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„Development of International Pharmacopoeia monographs for the analysis of dosage forms of selected essential antiretroviral drugs“

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List of Abbreviations

ACN	acetonitrile
AIDS	Acquired immunodeficiency syndrome
CD4	cluster of differentiation 4
cDNA	complementary DNA
CYP	cytochrome P450
EFV	efavirenz
FDA	Food and Drug Administration
FTC	emtricitabine
HAART	highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HPLC	high performance liquid chromatography
MeOH	methanol
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/ nucleotide reverse transcriptase inhibitor
Ph. Int.	International Pharmacopoeia
RSD	relative standard deviation
RT	reverse transcriptase
RTV	ritonavir
TDF	tenofovir disoproxil fumarate
TLC	thin-layer chromatography
WHO	World Health Organization

1 Introduction

1.1 The International Pharmacopoeia

1.1.1 Definition

The International Pharmacopoeia (Ph. Int.) is published by the World Health Organization (WHO) and per definitionem "(...) comprises a collection of recommended procedures for analysis and specifications for the determination of pharmaceutical substances, excipients and dosage forms that is intended to serve as source material for reference or adaptation by any WHO Member State wishing to establish pharmaceutical requirements." [The International Pharmacopoeia, 4th edition]

1.1.2 History

The history of the Ph. Int. can be tracked back to the year 1874, when the necessity of standardized terminology and specified drug composition was recognized. Different steps in this development, like the Agreement for the Unification of the Formulae of Potent Drugs (1902) and the Brussels Agreement (1925) in turn led to the constitution of an Expert Committee on the Unification of Pharmacopoeias by the Interim Commission of the WHO, the aim of which was to produce a draft international agreement for the unification of pharmacopoeias, continuing the work previously undertaken in this direction. In 1951 this committee became the Expert Committee on the International Pharmacopoeia and the first edition of the Ph. Int. was published in two volumes (1951 and 1955).

In 1967 the second edition was published, which, taking into account the development of new analytical techniques, revised the first edition, making numerous alterations and adding new analytical methods.

In 1975 the purpose of the Ph. Int. was reconsidered, putting the focus on the needs of developing countries and using simple classical chemical techniques in order to make the pharmacopoeia applicable also in laboratories without expensive equipment. Priority was henceforward given to drugs that are widely used throughout the world, part of WHO health programmes or likely to contain impurities due to degradation or difficulties in

their manufacturing process. Since 1979 the drugs included in the Ph. Int. have been selected from the WHO List of Essential Drugs.
The fourth and latest edition of the Ph. Int. was published in two volumes in 2006. It was updated by a first supplement in 2008 and a second one in 2011, which added monographs for pharmaceutical substances as well as dosage forms, mainly for antiretroviral, antimalarial, antituberculosis and anti-infectives medicines [The International Pharmacopoeia, 4th edition].

1.1.3 Intention

The aim of the Ph. Int. is to promote the overall quality of pharmaceutical substances and dosage forms. It contains quality specifications which are intended as basic requirements for pharmaceutical products and serve as source material for establishing pharmaceutical standards throughout the world. Therefore, it focuses on states that do not have pharmacopoeial compendia of their own, i.e. developing countries.
The Ph. Int. provides information on general requirements for pharmaceutical products and methods of analysis. In an attempt to minimize the use of expensive equipment it focuses on simple classical techniques. More sophisticated methods are included as well but usually accompanied by an alternative, less complex method. It contains monographs on drug substances and dosage forms. The latest version covers monographs on the following categories:

- HIV/AIDS
- Malaria
- Tuberculosis
- Anti-Infective
- Oral Rehydration therapy
- Radiopharmaceuticals
- Other medicines

The selection of monographs for inclusion in the Ph. Int. is based on the current WHO Model List of Essential Medicines and takes WHO disease programs into account [The International Pharmacopoeia, 4th edition].

1.2 The WHO Model List of Essential Medicines

The WHO Model List of Essential Medicines (formerly known as WHO list of essential drugs) is a compendium of medicines which "satisfy the priority health care needs of a population. They are selected with regard to disease prevalence, evidence of efficacy, safety, and comparative cost-effectiveness".
Since the development of the first list in 1977 it has been updated every two years. The latest version (March 2011) lists over 350 medicines for the treatment of priority conditions, such as HIV/AIDS, malaria, tuberculosis and reproductive health as well as chronic diseases like cancer and diabetes. Its purpose is to serve as a guide for establishing national essential medicines lists, which take local and regional needs into account. Especially in countries with less functional health systems it is supposed to help identify suitable medicines for priority health issues and thereby improve availability, accessibility, affordability and quality of essential drugs.
In 2007, a WHO Model List of Essential Medicines for Children was developed, which has also been updated every two years [WHO Factsheet Essential Medicines].

1.3 HIV/ AIDS

1.3.1 Overview

The occurrence of an acquired immunodeficiency syndrome (AIDS) was first described in 1981. A novel human retrovirus was isolated in 1983, soon identified as etiological agent and eventually named HIV [Sepkowitz 2001]. Human immunodeficiency viruses (HIV) are lentiviruses, a family of mammalian retroviruses. Its natural hosts are humans and nonhuman primates. It causes chronic persistent infections and progressively reduces the effectiveness of the immune system by destroying CD4+ T-helper cells. This in turn leads to an increasing susceptibility for opportunistic infections, which is called AIDS [Hare 2006].
There are two major groups of HIV: HIV-1, which is involved in most of the pandemic, and HIV-2, which is more closely related to the simian immunodeficiency virus (SIV) and occurs mostly in western Africa. Both of these groups can further be divided into subgroups, depending on their genomic sequence [HIV Sequence Database].

According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), almost 60 million people have been infected since the beginning of the epidemic and 25 million people have died of HIV-related causes [UNAIDS Global facts and figures 09].
The latest WHO global summary lists 2.6 million people newly infected with HIV in 2009, who add to a total of 33.3 million people living with HIV, about 97% of whom live in low or middle income countries [WHO Global summary of the AIDS epidemic 2009].

1.3.1.1 Characteristics of HIV

1.3.1.1.1 Structure

HIV consists of an RNA genome of 9300 base pairs, two copies of which are contained in a nucleocapsid core surrounded by a lipid bilayer envelope that is derived from the host cell's plasma membrane (Figure 1). The genome consists of nine genes which encode 15 proteins. The three most important are *gag*, which encodes the major structural proteins of the virus, *pol,* which encodes viral enzymes like integrase, protease and reverse transcriptase (RT), and *env,* which encodes the large transmembrane envelope protein. *Nef, rev, tat, vif, vpr, vpu* encode regulatory proteins that enhance virion production or antagonise host defenses. The viral particle also contains enzymes essential for the viral development such as integrase, protease and RT [Greene and Peterlin 2002].

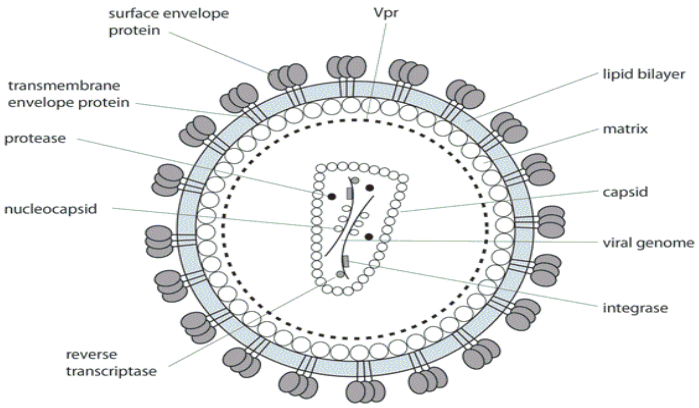


Figure 1: Schematic picture of an HIV virion

1.3.1.1.2 Life Cycle

HIV infects lymphocytes and macrophages that express the cluster of differentiation 4 glycoprotein (CD4) on their surface. The tropism for these cells is mediated by the envelope protein (env). Cell entry also requires binding to a coreceptor, generally the chemokine receptor CCR5 or CXCR4 [Greene and Peterlin 2002]. While CCR5 is present on macrophage lineage cells, CXCR4 is found on T-lymphocytes. HIV with CCR5-tropism is responsible for most of the naturally acquired infections, whereas a shift to CXCR4 tropism is usually associated with advancing disease and resulting in a higher affinity for CXCR4, infection of T-Lymphocytes and increased risk for immunosuppression [Berger et al. 1999].

HIV binds to the CD4 receptor and the coreceptor on the cell surface with its surface gp120 protein, which induces a conformational change and exposition of the gp41 protein which penetrates the target cell membrane and promotes fusion of virus and cell membrane. After entry of the viral capsid uncoating takes place and viral RNA and various enzymes are released. The reverse transcription complex is formed and, based on the viral RNA, a complementary DNA (cDNA) strand is produced, mediated by RT. The original RNA strand is degraded, allowing the creation of a double-stranded DNA copy of the virus. After completion of the reverse transcription, a preintegration complex containing the double-stranded viral DNA enters the cell nucleus, where it is inserted into the host genome by integrase. This provirus can remain latent, not producing new virions but replicating as the cell divides or it can be transcriptionally active, depending on the chromosomal milieu in which it is integrated. However, if more than one provirus is established in one cell, it is very likely that at least one is transcriptionally active. In the latter case it uses the host cell to produce viral RNA and proteins.

Structural viral proteins and RNA assemble within the cytoplasm to form new nucleocapsids whereas viral envelope proteins assemble at cholesterol rich lipid rafts at the cell surface. The capsid cores are then directed to those sites and bud through the cell membrane to form new virions [Goodman & Gilman's 2011].

A schematic replicate circle of HIV is given in Figure 2.

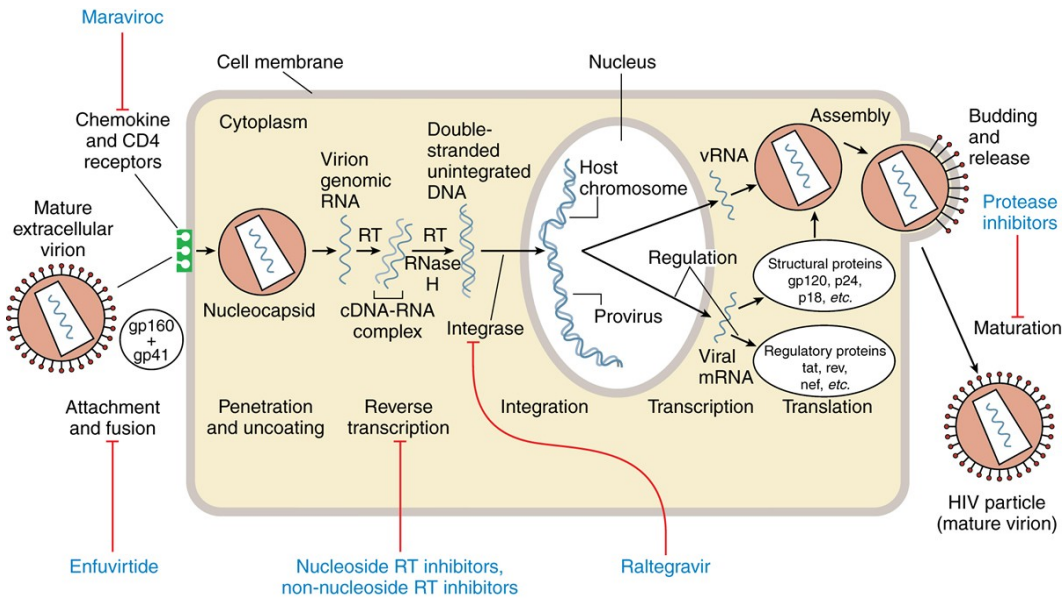


Figure 2: Replicate circle of HIV-1 showing the sites of action of available antiretroviral agents, from [Goodman & Gilman's 2011]
RT, reverse transcriptase; cDNA, complementary DNA; mRNA, messenger RNA; Rnase H, ribonuclease H; gp160+gp41, envelope glycoprotein.

1.3.1.2 HIV-Infection and development of AIDS

HIV can be transmitted through unprotected sexual intercourse, transfusion of contaminated blood, sharing of contaminated needles or between a mother and her infant during pregnancy, childbirth and breastfeeding.

Infection is followed by a burst in viral replication peaking after 2-4 weeks, which is associated with a massive decrease of the number of CD4+ T-lymphocytes and in most cases accompanied by influenza or a mononucleosis-like illness (Acute HIV infection) [Kahn and Walker 1998].

Symptoms of these usually vanish after 4 weeks as the plasma HIV RNA concentration declines, as a result of new host immune responses and depletion of target cells. The viral load reaches a quasi steady state which reflects the interaction between pathogenicity of HIV and host immunity (Chronic HIV infection). Eventually, the number of host CD4+ T-lymphocytes begin a steady decrease, whereas the plasma HIV concentration rises. Once the CD4 cell count falls below a threshold of 200 cell/µl, the risk of opportunistic infections increases [Goodman & Gilman's 2011].

Untreated, infection with HIV usually leads to clinical manifestation of AIDS within 5-10 years.

The WHO uses the following classification system to determine the progression of HIV infections [WHO Disease Staging System for HIV Infection and Disease]:

- Primary HIV infection - may be asymptomatic or experienced as acute retroviral syndrome
- Clinical stage 1 - asymptomatic or generalized swelling of the lymph nodes
- Clinical stage 2 - includes minor weight loss, minor mucocutaneous manifestations, and recurrent upper respiratory tract infections
- Clinical stage 3 - includes unexplained chronic diarrhoea, unexplained persistent fever, oral candidiasis or leukoplakia, severe bacterial infections, pulmonary tuberculosis, and acute necrotizing inflammation in the mouth
- Clinical stage 4 - includes 22 opportunistic infections or cancers related to HIV

The criteria used for AIDS diagnosis were established by the US Centers for Disease Control and Prevention (CDC) and include the occurrence of any of more than 20 opportunistic infections or HIV-related cancers. Also a CD4+ T-cell count below 200 cells/µl blood is defined as AIDS by the CDC [CDC revised classification system].

1.3.1.3 Treatment of HIV infection

So far, no curative treatment for HIV-infection has been found. Therapy focuses on slowing down the progression and delaying the manifestation of AIDS. Current treatment is based on the assumption that all aspects of the disease are a result of the HIV on host cells, mainly CD4+ T-lymphocytes. Successful therapy is therefore based on the inhibition of HIV replication and thereby increasing the levels of CD4+ T-lymphocytes. A conceivable positive effect by boosting host immunities, without additive antiretroviral remedies has had no reliable clinical benefit [Goodman & Gilman's 2011].
On the other hand, any treatment exerting long-term suppression of virus replication has been shown to be beneficial [Lee et al. 2001]. Thus, the credo of HIV-chemotherapy is to suppress HIV replication as much as possible for as long as possible.

An important question in the therapy of chronic HIV infection, which has caused extensive discussion, is when to start. Current guidelines by the US Department of Health and Human Services recommend starting in those patients with a CD4 count of 500 cells/µl or lower [Department of Health and Human Services 2011]. The European AIDS Clinical Society, on the other hand, recommends treatment at a CD4 count of less than 350 cells/µl and consideration of treatment at CD4 counts of more than 350 cells/µl if one or more comorbidities are present [European Guidelines for treatment of HIV infected adults in Europe 2009]. The WHO guidelines, however, recommend treatment of patients with WHO clinical stages 3 and 4 and at CD4 counts of 350 cells/µl or below, irrespective of the WHO clinical level [Antiretroviral Therapy for HIV infection in Adults and Adolescents 2010].

However, increasing evidence shows the clinical benefit of starting therapy at higher CD4 counts, beginning at CD4 counts of 500 cells/µl or lower [Kitahata et al. 2009].

In the foreseeable future, treatment may be recommended for all infected adults and children [Flexner 2007].

The current standard in therapy of chronic HIV infection is to use a combination of at least three drugs simultaneously for the duration of the treatment in order to achieve a highly effective antiretroviral effect and prevent HIV drug resistance. The aim of this Highly Active Antiretroviral Therapy (HAART) is to reduce the viral load to an undetectable level (<50 copies/ml) within 24 weeks after starting treatment. This treatment is effective against viral replication and infection of target cells, it cannot, however, eradicate HIV DNA integrated in host chromosomes. Consequently, life-long treatment is imperative to keep plasma HIV RNA levels low and inhibit progression of infection as well as reduce the risk of transmission.

While most antiretroviral therapy is used in treatment of established chronic HIV infection, this medication is also used in short courses to prevent mother-to-child transmissions and infection in post exposure setting [Goodman & Gilman's 2011].

1.3.1.3.1 Challenges in HIV treatment

Treating HIV infections faces a lot of challenges. One of the most prominent is the prevention of drug resistance. HIV RT lacks a proofreading function and is error prone, so mutation is quite frequent. It is estimated that out of every full-length replication, mutations occur at approximately three bases [Coffin 1995]. Given the large number of replication cycles in infected patients, mutations that lead to the emergence of drug

resistance are likely to occur, especially in untreated patients. Therefore, first-line therapy uses a combination of at least three antiretroviral agents, with different targets, usually two nucleoside/nucleotide RT inhibitors (NRTIs) and one non-nucleoside RT inhibitor (NNRTI), protease inhibitor or integrase inhibitor.

Treatment failure, which is defined as increasing plasma HIV RNA concentrations in a patient with a previously undetectable virus, requires a change of treatment, usually the implementation of a completely new combination of drugs. It is often the result of non-adherence, which depends on the percentage of prescribed doses taken, as well as the drugs used in the regimen [Goodman & Gilman's 2011]. In an effort to improve compliance, fixed dosage combinations, containing multiple antiretroviral agents, have been established and most antiretrovirals are administered orally once or twice daily. Enfuvirtide is the only approved antiretroviral drug that has to be administered parenterally and it is not used for first line therapy [Flexner 2007].

With more effective antiretroviral therapy and increasing life expectancy of HIV patients, long-term toxicity becomes of greater concern. A common consequence of long-term therapy is the development of HIV lipodystrophy syndrome, which has been observed in 10-40 per cent of patients receiving long-term treatment. The contribution of drug therapy to an increased cardiovascular risk associated with chronic HIV infection is not well defined either [Calmy et al. 2009].

A potential concern that applies to protease inhibitors and NNRTIs is pharmacokinetic drug interactions, as all agents of these classes can act as inducers or inhibitors of hepatic cytochrome P450 enzymes (CYPs) and other drug metabolizing enzymes [Goodman & Gilman's 2011]. A better safety profile will therefore play a more important role in the approval of new antiretroviral drugs.

However, one of the biggest challenges in fighting the HIV pandemic is to provide antiretroviral therapy to everyone who needs it. This is not so much of a problem in developed countries, whereas the coverage in antiretroviral therapy in low- and middle income-countries is only 36 per cent [WHO, UNICEF, UNAIDS progress report 2009].

1.3.1.3.2 Drugs used to treat HIV infection

1.3.1.3.2.1 Overview

Antiretroviral drugs can rationally be classified according to their viral targets. A large number of antiretrovirals target viral replication and the responsible viral enzymes, most eminently RT, protease and integrase. Inhibitors of RT and protease were the first available antiretroviral drugs and as per now, a relatively large number of drugs is available for both targets. For integrase, on the other hand, the first and up to now only inhibitor was approved in 2007. A newer approach targets viral entry in CD4+ cells, with two drugs available so far.

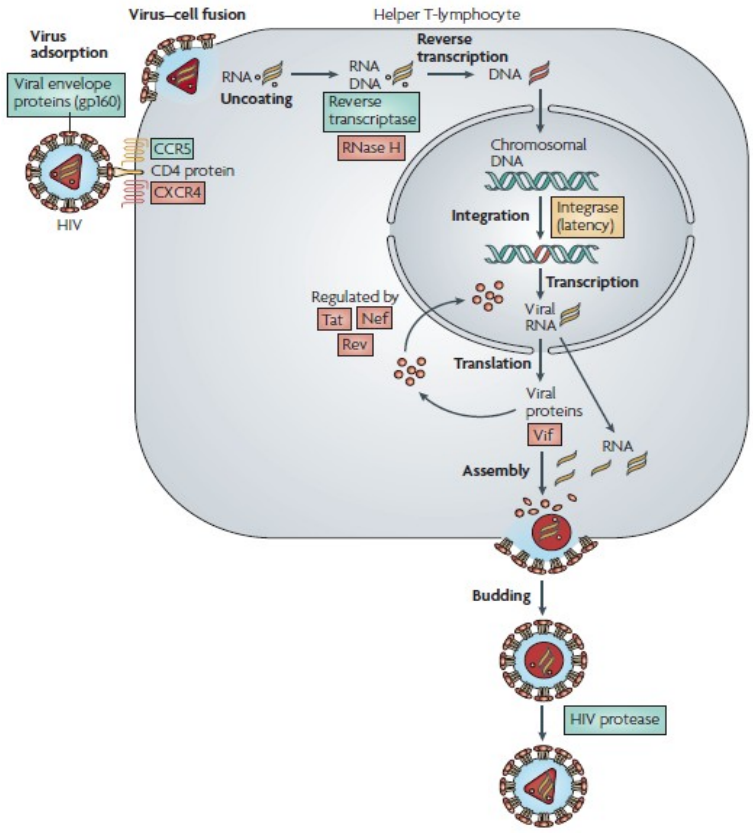


Figure 3: HIV replication cycle with current and possible targets for antiretroviral intervention, from [Flexner 2007]
current targets are blue and yellow, possible targets are coloured red.

Further potential targets include HIV regulatory proteins as well as host cell structures, like the CXCR4 receptor or host proteins involved in HIV replication and research in that direction is under way (Figure 3) [Flexner 2007].

1.3.1.3.2.2 Reverse transcriptase inhibitors

Inhibitors of RT, which converts viral RNA into viral DNA, can be subdivided into nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside inhibitors (NNRTIs)

1.3.1.3.2.2.1 Nucleoside /Nucleotide reverse transcriptase inhibitors

NRTIs structurally resemble natural nucleotides. They must enter cells and undergo phosphorylation in order to present synthetic substrates to RT. The phosphorylated analogues compete with native nucleotides for incorporation into the nascent proviral DNA and thereby inhibit proper reverse transcription. Due to the lack of a 3'-hydroxyl group they also terminate elongation [Goodman & Gilman's 2011].

Their selective toxicity depends on their ability to inhibit HIV RT without affecting host cell DNA polymerases. While all of these drugs have low affinity to human DNA polymerases α and β, some can inhibit human DNA polymerase γ, the mitochondrial enzyme. This toxicity can cause anemia, granulocytopenia, myopathy, peripheral neuropathy and pancreatitis [Calmy et al. 2009].

NRTIs are effective against both HIV-1 and HIV-2.

Table 1: List of antiretroviral agents approved for use, modified from [Goodman & Gilman's 2011]

Antiretroviral Agents Approved for Use in the U.S.	
GENERIC NAME [U.S. TRADE NAME]	ABBREVIATION; CHEMICAL NAMES
Nucleoside Reverse Transcriptase Inhibitors	
Zidovudine [RETROVIR, others][a]	ZDV; azidothymidine (AZT)
Didanosine [VIDEX; VIDEX EC, others]	ddI; dideoxyinosine
Stavudine [ZERIT]	d4T; didehydrodeoxythymidine
Zalcitabine [HIVID][c]	DDC; dideoxycytidine
Lamivudine [EPIVIR][a]	3TC; dideoxythiacytidine
Abacavir [ZIAGEN][a]	ABC; cyclopropylaminopurinylcyclopentene
Tenofovir disoproxil [VIREAD][a]	TDF; phosphinylmethoxypropyladenine (PMPA)
Emtricitabine [EMTRIVA][a]	FTC; fluorooxathiolanyl cytosine
Non-nucleoside Reverse Transcriptase Inhibitors	
Nevirapine [VIRAMUNE]	NVP
Efavirenz [SUSTIVA; STOCRIN][a]	EFV
Delavirdine [RESCRIPTOR]	DLV
Etravirine [INTELENCE]	ETV
Protease Inhibitors	
Saquinavir [INVIRASE]	SQV
Indinavir [CRIXIVAN]	IDV
Ritonavir [NORVIR]	RTV
Nelfinavir [VIRACEPT]	NFV
Amprenavir [AGENERASE; PROZEI][c]	APV
Lopinavir [KALETRA; ALUVIA][b]	LPV/r
Atazanavir [REYATAZ; ZRIVADA]	ATV
Fosamprenavir [LEXIVA; TELZIR]	FPV
Tipranavir [APTIVUS]	TPV
Darunavir [PREZISTA]	DRV
Entry Inhibitors	
Enfuvirtide [FUZEON]	T-20
Maraviroc [SELZENTRY; CELSENTRI]	MVC
Integrase Inhibitor	
Raltegravir [ISENTRESS]	RAL

[a]A number of fixed-dose co-formulations are available: zidovudine + lamivudine (COMBIVIR); zidovidine + lamivudine + abacavir (TRIZIVIR); abacavir + lamivudine (EPZICOM); tenofovir + emtricitabine (TRUVADA); tenofivir + efavirenz + emtricitabine (ATRIPLA). [b]Lopinavir is available only as part of a fixed-dose co-formulation with ritonavir (KALETRA/ALUVIA). [c]No longer marketed worldwide.

1.3.1.3.2.2.2 Non-nucleoside reverse transcriptase inhibitors

NNRTIs are a structurally varied class of substrates that bind to a hydrophobic pocket in the p66 subunit of HIV-1 RT. The pocket is distant from the active site and the NNRTI-binding is not essential for the enzyme activity. However, the NNRTI binding induces a conformational change in the enzyme that greatly decreases its activity. Unlike NRTIs, NNRTIs do act as non competitive inhibitors and do not need activation through phosphorylation. Their binding site however, is virus-strain-specific and the approved agents are only active against HIV-1. They have no activity against host cell DNA polymerases. Frequent side effects include rashes during the first four weeks of treatment and fat accumulation after long-term use [Goodman & Gilman's 2011].

1.3.1.3.2.3 HIV protease inhibitors

HIV protease inhibitors are peptidomimetic agents that competitively inhibit the action of the viral aspartyl protease, thus preventing proteolytic cleavage of HIV gag and pol precursor polypeptides which include essential structural and enzymatic components, and thereby anticipating virus maturation. Human aspartyl proteases are not significantly inhibited due to their different structure.
Most HIV protease inhibitors inhibit CYP 3A4 at clinically achieved concentrations, causing metabolic drug interactions. Common side effects are gastrointestinal effects during the first four weeks of treatment and an increased risk of insulin resistance and lipodystrophy in the long run [Goodman & Gilman's 2011].

1.3.1.3.2.4 Integrase inhibitors

These agents target integrase, the viral enzyme responsible for integration of proviral DNA into the host chromosomes. Integrase is an excellent antiretroviral target, as human DNA does not undergo excision and reintegration. Raltegravir, the only approved drug of this group, prevents covalent binding between host and viral DNA by interfering with essential divalent cations in the catalytic core of the enzyme. It is effective against both HIV-1 and HIV-2 and generally well tolerated [Goodman & Gilman's 2011].

1.3.1.3.2.5 Entry inhibitors

In this category there are currently two approved agents with different mechanisms.

1.3.1.3.2.5.1 Maraviroc

Maraviroc is a chemokine receptor antagonist and binds to the host cell CCR5 receptor and thereby blocks binding of the viral envelope protein gp120. It is the only approved antiretroviral drug that targets a host protein. It is effective against CCR5- tropic strains of HIV and has no activity against CXCR4- or dual-tropic strains. It is generally well tolerated, but there is some theoretical concern that CCR5 inhibition might interfere with immune function [Goodman & Gilman's 2011].

1.3.1.3.2.5.2 Enfuvirtide

Enfuvirtide is a synthetic peptide and inhibits fusion of viral and cell membranes mediated by gp41 and CD4-receptors. It binds gp41 and inhibits conformational changes essential for membrane fusion. Its amino acid sequence is derived from the transmembrane gp41 region of HIV-1, therefore it is only active against HIV-1. Enfuvirtide has to be administered parenterally. The most common side effects are injection-site reactions, like pain and erythema [Goodman & Gilman's 2011].

1.3.2 Drugs used in this study

This study deals with ritonavir (RTV), efavirenz (EFV), emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF).

1.3.2.1 Ritonavir

Ritonavir (RTV) is a peptidomimetic HIV protease inhibitor with a chemical formula of $C_{37}H_{48}N_6O_5S_2$ and a molecular weight of 720.96 [The Merck Index]. It is a white or almost white powder and may exhibit conformational polymorphism which necessitates special precautions in the manufacturing process, as the unwanted RTV form II is

therapeutically ineffective [Bauer et al. 2004]. The structural formula of RTV is depicted in Figure 4.

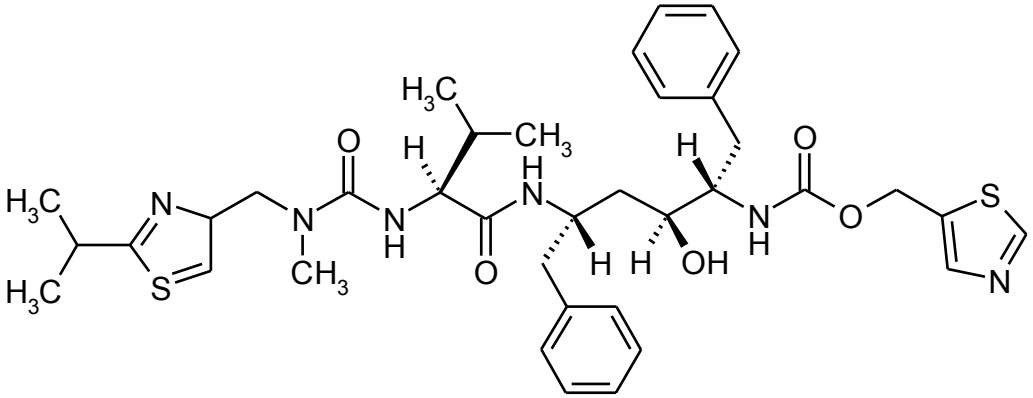


Figure 4: Structural formula of ritonavir

RTV was one of the first approved HIV protease inhibitors and is effective against both HIV-1 and HIV-2. However, it is also a strong inhibitor of CYP3A4 and weak inhibitor of CYP2D6. Special attention should therefore be paid to possible drug interactions with other substrates of these enzymes.

Major side effects of RTV are gastrointestinal (GI) and include nausea, vomiting, diarrhea, anorexia, abdominal pain and taste perversion. In therapeutic doses of 600 mg twice daily, peripheral and perioral paresthesias can occur. RTV can also cause an increase in total serum cholesterol and triglycerides and probably the long-term risk for atherosclerosis.

Nowadays RTV is seldom used as sole protease inhibitor but mostly in combination with other agents of this class to boost their effectivity. RTV inhibits the metabolism of all current HIV protease inhibitors and enhances their pharmacokinetic profile, allowing for a reduction dose and dosing frequency of coadministered drugs. The doses used to boost other HIV protease inhibitors are usually 100 or 200 mg once or twice daily, which is as effective at inhibiting CYP3A4 and much better tolerated than the 600 mg treatment [Goodman & Gilman's 2011].

1.3.2.2 Efavirenz

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor with potent activity against HIV-1. Its chemical formula is $C_{14}H_9ClF_3NO_2$ and its molecular weight is 315.68. [The Merck Index]. The structural formula of EFV is depicted in Figure 5.

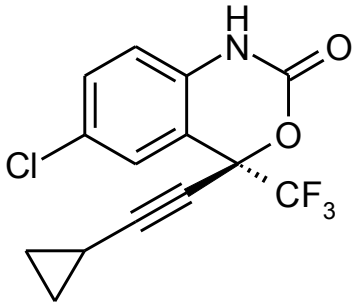


Figure 5: Structural formula of efavirenz

EFV binds HIV-1 RT and induces a conformational change that greatly reduces its activity. It has no activity against host cell DNA polymerases.
Common adverse effects of EFV are central nervous symptoms like dizziness, insomnia and vivid dreams. Rash occurs frequently during the first few weeks of treatment. A rare but life-threatening side effect is Steven-Johnson syndrome. EFV is teratogenic [Calmy et al. 2009].
The usual dose of EFV is 600 mg once daily.

1.3.2.3 Emtricitabine

Emtricitabine (FTC) is a nucleoside reverse transcriptase inhibitor with a structure derived from cytidine. The carbon atom in position 3´ of the ribose is substituted by a sulfur atom, thus preventing chain elongation in reverse transcription. FTC has a chemical formula of $C_8H_{10}FN_3O_3S$ and a molecular weight of 247.25 [The Merck Index]. The structural formula of FTC is depicted in Figure 6.

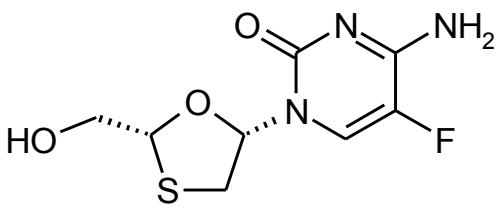


Figure 6: Structural formula of emtricitabine

FTC itself is a prodrug and exerts its antiretroviral activity only after activation by cellular kinases. Its active metabolite, emtricitabine 5´-triphosphate, inhibits HIV RT. FTC is effective against HIV-1, HIV-2 and HBV and has low affinity to human DNA polymerases, explaining its low toxicity to the host [Goodman & Gilman's 2011]. Significant adverse events are rare and include hyperpigmentation of the skin, especially in sun-exposed areas. Caution should be exercised in patients with hepatitis B infection, as severe exacerbations have been reported in patients who discontinued FTC [Calmy et al. 2009].
FTC is usually used in doses of 200 mg once daily.

1.3.2.4 Tenofovir disoproxil fumarate

Tenofovir is a nucleotide reverse transcriptase inhibitor, a derivate of adenosine 5´-monophosphate and the only nucleotide analogue currently marketed for treatment of HIV infection [Goodman & Gilman's 2011]. Unlike adenosine 5´-monophosphate it does not have a complete ribose ring, lacking the C3´. Its chemical formula is $C_9H_{14}N_5O_4P$ and it has a molecular weight of 287.21. Tenofovir itself has very poor oral bioavailability, so a disoproxil prodrug is used, which significantly improves oral absorption as well as cellular penetration. It is most commonly used as fumarate salt. Tenofovir disoproxil fumarate (TDF) occurs as white, fine powder-like crystals and has a chemical formula of $C_{19}H_{30}N_5O_{10}P.C_4H_4O_4$ [The Merck Index]. The structural formula of TDF is depicted in Figure 7.

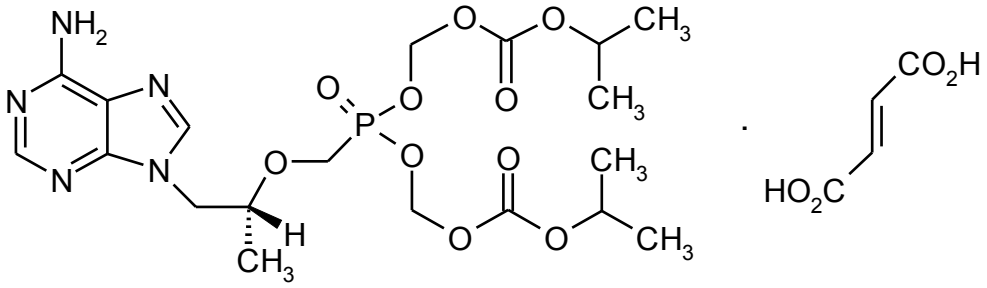


Figure 7: Structural formula of tenofovir disoproxil fumarate

The prodrug is hydrolyzed and tenofovir is phosphorylated by cellular kinases to its active form, tenofovir diphosphate, which is a competitive inhibitor of viral RT and terminates elongation of nascent HIV DNA chains. Tenofovir is effective against HIV-1 and HIV-2 as well as Hepatitis B virus (HBV) and has low affinity to human DNA-polymerases.

Tenofovir is generally well tolerated. However it should be used with caution in patients with preexisting renal diseases, as it is associated with small transient reductions of the estimated glomerular filtration rate and rare episodes of renal failure have been reported with tenofovir [Calmy et al. 2009].

TDF is usually used in doses of 300 mg, which corresponds to 245 mg of tenofovir disoproxil, once daily.

1.4 Aim of study

The aim of the study is twofold. The first part is to develop a monograph for RTV tablets for the Ph. Int. It should specify requirements for these tablets and provide methods of analysis for the verification of identity, dissolution testing, the detection of impurities and the quantification of the active ingredient.
Methods from the monograph of RTV drug substance in the Ph. Int. were checked for applicability to tablets, if necessary modified to meet the different requirements of this dosage form and applied to commercial samples of tablets. Other pharmaceutical compendia and scientific publications were consulted for additional information.
In accordance with the aims and guidelines of the Ph. Int., simple techniques were employed where possible.

The second part deals with proposed monographs of two fixed dosage combinations that have previously been developed in our laboratory. The assay methods for tablets containing FTC and TDF and for a combination of EFV, FTC and TDF were validated. ICH guidelines were used to identify the parameters of interest and establish a validation plan. Analyses were carried out to determine sensitivity, linearity, precision, including repeatability and intermediate precision, accuracy and range of the respective methods.

2 Materials and Methods

2.1 Reagents and chemicals

Dichloromethane and sodium hydroxide pellets were purchased from Fisher scientific (Erembodegem, Belgium). HPLC-grade acetonitrile (ACN), methanol (MeOH) and potassium permanganate were purchased from VWR (Leuven, Belgium). Ammonium hydroxide solution and decaethylene glycol monododecyl ether were obtained from Sigma-Aldrich (Seelze, Germany) and hydrochloric acid from J.T. Baker (Deventer, the Netherlands). Sodium dihydrogen phosphate dihydrate and sodium hexanesulfonate were purchased from Acros Organics (Geel, Belgium). Phosphoric acid and sulfuric acid were purchased from Chem Lab (Zedelgem, Belgium). Potassium dihydrogen phosphate was obtained from Merck (Darmstadt, Germany).
Demineralized water was purified in our laboratory by filtering through an ultrapure Milli-Q (Millipore, Milford, MA, USA). RTV, EFV, FTC and TDF drug substances, as well as RTV 100 mg tablets and EFV 600 mg/ FTC 200 mg/ TDF 300 mg tablets and FTC 200 mg/ TDF 300 mg tablets were donated by the WHO (Geneva, Switzerland).

2.2 Preparation of sample solutions

Sample solutions were prepared using a quantity of powdered tablets containing the amount of active ingredient given in the corresponding method. Therefore, 20 tablets were weighed and powdered. The average mass was calculated and used for determining the required amount of powdered tablets, which was then dissolved in the solvent, sonicated and filtered through a 0.45 µm membrane (Chromafil® RC-45/25, Macherey-Nagel, Düren, Germany).

2.3 Instrumentation and LC-conditions

2.3.1 Ritonavir tablets

Thin-layer chromatography (TLC) was performed using pre-coated TLC plates Silica gel 60/ Kieselguhr with fluorescence indicator (F_{254}) (Merck, Darmstadt, Germany) and Chromato-Vue Model CC-20 (Ultra-Violet Products Inc., Cambridge, UK) for examination in ultraviolet light (254 nm).

Ultraviolet (UV) - Spectra were generated using a Philips PU8740 UV/VIS scanning spectrophotometer.

A SR8 Plus Dissolution test station (Hanson Virtual Instruments, Chatsworth, USA) was used for dissolution testing.

Liquid chromatography (LC) analyses were carried out on a LaChrome Elite LC apparatus consisting of an L-2130 Pump (Merck-Hitachi), an L-2200 Autosampler (Merck-Hitachi), an L-2400 UV-Detector (Merck-Hitachi) and an Organizer (Merck-Hitachi). EZChrom Elite Version 3.1.6 (Scientific Software Inc., Lincolnwood, USA) was used for data acquisition. A Hypersil base deactivated silica (BDS) C18 column (250 x 4.6 mm I.D.), 5 µm (Thermo Hypersil-Keystone, Cheshire, UK) was used as stationary phase. The column was immersed in a water bath and the temperature was kept at 35 °C by a Julabo EM heating immersion circulator (Seelbach, Germany). The flow rate was 1.0 ml per minute and the UV-detector was set at 240 nm. The injection volume was 20 µl.
pH measurements were performed with a Metrohm 691 pH-Meter (Metrohm, Antwerp, Belgium).

2.3.2 Efavirenz, emtricitabine and tenofovir disoproxil fumarate tablets

Liquid chromatography (LC) analyses were carried out on two different LC apparatus. The first one was a LaChrome apparatus, consisting of an L-7100 Pump (Merck-Hitachi), an L-7200 Autosampler (Merck-Hitachi), an L-7400 UV-Detector (Merck-Hitachi), a D-

7000 Interface (Merck-Hitachi) and an L-7614 Degasser (Merck). EZChrom Elite Version 3.1.6 (Scientific Software Inc., Lincolnwood, USA) was used for data acquisition. LC-apparatus II was an UltiMate 3000-system, comprising Pump, Autosampler and Diode Array Detector, using Chromeleon 6.80 for data acquisition. A Hypersil BDS C18 column (250 x 4.6 mm I.D.), 5 µm (Thermo Hypersil-Keystone, Cheshire, UK) was used as stationary phase. Also Brava BDS (250 x 4.6 mm I.D.), 5 µm (Alltech, Deerfield, Illinois, USA), HyPurity Elite C18 (150 x 4.6 mm I.D.), 3 µm, HyPurity C18 (250 x 4.6 mm I.D.), 5 µm, HyPurity Elite C18 (150 x 4.6 mm I.D.), 5 µm (ThermoQuest, Cheshire, UK) and Discovery C18 (250 x 4.6 mm I.D.), 5 µm (Supelco, Bellefonte, PA, USA) columns were tested. The columns were immersed in water baths and the temperature was kept at 35 °C by Julabo EM heating immersion circulators (Seelbach, Germany). The flow rate was 1.0 ml per minute, the UV-detectors were set at 280 nm. The injection volume was 20 µl.

2.4 Mobile phases

2.4.1 Ritonavir tablets

Gradient elution was employed, using two mobile phases with different concentrations of ACN, phosphate buffer pH 4.0 and water. Mobile phase A consisted of 35 volumes of ACN, 28 volumes of phosphate buffer pH 4.0 and 37 volumes of water. Mobile Phase B contained 70 volumes of ACN, 28 volumes of phosphate buffer pH 4.0 and 2 volumes of water.

The phosphate buffer was prepared by dissolving 7.8 g of sodium dihydrogen phosphate dihydrate and 1.88 g of sodium hexanesulfonate in 800 ml of purified water, adjusting the pH to 4.0 by adding phosphoric acid (~ 105 g/l) and then diluting to 1000 ml with purified water. Phosphoric acid (~ 105 g/l) was prepared by diluting 115 g of phosphoric acid 85% with purified water to make 1000 ml solution.

Table 2: Gradient programme for LC analysis of ritonavir tablets

Time (min)	Mobile phase A (% v/v)	Mobile Phase B (% v/v)	Comments
0-20	70	30	Isocratic
20-30	70 to 0	30 to 100	Linear gradient
30-40	0	100	Isocratic
40-45	0 to 70	100 to 30	Return to initial composition
45-50	70	30	Re-equilibration

2.4.2 Efavirenz, emtricitabine and tenofovir disoproxil fumarate tablets

A gradient programme, as given in the monograph was used. Mobile phase A consisted of 5 volumes of phosphate solution and 95 volumes of water. Mobile Phase B contained 70 volumes of ACN, 5 volumes of phosphate solution and 25 volumes of water. The phosphate solution was prepared by dissolving 27.22 g of potassium dihydrogen phosphate in 1000 ml of purified water.

Table 3: Gradient programme for LC analysis of EFV/FTC/TDF tablets

Time (min)	Mobile phase A (% v/v)	Mobile Phase B (% v/v)	Comments
0-9	93	7	Isocratic
9-15	93 to 0	7 to 100	Linear gradient
15-19	0	100	Isocratic
19-19.1	0 to 93	100 to 7	Return to initial composition
19.1-30	93	7	Re-equilibration

3 Results and discussion

3.1 Development of a monograph for ritonavir tablets

The monograph for RTV drug substance in the Ph. Int. was used as a starting point for developing a monograph for RTV tablets [The International Pharmacopoeia, 4th edition]. General information was gathered according to guidelines for the development of monographs for the Ph. Int. by consulting the corresponding parts of the Indian Pharmacopoeia (IP) [Indian Pharmacopoeia, 5th edition], European Pharmacopoeia [European Pharmacopoeia, 7th edition] and the WHO list of essential medicines[WHO Model List of Essential Medicines]. Specific tests for identity, impurities and content were taken from the drug substance monograph in the Ph. Int. and, as far as they were applicable to tablets, applied to the sample. It was found necessary for some of these tests to make minor adjustments in order to use them for the analysis of RTV tablets, whereas others were found to be not suitable for this dosage form.
To fulfill the requirement of a dissolution test, the method from the RTV tablet monograph in the IP [Indian Pharmacopoeia, 5th edition] and a method taken from the FDA-Database [FDA-dissolution database] for dissolution tests were adapted and applied to the sample, the former of which was included in the proposed monograph for reasons of availability of chemicals used for medium preparation and easier handling.

3.1.1 The Proposed Monograph

The monograph as proposed for inclusion in the Ph. Int.:

Ritonaviri Compressi

Ritonavir Tablets

Category. Antiretroviral (Protease Inhibitor).

Storage. Ritonavir tablets should be stored at temperatures not exceeding 30 °C.

Additional information. Strength in the current WHO Model list of essential medicines: 25 mg, 100 mg of ritonavir.

Requirements

Comply with the monograph for "Tablets".

Definition. Ritonavir tablets contain not less than 90.0% and not more than 110.0% of the amount of ritonavir ($C_{37}H_{48}N_6O_5S_2$) stated on the label.

Identity tests

A. Carry out test A.1 or, where UV detection is not available, test A.2.

A.1 Carry out the test as described under 1.14.1 Thin-layer chromatography, using silica gel R6 as the coating substance and a mixture of 67 volumes of dichloromethane R, 20 volumes of acetonitrile R, 10 volumes of methanol R and 3 volumes of ammonia (~260 g/l) TS as the mobile phase. Apply separately to the plate 10 µl of each of the following solutions. For solution (A) shake a quantity of the powdered tablets equivalent to 25 mg of ritonavir with 5 ml methanol and filter. Add 0.5 ml of ammonia (~260 g/l) to 2.0 ml of the filtrate and shake. For solution (B) use 5 mg of ritonavir RS per ml, add 0.5 ml of ammonia (~260 g/l) to 2.0 ml of the solution and shake. After removing the plate from the chromatographic chamber, allow it to dry exhaustively in a current of cool air. Examine the chromatogram in ultraviolet light (254 nm).

The principal spot obtained with solution A corresponds in position, appearance and intensity with that obtained with solution B.

A.2 Carry out the test as described under 1.14.1 Thin-layer chromatography, using the conditions described above under test A.1 but using silica gel R5 as the coating substance. Spray lightly with basic potassium permanganate (5 g/l) TS and examine the chromatogram in daylight.

The principal spot obtained with solution A corresponds in position, appearance, and intensity with that obtained with solution B.

B. See the test described under Assay. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that in the chromatogram obtained with the reference solution.

Dissolution. Carry out the test as described under 5.5 Dissolution test for solid oral dosage forms, using as the dissolution medium, 900 ml of hydrochloric acid (0.1 mol/l) VS, and rotating the paddle at 100 revolutions per minute. At 60 minutes withdraw a sample of 10 ml of the medium through an in-line filter. Allow the filtered sample to cool down to room temperature.

Determine the content of ritonavir ($C_{37}H_{48}N_6O_5S_2$) in the medium by 1.14.4 High-performance liquid chromatography, using the conditions described under Assay using a suitable ritonavir RS solution as a reference solution.

For each of the six tablets tested, calculate the total amount of ritonavir ($C_{37}H_{48}N_6O_5S_2$) in the medium from the results obtained. The amount in solution for each tablet is not less than 80% of the amount stated on the label. If the amount obtained for one of the six tablets is less than 80%, repeat the test using a further six tablets; the average amount for all 12 tablets tested is not less than 75% and the amount obtained for no tablet is less than 60%.

Related substances

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using the conditions described under Assay.

Prepare the following solutions using a mixture of 70 volumes of mobile phase A and 30 volumes of mobile phase B as diluent. For solution (1) shake a quantity of powdered tablets containing 25 mg of ritonavir with 50 ml of the diluent, filter and use the clear filtrate. For solution (2) dilute a suitable volume of solution (1) to obtain a concentration of 0.5 µg of ritonavir per ml.

For the system suitability test: prepare solution (3) using 5 ml of solution (1) and 1 ml of sulfuric acid (475 g/l), heat in a boiling water bath for 20 minutes.

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of 240 nm.

Maintain the column temperature at 35 °C.

Inject 20 µl of solution (3). The test is not valid unless the resolution between the principal peak (retention time about 22 minutes) and the peak with a relative retention of about 0.8 is not less than 2.0. The test is also not valid unless the resolution between the principal peak and the peak with a relative retention of about 1.5 is not less than 6.5. If necessary adjust the amount of acetonitrile in both mobile phases A and B, or adjust the gradient program.

Inject alternatively 20 µl each of solutions (1) and (2).

In the chromatogram obtained with solution (1), the area of any peak, other than the principal peak, is not greater than three times the area of the principal peak obtained with solution (2) (0.3%). In the chromatogram obtained with solution (1), the areas of not more than two peaks, other than the principal peak, are greater than twice the area of the principal peak obtained with solution (2) (0.2%) and the areas of not more than four such peaks are greater than the area of the principal peak obtained with solution (2) (0.1%). The sum of the areas of all peaks, other than the principal peak, is not greater than ten times the area of the principal peak obtained with solution (2) (1%). Disregard any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with solution (2) (0.05%). Disregard any peak with a retention time less than the retention time of the peak obtained in the system suitability test with a relative retention of about 0.5.

Assay

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with base-deactivated particles of silica gel the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm)[1].

Use the following conditions for gradient elution:

Mobile phase A: 35 volumes of acetonitrile R, 28 volumes sodium phosphate buffer pH 4.0 and 37 volumes of purified water.

Mobile phase B: 70 volumes of acetonitrile R, 28 volumes sodium phosphate buffer pH 4.0 and 2 volumes of purified water.

Prepare the sodium phosphate buffer pH 4.0 by dissolving 7.8 g of sodium dihydrogen phosphate dihydrate R and 1.88 g of sodium hexanesulfonate R in 800 ml of purified water, adjust the pH to 4.0 by adding phosphoric acid (~105 g/l) TS and dilute to 1000 ml with purified water.

[1] Hypersil BDS C18 has been found suitable

Time (min)	Mobile phase A (% v/v)	Mobile Phase B (% v/v)	Comments
0-20	70	30	Isocratic
20-30	70 to 0	30 to 100	Linear gradient
30-40	0	100	Isocratic
40-45	0 to 70	100 to 30	Return to initial composition
45-50	70	30	Re-equilibration

Prepare the following solutions using a mixture of 70 volumes of mobile phase A and 30 volumes of mobile phase B as diluent. For solution (1) shake a quantity of powdered tablets containing 25 mg of ritonavir with 50 ml of the diluent, filter and use the clear filtrate. For solution (2) use 0.5 mg of ritonavir RS per ml of the diluent.

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of 240 nm.

Maintain the column temperature at 35 °C.

For the system suitability test: prepare solution (3) using 5 ml of solution (1) and 1 ml of sulfuric acid (475 g/l), heat in a boiling water bath for 20 minutes.

Inject 20 µl of solution (3). The test is not valid unless the resolution between the principal peak (retention time about 22 minutes) and the peak with a relative retention of about 0.8 is not less than 2.0. The test is also not valid unless the resolution between the principal peak and the peak with a relative retention of about 1.5 is not less than 6.5.

Inject alternatively 20 µl each of solutions (1) and (2).

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of ritonavir ($C_{37}H_{48}N_6O_5S_2$) in the tablets.

3.1.2 Development of the monograph

3.1.2.1 Requirements

The general monograph for tablets in the Ph. Int. provides definitions, information and requirements regarding manufacture, labeling and storage as well as some tests, such as visual inspection, uniformity of mass, uniformity of content and a dissolution test. Specific information not given in the general monograph was included in the proposed monograph.
20 random tablets were subjected to visual inspection to see if they are undamaged, smooth and of uniform color. The same tablets were weighed singly to perform a uniformity of mass test. The average mass and the deviation of the mass of each tablet from the average mass were calculated. The mass of a minimum of 18 tablets must not deviate more than 5% from the average mass, and the mass of not more than two tablets may deviate by more than 10% from the average mass.
The tablets examined complied with these specifications. Detailed results are given in the annex.

3.1.2.2 Definition

The requirements given under definition were established in accordance with the limits generally used for dosage forms.

3.1.2.3 Identity tests

The Ph. Int. monograph for RTV drug substance does give four Identity tests [The International Pharmacopoeia, 4th edition]. Two of them employ TLC, one UV-Spectrophotometry and one infrared (IR) - Spectroscopy.

3.1.2.3.1 Thin-Layer chromatographic methods

Identity Test A.1 was applied to the sample using pre-coated TLC plates Silica gel 60/ Kieselguhr F_{254} and a mixture of 67 volumes of dichloromethane, 20 volumes of ACN, 10 volumes of methanol and 3 volumes of ammonium hydroxide solution 25% as the mobile phase. The sample solution was prepared by shaking a quantity of powdered tablets equivalent to 25 mg of RTV with 5 ml of methanol and filtering it. A RTV drug substance solution of 5 mg/ml in methanol was used as reference solution. 10 µl of each solution were applied to the plate using a Hamilton syringe. After developing the chromatograms, the plates were allowed to dry and examined under ultraviolet light (254 nm).

Identity test A.2 was carried out using the conditions described under test A.1 and examined in daylight after spraying with basic potassium permanganate solution (5 g/l). The basic potassium permanganate solution was prepared by dissolving 4 g of sodium hydroxide in 100 ml of purified water and adding 0.5 g of potassium permanganate.

The principal spot obtained with the sample solution should correspond to the spot obtained with the reference solution in position, appearance and intensity. However, the chromatogram exhibited strong tailing for the sample (Figure 8).

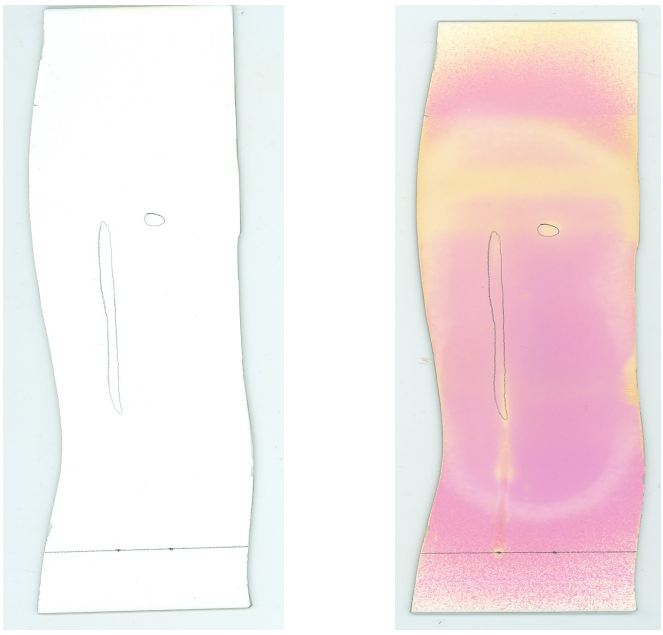


Figure 8: TLC-chromatograms obtained from Identity Test A.1. (left) and A.2. (right) with the method from the monograph of ritonavir drug substance

pH-values of both solutions were measured to see if there is any difference but were found to be 5 for both solutions.
It was decided to add ammonium hydroxide solution 25% to the sample solution, under the assumption that the tablets contain RTV in its protonated form and this being the reason for the strong tailing.
The tailing was significantly reduced by adding ammonia, whereupon different amounts of ammonia were tried, and a quantity of 0.5 ml ammonia per 2 ml of sample solution was found to be reasonable. To achieve the same degree of dilution for both the sample and the reference solution, the addition of ammonia to both solutions was included in the proposed monograph (Figure 9).

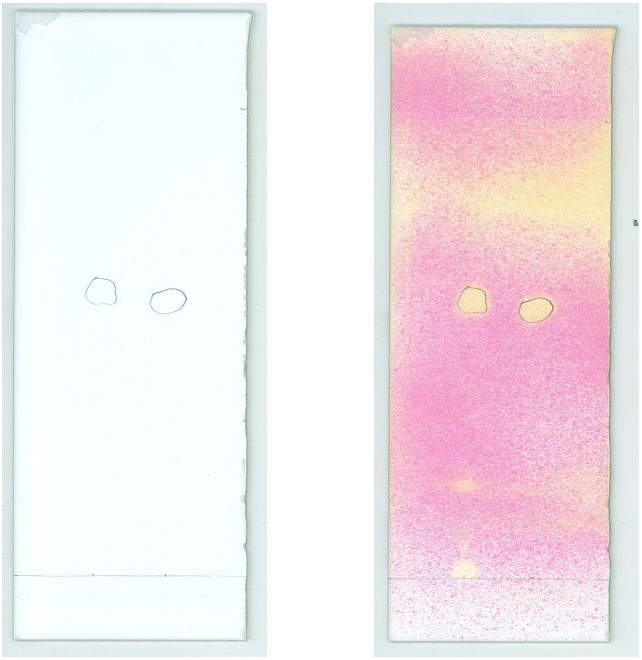


Figure 9: TLC-chromatograms obtained from Identity Test A.1. (left) and A.2. (right) with the method from the proposed monograph

3.1.2.3.2 Spectrophotometric methods

UV-Spectrophotometry

Two reference solutions were prepared by dissolving 20 mg of RTV drug substance in 100 ml of methanol each and further diluting 5 ml of each solution to 25 ml with the

same solvent. Spectra were recorded in triplicate for each solution. A typical reference spectrum is shown in Figure 10.

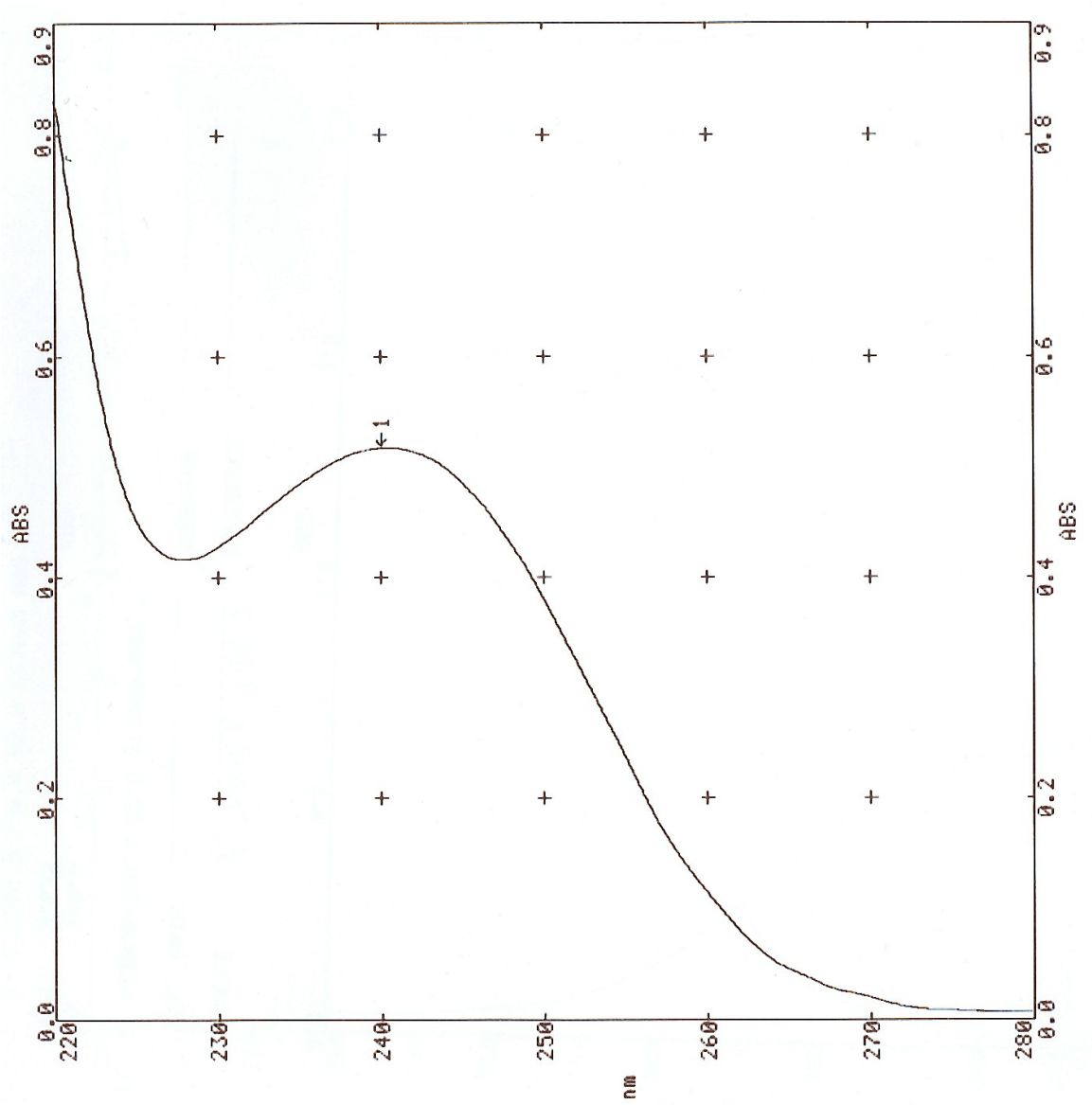


Figure 10: UV-spectrum obtained with reference solution

Two sample solutions with a concentration of 40 µg/ml RTV in methanol were prepared by dissolving a quantity of powdered tablets equivalent to 20 mg of RTV in methanol and then diluting 5 ml of the obtained solution to 25 ml with methanol. Spectra in the range of 220 to 280 nm were recorded in triplicate for both solutions. According to the RTV drug substance monograph, the spectrum exhibits a maximum at 240 nm. The spectrum

obtained with the sample solution does, however, not display this maximum (Figure 11). This is probably due to the influence of excipients contained in the dosage form.

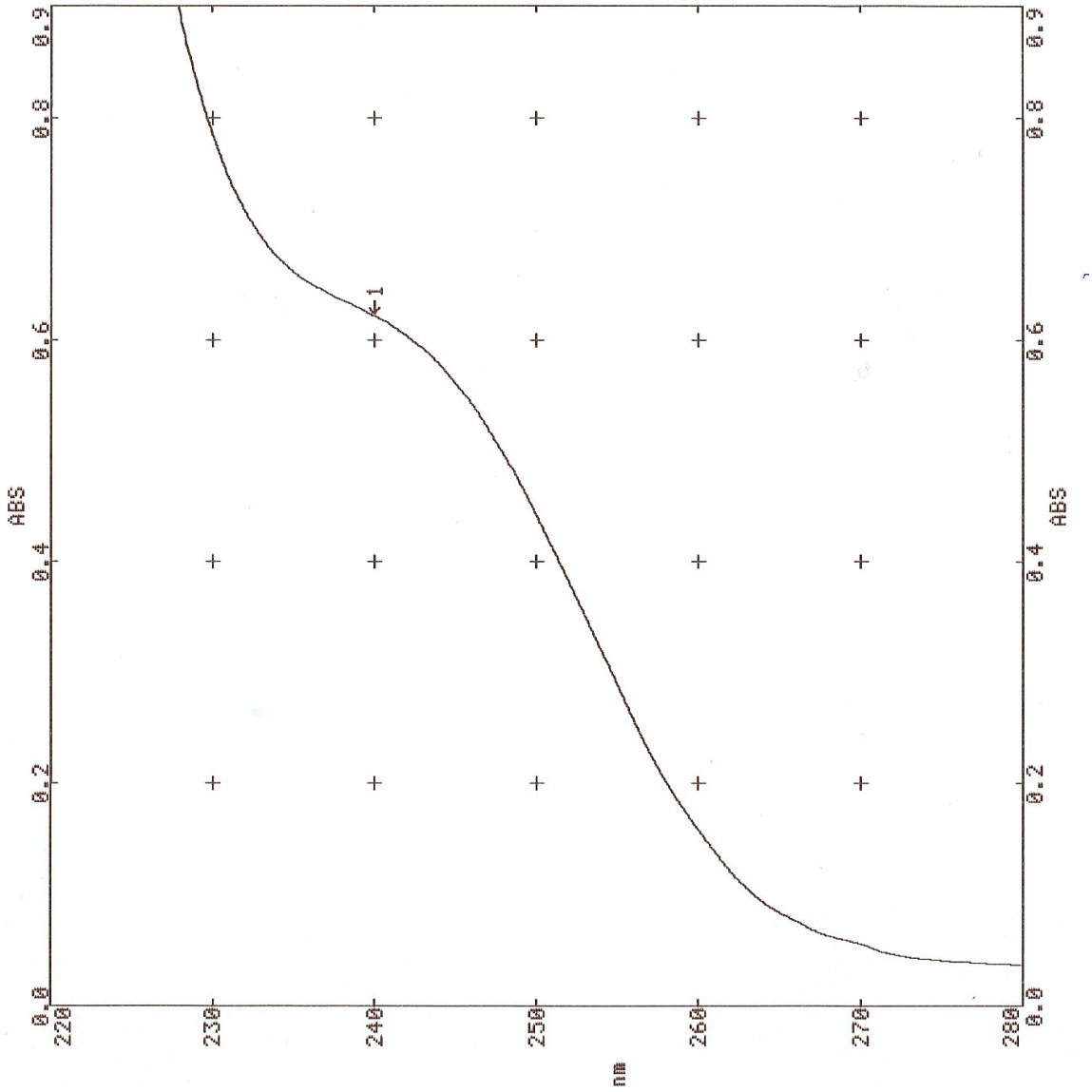


Figure 11: UV-spectrum obtained with sample solution

Unlike the spectrum obtained with the sample solution, the reference spectrum clearly exhibits a maximum at 240 nm, as described in the monograph for RTV drug substance. It was concluded that the UV-Spectrophotometric method described in the drug substance monograph is not appropriate for identification of RTV tablets and therefore not included in the proposed monograph.

IR-Spectroscopy

The IR-spectroscopic method provided by the RTV drug substance monograph was not applied to the tablets, as it is well established that due to the influence of excipients the spectrum obtained with this dosage form does clearly differ from a spectrum obtained with the drug substance or a reference spectrum.

LC

To provide an alternative to the TLC-tests, it was proposed to use the LC-method described under Assay for identification of RTV tablets by comparing the retention times of the principal peaks obtained with the sample solution and reference solution. A corresponding suggestion is included in the proposed monograph as Identity test B. An overlay of chromatograms of sample solution and reference solution obtained with the assay method is shown in Figure 12.

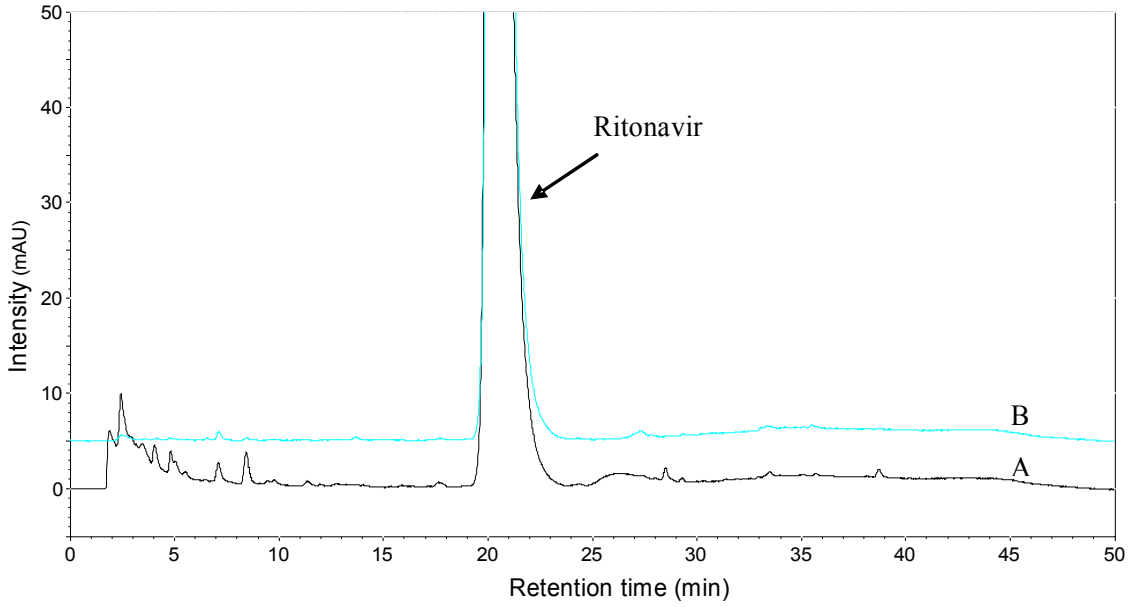


Figure 12: Overlay of chromatograms of sample solution (black) and reference solution (blue) obtained with the assay method

3.1.2.4 Dissolution test

Two different methods for dissolution tests were applied to the sample. The first one was taken from the IP monograph for RTV tablets [Indian Pharmacopoeia, 5th edition]. It

uses the “paddle”-apparatus, with 900 ml of hydrochloric acid (0.1 mol/l) as dissolution medium and the paddle rotating at 100 revolutions per minute. The dissolution medium was prepared by diluting 9.85 g of hydrochloric acid 37% to 1000 ml with purified water. After 60 minutes, samples were drawn, filtered, allowed to cool down to room temperature and analyzed by LC, using the method described under assay in the Ph. Int. drug substance monograph. A 0.1 mg/ml RTV drug substance solution, which was prepared by dissolving 20 mg of RTV drug substance in 20 ml of methanol and diluting 5 ml of this solution to 50 ml with the dissolution medium, was used as reference solution. The amount of RTV in the dissolution medium was calculated using the following equation:

$$X = a * 90 / b$$

with X:	amount of RTV in the dissolution medium (mg/ml)
a:	response of the sample solution (normalized area)
b:	response of the reference solution (normalized area)

The acceptance criterion given in the IP is not less than 80% for each tablet. The Ph. Int. does provide general acceptance criteria for dissolution tests in the “Methods of Analysis” part, which consist of three stages [The International Pharmacopoeia, 4th edition].
The average total amount of RTV in the dissolution medium for six tablets was 85.16 mg, which corresponds to 85.16% of the amount stated on the label. However, the amount for one tablet was below 80% and by using the limit from the IP, which was found to be reasonable, and by applying the criteria from the Ph. Int., another 6 tablets were analyzed to see whether the sample complies with the second stage. To meet these criteria, the average of 12 tablets has to be equal to or greater than 75%, and the amount of no tablet must be less than 60%. The average of all 12 tablets was 83.64% and the amount of no tablet was below 60%. Thus the sample complied with the second stage.

The second method was taken from the FDA-database for dissolution methods [FDA-dissolution database]. It also employs the “paddle”-apparatus but uses 900 ml of 60 mM Polyoxyethylene 10 laurylether as dissolution medium and 75 revolutions per minute. Samples were drawn after 60 minutes, filtered and allowed to cool down to room temperature. Quantification was done by LC, using the same method as described above. The average total amount of RTV in the dissolution medium for six tablets was 84.29

mg/ml, which corresponds to 84.29%. When applying the same criteria as in the first method, two tablets failed, so another six tablets were analyzed. The average of all 12 tablets was 90.05% and the amount of no tablet was below 60%.

Due to easier availability of chemicals for medium preparation and easier handling, the first method was preferred for inclusion in the proposed monograph. The limit for acceptance given in the IP was found to be reasonable and, combined with the general criteria from the Ph. Int., included as specific information in the proposed monograph.

3.1.2.5 Related substances

The method given in the Ph. Int. monograph for RTV drug substance was applied to the sample [The International Pharmacopoeia, 4th edition]. Sample solution (1) was prepared by shaking a quantity of powdered tablets containing 25 mg of RTV with 50 ml of mobile phase A, sonicating and filtering, to obtain a concentration of 0.5 mg RTV per ml.

When preparing the sample solutions, a solubility problem was encountered, which also occurred when preparing solutions with RTV drug substance. In spite of excessive sonication, the samples did not dissolve properly. Therefore, the dissolution medium was changed to a mixture of 70 volumes of mobile phase A and 30 volumes of mobile phase B, which corresponds to the initial composition of the mobile phase in the LC-method. The modified preparation of solutions was included in the proposed monograph accordingly.

To perform the system suitability test, solution (3) was prepared by adding 1 ml of sulfuric acid (~ 475 g/l) to 5 ml of solution (1) and heating in a boiling water bath for 20 minutes. The sulfuric acid was prepared by diluting 4.95 g of sulfuric acid 96% to 10 ml with water. This solution was then analyzed with the given LC-method. A typical chromatogram of RTV tablets solution obtained under the system suitability test conditions is shown in figure 13.

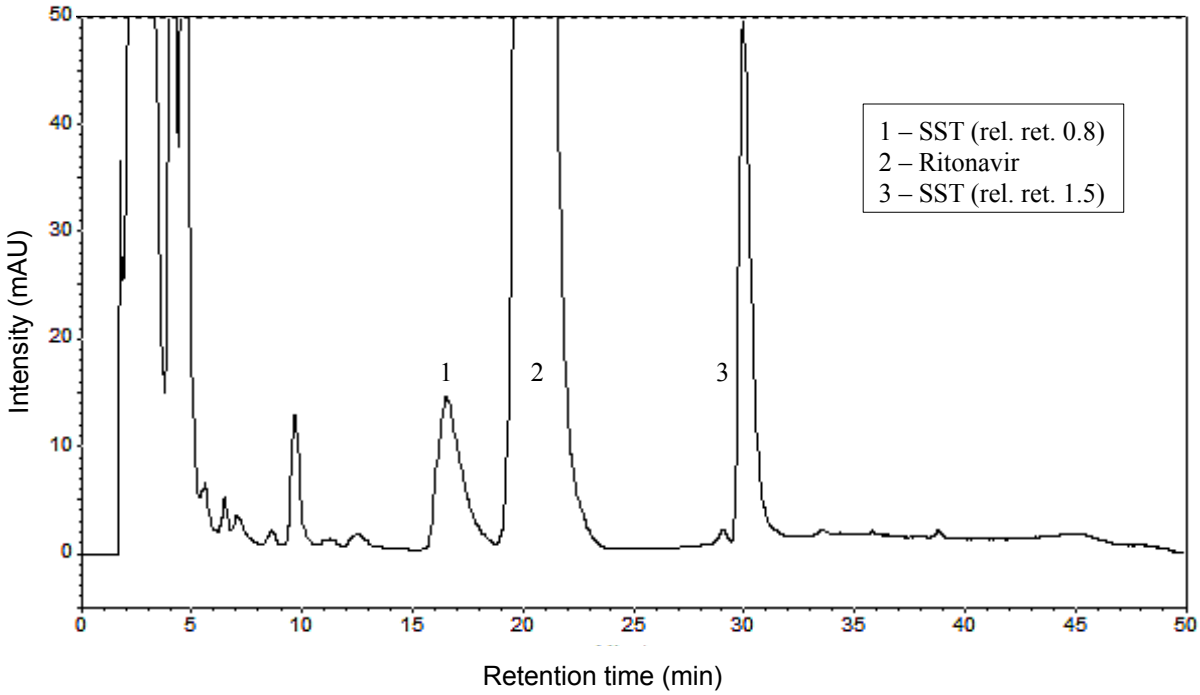


Figure 13: A typical chromatogram of ritonavir tablets solution obtained under the system suitability test conditions

According to the Ph. Int. monograph for RTV drug substance, the test is not valid unless the resolution between the principal peak and the peak with a relative retention of about 0.8 is not less than 3.5 and unless the resolution between the principal peak and the peak with a relative retention of about 1.5 is higher than 9.0 [The International Pharmacopoeia, 4th edition].

It was, however, not possible to meet these requirements, in spite of repeating the degradation and analyzing the degraded solution several times and using other columns of the same type. Finally system suitability criteria were adapted in accordance with the results obtained and with respect to reasonable limits for resolution.

Next, solution (2) was prepared by diluting solution (1) with a mixture of mobile phases A/B 70:30 (v/v), to obtain a concentration of 0.5 µg RTV per ml, which corresponds to 0.1% RTV. Both solutions were prepared in duplicate and analyzed with LC in triplicate (Figures 14 and 15).

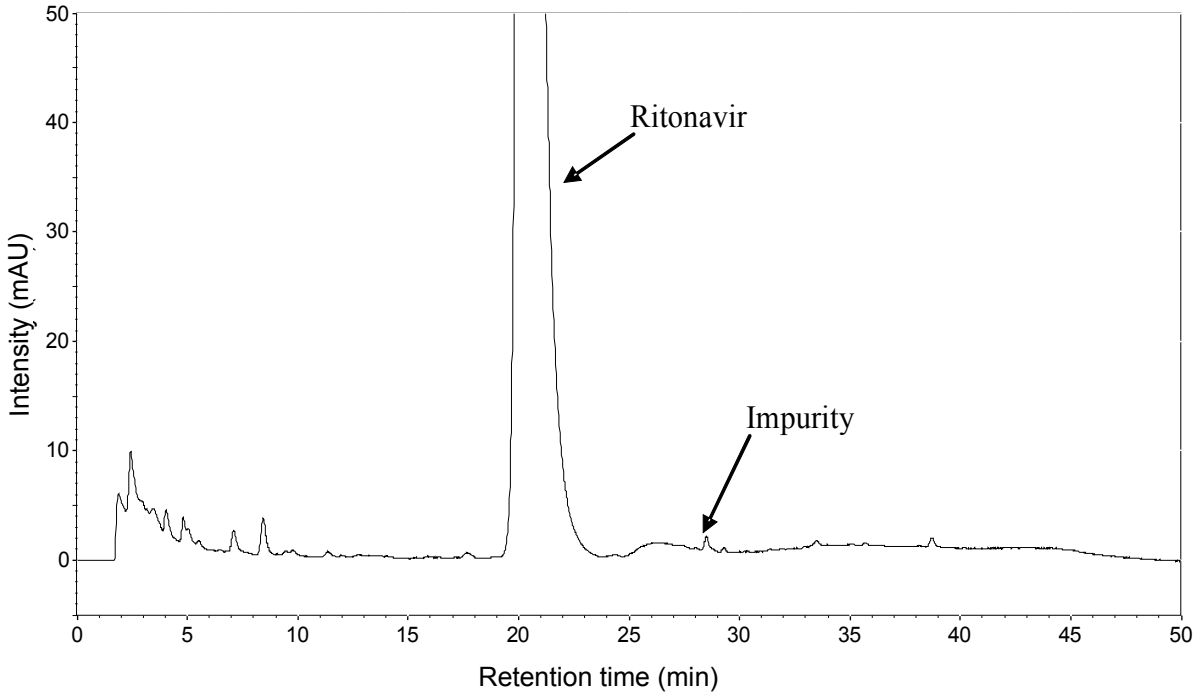


Figure 14: A typical chromatogram obtained with solution (1), 0.5 mg/ml RTV

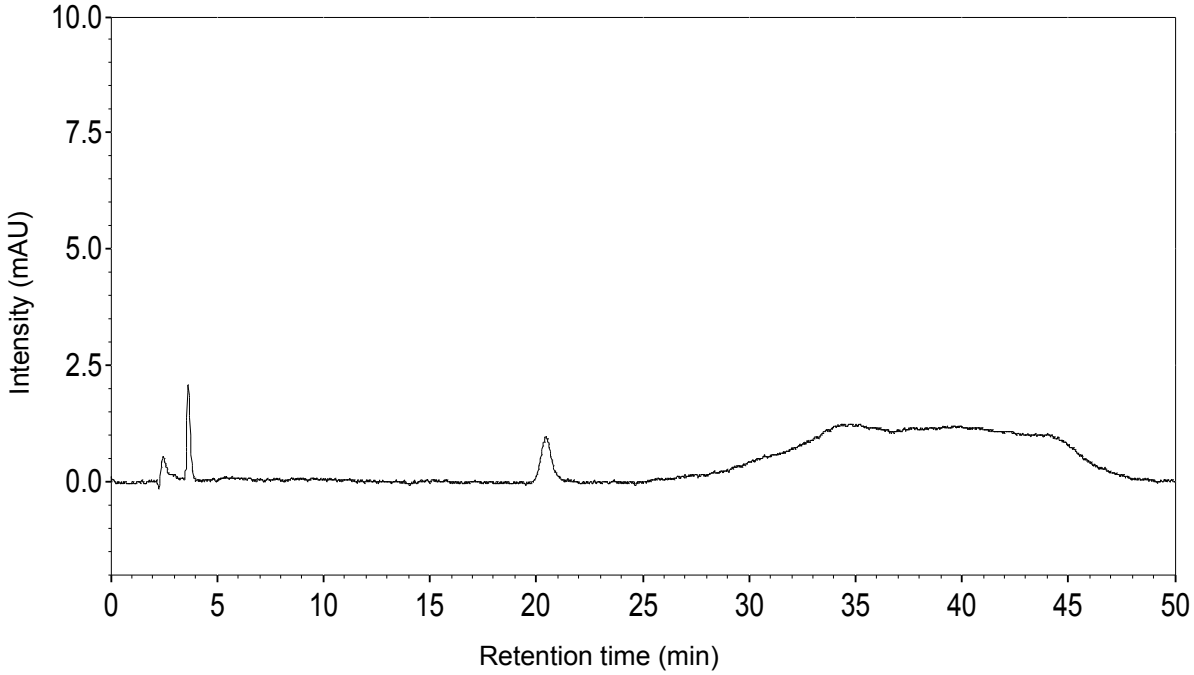


Figure 15: A typical chromatogram obtained with solution (2), 0.5 µg/ml RTV

The content of each impurity found with solution (1) was then calculated using the response obtained with solution (2) as a reference of 0.1%. The results were then compared to the criteria in the RTV monograph in the Ph. Int.:

"In the chromatogram obtained with solution (1), the area of any peak, other than the principal peak, is not greater than three times the area of the principal peak obtained with solution (2) (0.3%). In the chromatogram obtained with solution (1), the areas of not more than two peaks, other than the principal peak, are greater than twice the area of the principal peak obtained with solution (2) (0.2%) and the areas of not more than four such peaks are greater than the area of the principal peak obtained with solution (2) (0.1%). The sum of the areas of all peaks, other than the principal peak, is not greater than ten times the area of the principal peak obtained with solution (2) (1.0%). Disregard any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with solution (2) (0.05%)."[The International Pharmacopoeia, 4th edition]

It was furthermore decided to disregard peaks with a retention time less than the retention time of the peak obtained in the system suitability test (SST) with a relative retention of about 0.5, as a lot of peaks in that area are probably due to excipients and not well separated. A typical chromatogram obtained under the SST conditions with relevant SST peaks is shown in Figure 16.

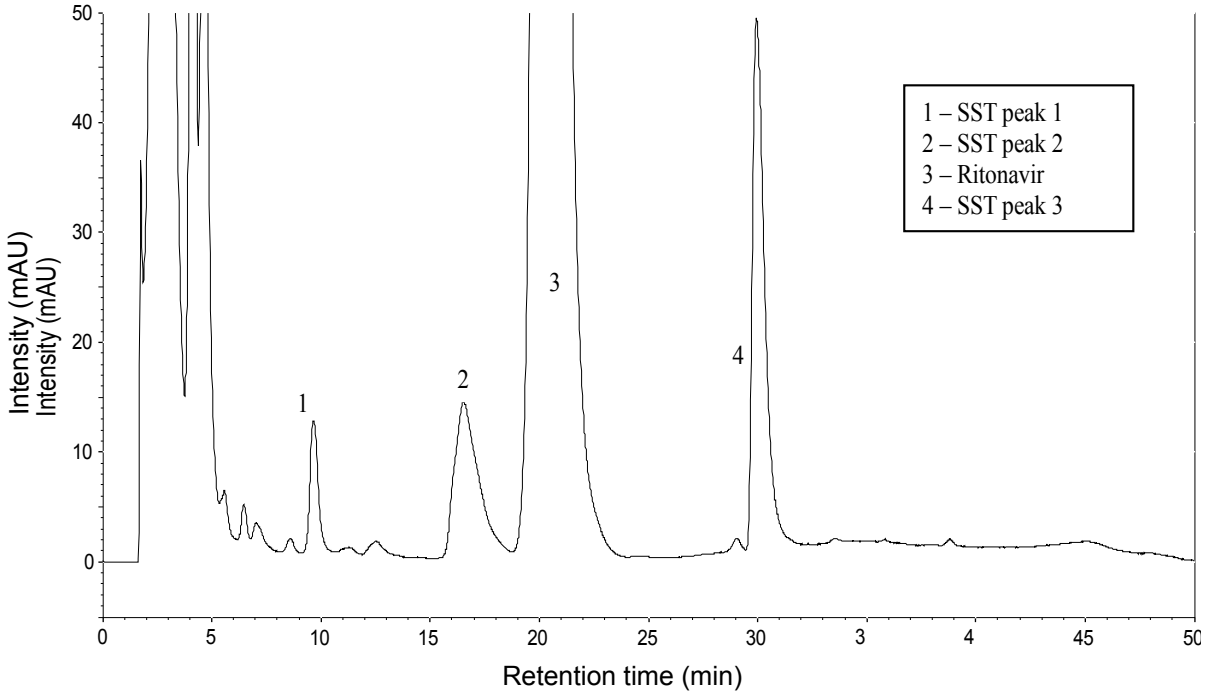


Figure 16: A typical chromatogram of ritonavir tablets solution obtained under the system suitability test (SST) conditions with numbered SST peaks

In the sample analyzed, one impurity above the disregard limit was found (Figure 14), with a content of 0.08%. Thus, applying the criteria of the proposed monograph, the sample complies.

3.1.2.6 Assay

The RTV drug substance monograph in the Ph. Int. does prescribe a titration method using perchloric acid to determine the content of RTV in the sample [The International Pharmacopoeia, 4th edition]. However, due to the influence of excipients the method used for the drug substance is not applicable to the tablets.

The LC method was applied for quantification, using a sample solution with a concentration of 0.5 mg RTV per ml in a mixture of mobile phases A/B 70:30 (v/v). An RTV drug substance solution of the same concentration in the same solvent was used as reference. Solutions were prepared in duplicate and analyzed in triplicate. An overlay of typical chromatograms of sample solution and reference solution is shown in Figure 12.

The average content of RTV in the sample tablets was then calculated using the normalized area values of all four solutions. The sample complies with the limits given in the proposed monograph, the content of RTV in the sample was found to be 101.3%.

Using the UV-Spectroscopic method given in the Ph. Int. monograph for RTV drug substance under Identity test as an alternative was also considered, but, as has been described under Identity test, this turned out to be not feasible, as the difference between the spectrum obtained with the sample solution of the drug product and the reference spectrum is too big. Furthermore calculations were done to determine the content of RTV in the sample. The equation

$$a = A/(b*c)$$

with a: absorptivity
A: absorbance
b: absorption path length (in cm)
c: concentration of the substance (in g/l)

was used to calculate the concentrations of both sample and reference solution. The content calculated this way was found to be higher than can reasonably be expected and clearly different from the results obtained with the LC method. Therefore no UV-spectroscopic method for quantification was included in the proposed monograph.

3.2 Validation of assay methods

The second part of the project was to validate assay methods of monographs that had previously been developed in our laboratory and proposed for inclusion in the Ph. Int. The first monograph is for tablets containing a combination of EFV/FTC/TDF and the second one for tablets containing FTC/TDF.

The LC method used had been developed for the related substances test of FTC drug substance and was then applied to different dosage forms and fixed-dose combinations of this drug, amongst others the aforementioned. It had been selected because it offers good separation at a reasonable runtime. It was used for determining the content of FTC/TDF tablets and in a modified way also for the assay of EFV/FTC/TDF tablets.

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines for validation of analytical procedures were consulted and a plan for the validation was drafted [ICH guidelines 2005]. It was decided to carry out tests to determine the following parameters:

- Sensitivity
- Linearity
- Precision
    - Repeatability
    - Intermediate Precision
- Accuracy
- Range

3.2.1 EFV/FTC/TDF tablets

The assay method extracted from the Ph. Int. monograph is given as follows:

Assay

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with base-deactivated particles of silica gel the surface of which has been modified with chemically bonded octadecylsilyl groups (5 μm).

The mobile phases for gradient elution consist of a mixture of Mobile phase A and Mobile phase B, using the following conditions:

Mobile phase A: 5 volumes of phosphate solution and 95 volumes of water R.

Mobile phase B: 70 volumes of acetonitrile R, 5 volumes of phosphate solution and 25 volumes of water R.

Prepare the phosphate solution by dissolving 27.22 g of potassium dihydrogen phosphate R in 1000 ml of water R.

Table 4: Gradient programme for LC analysis of EFV/FTC/TDF tablets

Time (min)	**Mobile phase A (% v/v)**	**Mobile phase B (% v/v)**	**Comments**
0 – 9	93	7	Isocratic
9 – 15	93 to 0	7 to 100	Linear gradient
15 – 19	0	100	Isocratic
19 – 19.1	0 to 93	100 to 7	Return to initial composition
19.1-30	93	7	Re-equilibration

After preparation, keep the solutions at about 6 °C, or use an injector with cooling.

Prepare the following solutions using a mixture of 20 volumes of acetonitrile and 80 volumes of water R as a diluent. For solution (1) weigh and powder 20 tablets. Disperse a quantity of the powder containing about 10 mg of tenofovir disoproxil fumarate, accurately weighed in 100 ml of the diluent and filter. For solution (2) dissolve quantities of efavirenz RS, tenofovir disoproxil fumarate RS and emtricitabine RS in the diluent to obtain a concentration of 0.2 mg/ml, 0.1 mg/ml and 66.7 µg/ml of efavirenz, tenofovir disoproxil fumarate and emtricitabine, respectively. For solution (c) use 0.02 mg of fumaric acid R per ml of water R.

If needed, adapt the concentration of solution (2) in function of the tablet composition.

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of 280 nm.

Maintain the column temperature at 35°C.

Inject alternatively 20 µl each of solutions (1), (2) and (3).

The test is not valid unless in the chromatograms obtained with solutions (1) and (2), four principal peaks **with the indicated retention times** are shown: **fumarate (about 2.5 minutes), emtricitabine (about 9 minutes), tenofovir disoproxil (about 18 minutes) and efavirenz (about 22 minutes).**

Calculate the contents of efavirenz ($C_{14}H_9ClF_3NO_2$), emtricitabine ($C_8H_{10}FN_3O_3S$) and tenofovir disoproxil fumarate ($C_{19}H_{30}N_5O_{10}P,C_4H_4O_4$) in the tablets from the declared contents of efavirenz RS, emtricitabine RS and tenofovir disoproxil fumarate RS.

3.2.1.1 Sensitivity

The sensitivity of the method was determined with respect to the limit of detection (LOD) and the limit of quantification (LOQ). LOQ for EFV, FTC and TDF were 0.49%, 0.06% and 0.50% and LOD 0.15%, 0.02% and 0.15%, respectively. The percentages were calculated with respect to the main component nominal value (200 µg/ml = 100% for EFV, 66.7 µg/ml = 100% for FTC and 100 µg/ml = 100% for TDF, 20 µl injected).

3.2.1.2 Linearity

Linearity was established from 0.5 to 125% of the test concentration, using 5 different concentrations. A solution of EFV, FTC and TDF in MeOH:H_2O, 50:50 (v/v) with concentrations corresponding to 250% of the test concentration was prepared and then mixed 50:50 with the solvent to obtain a 125% solution. This was in turn diluted with the solvent to obtain solutions with concentrations of 100, 50, 5 and 0.5% of the test concentration (100 % = 0.2mg/ml EFV, 66.7 µg/ml FTC and 0.1 mg/ml TDF). The solutions were analyzed in triplicate, the average response for each solution was calculated and used to create a plot depicting the response as a function of concentration (Figure 17).

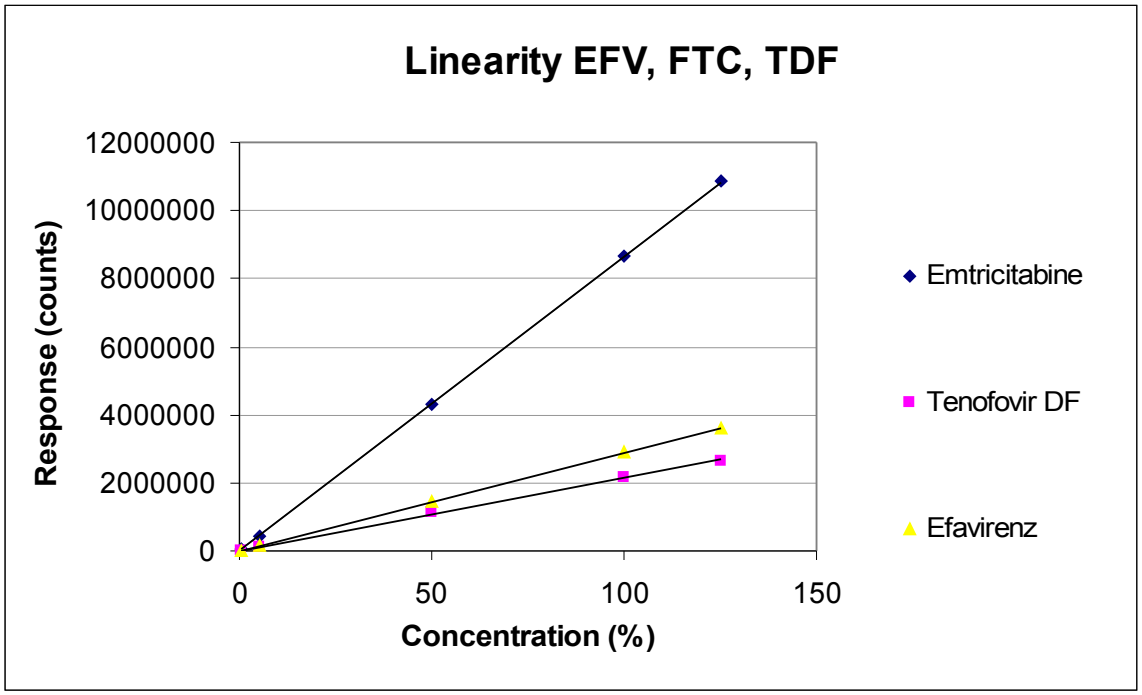


Figure 17: Linearity of the assay method for EFV/FTC/TDF tablets

Table 5: Findings for linearity of the assay method for EFV/FTC/TDF tablets

	EFV	FTC	TDF
Concentration (µg/ml)	1-250	0.33-83.3	0.5-125
Coefficient of determination (R^2)	1	1	0.9996
Regression equation	y = 28847x + 269.89	y = 86762x - 5669.2	y = 21367x + 15125

As can be seen from Figure 17 and Table 5, the method is linear in the investigated range.

3.2.1.3 Precision

Precision was determined by assessing repeatability and intermediate precision.

3.2.1.3.1 Repeatability

Repeatability was assessed using six determinations at 100% of the test concentration. Therefore, a reference solution containing EFV, FTC and TDF at 100% of the nominal test concentration was prepared and injected six times, as well as a sample solution. The sample solution was prepared by dissolving EFV, FTC and TDF in MeOH:H_2O, 50:50 (v/v), to obtain concentrations of 0.2 mg EFV, 66.7 µg FTC and 0.1 mg TDF per ml. After normalizing the response areas, the average content of EFV, FTC and TDF in the sample was calculated, and standard deviation and relative standard deviation (RSD) were determined. The results are given in Table 6.

Table 6: Findings for repeatability of the assay method for EFV/FTC/TDF tablets

	EFV	FTC	TDF
Average content	102.1%	97.5%	96.2%
Standard deviation	0.5	0.4	0.3
%RSD	0.5	0.4	0.3

As can be seen from Table 6, the RSD values for each component were not more than 0.5%, indicating that the method is repeatable.

3.2.1.3.2 Intermediate Precision

Intermediate precision was established by injecting a reference solution of drug substances at a concentration of 100% of the nominal test concentration and the sample solution six times each, over four days. The analyses of days 1 and 2 were carried out on LC-apparatus I, those of days 3 and 4 on LC-apparatus II and with a different column. After normalization, the average content of EFV, FTC and TDF, standard deviation and RSD were calculated for each day (Table 7). RSD was also determined for days 1+2, 3+4 and all four days (Table 8).

Table 7: Findings for intermediate precision of the assay method for EFV/FTC/TDF tablets

	Day 1			Day 2		
	EFV	FTC	TDF	EFV	FTC	TDF
Average content	102.1%	97.5%	96.2%	104.8%	97.5%	96.1%
Standard deviation	0.5	0.4	0.3	0.8	0.6	0.5
%RSD	0.5	0.4	0.3	0.8	0.6	0.5
	Day 3			Day 4		
	EFV	FTC	TDF	EFV	FTC	TDF
Average content	104.2%	97.4%	96.5%	105.4%	98.4%	96.9%
Standard deviation	0.05	0.4	0.06	0.2	0.5	0.1
%RSD	0.05	0.4	0.06	0.2	0.5	0.1

Table 8: Summarized findings for intermediate precision of the assay method for EFV/FTC/TDF tablets

	Days 1+2			Days 3+4			All 4 days		
	EFV	FTC	TDF	EFV	FTC	TDF	EFV	FTC	TDF
%RSD	0.6	0.5	0.4	0.1	0.4	0.09	0.4	0.5	0.2

As can be seen from Tables 7 and 8, the RSD values for each component were not more than 0.5%, indicating that the method is precise.

3.2.1.4 Accuracy

According to the ICH guidelines, several methods are available for determining the accuracy of a method for the assay for drug products [ICH guidelines 2005]. As not all components of the drug product were known, it was decided to determine the accuracy of the method by using the standard addition method. The sample was assayed, known amounts of active ingredients were added and the spiked solutions were assayed again. Accuracy was then established by calculating the recovery, which is defined as the ratio of the observed result to the expected result expressed as a percentage. Nine determinations at three different concentration levels were carried out.

It was decided to spike sample solutions with a concentration of 0.2 mg EFV, 66.7 µg FTC and 0.1 mg TDF per ml, which corresponds to 100% of the test concentration, with drug substances to obtain solutions with concentrations of 180%, 200% and 220% of the test concentration. Therefore a sample solution with a concentration of 0.4 mg EFV, 133.4 µg FTC and 0.2 mg TDF per ml in MeOH:H_2O, 50:50 (v/v) was prepared. A drug substance solution containing 0.48 mg EFV, 160.08 µg FTC and 0.24 mg TDF per ml was prepared and further diluted to also obtain concentrations corresponding to 200% and 160% of the test concentration. The sample solution was then in turn mixed 50:50 with the drug substance solutions to obtain concentrations equivalent to 220%, 200% and 180% of the test solution corresponding to an addition of 120, 100 and 80% respectively. The solutions were analyzed in triplicate and the average content of EFV, FTC and TDF was calculated using a drug substance solution with a concentration of 0.2 mg EFV, 66.7 µg FTC and 0.1 mg TDF per ml as a reference. The amount of EFV, FTC and TDF in the unspiked sample solution was determined and used to calculate recovery for the spiked solution with the formula:

$$R = a * 100 / b$$

with R: recovery
a: observed result
b: expected result

Table 9: Findings for accuracy of the assay method for EFV/FTC/TDF tablets - part 1

	Recovery (%)		
Spike solution	EFV	FTC	TDF
80%	93.5	101.5	95.0
100%	93.9	101.7	93.9
120%	93.7	101.0	92.0

However, the results do not meet the limits given in literature, which states that dosage form assays usually provide accuracy within 3-5% of the true value, and due to the fact that the recovery is worse for spiked solutions with higher concentrations it was concluded that there might be some solubility problems. As a next step, solutions with lower concentrations were used.

A sample solution with a concentration of 0.2 mg EFV, 66.7 µg FTC and 0.1 mg TDF per ml in MeOH:H_2O, 50:50 (v/v) was prepared. A drug substance solution containing 0.28

mg EFV, 93.38 µg FTC and 0.14 mg TDF per ml was prepared and further diluted to also obtain concentrations corresponding to 100% and 60% of the test concentration. The sample solution was then in turn mixed 50:50 with the drug substance solutions to obtain concentrations equivalent to 120%, 100% and 80% of the test solution. The solutions were analyzed in triplicate and the average content of EFV, FTC and TDF was calculated using a drug substance solution with a concentration of 0.2 mg EFV, 66.7 µg FTC and 0.1 mg TDF per ml as a reference. The amount of EFV, FTC and TDF in the unspiked sample solution was determined and used to calculate recovery for the spiked solution by using the formula given above.

Table 10: Findings for accuracy of the assay method for EFV/FTC/TDF tablets - part 2

	Recovery		
Spike solution	EFV	FTC	TDF
80%	102.1	98.9	100.3
100%	101.3	99.1	99.9
120%	100.3	98.7	98.9

As can be seen from the results compiled in Tables 9 and 10, the second approach clearly meets the requirements. It was therefore concluded that the unsatisfactory results with the more highly concentrated solutions were due to solubility problems and the accuracy of the method is good.

3.2.1.5 Range

The range was established from 80 to 120% of the test concentration (EFV 160-240 µg/ml, FTC 53-80 µg/ml, TDF 80-120 µg/ml) by proving that linearity, precision and accuracy of the assay within these limits are good.

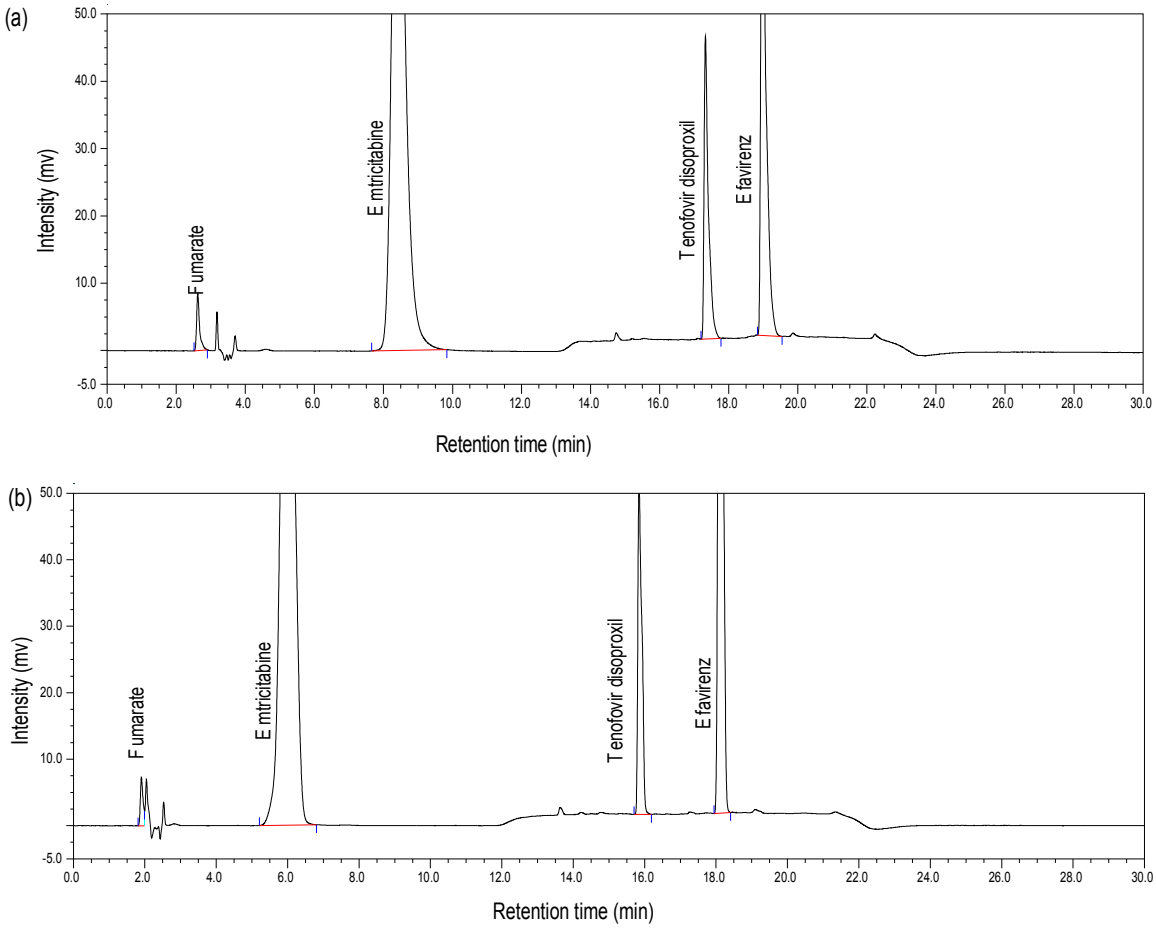
3.2.1.6 Evaluation of different stationary phases for selectivity

No tests for robustness were carried out, as the resolution between the peaks is high enough to presume that the reliability of the method is not susceptible to minor variations in method parameters. However, the method was tested using different columns to verify the applicability. For this purpose, the database of the column classification system for HPLC columns developed by our laboratory was used to find columns similar to the one

used [Column Classification System]. The five most similar were identified by their F-values. The lower the F-value, the higher the similarity to the column selected as reference, which was in our case the column used for method development. These five columns were:

1. Brava BDS 5, 250 x 4.6 mm I.D., 5µm (F = 0.152)
2. HyPurity Elite C18, 150 x 4.6 mm I.D., 3 µm (F = 0.330)
3. HyPurity C18, 250 x 4.6 mm I.D., 5 µm (F = 0.343)
4. HyPurity Elite C18, 150 x 4.6 mm I.D., 5 µm (F = 0.357)
5. Discovery C18, 250 x 4.6 mm I.D., 5 µm (F = 0.404)

As described in the monograph, a sample solution containing 0.2 mg EFV, 66.7 µg FTC and 0.1 mg TDF per ml in MeOH:H_2O, 50:50 (v/v) was prepared and analyzed on each of these columns. The resulting chromatograms are given in Figure 19.



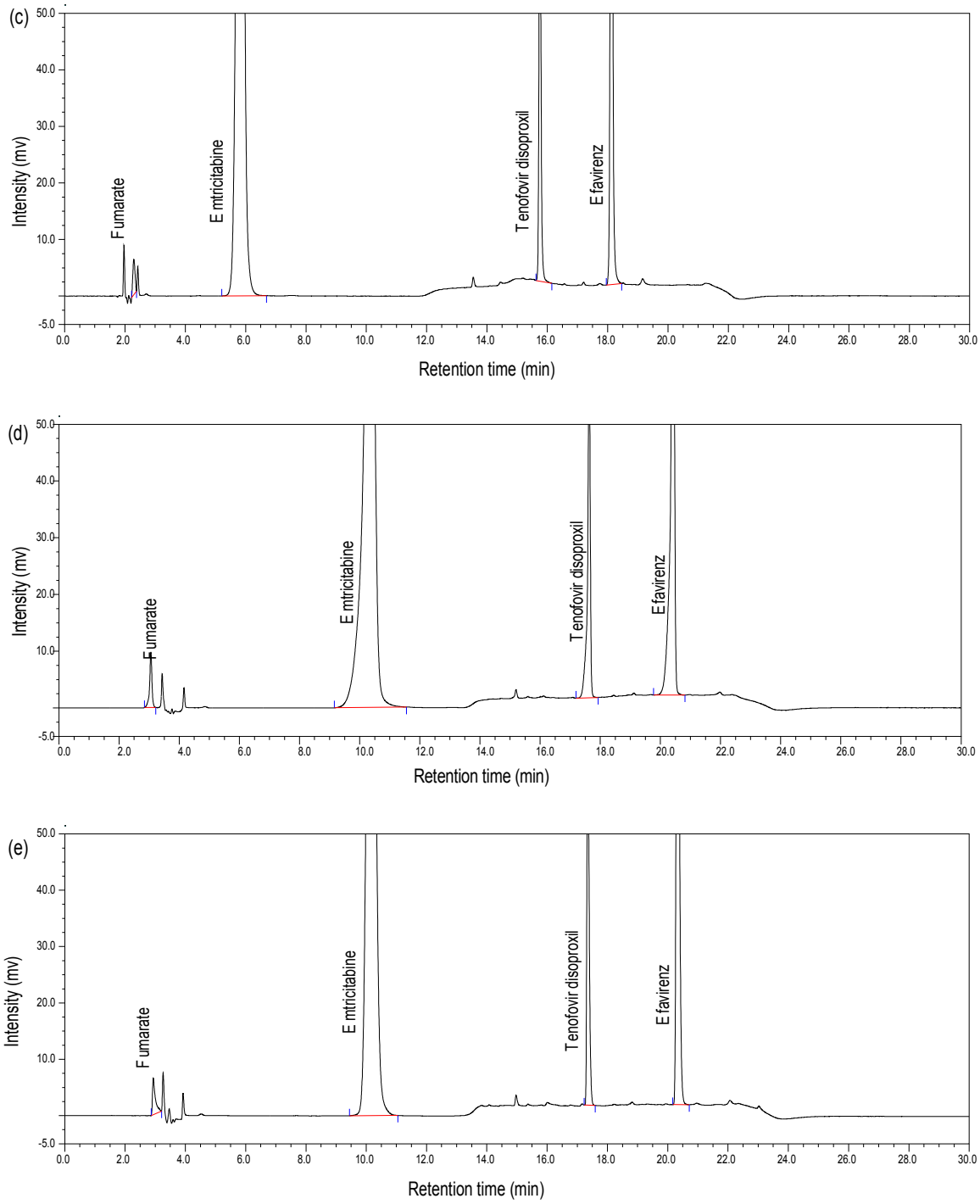


Figure 18: Chromatograms for separation of EFV/FTC/TDF tablets obtained on different columns, (a) Brava BDS (250 x 4.6 mm I.D., 5 µm, F = 0.152), (b) HyPurity Elite (150 x 4.6 mm I.D., 3 µm, F = 0.330), (c) HyPurity (250 x 4.6 mm I.D., 5 µm, F = 0.343), (d) HyPurity Elite (150 x 4.6 mm I.D., 5 µm, F = 0.357) and (e) Discovery (250 x 4.6 mm I.D., 5 µm, F = 0.404).

These columns give similar results as the reference column but for some of them the fumarate peak coelutes with a solvent peak.

3.2.2 FTC/TDF tablets

The assay method extracted from the Ph. Int. monograph is given as follows:

Assay

Carry out the test as described under 1.14.4 High-performance liquid chromatography, using a stainless steel column (25 cm x 4.6 mm) packed with base-deactivated particles of silica gel the surface of which has been modified with chemically bonded octadecylsilyl groups (5 µm).

The mobile phases for gradient elution consist of a mixture of Mobile phase A and Mobile phase B, using the following conditions:

Mobile phase A: 5 volumes of phosphate solution and 95 volumes of water R.

Mobile phase B: 70 volumes of acetonitrile R, 5 volumes of phosphate solution and 25 volumes of water R.

Prepare the phosphate solution by dissolving 27.22 g of potassium dihydrogen phosphate R in 1000 ml of water R.

Table 11: Gradient programme for LC analysis of FTC/TDF tablets

Time (min)	**Mobile phase A (% v/v)**	**Mobile phase B (% v/v)**	**Comments**
0 – 9	93	7	Isocratic
9 – 15	93 to 0	7 to 100	Linear gradient
15 – 19	0	100	Isocratic
19 – 19.1	0 to 93	100 to 7	Return to initial composition
19.1-30	93	7	Re-equilibration

After preparation, keep the solutions at about 6 °C, or use an injector with cooling.

Prepare the following solutions, using a mixture of 20 volumes of acetonitrile R and 80 volumes of water R as a diluent. For solution (1) weigh and powder 20 tablets. Disperse a quantity of the powder containing about 10 mg of Tenofovir disoproxil fumarate, accurately weighed in 100 ml of the diluent and filter. For solution (2) dissolve quantities of tenofovir disoproxil fumarate RS and emtricitabine RS in the diluent to obtain a concentration of 0.1 mg/ml and 66.7 µg/ml of tenofovir disoproxil fumarate and emtricitabine, respectively. If necessary, adapt the concentration of solution (2) according to the ratio of Emtricitabine and Tenofovir disoproxil fumarate in the tablets. For solution (3) use 0.02 mg of fumaric acid R per ml of water R.

Operate with a flow rate of 1.0 ml per minute. As a detector use an ultraviolet spectrophotometer set at a wavelength of 280 nm.

Maintain the column temperature at 35°C.

Inject alternatively 20 µl each of solutions (1), (2) and (3).

The test is not valid unless in the chromatograms obtained with solutions (1) and (2), three principal peaks are shown and the resolution factor between those peaks is at least 5.

Measure the areas of the peak responses obtained in the chromatograms from solutions (1) and (2), and calculate the content of emtricitabine ($C_8H_{10}FN_3O_3S$) and tenofovir disoproxil fumarate ($C_{19}H_{30}N_5O_{10}P$,$C_4H_4O_4$) in the tablets.

3.2.2.1 Sensitivity

LOQ values for EFV, FTC and TDF were 0.49%, 0.06% and 0.50% and LOD 0.15%, 0.02% and 0.15%, respectively. The percentages were calculated with respect to the main component nominal value (200 µg/ml = 100% for EFV, 66.7 µg/ml = 100% for FTC and 100 µg/ml = 100% for TDF, 20 µl injected).

3.2.2.2 Linearity

Linearity was established from 0.5 to 125% of the test concentration, using 5 different concentrations. An FTC solution and a TDF solution in ACN:H_2O, 20:80 (v/v) with concentrations corresponding to 250% of the test concentration were prepared and then

mixed 50:50 with the solvent to obtain a 125% solution. This was in turn diluted with the solvent to obtain solutions with concentrations of 100, 50, 5 and 0.5% of the test concentration (100 % = 66.7 µg/ml FTC and 0.1 mg/ml TDF). The solutions were analyzed in triplicate, the average response for each solution was calculated and used to create a plot depicting the response as a function of concentration (Figure 19).

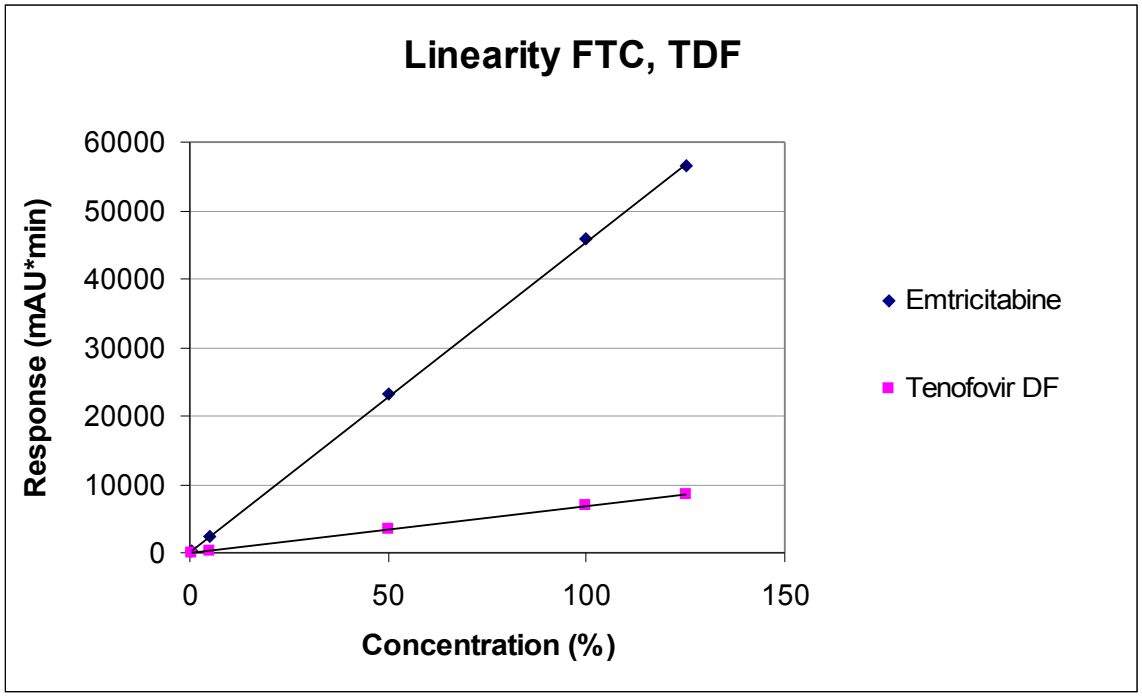


Figure 19: Linearity of the assay method for FTC/TDF tablets

Table 12: Findings for linearity of the assay method for FTC/TDF tablets

	FTC	TDF
Concentration (µg/ml)	0.33-83.3	0.5-125
Coefficient of determination (R^2)	0.9999	0.9995
Regression equation	y = 454.32x + 127.05	y = 68.111x + 57.565

As can be seen from Figure 19 and Table 12, the method is linear in the investigated range.

3.2.2.3 Precision

Precision was determined by assessing repeatability and intermediate precision.

3.2.2.3.1 Repeatability

Repeatability was assessed by using six determinations at 100% of the test concentration. Therefore, a reference solution containing FTC and TDF with 100% of the nominal test concentration was prepared and injected six times, as well as a sample solution. The sample solution was prepared, as described in the monograph, to obtain a concentration of 66.7 µg FTC and 0.1 mg TDF per ml. After normalizing the response areas, the average content of FTC and TDF in the sample was calculated, and standard deviation and RSD were determined. The results are given in Table 13.

Table 13: Findings for repeatability of the assay method for FTC/TDF tablets

	FTC	TDF
Average content	100.1	99.6
Standard deviation	0.1	0.2
%RSD	0.1	0.2

As can be seen from Table 13, the RSD value for each component is not more than 0.2%, indicating the method is repeatable.

3.2.2.3.2 Intermediate Precision

Intermediate precision was established by injecting a reference solution of drug substances with a concentration of 100% of the nominal test concentration and the sample solution six times, over four days. The analyses of days 1 and 2 were carried out on LC-apparatus II, those of days 3 and 4 on LC-apparatus I and with a different column. After normalization, average content of FTC and TDF, standard deviation and RSD were

calculated for each day (Table 14). RSD was also determined for days 1+2, 3+4 and all four days (Table 15).

Table 14: Findings for intermediate precision of the assay method for FTC/TDF tablets

	Day 1		Day 2		Day 3		Day 4	
	FTC	TDF	FTC	TDF	FTC	TDF	FTC	TDF
Average content	100.7	99.6	101.3	102.0	98.6	97.6	98.0	97.4
Standard deviation	0.1	0.2	0.2	0.05	0.5	0.2	0.3	0.2
%RSD	0.1	0.2	0.2	0.05	0.5	0.2	0.3	0.2

Table 15: Summarized findings for intermediate precision of the assay method for FTC/TDF tablets

	Days 1+2		Days 3+4		All 4 days	
	FTC	TDF	FTC	TDF	FTC	TDF
%RSD	0.1	0.1	0.4	0.2	0.3	0.2

As can be seen from Tables 14 and 15, the RSD values for each component are not more than 0.5% indicating that the method is precise.

3.2.2.4 Accuracy

Like for the triple combination, accuracy was determined by spiking sample solutions with known amounts of the drug substances. Therefore nine determinations at three different concentration levels were carried out.

A sample solution with a concentration of 66.7 µg FTC and 0.1 mg TDF per ml in ACN:H_2O, 20:80 (v/v) was prepared. A drug substance solution containing 93.38 µg FTC and 0.14 mg TDF per ml was prepared and further diluted to also obtain concentrations corresponding to 100% and 60% of the test concentration. The sample solution was then in turn mixed 50:50 with the drug substance solutions to obtain concentrations equivalent to 120%, 100% and 80% of the test solution. The solutions were analyzed in triplicate and the average content of FTC and TDF was calculated using a drug substance solution with a concentration of 66.7 µg FTC and 0.1 mg TDF per ml as a reference. The amount of FTC and TDF in the unspiked sample solution was determined and used to calculate recovery for the spiked solution with the formula:

$$R = a * 100 / b$$

with R: recovery
a: observed result
b: expected result

Table 16: Findings for accuracy of the assay method for FTC/TDF tablets

Spike solution	80 %	100 %	120 %
Recovery (%)			
FTC	98.2	98.7	99.4
TDF	98.8	98.3	98.3

The results for recovery indicate that the method is accurate.

3.2.2.5 Range

The range was established from 80 to 120% of the test concentration (FTC 53-80 µg/ml, TDF 80-120 µg/ml) by proving that linearity, precision and accuracy of the assay within these limits are good.

4 Conclusion

A monograph for RTV tablets was developed for inclusion in the Ph. Int. It contains basic information and methods of analysis to verify identity and allow for the determination of related substances. A method for dissolution testing was established and quantification of samples is included. Although the Ph. Int. focuses on simple operations, that can easily be performed, it was not always possible to meet this requirement and LC methods are widely used . Simpler methods, which are used for analysis of RTV drug substance, were used to test the sample tablets, but were found to be not suitable for the dosage form.

A report of the proposed monograph has been submitted to the WHO and is open for discussion. Future work has to be done to check the universal applicability of the methods, as the piece of work was subject to some limitations. The excipients of the tablets were not known, which made the work more complicated. It has to be pointed out that, due to the lack of availability, the whole development was based on one batch of sample tablets only. Verifying the methods with different samples will therefore be of great importance.

For the validation of the monographs for EFV/FTC/TDF tablets and FTC/TDF tablets, the parameters of interest were identified and tests were carried out accordingly. Minor difficulties were encountered in the process, and small adjustments were made. The tests for the assay methods showed good results, the methods are very suitable for the quantification of these fixed dosage combinations.

5 Abstract

The human immunodeficiency virus (HIV) is a retrovirus that causes chronic infection and progressively destroys host immunities. This in turn leads to a weakened immune system and higher risk of opportunistic infections called acquired immunodeficiency syndrome (AIDS). According to UNAIDS statistics, 60 million people have been infected with HIV since the beginning of the epidemic in the 1980s and 25 million people have died of HIV-related causes. This makes HIV one of the main health issues in the new millennium.

97% of people with HIV live in low and middle income-countries and although there is a range of medicines approved for treatment of HIV infection, the coverage of antiretroviral therapy in these countries is only 36%. One of the biggest challenges in fighting HIV is therefore providing antiretroviral medicines to those in need.

In an effort to improve the overall quality of, not only antiretroviral medicines, the International Pharmacopoeia provides information on pharmaceutical specifications and methods of analysis. It also includes monographs on antiretroviral medicines, and dosage forms.

In this work a monograph for ritonavir tablets has been developed and proposed for inclusion in the International Pharmacopoeia. Furthermore, two assay methods of proposed monographs for fixed dosage combinations of efavirenz, emtricitabine and tenofovir disoproxil fumarate as well as emtricitabine and tenofovir disoproxil fumarate were validated.

6 Zusammenfassung

Das Humane Immundefizienz Virus (HIV) ist ein Retrovirus, das chronische Infektionen verursacht und dabei in fortschreitendem Maße das Immunsystem des Wirtes zerstört. Durch diese Schwächung der Immunabwehr kommt es zu einer Häufung von opportunistischen Infektionen und einer generellen schlechten Immunlage, welche als acquired immunodeficiency syndrome (AIDS) bezeichnet wird. Laut UNAIDS Schätzungen infizierten sich seit Beginn der Epidemie Mitte der 1980er Jahre 60 Millionen Menschen mit HIV und 25 Millionen Menschen kamen durch HIV-assoziierte Komplikationen zu Tode. HIV stellt damit eine der größten Herausforderungen im Gesundheitswesen im neuen Millennium dar.

97 Prozent der HIV-Erkrankten leben in einkommensschwachen Ländern und Schwellenländern. Trotz der vorhandenen Auswahl an antiretroviralen Arzneistoffen beträgt die Behandlungsquote in diesen Staaten nur 36 Prozent. Eine der größten Herausforderungen im Kampf gegen HIV ist somit die Bereitstellung von antiretroviralen Arzneimitteln für jene, die sie benötigen.

Die Internationale Pharmacopöe ist ein Ansatz, um die Qualität von pharmazeutischen Produkten, unter anderem auch antitetroviralen Medikamenten, zu verbessern, und bietet Informationen zu Anforderungen und Qualitätsstandards sowie Analysemethoden. Sie enthält Monographien zu Arzneistoffen und Darreichungsformen unterschiedlicher Anwendungsgebiete.

In dieser Arbeit wird die Ausarbeitung einer Monographie für Ritonavir Tabletten beschrieben und der Monographievorschlag vorgestellt. Darüber hinaus wurden zwei assay-Methoden für Kombinationspräparate, die Efavirenz, Emtricitabin und Tenofovirdisoproxilfumarat bzw. Emtricitabin und Tenofovirdisoproxilfumarat enthalten, validiert.

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10 Curriculum Vitae

Name: Mattias Andreas Ungerböck

Date of birth: July, 27th 1987

Nationality: Austria

Address: Ingen-Housz-Gasse 4/3
1090 Vienna

Academic studies:

2005 - 2011	Diploma study pharmacy at the University of Vienna
Feb 2011 - Jun 2011	Erasmus student at the Katholieke Universiteit Leuven
	Laboratory for Pharmaceutical Analysis
	Diploma thesis: "Development of International Pharmacopoeia monographs for the analysis of dosage forms of selected essential antiretroviral drugs"

Education:

1993 - 1997	Primary School: VS Dunkelstein, 2630 Ternitz
1997 – 2005	Grammar School: BG und BRG Neunkirchen, 2620 Neunkirchen

Work experience:

Jul 2006, Aug 2007,Jul 2008, Jul 2009	Internships at Apotheke "Zum Heiligen Peter und Paul", 2630 Ternitz

Appendix

Ritonavir - Uniformity of mass

tablet no.	mass (g)	Deviation (%)
1	1.11746	-1.31
2	1.14255	0.90
3	1.13829	0.53
4	1.13640	0.36
5	1.12910	-0.28
6	1.12537	-0.61
7	1.13462	0.20
8	1.13944	0.63
9	1.13374	0.13
10	1.13930	0.62
11	1.13984	0.66
12	1.13167	-0.06
13	1.12303	-0.82
14	1.12915	-0.28
15	1.14250	0.90
16	1.12330	-0.80
17	1.14100	0.77
18	1.12130	-0.97
19	1.13335	0.09
20	1.12491	-0.65
Mean	1.132316	

Ritonavir – UV absorbance

Sample	weight	absorbance	calculated conc.	normalized conc.	mean	per cent
Tablet 1	228.1	0.630	0.0516	0.0513	0.0513	121.88
Tablet 2	226.9	0.625	0.0512	0.0512		
drug substance 1	20.11	0.511	0.0419	0.0417	0.0421	100
drug substance 2	19.99	0.518	0.0425	0.0425		

| drug substance 2 | 19.99 | 0.518 | 0.0425 | 0.0425 | | |

Ritonavir Dissolution Test

First 6 tablets

Indian Pharmacopoeia:

Apparatus: Paddle
Medium: 900ml of 0,1M hydrochloric acid
Speed and time: 100rpm; 60 min

Reference solution 1	
injection	area
1	27771404
2	27836575
3	27762456
Mean	27790145
SD	40457.70
RSD	0.15
weighed mass	20,14mg
normalized area	27596966

Reference solution 2	
injection	area
1	28011464
2	27980673
3	27966742
Mean	27986293
SD	22884.55
RSD	0.08
weighed mass	20,07mg
normalized area	27888683

Ref. Sol: 1+2	27742824

Tablets	No.					
replicate	1	2	3	4	5	6
1	27851446	26457058	25622455	26686628	22541944	28337820
2	27827655	26428650	25871063	26441714	22664086	28179147
3	27892332	26452063	25846484	26627261	22473257	28316691
Mean	27857144	26445924	25780001	26585201	22559762	28277886
SD	32712.87	15166.48	136990.91	127759.54	96654.26	86160.61
RSD	0.12	0.06	0.53	0.48	0.43	0.30

content of ritonavir	100.41	95.33	92.92	95.83	81.32	101.93
ritonavir in medium	90.37	85.79	83.63	86.24	73.19	91.74
Mean in mg (= % of stated amount):			85.16			

6 more tablets

Indian Pharmacopoeia:

Apparatus: Paddle
Medium: 900ml of 0,1M hydrochloric acid
Speed and time: 100rpm; 60 min

Reference solution 1	
injection	area
1	27581374
2	27631871
3	27606339
Mean	27606528
SD	25249.03
RSD	0.09
weighed mass	20,05mg
normalized area	27537684

Reference solution 2	
injection	area
1	27717701
2	27704385
3	27834374
Mean	27752153.3
SD	71515.78
RSD	0.26
weighed mass	19,94mg
normalized area	27835660

Ref. Sol; 1+2	27686672

Tablets	No.					
replicate	1	2	3	4	5	6
1	23338755	23108448	27263006	24380954	23610379	29845435
2	23370795	23125986	27458202	24358571	23630527	29885517
3	23239324	23141736	27265992	24356576	23535090	29812860
Mean	23316291	23125390	27329067	24365367	23591999	29847937
SD	68553.76	16652.00	111844.44	13535.54	50303.41	36393.08
RSD	0.29	0.07	0.41	0.06	0.21	0.12

content of ritonavir	84.21	83.53	98.71	88.00	85.21	107.81
ritonavir in medium	75.79	75.17	88.84	79.20	76.69	97.03
Mean in mg (= % of stated amount):			82.12			

average of all 12 tablets:	83.64

Ritonavir Dissolution Test

First 6 tablets

FDA-database-Method:

Apparatus: Paddle
Medium: 900ml of 60mM Polyoxyethylene(10)laurylether
Speed and time: 75rpm; 60 min

Reference solution 1	
injection	area
1	27704246
2	27883610
3	27778657
Mean	27788838
SD	90114.35
RSD	0.32
weighed mass	20,14mg
normalized area	27595668

Reference solution 2	
injection	area
1	27893672
2	27955173
3	27979380
Mean	27942742
SD	44185.62
RSD	0.16
weighed mass	20,07mg
normalized area	27845283

Ref. Sol: 1+2	27720476

Tablets	No.					
replicate	1	2	3	4	5	6
1	27866635	23062732	24985620	28731109	28967573	22296068
2	27955505	23059441	25059863	28791831	28876286	22225613
3	27795509	23006038	24880295	28885421	28809221	22062291
Mean	27872550	23042737	24975259	28802787	28884360	22194657
SD	80161.82	31824.83	90231.23	77737.21	79484.16	119923.36
RSD	0.29	0.14	0.36	0.27	0.28	0.54

content of ritonavir	100.55	83.13	90.10	103.90	104.20	80.07
ritonavir in medium	90.49	74.81	81.09	93.51	93.78	72.06
Mean in mg (= % of stated amount):			84.29			

6 more tables

FDA-database-Method:

Apparatus: Paddle
Medium: 900ml of 50mM Polyoxyethylene(10)laurylether
Speed and time: 75rpm; 60 min

Reference solution 1	
injection	area
1	26586896
2	26822446
3	26693772
Mean	26701038
SD	117942.98
RSD	0.44
weighed mass	20.05mg
normalized area	26634452

Reference solution 2	
injection	area
1	26037454
2	25991990
3	26049971
Mean	26026472
SD	30510.78
RSD	0.12
weighed mass	20.14mg
normalized area	25845553

Ref. Sol: 1+2	26240002

Tablets	No.					
replicate	1	2	3	4	5	6
1	29141186	25422652	25404043	29212019	29168473	29178604
2	29051586	25390454	25427266	29210993	29284369	29173404
3	29130742	25397806	25363789	29325634	29070859	29406874
Mean	29107838	25403637	25398366	29249555	29174567	29252961
SD	48994.74	16872.50	32117.03	65922.70	106885.37	133313.21
RSD	0.17	0.07	0.13	0.23	0.37	0.46

content of ritonavir	110.93	96.81	96.79	111.47	111.18	111.48
ritonavir in medium	99.84	87.13	87.11	100.32	100.07	100.33
Mean in mg (= % of stated amount):			95.80			

average of all 12 tablets:	90.05

Ritonavir - Related Substances

Solution 2 (0.1%)

I	
injection no.	area
1	112544
2	115262
3	112495
Mean	113433.67
SD	1583.57
RSD	1.40

II	
injection no.	area
1	111383
2	110709
3	112295
Mean	111462.33
SD	795.97
RSD	0.71

Solution 1 (0.5mg/ml)

I									
injection no.	peak 1 (RT=7.	peak 2 (RT=8.4min)	peak 3 (RT=9.8min)	peak 4 (RT=17.7min)	peak 5 (ritonavir)	peak 6 (RT=28.5min)	peak 7 (RT=29.3min)	peak 8 (RT=33.5min)	peak 9 (RT=38.7min)
1	123102	207924	30217	36396	140816782	84810	16923	17604	45633
2	109576	207655	32089	43168	140364574	76483	19580	15426	40785
3	114244	205119	32687	40405	139675465	78981	17240	19476	44614
Mean	115641	206899	31664	39990	140285607	80091	17914	17502	43677
SD	6870.31	1547.67	1288.60	3405.05	574741.65	4273.10	1451.19	2026.93	2556.13
RSD	5.94	0.75	4.07	8.51	0.41	5.34	8.10	11.58	5.85
amount in %	0.10	0.18	0.03	0.04	123.67	0.07	0.02	0.02	0.04

II									
injection no.	peak 1 (RT=7.	peak 2 (RT=8.4min)	peak 3 (RT=9.8min)	peak 4 (RT=17.7min)	peak 5 (ritonavir)	peak 6 (RT=28.5min)	peak 7 (RT=29.3min)	peak 8 (RT=33.5min)	peak 9 (RT=38.7min)
1	104757	210212	58171	44818	140703656	80964	16888	28008	43012
2	107736	209766	61438	43250	141094905	94493	17879	28525	44412
3	104238	204891	52332	40978	140387338	94113	17037	27426	42418
Mean	105577	208290	57314	43015	140728633	89857	17268	27986	43281
SD	1887.67	2951.77	4613.14	1930.73	354444.15	7703.62	534.36	549.82	1023.79
RSD	1.79	1.42	8.05	4.49	0.25	8.57	3.09	1.96	2.37
amount in %	0.09	0.19	0.05	0.04	126.26	0.08	0.01	0.02	0.04

Ritonavir - Assay

Reference-solution 1	
injection no.	area
1	140486335
2	140913453
3	140521094
Mean	140640294
SD	237200.18
RSD	0.17
weighed mass	12.66
normalized area	138862850

Reference-solution 2	
injection no.	area
1	139823372
2	139897986
3	140743328
Mean	140154895
SD	510961.41
RSD	0.36
weighed mass	12.65
normalized area	138492980

Reference solutions 1+2	
area	138677915
SD	343228.689
RSD	0.24750061

Tablet-solution 1	
injection no.	area
1	140816782
2	140364574
3	139675465
Mean	140285607
SD	574741.65
RSD	0.41
weighed mass	283.80
normalized area	140132370
content of tablet 1	101.05

Tablet-solution 2	
injection no.	area
1	140703656
2	141094905
3	140387338
Mean	140728633
SD	354444.15
RSD	0.25
weighed mass	283.47
normalized area	140738562
content of tablet 2	101.49

Average content (%)	101.27

Validation efavirenz, emtricitabine, tenofovir disoproxil fumarate - Linearity

Emtricitabine	area				
Injection #	0,5%-solution	5%-solution	50%-solution	100%-solution	125%-solution
1	42635	438816	4311666	8637643	10827874
2	42884	433918	4320884	8634155	10874353
3	41741	432231	4327233	8683020	10875769
Mean	42420	434988	4319928	8651606	10859332
SD	601.07	3420.49	7827.44	27261.16	27252.63
RSD	1.42	0.79	0.18	0.32	0.25

Tenofovir DF	area				
Injection #	0,5%-solution	5%-solution	50%-solution	100%-solution	125%-solution
1	10167	114662	1117929	2155042	2658535
2	10304	113620	1118698	2160574	2662751
3	10287	111940	1121735	2171476	2669348
Mean	10253	113407	1119454	2162364	2663545
SD	74.67	1373.41	2012.48	8361.95	5450.02
RSD	0.73	1.21	0.18	0.39	0.20

Efavirenz	area				
Injection #	0,5%-solution	5%-solution	50%-solution	100%-solution	125%-solution
1	9758	147180	1444389	2874342	3600400
2	9971	147968	1450613	2874159	3606362
3	10162	145947	1448880	2892918	3616054
Mean	9964	147032	1447961	2880473	3607605
SD	202.10	1018.63	3212.23	10778.07	7900.72
RSD	2.03	0.69	0.22	0.37	0.22

Validation efavirenz, emtricitabine, tenofovir disoproxil fumarate - Intermediate Precision

Day 1						
Emtricitabine	area	mass:25,1mg	Emtricitabine	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	8626660		1	8480367	8464979	97.72
2	8631655		2	8454295	8438954	97.42
3	8677862		3	8428069	8412776	97.11
4	8630144		4	8460123	8444772	97.48
5	8655785		5	8515957	8500504	98.13
6	8942031		6	8439534	8424220	97.25
Mean	8694023		Mean	8463058	8447701	97.52
SD	123076.69		SD	31498.95	31441.79	0.36
RSD	1.42		RSD	0.37	0.37	0.37
mean normalized	8662849.05		mean normalized	8447700.84		
Tenofovir DF	area	mass:37,45mg	Tenofovir DF	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	2154628		1	2106329	2102507	96.61
2	2160574		2	2091342	2087547	95.92
3	2171476		3	2088747	2084957	95.80
4	2159210		4	2101456	2097643	96.38
5	2164439		5	2105295	2101475	96.56
6	2230309		6	2092498	2088701	95.97
Mean	2173439		Mean	2097611	2093805	96.21
SD	39625.89		SD	6566.92	6555.01	0.30
RSD	1.82		RSD	0.31	0.31	0.31
mean normalized	2176341.12		mean normalized	2093804.94		
Efavirenz	area	mass:37,55mg	Efavirenz	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	2874342		1	2980118	2974710	102.24
2	2874177		2	2969001	2963614	101.86
3	2893094		3	2971477	2966085	101.95
4	2880184		4	2972782	2967388	101.99
5	2881674		5	2990848	2985421	102.61
6	2978061		6	2964880	2959500	101.72
Mean	2913306		Mean	2976170	2970770	102.06
SD	56084.13		SD	13311.39	13287.24	0.46
RSD	1.93		RSD	0.45	0.45	0.45
mean normalized	2909427.10		mean normalized	2970769.58		

Day 2						
Emtricitabine	area	mass: 25,1mg	Emtricitabine	area	mass: 55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	8694321		1	8471315	8455943	97.11
2	8713420		2	8488251	8472849	97.30
3	8793190		3	8468867	8453500	97.08
4	8734155		4	8459445	8444095	96.97
5	8746662		5	8544768	8529263	97.95
6	8752497		6	8581791	8566219	98.38
Mean	8739041		Mean	8502406	8486978	97.47
SD	34209.49		SD	49465.23	49375.47	0.57
RSD	0.39		RSD	0.58	0.58	0.58
mean normalized	8707705.63		mean normalized	8486978.10		
Tenofovir DF	area	mass: 37,45mg	Tenofovir DF	area	mass: 55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	2183624		1	2107222	2103398	95.89
2	2178358		2	2111779	2107947	96.10
3	2204189		3	2109498	2105670	96.00
4	2185712		4	2102825	2099009	95.69
5	2201966		5	2112757	2108923	96.15
6	2189278		6	2122934	2119082	96.61
Mean	2190521		Mean	2111169	2107338	96.07
SD	8542.97		SD	10054.75	10036.50	0.46
RSD	0.39		RSD	0.48	0.48	0.48
mean normalized	2193445.76		mean normalized	2107338.34		
Efavirenz	area	mass: 37,55mg	Efavirenz	area	mass: 55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	2891866		1	3042470	3036949	104.50
2	2896158		2	3040784	3035266	104.44
3	2937007		3	3036894	3031383	104.31
4	2902166		4	3039035	3033521	104.38
5	2916622		5	3055771	3050226	104.96
6	2916521		6	3085868	3080269	105.99
Mean	2910057		Mean	3050137	3044602	104.76
SD	8317.17		SD	23732.02	23688.96	0.82
RSD	0.29		RSD	0.78	0.78	0.78
mean normalized	2906181.76		mean normalized	3044602.37		

Day 3						
Emtricitabine	area	mass:25,1mg	Emtricitabine	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	45724		1	44897	44816	98.13870172
2	45733		2	44545	44464	97.36927786
3	45961		3	44498	44417	97.266542288
4	45971		4	44458	44377	97.179107759
5	45693		5	44552	44471	97.384578903
6	45897		6	44482	44401	97.231568477
Mean	45830		Mean	44572	44491	97.43
SD	127.24		SD	163.29	163.00	0.36
RSD	0.28		RSD	0.37	0.37	0.37
mean normalized	45665.50		mean normalized	44491.12		
Tenofovir DF	area	mass:37,45mg	Tenofovir DF	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	6619		1	6452	6440	97.035964433
2	6622		2	6400	6388	96.253901483
3	6617		3	6417	6405	96.509575909
4	6634		4	6414	6402	96.464456893
5	6637		5	6409	6397	96.389258532
6	6640		6	6417	6405	96.509575909
Mean	6628		Mean	6418	6407	96.53
SD	3.00		SD	4.04	4.03	0.06
RSD	0.05		RSD	0.06	0.06	0.06
mean normalized	6637.02		mean normalized	6406.52		
Efavirenz	area	mass:37,55mg	Efavirenz	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	17296		1	17564	17532	104.81107307
2	16597		2	17457	17425	104.17256334
3	16607		3	17430	17398	104.01144406
4	16657		4	17428	17396	103.9995093
5	16672		5	17432	17400	104.02337883
6	16669		6	17445	17413	104.10095477
Mean	16750		Mean	17459	17428	104.19
SD	7.94		SD	8.89	8.87	0.05
RSD	0.05		RSD	0.05	0.05	0.05
mean normalized	16727.36		mean normalized	17427.65		

Day 4						
Emtricitabine	area	mass:25,1mg	Emtricitabine	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	45551		1	45237	45155	99.26
2	45724		2	44765	44684	98.23
3	45716		3	44817	44736	98.34
4	45739		4	44727	44646	98.15
5	45750		5	44688	44607	98.06
6	45440		6	44676	44595	98.033172
Mean	45653		Mean	44818	44737	98.35
SD	127.74		SD	211.53	211.14	0.46
RSD	0.28		RSD	0.47	0.47	0.47
mean normalized	45489.64		mean normalized	44737.01		
Tenofovir DF	area	mass:37,45mg	Tenofovir DF	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	6573		1	6482	6470	97.82
2	6612		2	6432	6420	97.07
3	6618		3	6428	6416	97.01
4	6624		4	6395	6383	96.51
5	6623		5	6408	6396	96.70
6	6583		6	6396	6384	96.52
Mean	6606		Mean	6424	6412	96.94
SD	23.39		SD	7.23	7.22	0.11
RSD	0.35		RSD	0.11	0.11	0.11
mean normalized	6614.32		mean normalized	6411.84		
Efavirenz	area	mass:37,55mg	Efavirenz	area	mass:55,11mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	16579		1	17654	17622	106.16
2	16643		2	17520	17488	105.35
3	16705		3	17525	17493	105.38
4	16620		4	17468	17436	105.04
5	16621		5	17520	17488	105.35
6	16564		6	17479	17447	105.10
Mean	16622		Mean	17528	17496	105.40
SD	32.62		SD	27.40	27.35	0.16
RSD	0.20		RSD	0.16	0.16	0.16
mean normalized	16599.87		mean normalized	17495.86		

Emtricitabine	Tenofovir	Efavirenz
RSD days 1+2		
0.48	0.39	0.61
RSD days 3+4		
0.42	0.09	0.10
RSD total		
0.45	0.24	0.36

Validation efavirenz, emtricitabine, tenofovir disoproxil fumarate - Accuracy I

tablet solution I			
Mass: 110.09mg			
Emtricitabine	area		
injection #	spiked 80%	spiked 100%	spiked 120%
1	15613709	17322315	19044611
2	15641944	17372139	19028279
3	15652127	17564243	19069281
Mean	15635927	17419566	19047390
SD	19903.31	127746.84	20641.81
RSD	0.13	0.73	0.11
mean normalized:	15625985	17408490	19035279
content found	178.47	198.83	217.41
content found in assay (day 2):		97.47	
difference	81.00	101.36	119.94
recovery	100.56	100.69	99.97
Tenofovir DF	area		
injection #	spiked 80%	spiked 100%	spiked 120%
1	3670844	4021026	4364794
2	3664911	4032774	4356474
3	3679224	4059112	4367987
Mean	3671660	4037637	4363085
SD	7191.28	19503.20	5943.72
RSD	0.20	0.48	0.14
mean normalized:	3669325.066	4035070.0283	4360310.7612
content found	165.89	182.43	197.13
content found in assay (day 2):		96.07	
difference	69.82	86.36	101.06
recovery	94.22	93.04	91.23
Efavirenz	area		
injection #	spiked 80%	spiked 100%	spiked 120%
1	5113302	5624588	6182258
2	5040776	5638379	6175194
3	5087059	5647801	6182841
Mean	5080379	5636923	6180098
SD	36721.55	11674.82	4256.69
RSD	0.72	0.21	0.07
mean normalized:	5077149	5633338	6176168
content found	173.11	192.07	210.58
content found in assay (day 2):		105.05	
difference	68.06	87.02	105.53
recovery	93.55	93.67	93.57

tablet solution II			
mass: 110.04mg			
Emtricitabine	area		
injection #	spiked 80%	spiked 100%	spiked 120%
1	15915683	17776501	19343879
2	15920431	17790640	19484423
3	15881505	17706832	19407076
Mean	15905873	17757991	19411793
SD	21236.42	44865.47	70390.62
RSD	0.13	0.25	0.36
mean normalized:	15902982	17754763	19408265
content found	181.63	202.78	221.67
content found in assay (day 2):		97.47	
difference	84.16	105.31	124.20
recovery	102.35	102.69	101.93
Tenofovir DF	area		
injection #	spiked 80%	spiked 100%	spiked 120%
1	3732762	4124728	4434354
2	3740552	4113262	4458031
3	3726287	4101818	4419983
Mean	3733200	4113269	4437456
SD	7142.59	11455.00	19212.74
RSD	0.19	0.28	0.43
mean normalized:	3732521.816	4112521.738	4436649.4831
content found	168.75	185.93	200.58
content found in assay (day 2):		96.07	
difference	72.68	89.86	104.51
recovery	95.84	94.83	92.83
Efavirenz	area		
injection #	spiked 80%	spiked 100%	spiked 120%
1	5066006	5669478	6191538
2	5076984	5677055	6205845
3	5073519	5643443	6182332
Mean	5072170	5663325	6193238
SD	5612.01	17630.46	11848.36
RSD	0.11	0.31	0.19
mean normalized:	5071248	5662296	6192113
content found	172.91	193.06	211.13
content found in assay (day 2):		105.05	
difference	67.86	88.01	106.08
recovery	93.44	94.15	93.81

reference solution	
Emtricitabine	Mass: 25.10mg
injection #	area
1	8711995
2	8896553
3	8752839
Mean	8787129
SD	96939.50
RSD	1.10
mean normalized:	8755621
Tenofovir DF	mass: 37.45mg
injection #	area
1	2192556
2	2235518
3	2198758
Mean	2208944
SD	23221.74
RSD	1.05
mean normalized	2211893
Efavirenz	mass: 37.55mg
injection #	area
1	2914708
2	2975081
3	2920632
Mean	2936807
SD	33278.34
RSD	1.13
mean normalized	2932896

mean recovery								
Efavirenz			Emtricitabine			Tenofovir disoproxil fumarate		
spiked 80%	spiked 100%	spiked 120%	spiked 80%	spiked 100%	spiked 120%	spiked 80%	spiked 100%	spiked 120%
93.49	93.91	93.69	101.45	101.69	100.95	95.03	93.93	92.03

Validation efavirenz, emtricitabine, tenofovir disoproxil fumarate - Accuracy II

Assay						
Emtricitabine	area	mass: 28.05mg	Emtricitabine	area	mass: 55.10mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	45864		1	46469	46393	102.65
2	44884		2	46540	46464	102.81
3	45108		3	46420	46344	102.54
4	45231		4	46467	46391	102.65
5	45277		5	46477	46401	102.67
6	45156		6	46340	46264	102.37
Mean	45253		Mean	46452	46376	102.61
SD	328.93		SD	67.01	66.90	0.15
RSD	0.73		RSD	0.14	0.14	0.14
mean normalized	45195		mean normalized	46376		
Tenofovir DF	area	mass: 21.02mg	Tenofovir DF	area	mass: 55.10mg	
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	6728		1	6706	6695	101.37
2	6596		2	6738	6727	101.86
3	6617		3	6699	6688	101.27
4	6616		4	6740	6729	101.89
5	6612		5	6711	6700	101.45
6	6604		6	6714	6703	101.49
Mean	6611		Mean	6722	6711	101.55
SD	6.11		SD	15.95	15.92	0.24
RSD	0.09		RSD	0.24	0.24	0.24
mean normalized	6604		mean normalized	6711		
Efavirenz	area	mass: 21.02mg	Efavirenz	area	mass: 55.10mg	
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	16360		1	16134	16108	100.14
2	16022		2	16201	16175	100.56
3	16063		3	16208	16182	100.60
4	16101		4	16213	16187	100.64
5	16103		5	16199	16173	100.55
6	16095		6	16152	16126	100.26
Mean	16100		Mean	16188	16162	100.46
SD	4.16		SD	31.95	31.90	0.20
RSD	0.03		RSD	0.20	0.20	0.20
mean normalized	16084		mean normalized	16162		

tablet solution I			
mass: 55.10mg			
Emtricitabine	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	37159	45828	54316
2	36746	45791	54418
3	36671	45784	54747
Mean	36859	45801	54494
SD	262.79	23.64	225.24
RSD	0.71	0.05	0.41
mean normalized:	36798	45726	54405
content found	81.42	101.17	120.38
content found in assay:		102.61	
difference	-21.19	-1.44	17.76
recovery	100.14	99.87	99.23
Tenofovir DF	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	5415	6666	7881
2	5360	6662	7883
3	5350	6666	7929
Mean	5375	6665	7898
SD	35.00	2.31	27.15
RSD	0.65	0.03	0.34
mean normalized:	5366	6654	7885
content found	81.25	100.75	119.39
content found in assay:		101.55	
difference	-20.30	-0.81	17.83
recovery	100.59	99.97	98.85
Efavirenz	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	13158	16154	19163
2	12988	16159	19181
3	12962	16158	19264
Mean	13036	16157	19203
SD	106.45	2.65	53.87
RSD	0.82	0.02	0.28
mean normalized:	13015	16131	19171
content found	80.92	100.29	119.19
content found in assay:		100.46	
difference	-19.54	-0.17	18.73
recovery	100.86	100.06	99.14

tablet solution II			
mass: 55.06mg			
Emtricitabine	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	36480	45472	53849
2	35688	44907	53755
3	35675	44760	54002
Mean	35948	45046	53869
SD	461.06	375.89	124.67
RSD	1.28	0.83	0.23
mean normalized:	35915	45005	53820
content found	79.47	99.58	119.08
content found in assay:		102.61	
difference	-23.15	-3.03	16.47
recovery	97.74	98.30	98.17
Tenofovir DF	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	5423	6704	7910
2	5313	6616	7883
3	5296	6613	7883
Mean	5344	6644	7892
SD	68.94	51.69	15.59
RSD	1.29	0.78	0.20
mean normalized:	5339	6638	7885
content found	80.84	100.51	119.39
content found in assay:		101.55	
difference	-20.71	-1.04	17.83
recovery	100.08	99.74	98.85
Efavirenz	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	13532	16678	19661
2	13274	16493	19598
3	13242	16464	19635
Mean	13349	16545	19631
SD	159.00	116.09	31.66
RSD	1.19	0.70	0.16
mean normalized:	13337	16530	19614
content found	82.92	102.77	121.94
content found in assay:		100.46	
difference	-17.54	2.31	21.48
recovery	103.35	102.54	101.42

mean recovery								
Efavirenz			Emtricitabine			Tenofovir disoproxil fumarate		
spiked 30%	spiked 50%	spiked 70%	spiked 30%	spiked 50%	spiked 70%	spiked 30%	spiked 50%	spiked 70%
102.10	101.30	100.28	98.94	99.08	98.70	100.33	99.85	98.85

Validation emtricitabine, tenofovir disoproxil fumarate - Linearity

Emtricitabine	area				
Injection #	0,5%-solution	5%-solution	50%-solution	100%-solution	125%-solution
1	223	2331	23084	45935	56937
2	234	2316	23008	45874	56370
3	225	2279	23142	45943	56313
Mean	227	2309	23078	45917	56540
SD	5.86	26.76	67.20	37.74	344.99
RSD	2.58	1.16	0.29	0.08	0.61

Tenofovir DF	area				
Injection #	0,5%-solution	5%-solution	50%-solution	100%-solution	125%-solution
1	47	375	3553	6967	8525
2	42	373	3543	6955	8434
3	47	369	3560	6947	8442
Mean	45	372	3552	6956	8467
SD	2.89	3.06	8.54	10.07	50.39
RSD	6.37	0.82	0.24	0.14	0.60

Validation emtricitabine, tenofovir disoproxil fumarate - Intermediate Precision

Day 1						
Emtricitabine	area	mass: 33,36mg	Emtricitabine	area	mass: 34,69mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	45935		1	46050	45909	100.02
2	45874		2	46075	45934	100.07
3	45943		3	46018	45877	99.95
4	45928		4	46153	46012	100.24
5	45925		5	46085	45944	100.10
6	45860		6	46054	45913	100.03
Mean	45911		Mean	46073	45932	100.07
SD	34.80		SD	45.74	45.60	0.10
RSD	0.08		RSD	0.10	0.10	0.10
mean normalized	45900		mean normalized	45932		
Tenofovir DF	area	mass: 25.04mg	Tenofovir DF	area	mass: 34,69mg	
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	6967		1	6924	6903	99.57
2	6955		2	6921	6900	99.53
3	6947		3	6921	6900	99.53
4	6943		4	6938	6917	99.77
5	6934		5	6922	6901	99.54
6	6915		6	6918	6897	99.49
Mean	6944		Mean	6924	6903	99.57
SD	14.29		SD	10.58	10.55	0.15
RSD	0.21		RSD	0.15	0.15	0.15
mean normalized	6932		mean normalized	6903		

Day 2						
Emtricitabine	area	mass: 26.63mg	Emtricitabine	area	mass: 34,63mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	45155		1	45743	45682	100.96
2	45082		2	45949	45888	101.42
3	45226		3	45940	45879	101.40
4	45119		4	45845	45784	101.19
5	45206		5	45866	45805	101.23
6	45188		6	45928	45867	101.37
Mean	45163		Mean	45879	45818	101.26
SD	54.78		SD	78.54	78.43	0.17
RSD	0.12		RSD	0.17	0.17	0.17
mean normalized	45247		mean normalized	45818		
Tenofovir DF	area	mass: 20,13mg	Tenofovir DF	area	mass: 34,63mg	
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	7139		1	7224	7214	101.81
2	7123		2	7252	7242	102.21
3	7133		3	7245	7235	102.11
4	7129		4	7237	7227	101.99
5	7134		5	7235	7225	101.97
6	7135		6	7242	7232	102.06
Mean	7132		Mean	7239	7230	102.02
SD	3.21		SD	3.61	3.60	0.05
RSD	0.05		RSD	0.05	0.05	0.05
mean normalized	7086		mean normalized	7230		

Day 3						
Emtricitabine	area	mass: 26.65mg	Emtricitabine	area	mass: 34.62mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	8432621		1	8366523	8357823	98.03
2	8495053		2	8381563	8372847	98.21
3	8503752		3	8400724	8391988	98.43
4	8522359		4	8432389	8423620	98.81
5	8562045		5	8457723	8448928	99.10
6	8579499		6	8471487	8462678	99.26
Mean	8515888		Mean	8418402	8409648	98.64
SD	52408.92		SD	42239.43	42195.51	0.49
RSD	0.62		RSD	0.50	0.50	0.50
mean normalized	8525475		mean normalized	8409648		
Tenofovir DF	area	mass: 20.2mg	Tenofovir DF	area	mass: 34.62mg	
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	2103656		1	2058963	2056822	97.13
2	2179588		2	2062144	2060000	97.28
3	2129051		3	2066005	2063857	97.46
4	2129440		4	2073919	2071762	97.83
5	2142152		5	2073556	2071400	97.82
6	2149131		6	2079935	2077772	98.12
Mean	2138836		Mean	2069087	2066935	97.60
SD	9983.63		SD	3582.73	3579.00	0.17
RSD	0.47		RSD	0.17	0.17	0.17
mean normalized	2117660		mean normalized	2066935		

Day 4						
Emtricitabine	area	mass: 20.13mg	Emtricitabine	area	mass: 34.65mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	8643295		1	8399523	8383524	97.38
2	8605195		2	8451189	8435091	97.98
3	8618951		3	8453135	8437034	98.00
4	8637231		4	8464625	8448502	98.13
5	8649539		5	8466501	8450374	98.15
6	8656238		6	8477903	8461755	98.29
Mean	8635075		Mean	8452146	8436047	97.99
SD	19401.52		SD	27554.63	27502.15	0.32
RSD	0.22		RSD	0.33	0.33	0.33
mean normalized	8609260		mean normalized	8436047		
Tenofovir DF	area	mass: 20.15mg	Tenofovir DF	area	mass: 34.65mg	
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	1678732		1	1617440	1614359	96.90
2	1671649		2	1621726	1618637	97.15
3	1675994		3	1627470	1624370	97.50
4	1679378		4	1629911	1626806	97.64
5	1682131		5	1626991	1623892	97.47
6	1683500		6	1634610	1631496	97.92
Mean	1678564		Mean	1626358	1623260	97.43
SD	2099.37		SD	3843.96	3836.64	0.23
RSD	0.13		RSD	0.24	0.24	0.24
mean normalized	1666068		mean normalized	1623260		

Emtricitabine	Tenofovir DF
RSD days 1+2	
0.14	0.10
RSD days 3+4	
0.41	0.20
	RSD total
0.27	0.15

Validation emtricitabine, tenofovir disoproxil fumarate- Accuracy

Assay						
Emtricitabine	area	mass: 26.63mg	Emtricitabine	area	mass: 55.10mg	content
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	45155		1	45743	45682	100.96
2	45082		2	45949	45888	101.42
3	45226		3	45940	45879	101.40
4	45119		4	45845	45784	101.19
5	45206		5	45866	45805	101.23
6	45188		6	45928	45867	101.37
Mean	45163		Mean	45879	45818	101.26
SD	54.78		SD	78.54	78.43	0.17
RSD	0.12		RSD	0.17	0.17	0.17
mean normalized	45247		mean normalized	45818		
Tenofovir DF	area	mass: 20.13mg	Tenofovir DF	area		
Injection #	100%-solution		Injection #	tablet solution	normalized	
1	7139		1	7224	7214	101.81
2	7123		2	7252	7242	102.21
3	7133		3	7245	7235	102.11
4	7129		4	7237	7227	101.99
5	7134		5	7235	7225	101.97
6	7135		6	7242	7232	102.06
Mean	7132		Mean	7239	7230	102.02
SD	3.21		SD	3.61	3.60	0.05
RSD	0.05		RSD	0.05	0.05	0.05
mean normalized	7086		mean normalized	7230		

tablet solution I			
mass: 34,69mg			
Emtricitabine	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	36908	45811	55300
2	36585	45831	55290
3	36667	45789	55255
Mean	36720	45810	55282
SD	167.90	21.01	23.63
RSD	0.46	0.05	0.04
mean normalized:	36608	45670	55113
content found	80.91	100.93	121.80
content found in assay:		101.26	
difference	-20.35	-0.33	20.54
recovery	100.34	100.30	100.97
Tenofovir DF	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	5821	7158	8556
2	5775	7156	8557
3	5774	7152	8556
Mean	5790	7155	8556
SD	26.85	3.06	0.58
RSD	0.46	0.04	0.01
mean normalized:	5772	7133	8530
content found	81.46	100.67	120.38
content found in assay:		102.02	
difference	-20.56	-1.36	18.35
recovery	100.55	99.66	99.48

tablet solution II			
mass: 34,62mg			
Emtricitabine	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	35029	44280	53566
2	35110	44224	53502
3	35200	44216	53412
Mean	35113	44240	53493
SD	85.54	34.87	77.36
RSD	0.24	0.08	0.14
mean normalized:	35076	44194	53438
content found	77.52	97.67	118.10
content found in assay:		101.26	
difference	-23.74	-3.59	16.84
recovery	96.14	97.06	97.90
Tenofovir DF	area		
injection #	spiked 30%	spiked 50%	spiked 70%
1	5572	6946	8341
2	5581	6943	8331
3	5585	6937	8314
Mean	5579	6942	8329
SD	6.66	4.58	13.65
RSD	0.12	0.07	0.16
mean normalized:	5574	6935	8320
content found	78.65	97.86	117.41
content found in assay:		102.02	
difference	-23.37	-4.16	15.39
recovery	97.09	96.88	97.03

mean recovery					
Emtricitabine			Tenofovir disoproxil fumarate		
spiked 30%	spiked 50%	spiked 70%	spiked 30%	spiked 50%	spiked 70%
98.24	98.68	99.44	98.82	98.27	98.25