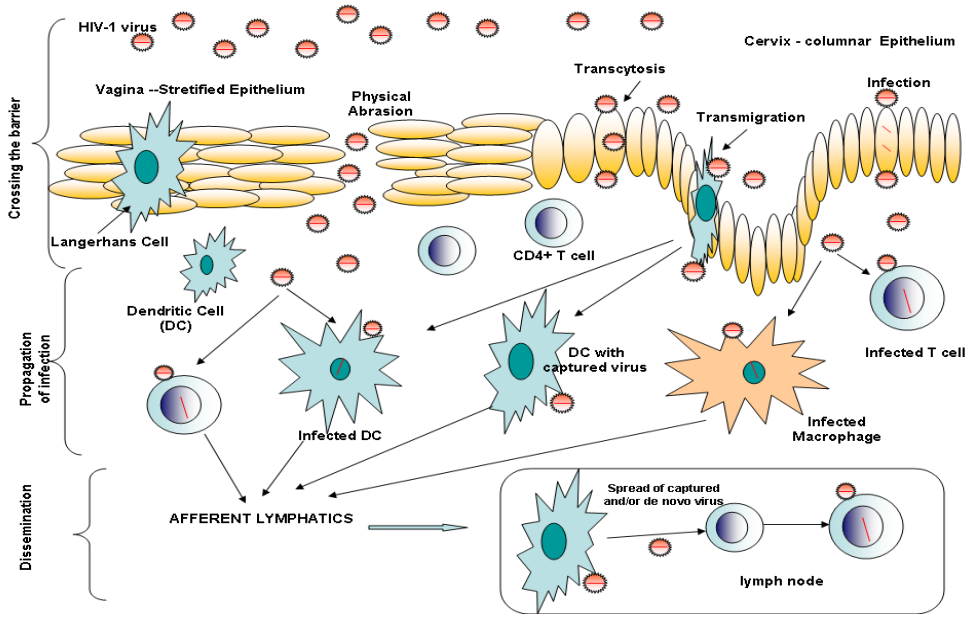


Figure 1-6. Potential pathway for HIV-1 crossing mucosal barriers.

HIV can cross the vaginal epithelia through physical abrasion or upon interaction with cells in the epithelium (DCs, T cells, epithelial cells). HIV infected cells or HIV-carrying cells can then migrate to the lymph nodes via the afferent lymphatics resulting in virus dissemination to and amplification in resident CD4+ T cells.

Importantly, heterosexual transmission is strain dependent. M-tropic HIV-1 strains (using the CCR5 co-receptor) are implicated in about 90% of sexual transmissions of HIV; CXCR4-using (T-tropic) strains are comparatively rare in sexual transmission (Moore,1997). It was reported that epithelial cells account for this preferential transmission of the R5 HIV-1 strain due to their selection of capturing R5 HIV-1 and then transferring the infection to CCR5-expressing target cells underneath the epithelia (Meng et al.,2002; Saïdi et al.,2007).

HIV-1 can invade mucosal barriers as either a cell-free or a cell-associated virus. For cell-free viral entry, HIV-1 virus has to cross the primary genital epithelial layer using unconventional mechanisms due to the natural barrier against pathogens invasion of vaginal epithelium. HIV may directly cross the epithelium through the ruptures in the epithelial surface and then infect target cells. Another route for HIV transfer across the epithelium layer is through the transcytotic pathway, a vesicular pathway which is hypothesized as one by which HIV-1

could cross an intact barrier to infect susceptible host cells in the underlying tissues (Bomsel,1997; Schacker et al.,1996). Fortunately, this route can be neutralized with secretory antibodies (Bélec et al.,2001; Bomsel et al.,1998). Furthermore, the efficiency of transcytosis is also extremely poor (less than 0.02% of the initial inoculums) (Bobardt et al.,2007).

HIV-1 can also enter DCs through the endocytosis using Dendritic Cell-Specific Intercellular Adhesion Molecule Grabbing Nonintegrin (DC-SIGN) receptors resulting in HIV-1 entry through the mucosal route (Geijtenbeek et al.,2000). However, about 90% of HIV-1 will be destroyed by this route in DCs (Arrighi et al.,2000). This interaction of HIV-DC-SIGN can be affected by environmental pH change (Davis et al.,2003).

For cell-associated virus transmission, DCs are currently thought to play an important role by trafficking HIV to T cells once DCs have captured HIV in the mucosa. In the vagina, HIV-1 can be captured by DCs and then presented to T-cells, resulting in a more efficient virus infection due to “trans transfer” through the formation of a 'viral synapse' (DC–T cell receptor–mediated binding) (Arrighi et al.,2000; Geijtenbeek et al.,2000). Thus, these virus-carrying DCs disseminate HIV infection to other CD4+ T cells, resulting in amplifying the infection. Moreover, they will further disseminate the infection to induce an immune response by carrying out their normal function of conveying and presenting pathogens or antigens to draining lymphatic tissues.

After crossing the epithelial barrier, HIV-1 can infect CCR5-expressing DCs, macrophages, and T cells underneath mucosal tissues to initiate its local propagation. Dendritic cells are widely found in skin and mucosal tissue. They capture microorganisms that enter peripheral mucosal tissues and then migrate to secondary lymphoid organs. DCs and CD4+ T lymphocytes are the predominant cell populations targeted by HIV-1 in both intraepithelial and

submucosal tissues. However, the exact role of cell-free *vs.* cell-associated virus in mucosal transmission remains unresolved. More research is still necessary to further understand mucosal transmission of HIV.

1.2.2.2 Microbicides for Prevention of Heterosexual Transmission of HIV-1

Numerous efforts have been applied in attempt to bring the HIV/AIDS epidemic into control. An effective vaccine could be the eventual solution—preventing HIV transmission and saving the lives of millions. Even a 50% effective vaccine could reduce HIV infections by more than half in 15 years in developing countries (IAVI,November, 2006). However, the development of an HIV vaccine has been elusive. Therefore, other methods must be developed for prevention of HIV sexual transmission.

A male-controlled method, the male condom, is quite effective in preventing HIV and other STIs. However, the male condoms usage rate is still not very high. In low-income drug abusing populations, condom use is only 19% (Malow et al.,2000). In addition to condom slippage and breakage during intercourse accounting for a 1.9% failure rate (Grady and Tanfer,1994), social and culture barriers hindering the use of condoms may put people, particularly women, at high risk of HIV infection in some regions(Mahoney,2000; Trussell et al.,1992). The female condom has also been applied for the prevention of HIV transmission. However, the female condom is less accepted; it was reported that most women (87%) and their partners (91%) disliked using the female condom (Andrzej et al.,2007). Thus, other effective, female-controlled methods need to be developed to prevent heterosexual HIV transmission. For all these reasons, microbicide represents the best near-term solution for protecting not only women, but also men from heterosexual transmission of HIV. Researchers are investigating the use of antiretroviral drugs as microbicide for pre-exposure prophylaxis (PrEP) to prevent

HIV infection in women (Mauck et al.,1997; Roddy et al.,1998). It was estimated that even a partially effective microbicide could prevent 2.5 million cases of HIV infection in three years in low-income countries (Katz,2007). Microbicide products are estimated much cheaper than regular HAART as mentioned previously. It only costs from $1 to $8 per daily dose (Klasse et al.,2006), a price that will make it accessible to a larger population, including low-income populations.

Microbicides, defined as antiviral products that can be applied topically for the prevention of STIs, particularly for HIV, may offer an alternative to condoms as the most feasible method for primary prevention of HIV infection. Unlike male or female condoms, microbicides are a potentially preventive option that women can easily control which do not require the cooperation, consent, or even knowledge of the partner. A successful microbicide product can offer a number of advantages and conveniences for female use(National Institute of Allergy and Infectious Diseases,2003). These advantages include that they can be fast-acting, long-lasting, and non-irritating; effective against multiple STDs, including HIV/AIDS; low cost and safe to use more than once a day and for long periods of time without affecting the vagina's physiological barrier and its natural flora; colorless, odorless and undetectable to a sexual partner.

Microbicides can be developed with various mechanisms of action as shown in Figure 1-7. These products are generally divided into two categorises, non-specific and specific (Table 1-2), according to their strategies for targeting more generalized features of the virus or blocking the viral replication cycle at a specific step (Balzarini and Damme,2005; Bass,2004). Non-specific microbicides consist of buffering agents, detergents, or surfactants, and a variety of anionic substances that target the adsorption and fusion process of the virus. Buffer agents can maintain the normal vaginal acidic pH, which results in inactivation of the pathogenic

microorganisms and viruses. Detergents, such as C31G and Nonoxynol-9 (N9), destroy the biomembrane of HIV-1 to disrupt and destroy the virus; Anionic substances target the adsorption and fusion process of the virus by coating the viral envelope through their negative charges and then blocking its cell entry. Pro2000, which is a sulfonated polyanion, significantly impairs virus capture by monocyte-derived Dendritic Cells (MDCCs) and envelope-mediated cell-cell fusion for both R5 and X4 HIV-1 virus (Teleshova et al.,2008).

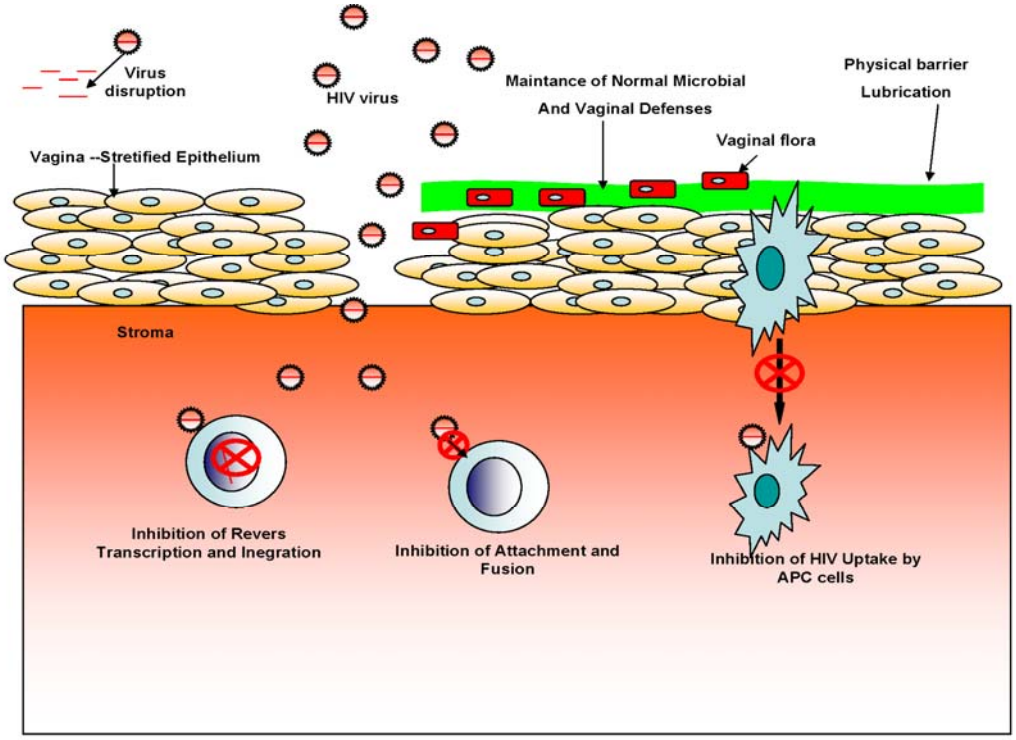


Figure 1-7. Potential anti-HIV mechanisms of microbicide compounds.

There are several mechanisms by which microbicids can provide protection as shown in Figure 1-7. Microbicide products are designed to provide a physical and/or chemical barrier against HIV-1 infection. They can act as a lubricant by coating the epithelial surface providing a physical barrier to entry. Some microbicide drug candidates act by lysing the HIV virus in the vaginal lumen prior to its entry. Other microbicide drug candidates offer a chemical barrier to protect target cells against HIV infection by interfering with virus adsorption and fusion or interfering with virus reverse transcription or integration into the host cell genome.

Table 1-2. Microbicide candidates in development for HIV prevention

Non-specific microbicides	**Detergents or surfactants**	•Nonionic compounds (e.g., nonoxynol-9, chlorhexidine) • Anionic compounds (e.g., SLS, SDS, monocaprin) •Cationic compounds (e.g., benzalkonium chloride,GEDA Plus) • Amphoteric compounds(e.g, C31G)
	Buffering agents	•BufferGel; •ACIDFORM
	Anionic polymers	•Sulfated polysaccharides (e.g., dextrin sulfate, heparin, carrageenan) •Sulfonated polymers (e.g., PRO-2000, PAVAS, PVAS) •Polycarboxylatesr†
Specific anti-HIV microbicides	**Entry inhibitors**	• Soluble CD4 (e.g., soluble CD4-IgG) • CXCR4 antagonists (e.g., SFD-1, bicyclams) • CCR5 antagonists (e.g., RANTES derivatives,) • Glycoprotein 120 (gp120)-recognizing agents (e.g., neutralizing antibodies,) • gp41-interacting agents (i.e., T-20 [enfuvirtide])
	Reverse transcriptase inhibitors	• NRTIs (e.g., [R]-PMPA [tenofovir]) • NNRTIs (e.g., UC781, TMC-120 [dapivirine])
	Integrase inhibitors	• Variety of compounds S-1360; C-731,

HIV-specific drug candidates should effectively block the viral replication cycle at a step before integration of the HIV genome into the target cell. Several steps of the viral life cycle (attachment, fusion, reverse transcription, and integration) can be a target for microbicidal intervention. A combination of several drugs, which target different steps in the virus infection cycle, may provide the advantage of decreasing their dosing level and toxicity, less viral resistance development, and more anti-HIV efficacy. Although microbicides provide a very promising strategy for HIV-1 prevention, there are still many challenges before a safe, cheap and effective microbicide product becomes commercially available.

1.2.2.3 NNRTIs: Optimal Drug Candidates for Microbicide Products

In order to develop a safe and effective microbicide product, only those compounds that have the ability to neutralize HIV virus, block HIV attachment/fusion, or prevent intracellular HIV replication will be considered acceptable drug candidates for microbicide products (Stone,2002). Various compounds, such as Cellulose sulfate (CS) , Vivigel®, PRO 2000®, and Cyanoviran® have been considered as microbicides for preventing heterosexual transmission of

HIV attachment/fusion intervention strategies (Pauwels and De Clercq,1996; Shattock and Moore,2003).

NNRTIs provide unique benefit in AIDS therapy. NNRTIs specifically bind to the "NNRTI binding pocket" in HIV-1 RT and inhibit the RT function as non-competitive inhibitors (De Clercq,1998). RT itself does not exist in host cells. The inhibition of RT by NNRTI will have less interference with the host cell, which leads to its unique property to be a good target for a microbicide. NNRTIs can prevent intracellular HIV replication at an early stage of HIV infection and do not require intracellular metabolic activation as do protease inhibitors (PIs). In addition to their high potency, they can effectively suppress HIV infection when used in combination with other anti-HIV agents (Drake,2000; Robbins et al.,2003; Staszewski et al.,1999). NNRTIs based regimens have also been reported to be better than PI based regimens for better tolerance and less risk for metabolic disorders (Chou et al.,2006; De Clercq,2004). Importantly, NNRTIs (such as UC781) can directly inactivate the reverse transcriptase in the virus particle (Balzarini and Van Damme,2005; Hossain and Parniak,2006; Stone,2002), create a local chemical barrier (Borkow et al.,1997), and provid extended protection due to "memory effect" (Barnard et al.,1997; Borkow et al.,1997). NNRTIs are promising candidates for the prevention of HIV infection as microbicide products due to their extremely potent antiretroviral activity and unique specificity for HIV-1 (Ren et al.,2002).

A limitation for NNRTIs in HIV treatment is that a single mutation in the RT enzyme NNRTI-binding pocket may confer high-level resistance to one or all of the available NNRTIs in long-term treatment of AIDS. However, the resistance development of HIV-1 to NNRTIs would not be a major issue for the use of NNRTIs as topical microbicide products (De Clercq,2004). NNRTIs can still effectively inhibit NNRTI-resistant virus at a high concentration without

toxicity due to their wide therapeutic windows (Balzarini and Van Damme,2005; Buckheit et al.,1997b).

In summary, NNRTIs are very promising candidates for the development of anti-HIV microbicide products. They can be used either as a single agent or in combination products. They provide many desired anti-HIV properties with high potency and better tolerance than PIs. Three NNRTIs, nevirapine, delavirdine, and efavirenz, are approved for clinical use by the FDA. Three others, etravirine, dapivirine and rilpivirine are subjects of clinical phase I/II studies currently, either for potential microbicide use (dapivirine) or for systemic (etravirine) therapy of HIV-1.

1.2.3 Formulation Development of Microbicide Products

Microbicides are a new class of drug products being developed in the form of gels, creams, tablets, films, or rings to help prevent sexually transmitted infections of HIV/AIDS. With the steadily growing incidence of AIDS infections globally, microbicides offer the most promising method for the prevention of sexually transmitted infections (STIs), including HIV.

Many dosage forms had been investigated as vaginal drug delivery systems for microbicide products (Bader et al.,1991; Balzarini et al.,1996b; Garg,2005; Mauck et al.,1997; Roddy et al.,1998). However, only gels and vaginal rings have entered clinical trials as of yet. Each dosage form has it own advantages and disadvantages. Economical situation, social conditions, age, and consumer/patient preferences can greatly interfere with the products' acceptability and consequently, with the effectiveness of microbicides. Several clinical studies on the acceptability of vaginal formulation were conducted in several different countries (Table 1-3)

Table 1-3. Acceptability of vaginal formulations

Products tested	Countries (people)	Preference of Products	Reference
Film Foaming tablet	Kenya (75) Dominican Republic (65) Mexico (60)	Film, (86 %) Film (52 %) Film (58 %)	(Steiner et al.,1995)
Film Gel	Cote D'Ivoire (31) Zimbabwe (22) USA (31) Thailand (rural, 36) Thailand (Urban, 25)	Discomfort or Irritation rate Film 4% Gel 20% Suppository 12%	(Coggins et al.,1998)
Film Foaming tablet	Mexico (292) Ecuador (226) Guatemala (91) Ghana (90) USA ((66)	Film (55%) Tablet (50%)	(Raymond et al.,1999)
Female condom Foaming tablets Sponge Foam Film Gel	Uganda (146)	Sponge (25%) Foaming tablets (23%) Female condom (19%) Foam (16%) Gel (9%) Film (7%)	(Pool et al.,2000)
Film Gel (A,B,C) Suppository	USA (1536)	Film (41%) Gel A, B, C (46, 49, 43 %) Suppository (34%)	(Raymond et al.,2005)

Gel, tablet, suppository, sponge, and film are investigated for their acceptability in women over several countries. Compared with other formulations, film shows significant acceptability and lower rate of discomfort or irritation rate.

Vaginal films in the recent decade have been investigated as contraceptives and more recently as microbicide formulations (Garg,2005; Mauck et al.,1997; Roddy et al.,1998). Vaginal films show combined advantages from both tablet and gel formulations. In addition to their superior pharmaceutical properties, such as extended retention time and increased drug stability, they are economic, easily applied, and acceptably to users.

In acceptability studies conducted at the University of Alabama and at the University of Zambia (Elias and Coggins,2001), it was shown that film formulations are more likely to be accepted by women than are other vaginal formulations, such as gels, foams, or suppositories. Vaginal films are easier for women to use without the help of applicators, and are light and

convenient to be carried. The small package of film makes it easy to store and inexpensive per dose. More advantages and disadvantages of film formulation are identified in following lists.

Advantages of Vaginal film

- Convenient for women to use without applicator.
- Inexpensive manufacturing cost per dose.
- Lighter and easier to carry.
- Not as messy as other current vaginal gel products.
- Can in some cases increase the stability of the drug.
- Can be formulated with a combination of different drugs.

Disadvantages Vaginal film

- Microbicide effectiveness is diminished if the film does not completely dissolve.
- Some individuals may have an allergic reaction to vaginal film.
- For some drugs this dosage form may require coitally dependent use.

1.3 DEVELOPMENT OF CYCLODEXTRIN BASED DRUG DELIVERY SYSTEMS

1.3.1 History of Cyclodextrins

Cyclodextrins have long history of application. Cyclodextrins (CDs) were first described by Villiers in 1891 (Villiers,1891). However, they did not come into wide use until after the 1950's, when French and coworkers modified the chemical process for the production of CDs (French,1957). CDs are obtained from the enzymatic digestion of starch by cyclodextrin glycosyltransferase (CGTase) (Biwer et al.,2002; Freitas et al.,2004; Larsen et al.,1998). But the availability of cyclodextrins and high production costs greatly limited their research and application until the 1970's (Horikoshi,1971; Horikoshi,1979). The advancement of biotechnology has resulted in dramatic improvements in cyclodextrin production, which has

lowered their production costs, leading to the availability of highly purified cyclodextrins and cyclodextrin derivatives (Astakhova and Demina,2004) at relatively inexpensive cost.

Today, more than 30 different pharmaceutical products containing cyclodextrins are on the market worldwide (Table 1-4). More and more cyclodextrin based dosage forms are under development.

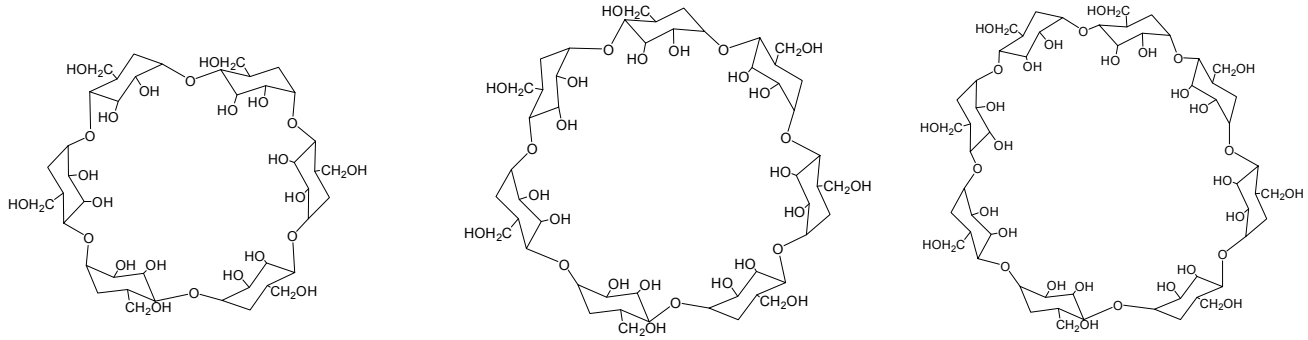
Table 1-4. βCD containing pharmaceutical products on the Market

Brand name	Drug / Cyclodextrin	Formulation	Company
Prostarmon E	PGE2/βCD	Sublingual tablet	Ono (Japan)
Caverject Dual	Alprostadil/ αCD	i.v. solution	Pfizer (USA)
Aerodiol	17-β-Estradiol / MβCD	Nasal spray	Servier (France)
Cetirizin	Cetirizine/ βCD	Chewable Tablet	Losan Pharma (Switzerland) UCB Pharma (USA)
Fluner	Flunarizine/ βCD	Tablet	Geno Pharmaceuticals)
Vitaseptol	Thiomersal/ βCD	Eye drop	Europhta (Monaco)
Mobitil	Meloxicam/ βCD	Tablet, suppository	Medical Union Pharmaceuticals (Egypt)
Abilify	Aripiprazole/ sulfobutyl-βCD	i. m. solution	Bristol-Myers Squibb (USA) Otsuka Pharm. (Japan, USA)
Cerenia	Maropitant/ sulfobutyl-βCD	Parental solution	Pfizer Animal Health (USA)
Dexocort	Hydrocortisone/ HPβCD	Solution	Actavis (EU)
Nicorette	Nicotine / βCD	Subligual tablet	Pfizer (USA)
Vfend	Voriconazole/ sulfobutyl ether - βCD	i.v. solution	Pfizer (USA)
MitoExtra	Mitomycin / HPβCD	i.v. Infusion	Novartis (Switzerland)
Voltaren OPHTHA	Diclofenac (INN) sodium / HPβCD	Eye drop	Novartis (Switzerland)

1.3.2 Chemical Structure of Cyclodextrins and Complexation Phenomenon

The family of cyclodextrins (CDs) comprises of a series of cyclic oligosaccharides compounds, and several members of this family are used industrially in pharmaceutical, chemical, and food science applications. CDs are generally crystalline, water-soluble, cyclic,

homogeneous, non-reducing, oligosaccharides built up from glucopyranose (Glc) units. The three commonly used CDs are α-cyclodextrin comprised of six glucopyranose units, βCD comprised of seven units and γCD comprised of eight such units (Figure 1-8). Larger CDs, containing more than eight glucopyranose units in the molecule, have also been studied for their complexation phenomenon (Maestre et al.,2007).



αCD βCD γCD

Figure 1-8. Chemical Structure of CDs

Structures of αCD, βCD, and γCD are shown in Figure 1-8 (from left to right). Chair conformation of glucopyranose units is presented. The number of glucopyranose units is six for αCD, seven for βCD, and eight for γCD resulting in a size different in three CDs.

The most important property of CDs is their ability of "entrapping" hydrophobic guest molecules into their cavity in the aqueous phase as shown in Figure 1-9. This complexation ability is due to their chemical structure and the glucopyranose units conformation. In cyclodextrin molecules, the glucopyranose units are present in the chair conformation. Therefore, the hydroxyl functional groups are orientated to the cone exterior with the primary hydroxyl groups of the sugar residues at the narrow and wider edges, which gives it a hydrophilic outer surface. The central cavity is formed by the skeletal carbons and ethereal oxygens of glucose residues, which gives the CD molecule a comparatively hydrophobic inner cavity. The polarity

of this cavity has been estimated to be similar to that of an aqueous ethanolic or methanolic solution (Connors,1997; Groom et al.,2003).

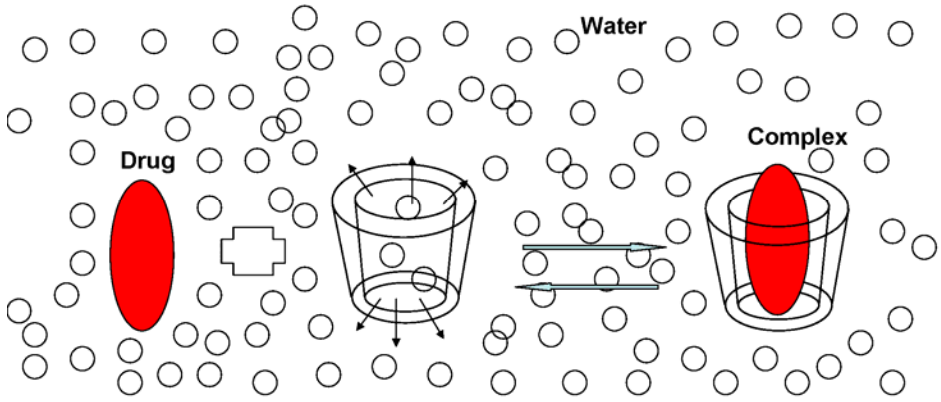


Figure 1-9. Complexation process of CDs with drugs

Schematic presentation of the process of complex formation. Small circles represent water molecules, red ellipses represent drug mulecules. Water molecules are repulsed both by thehydrophobic drug molecules and the hydrophobic cavity of the truncated CD cylinder. The main driving force for inclusion is mainly the substitution of the polar–apolar interactions (between the apolar CD cavity and polar water) for apolar–apolar interactions (between the drug and the CD cavity).

The main driving force for complex formation is thought to be the release of enthalpy-rich water from the cavity due to the entrapping of guest molecules of CDs (Guo et al.,1998; Loftsson and Brewster,1996; Loftsson and Masson,2001). Weak Van der Waals forces, hydrogen bonds, and hydrophobic interactions keep the complex together. No covalent bonds are formed or broken during drug-CD complex formation. Therefore, the complexation process can be considered as a replacement of water molecules with drug molecules.

Generally, in an aqueous solution, the cyclodextrin cavity (slightly apolar) is occupied by water molecules, which is thermodynamically unfavorable (polar-apolar interaction). Therefore, the water molecules inside the cavity have less tendency to form hydrogen bonds in the same way as in solution and result in a higher enthalpy and higher energy. When hydrophobic guest molecules are incorporated into this system, the energy of the system is lowered by substituting

these enthalpy-rich water molecules with those hydrophobic guest molecules to form the complex of CDs and "guest molecules".

In aqueous solution, an equilibrium is reached with the formation of a complex of the drug and CD and with the dissociation of the complexes. Therefore, the complexation can be studied with methods as chemical reactions. Most frequently, the complexation happens between one cyclodextrin and one guest (1:1 ratio) molecule. However, 2:1, 1:2, 2:2, and higher order complex equilibria always exist simultaneously in the system. Phase solubility diagrams are normally used to analyze the complexation stoichiometry.

In addition, the complexation is determined both by the CDs' inner cavity size and by the appropriate size of those organic compounds or guest molecules (Szejtli,1998b). Only those guest molecules with suitable shape and size can be incorporated into the CDs' inner cavity to form inclusion complexes. The cavity size of CDs is dependent on the number of glucose in the molecule as shown in Figure 1-10 and Table 1-5. The cavity size of αCD is the smallest of the three CDs and insufficient for many drugs. γCD has the largest cavity size of all three CDs. However, it is much more expensive than the other CDs. Therefore, βCD is most widely used in research and manufacturing due to its cost and suitable cavity size for most drug molecules. (Loftsson and Brewster,1996; Szejtli,1998a).

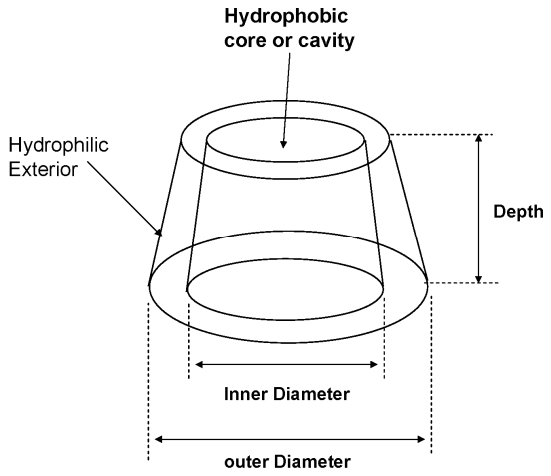


Figure 1-10. Dimensions and hydrophilic/hydrophobic regions of the CD molecules.

Table 1-5. Characteristics of α, β and γ-CDs

	α	β	γ
Number of Glucose units	6	7	8
Molecular Weight	972	1135	1297
H_2O solubility [g/100mL]	14.5	1.85	23.2
pK_a	12.33	12.2	12.08
Inner diameter [nm]	0.45-0.57	0.62-0.78	0.79-0.95
Outer diameter [nm]	1.37	1.53	1.69
Depth / Height [nm]	0.79	0.79	0.79
Cavity volume [nm^3]	0.174	0.262	0.472

Parameters of three cyclodextrins were reported by Szjtli *et al* (Szejtli,1998a)

Due to the limitation of size and apolar character of the CD cavity, the complexation is obviously not suitable for all drugs. For example, inorganic salts such as KCl and NaCl are generally recognized as not suitable for CD complexation. Generally, with the consideration of CD molecule characteristics, drug molecules should fit the following requirements but not without exception to form an applicable complex with βCD.

- more than 5 atoms (C, P, S, and N) form the skeleton of the drug molecule;
- solubility in water of should be less than 10 mg/ml;
- melting point temperature is below 250 ºC;

- the molecule consists of less than 5 condensed rings;

- a molecular weight between 100 and 400;

This solubilization strategy using cyclodextrin complexation is not suitable for very small compounds, or compounds that are too large such as peptides, proteins, enzymes, sugars, polysaccharides. However, the side chain in macromolecules may contain suitable groups which can react with CDs in aqueous solutions and form a partial complexes with CDs such as insulin (Lovatt et al.,1996).

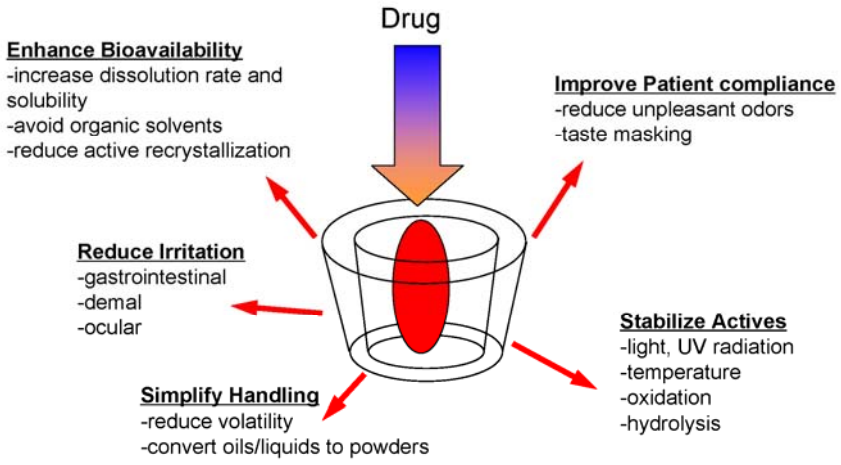


Figure 1-11. Application of βCD in Pharmaceutics

Schematic representation of the application of βCD in pharmaceutical industries for improving the drug performance in formulations. βCD complexation can improve bioavailability, reduce irritation, improve patient compliance, stabilize actives, and simplify handling.

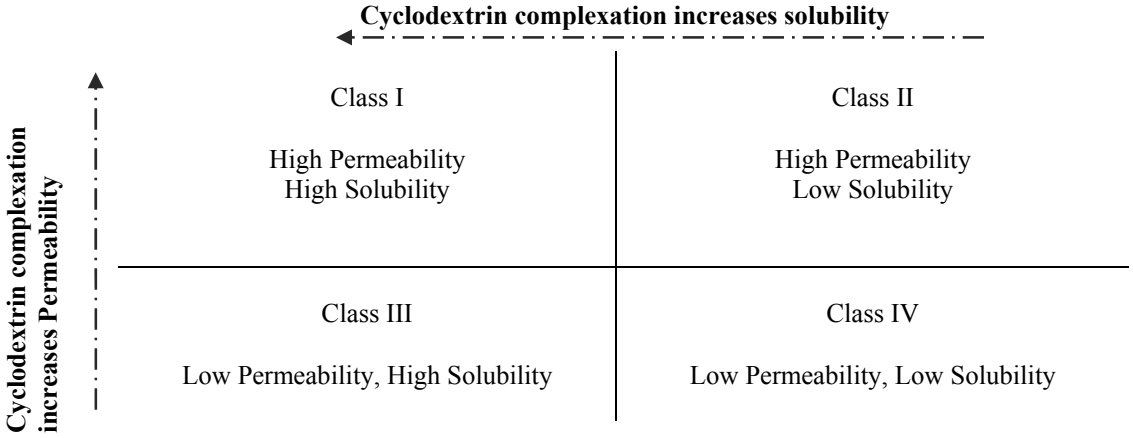
1.3.3 Pharmaceutical Application of Cyclodextrins Complexation

The formation of inclusion complexes provides numerous advantages in pharmaceutical formulation development (Figure 1-11). βCD was reported to increase bioavailability of poorly

soluble drugs by increasing the drug solubility. (Barone et al.,1998; Nasongkla et al.,2003). Light, thermal and oxidative stability of drug molecules can be improved through the formation of cyclodextrin complexes (Cwiertnia et al.,1999; Tirucherai and Mitra,2003). Cyclodextrins have also been used to reduce dermal, gastrointestinal, or ocular irritation, mask unpleasant tastes or odors, and prevent adverse drug-ingredient interactions (Loftsson and Jarvinen,1999; Redenti et al.,2001).

One major application of drug complexation with cyclodextrin is to increase the drug bioavailability in formulations. The solubility and permeability behavior of drug molecules has been extensively studied due to the significant impact on drug absorption. FDA and other drug regulatory organizations have defined a Biopharmaceutical Classification System in which drugs are sorted into four Classes based on their solubility and permeability (Amidon et al.,1995; Chen et al.,2001) as shown in Table 1-6. Numerous drug candidates with great potency belong to Class II or Class III with significant difficulties in low solubility or permeability. βCD complexation is an important technology for increasing the bioavailability of compounds belonging to Class II and III (Loftsson,2002; Loftsson et al.,2004). Drugs from Class II and Class III can be shifted to Class I by forming a complex with βCD(Loftsson,2002). This complexation can enhance the apparent water solubility and the permeability of these insoluble, hydrophobic drugs by increasing the amount of dissolved drug in bioliquid and biological membranes, leading to the increase of bioavailability.

Table 1-6. Effect of cyclodextrin complexation on the classification of drug substance.



βCD complexation process in Pharmaceutical industries for improving the performance of drug in formulations. βCD complexation can improve bioavailability, reduce irritation, improve patient compliance, stabilize actives, and simplify handling.

UC781 is a very potent NNRTI and a promising microbicide candidate with extremely low water solubility. It is important to formulate UC781 into a suitable dosage form for vaginal delivery. One of the major challenges in the development of UC781 formulation is to increase its solubility. For these reasons, this complexation technique may provide a potential strategy for the development of UC781 as a microbicide product.

1.4 HYPOTHESIS AND SPECIFIC AIMS

Microbicides are topically used antiviral products for the prevention of sexually transmitted infections (STIs), including HIV infection. They may provide an alternative to condoms as the most feasible method for primary prevention of HIV infection, particularly for women. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are considered potential candidates for microbicide products.

UC781 is a NNRTI, which provides a chemical barrier against HIV-1 infection by inhibiting its (HIV-1) reverse transcriptase (RT) activity with marked potency *in vitro*. Although it shows good permeability in the intestinal model (Deferme et al.,2002), as a ClassII drug, the extremely low water solubility (<29ng/ml, unpublished data) of the UC781 molecule poses a great challenge for its administration. Therefore, a suitable vaginal drug delivery system, which can increase the solubility of UC781, needs to be developed for formulation purposes.

β-cyclodextrin (βCD) is a cyclic (α-1, 4)-linked oligosaccharide of α-D-glucopyranose containing a relatively hydrophobic central cavity and hydrophilic outer surface. Due to its molecular structure and shape, βCD possesses a unique ability to act as a molecular container by entrapping guest molecules in its internal cavity.

Considering the highly hydrophobic nature of UC781 and βCD's ability of entrapping guest molecules, *we hypothesize that UC781 will be reversibly entrapped into the hydrophobic cavity of βCD due to its hydrophobic nature, and the formed UC781:βCD complex will not interfere with the interaction between UC781 and HIV-1 reverse transcriptase. Therefore, UC781 will be solubilized in the form of molecular complex with βCD in aqueous solution while maintaining its activity of RT inhibition, thus inhibiting HIV infection.*

Based on this hypothesis, we predict that (1) UC781 will form a complex with β-cyclodextrin in both liquid and solid state; (2) UC781: βCD complex will enhance the solubility of UC781 in aqueous media. The complexation process of UC781: βCD can be optimized by modifying the preparation methods and complexation conditions; and (3) UC781: βCD complex will facilitate the release of UC781 from formulation, due to the solubility increase of UC781,

and will maintain the same potency against HIV *in vitro* as UC781 alone. The hypothesis was tested with the following three specific aims:

Aim 1: Determine whether βCD can form an inclusion complex with UC781 and whether different cyclodextrins can affect the thermodynamic behavior of the complex (Chapter 2 and Chapter 3)

UC781: βCD complexes in the liquid and solid state were prepared respectively and evaluated using UV and NMR for the liquid state and Differential Scanning Calorimetry (DSC) and IR for the solid state. UC781 alone, βCD and UC781/βCD physical mixtures were also examined for comparison. The thermodynamic properties of UC781:βCD complexes were investigated with an HPLC method. Results obtained from these experiments provide evidence that UC781 and βCD can form inclusion complexes and elucidate the mechanism for the driving force for the reaction.

Aim 2: Determine whether UC781: βCD complexes increase the aqueous solubility of UC781 and investigate the impact of different experimental conditions on complexation (Chapter 4)

Solubility studies of the UC781: βCD complex were conducted using HPLC methods. Complexes were prepared using kneading, shaking, lyophilization, and autoclave methods. All samples were dissolved in Milli-Q water and filtered with a 0.45μm filter for quantitative HPLC assay. pH change as well as water-soluble polymer (HPMC, HEC, PVA, and PVP) incorporation were investigated as methods to enhance the solubility of UC781 in cyclodextrin solutions. Results obtained from these experiments revealed that the complexation of UC781 with βCD can

be enhanced using an autoclave method for manufacture, incorporation of water-soluble polymers during processing, or pH adjustment.

Aim 3: Determine whether complexed UC781 is released faster than non-complexed UC781 from formulations and whether the UC781: cyclodextrins complex maintains the same potency against HIV-1 *in vitro* as UC781 alone (Chapter 5)

Three formulations were developed for complexed and non-complexed UC781. The release of UC781 in both the complexed and non-complexed form was evaluated using a dissolution apparatus. The amount of UC781 released was determined using a quantitative HPLC assay. Mean dissolution time was determined for comparison. The ability of formulated non-complexed UC781 and complexed UC781 to maintain biological activity was also evaluated. Results showed that RT inhibition activity and virus replication inhibition can be maintained in the formulated state. Furthermore, the incorporation of complexed UC781 in formulations results in significant enhancement of UC781 release from the formulated product.

2.0 CHARACTERIZATION STUDIES FOR UC781: βCD COMPLEXES

2.1 INTRODUCTION

UC781, (N-[4-chloro-3-(3-methyl-2-butenyloxy) phenyl]-2-methyl-3-furancarbothioamide) (Figure 2-1), is a tight-binding HIV-1 non-nucleoside reverse transcriptase (RT) inhibitor (NNRTI). UC781 has been identified as an extremely potent inhibitor of HIV-1 replication in cell culture [50% effective concentration (EC_{50}), approximately 3 ng/ml] (Balzarini et al.,1996b; Balzarini et al.,1998; Barnard et al.,1997). It belongs to the class of (thio)carboxanilide derivatives, the prototype of which (UC-84) was previously reported (Bader et al.,1991).

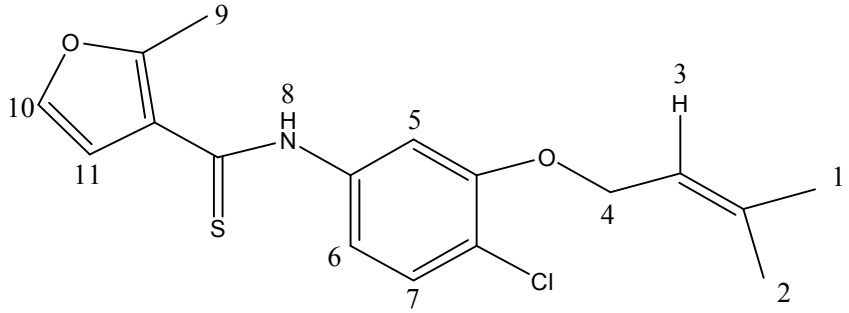


Figure 2-1. Structure of UC781

Structure of UC781: N-[4-chloro-3-(3-methyl-2-butenyloxy) phenyl]-2-methyl-3-furancarbothioamide (MW:335.48). Protons of UC-781 are shown for NMR purposes

UC781 can inhibit several laboratory and clinical strains of HIV-1 (Balzarini et al.,1996b), including both syncytium- and non syncytium-inducing phenotypes (Zussman et al.,2003). It has also been shown to inhibit HIV-1 strains which are resistant to nucleoside RT inhibitors with a potency similar to that for inhibition of wild-type virus (Borkow et al.,1999). Furthermore, UC781 targets HIV-1 RT and is effective against a variety of NNRTI resistant HIV-1 strains (Balzarini et al.,1996a; Balzarini et al.,1996b) and restores the anti viral activity of AZT to AZT resistant HIV-1 strains (Borkow et al.,1999). Importantly, high resistance to UC781 is only obtained when more than one mutation occurs in the NNRTI binding pocket (Balzarini et al.,1996a; Hossain and Parniak,2006). Thus, with the broad therapeutic index (>62,000) (Buckheit et al.,1997b), UC781 can effectively inhibit NNRTI-resistant virus isolates at a high dosing level without any toxicity.

UC781 is under consideration for use in microbicide formulations designed to minimize sexual transmission of HIV-1. This molecule exhibits a number of virologic properties that are important for anti-HIV microbicides (Borkow et al.,1997) including inactivation of isolated HIV virions and prevention of cell-to-cell transmission of HIV (Hossain and Parniak,2006; Zussman et al.,2003). Importantly, UC781 has two unique properties as a microbicide candidate. Firstly, UC781 takes effect very rapid. A very short tissue exposure to UC781 can offer enough protection against HIV-1 infection in cervical tissue models. Pretreatment of the cervical tissues with UC781 for 10 min at 10 μM offered complete protection from the HIV-1 containing semen (Zussman et al.,2003). These properties make UC781 an excellent candidate for a microbicide product. Secondly, it exhibits a "memory" effect in that pretreatment of uninfected cells renders the cells refractory to infection upon subsequent exposure to infectious HIV-1 despite the absence of exogenous drug (Borkow et al.,1997; Liu et al.,2005a). These results were confirmed

by Liu *et al* who also showed that the memory effect of UC781 was specific for drug-cell interaction and not to nonspecific binding of UC781 to the surfaces of culture plate wells (Liu et al.,2005a). This memory effect may result in the long cellular half life of UC781. The cellular half-life of UC781 in MT-2 cell line can reach 5.5 days (Borkow et al.,1997; Zussman et al.,2003). It was reported that HIV-1 virus still can be infective in the DC-SIGN-bound form for 4 days after exposure in the vagina (Geijtenbeek et al.,2000), but free HIV lost its infectivity very quickly with pH decrease. The half life for virus infectivity decreased from more than 120 min to 6 min at pH 4.5 and pH 3.5, respectively (Neurath et al.,2006; O'Connor et al.,1995). Therefore, UC781 will provide an effective protection window against HIV infection.

These properties of UC781 are likely related in part to the hydrophobicity of the compound. However, this hydrophobicity also poses great challenges in formulation and release of UC781 upon topical administration. UC781 is regarded as belonging to class II of the Biopharmaceutical Classification System (low solubility, high permeation across membranes) (Deferme et al.,2002). No aqueous parenteral formulation of UC781 is currently available for clinical use.

The purpose of this research is to develop a beta-cyclodextrin based drug delivery system for UC781 as an effective anti-HIV microbicide product. In this chapter, the ability of three different cyclodextrins, beta cyclodextrin (βCD), methyl-beta-cyclodextrin (MβCD) and 2-hydroxylpropyl-beta-cyclodextrin (HPβCD), to enhance the aqueous solubility of UC781 using phase-solubility diagram technique is evaluated. Characterization of these UC781-cyclodextrin complexes by a variety of physicochemical methods, including Ultraviolet (UV), Fourier Transform Infrared Spectroscopy (FTIR), and Differential Scanning Calorimetry (DSC) analysis is presented

Additionally, Nuclear Magnetic Resonance (NMR) spectroscopic studies of UC781 alone, βCD alone and UC781: CD complexes are presented to gain insight into interactions between UC781 and βCD in the complexed form. Finally the bioactivity of the UC781: HPβCD complex is demonstrated using an *in vitro* reverse transcriptase inhibition assay. Results from these studies showed that cyclodextrins provide substantial increases both in aqueous solubility and in biological activity of UC781, suggesting that UC781-cyclodextrin inclusion complexes may be important in the development of appropriate formulations of UC781 for use as a topical microbicide to prevent sexual transmission of HIV.

2.2 MATERIALS AND METHODS

2.2.1 Materials

UC781 for these studies was initially provided by Biosyn Co. Ltd. (Huntington, PA). However the licensing rights to this drug were transferred to CONRAD who provided subsequent supply as needed. βCD (MW 1134), MβCD (MW: Approx. 1320; Mean degree of substitution: 1.7-1.9), and HPβCD (MW: Approx. 1380 and Mean degree of substitution: 0.8) were purchased from Spectrum Chemical Company (Gardena, CA). Deuterated dimethylsulfoxide (DMSO-d6 99.6%) was obtained from Sigma Aldrich (St Louis, Missouri). All other reagents used were of reagent grade and all solvents were of HPLC grade. Milli-Q water was used to prepare buffer solutions and other aqueous solutions.

2.2.2 Methods

2.2.2.1 Phase Solubility Studies of UC781 Complexation

An excess amount of UC781 was added into a sealed 2 ml auto sampler screw-thread glass vial (Fisher Scientific, Hampton, NH) containing 1 ml distilled water with various concentrations of βCD, MβCD, and HPβCD (from 0 to 0.52 M). The vials were shaken on a horizontal rotary shaker at a speed of 50 rpm at ambient temperature (25 °C) for seven days. The solutions were then filtered through a 0.45 μm nylon disc filter (Millipore Co., Billerica, MA) to collect a clear solution. All samples were prepared in triplicate. The concentration of UC781 in the UC781: CD inclusion complex solution was determined using high performance liquid chromatography (HPLC). The assay used the following conditions: a Waters 510 pump and Waters 2487 dual wavelength absorbance detector at 275 nm; column ODS-C18 (4.6 × 250 mm, 5μm; Alltech, Columbia, MD); a mobile phase of acetonitrile/water (75:25 v/v); a flow rate of 1 ml/min.

The complexation constant ($K_{1:1}$), according to the hypothesis of 1:1 stoichiometric ratio of complexes, was calculated from phase-solubility diagrams (Higuchi and Connors,1965) using the following Equation (2-1).

$$K_{1:1} = \frac{\text{slope}}{S_o(1 - \text{slope})} \qquad \textbf{Equation 2-1}$$

In this equation, $K_{1:1}$ is the complexation constant, S_o is the intrinsic solubility, and the slope is calculated from a graph of the dissolved drug concentration verses βCD concentration in the medium. The intrinsic solubility value (S_o) of UC781 in the absence of βCD was determined directly in aqueous media.

2.2.2.2 UV Absorption Spectrophotometry of UC781 and Its Complexes

The effect of each βCD on the UV spectra of UC781 was studied using a NanoDrop® ND-1000 spectrophotometer (Nanodrop technologies Inc, Wilmington, DI). All measurements were performed at ambient temperature. Different amounts of MβCD (0 to 20%, w/v) and HPβCD (0 to 20%, w/v) were added into a 50% (v/v) aqueous ethanol solution with a fixed UC781 concentration (1×10^{-4}M). UV absorption profiles for UC781 at different CD concentrations were obtained between wavelengths of 220 to 410 nm. A UC781 solution without cyclodextrin was used as a control for these studies.

The stoichiometric proportion and complexation constant ($K_{1:1}$) were evaluated using the Scott Equation (2-2) (Brandao et al.,2003):

$$\frac{C_{CD}}{\Delta A} = \frac{1}{C_{UC781} \times \Delta\varepsilon} C_{CD} + \frac{1}{C_{UC781} \times \Delta\varepsilon \times K_{1:1}} \qquad \textbf{2-2}$$

where C_{CD} and C_{UC781} are the total molar concentrations of cyclodextrin and UC781, ΔA is the change in absorbance after complexation of cyclodextrin ($\Delta A=A_{UC781}-A_{CD:\,UC781}$), $K_{1:1}$ is the complexation constant and Δε is the difference of the molar absorptivities for free and complexed UC781. If the plot of $C_{CD}/\Delta A$ against C_{CD} yields a straight line, a 1:1 complex between UC781 and cyclodextrins is indicated. Under this circumstance, Equation (2-2) can be simplified giving Equation (2-3)

$$\frac{C_{CD}}{\Delta A} = slope \bullet C_{CD} + \text{int}\, ercept \qquad \textbf{2-3}$$

The complexation constant $K_{1:1}$ (M^{-1}) can be mathematically calculated from Equation (2-3) using Equation (2-4):

$$K_{1:1} = (\text{slope}/\text{intercept}) \times 1000 \qquad \textbf{2-4}$$

2.2.2.3 2D ^{1}H-^{1}H Nuclear Magnetic Resonance ROESY of UC781: βCD Complex

2D ^{1}H-^{1}H Rotating frame Overhause Effect Spectroscopy (ROESY) was carried out on a Varian Mercury 400 (400MHz) spectrometer (Varian Inc, Walnut Creek, CA) with a 5mm sample tube, operating at 400 MHz at 25°C. Typically, deuterated water is used as a solvent in this type of study. However, given the poor aqueous solubility of UC781, DMSO-d6 was chosen as the solvent in these experiments. DMSO-d6 has a dielectric constant which is similar to that of water (Miyake et al.,1999). Additionally, it offers acceptable solubilization for both UC781 and βCD moieties. No internal reference was used to avoid interference with the complexation between UC781 and cyclodextrins; therefore, the solvent signal (2.50 ppm) was used as an internal reference. 2D ROESY was performed in the phase-sensitive mode with a spin-lock mixing time of 500 ms. Complexes of UC781 with βCD were prepared by dissolving 1.0×10^{-2}M of both UC781 and βCD in DMSO-d6 for the 2D ^{1}H-^{1}H ROESY experiment. UC781 and βCD were dissolved in DMSO-d6 separately (1.0×10^{-2}M) as controls. The ^{1}H-signals of βCD were assigned as described by Schneider *et al* (Schneider et al.,1998).

2.2.2.4 Preparation of UC781: βCDs physical mixtures and the solid complexes

Complexes of UC781 with each of βCD, MβCD, and HPβCD were prepared at a 1:3 molar ratio. The UC781 cyclodextrin mixture was added to a solution consisting of 1.5ml $NH_3\cdot H_2O$ and 0.8ml EtOH to dissolve both the cyclodextrin and the UC781. The solution was shaken vigorously and then frozen using liquid nitrogen. The frozen solution was then freeze-dried with a Labconco Freezone 6 Freeze-Dryer system (Labconco Corporation, Kansas City, MO) for at least 48 hr. The freeze-dried samples were gently ground into a fine powder with a mortar and pestle. The powder was placed onto a filter paper and then rinsed with 1ml of 100% acetonitrile

(ACN) to remove non-complexed UC781. The filter was then placed into a vacuum oven at 30 ^{o}C for 30 minutes to remove any residual solvent.

Physical mixtures of UC781 with three different β-cyclodextrin in 1:3 molar ratios were prepared by mixing freeze-dried UC781 powder with freeze-dried βCD, MβCD, or HPβCD for 2 min on a Fisher Vortex Genie 2® Mixer (Fisher Scientific International Inc. Hampton, NH).

2.2.2.5 Differential Scanning Calorimetry (DSC)

DSC analysis was carried out for pure UC781, the three different pure cyclodextrins, physical mixtures of UC781 with each cyclodextrin at a 1:3 molar ratio, and complexes of UC781 with each of βCD, MβCD, and HPβCD. The DSC patterns were recorded on a Perkin-Elmer DSC-7 (PerkinElmer. Inc, Boston, MA) equipped with a thermal analysis data station. Each sample (3 to 5 mg) was heated in crimped aluminum pans over a temperature range of 50 to 200 °C at a constant scanning rate of 5 °C/m in nitrogen purging (20 ml/min). A blank aluminum pan was used as a reference. The heat of fusion was calibrated using an indium standard obtained from the PerkinElmer company (indium purity: 99.99 %, melting point: 157.07 °C, ΔH = 20.37 J/g, heating rate: 5 °C/min).

2.2.2.6 Fourier Transform Infrared Spectroscopy (FTIR) Studies

The infrared spectra of UC781, and its complexes with the three different βCD in solid state were obtained using a Bio-Rad Excalibur series FTIR spectrometer FTS 3000MX (Bio-Rad Laboratories, Inc. Hercules, CA) equipped with a Digilab Merlin 3.3 workstation according to the thin solid film method described by Song *et al.*(Song and Wang,2003). A thin solid film of UC781, βCD, MβCD, HPβCD, physical mixtures of UC781 with each βCD at a 1:1 molar ratio, and complexes of UC781 with each of βCD, MβCD, and HPβCD were cast on a sodium chloride

(NaCl) plate from a solution of 70% ethyl alcohol. The selected wave-number range used in this study was from 500 to 4000 cm^{-1}. For comparison purposes, FTIR spectra was obtained for pure UC781, each βCD being studied, as well as physical mixtures of UC781 and each cyclodextrin.

2.2.2.7 Determination of RNA-dependent DNA Polymerase Activity of HIV-1 Reverse Transcriptase

HIV-1 RT DNA polymerase activity was determined by a fixed time assay, as previously described by Barnard *et al* (Barnard et al.,1997). Reaction mixtures (50µl total volume) contained 50 mM Tris-HCl (pH 7.8, 37ºC), 60mM KCl, 10mM $MgCl_2$, 5 µg/ml of either poly(rA)-oligo(dT)12-18 or poly(rC)-oligo(dG)12-18, and either 20µM [^{3}H]TTP or 10µM [^{3}H]dGTP. Reactions were initiated by the addition of 50-80 ng of RT (9-12 nM final concentration). Reaction mixtures were incubated at 37ºC for 20 min and then quenched with 250µl of ice-cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. Quenched samples were left on ice for 20 min, then filtered using 1.2 µm glass fiber Type C filter multi-well plates (Millipore), and washed sequentially with 10% TCA containing 20mM sodium pyrophosphate and with 100% ethanol. The extent of radionucleotide incorporation was determined by a Wallace 1450 Microbeta Jet liquid scintillation (Perkin Elmer, Waltham, MA) counting of the dried filters.

2.2.2.8 Statistical Analysis

IC_{50} values for UC781 in both the non-complexed and complexed forms were calculated using GraphPad Prism 4.0. Data was analyzed using one-way ANOVA analysis with Tukey comparison. $P<0.05$ represented significant differences.

2.3 RESULTS AND DISCUSSION

2.3.1 Phase solubility Studies of UC781 Complexes

The effect of cyclodextrin on the aqueous solubility of UC781 was evaluated using the phase solubility method. Figure 2-2 and Figure 2-3 show the phase diagrams for UC781 in the presence of the three different types of cyclodextrins in aqueous media. The intrinsic aqueous solubility of UC781 is almost negligible ($<3\times10^{-5}$ mg/ml). The presence of βCD in the aqueous phase resulted in increased apparent aqueous solubility of UC781 regardless of the βCD type used. UC781 solubility was increased 20 fold in the presence of 1.8% w/v βCD (1.7×10^{-6} M), nearly 4000 fold in the presence of 40% w/v MβCD (3.2×10^{-4} M), and 500 fold in the presence 40 %w/v HPβCD (4.1×10^{-5} M). The solubility of UC781 increased linearly as a function of βCD, MβCD, and HPβCD concentration under the experimental conditions. The phase solubility profiles show that complexation of UC781 with all three βCDs increases UC781 solubility in a linear pattern, displaying type A_L Phase diagrams according to the classification by Higuchi(Higuchi and Connors,1965). This indicates the formation of 1:1 inclusion complexes of UC781 with each of three cyclodextrins in solution. The correlation coefficient of the linear regression for each of the phase solubility curves was greater than 0.98, indicating a good fit for all regressions. The complexation constants ($K_{1:1}$) of UC781 with each cyclodextrin are 1119.5 M^{-1} for βCD, 2239.4 M^{-1} for HPβCD, and 13449.7 M^{-1} for MβCD using Equation 2-1.

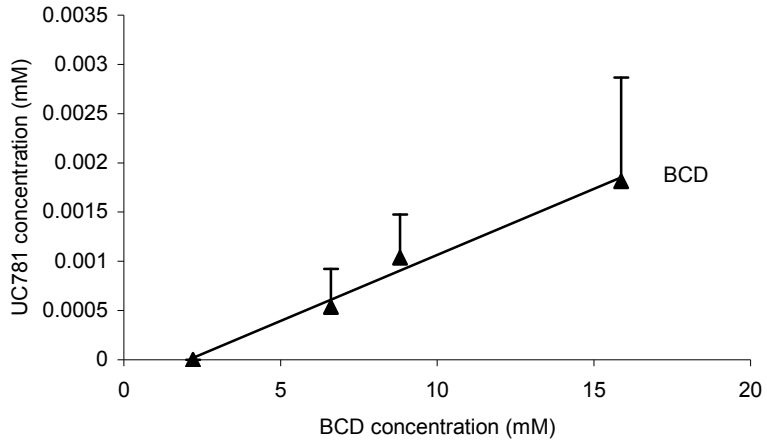


Figure 2-2. Phase-solubility diagram for UC781 with βCD in water

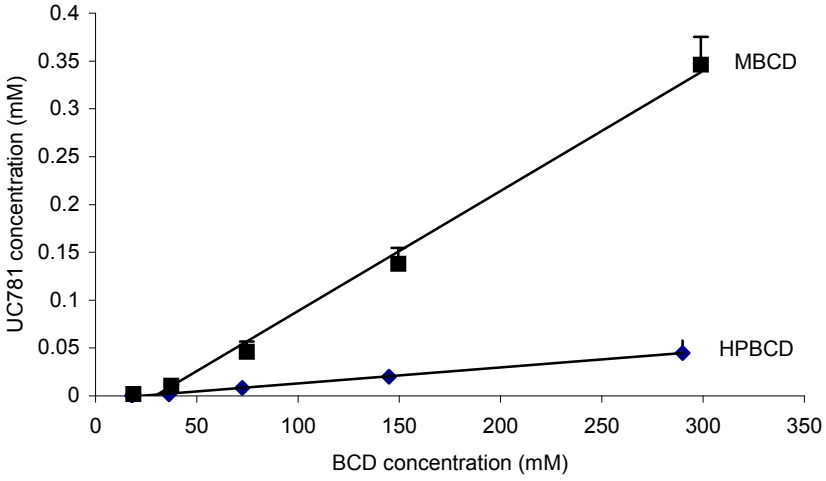


Figure 2-3. Phase-solubility diagrams for UC781: HPβCD and UC781: MβCD in water

For MβCD, inclusion constants were additionally calculated for a 1:2-complex ($K_{1:2}$) using Equation 2-5. This equation was derived by Lofftson *et al* (Loftsson et al.,2002).

$$S_t = S_0 + K_{1:1} \cdot S_0 \cdot [CD] + K_{1:1} \cdot K_{1:2} \cdot S_0 [CD]^2 \qquad \textbf{2-5}$$

In this equation, S_t and $[CD]_t$ correspond to total UC781 and MβCD concentration respectively, $K_{1:1}$ and $K_{1:2}$ represent the complexation constants for the 1:1 and 1:2 complexes respectively. The $K_{1:1}$ value for the MβCD complex was calculated as 5597 M^{-1}. This value was significantly higher than that calculated for $K_{1:2}$ for this complex (4.2 M^{-1}), and corresponds to the observed significant increase in aqueous solubility of UC781 in the presence of MβCD. The apparent low value of $K_{1:2}$ with respect to $K_{1:1}$ indicates that the 1:1 complex is the predominant form.

Increases in UC781 solubility in aqueous cyclodextrin solutions are consistent with the formation of inclusion complexes between UC781 and cyclodextrin molecules. Generally, the main driving force for complex formation is the hydrophobic interaction between a poorly water-soluble guest compound, such as UC781, and the apolar cavity of the cyclodextrin molecule. The hydrophobicity and geometry of the guest molecule as well as the cavity size and the derivative groups of the cyclodextrin are important for complex formation. In the current study, the enhancement of UC781 solubility is highly dependent on the type of cyclodextrin used. Based on the phase solubility diagrams, MβCD is more effective in solubilizing UC781 in aqueous media compare to HPβCD and βCD.

The different complexation constants found for the different cyclodextrin molecules studied indicate that the derivative groups on the cyclodextrins appear to play an important role in the incorporation of UC781 into the cyclodextrin cavity, since much higher complexation constants were obtained for MβCD and HPβCD. Specifically, physicochemical properties of cyclodextrins are greatly altered upon methylation. MβCD can be dissolved in both organic solvents and water with less hygroscopic and higher surface activity than underivatized cyclodextrins (Uekama and Otagiri,1987). Furthermore, the cyclodextrin cavity is lengthened upon for the methylation of the

OH (2) and OH (6) groups of the cyclodextrin rim without significant distortion of the ring, and this would enhance the complexation ability of MβCD (Sueishi et al.,2003; Suzuki et al.,1994). These properties lead to greater complexation ability for MβCD with UC781 than for βCD and HPβCD. Even though βCD is a good host molecule for UC781, the relatively poor aqueous solubility of βCD (16.3 mM) limits its utility for use in the enhancement of UC781 aqueous solubility. Our studies suggest that HPβCD and MβCD may be more appropriate candidates for the solubilization of UC781 for use in topical microbicide formulations.

2.3.2 UV Studies of UC781 Complexes

Ultraviolet spectroscopy was used to further confirm the complexation of UC781 with the three different cyclodextrins in solution. Figures 2-4 to 2-6 show the effect of βCD (Figure 2-4), HPβCD (Figure 2-5), and MβCD (Figure 2-6.) on the UV spectrum of UC781. Maximum UV absorption for UC781 solubilization in an aqueous ethanol solution was observed at 300 nm. The obtained maximum absorption of UC781 was enhanced with the addition of βCD, HPβCD, and MβCD. Results generated comparing varying concentrations of cyclodextrins showed that the most significant increase in enhancement of UC781 absorbance occurred at a concentration of 0.25% βCD, 2.5% HPβCD, and 10% MβCD. In addition, a broader UV peak and a slight blue shift (about 5 nm) was displayed in the UV absorption profile for UC781 under these conditions.

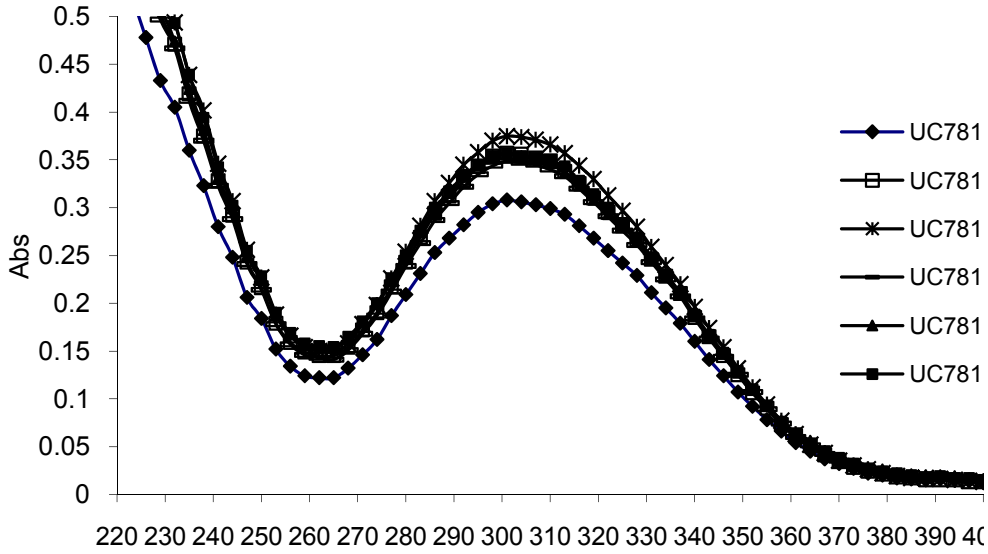


Figure 2-4. Effect of βCD concentration on the UV absorbance of UC781 in aqueous solution
The βCD concentration was 0%, 0.125%, 0.25%, 0.5%, 0.75%, and 1.0%. Solid diamond = UC781; blank square = UC781 with 0.125% βCD; black star=UC781 with 0.25% βCD; black dash=UC781 with 0.5% βCD; solid triangle = UC781 with 0.75% βCD; solid square= UC781 with 1% βCD.

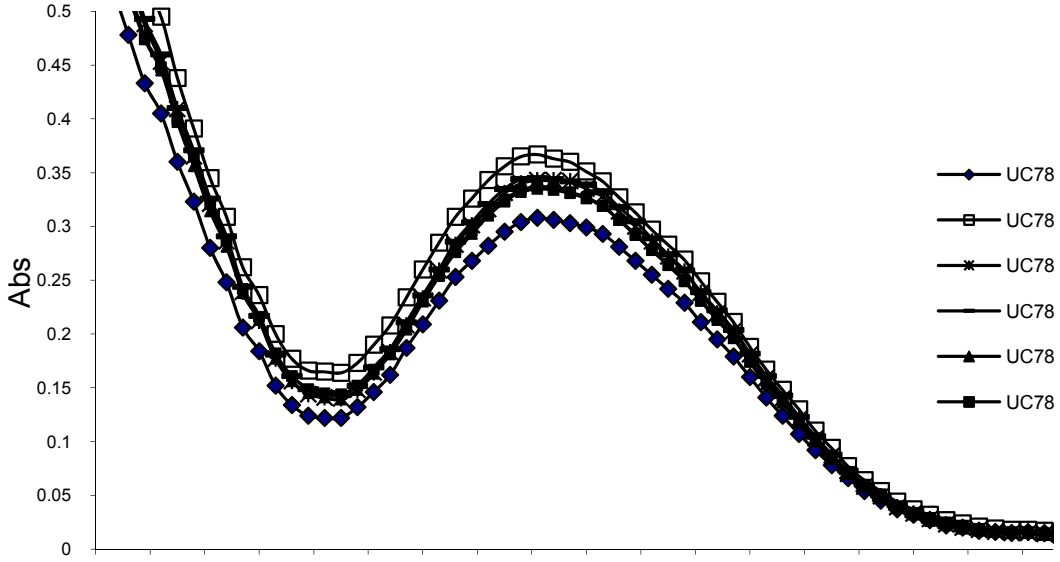


Figure 2-5. Effect of HPβCD concentration on the UV absorbance of UC781 in aqueous solution
The HPβCD concentration was 0%, 2.5%, 5%, 10%, 15%, and 20%. Solid diamond = UC781; blank square = UC781 with 2.5% HPβCD; black star=UC781 with 5.0% HPβCD; black dash=UC781 with 10% HPβCD; solid triangle = UC781 with 15% HPβCD; solid square= UC781 with 20% HPβCD.

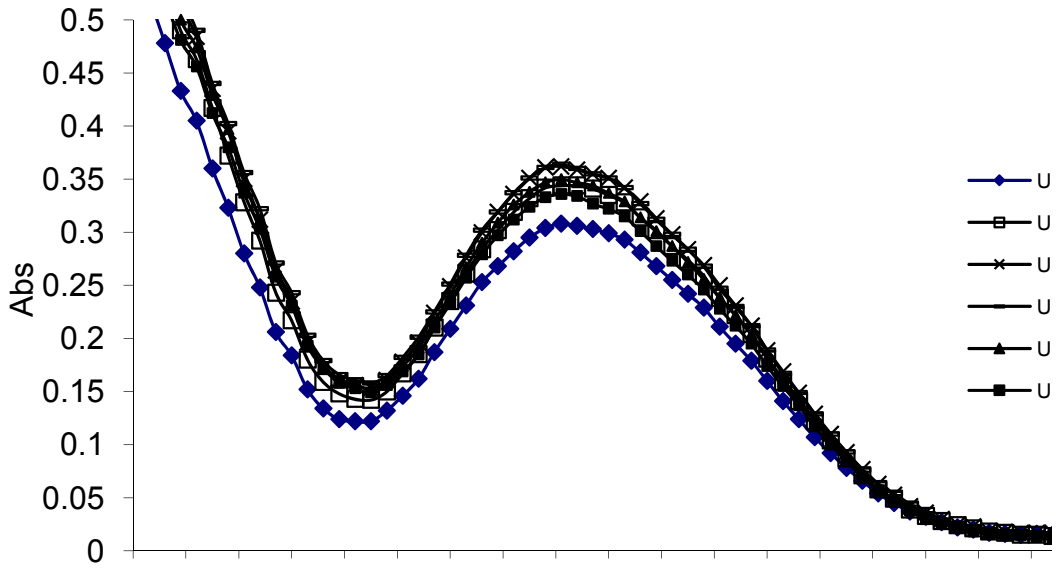


Figure 2-6. Effect of MβCD concentration on the UV absorbance of UC781 in aqueous solution The MβCD concentration was 0%, 2.5%, 5%, 10%, 15%, and 20%. Solid diamond = UC781; blank square = UC781 with 2.5% MβCD; black star=UC781 with 5.0% MβCD; black dash=UC781 with 10% MβCD; solid triangle = UC781 with 15% MβCD; solid square= UC781 with 20% MβCD.

UV results showed that the UC781 UV absorbance intensity increased in the presence of βCD, HPβCD, or MβCD. No baseline absorption was observed for any of the cyclodextrins studied under these experiment conditions. The baseline UV absorption profile for UC781 was modified in a concentration dependent manner upon addition of cyclodextrin. A maximum in UV absorption was observed in the presence of low cyclodextrin concentrations (0.25% βCD, 2.5% HPβCD and 10% MβCD). However, in the presence of higher cyclodextrin concentrations, no further increase in UV absorption was seen. This variation in absorption intensity observed at varied concentration of cyclodextrins may be due to the high concentration of alcohol in the solvent system (50%). Further studies are needed to clearly define this phenomenon.

The change in UV absorption of UC781 in the presence of the cyclodextrins indicated the formation of inclusion complexes, and suggested that this complexation involves the UC781 chromophore entering the cyclodextrin cavity (Chow and Karara,1986; Iglesias,2006; Liu et

al.,2006). A linear relationship of $C_{CD}/\Delta A$ *vs.* C_{CD} was observed indicating a 1:1 stoichiometric proportionality for the UC781: CD complexes (data not shown). The complexation constant values ($K_{1:1}$) calculated from the Scott Equation were found to be 17, 7, and 10 M^{-1} for βCD, MβCD, and HPβCD, respectively. The value of $K_{1:1}$ calculated from UV data was lower than that obtained from the phase-solubility data. This difference is most likely due to the presence of ethanol in the UV experiments. Ethanol was not present in the system for phase solubility studies.

2.3.3 DSC Analysis of UC781 Complexes

Differential scanning calorimetry (DSC) has been one of the most widely used calorimetric techniques for studies of the interactions between drugs and βCDs in the solid state (Ghorab and Adeyeye,2001). In this study, DSC was applied to evaluate the interaction between UC781 and each of the three different cyclodextrins in the solid state.

DSC thermograms for each pure component, physical mixtures, and UC781: cyclodextrin complexes are shown in Figure 2-7 to Figure 2-9. The DSC isotherms of the pure components were characterized by a sharp endothermic peak for UC781 at 131℃. No endothermic peaks were observed for MβCD and HPβCD due to their amorphous nature. The βCD endothermic peak was not observed due to the freeze-dry preparation. The broadening of the UC781 endothermic curve in DSC thermograms obtained for physical mixtures of either HPβCD or MβCD with UC781 may be due to carrier induced drug amorphorization. No characteristic UC781 endothermic curve was observed in DSC thermograms for solid complexes of UC781. This can be associated with the formation of UC781: βCD (Figure 2-7), UC781: HPβCD (Figure

2-8), and UC781: MβCD (Figure 2-9) inclusion complexes in the solid state. Although these results suggest complex formation, it is important to recognize that in these studies, uncomplexed UC781 in the amorphous state may also exist in this solid-state mixture. However, combined results obtained from DSC studies with those obtained from phase solubility and UV studies show that the complexed form of UC781 is the major product in the solid-state mixture.

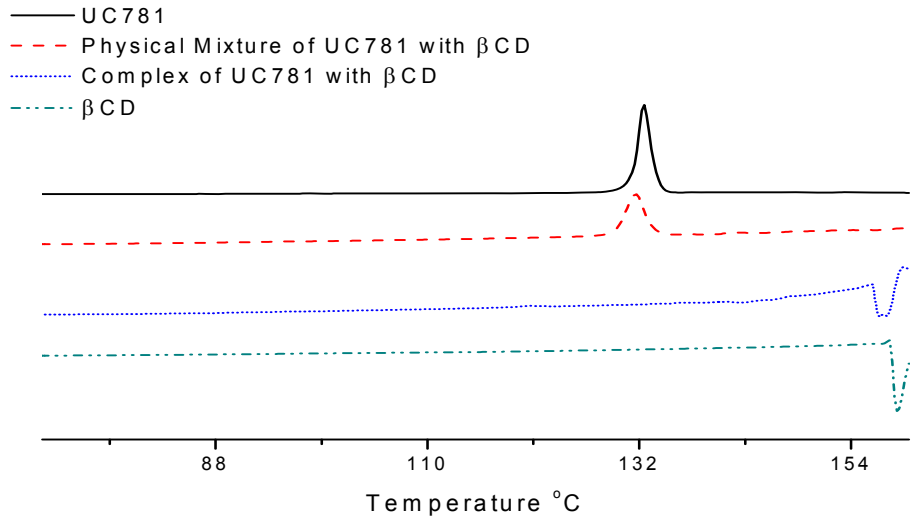


Figure 2-7. DSC thermograms for UC781: βCD physical mixture, βCD, UC781, and UC781: βCD complex.

Solid line = UC781; Dashed line =Physical Mixture of UC781 and βCD ; Dotted line =UC781 complex with βCD; dot and dash line = βCD.

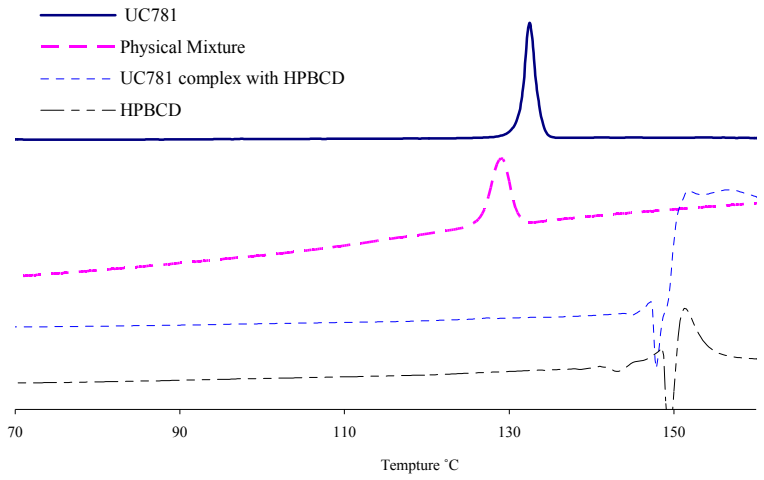


Figure 2-8. DSC thermograms for UC781, UC781: HPβCD physical mixture, UC781: HPβCD complex, and HPβCD.

Solid line = UC781; Dashed line =Physical Mixture of UC781 and HPβCD; Dotted line =UC781 complex with HPβCD; dot and dash line = HPβCD.

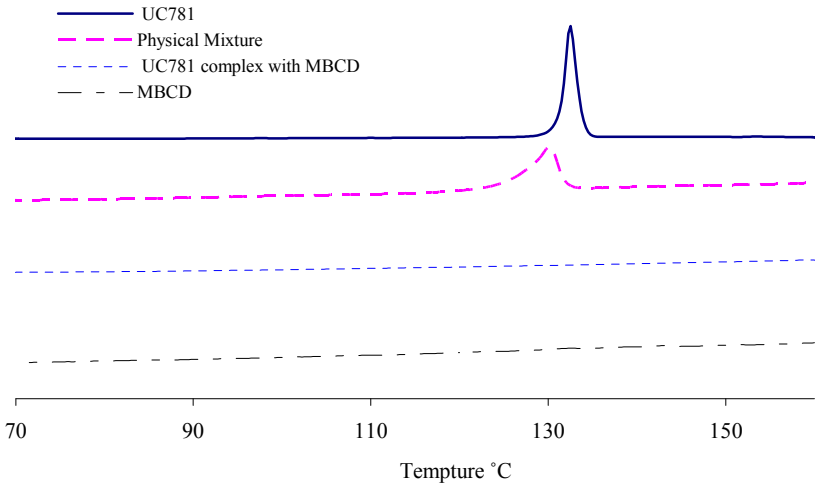


Figure 2-9. DSC thermograms for UC781: MβCD physical mixture, UC781: MβCD complex, MβCD, and UC781.

Solid line = UC781; Dashed line =Physical Mixture of UC781 and MβCD; Dotted line =UC781 complex with MβCD; dot and dash line = MβCD.

2.3.4 FTIR Studies of UC781 Complexes

Fourier Infrared spectrophotometry (FTIR) has been employed as a useful tool to identify drug excipient interactions (Hsiue et al.,1998; Sarisuta et al.,2006). In these reports, the use of FTIR spectroscopy to provide important information regarding the confirmation of inclusion complex formation of βCDs with drug molecules is demonstrated. Figure 2-10, Figure 2-11, and Figure 2-12 show the FTIR spectra of the three different UC781-βCD complexes as compared with spectra obtained for pure UC781, pure βCD, and a physical mixture of the two. In the FTIR spectrum for pure UC781, the absorption observed at 3437 cm^{-1} can be attributed to the stretching vibrations (*v*) of the N-H bond. The peak observed in this spectrum at 1645 cm^{-1} reflects N-H group bending vibrations (δ). FTIR spectrums for the complexed forms were shown to have a broadening in the *v* (N-H) and δ (N-H) bands obtained. In addition, a slight red shift was observed for each of these bands in the spectrums obtained for the complexes. These studies revealed that the N-H group of UC781 is specifically involved in the interaction between UC781 and each of the βCDs studied. The FTIR spectrums for the complexes showed a substantial decrease in intensity for the two bands associated with the UC781 N-H group. This result suggests the formation of new supramolecular compound. Additionally, no new peaks were observed in the spectra of all UC781-βCD complex systems, indicating no chemical bonds were created in the complex formation. Thus, the FTIR spectra indicate that UC781 molecule is partially (NH group) included into the βCD cavity to form the inclusion complex.

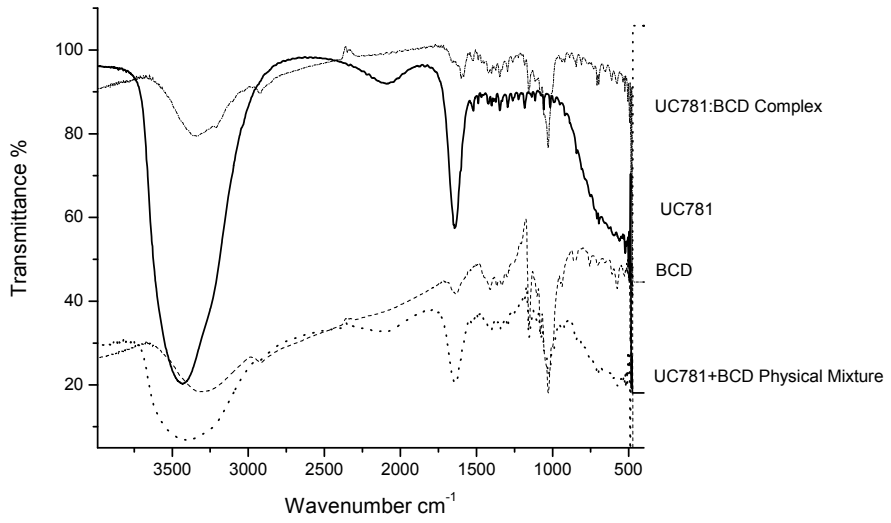


Figure 2-10. FTIR spectra of UC781, βCD and their complex.

Solid line = UC781; Dashed line =βCD; Dotted line = Physical Mixture of UC781 and βCD; Dot and dash line = UC781 complex with βCD

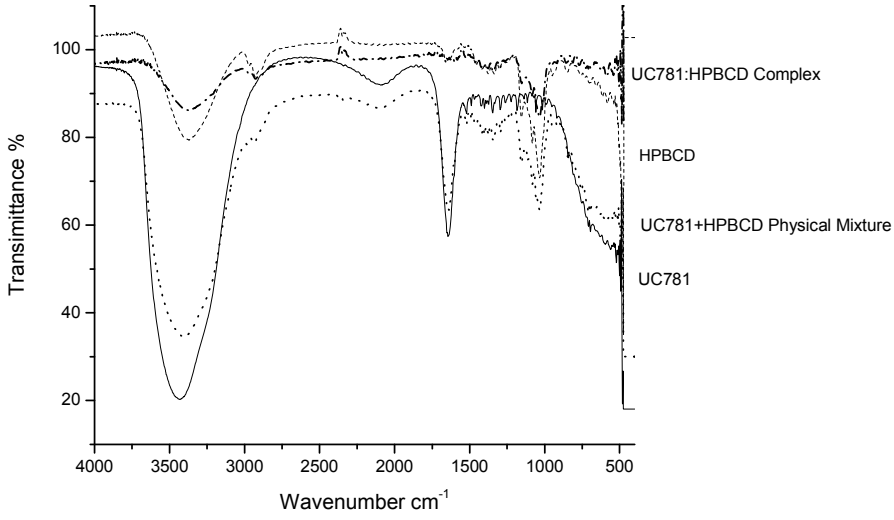


Figure 2-11. FTIR spectra of UC781, HPβCD and their complex.

Solid line = UC781; Dashed line =HPβCD; Dotted line = Physical Mixture of UC781 and HPβCD; Dot and dash line = UC781 complex with HPβCD

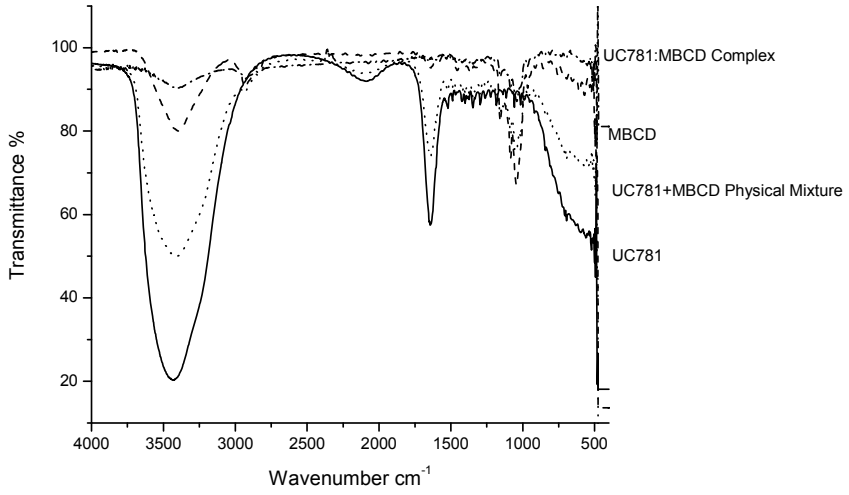


Figure 2-12. FTIR spectra of UC781, MβCD and their complex.

Solid line = UC781; Dashed line =MβCD; Dotted line = Physical Mixture of UC781 and MβCD; Dot and dash line = UC781 complex with MβCD

2.3.5 2D ROESY NMR Spectra Studies of UC781 Complexes

2D ROESY was conducted to predict the UC781: βCD complex structure in DMSO-d6. Figure 2-1 shows the numbered protons of the UC781 molecule. The entire NMR spectrum is shown in Figure 2-13. Cross-peaks were observed between the 5,6,7 protons on the benzene ring of UC781 and the 3',5' protons of βCD (Figure 2-14) and between the N-H proton of UC781 and OH_2, OH_3 of βCD (Figure 2-15) indicating very close interaction of UC781 and βCD in the liquid state. This suggests that the complex formed between UC781 and βCD involves the aromatic ring and NH group of UC781 entering the βCD cavity. These cross-peaks were not observed in spectra obtained for pure UC781 or pure βCD. Figure 2-16 shows a molecular simulation of the association of UC781 and βCD in the complex form.

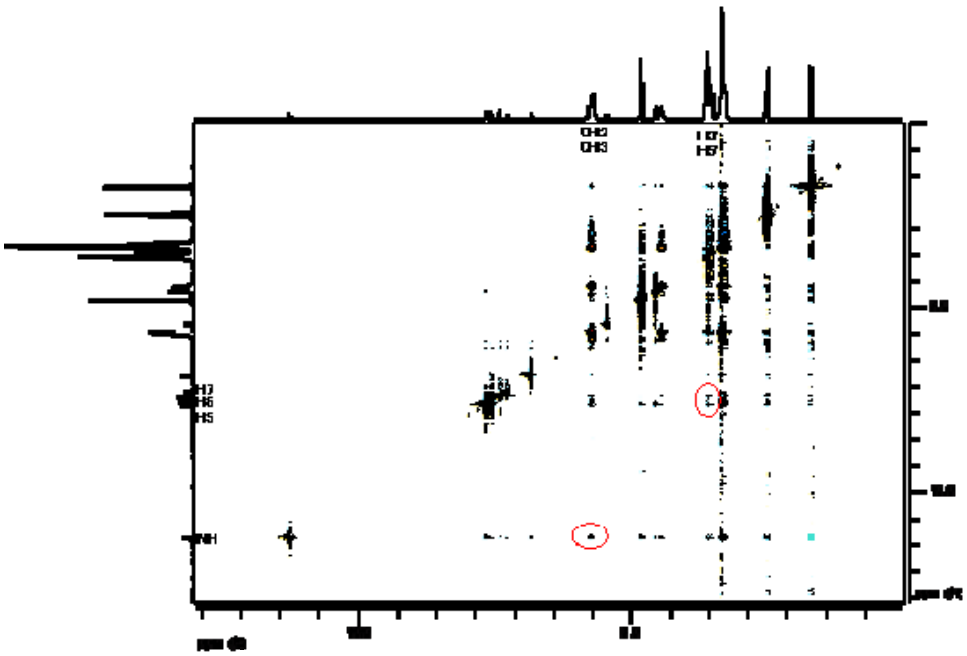


Figure 2-13. 2D ROESY spectrum of a mixture of 1.0×10^{-2}M UC781 and βCD.
Annotated cross-peaks indicate intermolecular interactions between UC781 and βCD.

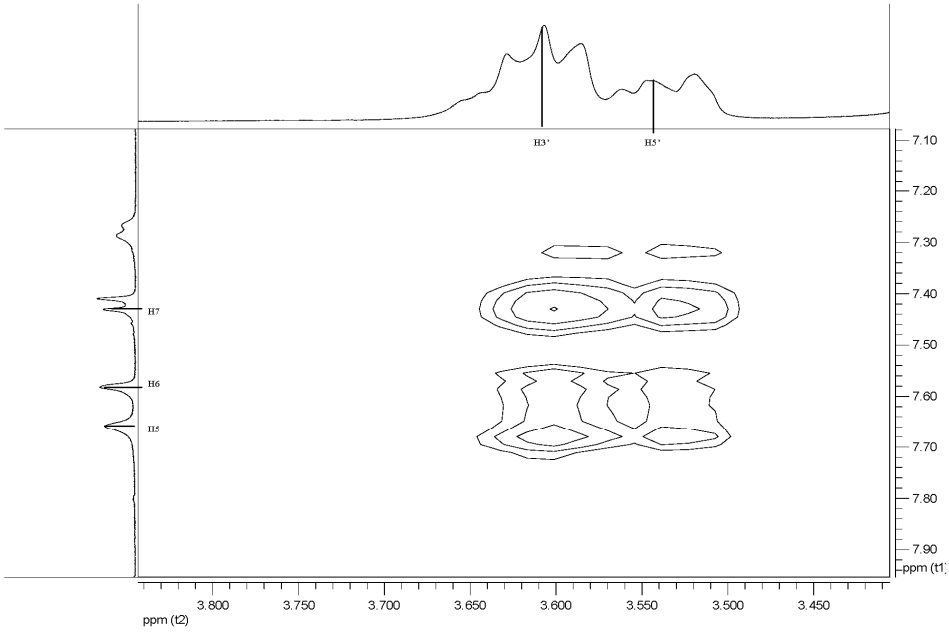


Figure 2-14. 2D ROESY spectrum of a mixture of 1.0×10^{-2}M UC781 and βCD.
Annotated cross-peaks indicate intermolecular interactions between benzene ring (H5, H6, H7) of UC781 and H3', H5' of βCD.

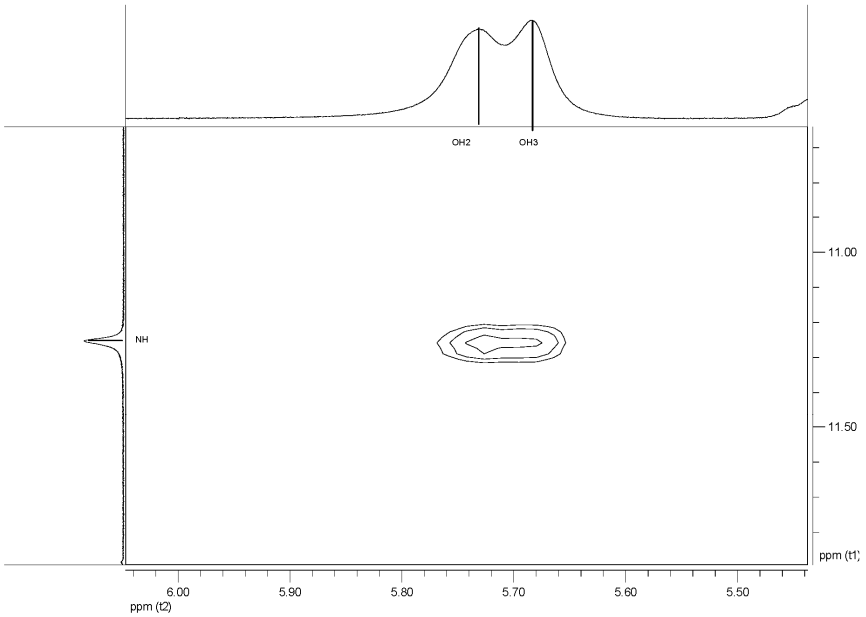


Figure 2-15. 2D ROESY spectrum of a mixture of 1.0×10^{-2}M UC781 and βCD.
Annotated cross-peaks indicate intermolecular interactions between NH of UC781 and OH2, OH3 of βCD.

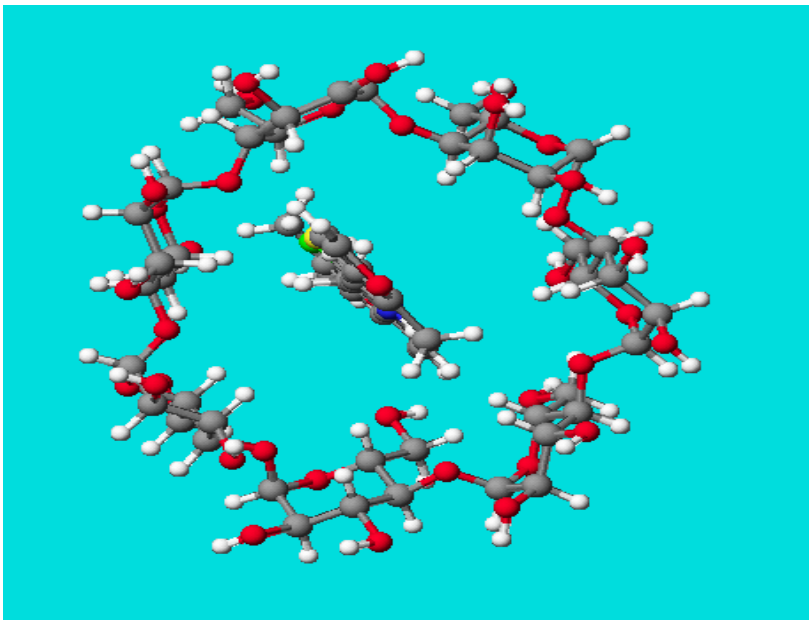


Figure 2-16. Molecular simulation of UC781:βCD complex created using CACHE docking software.

Due to random substitution of the βCD derivatives MβCD and HPβCD, NMR studies were conducted for the UC781: βCD complex only. Given the extremely low solubility of UC781 in

D_2O and the same for βCD in deuterated organic solvent, high-quality NMR spectra would not be possible using D_2O or other deuterated co-solvent. Therefore the drug and βCD were dissolved in DMSO-d6, as both βCD and UC781 have a high degree of solubility in this solvent. In addition, the relative magnitude of the dielectric constants of DMSO and H_2O (ε=46.8 and 80, respectively) are similar; therefore essential interactions of UC781 with βCD in DMSO should be similar to those in water (Matsui et al.,1994; Miyake et al.,1999). Combinding results from 2D ROESY, solubility, UV, IR spectroscopic, and DSC, it is showing that the benzyl ring of UC781 is included into the βCD cavity while the NH group of the drug interacts with OH_2 and OH_3 of βCD on the primary side as shown in Figure 2-16. This suggests that the molecule of UC781 enters cavity of beta-cyclodextrin when the inclusion complex is formed, hence demonstrating that UC781 has a preferred fixed orientation within the cyclodextrin cavity.

2.3.6 HIV Reverse Transcriptase (RT) Inhibition Analysis of UC781: HPβCD Complex

An *in vitro* HIV reverse transcriptase inhibition assay was used to study the anti-HIV activity of the complexed form of UC781 with HPβCD compared to the non-complexed form of UC781. These studies were conducted only for the UC781:HPβCD complex based on the cell toxicity observed for βCD and MβCD (Chapter 4 and 5). In these studies, the UC781 concentration in the complex was varied (1.5, 3.0, and 6.0μg/ml). No RT inhibition activity was observed for either the control (phosphate buffered saline) or cyclodextrin alone (2% HPβCD).

Figure 2-17 shows that the inhibition of RT by the UC781: HPβCD complex is dose-dependent. Currently, a UC781-containing gel product (50 μg/ml of UC781 dispersed in an aqueous based gel matrix) developed by Biosyn, Inc is being evaluated as a microbicide product

in Phase 1 clinical trials overseen by CONRAD. We used this gel to compare inhibitory activity with three different UC781:HPβCD complexes (containing 1.5, 3.0 or 6.0 μg/ml UC781). The half maximal inhibitory potency (IC_{50}) for each was calculated. As shown in Figure 2-18**,** the inhibitory activity of each of the UC781:HPβCD complexes was substantially better (up to 10-fold) than that of the gel formulated drug ($p<0.01$). We hypothesize that this enhanced bioactivity is due to the increase in aqueous solubility of the UC781:HPβCD complexes as compared to that of the non-complexed UC781 in the aqueous based gel matrix.

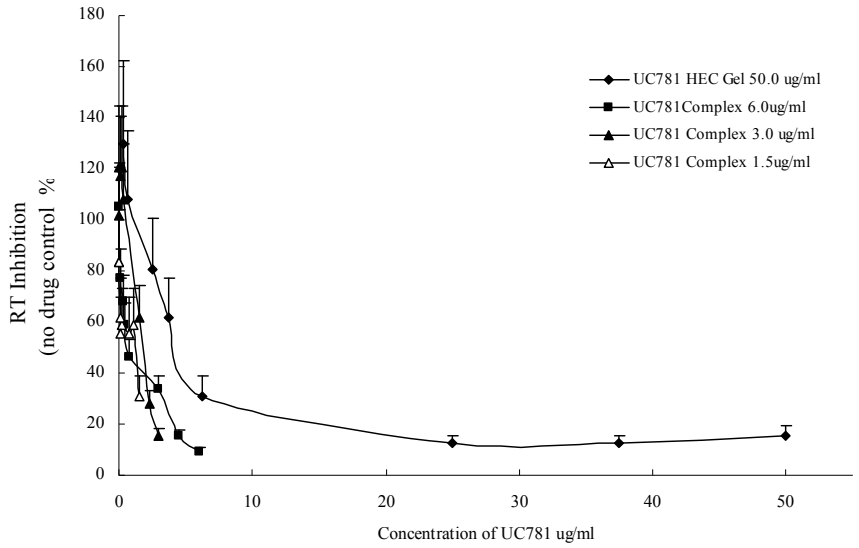


Figure 2-17. The reverse transcriptase inhibition activity of non-complexed UC781 gel *vs.* UC781: HPβCD complex.

UC781 HEC gel 50.0 μg/ml (♦); UC781 complex 6.0 μg/ml (■); UC781 complex 3.0 μg/ml (▲); UC781 complex 1.5 μg/ml (Δ)

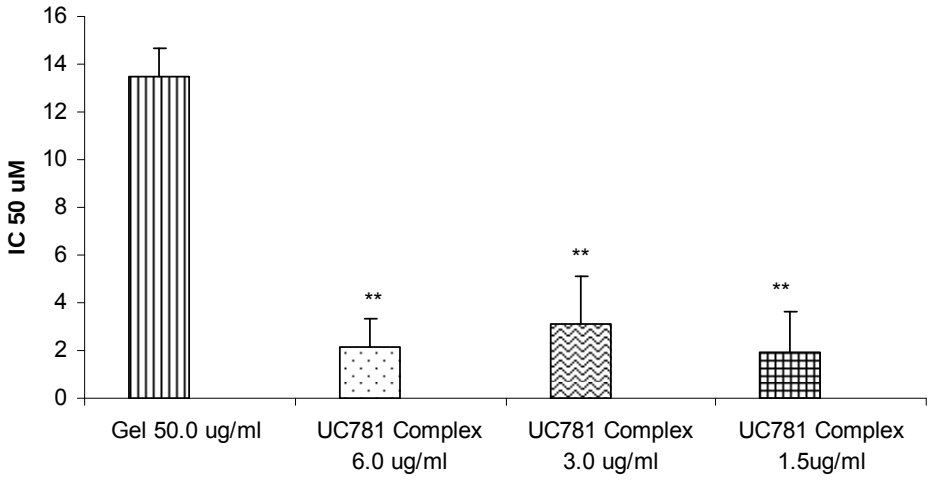


Figure 2-18. Calculated IC_{50} for UC781 in formulations and complex.

Non-complexed UC781 Gel formulation (50.0 μg/ml UC781) ( ); UC781:HPβCD complex (6.0 μg/ml) ( ); UC781:HPβCD complex (3.0 μg/ml) ( ); UC781:HPβCD complex (1.5 μg/ml) ( ). ** $p<0.01$ compared to Gel formulation of non-complexed UC781

Ultimately, it is important to demonstrate that the complex of UC781 still maintains the bioactivity of UC781. For this reason, a series of *in vitro* HIV reverse transcriptase inhibition studies were conducted. These studies showed that the complexed UC781 can greatly enhance the RT inhibition of UC781 as compared with that of non-complexed UC781. This enhanced bioactivity is most probably associated with the enhanced water solubility of UC781 obtained by the complexed form.

2.4 CONCLUSIONS

This study shows that UC781 can form inclusion complexes with various cyclodextrins including βCD, HPβCD, and MβCD, and that these complexes provide substantial increases in

the aqueous solubility of the virtually water-insoluble drug. Phase solubility and 2D ^{1}H NMR data suggest that the UC781: cyclodextrin interaction involves stable 1:1 stoichiometric complexes of the cyclodextrin and UC781. Importantly, the inhibitory potency of the cyclodextrin complexed form of UC781 was greatly increased in comparison to that of the non-complexed form in the aqueous based gel formulation currently being evaluated in Phase 1 clinical trials.

Our data suggest that the cyclodextrin complexation has the potential to greatly reduce the amount of UC781 required in a formulated biologically active microbicide product without significant loss of biological activity, leading to reduced cost per dose of the microbicide product. Furthermore, the dramatic increases in aqueous solubility of UC781 afforded by the complexation approach will enable new formulation technologies to be tested in the development of new more effective anti-HIV microbicide products.

3.0 THERMODYNAMIC PROPERTIES OF UC781: βCD COMPLEXES

3.1 INTRODUCTION

3.1.1 Determination of Thermodynamic Properties of UC781:Cyclodextrins Complexes Using HPLC

A β-cyclodextrin based drug delivery system was developed to enhance the aqueous solubility of UC781 was described in Chapter 2. We confirmed and characterized the formation of complexation of UC781 with βCD and its derivates. There are two questions which need to be addressed for the further development of this βCD based formulation: which cyclodextrin is the most efficient agent for the complexation of UC781? And, How can we maximize the solubility of UC781 using lower concentrations of cyclodextrin?

The complexation of UC781 with βCD must be further investigated before we can answer the questions above. As mentioned previously in Chapter 2, the main driving force for the complex formation is the replacement of water molecules in the cavity of CDs with drugs or guest molecules. This replacement process can be evaluated through thermodynamic parameters. Thermodynamic study of UC781: cyclodextrin complexation provides information needed to understand the behavior of complexation in the aqueous phase, optimize complexation

conditions, and choose the cyclodextrin with the best complexation efficiency. Furthermore, the understanding of the thermodynamic properties and mechanism of UC781:cyclodextrin complexation is a the key factors for the complexation processing during formulation, from bench scale to scaled-up manufacture.

The thermodynamic behavior of the βCD complexation with flavonoids using chromatographic methods had been previously reported. These studies used thin layer chromatography (Guo et al.,2004). The use of HPLC has been reported to study the lipophilicity of drugs directly (Cimpan et al.,2000; Liu et al.,2004; Musilek et al.,2008). Chapter 3 describes a newly developed high performance liquid chromatography (HPLC) method for the study of the thermodynamic properties of the complexation of UC781 with βCD and its derivatives. The primary advantage of the HPLC method is that it requires only small quantities of both drug and CDs. The secondary advantage is that thermodynamic assessment can be rapidly acquired. The complexation constants of UC781 with CDs were determined for comparison. The effect of βCD and its derivatives on lipophilicity change of UC781 was also investigated. These results facilitate comparison of the complexation ability of different CDs with UC781. The thermodynamic parameters of the complexation process were calculated and enthalpy-entropy (ΔH-ΔS) relationships were plotted. The results obtained for these thermodynamic studies provide a deeper understanding of the complexation of UC781 with βCD and its derivatives and offer information for the formulation development of βCD based drug delivery system for UC781.

3.1.2 HPLC Theory for The Complexation Study

High-performance liquid chromatography (or High Pressure Liquid Chromatography, HPLC) is a separation technique using column chromatography to separate, identify, and quantify compounds. Retention time of drugs in HPLC varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. In a given HPLC system, retention time (t_R) can be thought of as the character of the drug itself, reflecting the interaction between drug and the stationary phase. Retention time changes reflect differences in how the drug interacts with the stationary phase.

In addition to t_R, another important parameter used in this study is "capacity factor" (K_c). Historically, a slightly different retention parameter, K_c was introduced by the analogy based on the liquid partitioning theory and is widely accepted in chromatographic practice. K_c is dimensionless and independent of any geometrical parameters of the column or HPLC system. It can be considered a thermodynamic characteristic of the solid phase-compound-mobile phase system (Kazakevitch and Eltekov,1988). K_c is defined by Equation (3-1) (Swadesh,2000)

$$K_c = \frac{M_s}{M_m} = \frac{t_{drug} - t_m}{t_m} \qquad \textbf{3-1}$$

Where *s* is the solid phase and *m* is the mobile phase; M_s is the mass of analyte in the solid phase, M_m is the mass of analyte in the mobile phase; t is the retention time of drug (t_{drug}) and mobile phase (t_m).

In a given mobile phase, the following equilibrium can be reached between drug and solid phase. The mass balance can be expressed as Equation (3-2)

$$[\text{Drug}]+[\text{S}] \Leftrightarrow [\text{Drug-S}]$$

$$K_1 = \frac{[S - Drug]}{[Drug][S]} \qquad \textbf{3-2}$$

Where S is the solid phase of column; K_1 is the binding constant for drug and solid phase. When cyclodextrin is added into the mobile phase, another equilibrium can happen between drug and CD, expressed as Equation (3-3)

$$[\text{Drug}] + [\text{CD}] \Leftrightarrow [\text{Drug-CD}]$$

$$K_{1:1} = \frac{[CD - Drug]}{[Drug][CD]} \qquad \textbf{3-3}$$

$K_{1:1}$ is the complexation constant of drug and cyclodextrin; the total drug in the HPLC system can be given by Equation (3-4)

$$[\text{Drug}]_{\text{Total}} = [\text{Drug-S}] + [\text{Drug}]_m + [\text{Drug-CD}] \qquad \textbf{3-4}$$

Combining equation (3-4) and (3-1) obtains Equation (3-5) and (3-6)

$$\frac{M_s}{M_m} = \frac{[\text{Drug - S}]}{[\text{Drug}]_m + [\text{Drug - CD}]} = K_c = \frac{t_{drug} - t_m}{t_m} \qquad \textbf{3-5}$$

$$\frac{[\text{Drug}] \cdot [\text{S}] \cdot K_1}{[\text{Drug}]_m + [\text{Drug}] \cdot [\text{CD}] \cdot K_{1:1}} = K_c \qquad \textbf{3-6}$$

The drug concentration and retention time are now correlated with each other through K_c. Re-arranging the expression gives an Equation 3-7 and 3-8

$$\frac{1}{K_c} = \frac{t_m}{t_{drug} - t_m} = \frac{1 + K_{1:1} \cdot [CD]}{[S] \cdot K_1} \qquad \textbf{3-7}$$

$$\frac{t_m}{t_{drug} - t_m} = \frac{K_{11}}{[S] \cdot K_1} \cdot [CD] + \frac{1}{[S] \cdot K_1} \qquad \textbf{3-8}$$

Thus, plotting $\frac{t_m}{t_{drug} - t_m}$ *vs.* [CD] will results in a linear relationship between the retention time of Drug ($\frac{t_m}{t_{drug} - t_m}$) and βCD concentration ([CD]) where the slope $= \frac{K_{1:1}}{[S] \cdot K_1}$ and intercept $= \frac{1}{[S] \cdot K_1}$. $K_{1:1}$ under the experimental conditions can be calculated using Equation (3-9)

$$K_{1:1}=\text{slope/intercept} = \frac{K_{1:1}/([S] \cdot K_1)}{1/([S] \cdot K_1)} \qquad 3\text{-}9$$

3.2 MATERIALS AND METHODS

3.2.1 Materials

UC781 was provided by Biosyn Co. Ltd. (Huntington, PA). βCD (MW: 1134), MβCD (MW: Approx. 1320; Mean degree of substitution: 1.7-1.9) were purchased from Spectrum Chemical Company (Gardena, CA); HPβCD (MW: Approx. 1380 and Mean degree of substitution: 0.8) was obtained from Sigma Aldrich (St Louis, MO). All other reagents used were of reagent grade and all solvents were of HPLC grade. Milli-Q water was used to prepare buffer solutions and other aqueous solutions.

3.2.2 Methods

HPLC analysis for UC781 was conducted using a Waters HPLC system equipped with an auto injector model Waters 717, and a Waters 2487 dual wavelength (λ) absorbance detector at 300 nm (Waters Corporation, Milford, MA). Separation of the compound in the experiment was achieved by using an Alltech ODS-C8 column (4.6 mm i.d ×7.5 mm, 5μm, Columbia, MD). A mobile phase of acetonitrile (ACN) / Milli-Q water (30:70 v/v) containing different CD concentrations at a flow rate of 1.0 ml/min was used. The column temperature was controlled using a water bath with a range of ± 0.1 ^{o}C. UC781 was dissolved in ACN at a concentration of 3 μg/ml. A10 μl aliquot of UC781 ACN solution was injected for the thermodynamic study. The retention time of UC781 was recorded for calculation of the thermodynamic parameters calculation.

3.3 RESULTS AND DISCUSSION

3.3.1 Complexation Behavior of Cyclodextrins with UC781

Effect of cyclodextrins concentration on the retention time values for UC781 is shown in Table 3-1. A measurable decrease in UC781 retention time was observed with increasing cyclodextrins concentration in mobile phase. This result showed that the interaction between UC781 and the solid phase of the HPLC column was interfered with due to the complexation of UC781 with all three cyclodextrins in mobile phase. In addition, the retention time of UC781 decreased more quickly in MβCD containing mobile phase than in HPβCD containing mobile

phase at the same cyclodextrins concentration. This result showed a stronger complexation ability of MβCD with UC781 than that of HPβCD. It has been shown that both HPβCD and MβCD exhibit better solubility properties than native βCD in mobile phase. The effect of βCD on retention time was not compared with MβCD and HPβCD due to its limited solubility in mobile phase.

Table 3-1. Effect of CDs on the retention time of UC781 in HPLC

	Concentration %	**0.5**	**1**	**2**	**4**	**5**
MβCD	t_R (min)	34.18	31.36	26.47	19.81	17.65
HPβCD		32.89	31.49	30.62	28.52	27.25
	Concentration %	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
βCD	t_R (min)	36.99	34.67	33.68	31.84	32.20

3.3.2 Effect of Cyclodextrins on The Lipophilicity of UC781

In this chapter, a HPLC method for the determination of partition coefficient was used to evaluate the impact of different cyclodextrins on the lipophilicity of UC781.

The partition coefficient is a parameter that is used to describe the degree of lipophilicity of a compound typically expressed as log P. Specifically it is experimentally determined by evaluating the distribution of a compound between two immiscible phases (such as octanol and water). Drug molecules with larger log P values are more lipophilic and tend to be less able to be dissolved into water. This parameter is useful in estimating distribution and permeability of the drug within the body (Alhaique et al.,1977; Cross et al.,2003; Kretsos et al.,2004). However, the traditional shake-flask method (Cripe et al.,1987; Unger et al.,1978), does not provide a rapid determination of lipophilicity. In addition, this method requires a large amount of compound when the compound is very lipophilic. A HPLC method was used to rapidly determine log P

from K_w (Ayouni et al.,2005; Musilek et al.,2008). K_w is the capacity factor when the mobile phase is pure water. It has been demonstrated that the extrapolated logarithm of the capacity factor for a pure aqueous eluent (log k_w) gives the best correlation with log P (Liu et al.,2005b). Moreover, logarithm of the capacity factor (log K_c) and log K_w can be expressed through equation (3-10)

$$\log K_c = A \cdot [\mathrm{C}] + \log K_w \quad \textbf{3-10}$$

Where A is the slope, C is the volume fraction of organic solvent in mobile phase and log K_w the intercept of the regression curve. In these studies, the CD concentration change was used instead of the change in organic solvent for the calculation of log K_w since it was desired to determine the impact of CD on lipophilicity change of UC781. The log K_w is a fixed value obtained from equation (3-10) in these studies. Therefore, the slope values obtained from linear regression can be compared to evaluate the complexation ability of the three cyclodextrins with UC781.

Figure 3-1 represents the logarithm of the K_c of UC781 as a function of CD concentration in the mobile phase. In the presence of cyclodextrins in the mobile phase, log K_c of UC781 decreased with increasing CD concentration. Linear relationships between log K_c and CD concentration in the mobile phase were observed, indicating that a classical reverse phase elution mechanism is operating (Clarot et al.,2000; Horvath et al.,1976). The absolute value of slopes (Table 3-2) obtained in the linear regression treatment followed an order of MβCD > βCD >HPβCD, which also corresponds with the retention time changes shown in Table 3-1. This phenomenon can be explained by the alteration of hydrophobic interaction between UC781 and the solid phase in the HPLC column, which is induced by the complexation of UC781 with CD

in the mobile phase. The results also indicate that MβCD has a better ability to form a complex with UC781 under these experimental conditions.

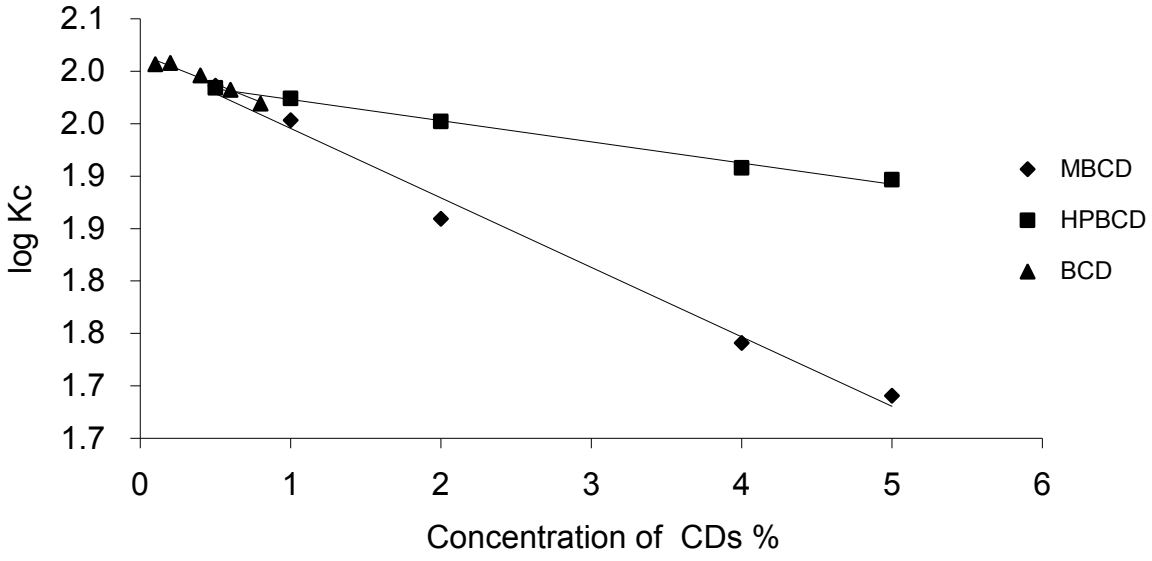


Figure 3-1. Influence of CDs on the lipophilicity of UC781 at 30°C

Table 3-2. Slopes, logK_w, and correlation coefficients (r^2) obtained for linear regression treatment of logK_c for different CDs in the mobile phase.

	slope	log K_w	r^2
βCD	-0.066	2.01	0.96
MβCD	-0.057	2.01	0.99
HPβCD	-0.020	1.99	0.99

3.3.3 Effect of Temperature on The Complexation of UC781 with Cyclodextrins

The effect of temperature on the inclusion constants obtained for UC781 with different CDs is shown in Figure 3-2. The Gibbs free energy (ΔG) values were calculated from the inclusion constants Equation (3-11) and are shown in Figure 3-3.

$$\Delta G = -RT \ln K_{1:1} \qquad \textbf{3-11}$$

Where R is the gas constant, and T is the temperature in Kelvin; $K_{1:1}$ is the complexation constant of UC781 with βCD. $K_{1:1}$ and absolute ΔG value decreased with increasing temperature. The results indicate that higher temperatures are unfavorable for the inclusion process. This can be explained by the increased movement of molecules at high temperatures, which leads to disassociation.

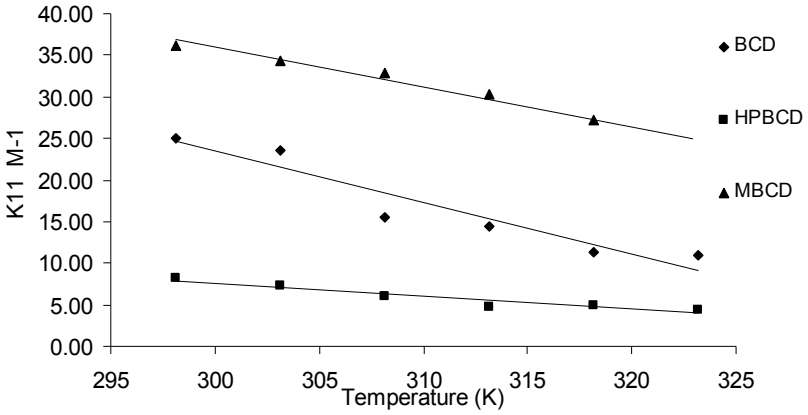


Figure 3-2. $K_{1:1}$ change of UC781 with CDs in HPLC at different temperature

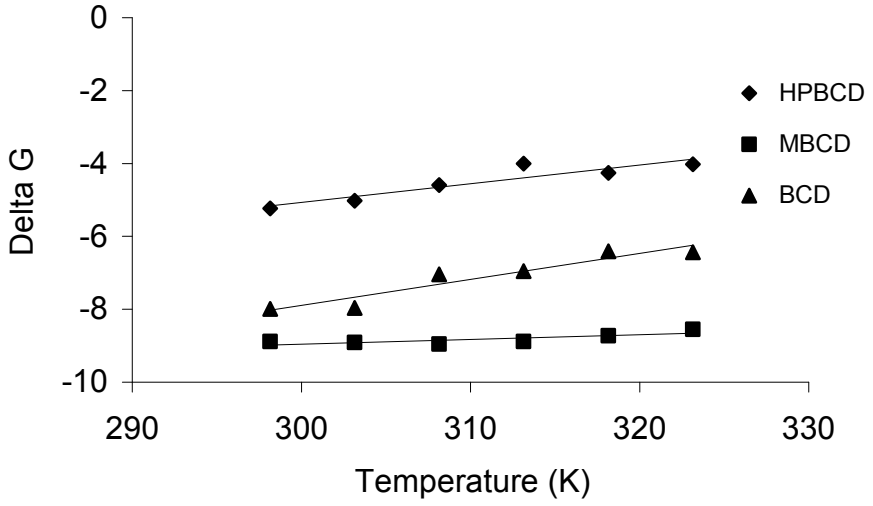


Figure 3-3. Temperature *vs.* Gibbs free energy change on the complexation process of UC781 with three cyclodextrins

All ΔG values obtained for inclusion processes as shown in Figure 3-3 are negative. This suggests that the inclusion process proceeds spontaneously. From ΔG values, other thermodynamic parameters can be obtained using the Van't Hoff Equation –(Equation (3-12)). Enthalpy (ΔH) and entropy (ΔS) changes for complexes can be calculated using this equation:

$$\frac{\Delta G}{T} = \Delta H \frac{1}{\mathrm{T}} - \Delta S \qquad \textbf{3-12}$$

From Equation (3-12), the slope and the intercept of the plot of ΔG/T vs. 1/T are −ΔS and ΔH, respectively. Figure 3-4 shows the plot of ΔG/T vs. 1/T for the three CDs studied. The ΔH and ΔS values obtained graphically are shown in Table 3-3. Negative values for ΔH mean that the inclusion process is exothermic; therefore, low temperatures are more suitable for the inclusion process. The absolute value of ΔH is much greater than ΔS, suggesting that the inclusion process of UC781 with CD is primarily an enthalpy driven process.

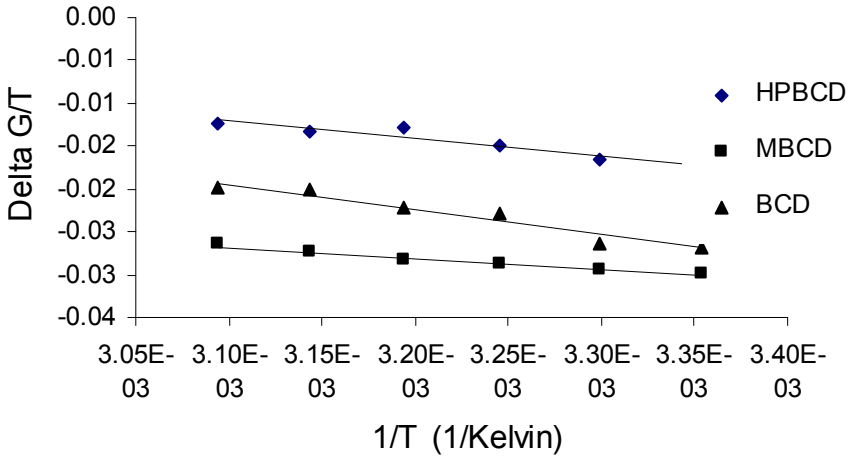


Figure 3-4. Van't Hoff equation for UC781 and CDs complex study

Table 3-3. Enthalpy-Entropy change of UC781 complex with CDs.

	ΔH (J/mol)	ΔS ($J \cdot mol^{-1} \cdot K^{-1}$)
βCD	-29.42	-0.072
MβCD	-11.02	-0.013
HPβCD	-20.64	-0.052

The enthalpy-entropy compensation relationship has been observed in these host-guest combinations for βCD complex (Connors,1997). Enthalpy-entropy compensation is the phenomenon in which the change in enthalpy is offset by a corresponding change in entropy resulting in a smaller net free energy change, and it is believed to play an important role in the reactions in solution (Inoue et al.,1993a; Inoue et al.,1993b). The Enthalpy-entropy compensation is also attributed to the interactions with water molecules in the βCD cavity (Lemieux et al.,2007). In this study, ΔH is plotted against ΔS to give a straight line (Figure 3-5). The slope is the compensation temperature, T=304 K. It demonstrates that the inclusion process also displays an enthalpy-entropy compensation effect (Luscher-Mattli,1982).

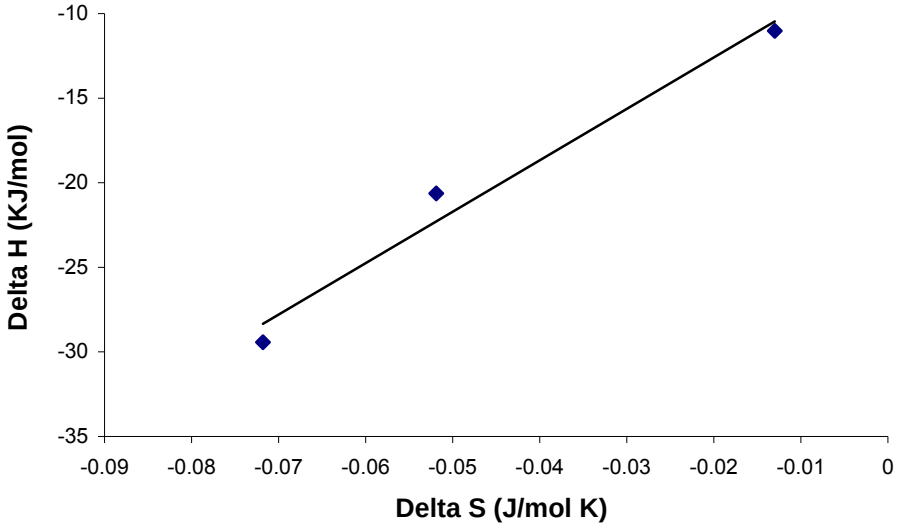


Figure 3-5. Enthalpy-entropy plots of UC781-CD inclusion complexes

3.4 CONCLUSIONS

The complexation interaction between UC781 and three different cyclodextrins was investigated systematically using HPLC method. This method provides a rapid evaluation of the thermodynamics of inclusion and requires minimal drug substance. The inclusion ability and hydrophilicity enhancement effect of cyclodextrins on UC781 were found to follow the rank order of MβCD > βCD > HPβCD. The thermodynamic behavior of UC781 and all three cyclodextrins studied were shown to be enthalpy driven processes. Enthalpy-entropy compensation was also observed for complexation of UC781 with cyclodextrins indicating a replacement of water molecules in cyclodextrin cavities with UC781. These results provide a better understanding of the inclusion process of UC781 with cyclodextrins. Additionally, the developed methods provide a novel means for evaluation of other hydrophobic drug candidates for their complexation ability with cyclodextrins.

4.0 EFFECT OF pH, POLYMERS, AND PREPARATION METHOD ON THE COMPLEXATION OF UC781

4.1 INTRODUCTION

As mentioned in Chapter 2 and Chapter 3, UC781 is a very hydrophobic compound belonging to Class II of the Biopharmaceutical Classification System (Deferme et al.,2002). Its intrinsic solubility is lower than 30 ng/ml. No aqueous parenteral formulation for UC781 is currently available for clinical use. Therefore, we are trying to develop a βCD based drug delivery system to enhance the solubility of UC781.

Cyclodextrins (CDs) are widely used in the pharmaceutical industry for drug complexation. Complexation with CDs is reported to enhance drug aqueous solubility (Loftsson and Brewster,1996), permeability (Sigurðoardóttir and Loftsson,1995) , and stability (Kang et al.,2002). Cyclodextrin containing dosage forms may also increase drug concentration in plasma, increase bioavailability, and create formulations that offer more effective and less frequent treatment schedules for hydrophobic molecules.

Although CDs can provide many benefits in the formulation development, there are some practical limitations on the application of CDs in formulations. For example, in solid oral dosage froms, the total amount of CD in each dosage cannot be over 600 mg. Generally, the weight of an oral solid dosage form is considered not to exceed 800 mg (Loftsson and O'Fee,2003).

Therefore, if a drug: βCD complex has only a very low complexation constant, more βCD is needed to form a complex with the drug than in the case of a high complexation constant in the formulation. It will be very hard to design a formulation with a large dosage form with drug:βCD complex due to the mass limitations of traditional oral dosage.

For vaginal formulations, mass limitation is more critical than in oral dosage due to the limitation of the vaginal cavity size and amount of bioliquid present. Too much cyclodextrin will not only cause difficulty in formulation design, but will also lead to toxicity and damage to the vaginal epithelium by depletion of cholesterol from cell membrane. Therefore, enhancement of the complexation capacity of the chosen CDs to reduce the amount of CDs in formulation is of practical importance.

The objective of these studies reported in this chapter is to optimize the complexation process and obtain the most efficient complexation condition for further formulation development. UC781: βCD complexes were developed and evaluated in Chapter 2 and Chapter 3. In this study, βCD and its derivatives (HPβCD and MβCD) were complexed with UC781 to increase the aqueous solubility of UC781. pH variation, complexation methods used, and incorporation water-soluble polymers on the complexation efficiency were investigated to optimize the complexation of UC781 with cyclodextrin. The *in vitro* RT inhibition activity of UC781: HPβCD and UC781: MβCD complexes was also determined to establish that formed complexes maintained bioactivity.

4.2 MATERIALS AND METHODS

4.2.1 Materials

UC781 was provided by Biosyn Co. Ltd. (Huntington, PA). βCD (MW:1134), ,MβCD(MW: 1320; Mean degree of substitution: 1.7-1.9) , HPβCD (MW: 1380 and mean degree of substitution: 0.8), hydroxypropyl methylcellulose (HPMC) (Mn: 10,000; viscosity: 6,000 mPa.s 2 % w/w in water), hydroxyethyl cellulose (HEC) (1,500 mPa.s; 5% w/w in water), polyvinyl alcohol (PVA) (MW: 9,000-10,000, 80% hydrolyzed), polyvinylpyrrolidone K30 (PVP K30) (MW: ~40,000), and PVP K90 (MW: ~360000) were purchased from Sigma Aldrich (St. Louis, MO). 2 ml autosampler screw-thread glass vials were obtained from FisherScientific Inc. (Waltham, MA). All other reagents used were of reagent grade and all solvents were of HPLC grade. Milli-Q water was used to prepare buffer solutions and other aqueous solutions.

4.2.2 Methods

4.2.2.1 High Performance Liquid Chromatography (HPLC) Analysis for UC781

HPLC analysis for UC781 was conducted as follows: A Waters HPLC system equipped with an auto injector model Waters 717 and a Waters 2487 dual wavelength (λ) absorbance detector at 275 nm was used (Waters Corporation, Milford, MA). Separation of the compound in experiment was achieved by using an Alltech ODS-C18 column (4.6 mm i.d ×250 mm, 5μm, Columbia, MD). A mobile phase of acetonitrile/ Milli-Q water (75:25 v/v) at a flow rate of 1.0 ml/min was used.

4.2.2.2 Methods for UC781: Cyclodextrins Complexation

Most cyclodextrin complexes are formed in the presence of water. Through repulsive interactions, water provides the main driving force for complexation. A secondary role for water is that it serves as a medium for dissolution of both the cyclodextrin and the drug molecule. A number of methods have been used for complex formation. These include coprecipitation, slurry or paste formation (ex. kneading), and dry mixing methods (ex. spray drying or lyophilization) (Hedges,1998). A heating method (autoclaving) has also been used for complex formation. This method offers an enthalpy driven process of complexation (Hassan et al.,1990). It has been previously shown that the choice of complexation method can impact complexation efficiency (Moyano et al.,1995). For this reason, it is important to evaluate several methods for complexation optimization.

In this chapter, four specific complexation methods were evaluated: kneading, shaking, autoclaving, and lyophilization. In the kneading method, 300 μg UC781 was mixed with 15 mg βCD, or 150mg HPβCD or 150 mg MβCD in an agate mortar. 50 μl ethanol was added as a wetting agent to form a slurry. The UC781 and βCD paste was then ground by pestle. 1 ml of Milli-Q water was added drop by drop while grinding until cyclodextrin was totally dissolved. The resulting solution was filtered with a 0.45 μm filter for HPLC analysis.

In the shaking method, 300 μg UC781 was mixed with 15 mg βCD, or 150 mg HPβCD or 150 mg MβCD in a 2 ml glass vial. 1 ml Milli-Q water was added with 50 μl ethanol as a wetting agent. All vials were shaken for 72 h at room temperature on a vertical shaker at a speed of 50 rpm. Solutions were then filtered with a 0.45 μm nylon filter for HPLC analysis.

In the autoclave method 300 μg UC781 was mixed with 15 mg βCD, or 150 mg HPβCD or 150 mg MβCD in a 2 ml glass vial. 1 ml Milli-Q water was added with 50 μl ethanol as a

wetting agent. All vials were autoclaved at 250 °F for 15 minutes followed by shaking for 24 h on a vertical shaker at a speed of 50 rpm. The solution was then filtered with a 0.45 μm nylon filter for HPLC analysis.

The lyophilization method involved mixing 300 μg UC781 with 15 mg βCD, or 150 mg HPβCD or 150 mg MβCD in a 2 ml glass vial. 1 ml $NH_3 \cdot H_2O$ was then added into the vial to dissolve cyclodextrin and 500 μl ethanol was added to dissolve the UC781. The resulting solution was then frozen at -80 °C for 6 h until totally frozen. All vials were lyophilized at -51 °C using a Labconco Freezone 6 lyophilizer (Kansas City, Missouri) for 48 h until all moisture was removed. Lyophilized powder was re-constituted into 1ml Milli-Q water. The solution was then filtered with a 0.45 μm nylon filter for HPLC analysis. All samples were prepared in triplicate. Data were expressed as mean ± SD.

4.2.2.3 Phase Solubility Studies of UC781 with Cyclodextrins

300 μg UC781 was added to sealed HPLC glass vials containing 1.0 ml of 10mM buffer (PBS for pH 7.0, boric acid buffer for pH 9.0, and PBS for pH 11.0) or distilled water with various concentrations of βCD, MβCD, and HPβCD (from 0 to 0.11 M). All vials were autoclaved for 15 min at 250 °F. The vials were then shaken on a horizontal rotary shaker at a speed of 50 rpm at ambient temperature (25 °C) for 24 h avoiding light. The solutions were filtered through a 0.45 μm filter for HPLC assay. Given that high pH systems are used in this set of experiments, it was necessary to replace the water portion of the mobile phase with 5.0 mM PBS buffer (pH 7.0) HPLC assay to keep the pH of the mobile phase stable. All samples were prepared in triplicate.

The complexation constant ($K_{1:1}$), according to the hypothesis of 1:1 stoichiometric ratio of complexation, was calculated from phase-solubility diagrams (Higuchi and Connors,1965) using the following Equation. (4-1):

$$K_{1:1} = \frac{\text{slope}}{S_0(1 - \text{slope})} \qquad \textbf{4-1}$$

In this equation $K_{1:1}$ is the complexation constant, S_o is the intrinsic solubility, and the slope is calculated from a graph of the dissolved drug concentration versus CD concentration in the medium. The intrinsic solubility value (S_o) of UC781 in the absence of CD was determined directly in aqueous media. Data were expressed as mean ± SD.

4.2.2.4 Polymer Effects on UC781 Solubility (in HPβCD and MβCD Solutions)

300μg UC781 were added into 1ml of 15% HPβCD and 15% MβCD solution with various concentrations of HPMC, HEC, PVA, PVP K30 or PVP K90 (from 0 to 1.0 %). 50 μl ethanol was used as a wetting agent. All vials were autoclaved at 121 ^{o}C (250 °F) for 15 min followed by shaking for 24 h at room temperature on a vertical shaker at a speed of 50 rpm. The solution was then filtered with a 0.45 μm filter for HPLC analysis. All samples were prepared in triplicate.

4.2.2.5 HIV-1 RT inhibition Assay for UC781 and Its Complexes in The Liquid State

HIV-1 RT activity was determined using a fixed time assay. HPβCD or MβCD was dissolved in 10 mM pH 7.2 PBS buffer to obtain a 15% w/w solution. To 1 ml of this solution, UC781 was added such that final concentration was either 5 or 10 μg/ml . The solution was then autoclaved at 250 °F for 15 min followed by rotation at 50 rpm overnight at room temperature to

form the UC781: HPβCD or UC781: MβCD complex at 5 or 10 μg/ml. UC781 was dissolved in DMSO and then quickly dispersed into 10 mM PBS buffer served as a positive control. PBS buffer was used as the negative control. Test samples were mixed with reaction solution (50 μl total volume) containing 50 mM Tris-HCl (pH 7.8, 37 ºC), 60 mM KCl, 10 mM $MgCl_2$, 10 UI/ml of either poly(rA)-oligo(dT)12-18 and 20 μM [^{3}H]TTP. Reactions were initiated by the addition of 50 ng of RT. Reaction mixtures were incubated at 37 ºC for 20 min and then quenched with 200 μl of ice-cold 10% trichloroacetic acid (TCA) containing 20 mM sodium pyrophosphate. Quenched samples were left on ice for 20 min, then filtered using 1.2 μM glass fiber Type C filter multi-well plates (Millipore) and with 100% ethanol wash. The extent of radionucleotide incorporation was determined by liquid scintillation spectrometry (Wallace 1450 Microbeta Jet Liquid scintillation, Perkin Elmer, Waltham, MA) of the dried filters. All samples were prepared in triplicate. Data were expressed as mean ± SD for analysis

4.2.2.6 Statistical Analysis

The 50% inhibitory concentration (IC_{50}) analysis was done using nonlinear regression analysis (GraphPad PRISM; GraphPad Software, Inc. La Jolla, CA). Data were expressed as mean ± sd and analyzed with one-way ANOVA using Tukey's multiple comparison.

4.3 RESULTS AND DISCUSSION

4.3.1 UC781 Cyclodextrins Complexes Formation Efficiency is Impacted by Method of Preparation

Our previous studies showed that the complexation of UC781 with CD is an enthalpy driven process. The complexation is more favorable at lower temperature. Therefore, a temperature-controlled method can be used to enhance the complexation of UC781 with CD. In addition, very poor solubility of UC781 greatly limited the interaction of UC781 and βCD molecules in solution. Therefore, the autoclave method was developed to offer a temperature controlled environment to enhance the solubility of UC781 and the interaction of UC781 with βCD in solution. Lyophilization method was also developed to be compared with traditional shaking and kneading methods for complexation optimization.

Autoclave, lyophilization, kneading, and shaking methods of preparation of UC781:βCD complexes with βCD, HPβCD or MβCD were investigated. Figure 4-1 shows that the processing method can greatly affect the aqueous solubility of UC781. An autoclave method was found to be the most efficient for UC781 complex preparation. It can increase UC781 solubility to 5 to10 times higher than other methods tested for βCD, 1 to 6 times for HPβCD, and 1 to 2 times for MβCD. The order of UC781 solubility enhancement obtained using different methods was autoclave > kneading > shaking > lyophilization for 1.5% βCD solution, autoclave > shaking > kneading >lyophilization for 15% HPβCD solution, and autoclave > kneading > lyophilization > shaking for 15% MβCD solution.

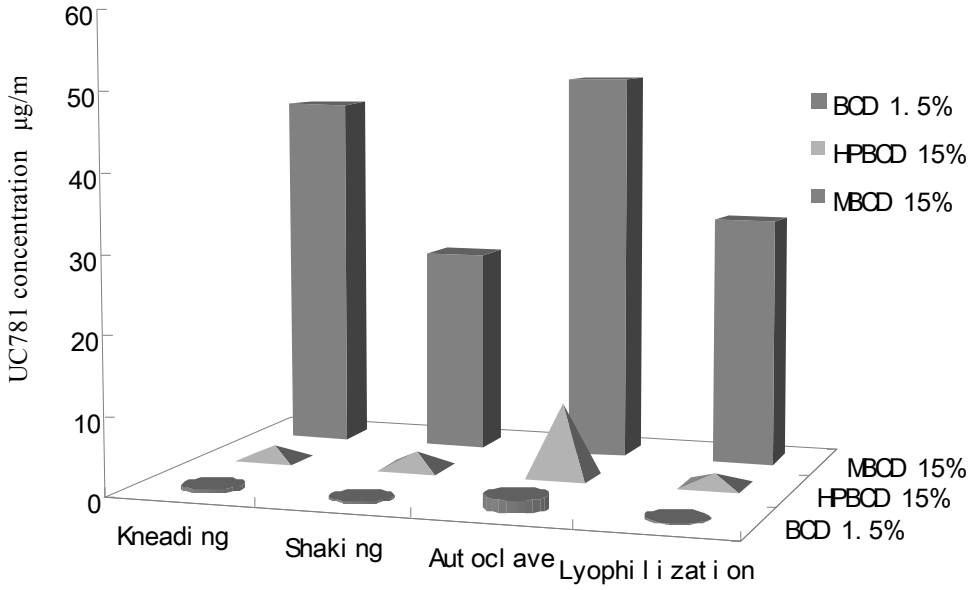


Figure 4-1. Methods of preparation for complexation of UC781 with cyclodextrins

The autoclave method is the most efficient preparation method for the complexation of UC781 with βCD, HPβCD or MβCD. The complexation of UC781 with βCD is an enthalpy-driven process. In the autoclave method, the displacement of water molecules in the cyclodextrin cavity with UC781 is enhanced during the cooling process of complex formation. Additionally, the solubility of UC781 may be increased at higher temperature (121 °C) increasing the interaction between UC781 and βCD molecules. Complexation of UC781 in aqueous media is highly associated with the CD species used in the experiment. In our study, MβCD showed the highest solubility enhancement of UC781 as compared to βCD and HPβCD.

With these studies, an autoclave method for complexation was successfully developed. This method was based on our previous studies on the thermodynamic properties of UC781

complexation with βCD. The autoclave method can dramatically enhance the formation of complexed UC781 with cyclodextrins over other methods tested.

4.3.2 pH Effect on the Complexation of UC781 with Cyclodextrins (Phase-Solubility Studies)

The ionized form of compounds dissolves more easily in aqueous media than non-ionized forms of compounds. Therefore, UC781 solubility may be increased due to an active proton in NH group in the molecular structure. This solubility increase will also enhance the interaction between UC781 and CDs to increase the complexation of UC781 with CDs. The ionization diagram of UC781 at different pH values was simulated with an online software-Marvin calculator as shown in Figure 4-2. The pKa of UC781 is 9.14 ± 0.66. Based on the simulation, we can predict that pH value must be over 9.14 to ionize the majority of UC781 hence enhancing the solubility of UC781.

Studies showed that solubility of UC781 is a function of pH (Figure 4-3). The solubility of UC781 was increased at high pH values. For this reason, studies were conducted to evaluate the impact of pH on the complexation of UC781 with cyclodextrins.

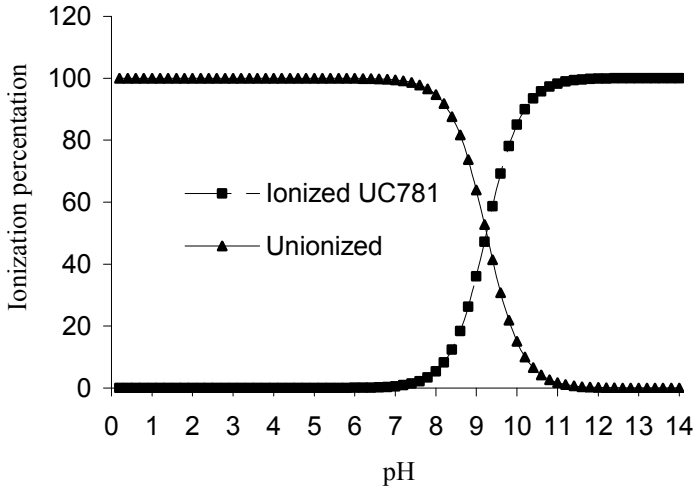


Figure 4-2. Relative proportions of UC781 and ionized UC781as a function of pH
Diamond line (◆) shows the change of non-ionized form of UC781 according to pH change in solution; square line (■) shows the change of ionized form of UC781 according to pH change in solution.

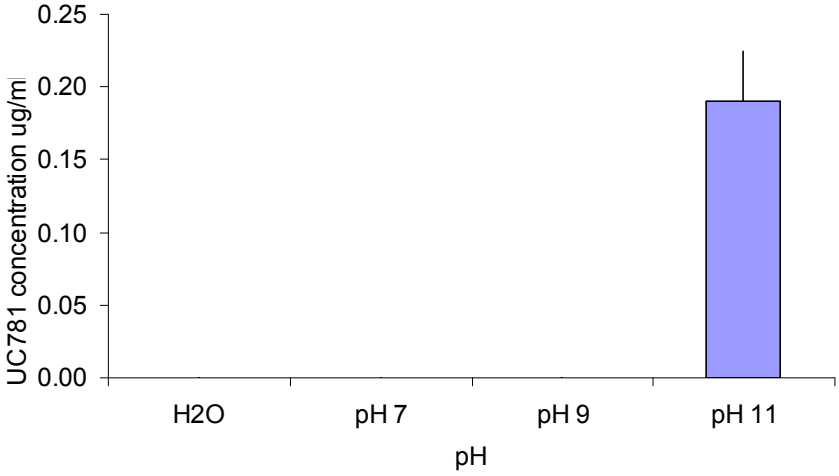


Figure 4-3. pH effect on UC781 solubility in water

The interaction of UC781 with βCD, HPβCD or MβCD in water and at pH 7.0, 9.0 and 11.0 were evaluated using phase-solubility analysis for βCD (Figure 4-4), HPβCD (Figure 4-5)

and MβCD (Figure 4-6). Qualitative assessment of relationships at all pH values studied shows a linear dependence of solubility on cyclodextrin concentration, indicating a linear increase in UC781 solubility behavior for both βCD and HPβCD. A positive curvature line was obtained for MβCD (The phase solubility diagram is described in the Appendix). This result suggests that first order cyclodextrin complexation occurs for βCD and HPβCD. Moreover, a higher order cyclodextrin complexation was observed for UC781: MβCD when high concentrations of MβCD were used in the system. In addition, higher pH values enhanced the complexation of UC781 with βCD by increasing UC781 solubility.

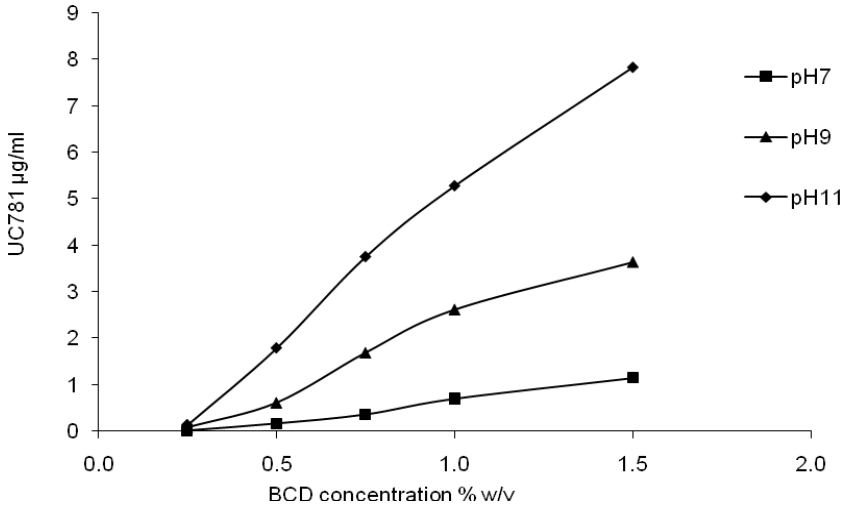


Figure 4-4. Phase solubility of UC781 with BCD at different pH values

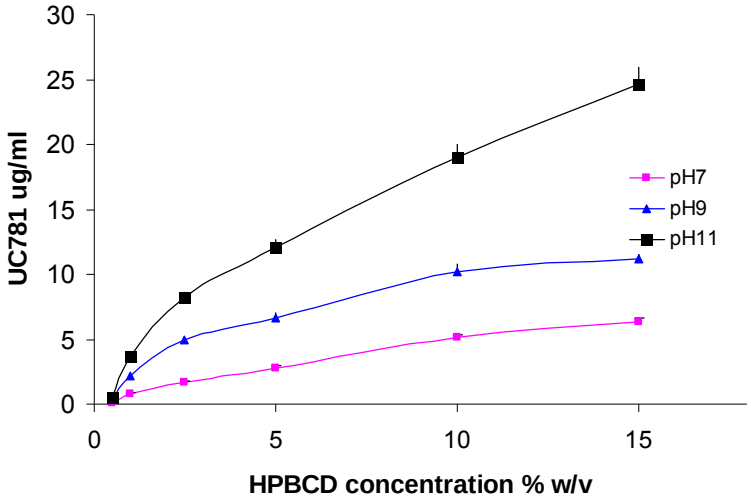


Figure 4-5. Phase solubility of UC781 with HPBCD at different pH values

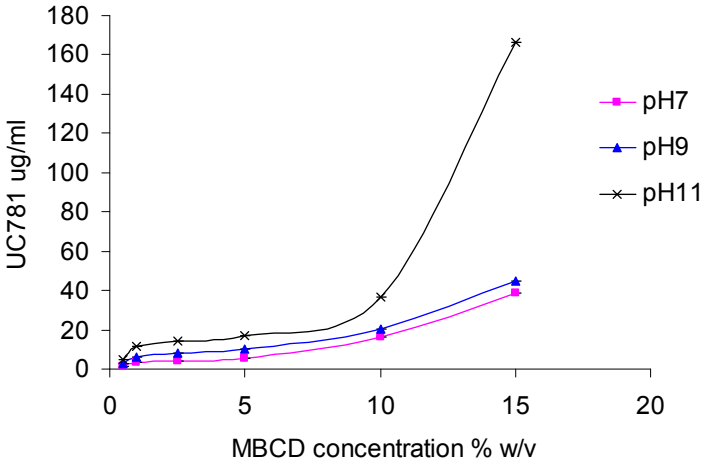


Figure 4-6. Phase solubility of UC781 with MBCD at different pH values

In the absence of cyclodextrin, the solubility of UC781 in water is almost negligible ($<3\times10^{-5}$ mg/ml). In the presence of cyclodextrins in pure water, UC781 solubility was increased over 50 fold with of 1.5% w/v βCD (1.58×10^{-3} mg/ml), nearly 874 fold with of 15% w/v MβCD (2.62×10^{-2} mg/ml), and 316 fold with 15% (w/v) HPβCD (9.48×10^{-3} mg/ml).

UC781 solubility increases with increasing pH, such as at pH 7, 9, and 11, concentrations of 1.14×10^{-3}, 3.63×10^{-3}, and 7.82×10^{-3} μg/ml in 1.5% βCD solution; 6.36×10^{-3},

11.19×10^{-3}, and 24.7×10^{-3} μg/ml in 15% HPβCD solution; 38.67×10^{-3}, 44.97×10^{-3}, and 50.30×10^{-3} μg/ml in 15% MβCD solution, respectively, were obtained.

The inclusion constants ($K_{1:1}$), calculated using Equation 4-1, which is described in the methods section of this Chapter, are reported in Table 4-1.

Table 4-1. Calculated $K_{1:1}$ values for UC781 complexes with CDs at different pH value

	pH 7	pH 9	pH 11
βCD	3388.25 ± 64.51	11364.34 ± 323.02	23499.42 ± 1121.37
HPβCD	2308.26 ± 129.00	5623.39 ± 64.73	11177.92 ± 1118.9
MβCD	4841.51±645.29	7859.42± 64.6	16028.15 ±646.58

For MβCD, inclusion constants were additionally calculated for a 1:2-complex ($K_{1:2}$) using Equation 4-2 (data shown in Table 4-2). This equation was derived by Loftson *et al* (Loftsson et al.,2002).

$$S_t = S_0 + K_{1:1}\cdot S_0 \cdot [CD] + K_{1:1}\cdot K_{1:2}\cdot S_0 [CD]^2 \quad \textbf{4-2}$$

In this equation, S_t and $[CD]_t$ correspond to total UC781 and MβCD concentration respectively; $K_{1:1}$ and $K_{1:2}$ represent the inclusion constants for the 1:1 and 1:2 complexes respectively. The MβCD complex displays a very high $K_{1:1}$ value compared with $K_{1:2}$; this corresponds to the observed significant increase in aqueous solubility in the presence of MβCD. The apparent low value of $K_{1:2}$ as compared with that of $K_{1:1}$ indicates that the 1:1 complex is the predominant form in the solution.

Table 4-2. Calculated $K_{1:1}$ and $K_{1:2}$ values for UC781:MβCD complex

		pH 7	pH 9	pH 11
1:1 ratio	$K_{1:1}$	4841.51±645.29	7859.42± 64.6	16028.15 ±646.58
1:2 ratio	$K_{1:1}$	2605.56 ±644.71	3387± 64.47	5955.56 ±1289.42
	$K_{1:2}$	41±7.09	39.25± 1.3	7.67 ±11.62

In the current study, the enhancement of UC781 solubility is highly dependent on the type of βCD molecule used and pH of the complexation environment shown in Table 4-2. The different inclusion constants found for the different cyclodextrin molecules studied indicate that the derivative groups in the cyclodextrin play an important role in the complexation of UC781 into the CD cavity. Therefore, MβCD and HPβCD appear to be better host molecules for UC781 inclusion. Additionally, due to the extremely low intrinsic solubility of UC781, the $K_{1:1}$ may be overestimated.

UC781 shows weak acidic property in aqueous solution based on the ionizable nitrogen in its structure. In solution, at pH equal to the drug's pKa, protonated forms of UC781 are present in equilibrium with nonprotonated forms. The pKa of UC781 is 9.14 ± 0.66 at ambient conditions. Data for phase solubility at different pH also suggest that the complexation may change as a function of UC781 ionization. At pH 7, the compound is essentially in the unionized form; $K_{1:1}$ is the smallest, exhibiting the lowest stability of the complex. The results from the phase solubility analysis indicate that the total drug solubility progressively increased with increasing pH. As expected, the $K_{1:1}$ values also increased with increasing pH. This can be explained by the progressive increase of the amount of drug in ionized form, which leads to increased solubility of UC781. These data suggested that complexation in a high pH enviroment enhanced the efficiency of UC781:βCD complexation due to the increased degree of ionization of UC781 and the increased solubility of UC781 in aqueous solution at high pH.

4.3.3 Water-soluble Polymers Enhance The Solubility of UC781 in Cyclodextrins Solutions

UC781 solubility was greater at extremely high pH values than lower pH. However, the high pH values required for enhancement are not suitable for vaginal formulations. Therefore, methods other than pH adjustment have to be investigated for enhancement of UC781:CD complexation. Polymers are widely used as excipients in most drug formulations. Therefore, it is worthy of interest to investigate the possible effect of polymer incorporation into the cyclodextrin complexation medium.

HPβCD and MβCD were selected in these experiments for their good water solutility. Effect of HPMC, HEC, PVA, PVP K30 and PVP K90 on UC781's solubility in cyclodextrin solutions were investigated with autoclave method, and are shown in Figure 4-7 for HPβCD and Figure 4-8 for MβCD. Quantitative measurement of UC781 concentration in cyclodextrin solutions was performed by HPLC. In the presence of polymers, the solubility of UC781 increased for both HPβCD and MβCD solutions (15%), especially in the presence of HPMC. Incorporation of HPMC resulted in greater solubility enhancement of UC781 for both HPβCD and MβCD over the other three polymers. The solubility enhancement effect on complexed UC781 is dependent on the presence of different polymers. A significant increase of HPβCD complexed UC781 solubility was obtained to a value of 33.71 μg/ml in the presence of 0.8 % HPMC, 15.8 μg/ml for 0.6%PVP K-30, 24.10 μg/ml for 1% PVP K-90 and 12.77 μg/ml for 1% PVA in 15% HPβCD, but only 8.28 μg/ml for 0.3% HEC. For 15% MβCD complexation, UC781 solubility was obtained to 185.9 μg/ml for 0.6% HPMC, 138 μg/ml for 1.0% PVP K-30, 123 μg/ml for 0.8% PVP K-90, 184 μg/ml for 0.8 % PVA and 181 μg/ml for 3% HEC. The

order of UC781 solubility enhancement effect of different polymers is HPMC> PVP K90 > HEC, PVP K30 and PVA in 15% HPβCD solution, and HPMC, HEC, and PVA > PVP K90 and PVP K30 in 15% MβCD solution.

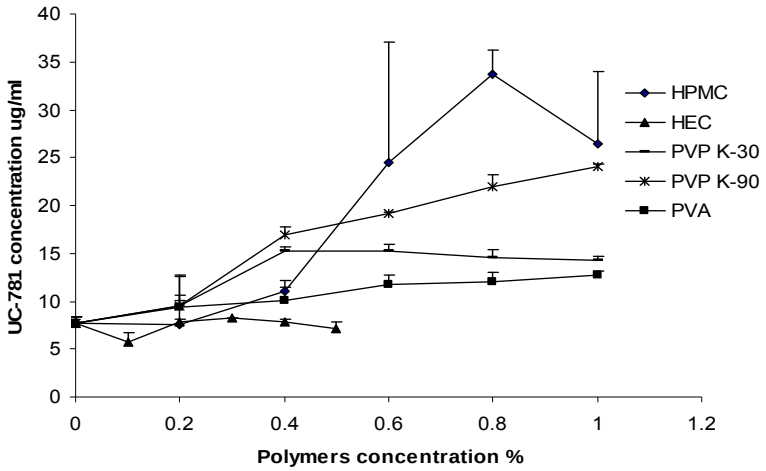


Figure 4-7. Polymer effect on UC781 solubility in a 15% HPβCD solution

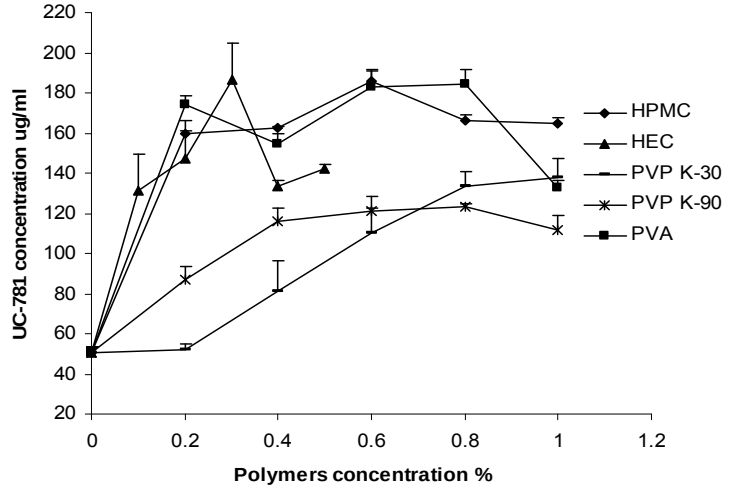


Figure 4-8. Polymer effect on UC781 solubility in a 15% MβCD solution

There are several possible explainations for the enhanced complexation efficiency observed in the presence of water-soluble polymers. Some polymers are known to be able to

interact with cycldoextrins via hydrophobic moieties in their polymer chains (Beheshti et al.,2007; Harada et al.,2005; Werner et al.,2004). Additionally, polymers were reported to inhibit the crystallization of water insoluble drugs (Khougaz and Clas,1999; Raghavan et al.,2001; Yeoh et al.,1994) into a super saturated state and to enhance the dissolution of drug into the aqueous phase. Therefore, βCD inclusion complexes can be impacted by their interaction with water-soluble polymers forming ternary complexes containing the drug molecule, βCD, and the polymer (Loftsson and Brewster,1996; Patel and Vavia,2006). At low concentrations, polymers increase the aqueous solubility of the compound. They also enhance the complexation ability of CDs as shown through an increase in the complexation constants of the drug-cyclodextrin complexes by forming a ternary complex of polymer: drug: cyclodextrins. Finally, low concentrations of polymers can enhance complexation of drug with cyclodextrins in solution (Chowdary and Srinivas,2006; Kristinsson et al.,1996; Sigurðoardóttir and Loftsson,1995; Valero et al.,2003).

Baseline aqueous solubility of UC781 in the presence of all polymers tested was lower than the HPLC limit of detection ($<3\times10^{-5}$ mg/ml), indicating a very limited aqueous solubility enhancement effect of polymers on UC781. Therefore, the impact of polymers on the solubility of UC781 would not contribute to their complexation enhancement effect. The ternary complex of polymer: UC781: cyclodextrins may be formed as to in aqueous phase to enhance the UC781: cyclodextrins complexation. It has been shown that the polymer partly or totally coats the inclusion complex, interacting with both the drug and the βCD molecule through hydrogen bonding in the ternary complex (Valero et al.,2003).

In these studies, different polymers showed different impacts on UC781 solubility in both HPβCD and MβCD solution. In both cyclodextrin systems, HPMC showed potent solubility

enhancement activity, indicating that it is a promising excipient to be considered in formulation to enhance the complex formation of UC781 and cyclodextrins.

4.3.4 HIV Reverse Transcriptase Inhibition Assay for UC781: Cyclodextrins Complexes

An *in vitro* HIV reverse transcriptase inhibition assay was used to study the anti-HIV activity of pure UC781 (dissolved in DMSO) as compared to the complex form of UC781 with HPβCD and MβCD. In these studies, the UC781 concentration in the complex was varied (5 and 10 μg/ml) with fixed amount of HPβCD (15% w/v) and MβCD (15% w/v). PBS (pH 7.2) was used as a control. A serial dilution of UC781 and its complex was tested to obtain the dilution effect for UC781 complexes.

Results showed that both HPβCD and MβCD have no baseline RT inhibition activity themselves (Figure 4-9). Inhibition of RT by the UC781 complexes with HPβCD or MβCD follows a similar pattern with the pure UC781 (with DMSO <1% as co-solvent) (Figure 4-10 and Figure 4-11). HPβCD and MβCD themselves did not show any RT inhibition at either 10% or 15% in PBS compared with control. The relative IC_{50} of UC781 is approximately 10 nM in the *in vitro* assay. No statistical difference in IC_{50} of UC781 was observed between UC781 and UC781: HPβCD complex or UC781:MβCD complex ($p>0.05$). (Figure 4-12).

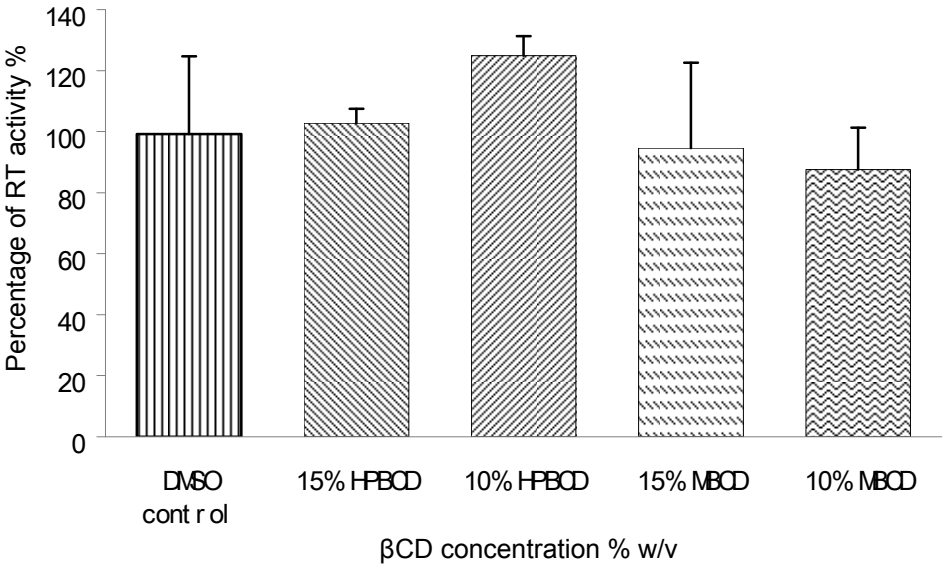


Figure 4-9. RT inhibition activity for HPβCD and MβCD

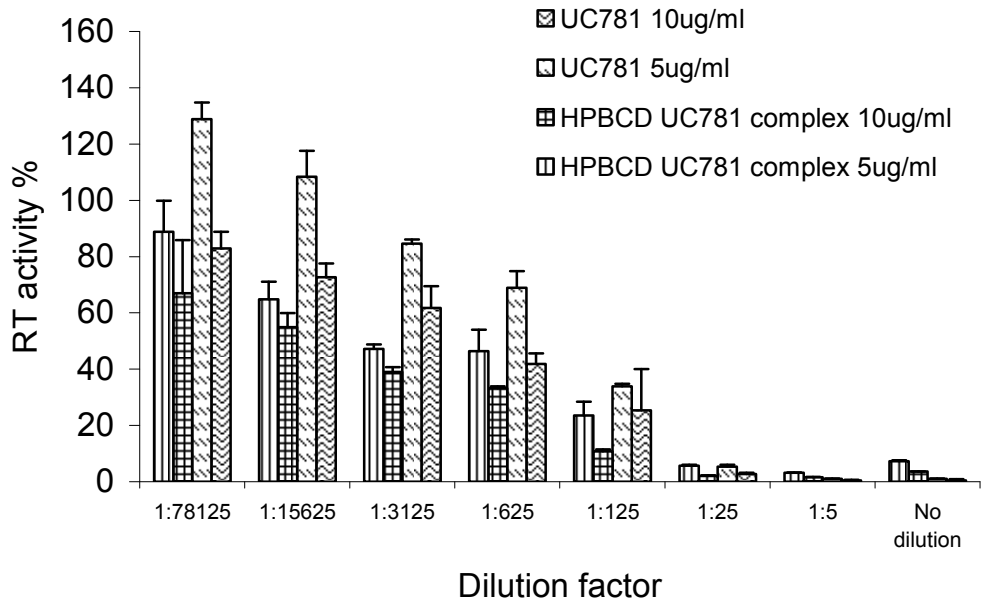


Figure 4-10. RT inhibition assay of UC781 complexed with 15% HPβCD

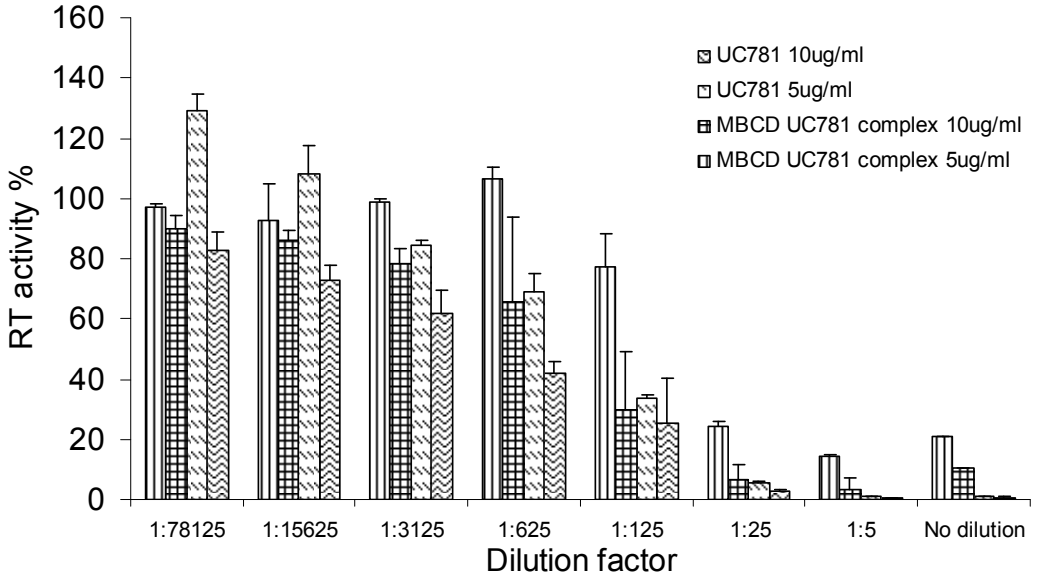


Figure 4-11. RT inhibition assay of UC781 complexed with 15% MβCD

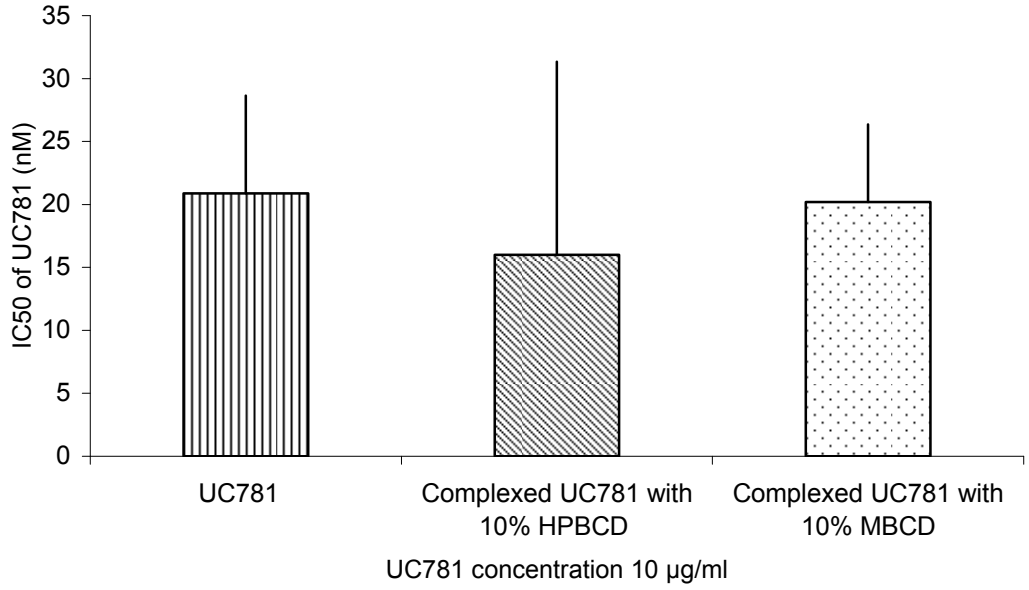


Figure 4-12. Comparison of IC_{50} of complexed *vs.* uncomplexed UC781 *in vitro* assay

No statistically significant differences in IC_{50} were found when comparing non-complexed UC781 with complexed UC781. However, complexation could result in competition for UC781 between CD and RT in the experimental setup. This would lead to a reduced effective

concentration of UC781 around RT, leading to a shift in IC_{50}. Some evidence for this phenomena may be seen in the MβCD data. HPβCD complex did not show this tendency. This can be explained by its weaker complexation ability with UC781 compared with MβCD. Thus, HPβCD may not interfere with the interaction between UC781 and RT.

It must be emphasized that MβCD will not enter the cell and only plays a role as a carrier to bring UC781 into cells. Therefore, MβCD will not affect the interaction between UC781 and RT inside HIV infected cells.

4.4 CONCLUSIONS

This study shows that UC781 can interact with βCD, HPβCD, or MβCD forming an inclusion complex in aqueous phase. The formation of an inclusion complex is dependent on cyclodextrin, different preparation methods, pH variance, and incorporation of polymers.

Autoclave method is the most efficient method for the preparation of UC781complex. Autoclave method is designed to provide a temperature controlled method for optimization of the complexation of UC781 with cyclodextrins due to the enthalpy driven process. In addition, the autoclave method may result in increased interaction between UC781 and βCD molecules. Compared to the other methods in experiments, the autoclave method provides a quick and efficient method to form UC781: cyclodextrins complexes.

pH variance can directly change the ionization degree of UC781 and enhance its solubility in water. Due to the high pKa value (pKa=9.14), UC781 can only be highly ionized at high pH leading to a higher solubility in aqueous phase. However, this pH is not applicable for the development of a vaginal formulation at pH 4.2.

In these studies, HPMC is the most potent solubility enhancer for UC781 in both 15% HPβCD and MβCD solution. In the presence of 0.8% HPMC and 15% MβCD, UC781 solubility can be increased to 33 μg/ml (4.2 times); with 0.6% HPMC and 15% HPβCD, UC781 solubility can be increased to 185 μg/ml (3.7 times). HPMC was also applied to protect cultured cells and human cervical epithelium against the toxicity of βCD as presented in Chapter 5.

MβCD showed the strongest complexation ability with UC781 in comparison with the other two β-cyclodextrins used in this experiment. Solubility of this complexed form is approximately 50 μg/ml in 15% w/v MβCD while only to 0.5 μg/ml for 1.5% βCD and 7.7 μg/ml for 15% HPβCD with the autoclave method. The RT inhibition activity of complexed UC781 was effectively maintained. MβCD showed better complexation ability with UC781. Further toxicity studies are necessary for the development of a formulation containing MβCD.

5.0 DEVELOPMENT OF VAGINAL GEL AND FILM FORMULATIONS FOR UC781 AND ITS β-CYCLODEXTRIN COMPLEXES AS TOPICAL VAGINAL MICROBICIDE PRODUCTS

5.1 INTRODUCTION

Vaginal drug administration has a long history dating back to the Middle Ages (Neves et al.,2008). This route of administration has long been employed for postmenopausal delivery of hormones such as estradiol (Van Laarhoven et al.,2002). Although the vagina can be used for both systematic and local drug delivery, the majority of vaginal products are intenede for local pharmacological activity. Compared with classical oral routes of administration, vaginal delivery is still not the preferred route for drug delivery. However, anatomical considerations for the transmission of HIV and other sexually transmitted diseases (STDs) require vaginal drug delivery for effective prevention of infection.

In these studies, UC781 was complexed with HPβCD or MβCD to enhance its aqueous solubility. The state of active agents in a formulation may change the physiological properties of the absorption site and affect drug efficacy by changing drug absorption and disposition (Bardelmeijer et al.,2002; Malingre et al.,2001). These changes may reduce the bioactivity of active agents in microbicide products and lead to an increase in toxicity. Therefore, the anti-HIV activity and toxicity of complexed UC781 should be evaluated.

Three formulations were developed as potential microbicide products for complexed UC781. These formulations include a methylcellulose/carbopol (MC) gel, hydroxyethycellulose (HEC) gel, and polyvinyl alcohol (PVA) film. The MC-based gel is based on the gel currently being evaluated clinically for delivery of UC781 in a suspended form. The HEC gel is based on the universal placebo designed for vaginal microbicide clinical trials (Tien et al.,2005). The PVA film provides a novel delivery system for UC781. PVA film systems are available on the market for vaginal cleansing and contraception. In these studies, these three formulations were developed as vaginal drug delivery systems for the evaluation of both complexed and non-complexed UC781.

The objective of this chapter is to evaluate the impact of complexation of UC781 with HPβCD or MβCD on the physical properties, toxicity profile, and the anti-HIV activity of the developed vaginal formulations.

Toxicity is an important concern in the successful development of a safe and effective microbicide product. Therefore, the toxicity of HPβCD or MβCD in the presence and absence of water-soluble polymers was investigated in a cell-based model (HeLa and A431) as well as in an excised human cervical epithelium model. In addition, the toxicity of each developed formulated product was assessed in the human cervical epithelium model. To assure that complexation did not result in any loss in product effectiveness, the inhibition of RT activity and HIV replication by UC781 was investigated. The anti-HIV activity of complexed UC781 in PVA film was also evaluated using a TZM-bl cell model.

5.2 MATERIALS AND METHODS

5.2.1 Materials

UC781 for these studies was provided by Biosyn Co. Ltd. (Huntington, PA). βCD (MW: 1134), MβCD (MW: approx. 1320; mean degree of substitution: 1.7-1.9), HPβCD (MW: approx. 1380; mean degree of substitution: 0.8) were purchased from Sigma Aldrich (St. Louis, MO). All other reagents used were of reagent grade and all solvents were of HPLC grade. Milli-Q water was used to prepare buffer solutions and other aqueous solutions. HeLa, A431, and TZM-bl cells were obtained from ATCC.

5.2.2 Characterization and Evaluation of Physicochemical Properties of UC781 Complexes Containing Formulations

5.2.2.1 Preparation of UC781: HPβCD and UC781: MβCD Complexes

The UC781: HPβCD complex solution was prepared using the following steps: 1) UC781 was mixed with HPβCD at either a 1:250 or 1:500 (w/w) ratio, and then dispersed into 5ml Milli-Q water; 2) The suspension was autoclaved at 120^{o}C (250°F) for 15 min, and then formulated into a HEC gel, MC gel, or PVA film after cooling down to room temperature. The UC781: MβCD complex was prepared following the same procedure.

5.2.2.2 Formulation of Complexed UC781 in a Hydroxyethycellulose (HEC) Gel

Formulation of the hydroxyethycellulose (HEC) gel was prepared based on the recipe of the universal placebo used in microbicide clinical trials (Tien et al.,2005). Briefly, HEC gel was

prepared by dissolving 0.1% (w/w) sorbic acid, 0.85% (w/w) sodium chloride, and 0.35% (w/w) 1 N sodium hydroxide (Spectrum Chemicals & Laboratory Products, Gardena, CA) in 96.0% of water. Next, 2.7% (w/w) hydroxyethylcellulose 250HX (Hercules Incorporated, Wilmington, DE) was slowly incorporated using an overhead motorized stirrer (RW20 Stirrer, IKA, Divitech Equipment Co., Cincinnati, OH). UC781 was dissolved into 2 ml of ethanol and then incorporated to 100 ml of gel, under constant stirring, to obtain a 0.01% UC781 containing gel. For complex containing gels, pre-prepared UC781: HPβCD or UC781: MβCD complex solution was incorporated into the gel under constant stirring to form UC781 complex containing gel. HEC gels containing no UC781 or its complex were used as control. The final pH was determined and adjusted if necessary to 4.4 ± 0.2 (pH values in normal vagina) using 1 N hydrochloric acid. Control gels containing no UC781 or βCD were also prepared.

5.2.2.3 Formulation of Complexed UC781 in a Methylcellulose/Carbopol (MC) Gel

Methylcellulose/Carbopol (MC) gel was prepared by dissolving 0.3% of Carbopol 974P (Carbopol ®, Noveon, Inc., Cleveland, OH) in water using an overhead motorized stirrer. UC781 was first dissolved into 2 ML of ethanol and then mixed into the Carbopol® solution. Methylparaben, propylparaben and 1.0% methylcellulose 4000 cps (Spectrum Chemicals & Laboratory Products, Gardena, CA) were mixed with glycerin, and then dispersed in the Carbopol® solution to obtain a 0.01% UC781 containing gels. For preparation of complex containing gel, pre-prepared UC781: HPβCD or UC781: MβCD solution was then incorporated into the gel under constant stirring to obtain UC781 complex containing gel. The final pH was adjusted to 5.2 ± 0.2 with 1 N sodium hydroxide (Spectrum Chemicals & Laboratory Products,

Gardena, CA) due to the optimal gellation pH for Carbopol®. Control gels containing no UC781 or βCD were also prepared.

5.2.2.4 Formulation of Complexed UC781 in a PVA Film

A PVA based fast dissolving film formulation was prepared by dissolving 3% PVA (cold water-soluble, molecule weight 30-70KD) (Sigma), 0.06% hydroxypropyl methylcellulose 6 mPa.s (Sigma), and glycerin (Spectrum) in water. 100 μg of UC781 was dissolved into 0.5 ml of ethanol and then mixed into 2.5 ml of PVA film solution, which had been previously transferred to a single well of an 8-well rectangular plate (Nalge Nunc International Inc. Rochester, New York). This process was repeated for each of the eight wells. Plates were then placed into an Isotemp® vacuum oven model 285A (Fisher Scientific, Hampton, NH) at 32^{o}C and -25 mHg for at least 48 h to remove moisture.

For the preparation of UC781 complex containing film, the complexed form of UC781 with HPβCD or MβCD, containing 100 μg of UC781, was prepared following the same method described for the HEC gel and MC gel formulations. A total of 0.5ml of ethanol was added to the complex containing films to be consistent with procedures used for non-complexed containing films. Control films containing no UC781 or βCD were also prepared.

5.2.2.5 Preparation of Vaginal Fluid Simulant (VFS)

Vaginal fluid simulant (VFS) was prepared as described by Owen and Katz (Owen and Katz,1999), using 3.51 g sodium chloride (Fisher Scientific, Hampton, NH), 1.4 g potassium hydroxide (Spectrum Chemicals & Laboratory Products, Gardena, CA), 0.222 g calcium hydroxide, 18 mg albumin bovine fraction V, 2.0 g lactic acid , 160 mg glycerol , 0.4 g urea

(Spectrum Chemicals & Laboratory Products, Gardena, CA), 1.0 g acetic acid glacial (Fisher Scientific, Hampton, NH), 5.0 g glucose (Spectrum Chemicals & Laboratory Products, Gardena, CA), and ultra-pure Milli-Q water (Millipore, Billerica, MA) to make 1000 ml of fluid. The pH of the vaginal fluid simulant was adjusted with 10% hydrochloric acid (FisherScientific, Hampton, NH) to pH 4.2 ± 0.1.

5.2.2.6 Evaluation of Physicochemical Properties of Formulations

The osmolality and viscosity of the MC gel and HEC gel, as well as the disintegration time of PVA film were evaluated for all formulations. All measurements were conducted in triplicate. Osmolality was determined using a Vapor Pressure 5520 Osmometer (Wescor, Inc., Logan, UT) calibrated with Opti-mole 290 and 1000 mmol/kg osmolality standards.

Evaluation of viscosity was conducted using the CP51 spindle on a cone/plate Brookfield Model DVIII+ viscometer (Brookfield Eng. Lab., Inc., Middleboro, MA) at 25^{o}C and 37^{o}C. Data were collected using Rheocalc software (Brookfield Eng. Lab. Inc., Middleboro, MA). Viscosity was measured using a program in which shear rate was increased from 0.2 to 30.0 s^{-1} and subsequently decreased to 0.2 s^{-1}. In order to compare data across samples, viscosity values acquired at 30.0 s^{-1} were used for analysis.

Disintegration times of PVA film were determined using a shaking method at room temperature. One PVA film was placed into 3 ml of Milli-Q water on multi-purpose rotator (Barnstead, USA), and shaken at 60 rpm until the film totally dissolved. Dissolving time based on visual observation was recorded for analysis.

5.2.2.7 Dissolution Testing of UC781 in the MC Gel, HEC Gel, and PVA Film

The enhancer cell (Distek Inc, North Brunswick, NJ) was used to conduct UC781 dissolution studies. These studies were conducted to assess UC781 release profiles from the formulation. In these studies, one film was inserted into a 100-mesh basket and then placed into 75 ml of 20 mM VFS (with 0.1% of sodium lauryl sulfate) at 37 ^{o}C ± 0.5 ^{o}C with continuous stirring at 50 rpm. Aliquots of 0.5ml buffer was taken at predetermined time points. These aliquots were then filtered using a 0.45 μm filter for HPLC analysis. For the evaluation of MC or HEC gels, 1g was loaded into an ointment cell flask, and the same procedure designed above was conducted using VFS (with 1.0% of sodium lauryl sulfate) as the dissolution media.

5.2.2.8 Kinetic Analysis of Dissolution Data

The release behavior of UC781 and its complexes from gel and film formulations was analyzed using the exponential equation, known as the power law shown as Equation 5-1 (Korsmeyer et al.,1983; Siepmann and Peppas,2001)

$$\frac{M_t}{M_\infty} = kt^n \qquad \textbf{5-1}$$

Here, M_t and M_∞ are the absolute cumulative amount of drug released at time t and infinite time respectively; k is a kinetic constant reflecting formulation characteristics; and n is an exponent characterizing the mechanism of drug release. When n <0.5, the drug is released with a quasi-Fickian diffusion mechanism, When n=0.5, the drug is released with a Fickian diffusion mechanism representing the special case of the power law, for 0.5<n<1, an anomalous transport drug release occurs and when n≥1, a non-Fickian Case II or zero order release kinetics is observed (Ritger and Peppas,1987; Siepmann and Peppas,2001). Dissolution data were fitted using SigmaPlot 10.0 software.

Mean dissolution time (MDT) was also considered to compare the drug release rate from the different formulation (Figure 5-1). MDT was estimated using the following equation 5-2:

$$MDT = \frac{\int_0 t \cdot W_t dt}{\int_0^\infty W_t dt} = \frac{ABC}{W_\infty} \qquad \textbf{5-2}$$

Here, ABC (area between curves) is the shaded area in Figure 5-1, Wt is the cumulative amount of drug released at any time interval, and W∞ is the actual quantity of drug released from formulation, which is available from the experiment. ABC was estimated using the trapezoidal rule in this study.

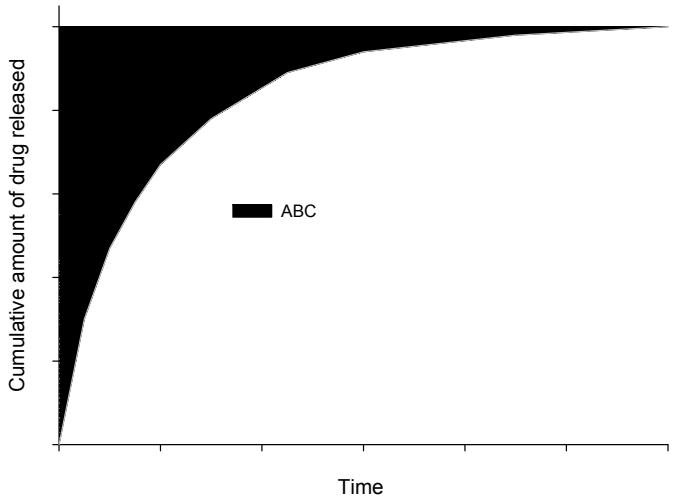


Figure 5-1. Graphical presentation of the parameters used to estimate the mean dissolution time (MDT)

Cumulative amount of UC781 released from formulations (μg) *vs.* drug release time (min) was plotted to illustrate the estimation of MDT using Equation 5-2. The black area represents the area between curves (ABC) used in the evaluation of MDT, which is calculated using the trapezoidal rule.

5.2.3 Preformulation Evaluation of *In Vitro* Toxicity of Cyclodextrins

5.2.3.1 Toxicity Evaluation of Cyclodextins in Hela and A431 Cell Models

The viability of HeLa cells (human epithelial cells from cervical cancer) and A431 cells (human epithelial cells from squamous carcinoma) was measured by quantification of the mitochondrial dehydrogenase activity by reducing the tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) (Coligan et al.,2007). HeLa cells or A431 cells were cultured in a 96 well-plate to a density of 1×10^5 cells in 100 μl of media in each well Cells were incubated with HPβCD or MβCD in Dulbecco's Modification of Eagle's Medium (DMEM) either in the presence or absence of several water-soluble polymers which are commonly used in vaginal formulations (0.5% and 1% HPMC, PVA, HEC, and PVP K30) at 37 °C with 5% CO_2 at saturated humidity. After the incubation period, cells were washed with DMEM, followed by incubation with 100 μl DMEM containing 20 μl (5mg/ml) MTT solution for an additional 4 h at 37°C with 5% CO_2 at saturated humidity. After incubation, 120 μl of stop solution was added into each culture well. After extraction of formazan crystals by isopropanol, optical density of the extracted solution was measured at 595 nm. A Tukey's multiple comparison test (GraphPad software, version 4) was used to determine significant differences in MTT levels.

5.2.3.2 Toxicity Evaluation of Cyclodextrins in an Excised Human Cervical Tissue Model

Viability of the human cervical tissue was quantified by MTT after direct exposure to HPβCD or MβCD in the presence or absence of polymers.

HPβCD or MβCD was dissolved into 1ml DMEM containing either 0.5% or 1.0% of each of the tested polymers (HPMC, HEC, PVA, and PVP K30) to obtain a final cyclodextrin concentration from 1% to 20%. Briefly, human cervical explants (3-mm diameter) were exposed directly to 200 µl of solution containing various concentrations of HPβCD or MβCD. DMEM was used as control in these experiments. After a 2 h exposure, explants were washed two times with fresh DMEM. Tissues were immediately incubated in DMEM containing MTT (500 µg/ml) for an additional 3 h at 37°C. Tissue viability was determined by dividing the optical density of the formazan product in isopropanol (595 nm) by the weight of the explants. The impact of each test sample on tissue viability was determined by comparing the viability of the treated explants to the DMEM treated tissue control. All samples were prepared in triplicate. Extra tissues treated with the same treatment were also fixed for histological study. A Tukey's multiple comparison test (GraphPad software, version 4) was used to determine significant differences in MTT levels.

5.2.4 Preformulation Evaluation of *Ex Vivo* Toxicity of Cyclodextrins and Uc781:Cyclodextrins Complexes Containing Formulations

5.2.4.1 Toxicity Evaluation of HPβCD or MβCD Containing Formulations in an Excised Human Tissue Model

Viability of human cervical tissue for the three formulations containing HPβCD or MβCD were evaluated using an MTT assay. The toxicity of the formulations was quantified by the reduction of the tetrazolium salt 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT). In these experiments, film products were dissolved into 1ml DMEM cell culture media to obtain

an equivalent concentration of cyclodextrin used in the gels. Briefly, human cervical explants (3-mm diameter) were exposed directly to 200 μl HEC gel, MC gel, or PVA film solution containing HPβCD or MβCD at concentrations of 5%, 10%, and 20%. DMEM was used as a control. The MTT assay was conducted as described in section 5.2.2.2. All samples were tested in triplicate.

5.2.4.2 Toxicity Evaluation of UC781:Cyclodextrins Complexes Containing Formulations in an Excised Human Tissue Model

Viability of the human cervical tissue for the three formulations was evaluated using the Multiple Exposure Device (MED) developed in our lab. This toxicity evaluation differs from the MTT described in section 5.2.4.1, in that the tissues are polarized. Formulation toxicity was quantified by MTT assay. In these experiments, film products were dissolved into 1ml DMEM cell culture media to obtain an equivalent concentration of cyclodextrin used in the gels. Briefly, Human cervical explants (6-mm diameter) were exposed directly to 200 μl HEC gel, MC gel, or PVA film solution containing UC781:HPβCD complex or UC781:MβCD complex. DMEM was used as a control. The MTT assay was conducted as described in section 5.2.2.2. All samples were tested in triplicate.

5.2.5 *In Vitro* Bioactivity Studies of UC781: Cyclodextrins Complexes

5.2.5.1 Effects of UC781:Cyclodextrins Complexes on RNA-dependent DNA Polymerase Activity of HIV-1 Reverse Transcriptase

HIV-1 RT DNA polymerase activity was determined by a fixed time assay. Briefly, HPβCD or MβCD were dissolved in 10mM pH 7.2 PBS buffer to obtain a 15% w/w solution.

UC781 was added into 1ml of a 15% HPβCD or a 15% MβCD solution to prepare a 5μg/ml, or 10μg/ml UC781 complex solution. The solution was then autoclaved at 250 °F for 15 min followed by a shaking in a Labquake shaker (Barnstead Thermolyne, USA) at 50 rpm overnight at room temperature to form the UC781:HPβCD and UC781:MβCD complexes containing 5μg/ml or 10μg/ml UC781. Non-complexed UC781 was dissolved using DMSO and quickly dispersed into 10mM PBS buffer as positive control (DMSO amount equal to 1% or less). PBS buffer was used as the negative control. The remaining procedure details are as described previously in section 4.2.2.5. All samples were tested in triplicate.

5.2.5.2 Effects of UC781:cyclodextrins Complexes Containing Formulations on RNA-dependent DNA Polymerase Activity of HIV-1 Reverse Transcriptase

UC781:HPβCD or UC781: MβCD complexes containing formulations were dissolved into PBS before experiments. These solutions were used for RT inhibition analysis as described in Section 5.2.5.1. The remaining procedure details are as described previously in Section 4.2.2.5. All samples were tested in triplicate.

5.2.5.3 Effect of UC781: Cyclodextrins Complexes Containing Film Formulations on The Replication of HIV in TZM-bl Cell Model.

PVA film containing non-complexed UC781 (100 μg/film) and complexed UC781 (100μg/ film) were dissolved into PBS for HIV inhibition testing. For UC781: HPβCD complex containing PVA films, the complexes were prepared with UC781:HPβCD at a 1:250 ratio and UC781:HPβCD at a 1:500 mass ratio. UC781:MβCD complex films were prepared following the same procedure.

A TZM-bl cell-based assay (TZM-bl cell is a genetically engineered HeLa cell line that express CD4, CXCR4 and CCR5 and contains Tat-inducible Luc and β-Gal reporter genes) for evaluation of HIV infection has been described in detail as previously reported(Derdeyn et al.,2000). The assay was briefly described as follows: TZM-bl cells were seeded into 96-well Packard ViewPlate at a density of 5×10^3 cells/well. 50 μl film solution was added into each well and then mixed with 40 μg/ml diethylaminoethyl-dextran (DEAE-Dextran) solution 50 μl containing HIV-1_{Bal} or HIV-1_{LAV}. Another 150 μl DMEM were added after two hours incubation at 37 °C. All cells were then cultured for 48 hthen carefully rinsed with blank Hank's Buffered Salt Solution (HBSS) to remove virus and residual film solution. TZM-bl cells were then lysed using 100 μl Bright-Glo reagent (Promega Corporation, Madison, WI), and the mixture was shaken for two min. The light intensity of each cell lysate was measured by DTX 800/880 Series Multimode Detectors (Beckman Coulter, Inc. Fullerton, CA). Samples were treated and measured in triplicate. The percentage of HIV-1 inhibition was expressed as mean ± SD.

5.2.6 Histology Studies on Excised Human Cervical Tissue

Human cervical tissue was fixed following exposure to test products using the following procedure for toxicity evaluation. Tissues were put in a cartridge with a foam insert and placed in Clark's solution of acetic acid and ethanol (1 part to 3 parts) for 12 hr. After this time, Clark's solution was replaced with 100% EtOH. The 100% EtOH was changed every hour for three hours following which the cartridge was removed from the EtOH and placed into 100% xylene, which was changed every 30 min for a total of one hour. Tissues were put in xylene overnight at -20°C prior to embedding in wax. Once embedded, tissues were cut in 5μM sections using a

microtome (Microm HM320, Brodersen, USA), and then stained with hematoxylin and eosin (H&E), and evaluated microscpically for gross morphological changes to the tissue.

5.2.7 Statistical Analysis

Data for physicochemical analysis (osmolality and viscosity) and disintegration time of PVA film were expressed as the mean ± SD. Tissue viability in percentage was expressed as mean ± SD. IC_{50} values determined for UC781 in each formulation were expressed as mean ± SD. IC_{50} was calculated with GraphPad software 4. Data were analyzed with one-way ANOVA using Tukey's multiple comparison tests (GraphPad software 4). Obtained $p < 0.05$ were considered to be statistically significant.

5.3 RESULTS AND DISCUSSION

5.3.1 Characterization and Evaluation of Physicochemical Properties of UC781:Cyclodextrins Complexes Containing Formulations

5.3.1.1 Osmolality Evaluation of MC and HEC Gels

Osmolality is an important parameter for the characterization of semisolid formulations. The osmolality (mmol/kg) results of MC gel and HEC gel are listed in Table 5-1. Figure 5-2 (HEC gel) showed that the osmolality increased with the incorporation of either HPβCD or MβCD complexed UC781. Osmolality results for the HEC gel without complex were 319 mmol/kg. Only a slight change of osmolality was observed in UC781 containing HEC gel (323

mmol/kg). The incorporation of cyclodextrin complexed UC781 into the HEC gel resulted in a statistically significant increase in osmolality as compared to placebo. The osmolality increased to 358 mmol/kg (UC781: HPβCD =1:250) and 361 mmol/kg (UC781: HPβCD = 1:500) for UC781:HPβCD complex containing gels; 357 mmol/kg (UC781: MβCD =1:250) and 392 mmol/kg (UC781: MβCD = 1:500) for UC781 :MβCD complex containing gels.

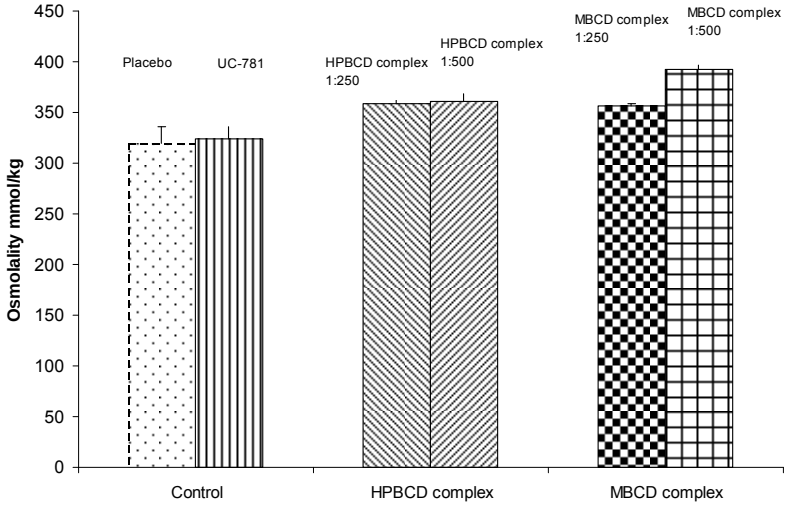


Figure 5-2. Effect of UC781 Complex on Osmolality (HEC gel)

Table 5-1. Impact of UC781 complex on osmolality change of HEC and MC gel

	Placebo Gel	UC781	UC781 HPβCD complex (1:250)	UC781 HPβCD complex 1:500)	UC781 MβCD complex (1:250)	UC781 MβCD Complex (1:500)
HEC Gel	319.67±16.26	323.67±11.93	358.67±4.16	361.00±7.94	356.67±1.53	392.00±4.58
MC Gel	585.67±4.04	573.33±6.66	616.00±4.58	677.00±4.36	667.00±11.79	696.00±6.24

Similar results in osmolality change were obtained in MC gels as shown in Figure 5-3. The osmolality value without complex was 585 mmol/kg for MC placebo gel and 573 mmol/kg for UC781 containing MC gel. The osmolality increased to 616 mmol/kg (UC781: HPβCD =1:250) and 677 mmol/kg (UC781: HPβCD = 1:500) for UC781: HPβCD complex containing

MC gels; 667 mmol/kg (UC781: MβCD =1:250) and 696 mmol/kg (UC781: MβCD = 1:500) for UC781: MβCD complex containing MC gels.

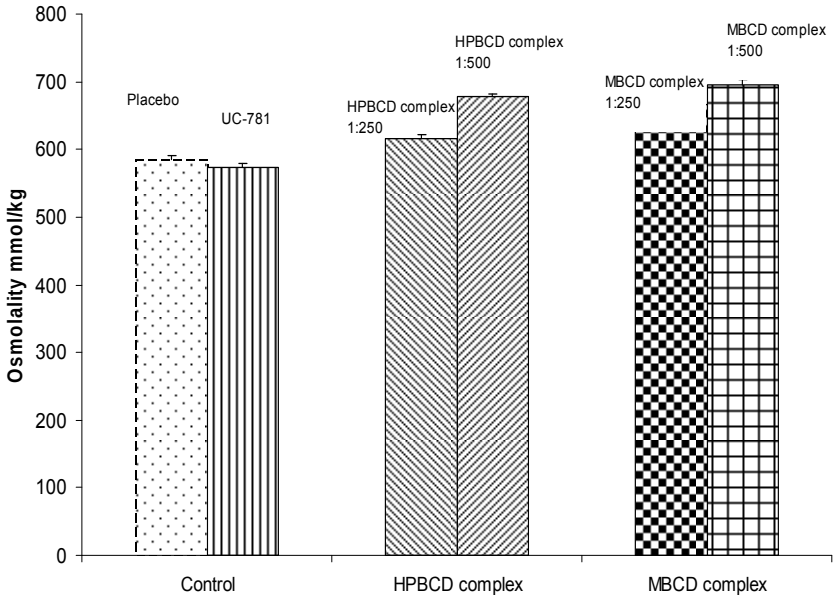


Figure 5-3. Effect of UC781 Complex on Osmolality (MC gel)

In these studies, cyclodextrin complexes with UC781 increased the osmolality of both gels. Osmolality changes were statistically significant ($p<0.05$) for all UC781 complex containing products tested compared with its placebo products. Greater amount of cyclodextrin in the complex leads to a higher osmolality value for the formulation indicating that cyclodextrin changes the osmolality of both MC and HEC gel formulations.

For gel formulations, osmolality not only reflects the physical characteristics of the gel product itself, but also affects the local vaginal environment. Products with too high osmolality value will increase the risk of HIV transmission by changing the barrier properties of vaginal epithelium (Fuchs et al.,2007). In addition, it was shown that products with high osmolality would leak more readily from the vagina than those that are isoosmolar. Viscosity will also affect the application of semisolid formulations. Lower viscosity products can lead to greater

leakage of the formulation from the vagina (Owen and Katz,1999). These data show that the incorporation of the complexed form of UC781 with HPβCD (2.5% to 5%) or MβCD (2.5% to 5%) into the formulation can statistically significantly increase the osmolality of both the MC gel and the HEC gel ($p < 0.05$). This observed increase is related to the amount of βCD in the gels. However, the osmolality of the complex containg gels are only 19% (HEC gel) or 35% (MC gel) of osmolality value for the marketed K-Y® gel product (containing 2% Nonoxynol 9 (N9)) listed in literature (Fuchs et al.,2007).

Comparison of these results suggests that the complex containing MC gel and HEC gel (even at the 5% level) are comparatively safe without potential for barrier alteration as a result of high osmolality.

5.3.1.2 Viscosity Evaluation of MC and HEC Gels

The viscosity of each gel used was experimentally determined at both 25 °C and 37 °C using the methods described in the methods section of the section 5.2.3.6. The viscosity results of products are shown in Figure 5-4 (MC gel 25 °C), Figure 5-5 (MC gel 37 °C), Figure 5-6 (HEC gel 25 °C), and Figure 5-7 (HEC gel 37 °C). A significant decrease in viscosity was observed for all MC products at both 25 °C and 37 °C when complexed UC781 was incorporated into the products compared to placebo gels ($p<0.05$). For the HEC formulation, viscosity significantly decreased only in UC781: MβCD complex containing gels compared to placebo gel at 25 °C. At 37 °C, the viscosity values significantly decreased in the complex containing gels of UC781: HPβCD complex (UC781:HPβCD=1:500), UC781:MβCD complex (UC781:MβCD=1:250), and UC781:MβCD complex (UC781:MβCD=1:500) compared to

placebo gel. This suggests that the viscosity may be both cyclodextrin and temperature dependent.

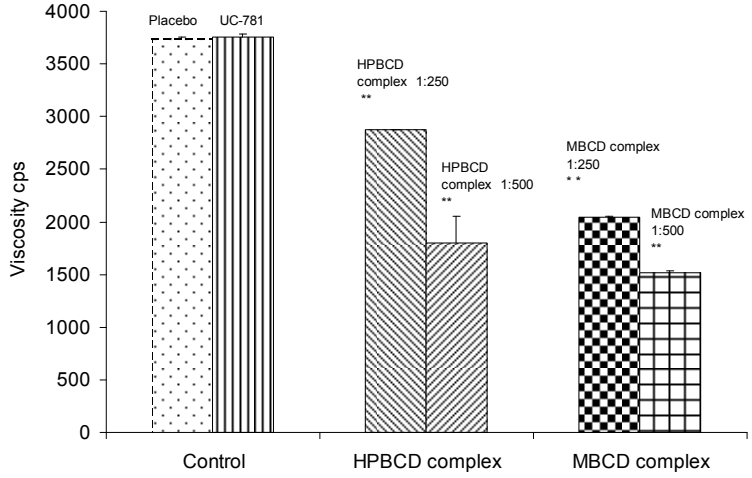


Figure 5-4. Effect of UC781 Complex on viscosity change in MC gel at 25 °C

*: $p<0.05$; **: $p<0.01$

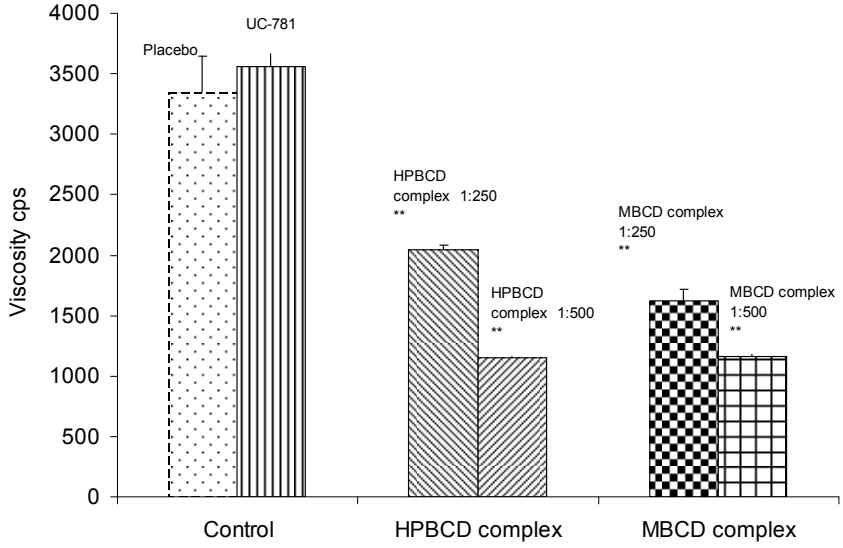


Figure 5-5. Effect of UC781 Complex on viscosity change in MC gel at 37 °C

*: $p<0.05$; **: $p<0.01$

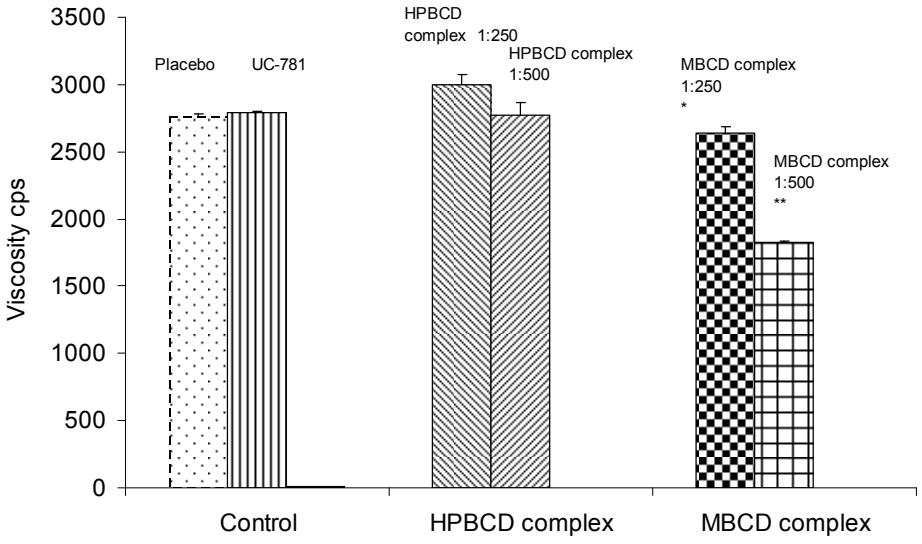


Figure 5-6. Effect of UC781 Complex on viscosity change in HEC gel at 25 °C

*: $p<0.05$; **: $p<0.01$

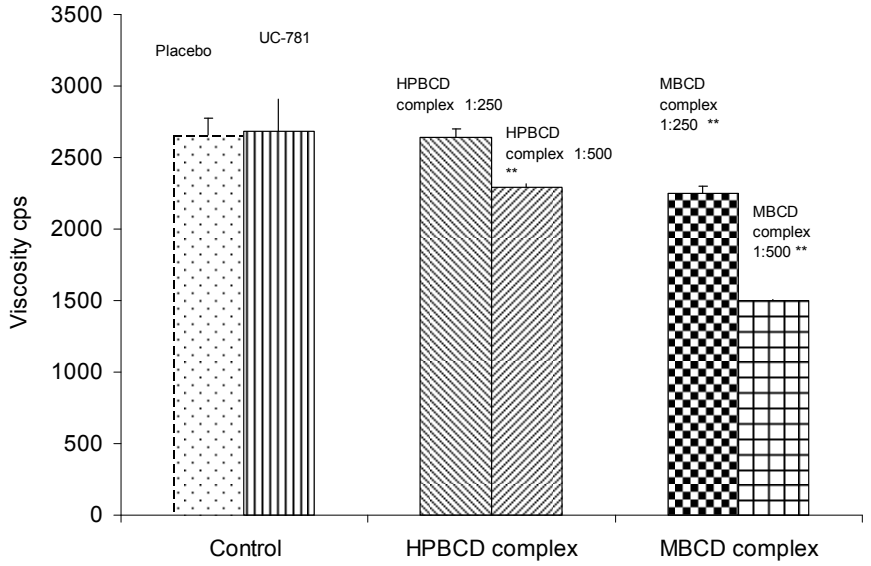


Figure 5-7. Effect of UC781 Complex on viscosity change in HEC gel at 37 °C

*: $p<0.05$; **: $p<0.01$

For vaginal microbicide products, viscosity plays an important role for product application and distribution over the vaginal mucosa. The viscosity of marked vaginal products is quite varied (Garga et al.,2001). The impact of HPβCD or MβCD on the gel viscosity was evaluated in our study. All HEC and MC gels showed a decrease in viscosity upon addition of complexed UC781 with HPβCD or MβCD into the formulation. With the incorporation of complexed form of UC781 with HPβCD (2.5% to 5%) and MβCD (2.5% to 5%) into the MC gel or the HEC gel, the viscosity of the gels greatly decreased as the β-cyclodextrin amount increased in complex. This result coincided with that observed in several previously published studies (Beheshti et al.,2007; Boulmedarat et al.,2003). This viscosity decrease indicates the interaction between the complex and the polymer chains of the viscosity increasing agents used in the formulation. This interaction could affect the binding force (hydrogen bonding) or decouple the hydrophobic interactions between polymer molecules. In addition, cyclodextrins inclusion complexes are known to interact with water-soluble polymers forming ternary complexes consisting of the drug molecule, cyclodextrins, and the polymer chain (Hsiue et al.,1998; Kim et al.,2002; Kristinsson et al.,1996; Loftsson and Brewster,1996; Patel and Vavia,2006). In the ternary complex, the polymer partly or totally coats the inclusion complex, interacting with both the drug and the βCD molecule through hydrogen bonding (Valero et al.,2003). This inclusion can reduce the interaction between polymer chains and therefore, the polymer chains are wholly disentangled and well aligned in the direction of flow, resulting in a lower viscosity.

The viscosity decrease provides both advantages and disadvantages for formulation development. The lower viscosity microbicide product can be more easily applied in the vagina,

but may lead to more leakage and thus decrease the dosing level. The ideal viscosity for a microbicide product still needs to be further investigated

5.3.1.3 Disintegration Evaluation of PVA Film

Disintegration test results for PVA films are shown in Figure 5-8. The disintegration time was shortened significantly with the incorporation of UC781 complexes compared to placebo film ($p<0.05$), decreasing from 34 min for placebo film to 14 min for UC781:MβCD complex containing films. This decrease in disintegration time is in accordance with the mass ratio increase in complex, indicating that cyclodextrin enhances the disintegration of the PVA film during the disintegration process. Notable, the data indicate that MβCD has a more significant impact on the disintegration time of PVA films than HPβCD.

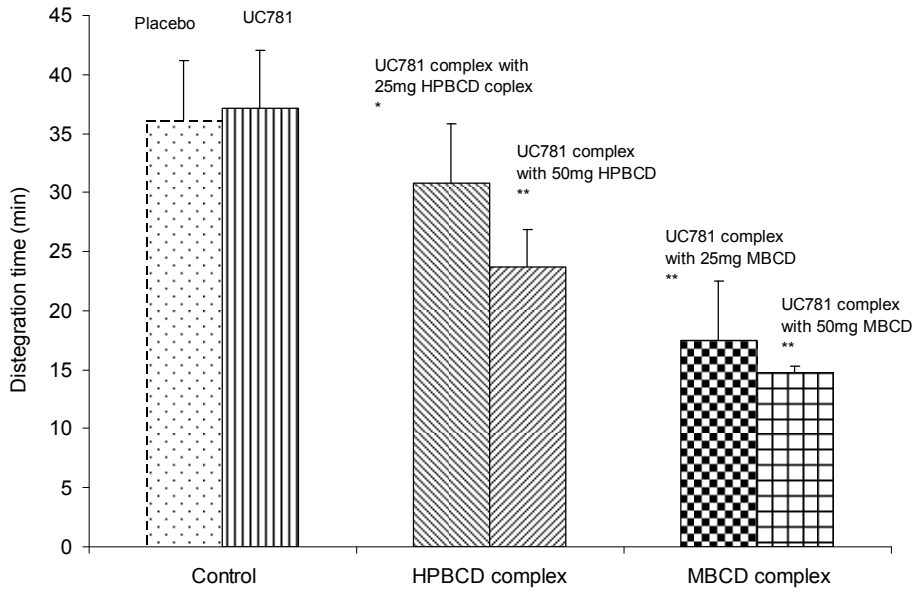


Figure 5-8. Disintegration time of PVA film

*: $p<0.05$; **: $p<0.01$

Disintegration is the first step toward dissolution of a solid dosage form. A complete disintegration is defined as that state in which any residue of the unit remaining on the screen of

the test apparatus is a soft mass having no palpably firm core (USP,2000). The official disintegration test for vaginal preparations requires a large volume of disintegrating fluid (1L of water). In our study, 3 ml of water were used. This was sufficient volume to keep test PVA film immersed, but provided a low volume that more closely represents the physical environment of the vagina (Owen and Katz,1999). The disintegration process was accelerated significantly when the complexed form of UC781 was incorporated into the film for both HPβCD and MβCD. This can be explained by the interaction between cyclodextrin and the PVA chains, leading to a weaker interaction between polymer chains.

Our studies suggest that the disintegration time of PVA films could be improved by the incorporation of βCD into films. βCD can enhance the disintegration of PVA film through reducing the interaction among PVA molecules. This effect of βCD on PVA film will help to improve the release of UC781 from the film due to the hydrophobic properties of UC781 itself. However, additional studies are required to fully characterize the mechanism for the enhanced disintegration of PVA film containing CD.

5.3.1.4 Dissolution Evaluation of UC781 Release from MC Gel, HEC Gel and PVA Film

The purpose of complexation of UC781 with cyclodextrins is to enhance the solubility of UC781 and obtain a rapid release of UC781 from the formulation in order to provide more immediate protection from sexual HIV transmission. Studies were conducted to investigate the impact of cyclodextrin on the viscosity, osmolality, and disintegration of PVA film. The results indicate that complexation of UC781 with HPβCD or MβCD may improve the dissolution of UC781. The dissolution of UC781 from three different formulations was investigated in vaginal

fluid simulant (VFS) at 37 °C. VFS was used to mimic the physical environment of the vagina. Released UC781 in VFS was expressed as a percentage of loaded UC781 as mean ± SD.

Incorporation of the complexed form of UC781 in all three formulations showed greatly enhancement on the release of UC781. All formulations containing non-complexed UC781 showed a relatively slower initial release of the drug during initial stages of dissolution testing than that of complexed UC781 containing formulations.

For MC and HEC gel formulations, UC781 was only found in the dissolution medium after the first 3 min for non-complexed UC781 containing MC gel (Figure 5-9 and Figure 5-10) and after 5 min for HEC gel (Figure 5-11 and Figure 5-12) in non-complexed UC781 gels. Conversely, over 8% of UC781 was released within 3 min ($p<0.05$) from all HPβCD or MβCD complexed UC781 containing formulations. For PVA film formulations, more than 20% of UC781 was released from complex containing films compared to around 10% of UC781 released from non-complexed UC781 containing film within 3 min ($p<0.05$) as shown in Figure 5-13 and Figure 5-14.

In all non-complexed UC781 containing formulations (gels and film), no more than 40% of UC781 was released over a 60 min time frame. Conversery, about 80% of UC781 was released in all complex containing formulations in this same period.

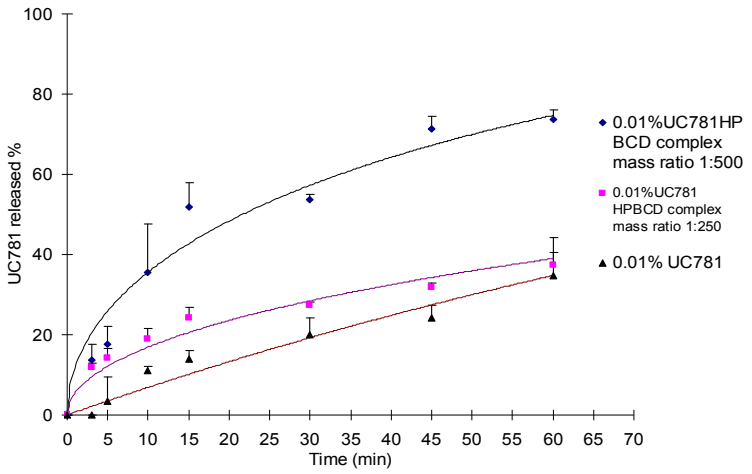


Figure 5-9. Effect of UC781:HPβCD complex on UC781 release from MC gel

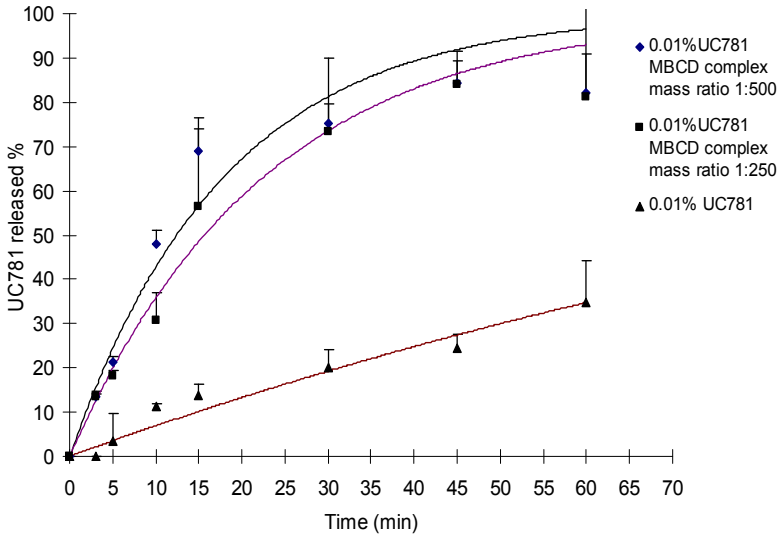


Figure 5-10. Effect of UC781:MβCD complex on UC781 release from MC gel

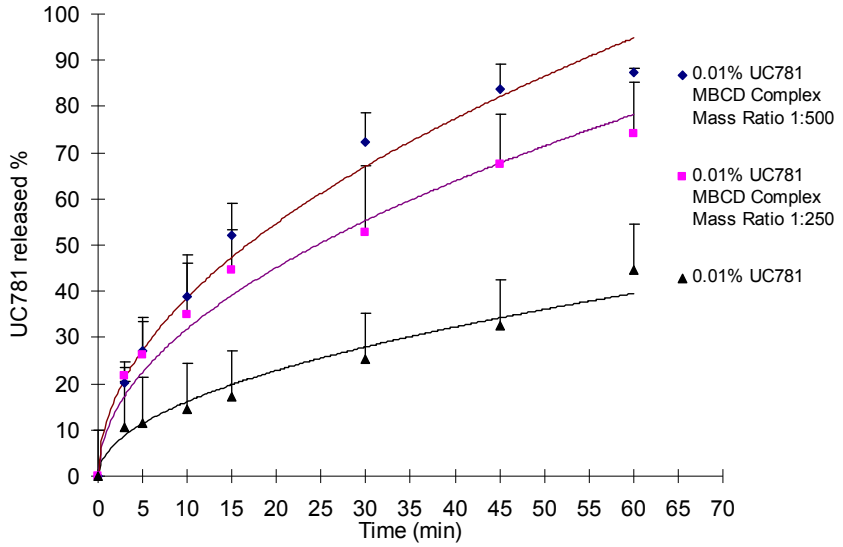


Figure 5-11. Effect of UC781:HPβCD complex on UC781 release from HEC gel

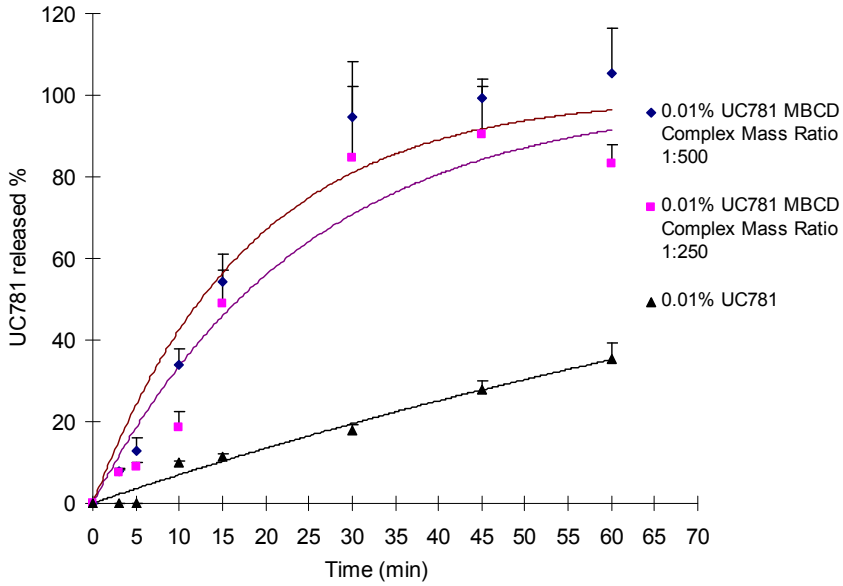


Figure 5-12. Effect of UC781:MβCD complex on UC781 release from HEC gel

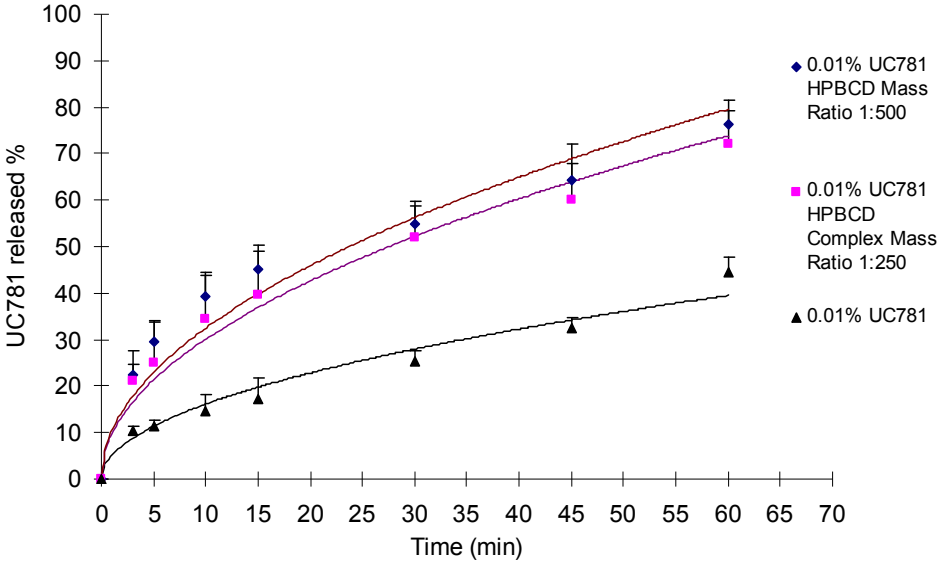


Figure 5-13. Effect of UC781:HPβCD complex on UC781 release from PVA Film

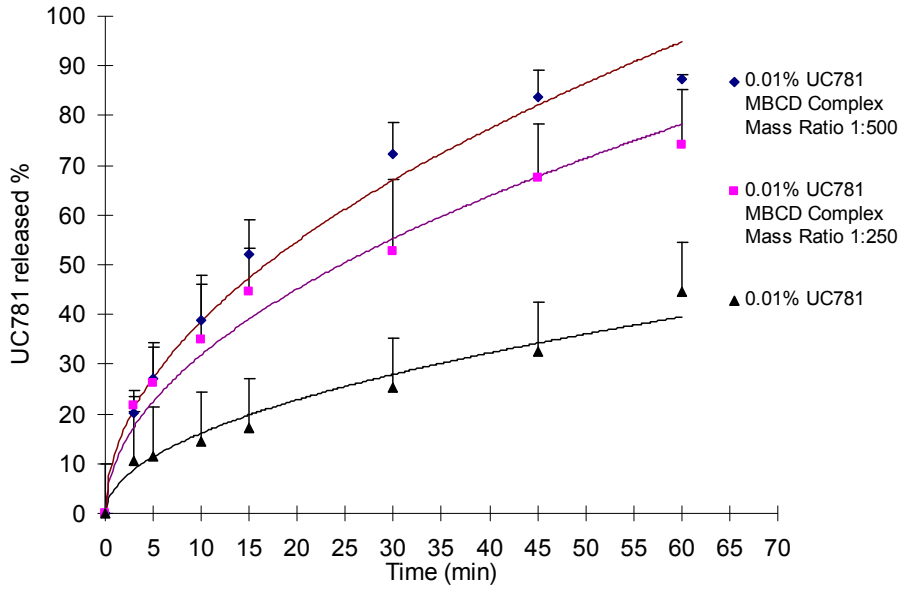


Figure 5-14. Effect of UC781: MβCD complex on UC781 release from PVA Film

Accumulated drug release at 10 min (Table 5-2) and 60 min (Table 5-3) was selected for comparison. From our studies, the complexation of UC781 can significantly improve the release

of UC781 at 10 min ($p<0.05$, t-test) and 60 min ($p<0.05$, t-test) in MC gel, HEC gel, and PVA films in MC gel. The data suggested that the complexation of UC781 could greatly enhance both extent and speed of drug release. These attributes would provide great advantage for microbicide products. This would result in a rapid drug release and more immediate protection against HIV transmission.

Table 5-2. Accumulated UC781 release in 10 min (percentage %)

	MC gel	HEC gel	PVA film
0.01% UC781	11.18±0.85	10.13±0.40	14.45±3.79
0.01% UC781 HPBCD complex (1:250)	18.93±2.60*	14.45±0.87*	34.28±10.12**
0.01% UC781 HPBCD complex (1:500)	35.45±12.24 **	35.45±12.24**	39.13±4.73*
0.01% UC781 MBCD complex (1:250)	30.73±6.19 **	18.63±4.04*	35.08±11.07**
0.01% UC781 MBCD complex (1:500)	47.88±3.16 **	34.03±3.88**	38.88±8.90*

*: $p<0.05$; **: $p<0.01$

Table 5-3. Accumulated UC781 release in 60 min (percentage %)

	MC gel	HEC gel	PVA film
0.01% UC781	34.68±9.44	35.50±3.89	44.57±3.11
0.01% UC781 HPBCD complex (1:250)	37.50±3.08	57.05±1.06**	72.12±9.41**
0.01% UC781 HPBCD complex (1:500)	73.60±2.49**	73.60±2.49**	76.17±2.92**
0.01% UC781 MBCD complex (1:250)	81.10±20.24**	83.28±4.62**	74.15±11.08**
0.01% UC781 MBCD complex (1:500)	82.05±8.91**	105.31±11.18**	87.37±0.73**

*: $p<0.05$; **: $p<0.01$

The power law was used to analyze the UC781 release pattern according to Equation 5-1. The n value decreased in all three formulations with the increase in CD concentration in the complex. This indicates a change of UC781 release pattern caused by complexation. The parameters for fitting are shown in Table 5-4.

Table 5-4. Calculated *K* and *n* following Power law

	UC781		UC781 :HPβCD complex (1:250)		UC781 :HPβCD complex (1:500)		UC781 :MβCD complex (1:250)		UC781 :MβCD complex (1:500)	
	K	***n***	***K***	***n***	***K***	***n***	***K***	***n***	***K***	***n***
Film	3.84	0.58	13.06	0.41	15.71	0.38	13.79	0.41	13.98	0.46
MC gel	1.51	0.76	11.42	0.47	8.24	0.36	11.57	0.51	16.51	0.42
HEC gel	0.85	0.91	2.70	0.76	11.42	0.47	7.15	0.64	8.74	0.63

The *k* values reflect the drug dissolution rate from the formulation into the dissolution system. The larger the k value, the quicker the drug dissolution from the formulation. In all formulations, the *k* values for complexed UC781 containing formulations are much higher than those obtained for non-complexed UC781 formulations. In addition, the *k* value increase follows a dose dependent relationship with respect to cyclodextrin concentration in the complex.

The release of UC781 from all gels and film formulations containing non-complexed UC781 displayed an anomalous pattern (n values are between 0.5 and 1.0), most likely due to the relative contributions of drug diffusion, polymer relaxation, and matrix erosion. The release pattern of non-complexed UC781 from MC gel (n=0.91), HEC gel (n=0.76), and PVA film (n=0.58) indicate differences in mechanism of drug release for the three different formulations. The higher n value (non-Fickian release) obtained for the MC gel suggests that drug release is controlled by matrix erosion and drug dissolution in this formulation. For the PVA films, the range of n values is in the neighborhood of the exponent 0.5, which is the theoretical value for a diffusion controlled pattern (Fickian release). The lower n value obtained for the PVA film group compared to those of MC and HEC gels indicates that UC781 is released faster from the film formulation than from gel formulations.

For the formulations containing complexed UC781, n values decreased greatly in comparison to those of non-complexed UC781 containing formulations. These results suggest that UC781 release is predominately controlled by its enhanced dissolution due to the increased water enhancement of complexed UC781 with either HPβCD or MβCD.

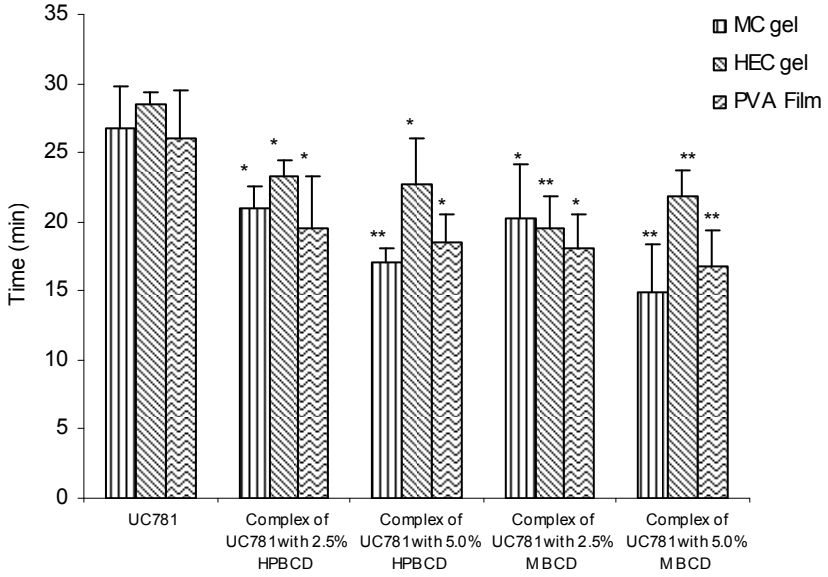
Mean dissolution time (MDT) of UC781 and its complexes was also evaluated and are shown in Table 5-5 and Figure 5-15. The complexed form of UC781 in all three formulations

greatly decreases the MDT of UC781 and follows a dose dependent relationship based on the amount of HPβCD or MβCD in the formulation ($p<0.05$, t-test, one tail).

Table 5-5. MDT (min) of complexed UC781 in different formulations

	UC781	UC781 :HPβCD complex (1:250)	UC781 :HPβCD complex (1:500)	UC781 :MβCD complex (1:250)	UC781 :MβCD complex (1:500)
Film	26.09±3.45	19.46±3.88*	18.47±2.11*	18.05±2.43*	16.84±2.49**
MC gel	26.80±3.02	20.93±1.63*	17.02±1.10**	23.36±7.36*	14.94±3.50**
HEC gel	28.43±0.95	23.35±1.11*	22.72±3.25*	19.52±2.29**	21.77±1.89**

*: $p<0.05$; **: $p<0.01$



*: $p<0.05$; **: $p<0.01$

Figure 5-15. MDT of complex UC781 in different formulations

MDT data also show that the complexed UC781 is more readily dissolved than non-complexed UC781 in all three tested formulations. This decrease in MDT is related to the βCD amount in UC781 complex. Greater amounts of βCD in the UC781 complex resulted in shorter MDT values. With respect to the PVA film dosage from containing HPβCD or MβCD complexed with UC781, this data shows that the film is fast dissolving with rapid release of UC781.

In our studies, the complexation of UC781 with HPβCD or MβCD not only increases the solubility of UC781 in the aqueous phase, but also changes the release behavior of UC781 from the formulations. The drug release rate and pattern were changed due to the incorporation of cyclodextrins complexed with UC781. The complexation of UC781 is an effective technique for the design of quick dissolving dosage forms with rapid drug release.

5.3.2 Preformulation evaluation of *in vitro* toxicity of cyclodextrins

5.3.2.1 Toxicity Evaluation of Cyclodextrins in HeLa and A431 Cell Models

The toxicity data obtained from studies in which HeLa cells or A431 cells were exposed to HPβCD or MβCD (1% to 20%) for 30 min, 1 h, or 2 h is shown in Figure 5-16 (HPβCD in HeLa cell), Figure 5-17 (MβCD in HeLa cell) and Figure 5-18 (HPβCD in A431 cell) and

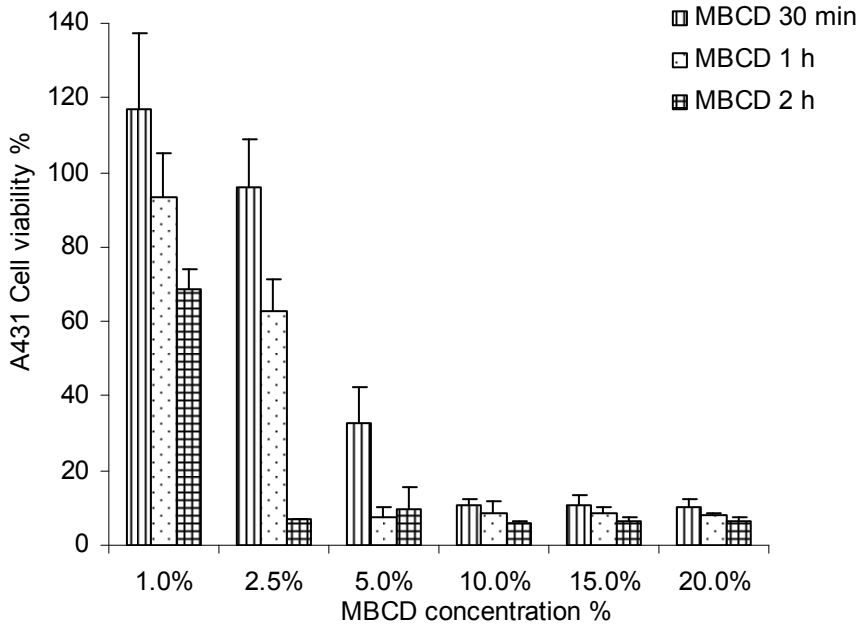
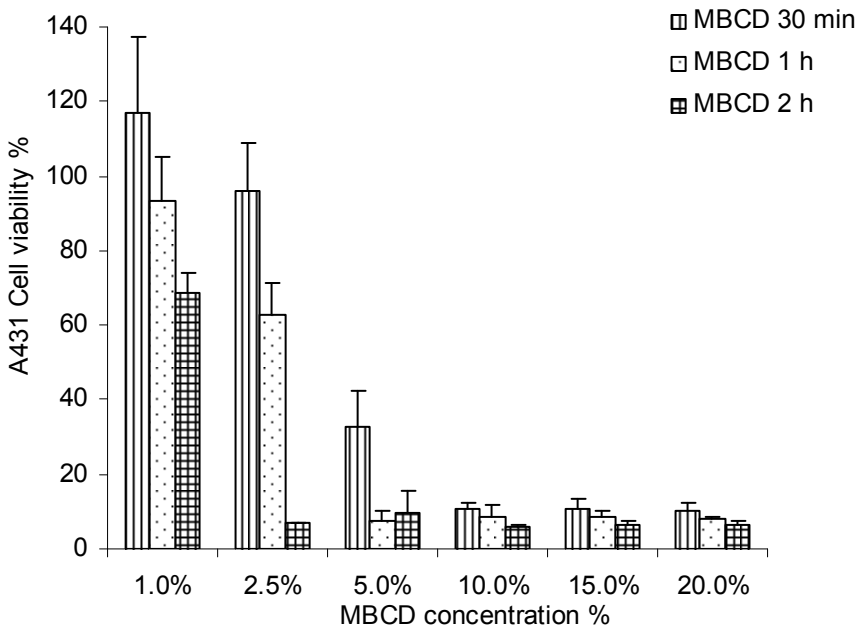


Figure 5-19 (MβCD in A431 cell). The data shows that cell viability decreases with both increased βCD concentration and exposure time. An 80% viability represents the damage threshold to evaluate the cytotoxic effect of HPβCD and MβCD according to other previous cytotoxicity studies (Kim et al.,2004) (Wang et al.,2004) (Maniratanachote et al.,2005). Using this threshold for toxicity, a 2 h exposure to 1% HPβCD (Figure 5-16) is comparatively safe. However, a 2 h exposure to 1% for MβCD is toxic (Figure 5-17).

In the A431 cell model, the safe concentration was found to be is 2.5% for HPβCD (Figure 5-18) and less than 1% MβCD



(

Figure 5-19) for 2 hour exposure.

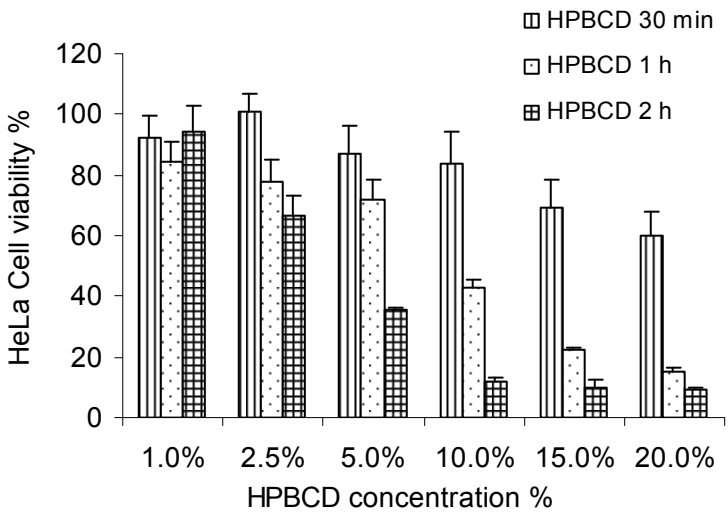


Figure 5-16. Cytotoxicity of HPβCD on HeLa cell

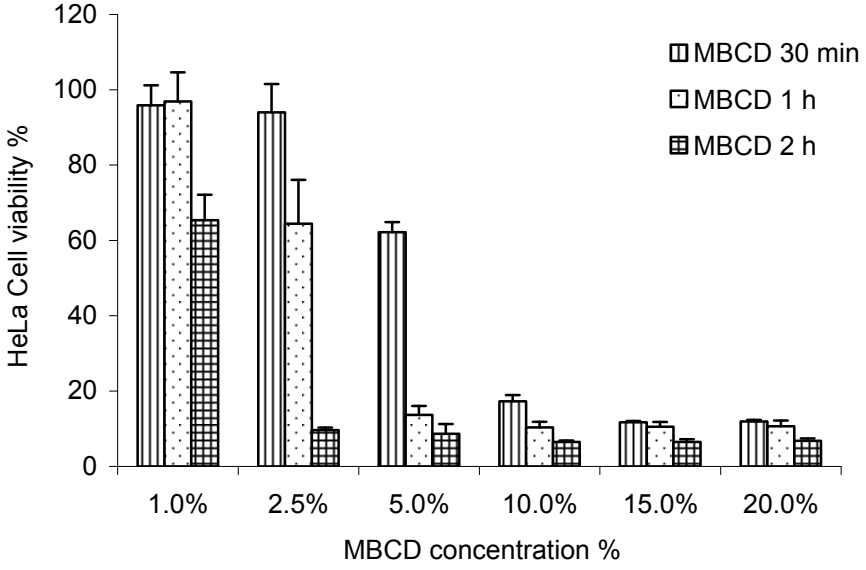


Figure 5-17. Cytotoxicity of MβCD on HeLa cell

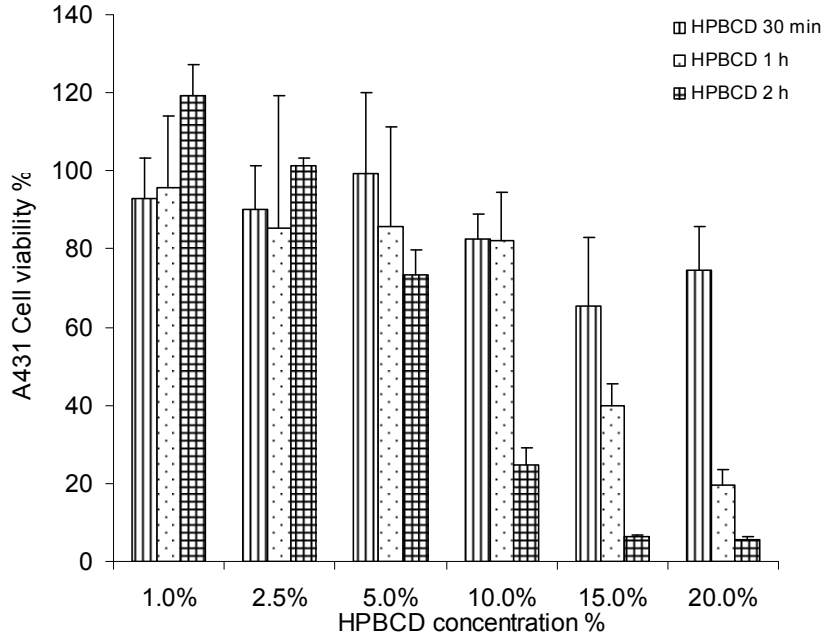


Figure 5-18. Cytotoxicity of HPβCD on A431 cell

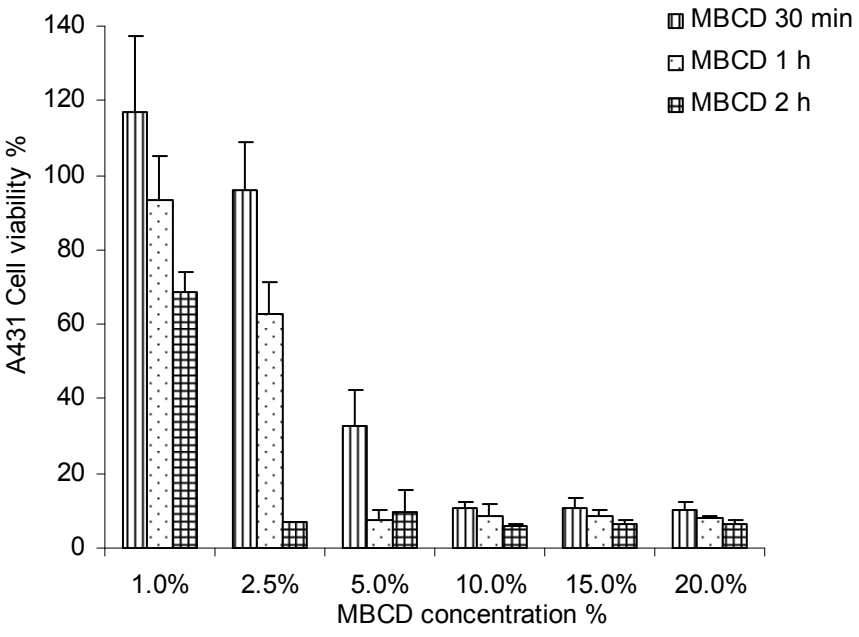


Figure 5-19. Cytotoxicity of MβCD on A431 cell

The impact of the presence of water-soluble polymers on toxicity following exposure to cyclodextrin for 2 h was evaluated in both the HeLa cell and the A431 cell model. These data show that all polymers evaluated in both the HeLa and A431 cell-based model reduced the toxicity of both HPβCD and MβCD.

The reduction in toxicity for water-soluble polymers as shown in the HeLa cell model is shown in Figure 5-20 (HPMC), Figure 5-21 (HEC), Figure 5-22 (PVA), and Figure 5-23 (PVP K30). The concentration of HPβCD can be increased to 5% while maintaining 80% cell viability when the exposure included the presence of either 0.5% or 1.0% of all four polymers used in experiments. In the absence of polymers, the HPβCD concentration must be 1% or less to maintain an 80% cell viability level. Moreover, recall that in the absence of polymers, the toxic concentration for MβCD was found to be less than 1%. Incorporation of water-soluble polymers with MβCD resulted in 80% cell viability being maintained even at the 1% level. Comparing individual polymers evaluated, It is observed that HPMC, HEC, and PVA can maintain cell viability of HeLa cells more effectively than PVP K-30 in the HPβCD treated groups. This indicates that HPMC, HEC, and PVA are safer components than PVP K30 in the formulations containing HPβCD.

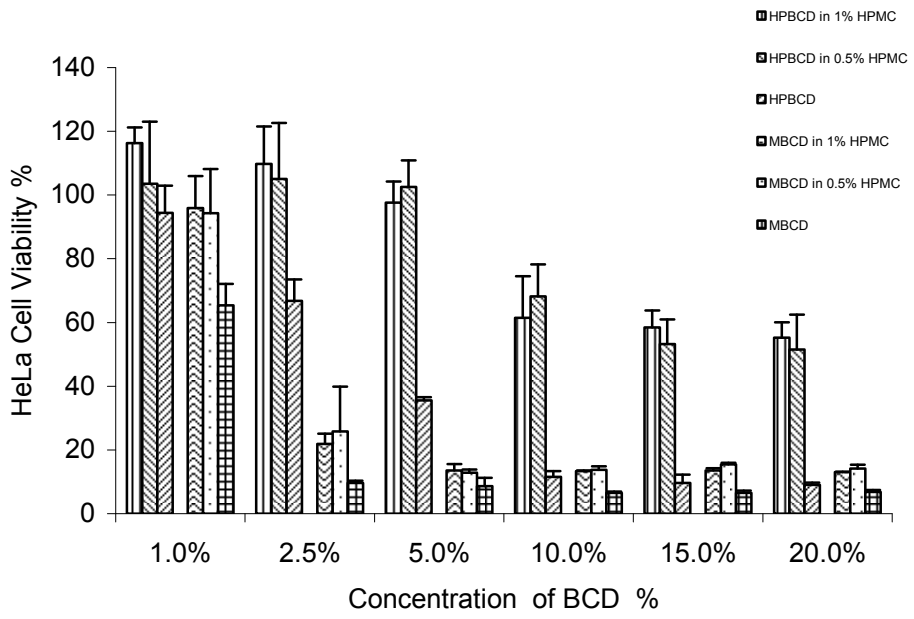


Figure 5-20. HPMC protection effect on cyclodextrin toxicity in HeLa cells in 2 hour exposure

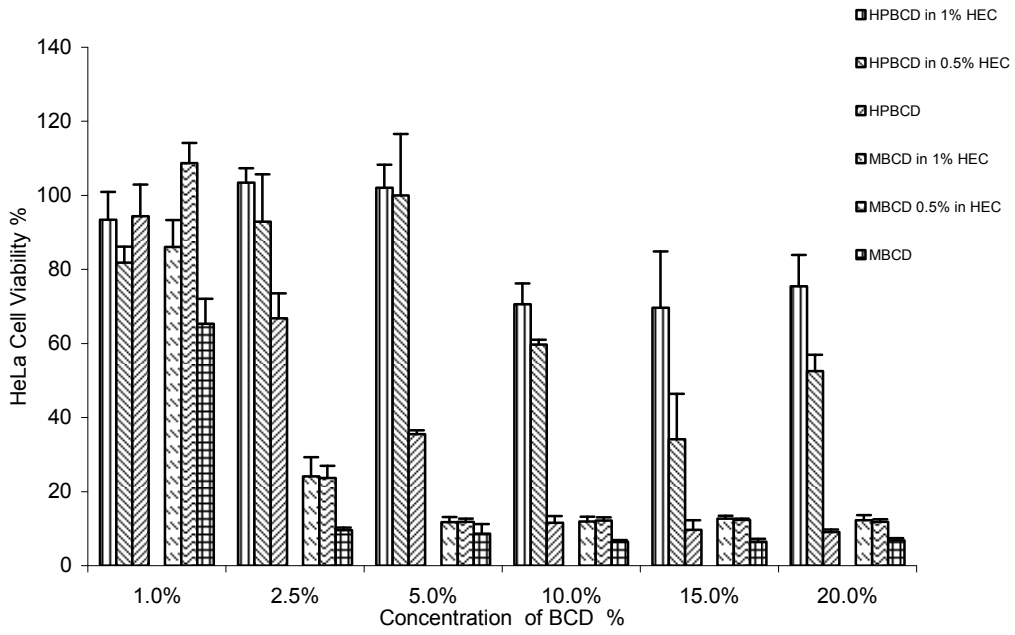


Figure 5-21. HEC protection effect on cyclodextrin toxicity in HeLa cells in 2 hour exposure

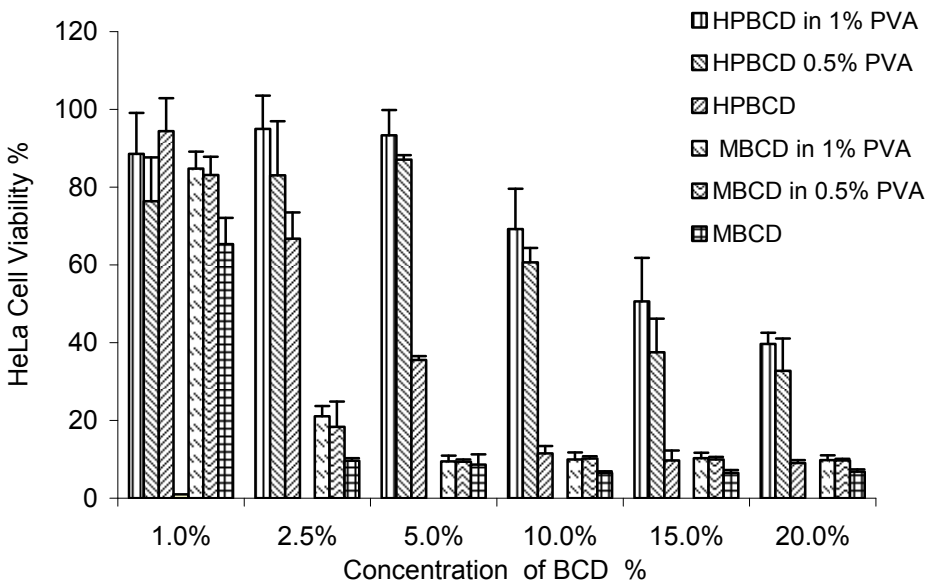


Figure 5-22. PVA protection effect on cyclodextrin toxicity in HeLa cells in 2 hour exposure

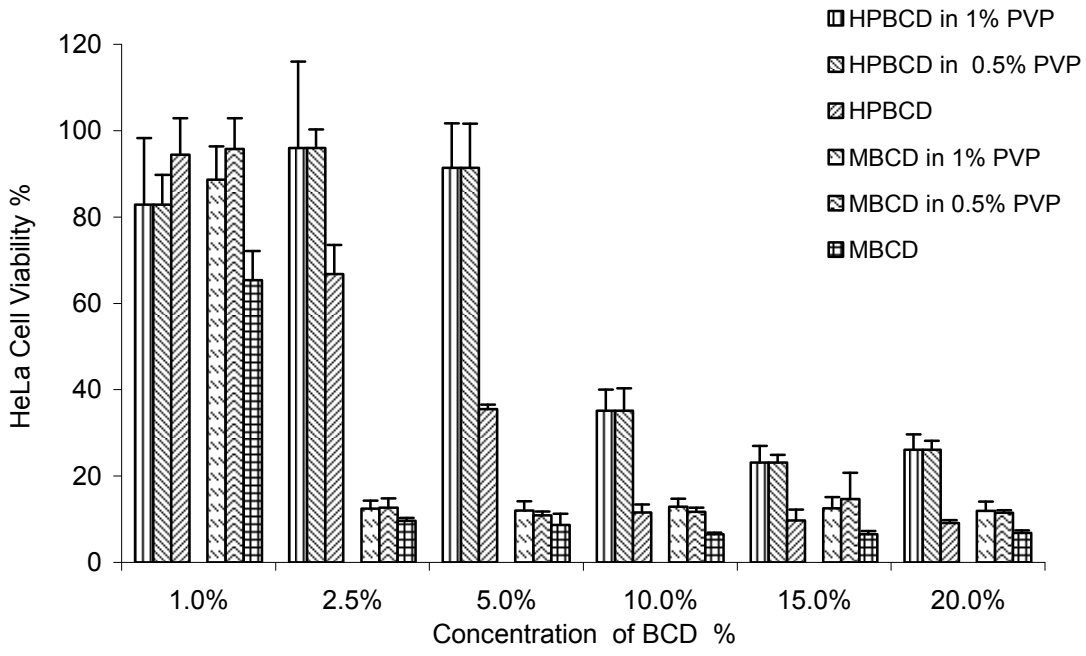


Figure 5-23. PVP K30 protection effect on cyclodextrin toxicity in HeLa cells in 2 hour exposure

In the A431 cells, similar results were observed as shown in Figure 5-24 (HPMC), Figure 5-25 (HEC), Figure 5-26 (PVA), and Figure 5-27 (PVP K30). Incorporation of water-soluble polymers leads to greater protection in the A431 cell model as compared to the HeLa cell model. The safe concentration of HPβCD (that which maintains 80% cell viability) was increased to 10 % in the presence of 1% of HPMC, HEC, PVP K-30 or PVA. Moreover, 2.5% of MβCD was shown to be safe in the A431 cell model in the presence of 1% water-soluble polymer. These results suggested that A431 cells are less susceptible to the toxic effect of either HPβCD or MβCD than the HeLa cells.

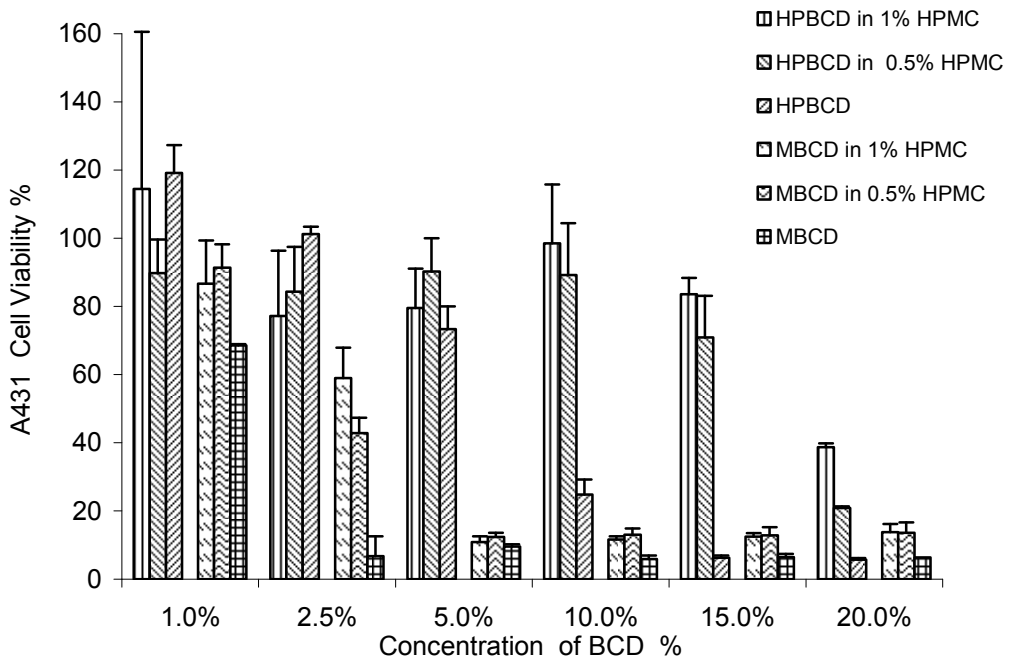


Figure 5-24. HPMC protection effect on cyclodextrins toxicity in A431 cells in 2 hour exposure

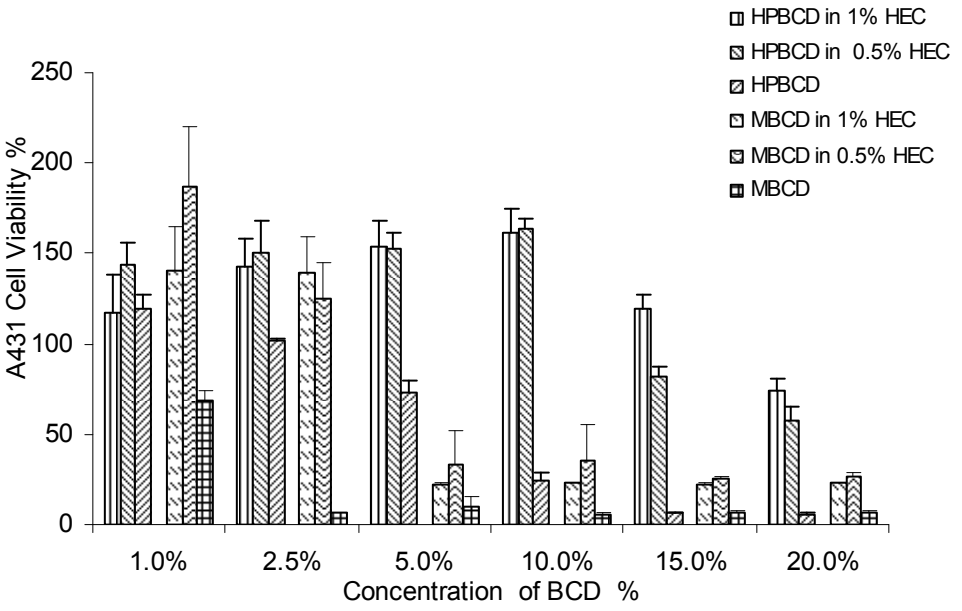


Figure 5-25. HEC protection effect on cyclodextrin toxicity in A431 cells in 2 hour exposure

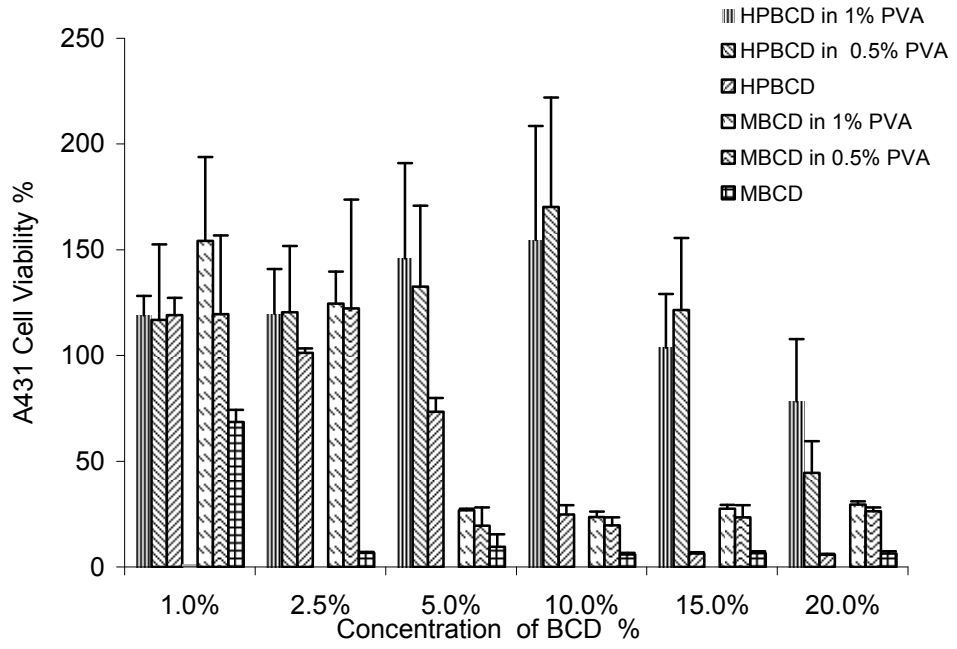


Figure 5-26. PVA protection effect on cyclodextrin toxicity in A431 cells in 2 hour exposure

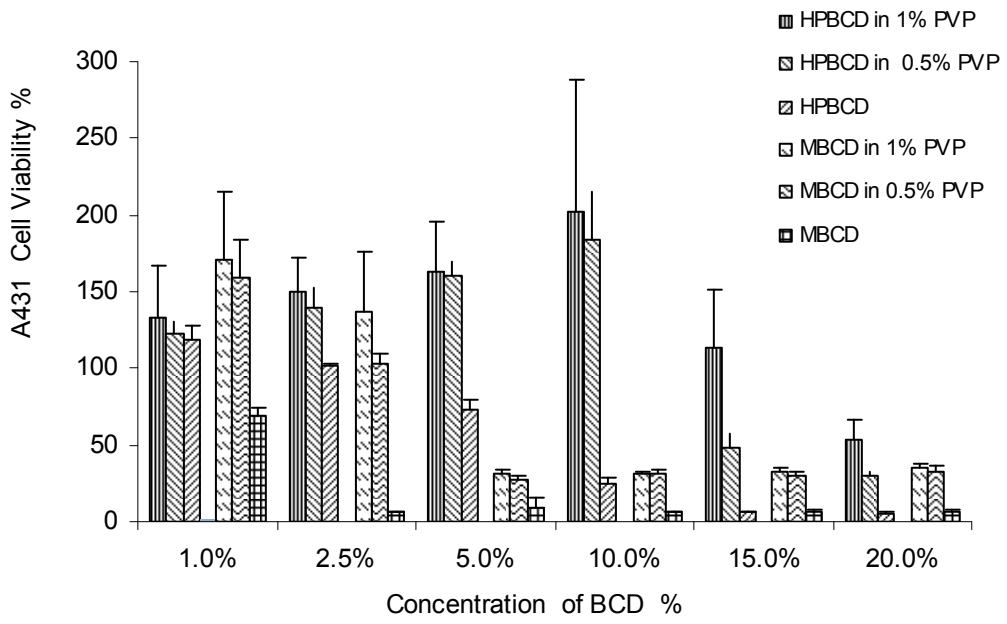


Figure 5-27. PVP K-30 protection effect on cyclodextrin toxicity in A431 cells in 2 hour exposure

Due to the polymeric matrix of most dosage forms, the impact of polymers in the formulations on active agents should be evaluated for their efficacy or toxicity. In our studies, a microbicide product was developed for UC781complexed with either HPβCD or MβCD for the prevention of HIV transmission; the toxicity of HPβCD or MβCD was a factor of concern in the formulation development. Using cell models, the impact of water-soluble polymers on HPβCD and MβCD was evaluated using an MTT assay. From these data, the water-soluble polymers were shown to provide protective effect from cyclodextrin toxicity. Therefore, incorporation of any of the four polymers evaluated into a formulated product for the delivery of cyclodextrins complexed UC781 will result in a formulation with a more acceptable toxicity profile.

5.3.2.2 Toxicity Evaluation of Cyclodextrins on Excised Human Cervical Tissue in The Presence of Water-Soluble Polymers

The protective effect of water-soluble polymers on cyclodextrins was shown in a cell-based model as described in section 5.3.1.1. However, the cell model utilized are only a first step toward toxicity assessment, since they provide only a simple system for toxicity evaluation. Further studies are necessary for toxicity evaluation of HPβCD and MβCD. To this end, studies were conducted in a human cervical epithelial model. This model provides a more comfort mechanism to evaluate toxicity in a more complex model, which is more physiologically relevant to the development of microbicide products.

Figure 5-28 to Figure 5-30 represent the data obtained for the toxicity evaluations in the excised human cervical tissue model. In these studies, excised human cervical tissue was exposed to either HPβCD or MβCD in the presence or absence of 1% water-soluble polymers in DMEM culture media for 2 hours. A DMEM exposure was used as a control. These results showed that toxicity is dependent on the cyclodextrin concentration used in the experiment. Water-soluble polymers included can effectively protect from the toxicity of HPβCD or MβCD at all concentrations used in experiment. Tissue viability was improved in the presence of polymers as compared to viability obtained in the absence of polymers. Importantly, in studies incorporation of 1% HPMC with the CD, no tissue toxicity was found at the 10% level of HPβCD exposure (Figure 5-29) or at 5% level of MβCD (Figure 5-30) exposure comparable to tissue viability obtained for the DMEM control group (containing no polymers) ($p>0.05$, unpaired t-test, one tail). These results indicated that HPMC could be safely used in microbicide products containing βCD based drug delivery systems.

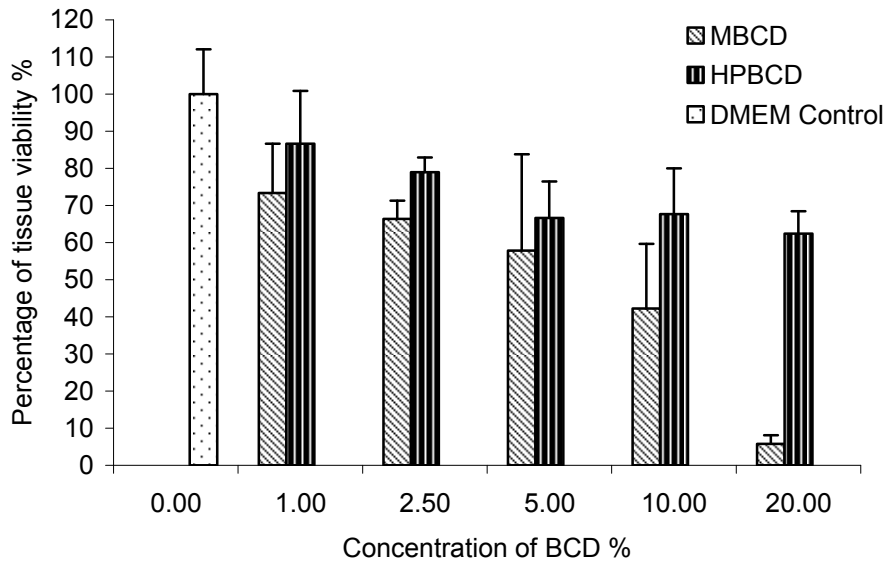


Figure 5-28. HPβCD and MβCD toxicity on Human Cervical Tissue in 2 horu exposure

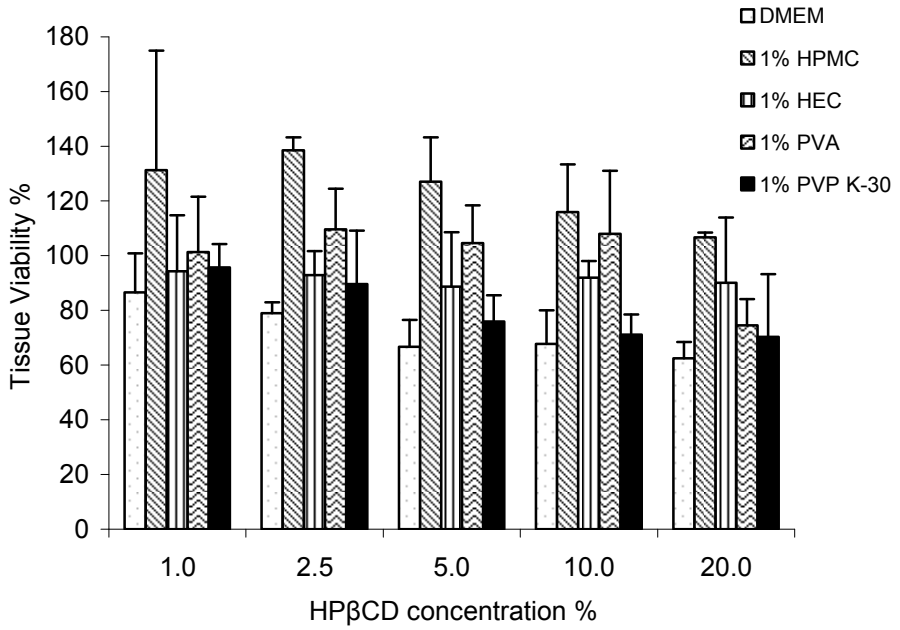


Figure 5-29. Polymers protection effect on HPβCD toxicity on Human Cervical Tissue in 2 hour exposure

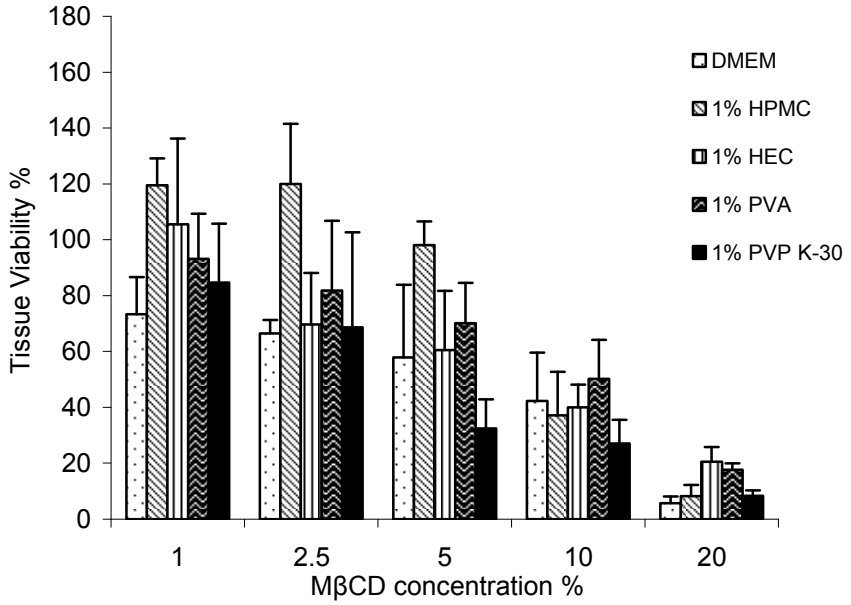


Figure 5-30. Polymers protection effect on MβCD toxicity on Human Cervical Tissue in 2 hour exposure

Our data suggested that water-soluble polymers can greatly protect cells and excised human cervical tissue against HPβCD and MβCD. These polymers may reduce the toxicity of CDs through their interaction with CD. HPMC is the most potent of all water-soluble polymers tested in these experiments in their ability to provide protection from toxicity.

Inactive excipients are always required to formulate drug substances into drug products. In our studies, all four water-soluble polymers were shown to effectively protect from the potential toxicity of HPβCD or MβCD in both cell and tissue models. Additionally, all four polymers are able to enhance the complexation efficiency of UC781 with HPβCD or MβCD.

Our data show that different excipients may play an important role in the toxicity profile of formulations as well as the complexation efficiency of UC781 with HPβCD or MβCD. With the toxicity and complexation information that is provided, optimization of complexed UC781 containing formulations with regard to toxicity is possible. However, it is important to assure

that the formulation maintains anti-HIV activity. These studies demonstrate that HPMC can be included in cyclodextrins complexed UC781 containing formulations to achieve a more acceptable toxicity profile and more efficient complexation of UC781.

5.3.3 *Ex vivo* Toxicity Evaluations of Cyclodextrin Alone and UC781: Cyclodextrins Complexes in The Formulated State

5.3.3.1 Toxicity Evaluation of HPβCD and MβCD Containing Formulations in an Excised Human Tissue Model

In addition to the physical and chemical properties of formulations, it is important to consider the toxicity of formulations to the vaginal epithelium, especially in the prevention of sexual transmission of HIV. Any toxicity from formulation may result in alteration of the epithelial barrier, which may facilitate pathogen entry. Cyclodextrins are known to modify membranes due to their complexation with cholesterol present in membranes (Ilangumaran and Hoessli,1998). The toxicity of any formulations on the vaginal epithelium must be evaluated for the development of a successful microbicide product.

Our MTT assay data show that 0.5% of N9 can cause great damage to cervical tissue. Different toxicity profiles for cervical tissues were observed for formulations containing either HPβCD or MβCD, as shown in Figure 5-31. When formulated in the MC gel, tissue viability decreased as a function of HPβCD or MβCD concentration, whereas formulation in the HEC gel or PVA film lead to a better toxicity profile in that toxicity was not observed even at levels of 20% HPβCD or 5% MβCD. MβCD showed significant toxicity when its concentration was greater than 10%, regardless of the formulation ($p<0.05$, t-test, one tail).

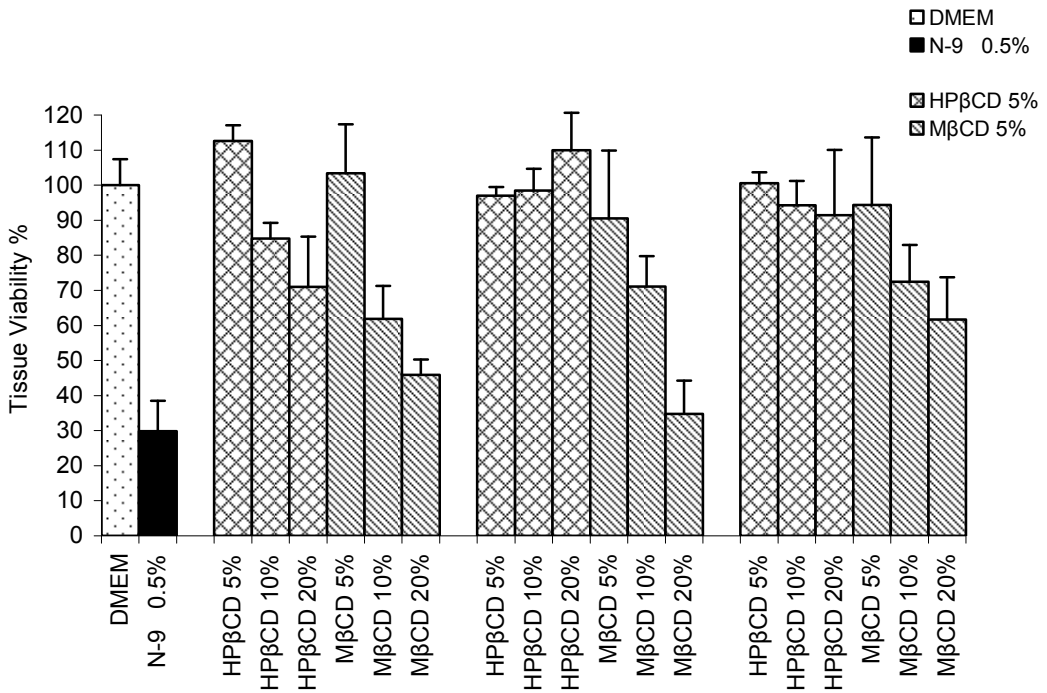


Figure 5-31. Toxicity of HPβCD and MβCD in differenct formulations

*: $p<0.05$; **: $p<0.01$

Above figure represents the data for excised human cervical tissue after exposure to formulated products containing HPβCD and MβCD. DMEM solution containing 0.5% N9was used as positive control.

For the formulations examined, a concentration-dependent and formulation selective toxicity was exhibited for HPβCD and MβCD. No toxicity was observed at the level of 5% MβCD or HPβCD in all tested formulations. For HEC gel and PVA film, no tissue damage was observed even at the level of 20% HPβCD. However, 10% or higher levels of MβCD may cause damage to epithelial cells. It is interesting that both HPβCD and MβCD showed a dose dependent pattern when formulated in MC gels, but not when formulated in HEC gels or PVA films. This suggests that the toxic effect of cyclodextrin is formulation dependent.

These results strongly suggested that the toxicity of cyclodextrins in the human cervical tissue model could be reduced in formulations compared to cyclodextrins solution. Although MβCD was more toxic than HPβCD in both cell and human cervical epithelium models, the toxicity of both cyclodextrins was decreased when formulated in MC gel, HEC gel, or PVA film. HEC gel and PVA film may provide better protection to cervical epithelium from βCD containing drug delivery systems than do MC gels. Thus, HEC gel or PVA film formulations should be considered as more suitable microbicide product platforms than MC gels for clinical use.

5.3.3.2 Toxicity Evaluation of UC781: HPβCD and UC781: MβCD Complexes Containing Formulations in An Excised Human Tissue Model

The toxicity of HPβCD or MβCD alone in three different formulations was evaluated in section 5.3.3.1. However, the toxicity profile for formulated products containing either HPβCD or MβCD complexed UC781 must also be evaluated since the solubility of UC781 is elevated in such formulations.

Figure 5-32 represents the data for tissue exposed to MC gel, HEC gel or PVA film containing UC781 complexed with either HPβCD or MβCD. No significant difference was observed between tissues treated with formulated product and DMEM treated tissues ($p>0.05$). No detectable epithelial damage was found upon exposure to any of the three formulations tested that contained complexed UC781with HPβCD or MβCD. This result correlates with the studies described in Section 5.3.3.1 that showed no observed toxicity at 5% concentrations of either HPβCD or MβCD. These results suggested that 50 mg of HPβCD or MβCD in film dosage forms or 5% HPβCD or MβCD in gel dosage forms is safe for vaginal delivery.

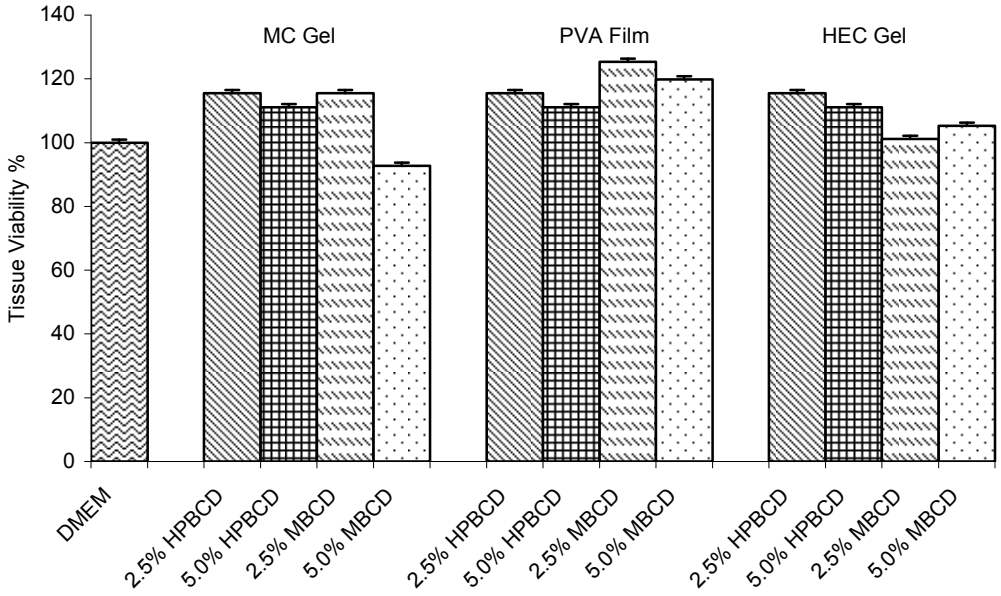


Figure 5-32. Toxicity of complexed UC781 (0.01% UC781) in MC gel, HEC gel, and PVA film

5.3.4 *In Vitro* Anti-Hiv Activity of Formulations Containing Complexed UC781

The anti-HIV activity of complexed and non-complexed UC781 needs to be evaluated in a formulated state. As an RT inhibitor, the *in vitro* bioactivity of UC781 should be investigated at both the enzyme level (RT inhibition) and the cell level (TZM-bl cell model).

5.3.4.1 RT Inhibition Assay of Formulations Containing UC781 Complexes

HIV reverse transcriptase inhibition results showed a complete RT inhibition for all UC781 containing formulations in our test HEC gel (Figure 5-33), MC gel (Figure 5-34), and PVA film (Figure 5-35). It should be noticed that some baseline RT inhitition activity was observed for all formulatios, but this activity could be quickly eliminated with product dilution.

In the MC and HEC gels, non-complexed UC781 containing formulations potently inhibited RT activity. However, this inhibition ability of formulated UC781 is lost quickly after dilution to 0.4 μg/ml or lower. Conversely, all complexed UC781 containing gels retain their RT inhibition activity as compared to non-complexed UC781with statistically significant difference ($p<0.01$).

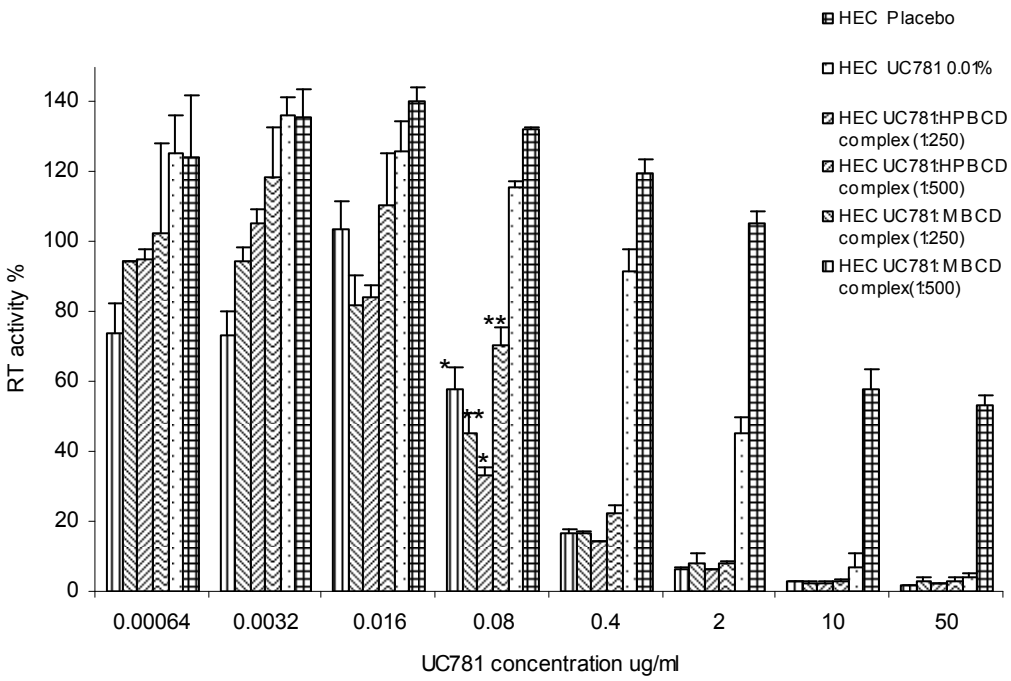


Figure 5-33. RT assay of UC781 and its complex in HEC gel

** $p<0.01$

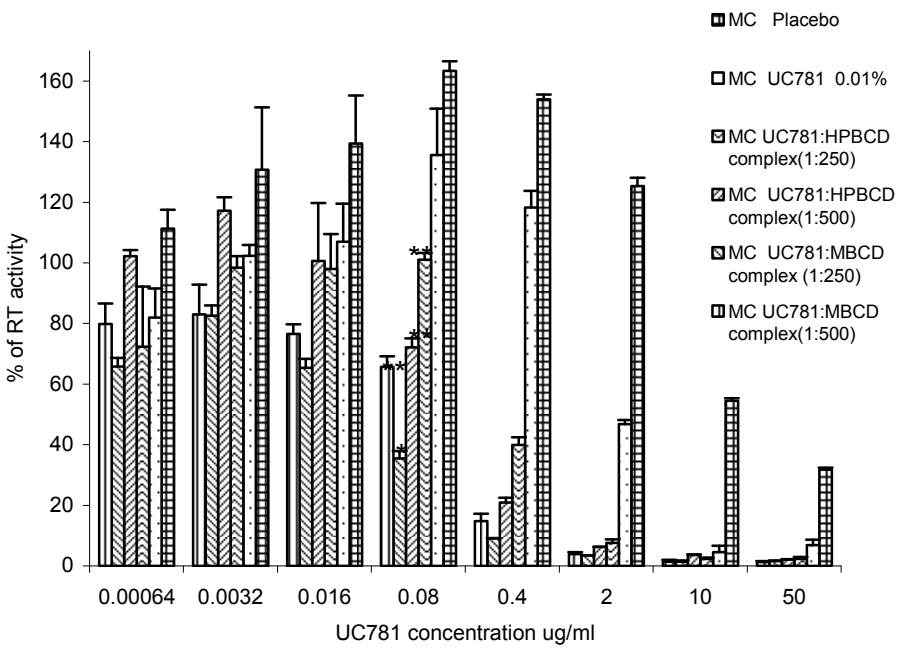


Figure 5-34. RT assay of UC781 and its complex in MC gel

**: $p<0.01$

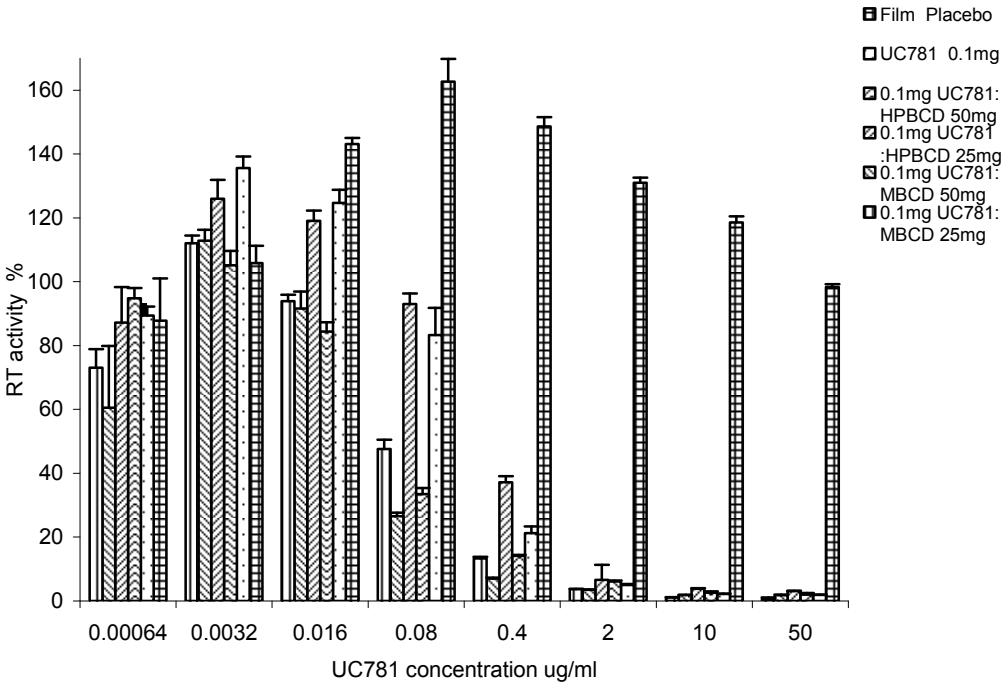


Figure 5-35. RT assay of UC781 and its complex in PVA film

** $p<0.01$

Similar RT inhibition results were observed for UC781 complex containing PVA film. Except for the complex of UC781: HPβCD (UC781: HPβCD = 1:250), all other UC781 complex containing PVA films can significantly increase the RT inhibition activity of UC781 as compared to noncompelxed UC781 ($p < 0.01$). There was no significant difference in RT inhibition between non-complexed UC781 and UC781 complexed with HPβCD (UC781: HPβCD =1:250). This may be explained by the low amount of HPβCD amount used for complexation with UC781 in this film formulation. Thus, greater amounts of HPβCD are needed for effective complexation with UC781 in the formulated state. This tendency is also observed in MC and HEC gel formulations.

The film formulation provided a better vehicle for delivery of non-complexed UC781. This is apparent given that, at a 625 times dilution, non-complexed UC781 in PVA film can still inhibit 17% RT activity as compared to no inhibition observed for MC and HEC gels at this dilution. This difference in results obtained for PVA film formulations and gel formulations may be caused by due to water cotent in formulations. PVA films formulations contain greatly less water content (less than < 1%) than that in gel formulations (over than 96% in HEC gel and over than 97% in MC gel). For this reason, the non-complexed UC781 is maintained in a highly dispersed state in PVA film as opposed to the gel formulations. This may lead to the increase of apparent solubility of UC781. Therefore, when UC781 concentrations were diluted to 0.08 μg/ml in the non-complexed UC781 containing formulations, gel formulations lost all these RT inhibition activity, whereas film formulation maintained 17% RT inhibition activity. These results suggested that PVA film formulations can maintain greater UC781 activity than gel formulations.

As detected IC_{50} value is a more sensitive index to compare the RT inhibition activity of UC781 in non-complexed and complexed form. IC_{50} values were calculated using GraphPad software for all three formulations containing either non-complexed or complexed UC781 as shown in Figure 5-36 and Table 5-6. For the MC and HEC gels, the IC_{50} of UC781 complexed with either HPβCD or MβCD is statistically significantly lower than that for non-complexedd UC781 ($p<0.05$). For PVA films, complexed UC781: HPβCD (UC781:HPβCD =1:500) and all MβCD compolexed UC781 can significantly decrease the IC_{50} values as compared to non-complexed UC781 ($p<0.01$). However, the complexed UC781:HPβCD (UC781:HPβCD =1:250) did not show the ability to improve the IC_{50} of UC781. This result suggests that the 1:250 mass ratio is not sufficient for UC781:HPβCD complexation in film formulation.

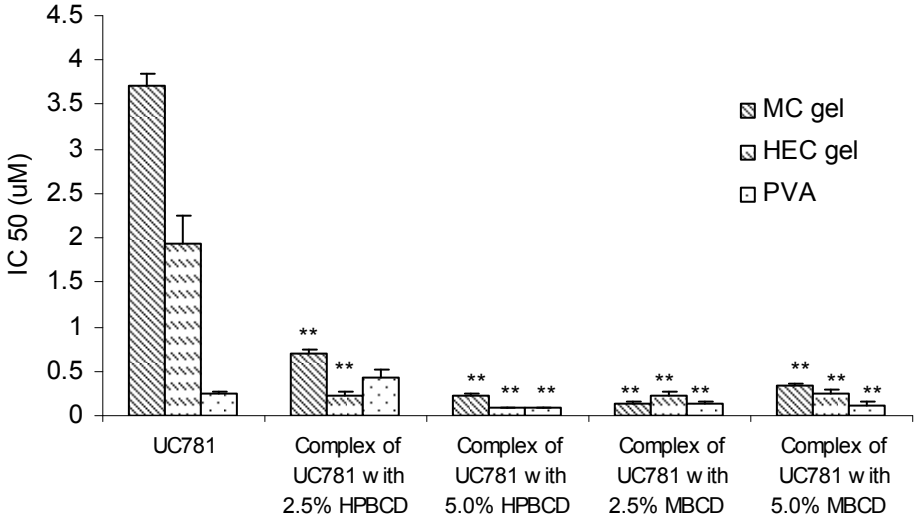


Figure 5-36. IC $_{50}$ of UC781 in MC gel, HEC gel, and PVA film

** $p<0.01$

Table 5-6. IC $_{50}$ of UC781 in MC gel, HEC gel, and PVA film

	IC_{50} μM				
	UC781	**UC781:HPβCD (1: 250).**	**UC781:HPβCD (1: 500).**	**UC781:MβCD (1: 250).**	**UC781:MβCD (1: 500).**
MC gel	3.72±0.13	0.71±0.04**	0.23±0.02**	0.13±0.03**	0.33±0.02**
HEC gel	1.94±0.30	0.22±0.05**	0.09±0.01**	0.22±0.05**	0.25±0.05**
PVA Film	0.25±0.01	0.43±0.08	0.09±0.01**	0.14±0.02**	0.11±0.04**

** $p<0.01$

The impact of formulation on RT inhibition was also observed in non-complexed UC781 containing formulations. The IC_{50} values decreased as ranked: PVA film (0.25 μM) < HEC gel (1.94μM) < MC gel (3.72 μM) ($p<0.01$). Therefore, the PVA film provides a better formulation for non-complexed UC781 as a microbicide product by maintaining higher anti-HIV activity. This can be explained by efficient solid dispersion formation of non-complexed UC781 in the PVA film formulation, which is likely caused by the processing method. In the preparation of PVA film, UC781 was dissolved into EtOH and incorporated into the polymeric film solution, which may lead to more efficient solid dispersion of UC781 increasing the solubility of UC781. This data can be supported by previous reports on UC781 solid dispersion (Damian et al.,2001; Damian et al.,2002).

In addition to these IC_{50} results, PVA film formulations can enhance the dissolution of complexed UC781 in VFS. Properties of the PVA film formulation containing complexed UC781 offer great benefit toward providing quick and effective protection against HIV infection, and greater efficacay as a microbicide product.

5.3.4.2 HIV Inhibition Assay of UC781 Complexes Containing Film Formulation in TZM-bl Cell Model

In addition to the evaluation of RT inhibition for different formulations containing complexed UC781. It is important to assess the *in vitro* inhibition of HIV replicaion prior to further *in vivo* evaluation. TZM-bl cell models were commonly used for these studies.

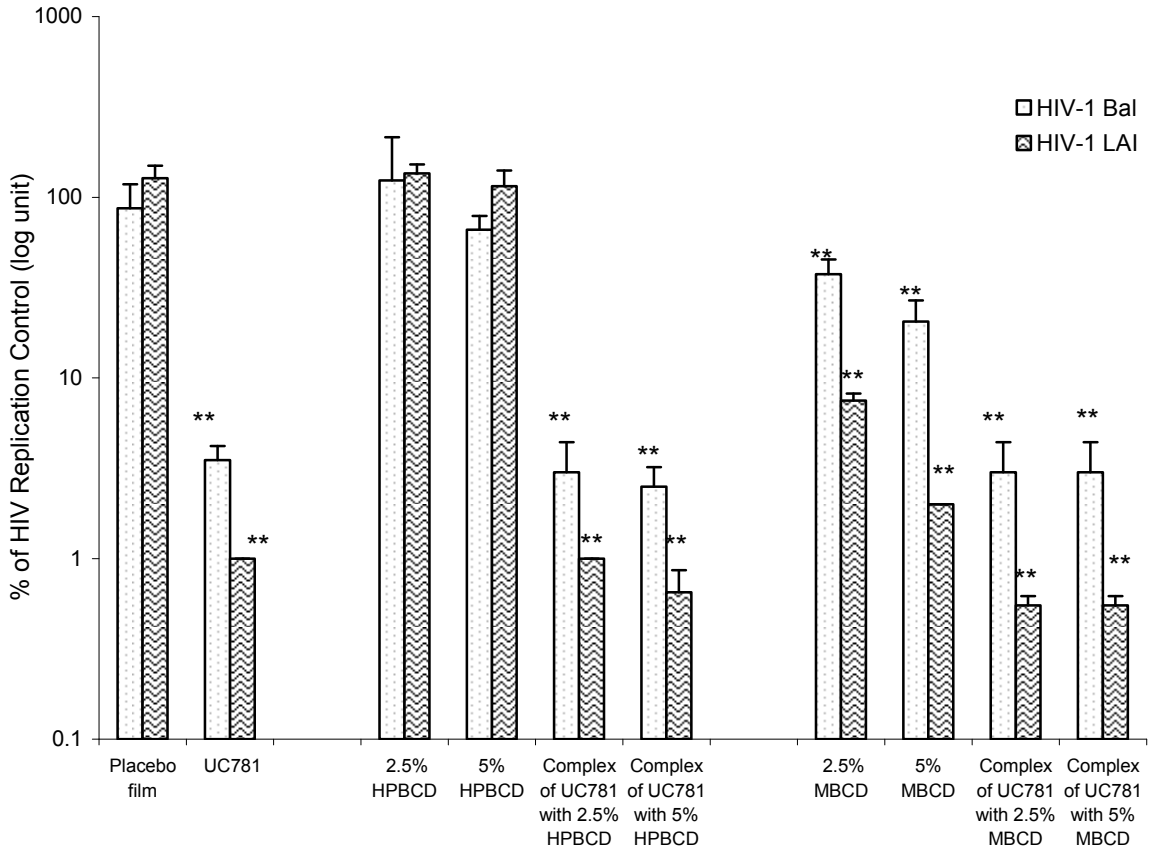


Figure 5-37. HIV inhibition assay of UC781:βCD complex film formulation in TZM-bl cells line model.
** $p<0.01$

Figure 5-37 shows results of obtained from the HIV-1 virus inhibition assay for complexed (HPβCD or MβCD) and non-complexed forms of UC781 in PVA film. Each film

contained 100 μg UC781. Films containing 25 mg or 50 mg of HPβCD or MβCD were used as comparison. PVA placebo film was used as a control. In these studies, TZM-bl cells, HIV, and UC781 were incubated together for 48 hours to obtain a full contact of all compournts.

These results showed that UC781 containing films can significantly reduce the infection of TZM-bl cells by HIV-1_{BAL} and HIV-1_{LAI} ($p<0.01$). Films containing only HPβCD exhibit no HIV-1 inhibition at concentration of 25 mg/film. But PVA film containing either 50 mg HPβCD /film, 25 mg MβCD /film or 50 mg MβCD /film greatly reduce HIV-1 infection ($p<0.01$). This observe baseline activity can be explained by the ability of CD to depelete cholesterol from viral and cell membranes (Doncel,2005; Kozak et al.,2002).

In our studies, the potency of film containing complexed UC781 was evaluated to be compared with non-complexed UC781 containing films. No significant difference was observed between non-complexed and complexed forms of UC781 (HPβCD or MβCD), which further validates the idea that the complexed form of UC781 maintaines anti-HIV activity *in vitro*.

5.3.5 Histologies Study of HPβCD and MβCD on Excised Human Cervical Tissue

5.3.5.1 *Ex Vivo* Toxicity Evaluation of HPβCD and MβCD Containing Formulations on Excised Human Cervical Tissue

Figure 5-38 shows the histology obtained from tissues exposed to either HPβCD or MβCD incorporated into the three developed formulations at the highest concentration evaluated. No gross morphological changes were observed for tissue exposed to 5%, 10%, or 20% of HPβCD or MβCD in the formulated state.

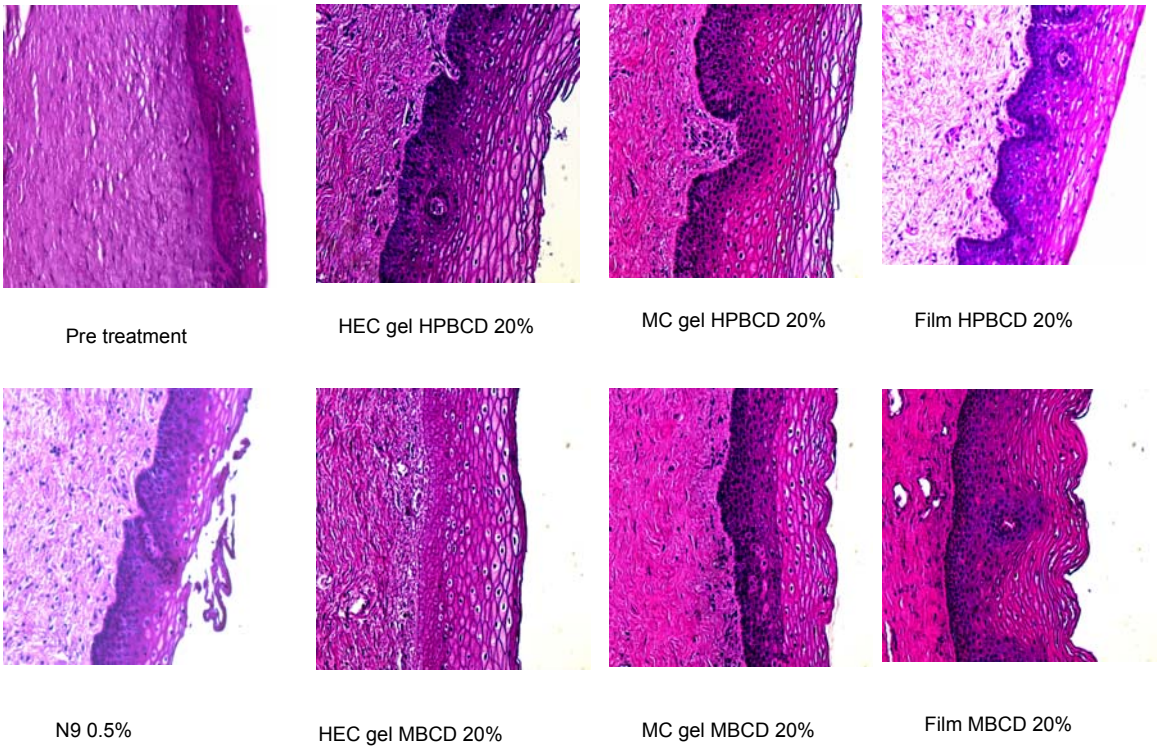


Figure 5-38. Histological results for excised human tissue exposed to 20% of HPβCD or MβCD formulated in a MC gel, HEC gel, and PVA film.
Pre treatment and N9 5% were used at placebo control and positive control for the histologies studies of cyclodextrins containing formulations. Pictures of highest concentration of cyclodextrins (20%)were shown here for comparison.

5.3.5.2 *Ex Vivo* Toxicity Evaluation of UC781:HPβCD or UC781:MβCD Complexes Containing Formulations on Excised Human Cervical Tissue

No toxicity was observed when complexed UC781 was incorporated into the developed formulations. No gross morphological changes were observed for either cyclodextrin evaluated (HPβCD or MβCD) at the 1:250 or 1:500 mass ratio (UC781: cyclodextrins) evaluated.

5.4 CONCLUSIONS

This research provides valuable information that can be used toward the development of beta-cyclodextrin based drug delivery systems. For mucosal administration, HPβCD and MβCD exhibit a concentration dependent toxic effect. However, this toxicity can be avoided by maintaining CD levels below 5% for HPβCD and below 1% for MβCD. In both cell-based and human cervical epithelial tissue models, water-soluble polymers can be used to reduce the toxicity of HPβCD and MβCD. HPMC was found to be the most effecive polymer for reduction of HPβCD and MβCD toxicity. Incorporation of this water-soluble polymer results in an increase in the amount of either HPβCD or MβCD (20% for HPβCD and 2.5% MβCD) that can be safely used. Interestingly, dosage form choice and excipient selection impact toxicity profile for HPβCD or MβCD. The HEC gel formulation and PVA film formulation offered better protective effects than the MC gel formulation. In both HEC gel formulations and PVA film formulations, 20% of HPβCD and 5% of MβCD can be used without gross toxicity.

These studies indicate that UC781 complexed with either HPβCD or MβCD can greatly enhance the release of UC781 from formulations by changing the release pattern from non-Fickian to Fickian release in VFS. Moreover, HPβCD or MβCD complexation increases the osmolality and decreases the viscosity of MC and HEC gel formulations. In addition, complexation resulted in shortened disintegration time for PVA film formulations due to the interaction between cyclodextrin and the polymer chains. These properties will facilitate distribution and rapid release of UC781 in the vagina, resulting in better protection against sexual HIV transmission.

Additionally, UC781 complexed with either HPβCD or MβCD greatly enhanced HIV RT inhibition activity as compared to non-complexed UC781 for all formulations. The IC_{50} values of complexed UC781 were greatly reduced as compared to non-complexed UC781. Importantly, complexed UC781 in a PVA film formulation can maintain the potency of UC781 and effectively reduce *in vitro* HIV-1 infection to almost zero. Finally, the toxicity study results of formulations indicate that HPβCD and MβCD are safe at 1:500 mass ratios (or 5% w/v) level in all three formulation.

Our studies showed that complexation with HPβCD or MβCD provides a safe and effective method for the development of UC781 drug delivery systems capable of overcoming UC781's innately poor water solubility. The complexed form of UC781 with either HPβCD or MβCD in a PVA film formulation provided quick release, potent HIV-1 inhibition, and low toxicity, all of which are essential criteria for a successful microbicide product. βCD-based drug delivery systems combined with PVA film formulation provides a promising solution for the formulation development of water insoluble drugs as effective microbicide products.

6.0 GENERAL DISCUSSION AND PERSPECTIVES

In these studies, β-cyclodextrin complexed with UC781 formulated in a PVA based fast dissolving film was developed as a microbicide product. The complexes of UC781 with βCD and its derivatives were systemically evaluated and characterized with UV, DSC, FTIR and NMR. These results confirm the formation of UC781: βCD complexes in both the liquid and solid state. The thermodynamic profile of the complexation of UC781 with three types of cyclodextrins was investigated using an HPLC method. The complexation ability of CDs with UC781 was found to follow the sequence of MβCD > βCD > HPβCD. An enthalpy driven process was identified for the complexation of UC781 with βCD, HPβCD, or MβCD. The enthalpy-entropy compensation was also observed for complexation of UC781 with CD, which is attributed to replacement of water molecules by UC781 in the βCD cavity.

The impact of pH variance, water-soluble polymers, and different preparation methods were investigated to optimize the complexation of UC781 with βCD, HPβCD and MβCD. Complexation of UC781 with CDs was conducted at pH 7.0, 9.0, or 11.0 to evaluate the impact of pH on complexation. pH 11.0 was shown to be the most efficient for complexation. This complexation enhancement effect resulted from the solubility increase of UC781 at pH 11.0 due to the increased degree of ionization at this pH. However, this condition cannot be applied in a vaginal formulation due to the extreme pH value. Kneading, shaking, autoclave, and lyophilization methods were evaluated for the complexation of UC781 with CDs. Comparison of

methods showed that the autoclave method is the most efficient method to form the complex of UC781 with CDs. Four water-soluble polymers, HPMC, HEC, PVA, and PVP K30, were incorporated during complexation of UC781 with HPβCD or MβCD. Although all polymers used in experiment resulted in enhanced complexation, maximal complexation of UC781 was obtained in the presence of HPMC.

Finally, MC gel, HEC gel, and PVA film formulations were developed and evaluated as carriers for complexed UC781 and non-complexed UC781. Complexed UC781 greatly increased osmolality and decreased viscosity for MC and HEC gel formulations. Complexed UC781 was also found to accelerate the disintegration rate of PVA film formulations. Importantly, complexed UC781 with HPβCD or MβCD can greatly enhance the dissolution and relative IC_{50} of UC781 in all three formulations tested without observed toxicity as evaluated in an excised human cervical epithelial tissue model. These results indicated that complexation of UC781 with HPβCD or MβCD provided a quicker and more potent protection from HIV infection han non-complexed UC781. Moreover, the PVA film formulation was found to be superior to the other two gel formulations with respect to tissue toxicity profile, UC781 release, and RT inhibition.

Although these studies provide proof of concept for utilization of a prototype fast dissolving film as a microbicide formulation for delivery of UC781 further evaluations are necessary. Formulation optimization is required to achieve a product with long-term stability, which can be manufactured using existing technologies. In addition, a thorough pharmacokinetic and pharmacodynamic evaluation will be required for the developed formulation. Finally, to achieve a more effective microbicide product the use of combination active agents in this dosage form should be explored.

6.1 FURTHER OPTIMIZATION AND OPTIMIZATION OF CYCLODEXTRIN BASED DRUG DELIVERY SYSTEMS

6.1.1 Consideration of Alternate Cyclodextrin Types and Modification To Manuafacturing Methods to Enhance Complexation Effiency of UC781 with Cyclodextrins

A number of potent, small anti-HIV molecules face the challenge of either poor water solubility or low permeability (BCS Class II or BCS Class III), which greatly hinders their clinical application. The development of many of these potent molecules is discontinued due to the lack of availability of applicable formulations. Therefore, a favorable formulation, which can enhance the solubility and permeability of these molecules, may help to bring more compounds into the therapeutic area of HIV prevention and treatment.

In our studies, we evaluated three cyclodextrin types for the formulation of UC781 as a microbicide product. βCD based drug delivery systems can greatly enhance the solubility of UC781 and accelerate the dissolution of UC781 while maintaining the anti-HIV activity of UC781. The membrane modulation effect of βCD may provide additional anti-HIV benefits for the microbicide product (Habeck,2001; Liao et al.,2001). It was reported that βCD can block the vaginal transmission of cell-associated HIV-1 in the mouse following a topical application (Khanna et al.,2002).

Although cyclodextrin can greatly improve the pharmaceutical profile of drug in formulation, it is important to incorporate as little cyclodextrin as possible in a pharmaceutical formulation due to the mass limitation in solid formulation, isotonicity of formulations, and its own toxicity (Loftsson et al.,1999; Loftsson et al.,2005b; Miller et al.,2007). Cyclodextrins have

an affinity for cholesterol and can extract it and other lipid membrane components from cells (Christian et al.,1997) leading to cellular toxicity at high concentration. Furthermore, considering the mass limitation of vaginal drug administration and the economic requirement for a low cost microbicide, complexation efficiency needs to be maximized to reduce the dose for both UC781 and cyclodextrin in the formulation while still maintaining the anti-HIV activity *in vivo*.

6.1.1.1 Use of Chemically Modified Cyclodextrins in UC781 Formulation (Captisol®)

The low intrinsic solubility (1.8%) of βCD itself greatly limits its application in pharmaceutical formulations. Thus, chemically modified cyclodextrins were developed to enhance the solubility of βCD. Functional groups are introduced at the 2-, 3- and 6-hydroxyl groups of the glucose residues to improve the solubility of βCD by breaking the 2-OH–3-OH hydrogen bonds and preventing crystallization of βCD. Thus, chemically modified cyclodextrins are amorphous products and are made up of isomers. Highly hydrophilic βCD derivatives, such as 2-hydroxypropyl–β-CD (HPβCD), provide a great increase on solubility as compared to βCD. Lipophilic cyclodextrin derivatives, such as the methyl-cyclodextrin (MβCD), are more efficient at extracting cholesterol (Christian et al.,1997) resulting in increased cell toxicity (Ulloth et al.,2007) leading to the limitation of its use.

Sulfobutyl ether–βCD (SBE-βCD) (Captisol®) is a new hydrophilic βCD derivative developed by CTD Inc. Captisol®, which has been intensively studied for its application in pharmaceutical research due to its low toxicity, water solubility, and organic solvent compatibility (CTD Inc,; Irie and Uekama,1997; Rajewskix and Stella,1996). Captisol® was reported to improve the ocular delivery of the pilocarpine prodrug (Jarho et al.,1996a). It has also

been successfully applied in the development of the formulation of Vfend® (voriconazole), a new antifungal agent developed and marketed by Pfizer Inc.

In future studies, the use of Captisol ® should be considered for UC781 complexation. Given the properties of this cyclodextrin derivative, an increased complexation efficiency with UC781 may be obtained with Captisol ®. In addition, a lower toxicity profile may be achieved as compared to that found for HPβCD in our previous studies.

6.1.1.2 Options for Improvement of Complexation Efficiency of UC781 with Cyclodextrins

Various methods to prepare drug/cyclodextrin complexes have been applied to enhance complexation efficiency, including co-precipitation, slurry, kneading, spray-drying and lyophilization (Hedges,1998). To obtain the liquid or solution state, cyclodextrin complexes are usually prepared by addition of an excess amount of drug to an aqueous cyclodextrin solution agitating at the desired temperature until equilibrium is achieved. The suspension is then filtered or centrifuged to form clear drug/cyclodextrin complex solution. For complex in the solid state, the water or solvent is removed from the drug/cyclodextrin solution by evaporation (e.g. spray-drying) or sublimation (e.g. lyophilization) (Pralhad and Rajendrakumar,2004).

In the studies described within this dessertation, the complexation of UC781 with cyclodextrin was found to be an enthalpy driven process. In addition, the autoclave method was the most efficient manufacturing process with respect to enhancement of the formation of UC781 complex. Spray-drying is a technique which can remove solvent from the system by evaporation at high temperature. It is applied in the preparation of solid dispersation for enhancing the solubility of water insoluble molecules (Otsuka et al.,1993; Paradkar et al.,2004). Therefore, the

spray-drying method may provide advantage with respect to the complexation efficiency of UC781 with cyclodextrins and should be further evaluated.

In addition to alternate manufacturing methods, other strategies can be used to enhance complexation efficiency. Such strategies include: addition of water-soluble polymers to the complexation media (Patel and Vavia,2006; Sigurðoardóttir and Loftsson,1995; Valero et al.,2003); adjustment of pH to change drug ionization and salt formation (El-Barghouthi et al.,2006; Peeters et al.,2002); addition of cosolvents (Miyake et al.,1999); or a combination of different methods as shown in Table 6-1.

Table 6-1. Methods for increasing cyclodextrin complex efficiency

Methods	Mechanism
Drug ionization	Ionization of a drug enhanced complexation through increase its apparent intrinsic solubility.(Peeters et al.,2002)
Salt formation	Salt formation can enhance apparent intrinsic solubility of a drug.(Mura et al.,2003; Redenti et al.,2001)
The acid/base ternary complexes	Some organic acids or bases are able to enhance the complexation efficiency by forming ternary drug/cyclodextrin/acid or base complexes.(Redenti et al.,2000)
Polymer complexes	Water-soluble polymers form a ternary complex with drug/cyclodextrin complexes increasing the apparent stability constant of the drug/cyclodextrin complex. (Faucci and Mura,2001; Loftsson et al.,1994)

In the studies described in this dissertation, four water-soluble polymers were evaluated for their impact on complexation efficiency of UC781 with both HPβCD and MβCD. It is possible to expand the number and type of polymers evaluated. Poloxamer 188 and PEG should also be investigated for their potential synergistic effect to enhance the complexation of UC781 with cyclodextrin and UC781's solubility (Chaudhari et al.,2007). However, the toxicity of Poloxamer-188 and PEG need to be carefully evaluated in cell and tissue models as described previously before their incorporation into formulations.

6.2 PROPOSED ADDITIONAL ASSESSMENTS OF UC781:CYCLODEXTRINS COMPLEXES BASED DRUG DELIVERY SYSTEMS

6.2.1 Biorelevant Evaluation of UC781 Release from Formulations

Rapid drug release is necessary for some microbicide products to enhance the efficiency with which they prevent HIV transmission. The main challenge for the evaluation of drug release behavior from microbicide products is the limited fluid present in the vaginal cavity. Normally, the daily production of vaginal fluid is around 6 g/day (Owen and Katz,1999). This fluid level is much less than the media levels (1000 ml media) typically used in USP dissolution testing (The United States Pharmacopeia,2000).

Within the studies conducted, a low volume dissolution model that uses vaginal fluid simulant (VFS) was used to evaluate the three developed formulations (75 ml). This model provides information on the rate and extent of drug release, as well as information toward the establishment of drug release mechanism in a comparatively low volume without the interference of organic solvents. It is a more physiologically relevant model for the evaluation of microbicide products as compared to the traditional USP method.

Considering differences between *in vitro* and *in vivo* conditions, the results obtained *in vitro* should be carefully considered prior to attempting to extrapolate to define *in vivo* properties of formulations. The dissolution of UC781 from formulations and its penetration through cervical tissue is currently being studies in order to predict the *in vitro – in vivo* relationship of formulations for this drug. Development of mathematical models is necessary to achieve more relevant predictions regarding *in vitro – in vivo* relationships of formulations.

6.2.2 Stability of Complexed vs. Non-Complexed UC781 in Formulations

The stability of a formulation or product under typical conditions of use is critical to its efficacy, shelf-life and ultimate success. Stability assessment is essential for pharmaceutical product evaluation. Therefore, with regard to the formulations developed within this dissertation work, it is critical to investigate the stability of the products containing UC781 complexed with cyclodextrins.

There are several reports showing that complexation with cyclodextrin can enhance the stability of drugs in formulation (Jarho et al.,1996b; Loftsson and Jarvinen,1999). Based on these reports we anticipate that the complexation of UC781 will result in enhanced stability of the drug in the formulation. Additional studies are needed to establish whether complexation results in increased stability of UC781 in product.

6.2.3 Pharmacokinetic (PK)/Pharmacodynamic (PD) Evaluation of UC781 Complexes Containing Film Formulations

Pharmacokinetic (PK) and pharmcodynamic (PD) studies of drug products are critical to evaluate clinical efficacy. PK data for UC781 established using a mouse model was reported (Buckheit et al.,1997a). In these studies a low oral bioavailability was observed for UC781 (31%) indicating either poor absorption or quick elimination of UC781 *in vivo*. Currently, no PK data is available for UC781 delivered in a film dosage form in human subjects. Therefore, investigation of PK properties for UC781 delivered via this dosage form is required.

In addition to the PK evaluation, clinically relevant studies on the *in vivo* PD properties of UC781 should be investigated. Animal models, especially small animal models, offer a

potential mechanism for studying the ability of a product to provide protection from HIV infection. However, a huge challenge in the development of NNRTIs is the lack of useful animal models.

Currently, rhesus macaque SIV (Simian Immunodeficiency Virus) models (Stellbrink,2003) and feline FIV (Feline Immunodeficiency Virus) models (Bendinelli et al.,1995) have been used as small animal models for HIV infection research. SIV and FIV are both similar to HIV-1 with regard to their genomic organization and biologic properties. *In vitro* models of FIV and SIV were used for anti-HIV studies (North et al.,1989; Witvrouw et al.,1999). However, FIV and SIV are not susceptible to NNRTIs. Therefore, modified FIVs or SIVs have to be developed for NNRTIs research. Reverse Transcriptase Chimeras of hybrids between HIV and feline immunodeficiency virus (FHIV) and hybrids between HIV and simian immunodeficiency virus (SHIV) were developed for NNRTIs evaluation (Auwerx et al.,2004; Hofman et al.,2004). In the monkey model for the evaluation of NNRTI activity, hybrid SIV strains in which the entire RT gene was replaced by the HIV-1 RT gene (designated RT-SHIV) have already been constructed (Hofman et al.,2004). However, both models have limitations. Hybrid FIV-FHIV greatly decreases the catalytic efficacy of RT (Auwerx et al.,2002). SHIV models in macaques may not directly correlate with the pathogenesis of HIV infection in humans.

As an alternative, transgenic rat models provide another method to evaluate anti-HIV-1 activity of fusion inhibitors and NNRTIs (Goffinet et al.,2007; Zhang et al.,2007). The humanized rat model is infectible with a broad range of HIV isolates using human CD4+ and CCR5 receptors and amenable to genetic manipulation of the rate recipient with relatively low cost (Goldstein,2008). Importantly, transgenic rats can be also used as a model to study

prevention of HIV-1 vaginal transmission (Di Fabio et al.,2003), offering an easyily controllable animal model for microbicide research.

The anti-HIV activity of the developed UC781 formulations should be evaluated in the humanized rat model. The PD data obtained will help to elucidate the mechanism of action for UC781 when used as a microbicide product in vagina. This model can also provide information toward effective dosing level choice.

6.2.4 Combination with Other Microbicide Candidates

Fusion inhibitors, RT inhibitors, and integrase inhibitors may block HIV-1 infection prior to irreversible cellular infection. Combined application of RT inhibitors with drug candidates with other mechanisms of action may result in synergistic activity. Some reports have shown that combination of UC781 with L-870812 (integrase inhibitor) or cellulose acetate 1,2-benzenedicarboxylate (CAP) results in significant synergistic effect on the inhibition of HIV-1 infection (Liu et al.,2005a; Terrazas-Aranda et al.,2008). Some advantages may be achieved by combining UC781 with other active drug candidates. We anticipate that the combination of UC781 with either fusion inhibitors or integrase inhibitors may potentially provide a microbicide product with increased potency and decreased drug-resistance.

6.2.5 Other Considerations for Formulation Optimization of UC781

Currently, more than 50 potential microbicides are being investigated for potential vaginal use. Some of those currently being evaluated in clinical trials are shown in Table 6-2. The main challenges to successful microbicide product development are generally recognized as

inadequate understanding of HIV transmission, uncertainty regarding which host cells to target, complexities of the genital and rectal environments, lack of adequate animal models, and inadequate funding. However, the impact of formulation on the effectiveness of a microbicide product can be easily underestimated. In fact, formulation may play a key role for drug availability from a microbicide product and furthermore can result in varied effectiveness or toxicity profile for anti-HIV agents. Our studies have demonstrated the feasibility of the use of a film formulation for delivery of complexed UC781. Additional studies on this formulation are required before its introduction to the clinic.

Table 6-2. Microbicide candidates in ongoing clinical trials

(Modified from Alliance for Microbicide Development 2008, www.microbicide.org)

Phase	*Candidate Name*	*Sponsor*[*]	*Sites by Country*
III	PRO 2000/5 gel	Indevus, MRC, DFID (Funder)	South Africa, Tanzania, Uganda, Zambia
II B	Tenofovir gel	CAPRISA, USAID, LIFElab, Gilead, FHI, CONRAD	South Africa
II/IIB	PRO 2000/5 gel (P) and BufferGel®	NIAID, Indevus, ReProtect	Malawi, South Africa, United States, Zambia, Zimbabwe
I	Dapivirine (TMC120) gel	IPM	Belgium
	Ethanol in Emollient Gel	NIAID	Kenya
	HEC/CS/N-9[†]	CONRAD/USAID	USA
	Tenofovir/PMPA gel	CONRAD, IPM/USAID	Dominican Republic, United States
	Tenofovir gel	NIAID	United States
	UC781 gel	NIAID, CONRAD	United States
	UC781 gel	UCLA, NIAID, CONRAD	United States
	UC781 gel	CDC, Thailand Ministry of Health, CONRAD	Thailand
	UC781	CONRAD	United States
	UC781	CONRAD, CDC, Emory University	United States
	VivaGel™ (SPL7013 gel)	DAIDS/NIAID, NICHD, Starpharma	Puerto Rico, United States
N/A	Placebo ring	IPM	Kenya, South Africa, Tanzania

Most excipients in pharmaceutical dosage forms are considered to be inactive ingredients. However, these excipients may have baseline toxicity or activity or they may interfere with the active or other excipients in the formulation. This may result in pharmacological profile changes.

In the development of a microbicide, the toxicity of excipients in the formulation as well as any potential impact imparted by the excipients on product bioactivity should be considered.

In the studies presented, a formulation dependent toxicity profile was observed for HPβCD and MβCD. Of the developed formulations the HEC gel and PVA film formulations showed greater protective effect with regard to toxicity of HPβCD and MβCD to human cervical epithelial tissue than did MC gel formulations. This result may be due to the excipents present in the different formulations. In the studies conducted, the major reason for observed differences in toxicity may result from the type of polymer present in the formulation as well as its concentration.

In addition, the impact of the polymer type used in the formulation on HIV infection should also be considered. Cellulose sulfate (CS), a polyanionic compound derived from cotton, has been proposed as a topical microbicide product to reduce the sexual transmission of HIV. It was reported as effective (Bourne et al.,1999; Keller et al.,2005) and safe (Smita et al.,2006; Van Damme et al.,2000) *in vivo*. However, recently it was found to enhance HIV infection under some circumstance (low concentration at < 10 μg/ml) (Tao et al.,2008). Developing a better understanding of the role of the excipients present in this product may help to explain the failure of the CS product in Phase III clinical trials.

The barrier function of the vagina should not be significantly affected after the application of a microbicide product for effective prevention of HIV transmission. Nonoxynol-9 is an example of the attempted use of a contraceptive product to prevent HIV transmission which resulted in failure (Richardson,2002). A vaginal formulation should not affect the integrity of vaginal epithelium nor should it negatively impact the normal vaginal flora. The normal flora of the vagina consists predominantly of lactobacilli species. A significant change in vaginal

lactobacilli, integrity of the vaginal epithelium, or alteration of the permeability of vaginal tissues may lead to the increased risk of HIV transmission . Therefore, the impact of microbicide products on vaginal lactobacilli, integrity of the vagina, and permeability of the vagina must be evaluated.

Semen viral load is an important risk factor for the vaginal HIV infection. It was reported that the mean viral load in semen is 3.9 log 10 RNA copies/ml (Medeiros et al.,2004). HIV RNA can be detected from treated patients with undetectable blood plasma HIV-1 RNA levels in HIV positive patients (Marcelin et al.,2008). In addition, human semen contains a peptide component- semen-derived amyloid fibrils, which can enhance HIV infection from 500 to 1000 times (Munch et al.,2007). Therefore, the activity of microbicide products should be evaluated in the presence of semen to provide information for appropriate dosing levels required in formulations.

The physicochemical properties of a formulation will play an important role in the interaction of the formulation with the vaginal/rectal tract. This ultimately will affect the pharmacological profile of the drug. Osmolality and viscosity are two common parameters evaluated for liquid and semisolid formulations. The osmolality of normal biofluid is around ~290 mOsm/kg. Products with extremely high osmolality values may potentially enhance HIV infection (Fuchs et al.,2007). However, no optimal osmolality value or viscosity has been set for microbicide products. The findings from these studies show that the components in a formulation can greatly change the osmolality of products. This may correspond to a reduction of the anti-HIV activity of the drug formulation. Further studies should be conducted to identify the appropriate range for osmolality with respect to safety for use in vaginal products.

In addition, high osmolality or low viscosity will lead to the leakage of microbicide products resulting in decreased patient acceptability. Incorporation of bioadhesive materials into formulations should be considered to overcome this problem. Bioadhesive materials when used in formulations maintain the formulation at the vaginal mucosal surface. In the studies described for UC781, HPMC, which is a bioadhesive material, provided an optimal choice for use in the formulation in that it imparts bioadhesiveness as well as complexation enhancement and reduction of toxic effects of CDs. Different types of HPMC should be evaluated for use in the PVA film formulation to assure that bioadhesion is optimized. It should also be noted that bioadhesive materials may hinder the drug release from formulations. For this reason, it is imperative that *in vitro* drug release testing be conducteded simultaneously to optimize the formulation.

In a summary, a promising prototype film formulation for the delivery of complexed UC781 was developed in these studies. Future studies are still necessary before this dosage form can be introduced into the clinic. Briefly, further formulation optimization, evaluation of the stability of complexed UC781, assessment of drug release from the product, and PK/PD characterization should be conducted prior to clinical evaluation of this new dosage form. Our studies have contributed to gain better insight into the development of βCD based drug delivery systems for water insoluble anti-HIV drug candidates into a successful microbicide product.

APPENDIX A

COMPLEXATION OF EFFECT OF CYCLODEXTRINS

Cyclodextrins (CDs) have aroused considerable attention in pharmaceutical application since the 1950's, due to their ability to form complexes with poorly water-soluble drugs and drug candidates, resulting in an increase in their apparent water solubility which can improve the pharmaceutical profile of these water insoluble drugs via complexation. Appendix A intends to give a general background for the principles and mechanism of cyclodextrin complexation.

Table A-1 Selected symbols used in Appendix

K	equilibrium constant
K_c	Complexation constant or stability constant (equilibrium constant for a drug-cyclodextrin interaction)
K_d	dissociation constant
$K_{1:1}$	complexation constant for the interaction of one drug molecule with one cyclodextrin molecule
$K_{n:m}$	complexation constant associated with the interaction of n drug molecules with m cyclodextrin molecules
S_o	intrinsic drug solubility
S_t	total drug in solution (complexed form + uncomplexed form)
CD_t	total cyclodextrin in solution (complexed and uncomplexed)
Cp	the solubility of the precipitated complex

A.1 PHASE SOLUBILITY

Conceptually, the complexation process of drug and cyclodextrin can be considered as a reversible chemical reaction at equilibrium. The stability or complexation constant (K_c or $K_{n:m}$) or its inverse, the dissociation constant (K_d) are crucial since these values provide an index of change of physicochemical properties for the whole complexation.

There is a equiblium exist in the complexation process,

$$n\text{Drug} + m\text{CD} \xleftarrow{K_c \text{ or } K_{n:m}} \text{Drug}_n \cdot \text{CD}_m \qquad \textbf{6-1}$$

The complexation constant/equilibrium constant ($K_{n:m}$) can be defined as:

$$K_{n:m} = \frac{[\text{Drug}_n \cdot \text{CD}_m]}{[\text{Drug}]^n \cdot [\text{CD}]^m} \qquad \textbf{6-2}$$

Therefore, the dissociation constant can be expressed as

$$K_d = \frac{[\text{Drug}]^n \cdot [\text{CD}]^m}{[\text{Drug}_n \cdot \text{CD}_m]} = \frac{1}{K_{n:m}} \qquad \textbf{6-3}$$

The complexation with cyclodextrins results in change in the physicochemical properties of guest molecules, such as apparent solubility, UV and IR profile, florescence properties, NMR chemical shifts, and DSC properties, and HPLC retention time (Chadha et al.,2004; Loftsson and Brewster,1996).

The solubility change may be the most convenient method to provide information on complexation process. Thus, the complexation of guest molecule can be addressed in solubility method – phase-solubility relationships.

Phase-Solubility Analysis

Phase-solubility analysis is a traditional isothermal approach to investigate the complexation phenomenon based on the solubility change in guest molecules in aqueous solution. Phase-solubility analysis can provide stability constant information as well as stoichiometry of the equilibrium with a simple diagram analysis, even only involves simple technology to form the complex.

The process of phase-solubility can be described as follows: Briefly, an excess amount of guest molecules is added to a CD solution at different concentrations to form a suspension. The suspension is then agitated at constant temperature until it reaches equilibrium. After removing the extra undissolved guest molecules using filtration, the total concentration of guest molecules in solution is then determined with an appropriate analytical technique. The phase-solubility diagram is then plotted as CD concentration [CD_t] *vs.* the apparent concentration of guest molecules [S_t].

Phase–solubility analysis was developed by Higuchi and Connors(Higuchi and Connors,1965). Based on the shape of the generated phase–solubility relationships, several types of behaviors can be identified. Phase–solubility diagrams can be classified into two major types: A and B as shown in Figure A 1 (Challa et al.,2005).

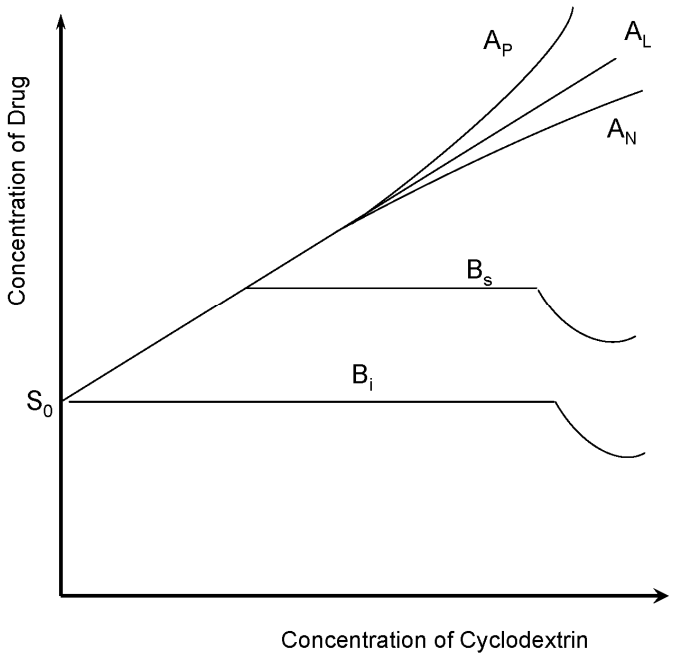


Figure A 1 Diagram of phase- solubility with A and B-type (A_P, A_L, A_N and B_S, B_i).

A and B type of phase-solubility diagrams were shown in above Figure. A type phase-solubility diagram reflects the soluble complex formed in system leading to an increase in drug concentration. B type phase-solubility diagram can be explained with the formation of insoluble complex in system.

A-type phase-solubility

A-type phase-solubility systems are most commonly scene in the complexation of cyclodextrin. In A-type phase-solubility systems, the apparent solubility of the substrate increases as a function of CD concentration. Three subtypes of phase-solubility diagrams are defined: an A_L profile is defined as a linear relationship of drug solubility *vs.* CD concentration (Senel et al.,1992), A_P system indicates a positive direction from linearity (Ventura et al.,2005) and A_N relationship suggests a negative deviation from linearity (Szafran and Pawlaczyk,1999).

A_L-type relationship is often assumed to be one-to-one relationship of complexation between guest molecule and cyclodextrin molecule in the system. However, a higher order

complexation may be formed with respect to the drug (i.e. Drug • CD, $Drug_2$ • CD, $Drug_3$ • CD, etc). A_P type profiles show the curvng pattern suggesting that the formation of higher order complexes with respect to the CD at high CD concentrations (i.e. Drug • CD, Drug • CD_2, Drug • CD_3, etc). CD is more effective for complexation at higher concentrations. The stoichiometry of the formed complexes can be obtained by using best-fit function. Thus, a best fit to different functions indicats the formation of different stoichiometric complex. For example, a quadratic function suggests the formation of a one-to-two (D • CD_2) complex; one best fit to a cubic function suggests a one-to-three complex (D • CD_3); and so forth. A_N type profiles indicate that the CD is less effective on complexation with drugs at higher concentrations. This phenomenon may caused by complex self-aggregation or self-association at high concentration and bulk changes imparted to the solvent by CD at various concentrations. Chemically modified cyclodextrins such as HPβCD can cause great change in volume as well as viscosity of its solution at 20% and above.

Although, A_p and A_N relationships show curving profiles of phase-solubility diagram, the linear part of the phase-solubility can still be used to estimate the complexation constant for one-to-one complex formation. Generally, a polynomial function is used for complexation constant estimation using the following Equations for mass balance:

$$[S] = S_0 \quad \textbf{6-4}$$

$$[S_t] = S_0 + m[S_m \cdot CD_n] \quad \textbf{6-5}$$

$$[CD_t] = [CD] + n[S_m \cdot CD_n] \quad \textbf{6-6}$$

Here, S_0 is intrinsic solubility of drug, S is free drug concentration, S_t is soluble drug concentration, CD is free cyclodextrin concentration, CD_t is total concentration of CD, and S_m CD_n is complex in solution. With Equation (4)-(6), $[D_t]$ can be expressed as

$$[S_t] = \frac{m \cdot K_{m:n} \cdot S_0^m}{1 + K_{m:n} \cdot S_0^m}[CD_t] + S_0 \qquad \textbf{6-7}$$

Thus, with a plot of $[S_t]$ *vs.* $[CD_t]$, the slope and y-intercept can be easily obtained. Therefore, from equation (7), the intercept from y-axis represents the S_0, and slope can be defined as

$$\text{Slope} = \frac{m \cdot K_{m:n} \cdot S_0^m}{1 + K_{m:n} \cdot S_0^m} \qquad \textbf{6-8}$$

When a one-to-one complex form (a drug interacts with one CD) happens in complexation system, m=n=1, the $K_{1:1}$ can given by

$$K_{1:1} = \frac{\text{slope}}{S_0(1 - \text{slope})} \qquad \textbf{6-9}$$

Similar procedure can be applied to one-to-two complex form (a drug interacts with two CD). The $K_{1:1}$ and $K_{1:2}$ can be expressed as following:

$$K_{1:1} = \frac{[S \cdot CD]}{[S] \cdot [CD]} \qquad \textbf{6-10}$$

$$K_{1:2} = \frac{[S \cdot CD_2]}{[S] \cdot [CD]^2} \qquad \textbf{6-11}$$

The mass balance equations are adjusted as

$$[S] = S_0 \quad \textbf{6-12}$$

$$[S_t] = S_0 + [S \cdot CD] + [S \cdot CD_2] \quad \textbf{6-13}$$

$$[CD_t] = [CD] + [S \cdot CD] + 2[S \cdot CD_2] \quad \textbf{6-14}$$

$$[S_t] = \frac{K_{1:1} \cdot S_0 + K_{1:1} \cdot K_{1:2} \cdot S_0[CD]}{1 + K_{1:1} \cdot S_0 + 2K_{1:1} \cdot K_{1:2} \cdot S_0[CD]}[CD_t] + S_0 \quad \textbf{6-15}$$

$$[S_t] = S_0 + K_{1:1} \cdot S_0[CD] + K_{1:1} \cdot K_{1:2} \cdot S_0[CD]^2 \quad \textbf{6-16}$$

Thus, the parameters of S_0, $K_{1:1}$, and $K_{1:2}$ can be easily calculated using a fitting curve from the phase-solubility diagram.

For one-to-three or higher order complex form, this equation can be derived in a similar way:

$$[S_t] = S_0 + K_{1:1} \cdot S_0[CD] + K_{1:1} \cdot K_{1:2} \cdot S_0[CD]^2 + K_{1:1} \cdot K_{1:2} \cdot K_{1:3} \cdot S_0[CD]^3 \quad \textbf{6-17}$$

B-type phase-solubility

The property of B-type phase-solubility is the formation of complexes with limited water solubility. Type B phase–solubility profiles are more easily observed in the complexation of naturally occurring CDs, especially βCD due to its limited the water solubility (1.8%) (Piel et al.,2006). The temperature impact on some chemically modified βCD can also lead to B-type phase-solubility diagram (Ventura et al.,2005). B-type diagrams can be classified into two subclasses: B_S and B_i systems.

For the BS-type phase-solubility, the following balance is assumed to occur in system as shown in Equation 18.

$$Drug_{solid} \overset{S_0}{\longleftrightarrow} Drug_{dissolved} + CD \overset{K}{\longleftrightarrow} Drug{\cdot}CD_{Dissolved} \overset{C_p}{\longleftrightarrow} Drug{\cdot}CD_{solid} \quad \textbf{6-18}$$

Where S_0 is intrinsic solubiligy of drug; K is the complexation constant; and C_p is the solubility of the precipitated complex (C_p). The solubility of complex observed in the systems is associated with the solubility of the precipitated complex.

With the βCD concentration increasing, the balance shifts to middle with increasing soluble complex forms, which increase the total solubility of the substrate. When the critical concentration of soluble complex is reached, the balance shifts to right to form insoluble complex leading to the plateau phase in phase-solubility diagram, which maintains the drug concentration S_t unchanged. After the drug is totally transferred into soluble from solid state, the extra CD added into the system will enhance precipitation, which results in the decrease of S_t in the system. The complex constant K can also be obtained from the initial ascending portion of a B_S-type phase-solubility with the same techniques used to assess A_L-type systems with the use of Equation (7)

In the B_i-type systems, the complex is also in insoluble form, which leads to the no ascension part in the phase-solubility diagram. The stoichiometry of complexes formed from B_S-type solubility can be obtained by analyzing the precipitated complex to get the stoichiometric relationships between drug and CD using job's plot.

Another benefit brought from phase-solubility method is to estimate the intrinsic drug solubility S_o from the y-intercept of the phase–solubility relationship. The results from this technique should be carefully treated when drug's intrinsic solubility is extremely low. It may cause underestimation of observed intrinsic solubility (Loftsson et al.,2005a). This will result in an overestimation of the K_c or $K_{1:1}$ value, especially for those highly lipophilic molecules.

As a traditional approach to determine the complexation of cylcodextin, phase-solubility analysis provides a simple and quick method to assess and evaluate the complexation phenomena of cyclodextrin with guest molecules.

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