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XX Ciclo

Determination of anti-HIV drugs in human plasma

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Contents

1. Introduction

1.1 Acquired Immunodeficiency Syndrome

Human immunodeficiency virus (HIV) is a retrovirus that can lead to *acquired immunodeficiency syndrome* (AIDS), a condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections. Previous names for the virus include human T-lymphotropic virus-III, lymphadenopathy-associated virus, and AIDS-associated retrovirus [1,2].

Infection with HIV occurs by the transfer of blood, semen, vaginal fluid, pre-ejaculate or breast milk. Within these bodily fluids, HIV is present as both free virus particles and virus within infected immune cells. The four major routes of transmission are unprotected sexual intercourse, contaminated needles, breast milk, and transmission from an infected mother to her baby at birth. Screening of blood products for HIV has largely eliminated transmission through blood transfusions or infected blood products in the developed world. HIV infection in humans is now pandemic. As of January 2006, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO) estimates that AIDS has killed more than 25 million people since it was first recognized on December 1, 1981, making it one of the most destructive pandemics in recorded history. It is estimated that about 0.6% of the world's population is infected with HIV [3]. In 2005 alone, AIDS claimed an estimated 2.4-3.3 million lives, of which more than 570,000 were children. A third of these deaths are occurring in sub-Saharan Africa, retarding economic growth and increasing poverty [4]. According to current estimates, HIV is set to infect 90 million people in Africa, resulting in a minimum estimate of 18 million orphans [5]. Antiretroviral treatment reduces both the mortality and the morbidity of HIV infection, but routine access to antiretroviral medication is not available in all countries [6].

HIV primarily infects vital cells in the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages, and dendritic cells. HIV infection leads to low levels of $CD4^+$ T cells through three main mechanisms: firstly, direct viral killing of infected cells; secondly, increased rates of apoptosis in infected cells; and thirdly, killing of infected $CD4^+$ T cells by CD8 cytotoxic lymphocytes that recognize infected cells. When $CD4^+$ T cell numbers decline below a critical level, cell-mediated immunity is lost, and the body becomes progressively more susceptible to opportunistic infections. If untreated, eventually most HIV-infected individuals develop AIDS and die; however, about one in ten remains healthy for many years (long term non-progressor with no noticeable symptoms) [7]. Treatment with anti-retrovirals increases the life expectancy of people infected with HIV. It is hoped that current and future treatments may allow HIV-infected individuals to achieve a life expectancy approaching that of the general public [8-10].

1.2 Classification

HIV is a member of the genus Lentivirus, part of the family of Retroviridae. Lentiviruses have many common morphologies and biological properties. Humans and animals can be infected by lentiviruses, which are responsible for long-duration illnesses with a long incubation period. Lentiviruses are transmitted as single-stranded, positive-sense, enveloped RNA viruses. Upon entry of the target cell, the viral RNA genome is converted to double-stranded DNA by a virally encoded reverse transcriptase that is present in the virus particle. The viral DNA is then integrated into the cellular DNA by a virally encoded integrase so that the genome can be transcribed. Once the virus has infected the cell, two pathways are possible: either the virus becomes latent and the infected cell continues to function, or the virus becomes active and replicates, and a large number of virus particles are liberated that can then infect other cells [11].

Two species of HIV infect humans: HIV-1 and HIV-2. HIV-1 is thought to have originated in southern Cameroon after jumping from wild chimpanzees (*Pan troglodytes troglodytes*) to humans during the twentieth century [12,13]. HIV-1 is the virus that was initially discovered and termed lymphadenopathy-associated virus. It is more virulent and relatively easy transmitted, it is the cause of the majority of HIV infections globally. The strains of HIV-1 can be classified into three groups: the

'major' group M, the 'outlier' group O and the 'new' group N. These three groups may represent three separate introductions of simian immunodeficiency virus into humans. Within group M there are known to be at least nine genetically distinct subtypes of HIV-1 (A to K). HIV-2 may have originated from the sooty mangabey (*Cercocebus atys*), an Old World monkey of Guinea-Bissau, Gabon, and Cameroon. HIV-2 is less transmittable than HIV-1 and is largely confined to West Africa [14].

1.3 Structure and genome

HIV is different in structure from other retroviruses. It is about 120 nm in diameter and roughly spherical [15]. It is composed of two copies of positive single-stranded RNA that codes for the virus nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24 [16]. The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and enzymes needed for the development of the virion such as reverse transcriptase, protease, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid ensuring the integrity of the virion particle [16]. This is, in turn, surrounded by the viral envelope which is composed of two layers of fatty molecules called phospholipids taken from the membrane of a human cell when a newly formed virus particle buds from the cell. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle [16]. This protein, known as Env, consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope [17]. This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle [17]. Both these surface proteins, especially gp120, have been considered as targets of future treatments or vaccines against HIV [10,18].

Of the nine genes that are encoded within the RNA genome, three of these genes, *gag*, *pol*, and *env*, contain information needed to make the structural proteins for new virus particles [16]. For example, *env* codes for a protein called gp160 that is broken down by the viral protease to form gp120 and gp41. The six remaining genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease [16]. The protein encoded by *nef*, for instance, appears necessary for the virus to replicate efficiently, and the *vpu*-encoded protein influences the release of new virus particles from infected cells [16]. The ends of each strand of HIV RNA contain an RNA sequence called long terminal repeat. Regions of the long terminal repeat act as switches to control production of new viruses, they can be triggered by proteins from either HIV or the host cell [16].

1.4 Tropism

The term viral tropism refers to which cell types HIV infects. HIV can infect a variety of immune cells such as $CD4^+$ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and $CD4^+$ T cells is mediated through interaction of the virion envelope gp120 with the CD4 molecule on the target cells and also with chemokine co-receptors [17].

Macrophage-tropic strains of HIV-1 and non-syncitia-inducing strains use the β-chemokine receptor CCR5 for entry and are thus able to replicate in macrophages and $CD4^+$ T cells. The CCR5 co-receptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype. Indeed, macrophages play a key role in several critical aspects of the HIV infection. They appear to be the first cells infected by HIV and perhaps the source of HIV production when $CD4^+$ cells become depleted in the patient. Macrophages and microglial cells are the cells infected by HIV in the central nervous system. In tonsils and adenoids of HIV-infected patients, macrophages fuse into multinucleated giant cells that produce huge amounts of virus [19].

T-tropic isolates or syncitia-inducing strains replicate in primary $CD4^+$ T cells as well as in macrophages and use the α-chemokine receptor CXCR4 for entry. Dual-tropic HIV-1 strains are thought to be transitional strains of the HIV-1 virus and thus are able to use both CCR5 and CXCR4 as co-receptors for viral entry [19,20].

The α-chemokine SDF-1, a ligand for CXCR4, suppresses replication of T-tropic HIV-1 isolates by down-regulating the expression of CXCR4 on the surface of these cells. HIV using only the CCR5 receptor are termed R5, those that only use CXCR4 are termed X4, and those that use both, X4R5. However, the use of co-receptor alone does not explain viral tropism. Indeed not all R5 viruses are able to use CCR5 on macrophages for a productive infection. Moreover, HIV can also infect a subtype of myeloid dendritic cells which do not have CXCR4 and CCR5 as co-receptors, these cells probably constitute a reservoir that maintains infection when $CD4^+$ T cell numbers have declined to extremely low levels [20,21].

Some people are resistant to certain strains of HIV. One example of how this occurs is people with the CCR5-Δ32 mutation; these people are resistant to infection with R5 virus as the mutation stops HIV from binding to this co-receptor, reducing its ability to infect target cells [22].

Sexual intercourse is the major mode of HIV transmission. Both X4 and R5 HIV are present in the seminal fluid which is passed from partner to partner. The virions can then infect numerous cellular targets and disseminate into the whole organism. However, a selection process leads to a predominant transmission of the R5 virus through this pathway [23-25]. How this selective process works is still under investigation, but one model is that spermatozoa may selectively carry R5 HIV as they possess both CCR3 and CCR5 but not CXCR4 on their surface and that genital epithelial cells preferentially sequester X4 virus [26,27]. In patients infected with subtype B HIV-1, there is often a co-receptor switch in late-stage disease and T-tropic variants appear that can infect a variety of T cells through CXCR4 [28]. These variants then replicate more aggressively with heightened virulence that causes rapid T cell depletion, immune system collapse, and opportunistic infections that mark the advent of AIDS [29]. Thus, during the course of infection, viral adaptation to the use of CXCR4 instead of CCR5 may be a key step in the progression to AIDS. A number of studies with subtype B-infected individuals have determined that between 40 and 50% of AIDS patients can harbor viruses of the SI, and presumably the X4, phenotype [30,31].

1.5 Replication cycle

HIV enters macrophages and $CD4^+$ T cells by the adsorption of glycoproteins present on its surface to receptors occurring on the target cell followed by fusion of the viral envelope with the cell membrane and the release of the HIV capsid into the cell [32,33].

Entry to the cell begins through interaction of the trimeric envelope complex (gp160 spike) with CD4 and chemokine receptors (generally either CCR5 or CXCR4, but others are known to interact) on the cell surface. The gp160 spike contains binding domains for both CD4 and chemokine receptors. The first step in fusion involves the attachment of the CD4 binding domains of gp120 to CD4. Once gp120 is bound with the CD4 protein, the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor. This allows for a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41 to penetrate the cell membrane. Repeat sequences in gp41, HR1, and HR2 then interact, causing the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid [32,33].

Once HIV has bound to the target cell, the HIV RNA and various enzymes, including reverse transcriptase, integrase, ribonuclease and protease, are injected into the cell [32].

HIV can infect dendritic cells (DCs) by this CD4-CCR5 route, but another route using mannose-specific C-type lectin receptors such as DC-SIGN can also be used. DCs are one of the first cells encountered by the virus during sexual transmission. They are currently thought to play an important role by transmitting HIV to T cells once the virus has been captured in the mucosa by DCs [34].

1.6 Replication and transcription

Once the viral capsid enters the cell, the viral reverse transcriptase liberates the single-stranded (+)RNA from the attached viral proteins and copies it into a complementary DNA. This process of reverse transcription is extremely error-prone and it is during this step that mutations may occur, such mutations may cause drug resistance. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate. The viral DNA is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out by the viral integrase [35].

The integrated viral DNA may then lie dormant, in the latent stage of HIV infection [35]. To actively produce the virus, certain cellular transcription factors need to be present, the most important of which is NF-κB, which is upregulated when T cells become activated. This means that those cells most likely to be killed by HIV are those currently fighting infection [36].

In this replication process, the integrated provirus is copied to mRNA which is then spliced into smaller pieces. These small pieces produce the regulatory proteins Tat (which encourages new virus production) and Rev. As Rev accumulates, it gradually starts to inhibit mRNA splicing. At this stage, the structural proteins Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles [37].

HIV-1 and HIV-2 appear to package their RNA differently; HIV-1 will bind to any appropriate RNA whereas HIV-2 will preferentially bind to the mRNA which was used to create the Gag protein itself. This may mean that HIV-1 is better able to mutate (HIV-1 infection progresses to AIDS faster than HIV-2 infection and is responsible for the majority of global infections) [38].

1.7 Assembly and release

The final step of the viral cycle, assembly of new HIV-1 virons, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion. This cleavage step can be inhibited by protease inhibitors. The mature virus is then able to infect another cell [39].

1.8 Treatment

There is currently no vaccine or cure for HIV or AIDS. The only known method of prevention is avoiding exposure to the virus. However, an anti-retroviral treatment, known as post-exposure prophylaxis, is believed to reduce the risk of infection if begun directly after exposure [40]. Current treatment for HIV infection consists of highly active anti-retroviral therapy, or HAART [41]. This has been highly beneficial to many HIV-infected individuals since its introduction in 1996, when the protease inhibitor- (PI-)based HAART initially became available [6]. Current HAART options are combinations (or ‘cocktails’) consisting of at least three drugs belonging to at least two types, or ‘classes’, of anti-retroviral agents. Typically, these classes are two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a PI or a non-nucleoside reverse transcriptase inhibitor (NNRTI). Because AIDS progression in children is more rapid and less predictable than in adults, particularly in young infants, more aggressive treatment is recommended for children than adults [42]. In developed countries where HAART is available, doctors assess their patients thoroughly: measuring the viral

load, how fast CD4 declines, and patient readiness. They then decide when to recommend starting treatment [10].

HAART allows the stabilisation of patient's symptoms and viremia, but it neither cures the patient, nor alleviates the symptoms; high levels of HIV-1, often HAART resistant, return once treatment is stopped [43,44]. Moreover, it would take more than a lifetime for HIV infection to be cleared using HAART [45]. Despite this, many HIV-infected individuals have experienced remarkable improvements in their general health and quality of life, which has led to a large reduction in HIV-associated morbidity and mortality in the developed world [6,46,47]. HAART sometimes achieves far less than optimal results, in some circumstances being effective in less than fifty percent of patients. This is due to a variety of reasons such as medication intolerance/side effects, prior ineffective anti-retroviral therapy and infection with a drug-resistant strain of HIV. However, non-adherence and non-persistence with anti-retroviral therapy is the major reason for which individuals fail to benefit from HAART [48]. The reasons for non-adherence and non-persistence with HAART are varied and overlapping. Major psychosocial issues, such as poor access to medical care, inadequate social supports, psychiatric diseases, and drug abuse contribute to non-adherence. The complexity of these HAART regimens, whether due to pill number, dosing frequency, meal restrictions or other issues along with side effects that create intentional non-adherence also, contribute to non-adherence and non-persistence [49,50,51]. The side effects include lipodystrophy, dyslipidaemia, insulin resistance, an increase in cardiovascular risks, and birth defects [52,53].

Anti-retroviral drugs are expensive, and the majority of the infected individuals do not have access to medications and anti-HIV and anti-AIDS treatments [54]. Research to improve current treatments includes decreasing side effects of current drugs, further simplifying drug regimens to improve adherence, and determining the best sequence of regimens to manage drug resistance. Unfortunately, only a vaccine is thought to be able to halt the pandemic. This is because a vaccine would cost less, thus being affordable for developing countries, and would not require daily treatment. The anti-HIV drugs act mainly as NRTIs, NNRTIs, and PIs; very recently, integrase inhibitors and fusion inhibitors have been developed [10,18,42,55-60].

NRTIs are intra-cellularly phosphorylated to their corresponding triphosphorylated derivatives, which compete with the corresponding natural nucleotide for binding to HIV reverse transcriptase inhibiting it. NRTIs inhibit the replication of the virus by blocking transcription of RNA viral into pro-viral DNA; they act replacing the nitrogen bases during transcription so that the new proviral DNA is incomplete and thus unable to rise new viral particles. NRTIs include abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, and zidovudine [61-65].

NNRTIs act as non-competitive inhibitors of the HIV reverse transcriptase blocking RNA transcription and pro-viral DNA synthesis. These drugs have good bioavailability and a long half-life, therefore they can be administered only once or twice a day. NNRTIs includes efavirenz and nevirapine [61-65].

PIs interfere with viral replication by inhibiting the viral protease, preventing maturation of the HIV virus and causing the formation of non-infection virions. PIs includes amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir [61-65].

Anti-HIV therapeutic strategy regimens require the administration of several antiretroviral drugs. The increasing number of anti-HIV drugs available rapidly increases the number of different combinations. Some very promising combination regimens contain PIs and NRTIs [61-65].

1.9 Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is the strategy by which the dosing regimen for a patient is guided by repeated measurements of the plasma drug concentration, enabling physicians to positively exploit drug-drug interactions to enhance exposure levels of anti-retroviral drugs, to avoid and to manage drug-related toxicity, and to sustain virological efficacy. Although, TDM consists of the individualizing dosages with the aim maximizing the efficacy of treatment while minimizing its toxicity. The combination of pharmacokinetic–pharmacodynamic relationships for antiretroviral therapy and the presence of a wide interpatient variability in drug exposure support the application of TDM in HIV-infected individuals. Prospective clinical trials assessing the clinical usefulness of this

strategy have shown contradictory results, pointing out the need to consider different issues when performing TDM [66-76].

1.10. References

[1] J. Coffin, A. Haase, J.A.vLevy, L. Montagnier, S. Oroszlan, N. Teich, H. Temin, K. Toyoshima, H. Varmus, P. Vogt, R.A. Weiss, What to call the AIDS virus? Nature 321 (1986) 10.

[2] D.L. Robertson, J.P. Anderson, J.A. Bradac, J.K. Carr, B. Foley, R.K. Funkhouser, F. Gao, B.H. Hahn, M.L. Kalish, C. Kuiken, G.H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P. M. Sharp, S. Wolinsky, B. Korber, HIV-1 Nomenclature Proposal:
A Reference Guide to HIV-1 Classification, 1999, available at: http://www.hiv.lanl.gov/content/sequence/HIV/REVIEWS/nomenclature/Nomen.html.

[3] Joint United Nations Programme on HIV/AIDS, Overview of the global AIDS epidemic, 2006, available at: http://www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/Default.asp.

[4] S. Gillespie, S. Kadiyala, R.Greener, Is poverty or wealth driving HIV transmission? AIDS 21 (2007) S5-S16.

[5] UNAIDS/WHO, AIDS epidemic update, 2007, available at: http://www.unaids.org/en/KnowledgeCentre/HIVData/EpiUpdate/EpiUpdArchive/2007/.

[6] F.J. Palella Jr, K.M. Delaney, A.C. Moorman, M.O. Loveless, J. Fuhrer, G.A. Satten, D.J. Aschman, S.D. Holmberg, Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators, N. Engl. J. Med. 338 (1998) 853-860.

[7] S.P. Buchbinder, M.H. Katz, N.A. Hessol, P.M. O'Malley, S.D. Holmberg, Long-term HIV-1 infection without immunologic progression, AIDS 8 (1994) 1123-1128.

[8] S, Walmsley, M. Loutfy, Can structured treatment interruptions (STIs) be used as a strategy to decrease total drug requirements and toxicity in HIV infection? J. Int. Assoc. Physicians AIDS Care (Chic. Ill.) 1(2002) 95-103.

[9] F. van Leth, B. Conway, H. Laplumé, D. Martin, M. Fisher, A. Jelaska, F.W. Wit, J.M. Lange; 2NN study group, Quality of life in patients treated with first-line antiretroviral therapy containing nevirapine and/or efavirenz, Antivir. Ther. 9 (2004), 721-728.

[10] Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents, 2008, available at: http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf.

[11] J.A. Lévy, HIV pathogenesis and long-term survival, AIDS 7 (1993) 1401-1410.

[12] F. Gao, E. Bailes, D.L. Robertson, Y. Chen, C.M. Rodenburg, S.F. Michael, L.B. Cummins, L.O. Arthur, M. Peeters, G.M. Shaw, P.M. Sharp, B.H. Hahn, Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*, Nature 397 (1999) 436-441.

[13] B.F. Keele, F. van Heuverswyn, Y.Y. Li, E. Bailes, J. Takehisa, M.L. Santiago, F. Bibollet-Ruche, Y. Chen, L.V. Wain, F. Liegois, S. Loul, E. Mpoudi Ngole, Y. Bienvenue, E. Delaporte, J.F.Y. Brookfield, P.M. Sharp, G.M. Shaw, M. Peeters, B.H. Hahn, Chimpanzee reservoirs of pandemic and nonpandemic HIV-1, Science Online 313 (2006) 523-526.

[14] J.D. Reeves, R.W. Doms, Human Immunodeficiency Virus Type 2, J. Gen. Virol. 83 (2002) 1253-1265.

[15] S.L. McGovern, E. Caselli, N. Grigorieff, B.K. Shoichet, A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening, J. Med. Chem. 45 (2002) 1712-1722.

[16] T. Leitner, B. Foley, B. Hahn, P. Marx, F. McCutchan, J.W. Mellors, S. Wolinsky, B. Korber, The Human Retroviruses and AIDS 2005 Compendium, 2005, Los Alamos National Laboratory, Los Alamos, NM, available at: http://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/2005compendium.html.

[17] D.C. Chan, D. Fass, J.M. Berger, P.S. Kim, Core structure of gp41 from the HIV envelope glycoprotein, Cell 89 (1997) 263-273.

[18] J. Wyman, Crystal structure of key HIV protein reveals new prevention, treatment targets, NIH, National Institute of Allergy and Infectious Diseases, 1998, available at: http://www3.niaid.nih.gov/news/newsreleases/1998/hivprotein.htm.
[19] E. Coakley, C.J. Petropoulos, J.M. Whitcomb, Assessing chemokine co-receptor usage in HIV, Curr. Opin. Infect. Dis. 18 (2005) 9-15.
[20] H. Deng, R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R.E. Sutton, C.M. Hill, C.B. Davis, S.C. Peiper, T.J. Schall, D.R. Littman, N.R. Landau, Identification of a major co-receptor for primary isolates of HIV-1, Nature 381 (1996) 661-666.
[21] S.C. Knight, S.E. Macatonia, S. Patterson, HIV I infection of dendritic cells, Int. Rev. Immunol. 6 (1990) 163-175.
[22] J. Tang, R.A. Kaslow, The impact of host genetics on HIV infection and disease progression in the era of highly active antiretroviral therapy, AIDS 17 (2003) S51-S60.
[23] T. Zhu, H. Mo, N. Wang, D.S. Nam, Y. Cao, R.A. Koup, D.D. Ho, Genotypic and phenotypic characterization of HIV-1 patients with primary infection, Science 261 (1993) 1179-1181.
[24] A.B. van't Wout, N.A. Kootstra, G.A. Mulder-Kampinga, N. Albrecht-van Lent, H.J. Scherpbier, J. Veenstra, K. Boer, R.A. Coutinho, F. Miedema, H. Schuitemaker, Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission, J. Clin. Invest. 94 (1994) 2060-2067.
[25] T. Zhu, N. Wang, A. Carr, D.S. Nam, R. Moor-Jankowski, D.A. Cooper, D.D. Ho, Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: evidence for viral compartmentalization and selection during sexual transmission, J. Virol. 70 (1996) 3098-3107.
[26] W. Berlier, T. Bourlet, P. Lawrence, H. Hamzeh, C. Lambert, C. Genin, B.Verrier, M.C. Dieu-Nosjean, B. Pozzetto, O. Delezay, Selective sequestration of X4 isolates by human genital epithelial cells: implication for virus tropism selection process during sexual transmission of HIV, J. Med. Virol. 77 (2005) 465-474.
[27] B. Muciaccia, F. Padula, E. Vicini, L. Gandini, A. Lenzi, M. Stefanini, β-chemokine receptors 5 and 3 are expressed on the head region of human spermatozoon, FASEB J. 19 (2005) 2048-2050.
[28] P. Clevestig, I. Maljkovic, C. Casper, E. Carlenor, S. Lindgren, L. Naver, A.B. Bohlin, E.M. Fenyo, T. Leitner, A. Ehrnst, The X4 phenotype of HIV type 1 evolves from R5 in two children of mothers, carrying X4, and is not linked to transmission, AIDS Res. Hum. Retroviruses 5 (2005) 371-378.
[29] J.P. Moore, Coreceptors: implications for HIV pathogenesis and therapy, Science 276 (1997) 51-52.
[30] A. Karlsson, K. Parsmyr, K. Aperia, E. Sandstrom, E.M. Fenyo, J. Albert, MT-2 cell tropism of human immunodeficiency virus type 1 isolates as a marker for response to treatment and development of drug resistance, J. Infect. Dis. 170 (1994) 1367-1375.
[31] M. Koot, A.B. van't Wout, N.A. Kootstra, R.E. de Goede, M. Tersmette, H. Schuitemaker, Relation between changes in cellular load, evolution of viral phenotype, and the clonal composition of virus populations in the course of human immunodeficiency virus type 1 infection, J. Infect. Dis. 173 (1996) 349-354.
[32] D. Chan, P. Kim, HIV entry and its inhibition, Cell 93 (1998) 681-684.
[33] R. Wyatt, J. Sodroski, The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens, Science 280 (1998) 1884-1888.
[34] M. Pope, A. Haase, Transmission, acute HIV-1 infection and the quest for strategies to prevent infection, Nat. Med. 9 (2003) 847-852.
[35] Y.H. Zheng, N. Lovsin, B.M. Peterlin, Newly identified host factors modulate HIV replication, Immunol. Lett. 97 (2005) 225-234.
[36] J. Hiscott, H. Kwon, P. Genin, Hostile takeovers: viral appropriation of the NF-κB pathway, J. Clin. Invest. 107 (2001) 143-151.
[37] V.W. Pollard, M.H. Malim, The HIV-1 Rev protein, Annu. Rev. Microbiol. 52 (1998) 491-532.
[38] Y.X. Feng, T.D. Copeland, L.E. Henderson, R.J. Gorelick, W.J. Bosche, J.G. Levin, A. Rein, HIV-1 nucleocapsid protein induces 'maturation' of dimeric retroviral RNA *in vitro*, Proc. Natl. Acad. Sci, USA 93 (1996) 7577-7581.

[39] H.R. Gelderblom, Fine structure of HIV and SIV, in HIV, 1997, available at: http://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1997/partIII/Gelderblom.pdf.

[40] H. Fan, R.F.Conner, L.P. Villarreal, Eds, AIDS: Science and Society, 4th edition, Boston, MA, Jones and Bartlett Publishers, 2005.

[41] J.G. Bartlett, A Pocket Guide to Adult HIV/AIDS Treatment, Johns Hopkins University School of Medicine, National Library of Medicine, Baltimore, MD, 2005.

[42] J. Oleske, G.B. Scott, P. Havens, Guidelines for the use of antiretroviral agents in pediatric HIV infection, 2006, available at: http://www.aidsinfo.nih.gov/contentfiles/PediatricGuidelines.pdf.

[43] J. Martinez-Picado, M.P. De Pasquale, N. Kartsonis, G.J. Hanna, J. Wong, D. Finzi, E. Rosenberg, H.F. Gunthard, L. Sutton, A. Savara, C.J. Petropoulos, N. Hellmann, B.D. Walker, D.D. Richman, R. Siliciano, R.T. D'Aquila, Antiretroviral resistance during successful therapy of human immunodeficiency virus type 1 infection, Proc. Natl. Acad. Sci. USA 97 (2000) 10948-10953.

[44] M. Dybul, A.S. Fauci, J.G. Bartlett, J.E. Kaplan, A.K. Pau.; Panel on Clinical Practices for Treatment of HIV, Guidelines for using antiretroviral agents among HIV-infected adults and adolescents, Ann. Intern. Med. 137 (2002) 381-433.

[45] J.N. Blankson, D. Persaud, R.F. Siliciano, The challenge of viral reservoirs in HIV-1 infection, Annu. Rev. Med. 53 (2002) 557-593.

[46] G. Chene, J.A. Sterne, M. May, D. Costagliola, B. Ledergerber, A.N. Phillips, F. Dabis, J. Lundgren, A. D'Arminio Monforte, F. de Wolf, R. Hogg, P. Reiss, A. Justice, C. Leport, S. Staszewski, J. Gill, G. Fatkenheuer, M.E. Egger and the Antiretroviral Therapy Cohort Collaboration, Prognostic importance of initial response in HIV-1 infected patients starting potent antiretroviral therapy: analysis of prospective studies, Lancet 362 (2003) 679-686.

[47] E. Wood, R.S. Hogg, B. Yip, P.R. Harrigan, M.V. O'Shaughnessy, J.S. Montaner, Is there a baseline CD4 cell count that precludes a survival response to modern antiretroviral therapy? AIDS 17 (2003) 711-720.

[48] S.L. Becker, C.M. Dezii, B. Burtcel, H. Kawabata, S. Hodder, Young HIV-infected adults are at greater risk for medication nonadherence, MedGenMed. 4 (2002) 21.

[49] C. Kleeberger, J. Phair, S. Strathdee, R. Detels, L. Kingsley, L.P. Jacobson, Determinants of heterogeneous adherence to HIV-antiretroviral therapies in the multicenter AIDS cohort study, J. Acquir. Immune Defic. Syndr. 26 (2001) 82-92.

[50] P. Nieuwkerk, M. Sprangers, D. Burger, R.M. Hoetelmans, P.W. Hugen, S.A. Danner, M.E. van Der Ende, M.M. Schneider, G. Schrey, P.L. Meenhorst, H.G. Sprenger, R.H. Kauffmann, M. Jambroes, M.A. Chesney, F. de Wolf, J.M. Lange. and the ATHENA Project (2001) Limited patient adherence to highly active antiretroviral therapy for HIV-1 infection in an observational cohort study. Arch Intern Med 161, 1962-1968.

[51] K.V. Heath, J. Singer, M.V. O'Shaughnessy, J.S.Montaner R.S. Hogg, Intentional nonadherence due to adverse symptoms associated with antiretroviral therapy, J. Acquir. Immune Defic. Syndr. 31 (2002) 211-217.

[52] V. Montessori, N. Press, M. Harris, L. Akagi, J.S. Montaner, Adverse effects of antiretroviral therapy for HIV infection, CMAJ 170 (2004) 229-238.

[53] A. Saitoh, A.D. Hull, P. Franklin, S.A. Spector, Myelomeningocele in an infant with intrauterine exposure to efavirenz, J. Perinatol. 25 (2005) 555-556.

[54] F. Ferrantelli, A. Cafaro, B. Ensoli, Nonstructural HIV proteins as targets for prophylactic or therapeutic vaccines, Curr. Opin. Biotechnol. 15 (2004) 543-556.

[55] J. Darbyshire, Therapeutic interventions in HIV infection - a critical view, Trop. Med. Int. Health 5 (2000) A26-A31.

[56] R.P. Van Heeswijk, Critical issues in therapeutic drug monitoring of antiretroviral drugs, Ther. Drug Monit. 24 (2002) 323-331.

[57] P. Clevenbergh, S. Mouly, P. Sellier, E. Badsi, J. Cervoni, V. Vincent, H. Trout, J.F. Bergmann, Improving HIV infection management using antiretroviral plasma drug levels monitoring: a clinician's point of view, Curr. HIV Res. 2 (2004) 309-321.

[58] A. Rouzes, K. Berthoin, F. Xuereb, S. Djabarouti, I. Pellegrin, J. L.Pellegrin, A.C. Coupet, S. Augagneur, H. Budzinski, M.C. Saux, D. Breilh, Simultaneous determination of the

antiretroviral agents: amprenavir, lopinavir, ritonavir, saquinavir and efavirenz in human peripheral blood mononuclear cells by high-performance liquid chromatography-mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 813 (2004) 209-216.

[59] M. Boffito, E. Acosta, D. Burger, C.V. Fletcher, C. Flexner, R. Garaffo, G. Gatti, M. Kurowski, C.F. Perno, G. Peytavin, M. Regazzi, D. Back, Current status and future prospects of therapeutic drug monitoring and applied clinical pharmacology in antiretroviral therapy, Antivir. Ther. 10 (2005) 375-392.

[60] S.F. Forsyth, P.D. French, E. Macfarlane, S.E. Gibbons, R.F. Miller, The use of therapeutic drug monitoring in the management of protease inhibitor-related toxicity, Int. J. STD AIDS 16 (2005) 139-141.

[61] J.A. Arnaiz, J. Mallolas, D. Podzamczer, J. Gerstoft, J.D. Lundgren, P. Cahn, G. Fätkenheuer, A. D'Arminio-Monforte, A. Casiró, P. Reiss, D.M. Burger, M. Stek, J.M. Gatell, BEST Study Team, Continued indinavir versus switching to indinavir/ritonavir in HIV-infected patients with suppressed viral load, AIDS 17 (2003) 831-840.

[62] M. Kurowski, T. Sternfeld, A. Sawyer, A. Hill, C. Möcklinghoff, Pharmacokinetic and tolerability profile of twice-daily saquinavir hard gelatine capsules and saquinavir soft gelatin capsules boosted with ritonavir in healthy volunteers, HIV Med. 4 (2003) 94-100.

[63] J.P. Lalezari, K. Henry, M .O'Hearn, J.S. Montaner, P.J. Piliero, B. Trottier, S. Walmsley, C. Cohen, D.R. Kuritzkes, J.J. Eron Jr, J. Chung, R. De Masi, L. Donatacci, C. Drobnes, J. Delehanty, M. Salgo; TORO 1 Study Group, Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America, N. Engl. J. Med. 348 (2003) 2175-2185. *Erratum* in: N. Engl. J. Med. 349 (2003) 1100.

[64] A. Lazzarin, B. Clotet, D. Cooper, J. Reynes, K. Arasteh, M. Nelson, C. Katlama, H.J. Stellbrink, J.F. Delfraissy, J. Lange, L. Huson, R. De Masi, C. Wat, J. Delehanty, C. Drobnes, M. Salgo; TORO 2 Study Group, Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia, N. Engl. J. Med. 348 (2003) 2186-2195.

[65] J.C. Gathe Jr, P. Ive, R. Wood, D. Schürmann, N.C. Bellos, E. DeJesus, A. Gladysz, C. Garris, J. Yeo, SOLO: 48-week efficacy and safety comparison of once-daily fosamprenavir/ritonavir versus twice-daily nelfinavir in naive HIV-1-infected patients, AIDS 18 (2004) 1529-1537.

[66] D. Back, G. Gatti, C. Fletcher, R. Garaffo, R. Haubrich, R. Hoetelmans, M. Kurowski, A. Luber, C. Merry, C.F. Perno, Therapeutic drug monitoring in HIV infection: current status and future directions, AIDS 16 (2002) S5-S37.

[67] P. Clevenbergh, R. Garraffo, J. Durant, P. Dellamonica, PharmAdapt: a randomized prospective study to evaluate the benefit of therapeutic monitoring of protease inhibitors: 12 week results, AIDS 16 (2002) 2311-2315.

[68] C.V. Fletcher, P.L.Anderson, T.N. Kakuda, T.W. Schacker, K. Henry, C.R. Gross, R.C. Brundage, Concentration-controlled compared with conventional antiretroviral therapy for HIV infection, AIDS 16 (2002) 551-560.

[69] R.E. Aarnoutse, J.M. Schapiro, C.A. Boucher, Y.A. Hekster, D.M. Burger, Therapeutic drug monitoring: an aid to optimising response to antiretroviral drugs? Drugs 63 (2003) 741-753.

[70] D.M. Burger, P.W. Hugen, R.E. Aarnoutse, R.M. Hoetelmans, M. Jambroes, P.T. Nieuwkerk, G. Schreij, M.M. Schneider, M.E. van der Ende, J.M. Lange, ATHENA Study Group, Treatment failure of nelfinavir-containing triple therapy can largely be explained by low nelfinavir plasma concentrations, Ther. Drug Monit. 25 (2003) 73-80.

[71] J.G. Gerber, E.P. Acosta, Therapeutic drug monitoring in the treatment of HIV-infection, J. Clin. Virol. 27 (2003) 117-128.

[72] P. Bossi, G. Peytavin, H. Ait-Mohand, C. Delaugerre, N. Ktorza, L. Paris, M. Bonmarchand, R. Cacace, D.J. David, A. Simon, C. Lamotte, A.G. Marcelin, V. Calvez, F. Bricaire, D. Costagliola, C. Katlama, GENOPHAR: a randomized study of plasma drug measurements in association with genotypic resistance testing and expert advice to optimize therapy in patients failing antiretroviral therapy, HIV Med. 5 (2004) 352-359.

[73] J. Moltó, B. Clotet, Therapeutic drug monitoring of antiretroviral agents scenario, J. HIV Ther. 9 (2004) 75-78.

[74] N.Y. Rakhmanina, J.N. van den Anker, S.J. Soldin, Therapeutic drug monitoring of antiretroviral therapy, AIDS Patient Care STDS 18 (2004) 7-14.

[75] S.H. Khoo, J. Lloyd, M. Dalton, A. Bonington, E. Hart, S. Gibbons, P. Flegg, J. Sweeney, E.G. Wilkins, D.J. Back, Pharmacologic optimization of protease inhibitors and non-nucleoside reverse transcriptase inhibitors (POPIN) - A randomized controlled trial of therapeutic drug monitoring and adherence support, J. Acquir. Immune Defic. Syndr. 41 (2006) 461-467.

[76] C. Torti, M.C. Uccelli, E. Quiros-Roldan, F. Gargiulo, V. Tirelli, G. Lapadula, M. Regazzi, P. Pierotti, C. Tinelli, A. De Luca, A. Patroni, N. Manca, G. Carosi, Prediction of early and confirmed virological response by genotypic inhibitory quotients for lopinavir in patients naïve for lopinavir with limited exposure to previous protease inhibitors, J. Clin. Virol. 35 (2006) 414-419.

2. Aim of the research

TDM is becoming increased in clinical care to determine the best dosage regimen adapted to each patient. Therefore, it has been incorporated into national HIV treatment guidelines in several countries there is a surprising lack of data to assess the benefit of TDM in routine clinical use. Prospective randomized clinical trials assessing the clinical usefulness of this strategy have shown contradictory results, pointing out the need to consider pharmacologic, analytical, and clinical criteria in order to interpret the values of the plasma drug concentration when performing TDM [1-11].

Here two new methods for the determination of the plasma levels of anti-HIV drugs (i.e., NRTIs, NNRTIs, and PIs) in HIV-infected patients are reported [12-14].

The HPLC-UV method allows to quantify simultaneously PIs (i.e., amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir), NRTIs (i.e., abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, and zidovudine), and NNTRIs (i.e., efavirenz and nevirapine) in human plasma [12]. The MALDI-TOF/TOF method allows to determine simultaneously the plasma concentration of PIs (i.e., amprenavir, lopinavir, and ritonavir), NRTIs (i.e., abacavir, didanosine, emtricitabine, lamivudine, and stavudine), and NNTRIs (i.e., efavirenz and nevirapine) [13,14].

For both methods, the volume of the plasma sample is 600 μL; moreover, they involve automated solid-phase extraction with Oasis HLB Cartridge 1 cc (divinylbenzene and N-vinylpyrrolidone) and evaporation in a water bath under nitrogen stream. The extracted samples are reconstituted with 100 μL methanol [12-14].

In the HPLC-UV method, 20 μL of the sample were injected into a HPLC-UV system, and the analytes were eluted on an analytical C18 SymmetryTM column (250mm×4.6mm I.D.) with a particle size of 5 μm. The mobile phase (0.01 M KH_2PO_4 and acetonitrile) was delivered at 1.0 mL/min with linear gradient elution. The total run time for a single analysis was 35 min, the anti-HIV drugs were detected by UV at 240 and 260 nm. The absolute recovery ranged between 88 and 120%. The calibration curves were linear up to 10 μg/mL. On these bases, a two to four analyte method has been tailored to the individual needs of the HIV-infected patient [12-14].

In the MALDI-TOF/TOF method, the extracted samples were mixed (1:1) with a saturated matrix solution (4-hydroxybenzoic acid in 50% acetonitrile-0.1% trifluoracetic acid), and spotted onto the MALDI-TOF/TOF sample target plate. The anti-HIV drug concentration was determined by standard additions analysis. Regression of standard additions was linear between 0.0025 and 5.0 pmol/μL. The absolute recovery ranged between 80 and 110%. MALDI-TOF/TOF method allows also the detection of the ritonavir metabolite R5 [12-14].

The HPLC-UV and MALDI-TOF/TOF techniques are useful to determine anti-HIV drug concentrations as low as 0.01 pmol/μL and 0.0025 pmol/μL, respectively. Values of the anti-HIV drug concentration determined by HPLC-UV and MALDI-TOF/TOF are in excellent agreement [12-14].

2.1. References

[1] D. Back, G. Gatti, C. Fletcher, R. Garaffo, R. Haubrich, R. Hoetelmans, M. Kurowski, A. Luber, C. Merry, C.F. Perno, Therapeutic drug monitoring in HIV infection: current status and future directions, AIDS 16 (2002) S5-S37.

[2] P. Clevenbergh, R. Garraffo, J. Durant, P. Dellamonica, PharmAdapt: a randomized prospective study to evaluate the benefit of therapeutic monitoring of protease inhibitors: 12 week results, AIDS 16 (2002) 2311-2315.

[3] C.V. Fletcher, P.L.Anderson, T.N. Kakuda, T.W. Schacker, K. Henry, C.R. Gross, R.C. Brundage, Concentration-controlled compared with conventional antiretroviral therapy for HIV infection, AIDS 16 (2002) 551-560.

[4] R.E. Aarnoutse, J.M. Schapiro, C.A. Boucher, Y.A. Hekster, D.M. Burger, Therapeutic drug monitoring: an aid to optimising response to antiretroviral drugs? Drugs 63 (2003) 741-753.

[5] D.M. Burger, P.W. Hugen, R.E. Aarnoutse, R.M. Hoetelmans, M. Jambroes, P.T. Nieuwkerk, G. Schreij, M.M. Schneider, M.E. van der Ende, J.M. Lange, ATHENA Study Group, Treatment

failure of nelfinavir-containing triple therapy can largely be explained by low nelfinavir plasma concentrations, Ther. Drug Monit. 25 (2003) 73-80.
[6] J.G. Gerber, E.P. Acosta, Therapeutic drug monitoring in the treatment of HIV-infection, J. Clin. Virol. 27 (2003) 117-128.
[7] P. Bossi, G. Peytavin, H. Ait-Mohand, C. Delaugerre, N. Ktorza, L. Paris, M. Bonmarchand, R. Cacace, D.J. David, A. Simon, C. Lamotte, A.G. Marcelin, V. Calvez, F. Bricaire, D. Costagliola, C. Katlama, GENOPHAR: a randomized study of plasma drug measurements in association with genotypic resistance testing and expert advice to optimize therapy in patients failing antiretroviral therapy, HIV Med. 5 (2004) 352-359.
[8] J. Moltó, B. Clotet, Therapeutic drug monitoring of antiretroviral agents scenario, J. HIV Ther. 9 (2004) 75-78.
[9] N.Y. Rakhmanina, J.N. van den Anker, S.J. Soldin, Therapeutic drug monitoring of antiretroviral therapy, AIDS Patient Care STDS 18 (2004) 7-14.
[10] S.H. Khoo, J. Lloyd, M. Dalton, A. Bonington, E. Hart, S. Gibbons, P. Flegg, J. Sweeney, E.G. Wilkins, D.J. Back, Pharmacologic optimization of protease inhibitors and non-nucleoside reverse transcriptase inhibitors (POPIN) - A randomized controlled trial of therapeutic drug monitoring and adherence support, J. Acquir. Immune Defic. Syndr. 41 (2006) 461-467.
[11] C. Torti, M.C. Uccelli, E. Quiros-Roldan, F. Gargiulo, V. Tirelli, G. Lapadula, M. Regazzi, P. Pierotti, C. Tinelli, A. De Luca, A. Patroni, N. Manca, G. Carosi, Prediction of early and confirmed virological response by genotypic inhibitory quotients for lopinavir in patients naïve for lopinavir with limited exposure to previous protease inhibitors, J. Clin. Virol. 35 (2006) 414-419.
[12] S. Notari, A. Bocedi, G. Ippolito, P. Narciso, L.P. Pucillo, G. Tossini, R. Perrone Donnorso, F. Gasparrini, P. Ascenzi, Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 831 (2006) 258-266.
[13] S. Notari, C. Mancone, M. Tripodi, P. Narciso, M. Fasano, P. Ascenzi, Determination of anti-HIV drug concentration in human plasma by MALDI-TOF/TOF, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 833 (2006) 109-116.
[14] S. Notari, C. Mancone, T. Alonzi, M. Tripodi, P. Narciso, P. Ascenzi, Determination of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine concentration in human plasma by MALDI-TOF/TOF, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. (2008) in press.

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Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography

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Abstract

Therapeutic drug monitoring (TDM) is pivotal to improve the management of HIV infection. Here, a HPLC–UV method has been developed to quantify simultaneously seven HIV protease inhibitors (amprenavir, atazanavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir; PIs), seven nucleoside reverse transcriptase inhibitors (abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, and zidovudine; NRTIs), and two non-nucleoside reverse transcriptase inhibitors (efavirenz and nevirapine; NNRTIs) in human plasma. The volume of the plasma sample was 600 μL. This method involved automated solid-phase extraction with Oasis HLB Cartridge 1 cc (divinylbenzene and *N*-vinylpyrrolidone) and evaporation in a water bath under nitrogen stream. The extracted samples were reconstituted with 100 μL methanol. Twenty microliters of these samples were injected into a HPLC–UV system, the analytes were eluted on an analytical C_{18} Symmetry™ column (250 mm × 4.6 mm I.D.) with a particle size of 5 μm. The mobile phase (0.01 M KH_2PO_4 and acetonitrile) was delivered at 1.0 mL/min with linear gradient elution. The total run time for a single analysis was 35 min, the anti-HIV drugs were detected by UV at 240 and 260 nm. The calibration curves were linear up to 10 μg/mL. The absolute recovery ranged between 88 and 120%. The in vitro stability of anti-HIV drugs (0.005–10 μg/mL) in plasma has been studied at 24.0 °C. On these bases, a two to four analyte method has been tailored to the individual needs of the HIV-infected patient. The HPLC–UV method here reported has been validated and is currently applied to monitor PIs, NRTIs, and NNRTIs in plasma of HIV-infected patients. It allows to monitor the largest number of anti-HIV drugs simultaneously, appearing useful in a routine laboratory, and represents an essential step to elucidate the utility of a formal therapeutic drug monitoring for the optimal follow-up of HIV-infected patients.

Keywords: HIV protease inhibitors; HIV nucleoside reverse transcriptase inhibitors; HIV non-nucleoside reverse transcriptase inhibitors; HPLC–UV; Therapeutic drug monitoring

1. Introduction

HIV nucleoside reverse transcriptase inhibitors (NRTIs) in combination with protease inhibitors (PIs) and/or with non-nucleoside reverse transcriptase inhibitors (NNRTIs) have transformed the short-term and prognosis of HIV-infected patients (see [1,2]). The aim of therapeutic drug monitoring (TDM) consists in individualizing dosages for maximizing the efficacy of treatment while minimizing its toxicity. The combination of pharmacokinetic–pharmacodynamic relationships for antiretroviral therapy and the presence of a wide interpatient variability in drug exposure supports the application of TDM in HIV-infected patients. Prospective clinical trials assessing the clinical usefulness of this strategy have shown contradictory results, pointing out the need to consider different issues when performing TDM. It may be useful in patient management because it contributes to ensure adequate and efficacy drug levels, avoiding or reducing, in many scenarios, the drug associated adverse effects. Then, TDM may warrant an adjustment of doses and combinations

Abbreviations: HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; SPE, solid-phase extraction; TDM, therapeutic drug monitoring

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to ensure an optimal therapy for HIV infected patients (see [3–8]).

Anti-HIV therapeutic strategy regimens require the administration of several antiretroviral drugs. The increasing number of anti-HIV drugs available rapidly increases the number of different combinations. Some very promising combination regimens contain PIs and NRTIs (see [9–13]). Therefore, an analytical method for anti-HIV drug determination in blood on a routine basis may represent a useful clinical tool, enabling the study of the relationship between plasma levels, metabolic disorders and virological response failure, and treatment fine-tuning. Moreover, it may contribute to ameliorate patient management, in particular in evaluating drug–drug interactions and indicating relationships between drug concentration and associated toxicity (see [14–16]).

Several HPLC–UV methods have been reported to quantify anti-HIV drugs in human biological fluids, e.g. abacavir (see [17]), amprenavir (see [18–20]), atazanavir (see [18,19]), didanosine (see [17]), efavirenz (see [18–21]), emtricitabine (see [22]), indinavir (see [23]), lamivudine (see [17]), lopinavir (see [19,20,24,25]), nelfinavir (see [18–20,24]), nevirapine (see [18–20,24]), ritonavir (see [23]), saquinavir (see [19,20]), stavudine (see [3]), zalcitabine (see [17]), and zidovudine (see [17]). Furthermore, each method (individual or simultaneous) involves a sample preparation procedure: liquid–liquid or solid–liquid extraction or protein precipitation. The application of solid-phase extraction (SPE) of analytes from biological matrix allows either higher recoveries or the elimination of some possible interferences from co-administrated drugs (see [3,17–25]).

Here, we report the setting up and validation of a HPLC–UV method for the simultaneous separation and quantitation of 16 anti-HIV drugs, i.e., abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamivudine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine, in plasma from HIV-infected patients. The determination of 16 anti-HIV drugs by a single method appears useful in a routine laboratory since different drug cocktails are administered to HIV-infected patients.

2. Materials and methods

2.1. Chemicals

Amprenavir (from Vertex/Kissei/Glaxo Wellcome), atazanavir (from Bristol-Meyers Squibb), abacavir (from Glaxo Wellcome), efavirenz (from Dupont Merck), didanosine (from Bristol-Myers Squibb), emtricitabine (from Triangle Pharmaceuticals), indinavir (from Merck), lamivudine (from Iaf Biochem. Int./Glaxo Wellcome), lopinavir (from Abbott), nelfinavir (from Agouron/Japan Tobacco), nevirapine (from Boehringer Ingelheim), ritonavir (from Abbott), saquinavir (from Roche), stavudine (from Bristol-Myers Squibb), zalcitabine (from Hoffman-La Roche), and zidovudine (from Glaxo Wellcome) were obtained through the NIH AIDS Research Reagent Program, Division of AIDS, NIAID, National Institute of Health (Bethesda, MD, USA). All anti-HIV drugs were of analytical grade and used without further purification. Acetonitrile, methanol, and KH_2PO_4 (from Carlo Erba reagenti, Rodano, Milano, Italy) were of HPLC grade. Deionized water (18.2 mΩ, total organic carbon <100 ppb) was produced on-site.

2.2. Chromatographic system

The chromatographic system consisted of a Waters 600 pump and a Waters autosample 717 PLUS equipped with a spectrophotometric UV–vis dual-wavelength system Waters 2487 set at 240 and 260 nm (Milford, MA, USA). Anti-HIV drug separation was performed at 24.0 °C on an analytical C_{18} Symmetry^{TM} column (250 mm × 4.6 mm I.D.) with a particle size of 5.0 μm (Waters) equipped with a Waters Sentry guard column (20 × 3.9 mm I.D.) filled with the same packing material (Waters). The 'Millenium' software (Waters) was used to pilot the HPLC–UV instrument and to process the data (i.e., area integration, calculation, and plotting of chromatograms) throughout the method validation and sample analysis.

2.3. Mobile phase solutions

The mobile phase is composed of solution A (0.01 M KH_2PO_4) and B (acetonitrile). Both solutions were degassed by sparging with helium. The injection volume was 20 μL. The mobile phase was delivered at 1.0 mL/min. The gradient program conditions are reported in Table 1.

2.4. Stock, working, and plasma solutions

Stock solutions of abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine (1.0 mg/mL) were prepared by dissolving 5.0 mg of each anti-HIV drug in 5.0 mL of methanol. Stock solutions were appropriately diluted with methanol for the preparation of working solutions (final concentration ranging between 0.005 and 10 μg/mL). The anti-HIV drug concentration in plasma calibration samples ranged between 0.005 and 10 μg/mL. All working solutions were stored at +4.0 °C and were stable for at least 6 months.

2.5. Sample preparation

According to the protocol approved by the Ethics Committee of the Istituto Nazionale per le Malattie Infettive I.R.C.C.S.

Table 1
Gradient elution program

Time (min)	Flow (mL/min)	Solution A (%)[a]	Solution B (%)[b]	Gradient curve profile[c]	pH
0	1	94	6	–	5.0
10	1	40	60	7	4.5
20	1	40	60	1	4.5
25	1	0	100	1	–
35	1	94	6	1	5.0
40	1	94	6	1	5.0

[a] 0.01 M KH_2PO_4.
[b] Acetonitrile.
[c] For details see [28].

'Lazzaro Spallanzani' (Roma, Italy) and with the written informed consent of the patients, blood samples were drawn from HIV-infected patients. Patients were instructed not to take their morning pills prior to the consultation. The patient selection criteria were pharmacological steady state and efficient response to the therapy.

Blood samples (6.0 mL) were collected in monovetters Li heparinate and centrifuged at 3000 rpm for 20 min at 24.0 °C. Then, human plasma was separated from blood cells and stored at −20.0 °C.

Human plasma samples were cleaned-up by off-line solid-phase extraction using Oasis HLB Cartridge 1 cc (30 mg) (Waters). The SPE cartridges were conditioned with 1.0 mL methanol followed by 1.0 mL water Milli-Q. One hundred microliters of methanol were added to 600 μL of human plasma, the solution was vortexed for 1.0 min and centrifuged at 13,000 rpm for 6.0 min, at 24.0 °C. The supernatant (ca. 650 μL) was diluted by adding water Milli-Q (1.0 mL) and loaded in the cartridge. Then, cartridges were washed with 1.0 mL of 5% (v/v) methanol in water Milli-Q. Analytes were eluted by washing cartridges with 550 μL 0.01 M KH_2PO_4 followed by 2.0 mL absolute methanol. The eluate was evaporated in a water bath at 36.0 °C under a stream of nitrogen. The extracted sample was reconstituted with 100 μL absolute methanol and transferred to an injection vial.

2.6. Calibration curves

The calibration curves were established over the 0.005–10 μg/mL range for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, the 0.025–10 μg/mL range for abacavir, didanosine, indinavir, and zidovudine, and the 0.10–10 μg/mL range for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine. Under all the experimental conditions, the response/amount ratio was linear.

2.7. Recovery

The efficiency of SPE was determined with control samples at 0.625, 5.0, and 10 μg/mL. The absolute recovery of abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine from plasma was obtained as the peak-area response of the processed samples, expressed as the percentage of the response of the anti-HIV drugs contained in the 20-μL injection volume and not subjected to SPE.

2.8. Development of a two to four analyte method

The liquid cromatography resources 'Gradient Scouting Run Evaluation' tool [26] was used to develop a two to four analyte method. The initial and final concentration (%) of the B mobile phase were calculated for each anti-HIV drug cocktail using the following parameters: length and diameter of the analytical C_{18} Symmetry™ column (250 mm × 4.6 mm I.D.), dwell volume (=0.10 mL), flow rate (=1.0 mL/min), initial and final concentration of the B mobile phase (=6 and 100%, respectively), and single run time (=35 min) for the simultaneous determination of 16 anti-HIV drugs, as well as the retention times given in Table 1. Chemicals, chromatographic system, mobile phases, sample preparation, calibration curves, and recovery of the two to four analyte method were identical to those used for the simultaneous determination of 16 anti-HIV drugs.

3. Results

3.1. Chromatograms

The HPLC method here reported provides a simple procedure to determine simultaneously the concentration of abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine in plasma by UV detection at 240 and 260 nm. The gradient program used for anti-HIV drug separation and the retention times of anti-HIV drugs are repoted in Tables 1 and 2, respectively. Data obtained at 240 nm (see Tables 1–7) are superimposable to those obtained at 260 nm (data not shown) within the experimental error.

Fig. 1 shows the chromatogram of a standard mixture of 16 anti-HIV PIs, NNRTIs, and NRTIs (10 μg/mL) (panel A), of a drug-free human plasma sample from a healthy donor (panel B), of a healthy donor plasma sample spiked with 100 μL of 16 anti-HIV drugs (10 μg/mL) (panel C), and of plasma samples from HIV-infected patients (panels D and E).

Table 3 shows the antiretroviral regimens and anti-HIV drugs plasma concentration of HIV-infected patients. Values here reported agree with literature (http://aidsinfo.nih.gov). This method was validated with regard to specificity, selectivity, linearity, limits of detection and quantification, recovery, precision, and accuracy.

3.2. Specificity and selectivity

Blank samples showed no interfering endogenous substances eluting at the retention time of anti-HIV drugs. The selectivity

Table 2
Retention time of anti-HIV drugs

Anti-HIV drug	Retention time (min)
Lamivudine	4.1
Zalcitabine	6.2
Emtricitabine	7.8
Didanosine	8.6
Stavudine	9.7
Abacavir	15.1
Zidovudine	16.2
Nevirapine	16.6
Indinavir	18.1
Saquinavir	19.2
Amprenavir	19.9
Nelfinavir	21.1
Ritonavir	23.1
Lopinavir	24.5
Efavirenz	28.4
Atazanavir	32.0

Table 3
Anti-HIV regimens and anti-HIV drug plasma concentration of HIV-infected patients[a]

Patient	Anti-HIV drug	Dose (mg)	Plasma concentration (μg/mL ± S.D.)
1	Atazanavir	400.0^{qid}	1.8173 ± 0.741
	Lamivudine	300.0^{qid}	0.3817 ± 0.114
	Ritonavir	100.0^{qid}	0.2633 ± 0.244
2	Lamivudine	150.0^{bid}	0.1736 ± 0.006
	Lopinavir	133.3^{bid}	0.5800 ± 0.221
	Ritonavir	33.30^{bid}	1.8543 ± 0.214
	Zidovudine	300.0^{bid}	0.3182 ± 0.013
3	Abacavir	300.0^{bid}	0.2533 ± 0.025
	Lopinavir	133.3^{bid}	1.0533 ± 0.125
	Ritonavir	33.30^{bid}	0.3138 ± 0.202
4	Lamivudine	150.0^{bid}	0.4351 ± 0.025
	Lopinavir	133.3^{bid}	0.1104 ± 0.082
	Ritonavir	33.30^{bid}	0.3138 ± 0.202
	Zidovudine	300.0^{bid}	0.2803 ± 0.010
5	Lamivudine	300.0^{bid}	0.0376 ± 0.019
	Lopinavir	133.3^{bid}	0.0284 ± 0.006
	Ritonavir	33.30^{bid}	0.3249 ± 0.494
6	Atazanavir	400.0^{qid}	1.0775 ± 1.016
	Lamivudine	300.0^{qid}	0.8841 ± 0.111
7	Efavirenz	600.0^{qid}	0.6561 ± 0.372
	Lamivudine	300.0^{qid}	0.6421 ± 0.008
8	Lamivudine	300.0^{qid}	0.0962 ± 0.016
	Lopinavir	133.3^{bid}	0.0383 ± 0.053
	Ritonavir	33.30^{bid}	0.3808 ± 0.375
9	Lamivudine	300.0^{bid}	0.0371 ± 0.040
	Lopinavir	133.3^{bid}	0.0618 ± 0.026
	Ritonavir	100.0^{bid}	0.2835 ± 0.243
10	Atazanavir	400.0^{qid}	0.4090 ± 0.345
	Lamivudine	300.0^{qid}	0.0884 ± 0.026
11	Lamivudine	300.0^{qid}	0.0363 ± 0.016
	Nevirapine	200.0^{bid}	0.0188 ± 0.005
12	Lamivudine	300.0^{qid}	0.1932 ± 0.126
	Ritonavir	100.0^{bid}	0.3132 ± 0.232
13	Abacavir	300.0^{bid}	0.1072 ± 0.076
	Lamivudine	300.0^{qid}	0.3300 ± 0.035
	Lopinavir	133.3^{bid}	0.0339 ± 0.011
	Ritonavir	33.30^{bid}	0.0320 ± 0.005
14	Atazanavir	400.0^{qid}	0.6858 ± 0.208
	Lamivudine	300.0^{qid}	0.5070 ± 0.392
	Ritonavir	100.0^{qid}	0.6971 ± 0.080
15	Atazanavir	400.0^{qid}	0.1583 ± 0.040
	Lamivudine	300.0^{qid}	0.0416 ± 0.031
16	Lopinavir	133.3^{bid}	0.0914 ± 0.125
	Ritonavir	33.30^{bid}	0.1108 ± 0.076
	Stavudine	40.00^{bid}	0.5245 ± 0.193
17	Didanosine	400.0^{bid}	0.6835 ± 0.392
	Lamivudine	300.0^{qid}	0.0692 ± 0.049
	Nelfinavir	250.0^{bid}	0.1021 ± 0.028
18	Lamivudine	150.0^{bid}	0.0165 ± 0.003
	Lopinavir	133.3^{bid}	0.6400 ± 1.031
	Ritonavir	33.30^{bid}	0.0949 ± 0.072
	Zidovudine	300.0^{bid}	0.1912 ± 0.138
19	Efavirenz	600.0^{qid}	1.0646 ± 0.006
	Lamivudine	150.0^{bid}	0.0793 ± 0.103
	Zidovudine	300.0^{bid}	0.3661 ± 0.097
20	Lamivudine	300.0^{qid}	0.0209 ± 0.002
	Lopinavir	133.3^{bid}	0.0424 ± 0.049
	Ritonavir	33.30^{bid}	0.0901 ± 0.062
21	Lamivudine	150.0^{bid}	0.0152 ± 0.003
	Lopinavir	133.3^{bid}	0.0271 ± 0.006
	Ritonavir	33.30^{bid}	0.1912 ± 0.044
	Zidovudine	300.0^{bid}	0.4396 ± 0.014
22	Abacavir	300.0^{bid}	0.0255 ± 0.005
	Lamivudine	300.0^{qid}	0.0131 ± 0.010
	Lopinavir	133.3^{bid}	0.0296 ± 0.001
	Ritonavir	33.30^{bid}	0.2540 ± 0.076
23	Abacavir	300.0^{bid}	0.1628 ± 0.028
	Amprenavir	1400^{bid}	0.7282 ± 0.185
24	Indinavir	800.0^{bid}	1.1987 ± 0.012
	Lamivudine	300.0^{qid}	1.7248 ± 0.603
	Ritonavir	100.0^{bid}	0.3308 ± 0.047
	Stavudine	30.00^{bid}	<LOD
25	Abacavir	300.0^{bid}	0.6104 ± 0.022
	Lamivudine	300.0^{qid}	7.6649 ± 0.087
	Ritonavir	100.0^{bid}	0.1144 ± 0.078
	Saquinavir	400.0^{bid}	1.6421 ± 0.058
26	Emtricitabine	200.0^{qid}	0.4729 ± 0.112
	Ritonavir	100.0^{bid}	0.2880 ± 0.258

^{qid}: once a day. ^{bid}: twice a day.
[a] Data referring to HIV-infected patients 1 and 4 correspond to those reported in panels D and E, respectively, of Fig. 1.

Table 4
Anti-HIV drug calibration curve parameters

Anti-HIV drug	Calibration curve	r^2
Lamivudine[a]	$y = 0.1314x + 0.0117$	0.9814
Zalcitabine[c]	$y = 0.2008x - 0.1034$	0.9738
Emtricitabine[c]	$y = 0.2563x - 0.0688$	0.9971
Didanosine[b]	$y = 0.2963x - 0.0599$	0.9903
Stavudine[c]	$y = 0.2653x - 0.0621$	0.9977
Abacavir[b]	$y = 0.6369x + 0.1339$	0.9919
Zidovudine[b]	$y = 0.0391x + 0.0028$	0.9862
Nevirapine[a]	$y = 0.0231x + 0.0174$	0.9928
Indinavir[b]	$y = 0.5681x + 0.0109$	0.9879
Saquinavir[a]	$y = 0.8278x - 0.0794$	0.9955
Amprenavir[a]	$y = 0.1011x - 0.0162$	0.9808
Nelfinavir[c]	$y = 0.1713x + 0.0195$	0.9994
Ritonavir[a]	$y = 1.2623x - 0.1744$	0.9855
Lopinavir[a]	$y = 1.1766x - 0.033$	0.9977
Efavirenz[c]	$y = 0.4849x - 0.0850$	0.9969
Atazanavir[a]	$y = 0.1242x - 0.0105$	0.9859

[a] The response range was 0.005–10 μg/mL.
[b] The response range was 0.025–10 μg/mL.
[c] The response range was 0.10–10 μg/mL.

Table 5
Recovery for each anti-HIV drug after extraction from human plasma

Anti-HIV drug	Recovery (% ± S.D.)[a]		
	0.625 μg/mL	5.0 μg/mL	10 μg/mL
Lamivudine	100.1 ± 6.4	107.8 ± 6.2	96.1 ± 0.6
Zalcitabine	99.1 ± 4.5	115.1 ± 5.8	104.5 ± 5.3
Emtricitabine	97.8 ± 2.3	113.0 ± 9.3	116.4 ± 5.1
Didanosine	99.8 ± 5.9	115.6 ± 4.8	98.4 ± 3.8
Stavudine	98.2 ± 2.9	105.2 ± 7.7	88.7 ± 3.9
Abacavir	98.1 ± 2.8	101.1 ± 10.0	93.6 ± 6.1
Zidovudine	100.9 ± 7.8	106.2 ± 7.2	93.8 ± 5.0
Nevirapine	98.1 ± 2.8	107.7 ± 6.3	105.4 ± 1.2
Indinavir	99.7 ± 5.6	114.8 ± 6.3	100.8 ± 6.2
Saquinavir	98.0 ± 2.7	107.4 ± 6.4	102.5 ± 6.1
Amprenavir	110.5 ± 8.1	116.3 ± 3.7	114.2 ± 8.4
Nelfinavir	99.8 ± 5.8	118.1 ± 1.7	99.3 ± 6.3
Ritonavir	120.4 ± 8.9	114.8 ± 6.3	104.7 ± 6.1
Lopinavir	117.8 ± 1.9	117.9 ± 1.8	90.5 ± 9.8
Efavirenz	113.9 ± 7.6	113.2 ± 3.4	96.9 ± 7.2
Atazanavir	119.5 ± 2.7	120.0 ± 6.8	91.4 ± 9.4

[a] Results are the mean of six experiments.

was determined by injecting onto the HPLC column all currently prescribed anti-HIV drugs and/or employed in the treatment/prophylaxis of opportunistic infections.

3.3. Linearity

The standard curves for abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine are satisfactorily described by unweighted least-squares linear regression. The response/amount ratio was linear between 0.005 and 10 μg/mL for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, between 0.025 and 10 μg/mL for abacavir, didanosine, indinavir, and zidovudine, and between 0.10 and 10 μg/mL for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine (Table 4). Data obtained dissolving drugs in methanol and plasma are superimposable. The calibration curves for the determination of amprevavir, lopinavir, nelfinavir, and saquinavir concentration are shown in Fig. 2.

3.4. Limits of detection and quantification

The limit of detection (LOD) in plasma of anti-HIV drugs was defined as the concentration that yields a signal-to-noise ratio of 3:1. For the concentration to be accepted as the lowest limit of quantification (LOQ), the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) has to be less than 20% [27]. LOQ values were 0.005 μg/mL for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, 0.025 μg/mL for abacavir, didanosine, indinavir, and zidovudine, and 0.10 μg/mL for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine.

3.5. Recovery

The absolute recovery was calculated by comparing the peak areas obtained from standard working solutions with the peak areas from standard extracts. Recovery experiments were carried out at 0.625, 5.0, and 10 μg/mL anti-HIV drug concentration in plasma samples. Unspiked samples were used as a control. Results are shown in Table 5.

3.6. Precision and accuracy

Intra- and inter-day precision and accuracy were studied at six different concentrations. The precision was calculated as the relative standard deviation (R.S.D.) within a single run (intra-day) and between different assays (inter-day):

$$\text{R.S.D. (\%)} = \left(\frac{\text{S.D.}}{\text{mean}}\right) \times 100$$

where S.D. is the standard deviation. The accuracy was calculated as the percentage of the deviation between the nominal and the found concentration:

$$\text{Accuracy (\%)} = \left(\frac{\text{found} - \text{nominal}}{\text{nominal}}\right) \times 100$$

results are shown in Table 6. For all anti-HIV drugs both precision and accuracy were <20%, according to literature [27].

3.7. The two to four analyte method

The two to four analyte method was tailored to the individual needs of the patient based on that developed for the simultaneous determination of 16 anti-HIV drugs. The gradient program parameters used for the separation of two to four anti-HIV drugs and the retention times of the anti-HIV drugs for infected patients are reported in Table 7. The calibration curves for abacavir, amprenavir, atazanavir, didanosine, efavirenz, emtricitabine, indinavir, lamividine, lopinavir, nelfinavir, nevirapine, ritonavir, saquinavir, stavudine, zalcitabine, and zidovudine are satisfactorily described by unweighted least-squares linear regression, overlapping those reported in Fig. 2 and Table 4. The anti-HIV drug plasma concentration of HIV-infected patients obtained by the two to four analyte method corresponds to that obtained by the simultaneous determination of 16 anti-HIV drugs.

4. Discussion

Here, we report a new fairly simple HPLC–UV method that provides the simultaneous determination of 16 anti-HIV drugs (i.e., PIs, NRTIs, and NNRTIs) in human plasma from HIV-infected patients. Note that no HPLC–UV methods are available for the simultaneous determination of PI, NRTI, and NNRTI plasma levels.

The volume of the plasma sample used here is 600 μL. Based on the different sensitivity of HPLC–UV methods for abacavir [17], amprenavir [18–20], atazanavir [18,19], didanosine [17], efavirenz [18–21], emtricitabine [22], indinavir [23], lamivudine

Table 6
Intra- and inter-day anti-HIV drug determination

Anti-HIV drug	Intra-day[a]				Inter-day[a]			
	Nominal concentration (μg/mL)	Found concentration (μg/mL)	Precision (%)	Accuracy (%)	Nominal concentration (μg/mL)	Found concentration (μg/mL)	Precision (%)	Accuracy (%)
Lamivudine	0.625	0.59 ± 0.04	7.6	5.2	0.625	0.62 ± 0.01	1.7	0.5
	5.0	4.81 ± 0.23	4.8	2.3	5.0	5.01 ± 0.10	2.1	−0.2
	10	8.41 ± 0.51	6.1	15.0	10	9.53 ± 0.39	4.1	4.6
Zalcitabine	0.625	0.58 ± 0.05	8.1	5.6	0.625	0.58 ± 0.02	0.3	7.1
	5.0	4.88 ± 0.22	4.5	2.2	5.0	4.71 ± 0.55	11.8	6.0
	10	9.09 ± 0.93	10.0	9.0	10	9.12 ± 1.02	11.1	8.7
Emtricitabine	0.625	0.57 ± 0.05	9.5	7.7	0.625	0.59 ± 0.02	4.3	4.8
	5.0	4.84 ± 0.31	6.4	3.1	5.0	4.52 ± 0.45	10.2	10.0
	10	9.02 ± 0.82	9.3	9.7	10	9.46 ± 0.49	5.2	5.4
Didanosine	0.625	0.58 ± 0.05	8.5	6.8	0.625	0.58 ± 0.03	6.2	6.4
	5.0	4.90 ± 0.19	4.1	1.9	5.0	5.11 ± 0.10	1.9	−2.1
	10	9.12 ± 0.72	8.1	9.1	10	9.66 ± 0.57	5.9	3.3
Stavudine	0.625	0.57 ± 0.05	9.2	7.5	0.625	0.57 ± 0.06	10.5	8.5
	5.0	4.86 ± 0.27	5.5	2.7	5.0	4.82 ± 0.32	6.6	3.0
	10	8.95 ± 0.55	6.5	10.5	10	9.45 ± 0.95	10.1	5.5
Abacavir	0.625	0.54 ± 0.02	4.6	12.0	0.625	0.56 ± 0.05	9.9	8.8
	5.0	4.83 ± 0.33	6.8	3.2	5.0	4.66 ± 0.30	6.5	6.7
	10	9.88 ± 0.81	8.9	1.2	10	9.4 ± 0.33	3.5	5.8
Zidovudine	0.625	0.55 ± 0.02	3.6	11.4	0.625	0.60 ± 0.02	3.0	3.2
	5.0	4.87 ± 0.25	5.3	2.5	5.0	4.80 ± 0.17	3.6	3.7
	10	8.81 ± 0.61	7.0	12.0	10	9.52 ± 0.45	4.7	4.8
Nevirapine	0.625	0.54 ± 0.02	3.7	12.5	0.625	0.63 ± 0.06	8.8	−2.1
	5.0	4.88 ± 0.23	4.8	2.3	5.0	4.93 ± 0.06	1.3	1.2
	10	9.91 ± 0.66	6.3	0.4	10	8.93 ± 0.12	1.4	10.6
Indinavir	0.625	0.55 ± 0.02	3.3	11.9	0.625	0.58 ± 0.04	7.3	6.4
	5.0	4.83 ± 0.25	5.3	3.3	5.0	4.90 ± 0.19	4.1	1.9
	10	9.89 ± 0.77	7.8	1.0	10	9.53 ± 0.39	4.1	4.6
Saquinavir	0.625	0.57 ± 0.06	10.0	7.7	0.625	0.58 ± 0.03	5.1	7.2
	5.0	4.66 ± 0.29	6.3	6.7	5.0	4.70 ± 0.28	6.0	5.9
	10	9.90 ± 0.71	7.2	0.8	10	8.78 ± 0.21	2.4	12.1
Amprenavir	0.625	0.58 ± 0.05	9.3	6.5	0.625	0.62 ± 0.01	1.7	0.5
	5.0	4.69 ± 0.28	6.0	6.0	5.0	4.90 ± 0.19	4.1	1.9
	10	10.1 ± 0.33	3.7	−0.8	10	9.32 ± 0.74	7.9	6.8
Nelfinavir	0.625	0.58 ± 0.05	9.7	6.7	0.625	0.56 ± 0.01	2.7	9.3
	5.0	4.73 ± 0.29	6.2	5.2	5.0	4.92 ± 0.12	2.4	1.5
	10	10.0 ± 0.52	5.4	−0.2	10	9.60 ± 0.70	7.4	3.9
Ritonavir	0.625	0.62 ± 0.05	9.0	0.2	0.625	0.58 ± 0.02	2.6	7.7
	5.0	4.66 ± 0.29	6.3	6.6	5.0	4.92 ± 0.12	2.4	1.5
	10	9.96 ± 0.62	6.0	0.3	10	9.41 ± 0.60	6.4	5.8
Lopinavir	0.625	0.61 ± 0.04	7.2	2.6	0.625	0.57 ± 0.03	5.2	8.8
	5.0	4.73 ± 0.29	6.2	5.4	5.0	4.99 ± 0.007	0.2	0.1
	10	9.68 ± 0.84	8.8	3.1	10	9.85 ± 0.07	0.7	1.4
Efavirenz	0.625	0.59 ± 0.04	8.3	4.1	0.625	0.57 ± 0.05	9.5	7.7
	5.0	4.70 ± 0.28	6.0	5.9	5.0	4.80 ± 0.10	2.1	4.0
	10	9.83 ± 0.82	9.1	1.6	10	9.91 ± 0.66	6.3	0.5
Atazanavir	0.625	0.61 ± 0.04	7.3	1.8	0.625	0.58 ± 0.05	8.5	6.8
	5.0	4.81 ± 0.40	8.4	3.6	5.0	5.13 ± 0.15	2.9	−2.6
	10	9.42 ± 0.87	9.1	5.7	10	9.88 ± 0.81	8.9	1.2

[a] Results are the mean of six experiments.

Table 7
Elution parameters for the two to four analyte method

Patient[a]	Solution B (%)		Anti-HIV drug	Retention time (min)
	Initial	Final		
1, 14	5	85	Lamivudine	5.1
			Ritonavir	22.7
			Atazanavir	28.2
2, 4, 18, 21	5	64	Lamivudine	2.3
			Zidovudine	4.6
			Ritonavir	5.8
			Lopinavir	7.9
3	34	64	Abacavir	2.8
			Ritonavir	6.9
			Lopinavir	7.3
5, 8, 9, 20	5	64	Lamivudine	2.4
			Ritonavir	5.7
			Lopinavir	7.5
6, 10, 15	5	85	Lamivudine	5.1
			Atazanavir	28.5
7	5	75	Lamivudine	2.3
			Efavirenz	23.0
11	5	43	Lamivudine	4.2
			Nevirapine	8.3
12	5	61	Lamivudine	2.4
			Ritonavir	6.0
13, 22	5	64	Lamivudine	2.4
			Abacavir	3.1
			Ritonavir	5.8
			Lopinavir	7.9
17	5	55	Lamivudine	3.2
			Didanosine	7.2
			Nelfinavir	8.2
23	34	52	Abacavir	2.7
			Amprenavir	3.5
16	20	64	Stavudine	3.0
			Ritonavir	5.9
			Lopinavir	7.7
19	5	75	Lamivudine	2.3
			Zidovudine	9.1
			Efavirenz	23.2
24	5	61	Lamivudine	2.4
			Stavudine	3.1
			Indinavir	4.4
			Ritonavir	5.8
25	5	61	Lamivudine	2.4
			Abacavir	3.2
			Saquinavir	4.5
			Ritonavir	5.8
26	15	61	Emtricitabine	2.9
			Ritonavir	8.6

[a] For details, see Table 3.

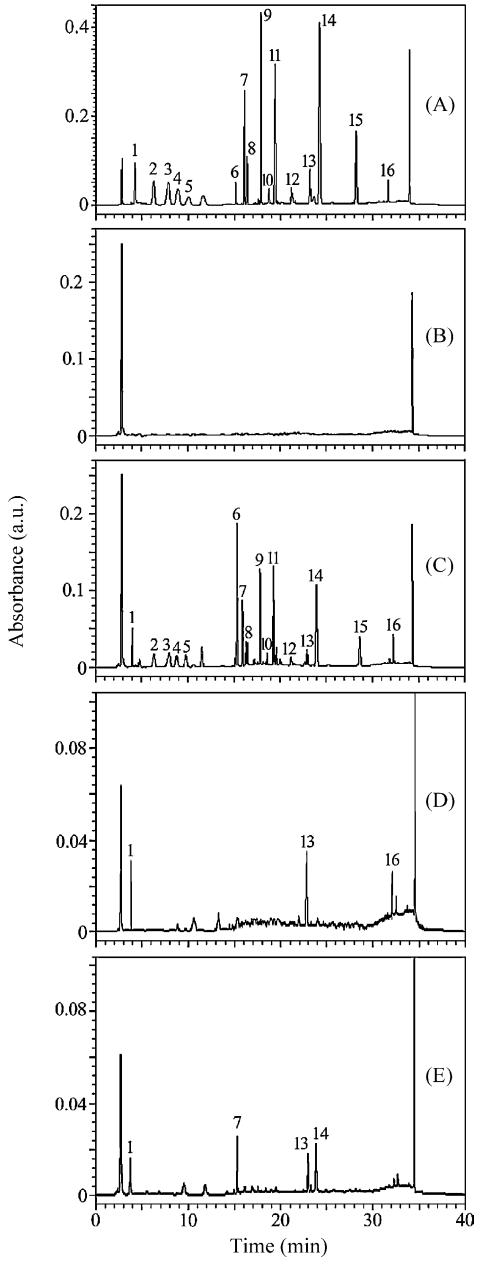

Fig. 1. Simultaneous detection of 16 anti-HIV drugs by HPLC–UV. Chromatogram of a standard mixture of 16 PIs, NNRTIs, and NRTIs (10 μg/mL) (panel A). Chromatogram of a drug-free human plasma sample from a healthy donor (panel B). Chromatogram of a healthy donor plasma sample spiked with 100 μL of 16 anti-HIV drugs (10 μg/mL) (panel C). Chromatogram of plasma samples from HIV-infected patients (panels D and E). Data shown in panels D and E correspond to those of HIV-infected patients 1 and 4, respectively, reported in Table 3. Lamivudine, 1; zalcitabine, 2; emtricitabine, 3; didanosine, 4; stavudine, 5; abacavir, 6; zidovudine, 7; nevirapine, 8; indinavir, 9; saquinavir, 10; amprenavir, 11; nelfinavir, 12; ritonavir, 13; lopinavir, 14; efavirenz, 15; and atazanavir, 16. For details, see text.

[17], lopinavir [19,20,24,25], nelfinavir [18–20,24], nevirapine [18–20,24], ritonavir [23], saquinavir [19,20], stavudine [3], zalcitabine [17], and zidovudine [17] determination, plasma volumes ranged between 500 and 1000 μL. Anti-HIV drug extraction was achieved by divinylbenzene and *N*-vinylpyrrolidone and evaporation in a water bath under nitrogen stream. The extracted samples were reconstituted with methanol and injected into a HPLC–UV system, the analytes were eluted on an analytical C_{18} Symmetry^{TM} column with a particle size of 5 μm. The C_{18} Symmetry^{TM} column gives good separation results (see Fig. 1, panels A and B) and the retention times (see Table 2) of

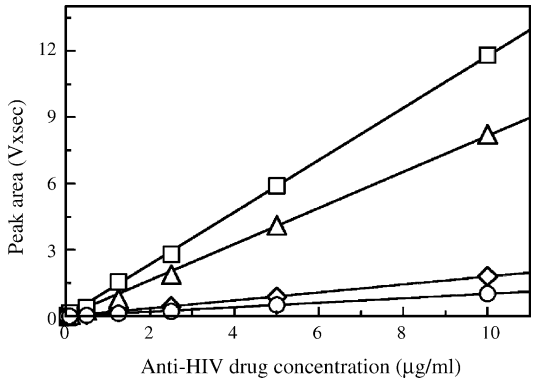

Fig. 2. Calibration curves for the determination of amprevavir (circles), lopinavir (squares), nelfinavir (diamonds), and saquinavir (triangles). The anti-HIV drugs were dissolved in plasma. Calibration curves obtained by dissolving the anti-HIV drugs in methanol are superimposable. The linearity of standard curves was excellent ($r^2 > 0.98$). For details, see text.

anti-HIV drugs are stable for a whole set of analytical runs (Δt_R <0.2 min in a 40-sample run). During the gradient chromatography, pH changes from 4.5 to 5.0 (see Table 1).

LOQ values achieved with this method were 0.005 μg/mL for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, saquinavir, and ritonavir, 0.025 μg/mL for abacavir, didanosine, indinavir, and zidovudine, and 0.10 μg/mL for efavirenz, emtricitabine, nelfinavir, stavudine, and zalcitabine. LOQ values here reported are somewhat lower than those given in the literature for amprenavir (0.025–0.2 μg/mL) [18–20], atazanavir (0.10–0.2 μg/mL) [18,19], lamivudine (0.015 μg/mL) [17], lopinavir (0.025–0.20 μg/mL) [19,20], nevirapine (0.010–0.40 μg/mL) [18–20], ritonavir (0.025–0.10 μg/mL) [19,20], and saquinavir (0.010–0.10 μg/mL) [19,20]. Present LOQ values are similar to those previously reported for abacavir (0.015 μg/mL) [17], didanosine (0.015 μg/mL) [17], and zidovudine (0.015 μg/mL) [17] determination. However, LOQ values obtained from literature for efavirenz (0.010–0.2 μg/mL) [18–20], indinavir (0.010–0.10 μg/mL) [19,20], nelfinavir (0.025–0.2 μg/mL) [18–20], stavudine (0.005 μg/mL) [3], and zalcitabine (0.015 μg/mL) [17] are lower than those here reported. Therefore, this method appears to be more sensitive than those previously reported for amprenavir, atazanavir, lamivudine, lopinavir, nevirapine, ritonavir, and saquinavir quantification.

According to recommendations [27], the linearity of standard curves was excellent ($r^2 > 0.97$) (Fig. 2 and Table 4), the absolute recovery of anti-HIV drugs ranged between 88 and 120% (Table 5), the standard deviation ranged between ±0.6 and ±10% (Table 5), and both precision and accuracy were always <20% (Table 6).

Based on the simultaneous determination of 16 anti-HIV drugs, a two to four analyte method was developed. By the algorithm 'Gradient Scouting Run Evaluation' the initial and final concentration (%) of the B mobile phase was rapidly identified being known the drug cocktail of the HIV-infected patient. Although the time of the single run and the anti-HIV drug retention time of the two to four analyte method are considerably shorter than those reported for the simultaneous determination of 16 anti-HIV drugs, the latter method appears useful in a routine laboratory since different drug cocktails are administered to HIV-infected patients. Moreover, the two to four analyte method needs specific column equilibration for each anti-HIV drug cocktail.

As a whole, the HPLC–UV method here reported is sensitive and specific, allowing the simultaneous determination of the largest number of anti-HIV drugs (i.e., 16 PIs, NRTIs, and NNRTIs). Therefore, it appears very promising to examine several anti-HIV drug combination regimens. This method is used routinely at the Istituto Nazionale per le Malattie Infettive I.R.C.C.S. 'Lazzaro Spallanzani' (Roma, Italy) for TDM in HIV-infected patients.

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References

[1] S. Walmsley, B. Bernstein, M. King, J. Arribas, G. Beall, P. Ruane, M. Johnson, D. Johnson, R. Lalonde, A. Japour, S. Brun, E. Sun, N. Engl. J. Med. 346 (2002) 2039.
[2] F. van Leth, P. Phanuphak, K. Ruxrungtham, E. Baraldi, S. Miller, B. Gazzard, P. Cahn, U.G. Lalloo, I.P. van der Westhuizen, D.R. Malan, M.A. Johnson, B.R. Santos, F. Mulcahy, R. Wood, G.C. Levi, G. Reboredo, K. Squires, I. Cassetti, D. Petit, F. Raffi, C. Katlama, R.L. Murphy, A. Horban, J.P. Dam, E. Hassink, R. van Leeuwen, P. Robinson, F.W. Wit, J.M. Lange, Lancet 363 (2004) 1253.
[3] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Chromatogr. B Biomed. Sci. Appl. 744 (2000) 227.
[4] D. Back, G. Gatti, C. Fletcher, R. Garaffo, R. Haubrich, R. Hoetelmans, M. Kurowski, A. Luber, C. Merry, C.F. Perno, AIDS 16 (2002) S5.
[5] R.E. Aarnoutse, J.M. Schapiro, C.A. Boucher, Y.A. Hekster, D.M. Burger, Drugs 63 (2003) 741.
[6] J.G. Gerber, E.P. Acosta, J. Clin. Virol. 27 (2003) 117.
[7] J. Molto, B. Clotet, J. HIV Ther. 9 (2004) 75.
[8] N.Y. Rakhmanina, J.N. van den Anker, S.J. Soldin, AIDS Patient Care STDS 18 (2004) 7.
[9] J.A. Arnaiz, J. Mallolas, D. Podzamczer, J. Gerstoft, J.D. Lundgren, P. Cahn, G. Fatkenheuer, A. D'Arminio-Monforte, A. Casiro, P. Reiss, D.M. Burger, M. Stek, J.M. Gatell, AIDS 17 (2003) 831.
[10] J.C. Gathe Jr., P. Ive, R. Wood, D. Schurmann, N.C. Bellos, E. DeJesus, A. Gladysz, C. Garris, J. Yeo, AIDS 18 (2004) 1529.
[11] M. Kurowski, T. Sternfeld, A. Sawyer, A. Hill, C. Mocklinghoff, HIV Med. 4 (2003) 94.
[12] J.P. Lalezari, K. Henry, M. O'Hearn, J.S. Montaner, P.J. Piliero, B. Trottier, S. Walmsley, C. Cohen, D.R. Kuritzkes, J.J. Eron Jr., J. Chung, R. DeMasi, L. Donatacci, C. Drobnes, J. Delehanty, M. Salgo, N. Engl. J. Med. 348 (2003) 2175 [Erratum in: N. Engl. J. Med. 349 (2003) 1100].
[13] A. Lazzarin, B. Clotet, D. Cooper, J. Reynes, K. Arasteh, M. Nelson, C. Katlama, H.J. Stellbrink, J.F. Delfraissy, J. Lange, L. Huson, R. DeMasi, C. Wat, J. Delehanty, C. Drobnes, M. Salgo, N. Engl. J. Med. 348 (2003) 2186.
[14] C. Marzolini, A. Telenti, T. Buclin, J. Biollaz, L.A. Decosterd, J. Chromatogr. B Biomed. Sci. Appl. 740 (2000) 43.
[15] E.P. Acosta, J.G. Gerber, AIDS Res. Hum. Retroviruses 18 (2002) 825.
[16] R.P. Van Heeswijk, Ther. Drug Monit. 24 (2002) 323.

[17] C.P. Verweij-van Wissen, R.E. Aarnoutse, D.M. Burger, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 816 (2005) 121.
[18] S. Colombo, N. Guignard, C. Marzolini, A. Telenti, J. Biollaz, L.A. Decosterd, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 810 (2004) 25.
[19] E. Dailly, F. Raffi, P. Jolliet, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 813 (2004) 353.
[20] N.L. Rezk, R.R. Tidwell, A.D. Kashuba, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 805 (2004) 241.
[21] L. Stahle, L. Moberg, J.O. Svensson, A. Sonnerborg, Ther. Drug Monit. 26 (2004) 267.
[22] A. Darque, G. Valette, F. Rousseau, L.H. Wang, J.P. Sommadossi, X.J. Zhou, Antimicrob. Agents Chemother. 43 (1999) 2245.
[23] J.R. King, J.G. Gerber, C.V. Fletcher, L. Bushman, E.P. Acosta, AIDS 19 (2005) 1059.
[24] D.T. Holland, R. DiFrancesco, J. Stone, F. Hamzeh, J.D. Connor, G.D. Morse, Antimicrob. Agents Chemother. 48 (2004) 824.
[25] C. Torti, E. Quiros-Roldan, V. Tirelli, M. Regazzi-Bonora, F. Moretti, P. Pierotti, A. Orani, P. Maggi, A. Cargnel, A. Patroni, A. De Luca, G. Carosi, AIDS Patient Care STDS 18 (2004) 629.
[26] Gradient Scouting Run Evaluation (2005) http://www.lcresources.com.
[27] Guidance for Industry: Q2B Validation of Analytical Procedures, Methodology (1996) http://www.fda.gov/cber/guidelines.htm.
[28] Waters 600E Multisolvent Delivery System, User's Guide (1993), Waters Co., Milford, MA.

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Determination of anti-HIV drug concentration in human plasma by MALDI-TOF/TOF☆

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Abstract

The antiretroviral therapeutic drug monitoring is becoming increased in clinical care to determine the best dosage regimen adapted to each patient. Here, the determination of the anti-HIV drugs lamivudine, lopinavir, and ritonavir concentration in the plasma of HIV-infected patients by MALDI-TOF/TOF is reported. The volume of the plasma sample was 600 μL. Plasma samples were extracted by solid-phase (divinylbenzene and *N*-vinylpyrrolidone) and evaporated in a water bath under a nitrogen stream. The extracted samples were reconstituted with methanol (100 μL), mixed (1:1) with a saturated matrix solution (4-hydroxybenzoic acid in 50% acetonitrile–0.1% trifluoracetic acid), and spotted onto the MALDI-TOF/TOF sample target plate. The lamivudine, lopinavir and ritonavir concentration was determined by standard additions analysis. Regression of standard additions was linear over the anti-HIV drug concentration ranges explored (lamivudine, 0.010–1.0 pmol/μL; lopinavir and ritonavir, 0.0025–0.50 pmol/μL). Moreover, emtricitabine (i.e., the fluorinated analog of lamivudine) was used as the internal standard to determine the lamivudine concentration. The calibration curve was linear on the emtricitabine concentration ranging between 0.050 and 5.0 pmol/μL. The absolute recovery ranged between 80 and 110%. Values of the lamivudine, lopinavir and ritonavir concentration determined by MALDI-TOF/TOF are in excellent agreement with those obtained by HPLC-UV and HPLC-MS/MS. MALDI-TOF/TOF experiments allowed also the detection of the ritonavir metabolite R5. Zidovudine was undetectable by MALDI-TOF/TOF analysis because also the minimal laser intensity may induce the anti-HIV drug photolysis. The MALDI-TOF/TOF technique is useful to determine very low concentrations of anti-HIV drugs (0.0025–0.010 pmol/μL).

Keywords: Anti-HIV drug determination; Human plasma; MALDI-TOF/TOF

1. Introduction

In HIV-infected individuals, the primary target of therapy is the human immunodeficiency virus (HIV), but most of the clinical manifestations are related to the effect of HIV on the immune system, which leads to progressive immunodeficiency. Recently, the introduction of highly effective combination regimens of antiretroviral drugs has led to substantial improvements in morbidity and mortality. The anti-HIV drugs include three different classes among nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and protease inhibitors. Nucleoside reverse transcriptase inhibitors are intra-cellularly phosphorylated to their corresponding triphosphorylated derivatives, which compete with

Abbreviations: CHCA, α-cyano-4-hydroxycinnamic acid; HBA, 4-hydroxybenzoic acid; HIV, human immunodeficiency virus; LOD, limit of detection; LOQ, limit of quantification; MALDI-TOF/TOF, matrix-assisted laser desorption ionization source and tandem-of-flight; R.S.D., relative standard deviation; S.D., standard deviation; SPE, solid-phase extraction; TDM, therapeutic drug monitoring

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the corresponding natural nucleotide for binding to HIV reverse transcriptase and inhibited it. Non-nucleoside reverse transcriptase inhibitors act as non-competitive inhibitors of the HIV reverse transcriptase. Protease inhibitors interfere with viral replication by inhibiting the viral protease, preventing maturation of the HIV virus and causing the formation of non-infection virions [1–6].

Therapeutic drug monitoring (TDM) consists of the individualizing dosages with the aim maximizing the efficacy of treatment while minimizing its toxicity. The combination of pharmacokinetic–pharmacodynamic relationships for antiretroviral therapy and the presence of a wide interpatient variability in drug exposure support the application of TDM in HIV-infected individuals. Prospective clinical trials assessing the clinical usefulness of this strategy have shown contradictory results, pointing out the need to consider different issues when performing TDM [7–11].

Several methods based on HPLC-UV and HPLC-MS/MS for quantitation of anti-retroviral drugs in human plasma have been developed [12–29]. Here, the determination of the anti-HIV drugs lamivudine, lopinavir, and ritonavir concentration in human plasma by MALDI-TOF/TOF is reported. Values of the anti-HIV drug concentration determined by MALDI-TOF/TOF are in excellent agreement with those obtained by HPLC-UV and HPLC-MS/MS. MALDI-TOF/TOF experiments allowed also the detection of the ritonavir metabolite R5. Zidovudine was undetectable by MALDI-TOF/TOF analysis because also the minimal laser intensity may induce the anti-HIV drug photolysis. The minimal anti-HIV drug concentration detectable by MALDI-TOF/TOF is 0.0025–0.010 pmol/μL.

2. Materials and methods

2.1. Chemicals

Lamivudine (from Iaf Biochem. Int./Glaxo Wellcome, London, UK), emtricitabine (from Triangle Pharmaceuticals, Durham, NC, USA), lopinavir (from Abbott, Abbott Park, IL, USA), ritonavir (from Abbott, Abbott Park, IL, USA), and zidovudine (from Glaxo Wellcome, London, UK) (Fig. 1) were obtained through the NIH AIDS Research Reagent Program, Division of AIDS, NIAID, National Institute of Health (Bethesda, MD, USA). α-Cyano-4-hydroxycinnamic acid (CHCA), 4-hydroxybenzoic acid (HBA), and trifluoracetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the other products were from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without purification.

2.2. MALDI-TOF/TOF system

Mass spectra were obtained between 50 and 1000 Da with 5100 laser shots intensity (Nd:YAG laser at 355 nm, 50 Shots/Sub-Spectrum for 2000 Total Shots/Spectrum) by reflectron positive mode on an Applied Biosystems 4700 Proteomics Analyzer mass spectrometer. For each sample, a data dependent acquisition method was created to select intense peaks, excluding those from the matrix. MS/MS spectra were acquired in positive mode with 6300 laser shots (Nd:YAG laser at 355 nm, 50 Shots/Sub-Spectrum for 2000 Total Shots/Spectrum) using atmospheric gas as the collision gas. Mass calibration of MALDI-TOF/TOF by using a standard mixture of peptides (Applied Biosystems Mass Standard Kit) in the mass range 900–3600 Da (see Table 1) allows the optimization of mass assignment, calibration, resolution, and sensitivity. Spectra were processed and analyzed by the GPS Explorer™ Software v.2.0 (Applied Biosystems, Foster City, CA, USA).

Table 1
MALDI-TOF/TOF calibration standards

Standard	Monoisotopic (Da)	Concentration (pmol/μL)
Des-Arg-Bradykinin	904.4681	1.0
Angiotensin I	1296.6853	2.0
Glu-Fibrinopeptide B	1570.6774	1.3
ACTH (clip 1–17)	2093.0867	2.0
ACTH (clip 18–39)	2465.1989	1.5
ACTH (clip 7–38)	3657.9294	3.0

2.3. Stock, working, and plasma solution

Stock solutions of emtricitabine, lamividine, lopinavir, ritonavir, and zidovudine were prepared by dissolving 5.0 mg of anti-HIV drug in 5.0 mL of methanol. Stock solutions were diluted with methanol to a final concentration ranging between 0.0050 and 10.0 pmol/μL. CHCA and HBA saturated solutions (≥2.0 g/L) were prepared by dissolving both matrices in 50% acetonitrile–0.1% trifluoracetic acid.

2.4. Sample preparation

According to protocol previously approved by the Ethics Committee of the Istituto Nazionale per le Malattie Infettive I.R.C.C.S. 'Lazzaro Spallanzani' (Roma, Italy) and with the written informed consent of the patients, blood samples were taken from HIV-infected patients. Patients were instructed not to take their morning pills prior to the consultation.

Blood samples (6.0 mL) were collected in monovetters Li heparinate and centrifuged at 3000 rpm for 20 min at room temperature. Then, human plasma was separated from blood cells and stored at −20.0 °C. Human plasma samples were cleaned-up by off-line solid-phase extraction (SPE) using Oasis HLB Cartridge 1cc (30 mg; divinylbenzene and *N*-vinylpyrrolidone) (Waters, Milford, MA, USA). The SPE cartridges were conditioned with 1.0 mL methanol followed by 1.0 mL Milli-Q water (Millipore, Bedford, MA, USA). Hundred microlitres of methanol were added to 600 μL of human plasma, the solution was vortexed for 1 min and centrifuged at 13,000 rpm for 6 min. The supernatant (ca. 650 μL) was diluted by adding Milli-Q water (1.0 mL) and loaded onto the cartridge. Then, cartridges were washed with 1.0 mL of 5% (v/v) methanol in Milli-Q water. Analytes were eluted by washing cartridges with 2.0 mL of absolute methanol. The eluate was evaporated in a water bath at

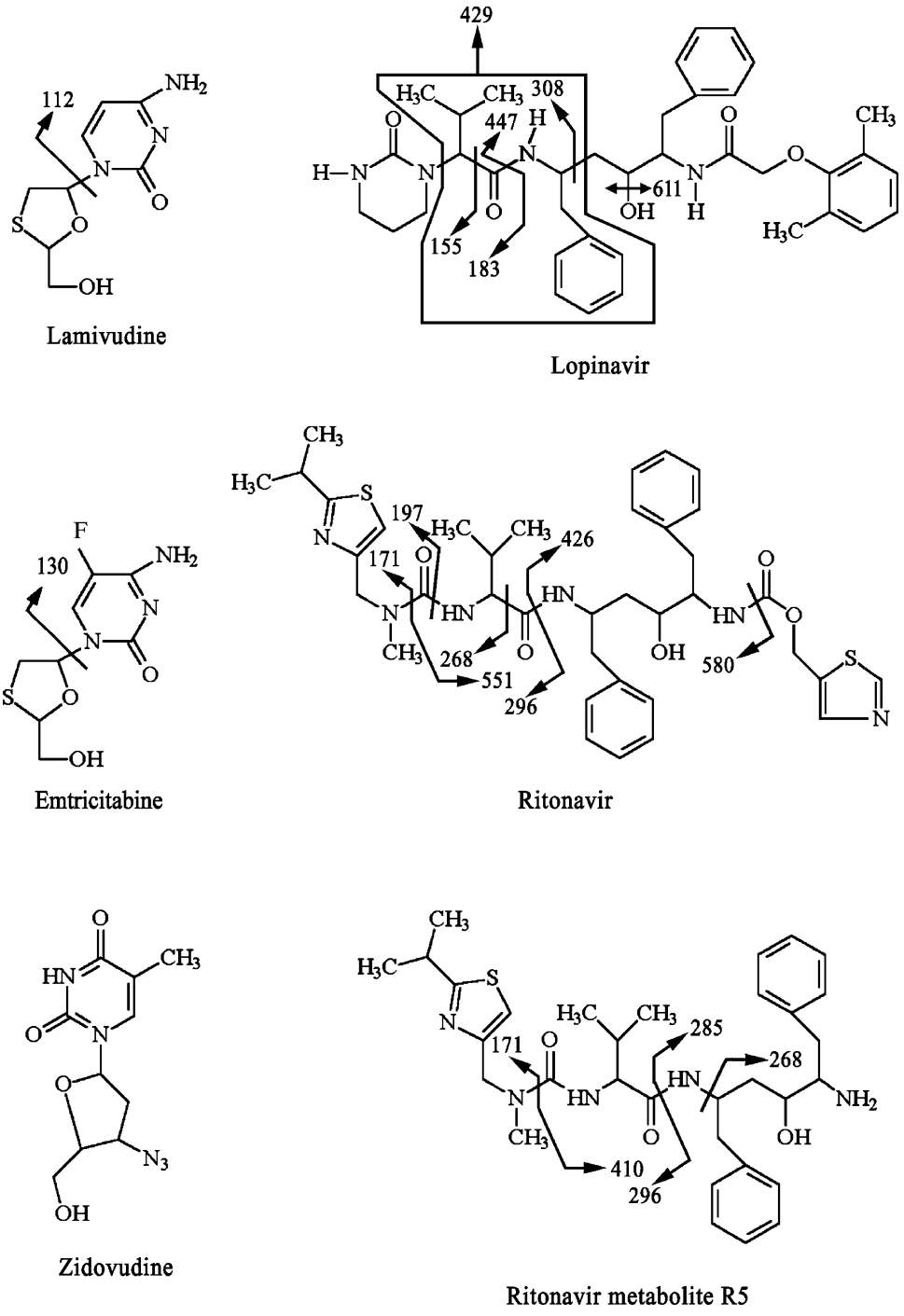

Fig. 1. Chemical structures of emtricitabine, lamivudine, lopinavir, ritonavir, ritonavir metabolite R5, and zidovudine. Fragment ions observed under MS/MS conditions are shown. For details, see text, Figs. 2 and 3 as well as Table 2.

36.0 °C under a stream of nitrogen. The extracted sample was reconstituted with 100 μL absolute methanol and spiked with known anti-HIV drug concentration (standard additions analysis) [30]. The lamivudine concentration ranged between 0.020 and 2.0 pmol/μL, and lopinavir and ritonavir concentration ranged between 0.0050 and 1.0 pmol/μL. 1.0 μL of each solution was mixed with matrix CHCA or HBA (1.0 μL). Furthermore, the sample was spotted onto the sample target plate of the MALDI (matrix-assisted laser desorption/ionization) tandem time-of-flight (TOF/TOF) 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) (MALDI-TOF/TOF).

2.5. Calibration curve to determine the lamivudine concentration using emtricitabine as the internal standard

The calibration curve to determine lamivudine concentration using its fluorinated analog emtricitabine as the internal standard was established over the 0.050–5.0 pmol/μL range. As

emtricitabine differs from lamivudine only for a fluorine atom instead of a hydrogen, emtricitabine has a molecular mass of 18 Da higher than lamivudine and shares with lamivudine the MS/MS fragmentation pattern. Under all the experimental conditions, the response/amount ratio was linear.

2.6. Recovery

The absolute recovery of emtricitabine, lamivudine, lopinavir, and ritonavir was calculated by comparing the peak intensity obtained from standard working solutions with the peak intensity from standard extract. Recovery experiments were carried out at the 0.10, 0.25, and 0.50 pmol/μL spiked levels in plasma sample. Unspiked samples were used as a control.

2.7. Determination of the lamivudine, lopinavir, and ritonavir concentration by HPLC-UV and HPLC-MS/MS

The chromatographic system for HPLC-UV consisted of a Waters 600 pump and a Waters autosampler 717 PLUS equipped with a spectrophotometric UV–vis dual-wavelength detector Waters 2487 set at 240 and 260 nm (Milford, MA, USA). Anti-HIV drug separation was performed at 24.0 °C on an analytical C_{18} Symmetry^{TM} column (250 mm × 4.6 mm i.d.) with a particle size of 5.0 μm (Waters) equipped with a Waters Sentry guard column (20 mm × 3.9 mm i.d.) filled with the same packing material (Waters). The Millenium software (Waters) was used to pilot the HPLC-UV instrument and to process the data (i.e., area integration, calculation and plotting of chromatograms) throughout the method validation and sample analysis. The mobile phases were 0.010 M KH_2PO_4 (solution A) and acetonitrile (solution B). The injection volume was 20.0 μL. The mobile phase was delivered at 1.0 mL/min. Gradient elution was performed by linearly increasing the percentage of acetonitrile from 6 to 100% in 35 min. The retention time for lamivudine, ritonavir, and lopinavir was 4.1, 23.1, and 24.5 min, respectively [29].

The chromatographic apparatus for HPLC-MS/MS was a Series 200 micro LC Pump (Perkin Elmer, Norwalk, CT, USA) equipped with a Series 200 Autosampler and a Series 200 Vacuum Degasser. Analytes were chromatographed on an Vydac column (250 mm × 1 mm i.d.) filled with 3.0 μm C_{18} (Lab Service Analytica, Bologna, Italy). The mobile phases were acetonitrile (solution A) and Milli-Q water (solution B), both phases contained 0.2% formic acid. The injection volume was 5.0 μL. The mobile phase was delivered at 70 μL/min. Gradient elution was performed by linearly increasing the percentage of acetonitrile from 5 to 100% in 16 min. The retention time for lamivudine, ritonavir, and lopinavir was 2.01, 14.47, and 14.99 min, respectively. A QSTAR Pulsar Hybrid Tandem-MS system (PE-Sciex, Concord, Canada) consisting of a quadrupole mass analyzer followed by a modified quadrupole as a collision cell and a reflectron TOF unit as a second mass analyzer, equipped with a TurboIon-Spray source operating in ion positive mode, was used. The mass spectrometry data handling system used was the AnalystQS software from PE-Sciex.

3. Results and discussion

Human plasma proteins were precipitated by addition of absolute methanol to the sample and removed by centrifugation. Then, samples were cleaned-up by SPE, a reliable way of eliminating interfering species. The recovery of the anti-HIV drugs lamivudine, lopinavir, and ritonavir ranged between 80 and 110% (data not shown).

Anti-HIV drugs analysis by MALDI-TOF/TOF was achieved accounting for both CHCA and HBA matrices. In the present study, HBA was used preferentially because it undergoes less fragmentation than CHCA under laser shot (data not shown).

For anti-HIV drugs, the precursor ions $[M+H]^+$ resulted from the addition of a proton to form the positively charged molecular ion. Fig. 2 shows the MALDI-TOF/TOF mass spectra of human plasma of a HIV-infected patient under therapeutical treatment with lamivudine (300 mg/day), lopinavir (800 mg/day), ritonavir (200 mg/day), and zidovudine (300 mg/day), in the absence (panel A) and presence (panel B) of the internal standard emtricitabine. The full scan mass spectral analyses showed protonated molecular ions of 230 *m/z*, 248 *m/z*, 629 *m/z*, and 721 *m/z*, corresponding to lamivudine,

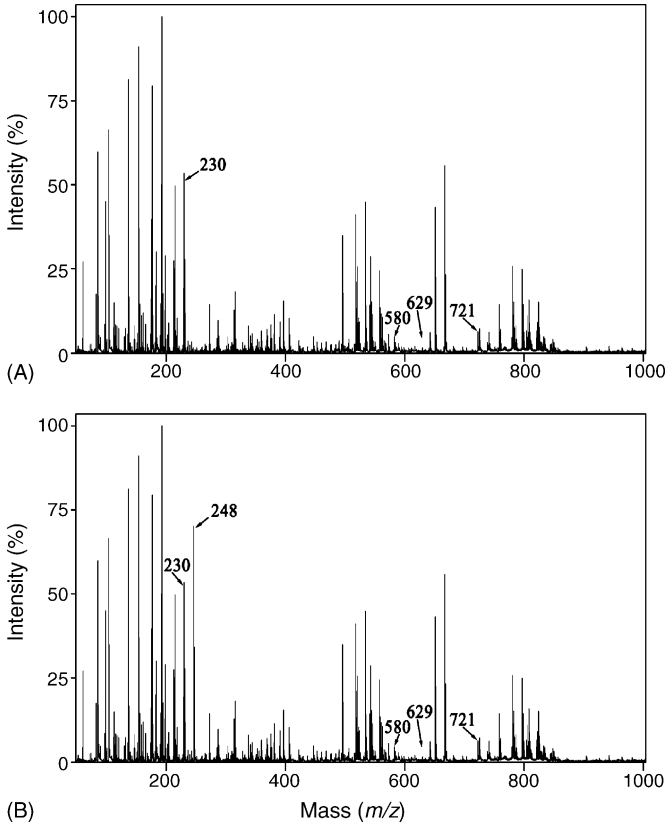

Fig. 2. MALDI-TOF/TOF MS spectrum of the plasma of a HIV-infected patient under therapeutical treatment with lamivudine, lopinavir, ritonavir, and zidovudine in the absence (panel A) and presence (panel B) of the internal standard emtricitabine. Zidovudine was undetectable. The full scan mass spectral analyses showed protonated molecule ions of 230 *m/z*, 248 *m/z*, 580 *m/z*, 629 *m/z*, and 721 *m/z*, corresponding to lamividine, emtricitabine, the ritonavir metabolite R5, lopinavir, and ritonavir, respectively. For details, see text, Figs. 1 and 3 as well as Table 2.

Table 2
Analyte fingerprints[a]

Analytes	Parent peak (*m/z*)	MS/MS peak (*m/z*)												
Lamivudine	230	112												
	230[b]	112[b]	30[b]	15[b]										
Emtricitabine	248	130												
Lopinavir	629	611	447	429	308	183	155							
	629[c]		447[c]		310[c]	183[c]	155[c]	120[c]						
Ritonavir	721	580	551		426		296		268	197	171			
	721[d]		551[d]	525[d]	426[d]		296[d]		268[d]	197[d]	171[d]	140[d]	98[d]	43[d]
Ritonavir metabolite R5	580					410	296	285	268		171			
	580[d]						296[d]	285[d]	268[d]	197[d]	171[d]	140[d]		

[a] For details, see Fig. 1 and text.
[b] Data obtained by a ESI micromass quattro II triple quadrupole mass spectrometer. From [19].
[c] Data obtained by a API 3000 tandem mass spectrometer. From [14].
[d] Data obtained by a TSQ 700 triple quadrupole or LCQ ion trap mass spectrometer. From [20].

emtricitabine, lopinavir, and ritonavir, respectively. The protonated molecular ion of 580 *m/z* corresponds to the ritonavir metabolite R5. Values of parent peaks and of protonated fragment ions of lamivudine, emtricitabine, lopinavir, and ritonavir as well as of the ritonavir metabolite R5 are given in Table 2.

The MALDI-TOF/TOF mass spectrum obtained in the positive reflectron mode is poorly resolved (Fig. 2), whereas the MS/MS mode allowed to detect unequivocally lamivudine, emtricitabine, lopinavir, and ritonavir as well as the ritonavir metabolite R5 (Fig. 3). The fingerprints of lamivudine, emtricitabine, lopinavir, and ritonavir as well as of the ritonavir metabolite R5 are reported in Table 2, the fragmentation patterns are shown in Fig. 1. The fragmentation patterns of lamivudine, emtricitabine, lopinavir, and ritonavir as well as of the ritonavir metabolite R5 determined by MALDI-TOF/TOF from human plasma of HIV-infected patients are in excellent agreement with those obtained from human plasma of a healthy donor spiked with anti-HIV drugs (data not shown), and/or with those reported from literature [14,19,20] (Table 2). The ritonavir metabolite R5 is naturally-occurring, it is absent in the plasma of a healthy donor spiked with ritonavir and examined after long incubation time (>1 week) (data not shown).

Zidovudine was undetectable by MALDI-TOF/TOF analyses. This anti-HIV drug may undergo photolysis also at the minimal laser intensity used (i.e., 5100 laser shots), this may reflect the photosensitivity of the highly reactive azide group.

In the absence of any available deuterated analog of the anti-HIV drugs being considered, the actual concentration in serum samples was performed by the standard additions method [30]. Moreover, concentration of lamivudine was determined by using its fluorinated analog emtricitabine as the internal standard.

The curves of standard additions analysis for lamividine, lopinavir, and ritonavir (Fig. 4) are satisfactorily described by unweighted least-squares linear regression. The response was linear between 0.010 and 1.0 pmol/μL for lamivudine, and between 0.0025 and 0.50 pmol/μL for lopinavir and ritonavir (Table 3).

The calibration curve for the determination of lamivudine concentration based on emtricitabine as the internal standard is shown in Fig. 5. The calibration curve is satisfactorily described by unweighted least-squares linear regression. The response was linear between 0.050 and 5.0 pmol/μL (Table 3).

Table 3
Anti-HIV drug parameters

Anti-HIV drug	Regression analysis	r^2
Lamivudine[a]	$y = 1.6466x + 0.2635$	0.9987
Lopinavir[b]	$y = 12.081x + 1.8740$	0.9902
Ritonavir[b]	$y = 4.1696x + 0.3969$	0.9890
Emtricitabine[c]	$y = 0.6143x + 0.00145$	0.9834

[a] The response range was 0.010–1.0 pmol/μL (see Fig. 3).
[b] The response range was 0.0025–0.50 pmol/μL (see Fig. 3).
[c] The response range was 0.050–5.0 pmol/μL (see Fig. 4).

Values of lamivudine, lopinavir, and ritonavir concentration determined by MALDI-TOF/TOF, HPLC-UV, and HPLC-MS/MS are in excellent agreement (Table 4). The lamivudine concentration obtained by standard additions analysis is identical to that determined by the calibration curve based on the internal standard emtricitabine (Table 4).

The limit of detection (LOD) in plasma of anti-HIV drugs was defined as the concentration that yields a signal/noise ratio of 3:1 [31]. For the concentration to be accepted as the lowest

Table 4
Values of lamivudine, lopinavir, and ritonavir concentration in the plasma of the HIV-infected patient determined by MALDI-TOF/TOF, HPLC-UV, and HPLC-MS/MS

Anti-HIV drug	Concentration (pmol/μL)		
	MALDI-TOF/TOF	HPLC-UV	HPLC-MS/MS
Lamivudine	0.15 ± 0.02[a] 0.15 ± 0.02[b]	0.16 ± 0.02	0.17 ± 0.02
Lopinavir	0.16 ± 0.02[a]	0.15 ± 0.02	0.15 ± 0.01
Ritonavir	0.083 ± 0.008[a]	0.073 ± 0.010	0.086 ± 0.008

[a] The lamivudine, lopinavir, and ritonavir concentration was obtained by standard additions analysis.
[b] The lamivudine concentration was determined by using the fluorinated analog emtricitabine as the internal standard.

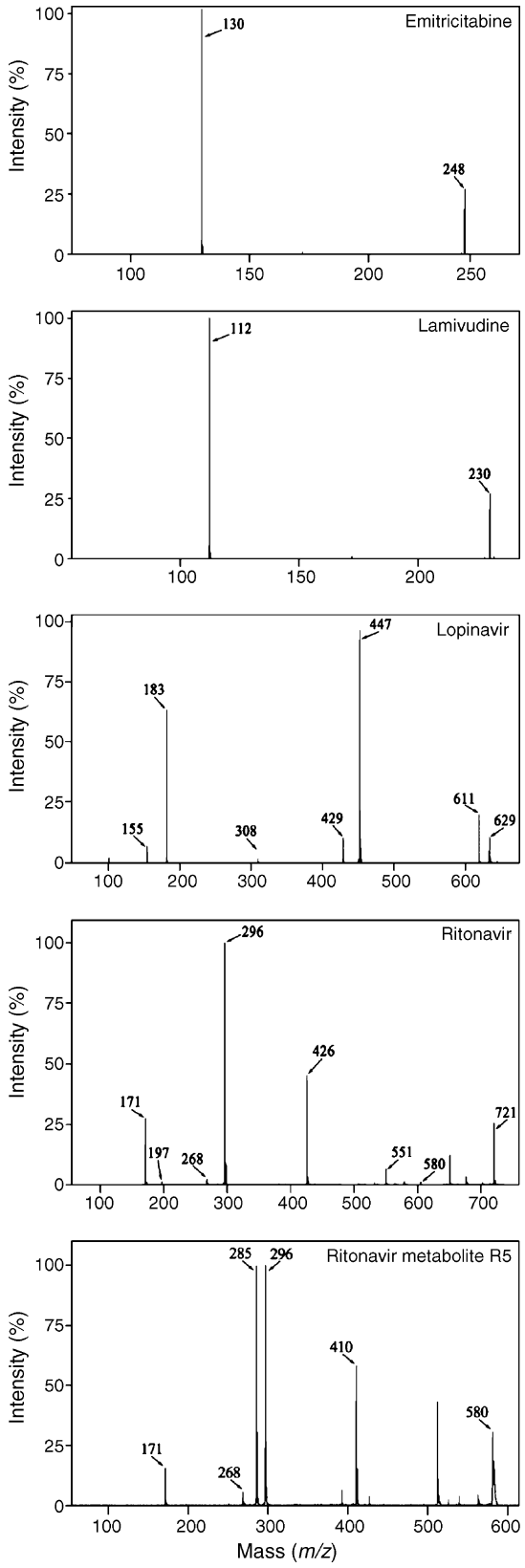

Fig. 3. MALDI-TOF/TOF MS/MS spectra of emtricitabine, lamivudine, lopinavir, ritonavir, and ritonavir metabolite R5 from the plasma of a HIV-infected patient spiked with emtricitabine. For details, see text, Figs. 1 and 2 as well as Table 2.

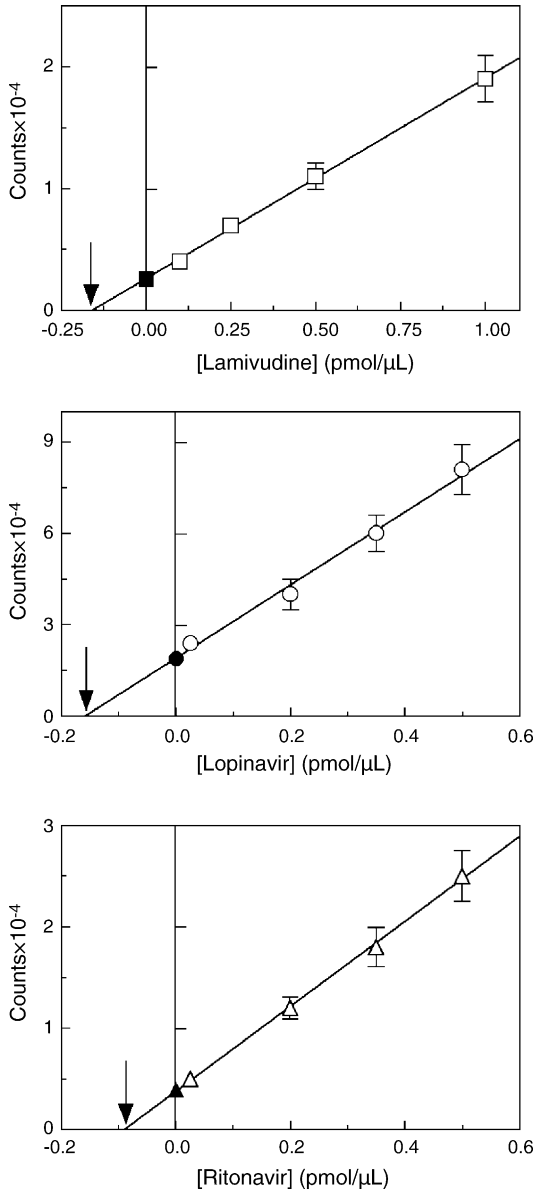

Fig. 4. Linear regression of standard additions of lamivudine, lopinavir, and ritonavir. Filled symbols on the *y*-axis indicate counts corresponding to the unknown anti-HIV drug concentration present in the plasma of the HIV-infected patient. The *x*-axis intercept of the regression straight line (arrow) indicates the anti-HIV drug concentration. The linearity of regression was excellent ($r^2 > 0.98$). Data were analyzed according to [30]. Averages and error bars were obtained from at least four repeats. Where error bars are not seen, they are smaller than the data point symbols. For details, see text and Table 3.

limit of quantification (LOQ), the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) have to be less than 20% [31]. LOQ values were 0.010 pmol/μL for lamivudine, and 0.0025 pmol/μL for lopinavir and ritonavir.

Intra-day and inter-day precision and accuracy were studied at different anti-HIV drug concentrations. The precision was calculated as the relative standard deviation (R.S.D.) within a single run (intra-day) and between different assays (inter-day):

$$\text{R.S.D.}\,(\%) = \left(\frac{\text{S.D.}}{\text{mean}}\right) \times 100$$

Table 5
Intra-day and inter-day anti-HIV drug determination

Anti-HIV drug	Intra-day[a]				Inter-day[a]			
	Nominal concentration (pmol/μL)	Found concentration (pmol/μL)	Precision (%)	Accuracy (%)	Nominal concentration (pmol/μL)	Found concentration (pmol/μL)	Precision (%)	Accuracy (%)
Lamivudine	0.25	0.24 ± 0.07	2.8	2.9	0.25	0.23 ± 0.01	7.2	4.0
	0.50	0.47 ± 0.01	3.2	4.6	0.50	0.48 ± 0.02	4.1	4.0
	1.0	0.89 ± 0.02	1.7	10.0	1.0	0.96 ± 0.10	11.0	4.3
Lopinavir	0.025	0.023 ± 0.001	2.6	5.6	0.025	0.024 ± 0.001	4.4	4.3
	0.35	0.33 ± 0.04	1.4	3.5	0.35	0.34 ± 0.05	1.7	2.0
	0.50	0.48 ± 0.01	1.2	3.4	0.50	0.49 ± 0.01	2.0	2.0
Ritonavir	0.025	0.024 ± 0.002	6.5	3.0	0.025	0.024 ± 0.001	6.5	3.0
	0.35	0.35 ± 0.01	4.0	1.0	0.35	0.33 ± 0.01	3.4	3.8
	0.50	0.47 ± 0.02	4.0	4.2	0.50	0.47 ± 0.02	5.4	5.5

[a] Results are the mean of four experiments.

where S.D. is the standard deviation. The accuracy was calculated as the percentage of the deviation between the nominal and the found anti-HIV drug concentration:

$$\text{Accuracy}\,(\%) = \left\{\frac{[\text{found}] - [\text{nominal}]}{[\text{nominal}]}\right\} \times 100$$

Results are shown in Table 5. According to literature [31], for all anti-HIV drugs both precision and accuracy were <20%.

The minimal anti-HIV drug concentration detectable by MALDI-TOF/TOF ranges between 0.0025 and 0.010 pmol/μL. The minimal drug concentration detectable by HPLC-UV and HPLC-MS/MS is about 0.010 pmol/μL [12,13,15,17,22,24,25,29] and 0.0010 pmol/μL [14,16,18–21,23,26–28], respectively. The minimal anti-HIV drug concentration detectable by a prototype MALDI-triple quadrupole instrument equipped with a high repetition rate laser is about 0.0010 pmol/μL [32].

As a whole, MALDI-TOF/TOF spectrometry allows the determination of the anti-HIV drugs lamivudine, lopinavir, and ritonavir concentration. Anti-HIV drug metabolites can be also detected as reported for the ritonavir metabolite R5. However, the laser shot may photolyze analytes as observed for zidovudine which is undetectable. MALDI-TOF/TOF spectrometry is a rapid and efficacious technology to detect drug(s) in biological samples (e.g., human plasma), both in terms of volume of human plasma required and number of specimens being loaded on the target plate simultaneously. Moreover, the time of the analysis in much faster than that of chromatographic methods, representing therefore a valuable contribution to TDM.

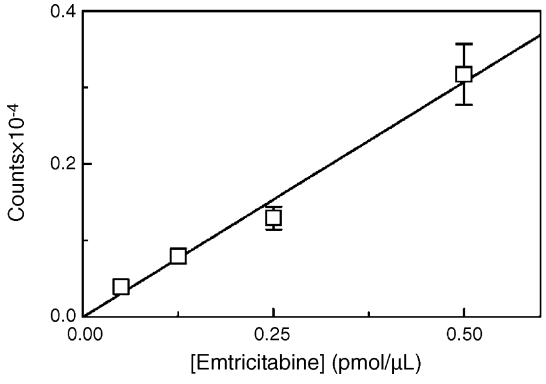

Fig. 5. Calibration curve for the determination of lamivudine concentration based on the fluorinated analog emtricitabine as the internal standard. Averages and error bars were obtained from at least four repeats. Where error bars are not seen, they are smaller than the data point symbols. For details, see text and Table 3.

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References

[1] J. Darbyshire, Trop. Med. Int. Health 5 (2000) A26.
[2] R.P. Van Heeswijk, Ther. Drug Monit. 24 (2002) 323.
[3] P. Clevenbergh, S. Mouly, P. Sellier, E. Badsi, J. Cervoni, V. Vincent, H. Trout, J.F. Bergmann, Curr. HIV Res. 2 (2004) 309.
[4] A. Rouzes, K. Berthoin, F. Xuereb, S. Djabarouti, I. Pellegrin, J.L. Pellegrin, A.C. Coupet, S. Augagneur, H. Budzinski, M.C. Saux, D. Breilh, J. Cromatogr. B 813 (2004) 209.
[5] M. Boffito, E. Acosta, D. Burger, C.V. Fletcher, C. Flexner, R. Garaffo, G. Gatti, M. Kurowski, C.F. Perno, G. Peytavin, M. Regazzi, D. Back, Antivir. Ther. 10 (2005) 375.
[6] S.F. Forsyth, P.D. French, E. Macfarlane, S.E. Gibbons, R.F. Miller, Int. J. STD AIDS 16 (2005) 139.
[7] D. Back, G. Gatti, C. Fletcher, R. Garaffo, R. Haubrich, R. Hoetelmans, M. Kurowski, A. Luber, C. Merry, C.F. Perno, AIDS 16 (2002) S5.
[8] R.E. Aarnoutse, J.M. Schapiro, C.A. Boucher, Y.A. Hekster, D.M. Burger, Drugs 63 (2003) 741.
[9] J.G. Gerber, E.P. Acosta, J. Clin. Virol. 27 (2003) 117.
[10] J. Molto, B. Clotet, J. HIV Ther. 9 (2004) 75.
[11] N.Y. Rakhmanina, J.N. van den Anker, S.J. Soldin, AIDS Patient Care STDS 18 (2004) 7.
[12] K.C. Marsh, E. Eiden, E. McDonald, J. Cromatogr. B 704 (1997) 307.
[13] A. Darque, G. Valette, F. Rousseau, L.H. Wang, J.P. Sommadossi, X.J. Zhou, Antimicrob. Agents Chemother. 43 (1999) 2245.

[14] G.N. Kumar, V. Jayanti, R.D. Lee, D.N. Whittern, J. Uchic, S. Thomas, P. Johnson, B. Grabowski, H. Sham, D. Betebenner, D.J. Kempf, J.F. Denissen, Drug Metab. Dispos. 27 (1999) 86.
[15] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Cromatogr. B 744 (2000) 227.
[16] K.B. Kenney, S.A. Wring, R.M. Carr, G.N. Wells, J.A. Dunn, J. Pharm. Biomed. Anal. 22 (2000) 967.
[17] C. Marzolini, A. Talenti, T. Buclin, J. Biollaz, L.A. Decosterd, J. Cromatogr. B 740 (2000) 43.
[18] A.S. Pereira, K.B. Kenney, M.S. Cohen, J.E. Hall, J.J. Eron, R.R. Tidwell, J.A. Dunn, J. Chromatogr. B Biomed. Sci. Appl. 742 (2000) 173.
[19] B. Fan, M.G. Bartlett, J.T. Stewart, Biomed. Chromatogr. 16 (2002) 383.
[20] E. Gangl, I. Utkin, N. Gerber, P. Vouros, J. Chromatogr. A 974 (2002) 91.
[21] K.M.L. Crommentuyn, H. Rosing, L.G.A.H. Nan-Offeringa, M.J.X. Hillebrand, A.D.R. Huitema, J.H. Beijnen, J. Mass Spectrom. 38 (2003) 157.
[22] J. Donnerer, M. Kronawetter, A. Kapper, I. Haas, H.H. Kessler, Pharmacology 69 (2003) 197.
[23] V.A. Frerichs, R. DiFrancesco, G.D. Morse, J. Chromatogr. B 787 (2003) 393.
[24] U.S. Justesen, C. Pedersen, N.A. Klitgaard, J. Chromatogr. B 783 (2003) 491.
[25] P.D. Walson, S. Cox, I. Utkin, N. Gerber, L. Crim, M. Brady, K. Koranyi, Ther. Drug Monit. 25 (2003) 650.
[26] L.D. Williams, L.S. Von Tungeln, F.A. Beland, D.R. Doerge, J. Chromatogr. B 798 (2003) 55.
[27] W. Egge-Jacobsen, M. Unger, C.U. Niemann, M. Baluom, S. Hirai, L.Z. Benet, U. Christians, Ther. Drug Monit. 26 (2004) 546.
[28] R.C.E. Estrela, M.C. Salvadori, G. Suarez-Kurtz, Rapid Commun. Mass Spectrom. 18 (2004) 1147.
[29] S. Notari, A. Bocedi, G. Ippolito, P. Narciso, L.P. Pucillo, G. Tossini, R. Perrone Donnorso, F. Gasparrini, P. Ascenzi, J. Chromatogr. B 831 (2006) 258.
[30] G.R. Bruce, P.S. Gill, J. Chem. Educ. 76 (1999) 805.
[31] Guidance for Industry: Q2B Validation of Analytical Procedure, Methodology, 1996, http://www.fda.gov/cber/guidelines.htm.
[32] L. Sleno, D.A. Volmer, Rapid Commun. Mass Spectrom. 19 (2005) 1928.

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Determination of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine concentration in human plasma by MALDI-TOF/TOF

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Abstract

The interest in therapeutic drug monitoring (TDM) of antiretroviral drugs has grown significantly since highly active antiretroviral therapy (HAART) became a standard of care in clinical practice. TDM is useful to determine the best dosage regimen adapted to each patient. Here, we apply MALDI-TOF/TOF technology to quantify abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine in the plasma of HIV-infected patients, by standard additions analysis. Regression of standard additions was linear over the whole anti-HIV concentration range explored (1.00×10^{-2}–1.00 pmol/μL). The absolute recovery ranged between 80% and 110%. Values of the drug concentration determined by MALDI-TOF/TOF were in the range of 1.00×10^{-2}–1.00 pmol/μL. The limit of quantification value was 1.00×10^{-2} pmol/μL for abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine.

Keywords: Human plasma; Anti-HIV drug determination; MALDI-TOF/TOF

1. Introduction

Antiretroviral therapy for treatment of human immunodeficiency virus type 1 (HIV-1) infection has improved steadily since the advent of combination therapy in 1996. The clinical treatment of patients with HIV-1 infection has been advanced by the development of the highly active antiretroviral therapy (HAART). HAART reduces plasma HIV-RNA below detectable limits in most cases. However, some patients do not have a sustainable antiviral response, even after experiencing a decrease in plasma HIV-RNA, due to the development of drug resistance and metabolic complications. This undesirable outcome may result from a failure to achieve effective antiretroviral drug plasma concentration. Therefore, monitoring plasma concentration of anti-HIV drugs is essential not only to evaluate drug pharmacokinetics in clinical trials but also to correlate plasma levels with efficacy and toxicity in the clinical setting. Prospective clinical trials assessing the clinical usefulness of this strategy have shown contradictory results, pointing out the need to consider different issues when performing therapeutic drug monitoring (TDM). Among others, the development of analytical methods for the identification and quantification of anti-HIV drugs in human plasma is a main goal [1–6].

Recently, using MALDI-TOF/TOF, we have developed a new and rapid method for the detection of lamivudine, lopinavir and ritonavir in the plasma of HIV patients [8]. Here, we apply MALDI-TOF/TOF technology to quantify several antiviral drugs, such as the nucleoside reverse transcriptase inhibitors (NRTIs) abacavir and stavudine, the non-nucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine, and

Abbreviations: HAART, highly active antiretroviral therapy; HBA, 4-hydroxybenzoic acid; HIV, human immunodeficiency virus; LOD, limit of detection; LOQ, limit of quantification; MALDI-TOF/TOF, matrix-assisted laser desorption ionization source and tandem-of-flight; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors; PI, protease inhibitor; RSD, relative standard deviation; SD, standard deviation; SPE, solid-phase extraction; TDM, therapeutic drug monitoring.

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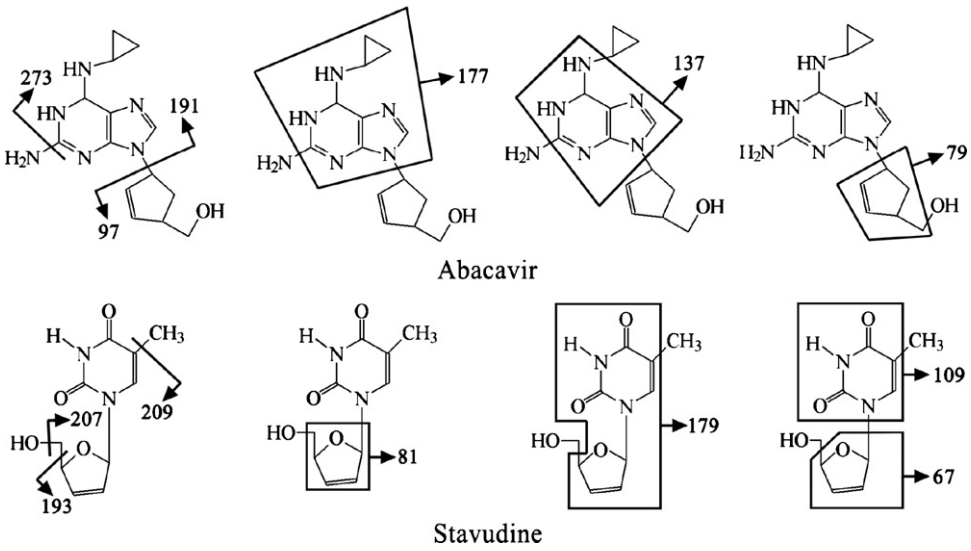

Fig. 1. Chemical structures of the HIV NRTIs abacavir and stavudine. Fragment ions observed under MS/MS conditions are shown. The mass of MS/MS fragments is expressed as *m/z*. For details see text.

the protease inhibitors (PIs) amprenavir and didanosine, in the plasma of HIV-infected patients.

2. Materials and methods

2.1. Chemicals

Abacavir (from Glaxo Wellcome, London, UK), amprenavir (Vertex/Kissei/Glaxo Wellcome, London UK), didanosine (from Bristol-Myers Squibb, Princeton, NJ, USA), efavirenz (from Dupont Merck, Wilmington, DE, USA), lamivudine (from Iaf Biochem. Int./Glaxo Wellcome, London, UK), lopinavir (from Abbott Park, IL, USA), nevirapine (from Boehringer Ingelheim, Ridgefield, CO, USA), ritonavir (from Abbott Park, IL, USA), and stavudine (from Bristol-Myers Squibb, Princeton, NJ, USA) (Figs. 1–3) were obtained through the NIH AIDS Research Reagent Program, Division of AIDS, NIAID, National Institute of Health (Bethesda, MD, USA). 4-Hydroxybenzoic acid (HBA) and trifluoracetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the other products were from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without purification.

Stock solutions of abacavir, amprenavir, didanosine, efavirenz, lamivudine, lopinavir, nevirapine, ritonavir, and stavudine were prepared by dissolving 5.0 mg of each anti-HIV drug in 5.0 mL of methanol. Stock solutions were diluted with methanol to a final concentration ranging between 2.00×10^{-2} pmol/μL and 2.00 pmol/μL.[1] The HBA saturated

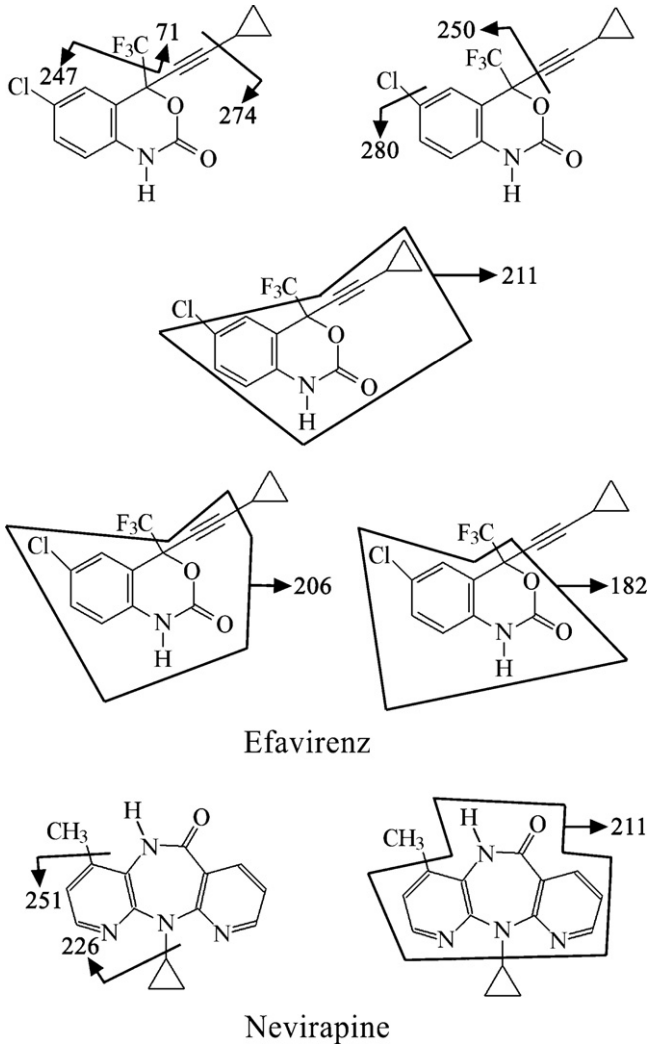

Fig. 2. Chemical structures of the HIV NNRTIs efavirenz and nevirapine. Fragment ions observed under MS/MS conditions are shown. The mass of MS/MS fragments is expressed as *m/z*. For details see text.

[1] For comparison with data concerning the determination of the concentration of anti-HIV drugs emtricitabine, lamivudine, lopinavir, and ritonavir in human plasma by MALDI-TOF/TOF [8], the pmol/μL unit has been used in the present study. Data here obtained in the pmol/μL unit can be converted to the ng/mL unit according to the following equation: ng/mL = pmol × m.w./μL, where m.w. represents the molecular weight of the drug. The molecular weights of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine are 286.333 g/mol, 505.628 g/mol, 236.227 g/mol, 315.675 g/mol, 266.298 g/mol, and 224.213 g/mol, respectively [26].

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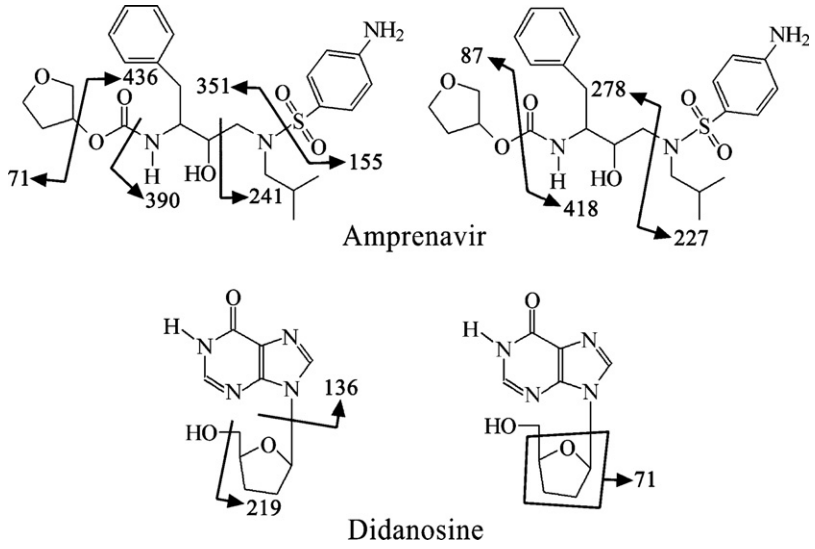

Fig. 3. Chemical structures of the HIV PIs amprenavir and didanosine. Fragment ions observed under MS/MS conditions are shown. The mass of MS/MS fragments is expressed as *m/z*. For details see text.

solution (≥2.0 g/L) was prepared by dissolving HBA in 50% acetonitrile–0.1% trifluoracetic acid.

2.2. Sample preparation

The regimens of HAART of the HIV-infected patients were: amprenavir and lamivudine (Patient 1; 700.0 mg, twice a day and 300.0 mg, once a day, respectively); abacavir, didanosine, lopinavir, and ritonavir (Patient 2; 300.0 mg, 400.0 mg, 133.3 mg, 33.3 mg, twice a day, respectively); efavirenz and lamivudine (Patient 3; 600.0 mg and 300.0 mg, once a day, respectively); didanosine and nevirapine (Patient 4; 400.0 mg and 200.0 mg, twice a day, respectively); didanosine, lopinavir, ritonavir, and stavudine (Patient 5; 400.0 mg, 133.3 mg, 33.3 mg and 40,0 mg twice a day, respectively).

According to the protocol previously approved by the Ethics Committee of the Istituto Nazionale per le Malattie Infettive I.R.C.C.S. 'Lazzaro Spallanzani' (Roma, Italy) and with the written informed consent of the patients, blood samples were taken from HIV-infected patients. Patients were instructed not to take their morning pills prior to the consultation.

Anti-HIV drug samples were prepared as previously reported [7,8]. Briefly, blood samples (6.0 mL) were collected in monovetters Li heparinate and centrifuged at 3000 rpm for 20 min at room temperature. Then, human plasma was separated from blood cells and stored at −20.0 °C. Human plasma samples were cleaned-up by off-line solid-phase extraction (SPE) using Oasis HLB Cartridge 1 cc (30 mg; divinylbenzene and *N*-vinylpyrrolidone) (Waters, Milford, MA, USA). The SPE cartridges were conditioned with 1.0 mL methanol followed by 1.0 mL milliQ water (Millipore, Bedford, MA, USA). One hundred microlitres of methanol was added to 600 μL of human plasma, the solution was vortexed for 1 min and centrifuged at 13,000 rpm for 6 min. The supernatant (*ca.* 650 μL) was diluted by adding milliQ water (1.0 mL) and loaded onto the cartridge. Then, cartridges were washed with 1.0 mL of 5% (v/v) methanol in milliQ water. Analytes were eluted by washing cartridges with 2.0 mL of absolute methanol. The eluate was evaporated in a water bath at 36.0 °C under a stream of nitrogen. The extracted sample was reconstituted with 100 μL absolute methanol and subjected to MALDI-TOF/TOF and HPLC-UV analysis.

2.3. Determination of anti-HIV drug concentration by MALDI-TOF/TOF

To determine the concentration of anti-HIV drugs by MALDI-TOF/TOF, the reconstituted samples were spiked with known anti-HIV drug concentration (standard additions analysis) [9]. The lamivudine, lopinavir, and ritonavir concentration was determined by MALDI-TOF/TOF as previously reported [8].

The abacavir, didanosine, efavirenz, nevirapine, and stavudine concentration ranged between 2.00×10^{-2} pmol/μL and 2.0 pmol/μL before mixing with the matrix HBA. The amprenavir concentration ranged between 2.00×10^{-2} pmol/μL and 1.00 pmol/μL before mixing with the matrix HBA. One microlitre of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine was mixed with 1.0 μL of the matrix HBA. The final abacavir, didanosine, efavirenz, nevirapine, and stavudine concentration was 1.00×10^{-2} pmol/μL, 1.00×10^{-1} pmol/μL, 3.00×10^{-1} pmol/μL, 6.00×10^{-1} pmol/μL, and 1.00 pmol/μL. The final amprenavir concentration was 1.00×10^{-2} pmol/μL, 5.00×10^{-2} pmol/μL, 1.50×10^{-1} pmol/μL, 3.00×10^{-1} pmol/μL, and 5.00×10^{-1} pmol/μL.

Then, each sample was spotted onto the sample target plate of the MALDI-TOF/TOF 4700 Proteomics Analyzer (Applied

Biosystems, Foster City, CA, USA) and mass spectra recorded [8].

Mass spectra of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine were obtained between 50 Da and 1000 Da with 4800 laser shots intensity (Nd:YAG laser at 355 nm, 50 Shots/Sub-Spectrum for 2000 Total Shots/Spectrum) by reflectron positive mode on an Applied Biosystems 4700 Proteomics Analyzer mass spectrometer. For each sample a data dependent acquisition method was created to select intense peaks, excluding those from the matrix. MS/MS spectra were acquired in positive mode with 5100 laser shots (Nd:YAG laser at 355 nm, 50 Shots/Sub-Spectrum for 2000 Total Shots/Spectrum) using atmospheric gas as the collision gas. Mass assignment, calibration, resolution, and sensitivity of MALDI-TOF/TOF was optimized by using a standard mixture of peptides (Applied Biosystems Mass Standard Kit) in the mass range 900–3600 Da (*i.e.*, Des-Arg-bradykinin, 904.4681 Da, 1.00 pmol/μL; angiotensin I, 1296.6853 Da, 2.00 pmol/μL; Glu-fibrinopeptide B, 1570.6774 Da, 1.30 pmol/μL; ACTH (clip 1–17), 2093.0867 Da, 2.00 pmol/μL; ACTH (clip 18–39), 2465.1989 Da, 1.50 pmol/μL; and ACTH (clip 7–38), 3657.9294 Da, 3.00 pmol/μL). Spectra were processed and analyzed by the GPS Explorer™ Software v.2.0 (Applied Biosystems, Foster City, CA, USA). Mass spectra of lamivudine, lopinavir, and ritonavir were obtained as previously reported [8]. For all anti-HIV drugs, the precursor ions $[M + H]^+$ resulted from the addition of a proton to form the positively charged molecular ion [8].

2.4. Determination of anti-HIV drug concentration by HPLC-UV

The abacavir, amprenavir, didanosine, efavirenz, lamivudine, lopinavir, nevirapine, ritonavir, and stavudine concentration by HPLC-UV was determined as follows [7]. Briefly, the chromatographic system for HPLC-UV consisted of a Waters 600 pump and a Waters autosampler 717 PLUS equipped with a spectrophotometric UV–vis dual-wavelength detector Waters 2487 set at 240 nm and 260 nm (Milford, MA, USA). Anti-HIV drug separation was performed at 24.0 °C on an analytical C_{18} Symmetry™ column (250 mm × 4.6 mm i.d.) with a particle size of 5.0 μm (Waters) equipped with a Waters Sentry guard column (20 mm × 3.9 mm i.d.) filled with the same packing material (Waters). The Millenium software (Waters) was used to pilot the HPLC-UV instrument and to process the data (*i.e.*, area integration, calculation, and plotting of chromatograms) throughout the method validation and sample analysis. The mobile phases were 1.00×10^{-2} M KH_2PO_4 (solution A) and acetonitrile (solution B). The injection volume was 20.0 μL. The mobile phase was delivered at 1.0 mL/min. Gradient elution was performed by linearly increasing the percentage of acetonitrile from 6% to 100% in 35 min. The retention times for lamivudine, didanosine, stavudine, abacavir, nevirapine, amprenavir, ritonavir, lopinavir, and efavirenz, are 4.1 min, 8.6 min, 9.7 min, 15.1 min, 16.6 min, 19.9 min, 23.1 min, 24.5 min, and 28.4 min, respectively [7].

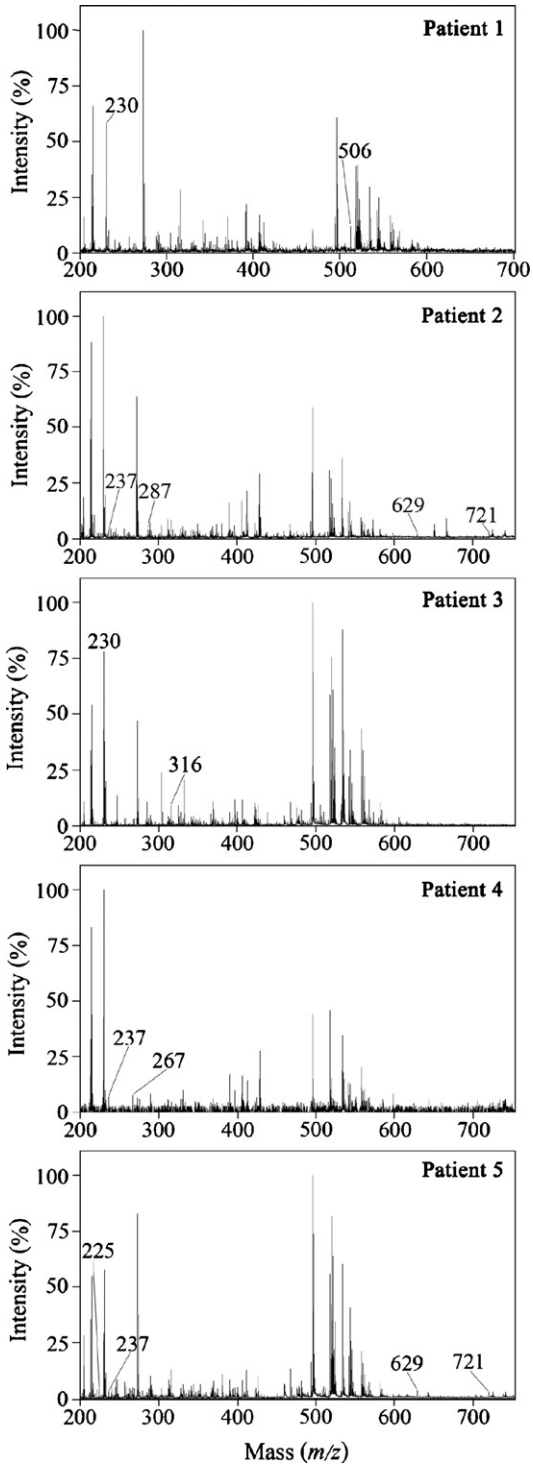

Fig. 4. MALDI-TOF/TOF MS spectra of the plasma of five HIV-infected patients under therapeutical treatment with amprenavir and lamivudine (Patient 1, see Table 2), abacavir, didanosine, lopinavir, and ritonavir (Patient 2, see Table 2), efavirenz and lamivudine (Patient 3, see Table 2), didanosine and nevirapine (Patient 4, see Table 2), and didanosine, lopinavir, ritonavir, and stavudine (Patient 5, see Table 2). The full scan mass spectral analyses showed protonated molecule ions of 225 *m/z*, 230 *m/z*, 237 *m/z*, 267 *m/z*, 287 *m/z*, 316 *m/z*, 506 *m/z*, 629 *m/z*, and 721 *m/z*, corresponding to stavudine, lamivudine, didanosine, nevirapine, abacavir, efavirenz, amprenavir, lopinavir, and ritonavir, respectively. For details, see text, Figs. 1–3 and 5.

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2.5. Recovery

The absolute recovery of anti-HIV drugs was calculated by comparing the peak intensity obtained from standard solutions with the peak intensity from standard extract. Recovery experiments were carried out at the following drug spiked levels 1.00×10^{-2} pmol/μL, 1.00×10^{-1} pmol/μL, 2.50×10^{-1} pmol/μL, and 5.00×10^{-1} pmol/μL in plasma samples. Unspiked samples were used as a control [7,8].

2.6. Data analysis

Statistical test for significance were performed using Graphpad Version 4. For the analysis of standard addition curves, the non-weighting linear fit was used.

3. Results and discussion

In order to extent the range of drugs detectable by MALDI-TOF/TOF technology, we analyzed the plasma of several HIV patients under different regimens of HAART.

Plasma proteins were precipitated by addition of absolute methanol to the sample and removed by centrifugation. Then, samples were cleaned-up by SPE, a reliable way of eliminating interfering species. The efficiency of SPE was determined with control samples at 1.00×10^{-2} pmol/μL, 1.00×10^{-1} pmol/μL, 2.50×10^{-1} pmol/μL, and 5.00×10^{-1} pmol/μL. The recovery of analyzed anti-HIV drugs from plasma ranged between 80% and 110%, with standard deviation ranging between 0.6 and 9.8 (data not shown). These values are in accord to recommendations [10].

Compounds derived from plasma of either HIV-infected patients, under different therapeutical treatments, or healthy donors spiked with drugs were analyzed by MALDI-TOF/TOF. Since HBA matrix undergoes low fragmentation under laser shot with respect to others (data not shown) [8], we used this matrix for the anti-HIV drugs analysis. Fig. 4 shows the mass spectra of compounds derived from plasma of HIV-infected patients. Among all the different samples we found protonated molecular ions of 287 *m/z*, 506 *m/z*, 237 *m/z*, 316 *m/z*, 267 *m/z*, and 225 *m/z*, corresponding to abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine, respectively (Fig. 4). As already reported [8], we also detected mass peaks corresponding to lamivudine, lopinavir and ritonavir (230 *m/z*, 629 *m/z* and 721 *m/z*, respectively; Fig. 4) (see also [8]).

Since the MS spectra obtained in the positive reflectron mode are poorly resolved (Fig. 4), and thus not allowing the unequivocally identification of the drugs, we take advantage of the MS/MS system. MS/MS analysis allowed to unambiguously detect abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine (Fig. 5) as well as lamivudine, lopinavir and ritonavir (data not shown) (see [8]). The laser-induced

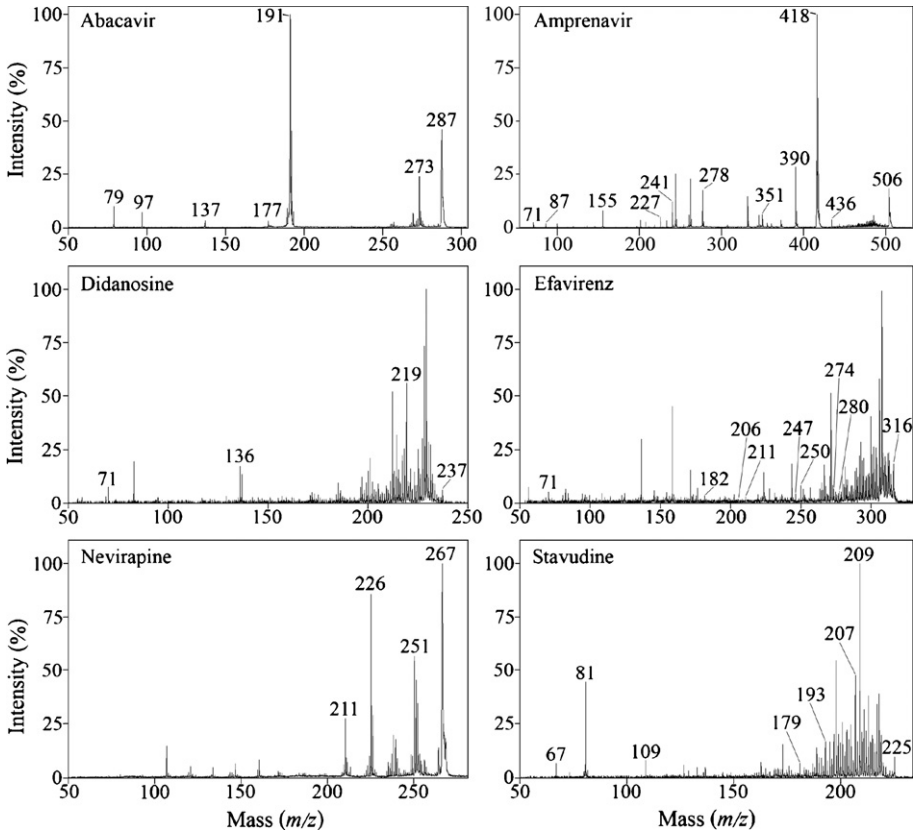

Fig. 5. MALDI-TOF/TOF MS/MS spectra of abacavir (Patient 2, see Table 2), amprenavir, (Patient 1, see Table 2) didanosine (Patient 2, see Table 2), efavirenz (Patient 3, see Table 2), nevirapine, (Patient 4, see Table 2), and stavudine (Patient 5, see Table 2) from the plasma of HIV-infected patients. For details, see text, Figs. 1–3.

fragmentation patterns for each antiviral drug are illustrated in Figs. 1–3, as calculated by ChemWindow software (Bio-Rad, Hercules, CA, USA). The mass peak values obtained from HIV-infected plasma correspond to those obtained from healthy donor plasma spiked with anti-HIV drugs (data not shown), and are in accordance to those reported in the literature [8,11–16].

The concentration of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine in the plasma of HIV-infected patients was determined by the standard additions method [9]. The calibration curves of standard additions for these antiviral drugs were satisfactorily described by unweighted least-squares linear regression (Fig. 6). The response was linear between 1.00×10^{-2} pmol/μL and 1.00 pmol/μL for abacavir, didanosine, efavirenz, nevirapine, and stavudine, and between 1.00×10^{-2} pmol/μL and 5.00×10^{-1} pmol/μL for amprenavir (r^2 ranged 0.998–0.999) (see Fig. 6 and Table 1).

Values of drug concentration obtained by the two different methods match each other very well (see Table 2), thus confirming that the MALDI-TOF/TOF is suitable for TDM (see also [8]). Indeed, the drug concentration in human plasma can be determined by both MALDI-TOF/TOF and HPLC-UV. However, the sensibility of MALDI-TOF/TOF is higher than that of HPLC-UV (see [7,8]).

The limit of detection (LOD) of anti-HIV drugs isolated from plasma is defined as the concentration that

Table 1
Linear response for anti-HIV drug determination[a]

Anti-HIV drug	Regression analysis	r^2
Abacavir[b]	$y = (3.10 \pm 0.02)x + (1.80 \pm 0.20) \times 10^{-1}$	0.999
Amprenavir[c]	$y = (1.97 \pm 0.01)x + (2.00 \pm 0.20) \times 10^{-2}$	0.999
Didanosine[b]	$y = (8.30 \pm 0.20) \times 10^{-1}x + (1.60 \pm 0.10) \times 10^{-1}$	0.999
Efavirenz[b]	$y = (5.40 \pm 0.10) \times 10^{-1}x + (1.00 \pm 0.03) \times 10^{-1}$	0.999
Nevirapine[b]	$y = (7.05 \pm 0.06)x + 1.41 \pm 0.03$	0.999
Stavudine[b]	$y = (3.10 \pm 0.10) \times 10^{-1}x + (5.00 \pm 0.40) \times 10^{-2}$	0.998

[a] Data obtained by MALDI-TOF/TOF in positive mode. Results are the mean of four experiments.
[b] The response range was 1.00×10^{-2} pmol/μL to 1.00 pmol/μL.
[c] The response range was 1.00×10^{-2} pmol/μL to 5.00×10^{-1} pmol/μL.

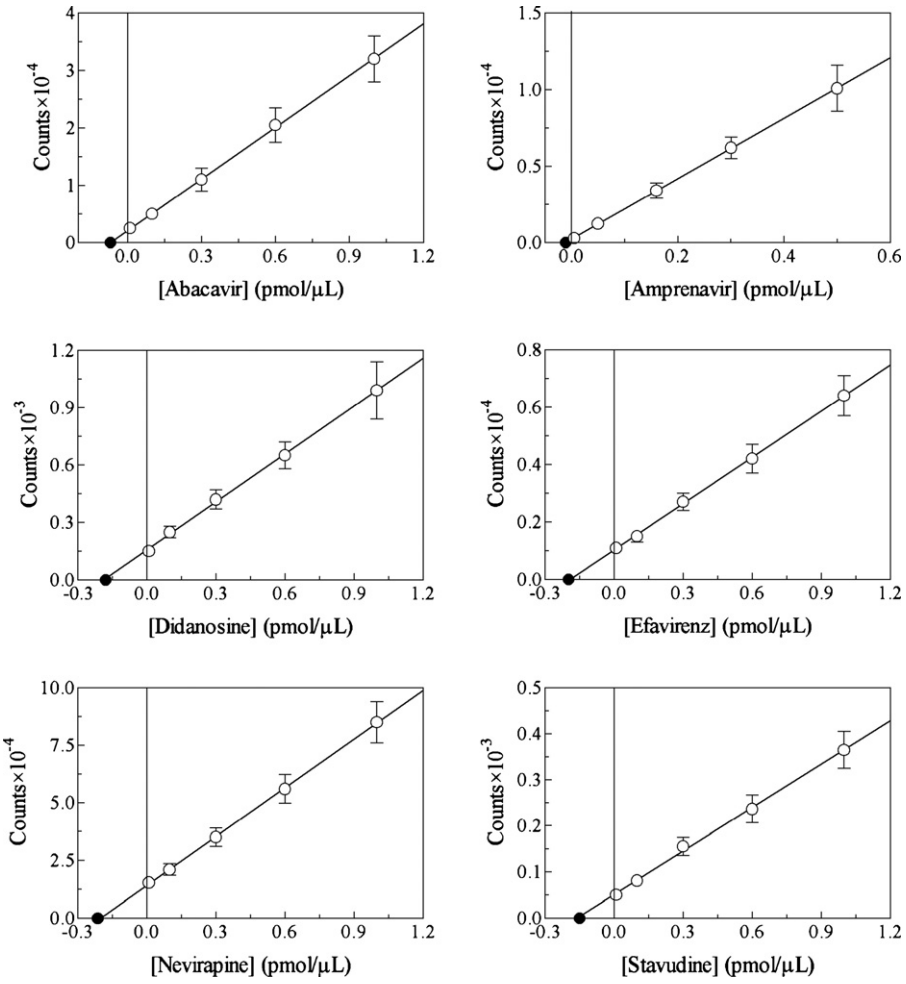

Fig. 6. Linear regression of standard additions of abacavir (Patient 2, see Table 2), amprenavir (Patient 1, see Table 2), didanosine (Patient 2, see Table 2), efavirenz (Patient 3, see Table 2), nevirapine (Patient 4, see Table 2), and stavudine (Patient 5, see Table 2). Filled symbols on the *x*-axis indicate to the unknown anti-HIV drug concentration present in the plasma of the HIV-infected patients. The linearity of regression was excellent (r^2 ranged between 0.998 and 0.999, see Table 1). Data were analyzed as previously reported [9]. Averages and error bars were obtained from at least four repeats. Where error bars are not seen, they are smaller than the data point symbols. For details, see text.

Table 2
Anti-HIV regimens and anti-HIV drug plasma concentration of HIV-infected patients[a]

Patient	Anti-HIV drug	Concentration (pmol/μL)	
		MALDI-TOF/TOF[b]	HPLC-UV
1	Amprenavir	$(1.17 \pm 0.13) \times 10^{-2}$	$(1.51 \pm 0.03) \times 10^{-2}$
	Lamivudine	$(1.61 \pm 0.18) \times 10^{-1}$	$(1.81 \pm 0.19) \times 10^{-1}$
2	Abacavir	$(4.25 \pm 0.09) \times 10^{-2}$	$(4.53 \pm 0.18) \times 10^{-2}$
	Didanosine	$(1.81 \pm 0.19) \times 10^{-1}$	$(1.81 \pm 0.04) \times 10^{-1}$
	Lopinavir	$(5.10 \pm 0.04) \times 10^{-2}$	$(5.00 \pm 0.01) \times 10^{-2}$
	Ritonavir	$(3.27 \pm 0.24) \times 10^{-2}$	$(3.32 \pm 0.30) \times 10^{-2}$
3	Efavirenz	$(1.97 \pm 0.30) \times 10^{-1}$	$(2.03 \pm 0.70) \times 10^{-1}$
	Lamivudine	$(4.71 \pm 0.90) \times 10^{-1}$	$(4.84 \pm 0.22) \times 10^{-1}$
4	Didanosine	$(4.56 \pm 0.90) \times 10^{-1}$	$(4.58 \pm 0.21) \times 10^{-1}$
	Nevirapine	$(2.03 \pm 0.12) \times 10^{-1}$	$(2.13 \pm 0.50) \times 10^{-1}$
5	Didanosine	$(4.95 \pm 0.60) \times 10^{-1}$	$(4.92 \pm 0.12) \times 10^{-1}$
	Lopinavir	$(1.07 \pm 0.20) \times 10^{-1}$	$(1.08 \pm 0.12) \times 10^{-1}$
	Ritonavir	$(1.83 \pm 0.10) \times 10^{-2}$	$(2.01 \pm 0.30) \times 10^{-2}$
	Stavudine	$(1.50 \pm 0.20) \times 10^{-1}$	$(1.50 \pm 0.10) \times 10^{-1}$

[a] Results are the mean of four experiments.
[b] The anti-HIV drugs concentration was obtained by standard additions analysis.

yields a signal/noise ratio of 3:1 [10]. For the concentration to be accepted as the lowest limit of quantification (LOQ), the percent deviation from the nominal concentration (measure of accuracy) and the relative standard deviation (measure of precision) have to be less than 20% [10]. The LOQ value was 1.00×10^{-2} pmol/μL for abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine (Table 2).

Values of LOQ here reported are lower than those given in the literature for abacavir (5.20×10^{-2} pmol/μL to 8.70×10^{-2} pmol/μL) [7,17], amprenavir (4.90×10^{-2} pmol/μL to 3.95×10^{-1} pmol/μL) [7,18,19], didanosine (6.30×10^{-2} pmol/μL to 1.05×10^{-1} pmol/μL) [7,17], efavirenz (3.10×10^{-2} pmol/μL to 6.32×10^{-1} pmol/μL [7,18,19], nevirapine (2.00×10^{-2} pmol/μL to 1.49 pmol/μL) [7,18,19], and stavudine (2.20×10^{-2} pmol/μL to 4.44×10^{-1} pmol/μL) [7,20]. Therefore, this method is sensitive enough for TDM of these anti-HIV drugs.

Values of intra-day and inter-day precision and accuracy were determined at different anti-HIV drug concentrations. The precision was calculated as the relative standard deviation (RSD) within a single run (intra-day) and between different assays (inter-day):

$$\text{RSD}\,(\%) = \left(\frac{\text{SD}}{\text{mean}}\right) \times 100$$

where SD is the standard deviation. The accuracy was calculated as the percentage of the deviation between the nominal and the found anti-HIV drug concentration:

$$\text{Accuracy}\,(\%) = \left\{\frac{([\text{found}] - [\text{nominal}])}{[\text{nominal}]}\right\} \times 100$$

As shown in Table 3 for all the antiviral drugs measured both precision and accuracy were, according to literature [10], <20%, thus confirming the reliability of the MALDI-TOF/TOF technology.

The minimal anti-HIV drug concentration detectable by MALDI-TOF/TOF ranges between 2.50×10^{-3} pmol/μL and 1.00×10^{-2} pmol/μL (see Table 3 and [8]). The minimal anti-HIV drug concentration detectable by HPLC-UV and HPLC–MS/MS is about 1.00×10^{-2} pmol/μL and 1.00×10^{-3} pmol/μL, respectively (see [8]). The minimal anti-HIV drug concentration detectable by a prototype MALDI-triple quadrupole instrument equipped with a high repetition rate laser is about 1.00×10^{-3} pmol/μL [21].

Although MALDI-TOF/TOF is not usually used as a quantitative instrument, due to limitations of its linearity, we demonstrate here, according to previous results [8], that it is a suitable technology to identify and measure the concentration of several anti-HIV drugs, such as abacavir, amprenavir, didanosine, efavirenz, lamivudine, lopinavir, nevirapine, ritonavir, and stavudine, in the human plasma. MALDI-TOF/TOF takes the great advantage with respect to HPLC–MS, since it allows to analyze a larger number of samples in a shorter time [22]. Quantitative HPLC–MS is performed by using isotope drug labelling and analyzing the mass shift [23]. HPLC–MS is extremely sensitive and accurate, but it is difficult to apply to drugs due to the scarce availability and the high cost of stable isotopes [24]. The standard additions analysis coupled to MALDI-TOF/TOF (see [8]) avoids these limitations. MALDI-TOF/TOF allows the direct analysis of samples and permits a high number of samples being loaded at the same time [22]. Moreover, the great level of automation of MALDI-TOF/TOF reduces the time of analysis to few minutes, allowing to carry out high-throughput studies [25].

In conclusion, we demonstrate that MALDI-TOF/TOF spectrometry represents a valuable technology to be applied for TDM studies.

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Table 3
Intra-day and inter-day anti-HIV drug determination

Anti-HIVdrug	Intra-day[a]				Inter-day[a]			
	Nominal concentration (pmol/μL)	Found concentration (pmol/μL)	Precision (%)	Accuracy (%)	Nominal concentration (pmol/μL)	Found concentration (pmol/μL)	Precision (%)	Accuracy (%)
Abacavir	1.00×10^{-2}	$(8.00 \pm 0.30) \times 10^{-3}$	3.1	4.3	1.00×10^{-2}	$(9.00 \pm 0.60) \times 10^{-3}$	8.8	3.1
	2.50×10^{-1}	$(2.40 \pm 0.05) \times 10^{-1}$	2.2	1.6	2.50×10^{-1}	$(2.40 \pm 0.10) \times 10^{-1}$	4.6	2.8
	5.00×10^{-1}	$(4.80 \pm 0.30) \times 10^{-1}$	5.4	4.1	5.00×10^{-1}	$(4.80 \pm 0.20) \times 10^{-1}$	3.1	2.7
	1.00	1.06 ± 0.12	11.2	6.3	1.00	1.06 ± 0.12	10.0	6.6
Amprenavir	1.00×10^{-2}	$(9.00 \pm 0.80) \times 10^{-3}$	8.2	6.1	1.00×10^{-2}	$(1.20 \pm 0.08) \times 10^{-2}$	3.4	5.1
	2.50×10^{-1}	$(2.60 \pm 0.30) \times 10^{-1}$	11.0	6.5	2.50×10^{-1}	$(2.50 \pm 0.60) \times 10^{-1}$	2.3	1.3
	5.00×10^{-1}	$(4.90 \pm 0.10) \times 10^{-1}$	2.3	1.3	5.00×10^{-1}	$(4.70 \pm 0.20) \times 10^{-1}$	5.4	5.3
Didanosine	1.00×10^{-2}	$(9.00 \pm 0.20) \times 10^{-3}$	4.5	2.2	1.00×10^{-2}	$(1.20 \pm 0.80) \times 10^{-2}$	10.5	1.9
	2.50×10^{-1}	$(2.40 \pm 0.10) \times 10^{-1}$	4.6	2.9	2.50×10^{-1}	$(2.30 \pm 0.10) \times 10^{-1}$	4.2	4.0
	5.00×10^{-1}	$(5.00 \pm 0.30) \times 10^{-1}$	6.0	0.1	5.00×10^{-1}	$(4.80 \pm 0.50) \times 10^{-1}$	11.0	4.0
	1.00	1.01 ± 0.04	3.5	1.6	1.00	1.02 ± 0.08	7.0	4.0
Efavirenz	1.00×10^{-2}	$(8.00 \pm 0.60) \times 10^{-3}$	4.6	12.0	1.00×10^{-2}	$(1.10 \pm 0.70) \times 10^{-2}$	3.8	1.6
	2.50×10^{-1}	$(2.60 \pm 0.20) \times 10^{-1}$	11.2	6.3	2.50×10^{-1}	$(2.40 \pm 0.10) \times 10^{-1}$	4.0	0.1
	5.00×10^{-1}	$(4.90 \pm 0.10) \times 10^{-1}$	2.0	2.0	5.00×10^{-1}	$(4.90 \pm 0.20) \times 10^{-1}$	5.0	1.0
	1.00	$(9.60 \pm 0.50) \times 10^{-1}$	6.0	3.4	1.00	$(9.80 \pm 0.30) \times 10^{-1}$	2.7	2.0
Nevirapine	1.00×10^{-2}	$(1.00 \pm 0.20) \times 10^{-2}$	5.5	2.7	1.00×10^{-2}	$(9.00 \pm 0.40) \times 10^{-3}$	8.1	9.1
	2.50×10^{-1}	$(2.50 \pm 0.10) \times 10^{-1}$	5.6	1.1	2.50×10^{-1}	$(2.40 \pm 0.10) \times 10^{-1}$	2.6	1.0
	5.00×10^{-1}	$(5.20 \pm 0.20) \times 10^{-1}$	4.8	4.6	5.00×10^{-1}	$(4.80 \pm 0.20) \times 10^{-1}$	3.7	4.0
	1.00	1.06 ± 0.50	5.2	6.3	1.00	$(9.60 \pm 0.30) \times 10^{-1}$	3.0	3.3
Stavudine	1.00×10^{-2}	$(1.10 \pm 0.40) \times 10^{-2}$	7.5	1.9	1.00×10^{-2}	$(1.10 \pm 0.20) \times 10^{-2}$	4.1	8.5
	2.50×10^{-1}	$(2.50 \pm 0.10) \times 10^{-1}$	3.1	1.6	2.50×10^{-1}	$(2.50 \pm 0.10) \times 10^{-1}$	6.1	1.4
	5.00×10^{-1}	$(4.80 \pm 0.20) \times 10^{-1}$	3.6	2.1	5.00×10^{-1}	$(5.20 \pm 0.60) \times 10^{-1}$	12.2	5.3
	1.00	$(9.20 \pm 0.60) \times 10^{-1}$	7.2	7.7	1.00	$(9.80 \pm 0.30) \times 10^{-1}$	3.0	2.0

[a] Results are the mean of four experiments.

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References

[1] D. Back, S. Khoo, S. Gibbons, AIDS 16 (Suppl. 1) (2002) S5.
[2] J.G. Gerber, E.P. Acosta, J. Clin. Virol. 27 (2003) 117.
[3] R.E. Aarnoutse, J.M. Schapiro, C.A. Boucher, Y.A. Hekster, D.M. Burger, Drugs 63 (2003) 741.
[4] J. Molto, B. Clotet, J. HIV Ther. 9 (2004) 75.
[5] N.Y. Rakhmanina, J.N. van den Anker, S.J. Soldin, AIDS Patient Care STDS 18 (2004) 7.
[6] Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents May 4 2006 http://aidsinfo.nih.gov.
[7] S. Notari, A. Bocedi, G. Ippolito, P. Narciso, L.P. Pucillo, G. Tossini, R.P. Donnorso, F. Gasparrini, P. Ascenzi, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 831 (2006) 258.
[8] S. Notari, C. Mancone, M. Tripodi, P. Narciso, M. Fasano, P. Ascenzi, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 833 (2006) 109.
[9] G.R. Bruce, P.S. Gill, J. Chem. Educ. 76 (1999) 805.
[10] Guidance for Industry: Q2B Validation of Analytical Procedure, Methodology 1996 http://www.fda.gov/cber/guidelines.htm.
[11] T.N. Clark, C.A. White, M.G. Bartlett, Rapid Commun. Mass Spectrom. 18 (2004) 405.
[12] S. Colombo, A. Beguin, A. Telenti, J. Biollaz, T. Buclin, B. Rochat, L.A. Decosterd, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 819 (2005) 259.
[13] S. Compain, D. Schlemmer, M. Levi, A. Pruvost, C. Goujard, J. Grassi, H. Benech, J. Mass Spectrom. 40 (2005) 9.
[14] B. Fan, M.G. Bartlett, J.T. Stewart, Biomed. Chromatogr. 16 (2002) 383.
[15] G.N. Kumar, V. Jayanti, R.D. Lee, D.N. Whittern, J. Uchic, S. Thomas, P. Johnson, B. Grabowski, H. Sham, D. Betebenner, D.J. Kempf, J.F. Denissen, Drug Metab. Dispos. 27 (1999) 86.
[16] E. Gangl, I. Utkin, N. Gerber, P. Kouros, J. Chromatogr. A. 974 (2002) 91.
[17] C.P. Verweij-van Wissen, R.E. Aarnoutse, D.M. Burger, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 816 (2005) 121.
[18] E. Dailly, F. Raffi, P. Jolliet, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 813 (2004) 353.
[19] N.L. Rezk, R.R. Tidwell, A.D. Kashuba, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 805 (2004) 241.
[20] G. Aymard, M. Legrand, N. Trichereau, B. Diquet, J. Chromatogr. B Biomed. Sci. Appl. 744 (2000) 227.
[21] L. Sleno, D.A. Volmer, Rapid Commun. Mass Spectrom. 19 (2005) 1928.
[22] K. Stülher, H.E. Meyer, Curr. Opin. Mol. Ther. 6 (2004) 239.
[23] W. Lu, E. Kimball, J.D. Rabinowitz, J. Am. Soc. Mass Spectrm. 17 (2006) 37.
[24] S. Ma, S.K. Chowdhury, K.B. Alton, Curr. Drug Metab. 7 (2006) 503.
[25] X. Zhang, D. Wei, Y. Yap, L. Li, S. Guo, F. Chen, Mass Spectrom. Rev. 26 (2007) 403.
[26] D.S. Wishart, C. Knox, A.C. Guo, S. Shrivastava, M. Hassanali, P. Stothard, Z. Chang, J. Woolsey, Nucleic Acids Res. 34 (2006) D668.

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6. Conclusion

Combining the information of resistance testing, TDM, and pharmacogenomics is likely to be of great clinical utility to individualize the antiretroviral regimen trough to a possibility to give the right amount of the right drug to the right person (see [1,2]).

The HPLC-UV and MALDI-TOF/TOF methods here reported are sensitive and specific, allowing the simultaneous determination of the largest number of anti-HIV drugs. Therefore, they appears very promising to examine several anti-HIV drug combination regimens [3-5].

Although MALDI-TOF/TOF is not usually used as a quantitative instrument, due to limitations of its linearity, we demonstrated here, that it is a suitable technology to identify and measure the concentration of anti-HIV drugs in the human plasma. MALDI-TOF/TOF takes the great advantage with respect to HPLC-MS(/MS) since allows to analyze a larger number of samples in a shorter time [6]. Moreover, the great level of automation of MALDI-TOF/TOF instruments reduces the time of analysis to few minutes, allowing to carry out high-throughput studies [7]. In contrast with MALDI-TOF/TOF, HPLC-MS(/MS) is difficult to apply to drugs due to the scarce availability and the high cost of their stable isotopes [8,9]. As a whole, MALDI-TOF/TOF spectrometry represents a valuable technology to be applied for TDM in HIV-infected patients [4,5].

Both the HPLC-UV and MALDI-TOF/TOF methods (see [3-5]) have been validated and are used routinely to monitor the plasma levels of PIs, NRTIs, and NNRTIs in HIV-infected patients at the Istituto Nazionale per le Malattie Infettive I.R.C.C.S. 'Lazzaro Spallanzani' (Roma, Italy).

6.1. References

[1] J. Moltó, B. Clotet, Therapeutic drug monitoring of antiretroviral agents scenario, J. HIV Ther. 9 (2004) 75-78.

[2] N.Y. Rakhmanina, J.N. van den Anker, S.J. Soldin, Therapeutic drug monitoring of antiretroviral therapy, AIDS Patient Care STDS 18 (2004) 7-14.

[3] S. Notari, A. Bocedi, G. Ippolito, P. Narciso, L.P. Pucillo, G. Tossini, R. Perrone Donnorso, F. Gasparrini, P. Ascenzi, Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 831 (2006) 258-266.

[4] S. Notari, C. Mancone, M. Tripodi, P. Narciso, M. Fasano, P. Ascenzi, Determination of anti-HIV drug concentration in human plasma by MALDI-TOF/TOF, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 833 (2006) 109-116.

[5] S. Notari, C. Mancone, T. Alonzi, M. Tripodi, P. Narciso, P. Ascenzi, Determination of abacavir, amprenavir, didanosine, efavirenz, nevirapine, and stavudine concentration in human plasma by MALDI-TOF/TOF, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. (2008) in press.

[6] K. Stühler, H.E. Meyer, MALDI: more than peptide mass fingerprints, Curr. Opin. Mol. Ther. 6 (2004) 239-248.

[7] X. Zhang, D. Wei, Y. Yap, L. Li, S. Guo, F. Chen, Mass spectrometry-based 'omics' technologies in cancer diagnostics, Mass Spectrom. Rev. 26 (2007) 403-431.

[8] W. Lu, E. Kimball, J.D. Rabinowitz, A high-performance liquid chromatography-tandem mass spectrometry method for quantitation of nitrogen-containing intracellular metabolites, J. Am. Soc. Mass Spectrom. 17 (2006) 37-50.

[9] S. Ma, S.K. Chowdhury, K.B. Alton, Application of mass spectrometry for metabolite identification, Curr. Drug. Metab. 7 (2006) 503-523.