UNIVERSITAT JAUME·I

Anàlisi de fàrmacs prescrits en tractaments de SIDA en plasma i sèrum mitjançant cromatografia líquida micel·lar

Tesis presentada per Inmaculada Casas Breva

per a obtindre el grau de Doctor

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CERTIFIQUEN

Que la present Memòria, ***Anàlisi de fàrmacs prescrits en tractaments de SIDA en plasma i sèrum mitjançant cromatografia líquida micel·lar*** constitueix la tesi doctoral de:

INMACULADA CASAS BREVA

Així mateix, certifiquen haver dirigit i supervisat les parts teòriques, metodològiques, instrumentals i aplicacions dels diferents treballs, així com també la seua redacció.

I perquè conste als efectes oportuns, signem la present.

Josep Esteve i Romero | Samuel Carda Broch | Juan Peris Vicente

Castelló de la Plana, 15 de Maig de 2015

A mi padre, a mi madre,

a mi marido, a mi hija y a mi hijo.

A todo lo que quiero.

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Destacar que en la vida uno tiene que tomar decisiones muy dificiles.

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Índex

Pròleg

El grup de Química Bioanalítica del Departament de Química Física i Analítica de la Universitat Jaume I, amb codi 029 de l'Oficina per a la Cooperació en Investigació i Desenvolupament Tecnològic (OCIT), s'inicià en la recerca sobre cromatografia líquida als anys 90, primer a la Universitat de València (UV) i seguidament a l'Institut de Recerca en Aliments de Norwich (UK). Posteriorment, s'especilitzà en cromatografia líquida micel·lar (MLC), la qual ràpidament va esdevindre la línea prioritaria de recerca. A l'inici, l'estudi estigué orientat als fonaments d'aquesta tècnica innovadora, que destacava per la seua gran versatilitat i la possibilitat d'injecció directa de mostres complexes, i després, a l'aplicació de l'MLC al anàlisi mediambiental, de salud alimentaria, forense i clínic.

El grup presenta una llarga experiència en el desenvolupament de mètodes analítics amb aplicació clínica. De fet, la majoria de publicacions, projectes i Tesis Doctorals dirigides estan relacionats amb aquest tema. En 1999, es va començar una fructífera cooperació amb el Hospital Virgen de los Lirios de Alcoi, que es va extendre posteriorment a hospitals més propers geogràficament: General, La Plana i Provincial de Castelló. Aquesta col·laboració consisteix en informació de quins son les aplicacions analítiques que demanden els Hospitals a cada moment, en la provisió de medicaments, de mostres i la presentació de projectes conjunts.

L'MLC és una variant de la HPLC que utilitza dissolucions micel·lars, és a dir de surfactant per damunt de la concentració micel·lar crítica (CMC), com a fases mòbils. Sota aquestes condicions, els monòmers de tensioactiu s'organitzen en agregats esfèrics: les micel·les, formant una dispersió col·loidal. El nucli de la micel·la està format per les cadenes carbonades, que busquen aïllar-se de l'aigua, mentre que a la superfície es sitúen les grups polars. La formació de la micel·la permet que tant la part hidrofòbica com hidrofílica estiguen en un entorn favorable. La interacció entres les cadenes lipofíliques estabilitza la micel·la, mentre que la interacció entre els grups polars i l'aigua manté a la micel·la suspesa en la dissolució aquosa i evita l'unió entre micel·les per a formar agregats més grans. En la majoria dels cassos, s'afegeix un volum generalment baix de disolvent orgànic per a disminuir la

polaritat de la fase mòbil, obtenint fases mòbils micel·lars híbrides. Els més utilitzats son alcohols de cadena curta i acetonitril.

Des del seu inici en 1980, l'MLC ha evolucionat fins a convertir-se en una alternativa real a la cromatografia líquida de fase inversa clàssica (RP-HPLC), que utilitza fases mòbils hidroorgàniques. Gràcies a la gran quantitat d'estudis realitzats, i a nombrosos llibres i articles publicats dirigits al coneixement d'aquesta tècnica, els fonaments de l'MLC estan actualment fermament establits.

A causa de les propietats singulars de les dissolucions micel·lars, l'MLC constitueix una excel·lent alternativa per a l'anàlisi de fluids biològics, especialment el plasma i el sèrum. Els monòmers de surfactant desnaturalitzen les proteïnes, que adopten la estructura lineal primaria. Les dissolucions micel·lar solubilitzen les cadenes peptídiques i altres compostos hidròfobs, mitjançant la seua interacció amb el nucli lipofílic de la micel·la. Així doncs, les mostres de plasma i sèrum, tot i ser suspensions complexes, es poden injectar directament sense risc de precipitació al mesclar-se amb la fase mòbil dins de la columna, l'agulla o els tubs del sistema cromatogràfic. Aquesta simplificació de la preparació de la mostra representa un fort avantatge front a la HPLC aquoorgànica, que exigeix la introducció de etapes intermèdies d'extració i neteja de mostra, que allargen i compliquen el protocol experimental, i poden ser causa de pèrdua d'analit i variabilitat en la recuperació. A més a més, els analits lligats a les proteïnes i macromolècules son desplaçats pels monòmers de tensioactiu, resultant en una major recuperació.

A les fases mòbils micel·lars s'estableix una varietat més ampla d'interaccions i d'equilibris de repartiment dels analits (entre la fase estacionaria, la fase mòbil, la superfície de la micel·la i l'interior de la micel·la) que a les fases mòbils homogènies hidroorgàniques (només entre la fase estacionaria i la fase mòbil), i, per tant, augmenta la versatilitat de l'MLC. Es poden resoldre mescles complexes de soluts de diversa natura (polars o apolars, carregats o sense càrrega) i de diferent hidrofobicitat, mitjançant elució isocràtica. Per altra banda, el comportament cromatogràfic dels analits a l'MLC és altament estable i reproduïble, i pot modelitzar-se amb gran exactitut mitjançant equacions matemàtiques, que prediuen els paràmetres cromatogràfics segons la composició de la fase mòbil. Així doncs, es pot optimizar simultàneament la concentració de surfactant i modificador orgànic mitjançant una

estrategia interpretativa, a partir de les dades experimentals obteses amb un número reduït de fases mòbils, disminuïnt l'esforç necessari. A més, l'MLC fa servir en el protocol experimental i en la fase mòbil una menor quantitat de volum de reactius i disolvents tòxics i inflamables que la HPLC hidroorgànica, millorant la seguretat al laboratori, i disminuint el vessament de contaminants al medi ambient. Totes aquestes característiques doten a l'MLC d'importants avantatges pràctics, com la capacitat del processament successiu de una gran quantitat de mostres per dia, alt grau d'automatització i reducció del preu dels anàlisis.

El síndrome de inmunodeficiencia adquirida (SIDA) és una greu enfermetat causada pel virus de inmunodeficiencia humana-1 (VIH-1). Provoca un debilitament general del sistema inmunològic, fent al pacient especialment sensible a qualsevol infecció. El virus va ser descobert en 1981, i es considera responsable de la mort de 39 mil·lions de persones. El VIH es transmet mitjançant l'intercanvi de fluids (sang, rel·lacions sexuals i de mare a fill) i s'estima que vora 35 mil·lions de persones hi estan infectades actualment. L'existència de la SIDA i les seues característiques han estat ampliament estesses pels mitjans de comunicació, a causa de la seua ràpida expansió, la seua virulència i la condició de malaltia de transmisió sexual. Això ha permés que la opinió pública siga conscient dels seus perill i de la necessitat de introduïr mesures de prevenció. Malgrat l'intent de governs i empreses farmacèutiques en erradicar la malaltia, encara no s'hi ha descobert cap cura definitiva.

En 1996, es va proposar la terapia HAART, que aconseguí resultats clínics impresionants en la lluita contra aquesta malaltia. Consisteix en l'administració simultànea de tres o cuatre antirretrovirals i/o antivirals, que actúen conjuntament per a suprimir l'activitat del VIH-1. No obstant, els tractaments HAART també presenten problemes. És una terapia difícil de presciure, ja que la dosis idònea pot variar d'un pacient a un altre. Per a ser efectiva, la quantitat de antirretrovirals i antivirals en sang s'ha de mantindre dins d'un "rang terapèutic". Una quantitat molt baixa pot estimular l'aparició de mutacions resistents, i una concentració massa elevada pot causar efectes secundaris, com l'increment de la concentració de triglicèrids i colesterol, i l'aparició de problemes renals i hepàtics. La quantitat de fàrmacs en sang no només depén de la dosi prescrita, sino també de cóm el pacient metabolitza els fàrmacs, i per tant depén de multitud de variables, com el fenotip/genotip, la fisiologia, del temps que pren el tractament, dels seus hàbits alimentaris i de vida, i de la injestió de altres medicaments. A més a més, s'ha d'administrar de per vida, ja que no elimina el

virus, sino que només l'atura. Això afavoreix la falta l'adherencia al tractament, que de fet és la primera causa de fracàs. A més a més, es tracta de una teràpia enormement costossa.

La monitoritació terapèutica de fàrmacs (TDM) consisteix en la mesura de la concentració de fàrmacs en plasma i/o sèrum a diversos temps després de la injesta del medicament. Aquesta informació permet esbrinar la seua farmacocinètica, previndre i explicar els efectes adversos i terapèutics, i determinar la dosi adecuada per a un pacient específic. També permet la detecció de quantitats sub- i superterapèutiques, i comprovar si el pacient effectivament prén els medicaments. Per tant, per a avançar en la lluita contra aquesta malatia és necessari que els Hospitals disposen de mètodes analítics que mesuren amb fiabilitat i a baix preu la concentració dels antirretrovirals i antivirals prescrits en HAART en plasma i sèrum sanguini.

En aquesta tesis s'aplica l'MLC per a la determinació de antirretrovirals i antivirals prescrits en HAART en plasma i sèrum. Es presentarà un mètode de cribat per a determinar els farmacs més habituals a HAART, i després cinc mètodes centrats en la detecció simultànea dels tres o cuatre antirretrovirals inclosos en els cinc tractaments HAART més prescrits. Els mètodes es validaran i s'aplicaran a mostres reals, per a que els metges puguen avaluar l'adherència del pacient, estudiar la farmacocinètica dels analits i comprobar si la quantitat de antirretroviral en sang està dintre del rang terapèutic. Així doncs, els treballs desenvolupats tindran un fort impacte social en el àmbit de la salud bioclínica i del tractament contra la SIDA. Els diferents treballs d'aquesta memòria s'emmarquen en la línia d'investigació que du a terme el grup de Química Bioanalítica i es basen en l'ampla experiència del grup de recerca en el desenvolupament de mètodes per MLC.

Aquesta memòria consta de set capítols. A l'introducció es fa una explicació extensa sobre les característiques i el àmbit d'aplicació dels antirretroviral i antivirals. També es descriuren les característiques de la cromatografia líquida micel·lar, sobre tot les que resulten d'interés per a l'anàlisis de les matrius estudiades. Posteriorment es presenten els objectius de la tesis i es discutixen els resultats obtinguts a cada estudi. Finalment s'indiquen els conclusions extretes de la totalitat del treball realitzat.

Per ser una Tesis escrita parcialment en anglés (Art. 24 de la *NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL RD 99/2011, EN LA UNIVERSITAT JAUME I*

(Aprovada pel Consell de Govern núm. 19 de 26 de gener de 2012)), aquesta tesi ha de contindre un apartat prou ampli en valencià o castellà, que ha de formar part de l'enquadernació de la tesi on s'incloga necessàriament:

- Objecte i objectius de la investigació
- Aportacions originals
- Conclusions obtingudes i futures línies d'investigació

Chapter 1

Introduction

1. Human Immunodeficiency Virus infection: an overview

The Human Immunodeficiency Virus-1 (HIV-1) is a *Lentivirus*, which attacks the immune system when introduced into the human body. The virus infection facilitates the occurrence of many diseases, and causes the Acquired Immunodeficiency Syndrome (AIDS), which is the last phase of the HIV infection [1]. In addition to the destruction of the immune system, the HIV infection stimulates the formation of tumors and infections caused by opportunist microorganisms. This diversity of clinical manifestations originates from the double tropism of HIV: as a *Lentivirus*, it infects the tissue cells of macrophage lineage, and also holds a special ability to affect the lymphocytes T cluster of differentiation 4 (CD4) [2].

AIDS is the expression of a wide range of organic alterations caused by the dysfunction of the immune system, both cellular and humoral, produced by the infection of the HIV-1. It was recognized as a specific clinical syndrome in 1981 by the US Centers for Disease Control and Prevention (CDC), when several healthy patients show serious infections by rare pathogens, which have been previously detected only in patients with strong cellular immunodeficiency. Since the descriptions of the first cases, AIDS became the first pandemic of the 21^{th} Century [3].

Since the start of the AIDS epidemic in 1981, more than 78 millions of people have been infected with HIV and approximately 35 million have died of the disease. Nowadays, it is estimated that nearly 39 millions of people are infected with HIV. The last year, it was appraised that 2.1 million were infected by the virus. Despite this, UNAIDS states that the incidence of AIDS is stabilizing in a global context. The number of infections per year has decreased since 1990. The number of deaths has also declined since 2005, because of the implementation of therapies based on antiretroviral (ARV) drugs [4].

The medical advances have reduced the mortality of the HIV infected patients, allowing, in many cases, an improvement of the quality of life. The determination of the plasmatic viral load, the finding of new and more effective combinations of ARV drugs and the development of preventive strategies have allowed that AIDS has turned to a manageable chronic process. However, it is estimated that only 37 % of the infected patient receive ARV therapies. Therefore, other diseases not related to HIV, such as chronic hepatitis, are causing

more problems than AIDS itself [4]. Besides, the toxicity of the long-term ARV therapy is currently one of the more relevant aspects of the monitoring of these patients [5].

2. Antirretroviral treatment

Nowadays, the treatment using antiretroviral (ARV) is the main factor that could influence the development of the VIH-1 infection. Therefore, the accessibility of this therapy by the patients to should be effective. The correct use of the ARV requires the knowledge of concepts related to the pathogenesis and therapy of VIH infection, as well as of the adequate criteria to select the beginning of the therapy and the kind of drugs to-be-used, the way to monitor the answer and when a change of therapy must be envisaged [6].

The prescription and dispensation of the ARV treatment are performed by the hospitalary department specialized in HIV. However, the primary health care centers also maintain a fundamental role and a strong responsibility in the scope of AIDS diagnosis, treatment and monitoring of patients. The intervention which the primary health care centre should develop, regarding on the ARV treatment, are: to acquire the basic knowledge about the drugs, and current guidelines of the treatment, to get a elemental comprehension about the adverse and side effects of the prescribed drugs, to know and watch over the pharmacological interaction between ARV and other coadministered drugs, as well as between other compounds usually consumed by HIV infected patients, to actively stimulate the compliance of the patients with the trearment, in order to avoid the appearance of drug resistance, and to collaborate to attain the desired therapeutic objectives (improvement of the lifespan and quality life, increment of the health level, to slow the progression of the disease, and diminishing the mortatiry rate) [7].

The HIV infection cannot be nowadays eradicated. Thus, the objective of the ARV treatment is to achieve the maximal suppression of the replication of the virus as long as possible, in order to avoid the progression of the disease (occurrence of symptoms and medical complications related to the HIV infection), to prevent the appearance of resistant HIV strains, avoid the decay of the immune system, and, if possible, accomplish the

recovery, improve the quality life and reduce the mortality rate [8]

2.1 The concept of highly active antiretroviral therapy

In 1987, the use of the first antiretroviral, the transcriptase inverse inhibitor zidovudine (AZT) was approved as monotherapy in HIV patients. Nevertheless, the monotherapy was found unsuccessful against HIV. Treatment of transcriptase inverse inhibitor was able to reduce the replication of the virus during a short period, but soon became ineffective. In fact, the virus shows a short life cycle (a half-life of nearly 6 h), and replicates and mutates rapidly, so that it easily develop resistance to the simple therapies. Therefore, the necessity to investigate new drugs to drastically reduce the viral load, in order to hinder its ability to mutate and replicate. In 1996, a new group of ARV able to inhibit the activity of the protease, the enzyme responsible of the replication of the virus in the lymphocyte was developed, the protease inhibitors (PIs) was developed. The combination of these others with those previously proposed lead to HAART [9].

The highly active antiretroviral therapy (HAART) is a treatment based on the simultaneous prescription of a regimen containing a set of at least three ARV drugs. Each ARV attack a different step in the reproduction cycle of the virus, or some related aspects, generating a better inhibition of the viral process and its replication. The most usual antiretroviral are: the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleotide reverse transcriptase inhibitor (NtRTIs) and protease inhibitors (PIs). Other antiretroviral, such as fusion inhibitors (FIs) and integrase strand transfer inhibitors (ISTIs) are included in lesser extent. The combined activity of the ARV is more effective than the same ARV in a monotherapy.

The usual HAART regimen combines three or more different drugs such as two nucleoside reverse transcriptase inhibitors (NRTIs) and a protease inhibitor (PI), two NRTIs and a non-nucleoside reverse transcriptase inhibitor (NNRTI) or other such combinations. These HAART regimens have proven to reduce the amount of active viruses and in some cases, the viral load has been decreased until it is undetectable by current blood testing

techniques [10].

HAART reached an impressive clinical result by suppressing the replication of HIV during a large period of time, ranging from months to years, in a significative number of patients. HAART also improves and can even restore the immune system of the patient. This would diminish the occurrence of opportunistic infections. The introduction of HAART has resulted in a significant reduction of the HIV activity, and the improvement of the lifespan and the quality of life [9].

2.2 ARV included in HAART

The main antiretroviral used in HAART are [10]:

- Nuceloside reverse transcriptase inhibitors (NRTIs): Zidovudine (AZT) (Retrovir ®, Zidovudina ® EFG), didanosine (ddl) (Videx ®), zalcitabine (ddC) (Hivid ®), lamivudine (3TC)(Epivir ®), stavudine (d4T)(Zerit ®), abacavir (ABC)(Ziagen ®) and emtricitabine (FTC) (Truvada ®; Emtriva ®).
- Nucleotide reverse transcriptase inhibitors (NtRTIs): tenofovir (Viread ®).
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs): nevirapine (NVP)(Viramune ®), efavirenz (EFV)(Sustiva ®) and delavirdine (DLV)(Rescriptor ®)
- Protease inhibitors (PIs): amprenavir (APV)(Agenerase ®), darunavir (Prezista ®) indinavir (IDV)(Crixivan ®), lopinavir (LPV) + ritonavir (RTV) (Kaletra ®), nelfinavir (NFV) (Viracept ®), saquinavir (SQV)(Invirase ®, Fortovase ®), atazanavir(ATV) (Reyataz ®), tipranavir and ritonavir (Norvir ®).
- Fusion inhibitors (FIs): enfuvirtide (Fuzeon ®)
- Integrase strand transfer inhibitors (INSTIs): elvitegravir, dolutegravir (Tivicay ®) and raltegravir (Isentress ®).

2.3 Sets of ARV commonly prescribed in HAART

There are different kinds of therapeutic regime, depending on the included ARV [6,11]:

2.3.1 Based on NNRTIs

- Efavirenz + 3TC + (AZT or tenofovir or d4T), not recommended during pregnancy.
- Efavirenz + 3TC + ddl, not recommended during pregnancy.
- Nevirapine + 3TC + (AZT or d4T or ddl).

These regimens have a well-documented virologic and immune efficiency, avoid the adverse side effects of PIs, and show less pharmacological interactions than these last ones. They are easier to take, and facilitate the adherence of the patient. However, using these regimens, the HIV have a higher probability to develop drug resistance.

2.3.2 Based on PIs

- Lopinavir + ritonavir + 3TC + (AZT or d4T)
- Amprenavir + ritonavir + 3TC + (AZT or d4T)
- Indinavir + 3TC + (AZT or d4T)
- Indinavir + ritonavir + 3TC + (AZT + d4T)
- Nelfinavir + 3TC + (AZT or d4T).
- Saquinavir + ritonavir + (AZT or d4T)

These regimens also hold a strong immune and virologic activity. They act at two levels of the replication cycle of HIV and require more mutation of the virus to develop resistance. The main disadvantage is the high number of tablets that the patient must take. This complicates the compliance of the patient with the therapy. Besides, they show important pharmacological interactions with all the drugs and compounds which are metabolized by the cytochrome P 450.

2.3.3 Triple regimen of NRTIs

- ABC + 3TC + AZT
- ABC + 3TC + d4T

These regimens have a weaker virologic efficiency than the others, and can be used as initial therapy in naïve patients, those have not previously received any treatment. They do not show interactions with other drugs or compounds being metabolized by the cytochrome P 450. These regimens involve the ingestion of a few tablets, and are then easy to administrate, facilitating the adherence of the patient to the therapy.

2.3.4 Not recommended ARV regimens

- Monotherapy, except AZT during the pregnancy to reduce the risk of vertical transmission.
- Double therapy, except in patients already following this therapy and show a good response.
- d4T + ddl, during pregnancy.
- EFV, during pregnancy.
- AZT + d4T, antagonist effect.
- ddC + (d4T or ddl), additive effect in the peripheral neuropathy.

2.4 Directives for the prescription of the HAART regimens

In the early stages of the implementation of HAART, the ARV treatment was prescribed using the strategy "*hit hard and early*", that means to prescribe the HAART regimen with stronger virologic activity as soon as possible. Nowadays, the optimum grade of disease progress initiates the therapy and the most adequate HAART regimens are established by considering, aside from the ability of the ARV to prevent the replication of the HIV, the influence in the quality of life and the medium- and long-term adverse effects

caused by the treatment [12].

The Table 1.1 shows the guidelines for the starting of HAART, based on the symptoms, the strenghness of the immune system, quantified by the number of lymphocytes T CD4, and progression of the HIV infection, measured as the viral load.

Table 1.1. Recommendations for the start of HAART in teenager and adult patients, with chronic infection of HIV.

Clinical	Number of CD4	Viral load	Recommendation
Symptomatic (AIDS or serious symptoms)	Any	Any	Start treatment
Asymptomatic, AIDS	< 200 mm^3	Any	Start treatment
Asymptomatic	200 - 350 mm^3	Any	Consider starting treatment
Asymptomatic	> 350 mm^3	> 30 000 copies/mL	Consider starting treatment
Asymptomatic	> 350 mm^3	> 30 000 copies/mL	Wait

We currently dispose of limited clinical data about the treatment of the infection at the acute phase of the disease (the six first months from the seroconversion). The benefits of HAART about the replication of the virus and the immune system of the patient are not enough to keep the disease in latency when the treatment is removed. Therefore, once the initiated the HAART, this one should be indefinitely maintained.

The progression degree of the disease at which the starting of HAART would be more effective must be taken considering the consequences of an early or late prescription, and can be different for each patient. Besides, a wrong decision can provoke therapy failure. For these reasons, it is recommended to individualize the starting of HAART at the early stage of the HIV infection [13].

2.4.1 Consequences of an early start of HAART

Advantages:

- The suppression of the viral replication is easier to achieve and maintain.
- To delay or even prevent the decay of the immune system.
- The risk of drug resistance is diminished, if the total suppression of the replication of the virus is accomplished.
- Reduction of the probability of transmission of HIV.

Disadvantages:

- The quality of life of the patient is worsened due to the treatment.
- The adverse effects increase.
- The virus easily develops drug resistance, if the viral replication is not totally prevented.
- To limit future alternative therapies.

2.4.2 Consequences of a late start of HAART

Advantages:

- To avoid the negative effects of the patient on the quality of life of the patient.
- To prevent the adverse outcomes of the treatments.
- To retard the appearance of drug resistance.
- To maintain more therapeutic options when the treatment starts

Disadvantages:

- It enables the irreversible deterioration of the immune system.
- The suppression of the viral load is more difficult to reach.
- The probability of HIV transmission is enhanced.

2.5 Evaluation of the efficiency of HAART

Once the HAART has been started, the efficiency of the treatment and the response by the patient is performed by monitoring the viral load in plasma. The therapy is considered as effective, when it is able to diminish the viral load (RNA-HIV) until the decimal logarithm of the initial viral load at 4 - 8 weeks, and to undetectable levels (< 50 copies /mL) at 4 - 6 months. On the contrary, or if the viral load increases (except by an intercurrent infection), it is considered a therapeutic failure, and the reasons must be investigated (poor adherence, inadequate bowel absorption, pharmacological interactions, or appearance of drug resistance), and/or propose a change of HAART regimen. If the HAART treatment is interrupted, it is important that the patient also stops the ingestion of all the prescribed ARV, in order to avoid the therapeutic failure [14]

The extensive use of HAART and the rapid replication of HIV stimulate the development of viral mutations, and the formation of strains resistant to the taken ARV. Therefore, the performing of resistance studies, when the therapy failure has been detected, can be useful to decide the alternative therapy, even if the interpretation of these studies is complex. There are several situations in which the resistance studies are should be performed before the initiation of the HAART regimen: for pregnant women with an acute HIV infection, if they have not previously taken another HAART regimen, and in the case of post-exposure prophylaxis; in already treat patients, when the previous treatment has failed, between the first and third failure. No resistance studies should be performed if the viral load is under 1000 copies/mL [15].

2.6 Compliance with the treatment

The current directives in the treatment of the HIV infection provoke that many patients, usually asymptomatic, receive HAART during the rest of their life. The capacity of the patient to correctly adhere to the therapy is fundamental to its success, and to prevent the development of strain virus resistant to the ARV. The therapeutic compliance rate for chronic

diseases is variable, with an average value of nearly 50 %, while the minimal rate considered as acceptable is around 80 %. In the treatment of HIV infection, the adherence requirements are higher, as the effectiveness of the HAART is maximal if the compliance rate is over 95 %. Nevertheless, HAART has most factors which difficult the adherence, such as excessive of tablets per day, tedious dosage schedule, side effects, large duration, *etc*. This represents a challenge for the patient and the medical staff. Altogether, the surveillance of the correct therapeutic compliance of the patients with HAART is a multidisciplinary task, in which the primary health care has a relevant role, because of the direct and continuous relationship with the patients [16].

The compliance level of the patient depends on the characteristics of the treatment and several psychological factors affecting the patients [17]. A high adherence is reached in the following cases:

- The HAART regimen is easy to take, that means a reduced umber of tablets few times a day.
- The therapeutic effects are noticed early.
- To enjoy emotional and practical support
- Adaptability of the dosage schedule with the quotidian routine.
- To be clearly aware that an incomplete adherence can favor the development of drug resistance.
- To be conscious about the importance of taking all the dosages.
- To be able to take the tablets in the presence of other people.

A poor compliance of the patient with HAART can be caused by many reasons. The main factors related to the adherence to the therapy can be grouped in the following categories:

- Related to the disease: the adherence to HAART depending on the occurrence of clinical symptoms. It increases during the symptomatic phases of the treatment, and it decreases when the patient does not advise the symptoms, because he is not aware of the severity of his situation.
- Related to the therapy: Several studies have proven that the adherence is reduced if the number of tablets, the frequency of ingestion and the duration of the treatment is high. The

incidence of adverse side effects can also provoke the early interruption of the treatment. The adherence is improved with the affordability of the pharmaceutical formulations.

- Related to the patient: mental disorders, such as anxiety, depression (usually associated with AIDS patients), and the consumption of toxic compounds (abuse drugs or alcohol), can jeopardize the adherence to the therapy. On the other hand, the conviction of the patient regarding on the efficacy of the therapy, the perception of the seriousness of the diseases, the ability to correctly follow the therapy and the skill of the medical staff are important factors. The adherence is improved is the patient is able to identify the different ARV and has a basic knowledge about the activity of the ARV.
- Related to the social situation of the patient: the social or familiar discrimination, the domestic violence, the family breakdown, the lack of an assistance network, the prejudice about some lifestyles popularly associated with AIDS patients and the absence of information can also difficult the compliance with the therapy.
- Related to the care team: a good relationship between the patient and the medical staff, in terms of trust, confidentiality, availability, flexibility and abidance are essential for reaching a high degree of adherence.

2.7 Evaluation of the compliance with the therapy

The most important methodologies to measure the compliance with HAART are [18]:

- Quantification of the ARV in biological fluids, especially plasma. The moment of the sampling within the dosage schedule must be optimized and maintained through the whole therapy. However, the concentration of the ARV can be modified by unusual interactions and malabsorption, thus providing low ARV amounts even with a high adherence.
- Interview with the patient: in a context of full confidence, the medical staff has a higher probability to detect the impediments to correctly follow the therapy and together try to overcome them and find the adequate motivation. This methodology can be easily implemented by the primary health care.
- Structured questionnaire: the patient is asked to fulfill a quizz about whether he has ingested the correct dosage at the correct time. The questionnaire is structured to detect false answers.

- Control the attendance to the scheduled appointments to receive the ARV pharmaceutical formulations, and with the doctor: the non attendance to these meetings is an indication of a low adherence.
- Clinical evolution and the analytical data: they give an idea about the compliance with HAART. However, this criterion is not applicable to patients undergoing unusual clinical effects.
- Count of the remaining tablets: it must be performed at the Hospital and at the patient's home. The date of starting of the therapy, the dosage schedule and the number of dispensed tablets must be known to apply this methodology. The compliance rate can be calculated as: (number of dispensed tablets minus number of remaining tablets)/number of expected ingested tablets, considering a total adherence.
- Electronic monitoring.

As seen, no methodology has a total reliability. These methodologies should be combined to obtain data about the real situation as accurately as possible. A complete strategy to measure of the compliance must hold these characteristics [19]:
- It must evaluate the accomplishment of the established dosage schedule, both numbers of tablets and ingestion frequency.
- The achievements of the proposed objectives, especially in terms of viral load.
- It must be expressed as a global adherence rate (*e.g.*, percentage of intake dosages, proportion of days with the correct number of dosages, ratio of doses taken at the correct time). The quantification of the compliance is very important. The patient attaining an adherence rate over 95 % is considered as "trustworthy", whereas an adherence rate < 95 % is a "partial trustworthy" patient.

2.8 Administration and pharmacological characteristics of the main ARV

The information about the administration (form, amount and schedule), special attention to avoid negative interactions with other drugs or meals, and the pharmacological characteristics of the main ARV used in HAART are in summarized in Tables 1.2; 1.3 and

1.4 [11].

Table 1.2. Administration and pharmacological properties of the main NRTIs

Drug	AZT	ddl	ddC	3TC	d4T	ABC
Dosage form	Capsule	Enteric capsule	Tablet	Tablet	Capsule	Tablet
Amount of drug per dosage piece	100 or 250 mg	250 or 400 mg	0.75 mg	150 mg	30 or 40 mg	300 mg
Diary dosage and number of intakes	500-600 mg in 2 or 3 intakes. Combined with 3TC (combivir®), 1 piece, twice a day	1 time, depending on the patient's weight: > 60 Kg: 400 mg < 60 Kg: 250 mg	0.75 mg, 3 times	150 mg, 2 times	2 times, depending on the patient's weight: > 60 Kg: 40 mg < 60 Kg. 30 mg	300 mg, 1 time
Influence of food	No need to consider the meals	Take 0.5 h before or 1 h after the meal. If combined with tenofovir, then take during meals	No need to consider the meals	No need to consider the meals	No need to consider the meals	No need to consider the meals. Alcohol increases up to 41 % the ABC amount
Oral bioavailability	60 %	30-40 %	85 %	86 %	86 %	83 %
Elimination	Metabolized to ATZ-glucoronide; renal excretion	50 % -renal excretion	70 % - renal excretion	Non-modified renal excretion	50 % - renal excretion	82 % - renal excretion of the metabolites

Table 1.3. Administration and pharmacological properties of the main NNRTIs

Drug	Nevirapine	Efavirenz	Delavirdine
Dosage form	Tablet	Capsule	Tablet
Amount of drug per dosage piece	200 mg	600 mg	200 mg
Diary dosage and number of intakes	200 mg in 1 intake for 14 days, and thereafter, 200 mg, twice	600 mg	400 mg, 3 times
Influence of food	No need to consider the meals	Avoid meals with a high content of fats, they entail an increase of 50 % of the EFV levels	No need to consider the meals
Oral bioavailability	> 90 %	No data available	85 %
Elimination	Metabolized by the cytochrome P 450; 80 % - renal excretion and 10 % - fecal excretion	Metabolized by the cytochrome P 450; 14-34 % - renal excretion and 61 % - fecal excretion	Metabolized by the cytochrome P450; 51 % - renal excretion and 44 % - fecal excretion

Table 1.4. Administration and pharmacological properties of the main PI

Drug	Indinavir	Ritonavir	Nelfinavir	Saquinavir	Amprenavir
Dosage form	Capsule	- Capsule - Oral solution	Tablet	- Capsules of hard or soft gelatine	Tablets
Amount of drug per dosage piece	400 mg	100 mg	250 mg	200 mg	150 mg
Dosage and number of intakes	800 mg/8 h: 1 h before or after the intake of ddl. The dose and fast depends on the coadministration of other PIs.	Gradual dosage: 300 mg/ 12 h and increase to 600/12 h for 14 days by steps of 100 mg/12 h. Take 1 h before or after ddl	750 mg/day in 3 intakes or 1250 mg/day in two intakes	1600 mg/day: in 3 intakes for hard gelatine capsules, or in 2 intakes for soft gelatine capsules	1200 mg in 2 ingestions
Influence of food	Food can provoke an amount of the drug level up to 77 %. Take 1 h or 2 h after the meal. It can be taken together with low-fat food.	Food increases the drug level a 15 %. Take with meals, to improve tolerance	The level of the drug can increase 2 or 3 times. Take with a snack or a meal	Hard gelatine capsules: no influence (it is taken with RTV) Soft gelatine capsules: the drug levels can 6-times increase, take with a main meal	High-fat foods must be avoided, as they entail a reduction of 21 % of the drug level It can be taken with or without other foods
Storage	Room temperature	Capsule in a fridge. Oral solution at room temperature	Room temperature	Hard gelatine capsules: in a fridge Soft gelatine capsules: in a fridge or at room temperature (max. 3 months)	Room temperature
Oral bioavailability	65 %	Not determined	20 - 80 %	Erratic or not determined	Not determined
Metabolization	Cytochrome P 450; inhibits the 3A4	Cytochrome P 450; strong inhibition of the 3A4	Cytochrome P 450; inhibits the 3A4	Cytochrome P 450; inhibits the 3A4	Cytochrome P 450; inhibits the 3A4

2.9 Side effects of the main ARV

The currently prescribed HAART regimens doubtlessly hold toxicity risks, as well as side and adverse effects. Their occurrence is variable and depends on the physical and psychological condition of the patient, the dosage amount and schedule, and the stage of the treatment. The side effects can appear at the early stage of the therapy, but they usually develop over the time. The occurrence of adverse effects in patients must be controlled, in order to correct them and/or propose a modification of the treatment [6,20]. Some of the side effects of the main ARV are below indicated:

- Zidovudine: anemia and/or neutropenia. It can cause gastrointestinal intolerance, headache, insomnia, asthenia and lactic acidosis with hepatic steatosis.
- Didanosine: pancreatitis, peripheral neuropathy, nausea and stomatisis. It can provoke lactic acidosis with hepatic steatosis.
- Zalcitabine: peripheral neuropathy and stomatisis. It can cause lactic acidosis with hepatic steatosis.
- Stavudine: peripheral neuropathy. It can cause lactic acidosis with hepatic steatosis.
- Lamivudine: minimal toxicity. Possibility of provoking lactic acidosis with hepatic steatosis.
- Abacavir: it can provoke a hypersensibility reaction, which can be highly serious. The symptoms are fever, rash, nausea, vomit, malaise, fatigue and loss of hunger. It can also cause lactic acidosis with hepatic steatosis.
- Nevirapine: rash, increment of the levels of hepatic transaminases, hepatitis.
- Delavirdine: rash, augmentation of the levels of hepatic transaminases, headache.
- Efavirenz: rash, symptoms of the central nervous system, increase of the levels of hepatic transaminases, false positive in the detection of cannabis derivatives.
- Indinavir: Nefrolithiasis, gastrointestinal intolerance, increment of the indirect bilirrubin, headache, asthenia, blurred vision, dizziness, exanthema, metallic taste, thrombocytopenia, hyperglycemia, lipodystrophy and lipid alterations. Possible rising of bleeding in hemophilia patients.
- Ritonavir: gastrointestinal intolerance, paresthesia around the throat and on the extremities,

hepatitis, asthenia, taste alterations, increasing of triglycerides over 200 %, transaminases, creatine kinase and acid uric, hyperglycemia, lipodystrophy and lipid alterations. Possible increase of bleeding in hemophilia patients.

- Nelfinavir: hyperglycemia, lipodystrophy and lipid alterations. Possible increment of bleeding in hemophilia patients.
- Saquinavir: gastrinal intolerance, headache, rise of the hepatic transaminases, hyperglycemia, lipodystrophy and lipid alterations. Possible increment of bleeding in hemophilia patients.
- Amprenavir: gastrointestinal intolerance, rash, paresthesia in mouth, liver damage, hyperglycemia, lipodystrophy and lipid alterations. Possible augmentation of bleeding in hemophilia patients.

2.10 Parmacological interactions of the ARV

The ARV can establish pharmacokinetic interactions with other drugs or compounds commonly taken, which can negatively interfere with HAART, by reducing its therapeutic activity or enhancing its adverse effects. These interferences are especially serious in HIV patients, because they become vulnerable to opportunistic infections, due to the weakening of the immune system induced by AIDS. The most usual diseases associated with AIDS are tuberculosis and hepatic disorders [4]. These negative interactions can eventually obstruct the treatment of the adverse effects of the ARV. Besides, some ARV also influence the activity of other ARV.

Most ARV are inhibitors or inductors of the cytochrome P 450, which is formed by many families and subfamilies of isoenzymes. The CYP3A4 is the one that metabolizes a higher number of drugs. The drugs and other compounds which interact with the cytochrome P 450 can increase or decrease the plasmatic concentration of the ARV metabolized by this same enzyme [21].

The topic of pharmacological interactions of the ARV is quite complex and constrain the adjustment of the dosage of the ARV and the concomitant drugs. In some cases, the

coadministration of specific pairs other drugs/ARV are contraindicated and even banned. Therefore, the medical staff must be aware whether the patient is taking a medication to treat other diseases to prescribe the most adequate HAART, and *vice versa* [6]. The following situations can be highlighted:

- Rifampicin and rifabutine are potent inductors of the cytochrome P 450. Therefore, their dosage must be modified, if they are coadministered with other ARV.

- Methadone shows important pharmacological interactions with the ARV (and also with the tuberculostatic ones). Thus, its dose must be adjusted in HIV patients also taking methadone, to avoid withdrawal and to prevent the toxic effects. For instance, the methadone dose must be increased if nevirapine, delavirdine, efavirenz or ritonavir are administered [22].

- Several natural products also have pharmacological interactions with the ARV. Orange juice is an inhibitor and *Hypericum* is a potent inductor of the cytochrome P 450. These compounds can be found in different pharmaceutical formulations and natural products.

- Some trendy drugs, such as sildenafil (Viagra ®) have strong interactions with the PIs. Therefore, the patient should not take more than 25 mg each 48 h to avoid its adverse effects.

Several associations of other drugs with ARV, which are contraindicated because of these pharmacological interactions, are shown in Table 1.5 and 1.6.

The patient must be aware about the consequences of some of the pharmacokinetic interactions on his health. A practical way to deal with this topic at the primary health care is to ask the patient to indicate the mediations he is currently taken or he has been recently taken. With this information, the patient and the medical staff can together evaluate the needing of dosage readjustment, as well as to establish some potentially dangerous situations which must be avoided [6].

Table 1.5. Incompatibilities of the main NNRTIs with other drugs

Drug category	Nevirapine	Delavirdine	Efavirenz
Hypolipemiant		Simvastatin Lovastatin	
Antimycobacterial	Rifapentine	Rifampicin Rifabunitin Rifapentine	Rifapentine
Antihistamine		Astemizole Terfenadine	Astemizole Terfenadine
Gastrointestinal		Cisapride Antihistamines H2 Inhibitors of the proton pump	Cisapride
Psychotropic		Alprazolam Midazolam Triazolam	Midazolam Triazolam
Ergotamine alkaloid		Dihydroergotamine Ergotamine	Dihydroergotamine Ergotamine
Others	Oral contraceptives Saquinavir Ketoconazole	Carbamazepine Phenytoin Phenobarbital Ketoconazole	Oral contraceptives Clarithromycin Saquinavir

Table 1.6. Incompatibilities of the main PIs with other drugs

Drug category	Indinavir	Ritonavir	Saquinavir	Nelfinavir	Amprenavir
Calcium agonist		Beripril			Beripril
Cardiac		Amiodarone Flecainide Propafenone Quinidine			
Hypolipemiant		Simvastatin and Lovastatin (for all the PIs)			
Antimycobacterial	Rifampicin Rifapentine	Rifapentine	Rifampicin Rifabutine Rifapentine	Rifampicin Rifapentine	Rifampicin Rifapentine
Antihistamine	Astemizole Terfenadine	Astemizole Terfenadine	Astemizole Terfenadine	Astemizole Terfenadine	Astemizole Terfenadine
Gastrointestinal	Cisapride	Cisapride	Cisapride	Cisapride	Cisapride
Neuroleptic	Pimozide	Pimozide	Pimozide	Pimozide	Pimozide
Psychotropic	Midazolam Triazolam	Midazolam Triazolam Diazepam Clozapina Zolpidem	Midazolam Triazolam	Midazolam Triazolam	Midazolam Triazolam
Ergotamine alkaloid		Dihydroergotamine and Ergotamine (for all the PIs)			
Others	*Hypericum*	Clorazepate Oral contraceptives Bupropion Dextropropoxyphene Disulfiram Piroxicam Methamphetamine *Hypericum* Ecstasy	Carbamazepine Phenytoin Phenobarbital Dexamethasone Efavirenz Nevirapine *Hypericum*	Oral contraceptives *Hypericum*	Oral contraceptives *Hypericum*

2.11 Considerations about HAART in HIV infected pregnant women

The objectives and the dosage schedule of HAART for women at childbearing age are the same as for young and adult patients. When dealing with a pregnant women, a new therapeutic aim is added: to avoid the transmission of the HIV infection to the future baby, and to avoid him any damage potentially caused by the treatment. The HAART regimens must include AZT, in order to reduce the transmission of the infection to the fetus [23].

The current ARV therapies are recommended in pregnant women, considering the virological, immunological and clinical state. The criteria to prescribe HAART are the same as for other patients, and it is even advisable if the viral load is > 1000 copies/mL. It is also recommended for pregnant women without a previous ARV treatment and with a viral load < 1000 copies/mL [24].

3. Micellar liquid chromatography

In 1976, Knox and Laird [25] developed ion-pair chromatography (IPC) by adding a small amount of ionic surfactant to the polar hydro-organic mobile phase in RPLC. In these conditions, the surfactant is adsorbed on the stationary phase and is able to associate with ionic solutes bearing an opposite charge. At IPC, retention factors (k) are proportional to the amount of counter-ion adsorbed on the stationary phase. The stationary phase, the nature and concentration of the ionic surfactant and organic solvent, the ionic strength and pH are the main factors that affect the retention behaviour. Methanol, propanol and acetonitrile are the most commonly used organic solvents in mixtures with water. Ionic surfactants with alkyl chains ranging from 6 (hexyl) to 16 (hexadecyl) methylene units are usual. The adsorbed amount of ionic surfactant increases with its concentration in the mobile phase and carbon number in the alkyl-chain. However, it decreases rapidly with the organic solvent contents, owing to the increased elution strength of the organic-rich mobile phase and desorption of surfactant from the stationary phase.

In IPC, the adsorption of surfactant makes column equilibration a critical step, which may give rise to non-reproducible results. Also, care should be taken to avoid exceeding the critical micellar concentration (CMC), since the retention behaviour changes drastically when micelles start to form. Not surprisingly, people working with IPC were not the developers of micellar liquid chromatography (MLC). This technique was proposed by researchers studying the use of surfactant solutions as new and original mobile phases with particular properties [26]. The relationships between both techniques (IPC and MLC) should not, however, be overlooked.

MLC is a reversed-phase liquid chromatographic (RPLC) mode with a mobile phase consisting of an aqueous solution of surfactant above its CMC [27,28]. The idea of using pure micellar solutions as mobile phases in RPLC is very attractive owing to the lower cost and toxicity, and the reduced environmental impact. In practice, however, the addition of a small amount of organic solvent to the micellar solution is needed to achieve retention in practical time windows, and improve peak efficiency and resolution.

Micellar mobile phases have been used with different bonded stationary phases (mostly C8, C18 and cyanopropyl). The most common surfactants are the anionic sodium dodecyl sulfate (SDS), cationic cetyltrimethylammonium bromide (CTAB), and non-ionic Brij-35. Several organic solvents have been used as modifiers, short/medium chain alcohols and acetonitrile being the most suitable. The presence of micelles contributes to keep these organic solvents in solution at concentrations well above their solubility in water. Also, the risk of evaporation is diminished.

Above the CMC, an increment of the surfactant concentration originates an increase in the concentration of micelles in the solution, whereas the number of monomers of surfactant in the mobile phase remains constant. Adsorption of an approximately fixed amount of surfactant monomers on the stationary phase is also produced, giving rise to a stable modified column and regular retention behaviour.

3.1 Stationary phase and mobile phase nature in MLC

MLC shares the basic components of RPLC systems, that is, a non-polar stationary phase and a polar aqueous mobile phase. However, hydro-organic mobile phases in conventional RPLC are homogeneous, whereas micellar solutions are microscopically heterogeneous, being composed of two distinct media: the amphiphilic micellar aggregates (micellar pseudophase) and the surrounding bulk water or hydro-organic mixture that contains surfactant monomers in a concentration approximately equal to the CMC. On the other hand, the stationary phase is modified by the adsorption of surfactant monomers, creating a structure similar to an open micelle.

In conventional hydro-organic RPLC, the surface composition of the bonded silica is more relevant to chromatographic processes than bare silica, since solute retention results from interaction with the outer few nanometers of the bonded silica [29]. This can be extended to the surfactant-coated RPLC modes. With non-ionic surfactants, only the polarity of the stationary phase changes, whereas with ionic surfactants, a net charge (positive or negative) appears on its surface, with major implications. Also, the adsorption of surfactant monomers on the stationary phase reduces silanophilic interactions.

Surfactant adsorption on the porous RPLC packing affects drastically chromatographic retention, owing to the change of diverse surface properties of the stationary phase (e.g. polarity, structure, pore volume and surface area). Surfactant molecules coat the stationary phase pores, reducing appreciably their volume In RPLC, organic solvents associate to the bonded phase, changing its structure and properties. The alkyl chains of 1-propanol and longer n-alcohols have been found to interpenetrate the alkyl chains of the bonded phase to form a single monolayer, adopting a structure similar to that found with the surfactant monomers in MLC, with the hydroxyl group oriented towards the aqueous phase [30].

Organic solvents are added to micellar mobile phases to improve peak efficiencies and reduce retention times, giving rise to the so-called hybrid micellar mobile phases. Competition between alcohols and surfactant molecules for adsorption sites on the stationary

phase explains the linear reduction in the amount of adsorbed surfactant with increasing concentration of alcohol in the mobile phase. Mobile phases rich in organic solvent can sweep completely the adsorbed surfactant molecules from the bonded phase.

The surface of porous silica is covered with silanol groups (Si–OH), which are ionized in slightly acidic and basic media [31], being responsible for the polarity of silica. Less polar packings are obtained by derivatisation of the silanol groups, although some remain underivatised. The ionic interaction of positively charged species with free silanols is the main reason of the reduced efficiency and peak tailing in the separation of basic compounds with conventional C8 and C18 columns. This effect can be prevented by reducing the pH of the mobile phase to suppress silanol ionization.

Silanols seem to play a less important role with micellar mobile phases of the anionic SDS, due to screening by the adsorbed surfactant. Positively charged basic drugs interact with the negatively charged surfactant layer (fast process), without penetrating too much the alkyl-bonded layer to interact with the buried silanols (slow process). This results in improved efficiencies and peak tailing suppression [32]. MLC with conventional C18 columns and SDS has been reported to yield good performance in the analysis of several groups of basic drugs, such as β-blockers [33], phenethylamines [34], tetracyclines [35], and tricyclic antidepressants [36].

Note that cationic surfactants, such as CTAB, would not be effective in the analysis of basic drugs. Although a significant fraction of ammonium groups of CTAB is buried inside the C18 layer, the stationary phase is positively charged, and would repel the protonated basic drugs.

Micelles provide hydrophobic and electrostatic (for ionic surfactants) sites of interaction. In the micelles, three sites of solubilisation can be identified: the core (hydrophobic), the surface (hydrophilic) and the palisade layer (the region between the surfactant head groups and the core). Solutes associated to micelles experience a microenvironment that is different from that of bulk solvent. This is reflected by micelle-induced perturbations in the solute physicochemical properties, including changes in solubility, acidity, photophysical properties, and reaction rates [37].

Hybrid micellar mobile phases contain micelles, surfactant monomers, molecules of organic solvent (free or associated to the surfactant), and water. The organic solvent decreases the polarity of the aqueous solution and alters the micelle structure. Although the separation mode is still predominantly micellar in nature, the micelle is perturbed by the organic solvent. This can change micellar parameters, such as the CMC and surfactant aggregation number. A high percentage of organic solvent can disrupt the micelle structure, the maximal allowable concentration depending on the organic solvent and surfactant nature.

Organic solvents partition to the micellar aggregates, and the degree of association increases with their hydrophobicity. The same as solutes, organic solvent molecules can be located outside the micelles, entry into the palisade, or into the micelle core. The first two effects might favour the formation of micelles, whereas the latter can substantially increase the amount of organic solvent inside the micelle and produce a microemulsion [37].

Short-chain alcohols added to an ionic micellar mobile phase show an interesting range of behaviours [38]. Methanol, with the shortest carbon chain, is more polar and soluble than the other alcohols. It can solvate surfactant monomers more easily, which hinders micelle formation, and consequently, a greater amount of surfactant is required to form the micellar assemblies. As a result, the CMC increases with added methanol. The effect of ethanol and propanol is opposed to methanol. These alcohols remain mainly outside the micelles, dissolved in the bulk liquid, but they interact with the micelle surface, reducing repulsion among the ionic heads of the surfactant monomers. This favours the formation of micelles and reduces the CMC. Finally, butanol and pentanol are inserted into the micellar assembly, owing to their particular structure that combines a polar group with a non-polar chain, similarly to surfactant molecules. These alcohols align with the surfactant molecules in the micelle palisade, the polar hydroxyl group of the alcohol oriented towards the Stern layer and the alkyl chain located in the non-polar micelle core. This gives rise to swollen mixed micelles. Finally, it should be noted that the effect of acetonitrile on the CMC is similar to methanol, in spite of the different polarities and structures.

3.2 Solute–micelle and solute–stationary phase interactions

The unique capabilities of micellar mobile phases are attributed to the ability of micelles to selectively compartmentalize and organize solutes at the molecular level. However, the association of the surfactant monomers to the bonded phase has deep implications with regard to retention and selectivity. The chromatographic behaviour in an RPLC system of a solute eluted with a mobile phase containing a surfactant above the CMC can be explained by considering three phases: stationary phase, bulk solvent and micellar pseudophase. Solutes are separated on the basis of their differential partitioning between bulk solvent and micelles in the mobile phase or surfactant-coated stationary phase. For water-insoluble species, partitioning can also occur via direct transfer of solutes between the micellar pseudophase and the modified stationary phase. The partitioning equilibria in MLC can be described by three coefficients: PWS (partition between aqueous solvent and stationary phase), PWM (between aqueous solvent and micelles), and PMS (between micelles and stationary phase). The coefficients PWS and PWM account for the solute affinity to the stationary phase and micelles, respectively, and have opposite effects on solute retention: as PWS increases the retention increases, whereas as PWM increases the retention is reduced due to the stronger association to micelles.

The retention behaviour depends on the interactions established by the solute with the surfactant-modified stationary phase and micelles. Neutral solutes eluted with non-ionic and ionic surfactants, and charged solutes eluted with non-ionic surfactants will only be affected by non-polar, dipole–dipole and proton donor–acceptor interactions [39]. Besides these interactions, charged solutes will interact electrostatically with ionic surfactants (*i.e.* with the charged surfactant layer on the stationary phase and the charged outer layer of micelles). In any case, the steric factor can also be important.

With ionic surfactants, two situations are possible according to the charges of solute and surfactant: repulsion or attraction (by both surfactant-modified stationary phase and micelles). In the case of electrostatic repulsion, charged solutes cannot be retained by the stationary phase and elute at the dead volume, unless significant hydrophobic interaction with the modified bonded layer exists. In contrast, combined electrostatic attraction and

hydrophobic interactions with the modified stationary phase may give rise to strong retention in MLC. Mixtures of polar and non-polar solutes can be resolved, provided an appropriate surfactant is chosen.

3.3 Direct injection

MLC appears as a useful technique for the direct injection of physiological samples. The compatibility with conventional RPLC column packings is particularly attractive. Surfactant monòmers and micelles tend to bind proteins competitively [40], thereby releasing protein-bound drugs, which are free to partition into the stationary phase. Meanwhile, the proteins rather than precipitating on the column, are solubilized and swept harmlessly away eluting with or shortly after the solvent front. Moreover, surfactants are non-toxic, non-flammable, biodegradable with low pollution impact, and inexpensive in comparison to aqueous-organic solvents. The use of surfactants in direct injection is also much less complex than column-switching procedures which require additional instrumentation (precolumns, switching valves and HPLC pumps), and accurate and precise timing of valve switching for a successful separation.

In MLC, untreated physiological fluids directly injected into the HPLC system have been reported to be simple, allowing repetitive serial injections with no increase in system pressure, no noticeable clogging of the injection valve or analytical column, no changes in retention factors, or system contamination evident. However, the analytical column should be protected with a guard precolumn to saturate the micellar mobile phase with silica. Control of the precipitation of proteinaceous material in the column can be made by monitoring the pressure of the system, and by the daily injection of a probe solute to check for possible changes in retention.

4. References

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Chapter 2

Objectives

L'objectiu principal de la investigació ha estat el desenvolupament de una sèrie de mètodes analítics senzills, ràpids, fiables i barats, basats en MLC, per a la identificació i quantificació de antirretrovirals i antivirals, inclosos en HAART, en plasma i sèrum, extrets de malats de SIDA. En primer lloc, es considera el desenvolupament de un mètode de cribat per a la detecció dels 13 fàrmacs més utilitzats en protocols hospitalaris en el tractament de la SIDA. Es podrà aplicar en pacients on no es conega o es tinguen dubtes dels medicaments que prenen. Posteriorment, es proposaran mètodes per a la monitorització de la mescla de fàrmacs (tres o quatre) inclosos als cinc tractaments HAART més prescrits. Aquestos mètodes es podran emprar per a fer el seguiment de pacients als que s'ha prescrit un determinat conjunt HAART. En aquestos casos, no cal usar el mètode de cribat, ja que només es poden trovar els antirretrovitals de un tractament HAART específic. La informació proporcionada pels mètodes té una gran importancia en el àmbit de la salud clínica, ja que pot ser utilitzada pels professionals mèdics per a determinar aspectes importants del tractament i estudiar el seu desenvolupament en pacients específics.

Els mètodes han de ser aplicables a l'anàlisi rutinari de aquestes mostres en laboratoris hospitalaris. És a dir, han de ser capaços de procesar successivament una gran quantitat de mostres de plasma i sèrum en poc de temps, amb un grau elevat de automatització. També resulta interesant minimitzar la quantitat de reactius tòxics utilitats, per a incrementar la seguretat al laboratori i reduïr l'impacte mediambiental dels anàlisis. Tanmateix, s'ha d'incidir en la disminució del cost de cada anàlisi, ja que, en aquestos temps de crisi econòmica, és important que els hospitals puguen reduir el seu pressupost sense que la qualitat dels serveis es veja afectada. Per altra banda, la sensibilitat ha de ser suficient per a quantificar els analits a les concentracions en la que es poden trobar en plasma i sèrum extret de malats de SIDA que prenguen aquestos fàrmacs.

La característica de l'MLC més útil per a l'anàlisi de mostres de plasma és la capacitat dels monòmers de SDS i de les micel·les de desnaturalitzar les proteïnes, portant-les a la seua estructura primaria, i la solubilització de compostos hidròfobs. Així doncs, la matriu es pot injectar directament, després de una simple dilució la dissolució micel·lar i filtració, al sistema cromatogràfic, sense el risc de precipitació en la columna. A més a més, la possibilitat de resolució de mescles complexes emprant fases mòbils circulant en mode

isocràtic és molt interessant. Aquestes característiques es poden considerar com a les que més avantatges aporten enfront d'altres tècniques revisades a la bibliografia, que, per les seues característiques, necessiten instrumentació més cara, llargs procediments previs d'extracció i ús de gradients. Això provoca un increment del temps necessari per a l'anàlisi, de la quantitat de reactius contaminants usats, i exigeix una manipulació més extensa de reactius i mostres part del personal de laboratori. Tot això provoca finalment un increment en el preu final de l'anàlisi, i introdueix més fonts de variança, disminuint la reproduïbilitat i la exactitut.

Un altre dels objectius ha estat la validació dels mètodes seguint els criteris definits per les guies oficials de validació. Aquesta etapa és clau en el desenvolupament del mètode, ja que és una proba de la fiabilitat de les concentracions de antirretrovirals i antivirals mesurats, i ens indica el rang de la concentració i la mastriu a la que es pot aplicar. Per tant, és imprescintible per a autoritzar el seu ús en mostres reals, considerant el cost econòmic i l'efecte de les decissions preses pels metges en funció de aquestes mesures a la salut del pacient.

Per a assolir aquestos objectius generals, es proposen les següents objectius específics, que son comuns a tots els treballs:

- Estudi dels paràmetres fisico-químics dels analits (pKa, hidrofobicitat, solubilitat, propietats espectrofotomètriques).
- Establir les condicions cromatogràfiques generals (fase estacionària, volum d'injecció, cabdal...)
- Optimització la composició de la fase mòbil (concentració de tensioactiu, modificador orgànic i pH) per a resoldre la mescla d'analits i evitar que es solapen amb compostos de la matriu, en el mínim temps d'anàlisi i circulant en mode isocràtic.
- Optimització de les condicions de dectecció (longituds d'ona màximes de absorció en medi micel·lar).
- Optimització de la dilució inicial òptima de la mostra amb el medi micel·lar.
- Selecció de la guia de validació adequada: les regulacions de la US Food and Drug Administration (FDA), i de la International Conference on Harmonization (ICH) Guideline, especialment dedicades a l'anàlisi de fàrmacs.
- Determinació dels paràmetres de validació: selectivitat, sensibilitat (límit de detecció i de

quantificació), linealitat, intèrval de calibratge, precissió i exactitut intra- e intedia, recuperació, robustessa i estabilitat. Les guies indiquen cóm es determina cada paràmetre, així com els valors que han de tindre per a considerar-se aptes. A més a més, el mètode ha de ser capaç de detectar les concentracions mínimes de antirretrovirals i antivirals que es puguen trobar al plasma i que siguen d'interés per als metges.

- Aplicar el mètode a mostres de plasma extret de malats de SIDA que prenen algún tractament HAART. Aquestes mostres estaran proporcionades per el Hospital La Plana de Vil·la-real, amb el consentiment de pacients i metges.

Chapter 3

Screening and monitoring antiretroviral and antiviral in the serum of acquired immunodeficiency syndrome patients by micellar liquid chromatography

Abstract

Thirteen different antiretroviral are commonly used in hospital protocols for suppressing the activity of the human immunodeficiency virus (HIV) and associated opportunistic diseases in patients with acquired immunodeficiency syndrome (AIDS). In this work, three micellar mobile phases are recommended for screening these substances, using UV detection, and the process can be performed in less than 18 min. The first mobile phase (sodium dodecyl sulfate or SDS 50 mM) is used for the group consisting of acyclovir, didanosine, ganciclovir, stavudine and zidovudine. The second mobile phase (SDS 120 mM/4.5% 1-propanol) is used for the group containing abacavir, lamivudine, nevirapine, valaciclovir and zalcitabine, whereas the third mobile phase (SDS 150mM/5% 1-pentanol) is used for efavirenz, indinavir and ritonavir. The use of micellar liquid chromatography (MLC) as an analytical tool allows serum samples to be injected directly. The method was validated over the range of 0–10 μg mL^{-1}. The limits of detection (signal-to-noise ratio of 3), which ranged from 6 to 30 ng mL^{-1}, were adequate for monitoring these substances. Intra- and interday relative standard deviations of the assay were below 3% for all compounds. The recoveries in spiked serum samples were in the 89.5–104.4% range. The method can be applied to the screening, monitoring and control of patients' treatment with antiretroviral and antiviral.

1. Introduction

Twenty-six years ago, reports were published on fatal cases of rare opportunistic infections. The disease, which was caused by the acquired immunodeficiency virus 1 (HIV-1), then became known as acquired immunodeficiency syndrome (AIDS) [1–4]. Various attempts have been undertaken since then to find a cure for the disease, but with limited success. In 1996, highly active antiretroviral therapy (HAART) was introduced, with impressive clinical results [5–8]. Generally speaking, HAART regimens (Fig. 3.1) contain two nucleoside reverse transcriptase inhibitors (NRTIs), such as abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, zalcitabine and zidovudine, one non-nucleoside reverse transcriptase inhibitor (NNRTI), such as nevirapine and efavirenz, or protease inhibitors (PI), such as amprenavir, atazanavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir [9]. On the other hand, due to the fact that viral infection with cytomegalovirus is one of the most serious problem for patients with AIDS, the drugs acyclovir, ganciclovir and valaciclovir are also coadministered to these patients [10].

Therapeutic drug monitoring (TDM) involves taking a blood sample to measure the amount of a particular PI and/or NNRTI. Most experts believe that measuring the levels of NRTIs will be of little value, as they block HIV inside the cell. Drug levels found in blood might not necessarily compare to those inside cells. TDM may be particularly useful for protease inhibitors, as their levels in blood can vary greatly from one individual to another because there are differences in howpeople break down (metabolize) these drugs. Ensuring that people stay within a “therapeutic range” may greatly improve the likelihood of a lasting anti-HIV response. TDM may also help determine the proper dose of a drug for a particular person [11].Moreover, studies have proved the existence of a series of relations between plasmatic levels of drug and (a) significantly increased levels of fasting triglyceride and cholesterol by the continued use of lopinavir/ritonavir; (b) renal disorders in the case of indinavir; and (c) liver toxicity for nevirapine. No such relationships were established with other conditions, such as exanthem in the case of NNRTIs or hyperlipidemia [12–14].

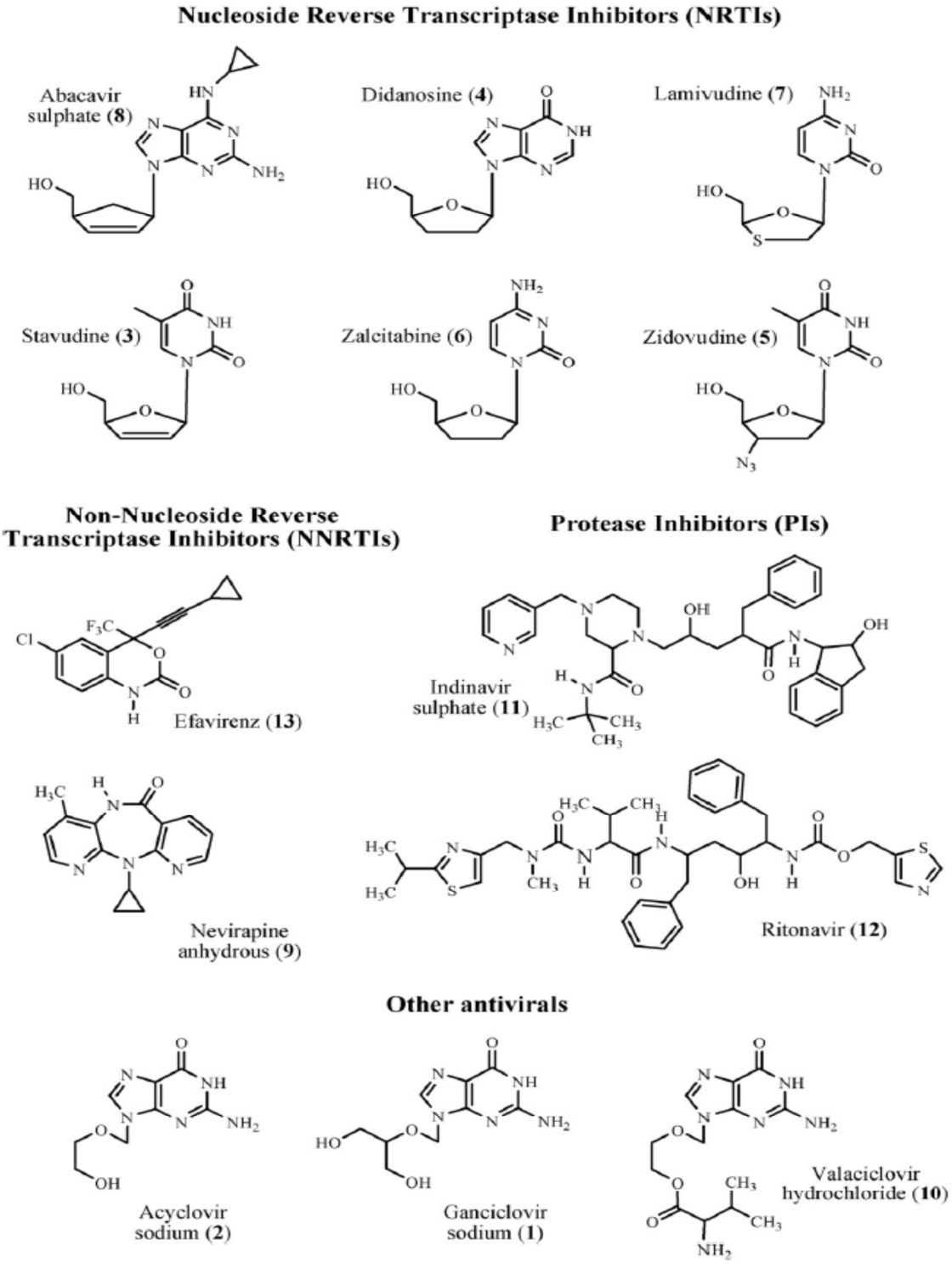

Fig. 3.1. Chemical structures of the investigated drugs.

On the other hand, the lack of compliance to HAART is the first cause of therapeutic

failure. The virological characteristics of the HIV are such that when there are subtherapeutic levels of antiretroviral drugs, the virus can multiply and develop resistance, which is why a level of treatment compliance above 95% is necessary [15–17]. In addition, poor compliance is associated with a poor immunological response [18] and a greater risk of mortality [15,19].

Specific and sensitive analytical methods are needed for simultaneously determining serum concentrations of as many HIV medications as possible. A clinician can use such methods to provide valuable information about several aspects of patient treatment, including malabsorption, drug interactions, fulfillment of the treatment and individual drug pharmacokinetics, as well as therapeutic drug level monitoring [20–22].

Therefore, an analytical method for their determination in serum on a routine basis could constitute a useful clinical tool. In this regard, analytical methods have already been described to quantify single [23,24] and combined [20–22,25–27] anti-HIV agents in human serum. Furthermore, each method (individual or simultaneous) involves a previous sample preparation procedure: liquid–liquid [22,23] or solid–liquid extraction [20,21,26,27] or deproteinisation [24,25]. Such methods increase the difficulty, in both time and costs, of quantifying all the drugs taken by one single HIV-infected patient undergoing multiple therapy with drugs from different therapeutic classes. In addition, many methods use HPLC instruments together with either column-switching techniques [26] or mass spectroscopy [28,29], which are not commonly available in conventional hospital laboratories.

Micellar liquid chromatography (MLC) is an alternative to these methods [30] for drug determination in physiological fluids. The use of surfactants in direct injection is much less complex. The sodium dodecyl sulfate (SDS) micelles tend to bind proteins competitively, thereby releasing protein-bound drugs. Therefore, the drugs are free in the stationary phase, whereas the proteins, rather than precipitating in the column, are solubilised and elute with or shortly after the solvent front. MLC has recently proved itself to be a useful technique in the control of diverse groups of substances in serum with direct injection of the samples, such as vitamins [31], antiepileptic drugs [32], benzodiazepines [33], barbiturates [34] and stimulants [35]. Finally, compared to other eluents, the micellar mobile phases [36,37] are less flammable, inexpensive, non-toxic, biodegradable, and can co-solubilise hydrophobic and

hydrophilic analytes in complex matrices like serum.

This paper describes the studies carried out to develop and validate a fast, simple method for monitoring 13 antiretroviral and antiviral used in the treatment of patients with AIDS, using three mobile phases that contained sodium dodecyl sulfate alone or with 1-propanol, 1-butanol or 1-pentanol and with direct injection of human serum.

2. Experimental

2.1 Chemicals and reagents

The antiviral studied were: abacavir sulfate, valaciclovir hydrochloride (Glaxo Smith Kline, Brentford, UK), acyclovir sodium, ganciclovir sodium (Roche Farma, Barcelona, Spain), didanosine, efavirenz, indinavir sulfate, lamivudine, nevirapine anhydrous, zalcitabine and zidovudine (Filaxis, Córdoba, Argentina), ritonavir (Abbot Laboratories, North Chicago, IL, USA) and stavudine (Bristol-Myers Squibb, New York, NY, USA). SDS, 1-propanol, 1-butanol, 1-pentanol, disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). Stock solutions and mobile phases were prepared in ultrapure water (Simplicity UV, Millipore Molsheim, France).

The blood samples used for spiking were taken from five healthy volunteers; these samples were centrifuged at 3000 rpm for 10 min and serum was finally separated and either used immediately or frozen and stored at −20 ºC. The antiviral, the antiretroviral and the blood samples were provided by the La Plana Hospital in Vilareal and the General Hospital in Castelló, after consent had been obtained from the Ethical Committee and from patients. Serum solutions were injected into the chromatography system with no treatment other than filtration. The solutions and the mobile phases were filtered through 0.45 μm nylon

membranes (Micron Separations, Westboro, MA, USA).

2.2 Instrumentation

The chromatographic system used for the optimization procedure and for method validation was an Agilent Technologies Model 1100 (Palo Alto, CA, USA). It was equipped with a quaternary pump, an autosampler with 2mL vials fitted with a Rheodyne valve (Fitatu, CA, USA) and a diode array detector (range 190–700 nm). A Kromasil 100 C18 column (5 μm particle size, 250 mm × 4.6 mm I.D.) from Scharlab (Barcelona, Spain) thermostated at 25 ◦C was used in the separations. The flow rate and injection volume were 1mL min^{-1} and 20 μL, respectively. The detection wavelengths were 260 nm for acyclovir, didanosine, ganciclovir, stavudine and zidovudine (group A) and abacavir, lamivudine, nevirapine, valaciclovir and zalcitabine (group B), and 214 nm for efavirenz, indinavir and ritonavir (group C). The signal was acquired by a personal computer connected to the chromatograph by means of a Hewlett-Packard Chemstation. When the mobile phase changed, a period of 30 min was required to reequilibrate the column and to obtain a stable baseline.

A GLP 22 potentiometer (Crison, Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode was used to measure pH values. The balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland).

2.3 Preparation of solutions, samples and mobile phases

For optimization studies, stock solutions of each antiretroviral or antiviral were prepared in methanol–water 5:95 (v/v). Working solutions were prepared by diluting these stock solutions in the mobile phase. All stock and working solutions stored at +4 ºC were stable for at least 3 months, which was confirmed by measuring the chromatographic signal. For preparation of the serum sample, 0.5 mL of serum was diluted with an appropriate

amount (0.01–1 mL) of the stock solutions and mobile phase (final volume of 5 mL) and injected into the chromatograph with no pretreatment other than filtration.

The micellar mobile phase was prepared using SDS, which was buffered with disodium hydrogen phosphate/sodium dihydrogen phosphate 10 mM at pH 7, and lastly 1-propanol, 1-butanol or 1-pentanol was added to achieve the desired concentration.

Solutions of potential interfering drugs were prepared from clear filtered extracts of the pharmaceutical formulations. These solutions were prepared in methanol–water 5:95 (v/v) and diluted with mobile phase before injection into the MLC system at concentrations of 2 μg mL^{-1}.

3. Results and discussion

3.1 Optimization strategy and mobile phase selection

In order to find the best mobile phase composition that allows the 13 antiretroviral and antiviral considered in this study to be analyzed simultaneously, each of them was injected in mobile phases at pH 7 containing SDS (mM)/modifier (%): SDS/1-pentanol (50/1; 50/5; 75/3; 100/3; 125/3; 150/1; 150/5); SDS/1-butanol (50/1, 50/7, 75/4.5, 100/4, 125/4.5, 150/1, 150/7); SDS/1-propanol (50/2.5, 50/12.5, 75/7.5, 100/7.5, 125/7.5, 150/2.5, 150/12.5) and finally, SDS (mM) pure micellar phase (50, 125, 100, 125 and 150). The peaks obtained in these mobile phases allowed us to obtain measurements of the chromatographic parameters for each antiretroviral and antiviral: retention time (t_R), capacity factor (k), efficiency (N) and asymmetry factor (B/A). These data were used together with a mathematical model and an interpretative optimization procedure to predict the behaviour of the peaks corresponding to the antiretroviral and antiviral [38]. For all the studied antiretroviral, the behaviour observed was: retention factors and efficiencies decreases when the concentration of the surfactant increases, but when the concentration of modifier increase, the retention factor decrease but

the efficiency increase.

When attempting to establish an optimization criterion, the possibility of using the same mobile phase to determine the 13 antiretroviral and antiviral is impossible because of the overlapping that occurs between some of the compounds: abacavir and stavudine, and zidovudine or indinavir with valaciclovir. Since many of the drugs share certain structural characteristics and have very similar physicochemical properties, a good resolution was not obtained between all of them and thus the decision was taken to form groups under the criteria of maximum resolution and efficiency and minimum analysis time.

In liquid chromatography, interpretive optimization strategies are more efficient and reliable than sequential approaches. The methodology followed by chromatographers can be mimicked by these strategies, which can be assisted by computer simulation, thus reducing the amount of time and effort required. MLC is capable of predicting the retention of compounds using simple equations. Different mathematical models can be used to describe the retention of analytes. The model employed in this work was the following Eq. (3.1) [36]:

$$k = \frac{K_{AS}\dfrac{1}{1 + K_{AD}\varphi}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}[M]} \qquad \textbf{(3.1)}$$

where [*M*] and φ are the concentrations of surfactant and modifier; K_{AS}, K_{AM}, K_{MD}, K_{SD}, and K_{AD} correspond to the equilibria between the solute (*A*) in the stationary phase (*S*), micelle (*M*), or bulk water (D). This equation was non-linearly fitted according to the Powell method [37] using the retention data from injections (*n*=3) of the drug solutions in several mobile phases. To avoid inconsistent results, the experimental design should have at least one mobile phase more than there are parameters in the equation; in this case, it consisted of seven mobile phases as described earlier. The prediction capacity of the mathematical model was evaluated as the differences between the experimental and predicted capacity factors for the antiaretroviral, expressed as the relative global error [38]. Using Eq. (3.1) and the

mathematical treatment described here, the relative global error in the prediction of retention factors was below 2% for the 13 substances studied. Adjusting the coefficients that were calculated to Eq. (3.1) for each substance allows the mobile phase composition to be predicted for any desired retention time and also provides a simple way to optimize the separation of mixtures. It must be noted that, in the analysis of physiological fluids, the retention time of the endogenous compounds and the protein band at the head of the chromatogram should be considered when selecting the mobile phase.

The optimization procedure, which maximized the resolution in the separation of the drugs, was used to select the most suitable mobile phases. The mobile phases allowing for the separation and quantification of the components in each group in an acceptable analysis time will be useful to analyze the serum samples.

The resolution diagram data (results unshown) are obtained by the option *Evaluate grid* in the Michrom program [36–38]. This option calculates the resolution matrix. This matrix may contain one, two or three experimental factors. In the present case, two experimental factors (surfactant and modifier) were studied and thus two-dimensional matrices were obtained. The maximal resolution value found is given during the calculation process, together with the composition of the corresponding mobile phase. Once the calculation of the matrix has finished, the active mobile phase for the simulations and other calculations will be the optimal phase that was found. The grid, thus calculated, has the extension GRD and contains all the information about the series needed to build the diagrams. The grids could be exported to allow retrieval by other programs such as *Surfer* (RockWare Europe, Cureglia, Switzerland).

After this mathematical treatment of the data for all the substances, the mobile phases chosen were: SDS 50mM for acyclovir, didanosine, ganciclovir, stavudine and zidovudine; SDS 120 mM/4.5% 1-propanol for abacavir, lamivudine, nevirapine, valaciclovir and zalcitabine, and SDS 150mM/5% 1-pentanol for efavirenz, indinavir and ritonavir. Using these mobiles phases and under the criteria of maximum resolution and efficiency–minimum analysis time, the values of the parameters for each antiviral are summarized in Table 3.1. All the asymmetry factors are around 0.75 and most of the efficiency values are above 6000, the

maximum efficiency being 11 127 for zidovudine.

3.2 Method validation

3.2.1 Linearity, limit of detection (LOD) and quantification (LOQ)

The standard curves for all compounds were satisfactorily described by unweighted least-square linear regression. Calibration curves were constructed using the areas of the chromatographic peaks (triplicate injections) obtained at eight different concentrations, in the 0–10 μg mL^{-1} range for all the compounds, in the line of real samples. This interval is adequate for these drugs, in which peak-valley concentrations are in the 0.5–5 μg mL^{-1} range. The detection limit (LOD) of a method, is the lowest antiretroviral concentration in serum, that produces a response that is detectable above the noise level of the system, typically taken as being three times the noise level (3 s *criterion*). The quantification limit (LOQ) of a method, is the lowest antiretroviral concentration in serum, that can be identified and quantitatively measured, typically taken as being 10 times the noise level (10s *criterion*). Table 3.2 shows the slopes, intercepts and regression coefficients (r^2 > 0.999) of the calibration curves, the LODs (using the 3 s *criterion*) and LOQs (with the 10s *criterion*) determined after measurements in 20 injections of a serum blank. The results of LOD and LOQ were based on the standard deviation of the response, and on the slope of a specific calibration curve containing the substance. The LOD and LOQ of each antiretroviral and antiviral ranged from 6.0 to 30.2 ng mL^{-1} and 20.1 to 100.5 ng mL^{-1}, respectively, and were therefore comparable to (or were even better than) reported methods. These values were good enough to monitor the compounds under study in serum matrices.

Table 3.1. Mean values (n = 3) for retention time, capacity factor, efficiency and asymmetry of the studied drugs.

Compound	t_R	K	N	B/A
Abacavir	4.1	1.75	5 200	0.73
Acyclovir	2.8	1.09	8 800	0.73
Didanosine	6.4	3.8	10 900	0.85
Efavirenz	1.7	1.06	6 350	0.81
Ganciclovir	2.4	0.74	8 700	0.77
Indinavir	8.8	5.0	5 350	0.77
Lamivudine	2.8	0.83	9 500	0.71
Nevirapine	7.1	3.7	6 500	0.76
Ritonavir	9.9	6.4	3 400	0.78
Stavudine	4.1	2.0	8 700	0.76
Valaciclovir	8.2	4.4	9 300	0.76
Zalcitabine	2.5	0.62	8 800	0.71
Zidovudine	7.8	5.5	11 150	0.75

Table 3.2. Calibration with 6 points (n = 6) and each point is the mean of three injections (m= 3): slope, intercept, regression coefficient (r^2), limit of detection (LOD, 3s criterion) and quantification (LOQ, 10s criterion) for the antiretroviral and antiviral that were studied.

Compound	Slope	Y-intercept	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
Abacavir	35.4 ± 0.5	3.3 ± 2.1	0.9993	150	450
Acyclovir	66.2 ± 0.7	8.3 ± 4.5	0.9995	60	180
Didanosine	38.2 ± 0.2	2.2 ± 0.2	0.9998	100	300
Efavirenz	55.3 ± 0.4	−9.4 ± 2.2	0.9995	80	240
Ganciclovir	64.6 ± 0.5	3.7 ± 5.1	0.9995	55	150
Indinavir	29.1 ± 0.4	−3.3 ± 5.8	0.9995	165	480
Lamivudine	35.5 ± 0.1	7.1 ± 1.8	0.9999	90	280
Nevirapine	30.2 ± 0.1	−5.6 ± 5.5	0.9997	120	360
Ritonavir	42.5 ± 0.5	12.2 ± 0.5	0.9995	75	210
Stavudine	51.1 ± 0.3	−4.4 ± 10.2	0.9995	65	200
Valaciclovir	48.1 ± 0.3	5.2 ± 3.3	0.9996	68	200
Zalcitabine	45.9 ± 0.4	−4.5 ± 1.1	0.9999	70	210
Zidovudine	42.2 ± 0.2	−6.4 ± 9.2	0.9997	72	220

3.2.2 Precision

Precision, defined as the relative standard deviation, was determined by intra- and inter-day assays. These were determined at low, medium and high concentrations, according to the calibration curve ranges (Table 3.2). The intra-day variability of the assay was tested on 20 samples (n = 20) over three different concentrations for each compound for 20 separate days over a 4-months period; inter-day variability was tested using the same procedure. The method proved to be precise and the intra-day results ranged from 0.17 to 1.89% and inter-day values ranged from 0.21 to 2.98%.

3.2.3 Selectivity

An interfering drug is defined as a molecule which exhibits a retention time close to 0.3 min with the substances studied here. The drugs that are most commonly prescribed together with the antiretroviral and antiviral are: acebutolol, acetylprocainamide, acetaminophen, acetylsalycilic acid, amiodarone, amoxicillin, ampicillin, atenolol, azithromycin, bromazepam, caffeine, captopril, carbamazepine, ciprofloxacin, clonazepam, chloramphenicol, cocaine, codeine, corticosterone, desipramine, dexamethasone, diazepam, diltiazem, doxycycline, ephedrine, flunitrazepam, furosemide, gentamicin, hydrocortisone, imipramine, levofloxacine, lidocaine, loratadine, medazepam, methotrexate, nicotine, nifedipine, norfloxacin, oxazepam, phenylefrine, propranolol, quinidine, sulphametoxazole, sulphathiazole, tetracycline, theophylline and valproic acid. In our laboratory, a solution containing 1 μg mL^{-1} of these substances was injected in the three mobile phases recommended for monitoring and quantifying antiretroviral and antiviral. In these studies and under the criteria previously defined, none of these substances were seen to interfere with the determination of the antiretroviral and antiviral studied in this work using the recommended micellar liquid chromatography conditions. Finally, after injection of real serum samples in the three mobile phases recommended in this work, and comparison with blank serum samples and spiked serum samples (containing the standard solutions of the 13 drugs), no

metabolites or impurities of the drugs could be detected in the chromatograms.

3.2.4 Analysis of spiked and real serum samples

The recoveries of all drugs were estimated by comparing peak areas in extracted spiked drug-free serum with those of standard solutions. MLC is a reliable way to eliminate material from serum, with satisfactory recovery rates (range from 92.2 to 104.4%), except for the relatively low recovery for indinavir (range from 84.5 to 94.1%). Table 3.3 summarizes the recoveries from serum samples at two different spiking levels. Fig. 3.2 shows chromatograms of spiked serum corresponding to the three groups of substances. In the first case, an endogenous peak at a t_R of less than 2 min (Fig. 3.2A) does not interfere with the peaks of the antiretroviral and antiviral. In the second group, in addition to this peak, there are other small peaks eluting between lamivudine and abacavir (Fig. 3.2B) that do not interfere with these compounds. Finally, in the third group, we can observe peaks in/with the solvent front, which do not interfere with their determination either. Some of the changes observed in Fig. 3.2 A–C are due to the fact that throughout the chromatograms some changes in wavelength are included in the chromatographic method.

Table 3.3. Recovery ± SD (%, n = 5) in spiked serum samples: c_1 and c_2 were 1 and 5 μg mL^{-1} for all substances except for efavirenz, indinavir and ritonavir (2 and 4 μg mL^{-1}).

Compound	c_1	c_2	Compound	c_1	c_2
Abacavir	97.9 ± 0.40	96.7 ± 1.1	Nevirapine	97.4 ± 0.15	100.5 ± 0.40
Acyclovir	94.0 ± 4.1	98.6 ± 0.10	Ritonavir	99.2 ± 4.2	104.4 ± 0.30
Didanosine	102.4 ± 1.6	101.2 ± 0.40	Stavudine	101.0 ± 0.75	100.2 ± 0.45
Efavirenz	101.4 ± 0.40	100.0 ± 0.88	Valaciclovir	95.9 ± 1.1	96.3 ± 0.85
Ganciclovir	104.1 ± 0.65	104.0 ± 1.5	Zalcitabine	96.4 ± 1.2	92.2 ± 0.10
Indinavir	84.5 ± 28.1	94.1 ± 3.9	Zidovudine	95.6 ± 0.10	96.5 ± 0.10
Lamivudine	101.7 ± 0.85	97.4 ± 0.50			

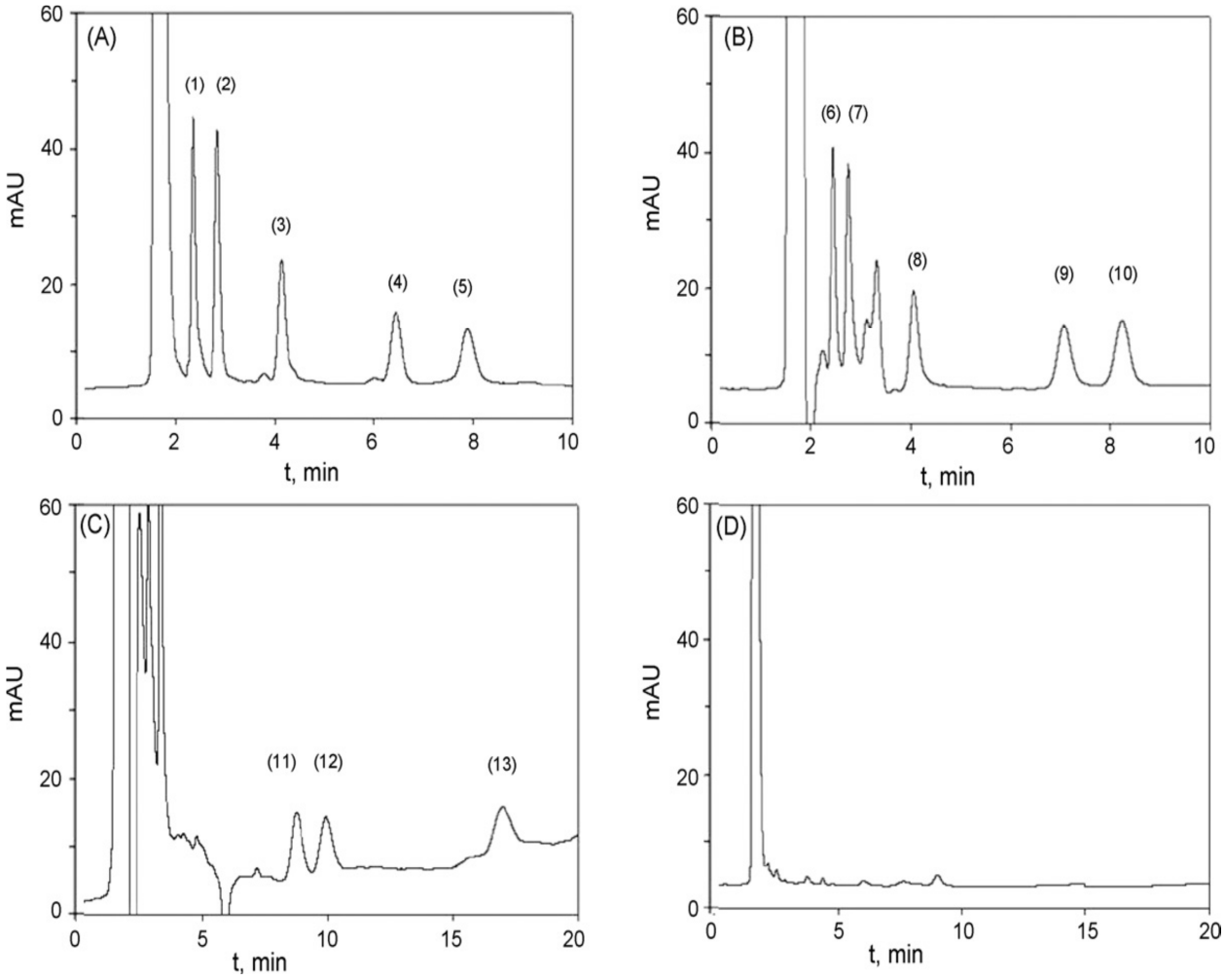

Fig. 3.2. Chromatograms showing spiked serum samples (A–C) and a serum blank (D). In (A) the mobile phase was SDS 50 mM and substances are (1) ganciclovir, (2) acyclovir, (3) stavudine, (4) didanosine, (5) zidovudine at 2 μg mL^{-1} for each antiretroviral or antiviral drugs; retention times: 2.3, 2.8, 4.1, 6.4 and 7.9, respectively. In (B) the mobile phase was SDS 120mM/4.5% 1-propanol, and the substances: (6) zalcitabine, (7) lamivudine, (8) abacavir, (9) nevirapine, (10) valaciclovir at 2 μg mL^{-1} for each antiretroviral or antiviral drugs, retention times: 2.4, 2.8, 4.1, 7.1 and 8.2, respectively. In (C) the mobile phase was SDS 150mM/5% 1-pentanol, and the substances (11) indinavir, (12) ritonavir, (13) efavirenz at 4 μg mL^{-1} for each antiretroviral drug, retention times: 8.8, 9.9 and 17.0, respectively.

Finally, the assay was used for the determination of these substances in HIV serum patients and results are shown in Table 3.4 and Fig. 3.3.

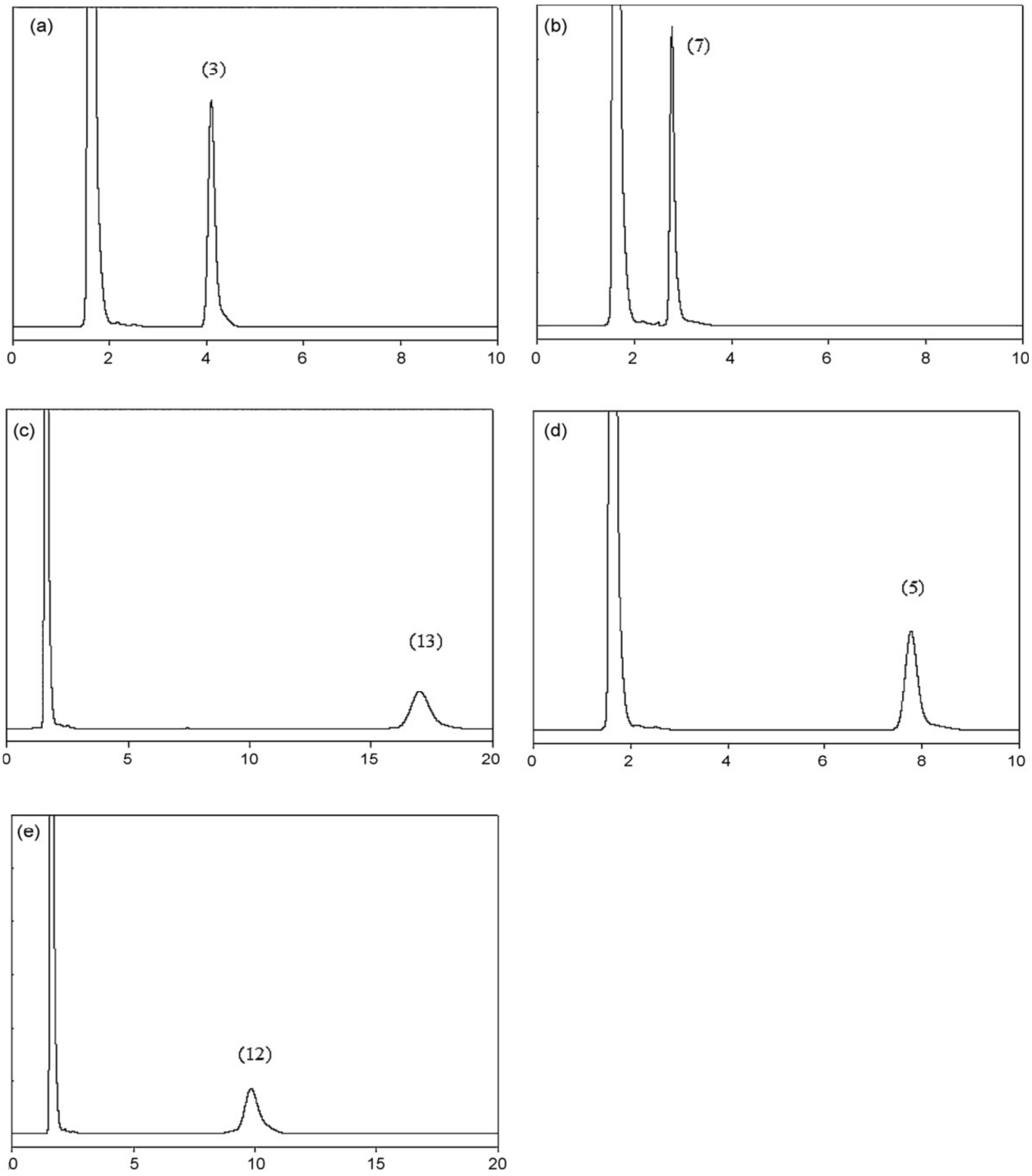

Fig. 3.3. Chromatograms showing the screening of real serum samples for two patients of AIDS—first patient: chromatograms (a), (b) and (c) show the separation of stavudine (3), lamivudine (7) and efavirenz (13), with mobiles phases A, B and C, respectively. For the second patient, chromatograms (d) and (e) show the separation of zidovudine (5), and ritonavir (12), with mobile phases A and C, respectively.

Table 3.4. Determination (n = 3) of antiviral and antiretroviral in the serum of HIV patients.

Patient	Compound	MLC (ng mL^{-1})	HPLC [a] (ng mL^{-1})
1	Zidovudine	708 ± 11	690 ± 18
	Ritonavir	627 ± 9	620 ± 12
2	Zidovudine	581 ± 15	565 ± 25
	Ritonavir	455 ±5	441 ± 10
3	Efavirenz	347 ± 11	325 ± 10
	Lamivudine	1007 ±5	998 ± 8
	Stavudine	871 ± 8	860 ± 15
4	Efavirenz	462 ±7	448 ± 16
	Lamivudine	873 ± 8	869 ± 9
	Stavudine	794 ± 18	781 ± 8

[a] Lamivudine and stavudine were determined using the method proposed in [21]; zidovudine, ritonavir and efavirenz were determined with the method described in [27].

4. Conclusions

The MLC assay described here offers an accurate, precise and highly reproducible procedure for the direct monitoring of 13 antiretroviral and antiviral drugs from different therapeutic groups in serum. Thanks to the absence of interferences, the methods are specific and reliable. This method does not require complex procedures such as sample extraction and/or sample cleaning. Chromatographic analyses require only 9, 9 and 18 min for the three recommended mobile phases, respectively. In addition, this method needs only simple chromatographic apparatus that is available in any hospital laboratory. Such monitoring is very important and useful to verify that correct dosages have been established, and also because patients' responses due to subtherapeutic and toxic concentrations are similar. These conditions make this method suitable for rapid monitoring and quantifying of antiretroviral and antiviral.

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Chapter 4

Monitoring of HAART regimen antiretroviral in serum of acquired immunodeficiency syndrome patients by micellar liquid chromatography

Abstract

A methodology based on MLC to monitor five antiretroviral drugs (lamivudine, stavudine, tenofovir, zidovudine and efavirenz) was proposed. Antiretroviral were studied in sets of three, corresponding to each highly active antiretroviral therapy (HAART) regimen, prescribed to acquired immunodeficiency syndrome (AIDS)-infected patients. Four aqueous micellar mobile phases buffered at pH 7 were optimized to separate these compounds, using sodium dodecyl sulfate as the tensioactive, and 1-propanol or 1-pentanol as organic modifier. The composition of each mobile phase was optimized for each antiretroviral. The common separation conditions were: C18 apolar column (125 x 4.6 mm, 5 μm particle size), UV detection set at 214 nm, and mobile phase running at 1 mL min^{-1} without controlling the temperature. The finally suggested method was validated for five analysed antiretroviral drugs following the US FDA guidelines in terms of: linearity between 0.5 and 50 μg/mL (r^2 > 0.9995), sensitivity (LOD lower than 0.25 μg/mL), intra- and inter-day precision (<7.1 and <5.2%, respectively) and accuracy (recovery 88.5–105.3% and 93.5–101.3%, respectively), as well as robustness (<6.5%). The proposed method was used to monitor the level of antiretroviral in the serum of AIDS patients. The suggested methodology was found to be useful in the routine analysis of antiretroviral in serum samples.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a disease provoked by the human immunodeficiency virus 1 (HIV-1), which strongly affects the human immunological system [1]. Currently, HIV/AIDS is the fourth greatest cause of death worldwide. It is estimated that 40 million people are infected with HIV and 22 million have died of the disease [2]. HIV-1 is spread between by blood contact and sexual relationships [3] and through blood, amniotic fluid and/or breast milk from a mother to her child [4] A definitive cure has not been found, but in 1996, the highly active antiretroviral therapy (HAART) was proposed, with impressive clinical results in suppressing the activity of HIV [5]. HAART involves different kinds of antiretroviral drugs, such as: nucleoside reverse transcriptase inhibitors (NRTIs): abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir, zalcitabine and zidovudine; non-nucleoside reverse transcriptase inhibitors (NNRTIs): delavirdine, nevirapine and efavirenz; and protease inhibitors (PIs): amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir and tipranavir [6,7].

HAART regimens contain three or four drugs, two NRTIs and one or two NNRTIs, or one or two PIs. The hydrophobicity is assessed by the quotient of the solubility in octanol and in water (Po/w). A high value of Po/w indicates a high hydrophobicity [8]. The mixtures more administrated are: (a) lamivudine (log Po/w = - 1.4), zidovudine (log Po/w = 0.05) and efavirenz (log Po/w = 4.46); (b) lamivudine, stavudine (log Po/w = - 0.8) and efavirenz; and (c) lamivudine, tenofovir (log Po/w = - 1.6) and efavirenz. For each patient, one HAART set of antiretrovirals is prescribed [6,9].

Therapeutic drug monitoring (TDM) consists of the determination of the amount of a drug in the blood. This information can be used by the clinician to establish many aspects of patient treatment, such as malabsorption, drug interactions, individual drug pharmacokinetics and the way each person metabolizes the drug [10] This can explain the different effects of the drug depending on the patient and the possible secondary effects. On the other hand, the presence of sub-therapeutic levels of antiretroviral drugs in the blood may provoke the virus to develop resistance [11]. This can help to determine the proper dose to be taken for a particular person [6] However, as each patient takes only one of the HAART regimens, the

screening of a wide amount of antiretrovirals is large. Clinics need analytical methods to quantify the three antiretrovirals belonging to the corresponding HAART. The be able to optimize the methodology for a low number of analytes instead of a high number will allow the development of a methodology with a lower analysis time and more selectivity, sensitivity and resolution [12].

We have previously developed a methodology based on micellar liquid chromatography for the screening of 13 antiretroviral drugs by the use of only three mobile phases [6]. The use of micellar solvents is especially useful in samples with serum matrices, because serum contains hydrophobic compounds which are harmful for the chromatographic system [13–15]. Micellar media denaturalize and solubilize hydrophobic substances, releasing possible interferent-analyte adducts and avoiding precipitation into the columns [8,16]. After a simple dilution and filtration, samples are able to be directly injected, expediting the experimental procedure [17]. Our previously developed analytical method [6] can be adapted by the modification of the mobile phases in order to analyze three determined antiretrovirals. This way, this modified methodology will keep the benefits of micellar liquid chromatography, including the use of a low amount of pollutant chemicals [18].

The aim of this work is to develop an analytical methodology to determine each of the most used sets of three antiretroviral drugs used in HAART in serum. The selected HAART sets of antiretrovirals were: lamivudine + zidovudine + efavirenz, lamivudine + stavudine + efavirenz and lamivudine + tenofovir + efavirenz. The methodology must be validated in terms of linear interval, sensitivity, selectivity, limits of detection and quantification, precision, accuracy and robustness, following the guidelines of the FDA [19]. The developed analytical method was used to quantify the analytes in human serum from AIDS patients.

2. Experimental procedure

2.1 Chemicals and reagents

Sodium dodecyl sulfate (SDS, 99% purity) was obtained from Merck (Darmstadt, Germany). $NaH_2PO_4.H_2O$ and HCl were ordered from Panreac (Barcelona, Spain). NaOH came from Scharlab (Barcelona, Spain). Methanol was bought from J.T. Baker (Deventeer, The Netherlands) and 1-propanol and 1-pentanol came from Scharlab. An ultrapure water device (Millipore S.A.S., Molsheim, France) was used to prepare the aqueous solutions and mobile phases.

The antiretroviral studied were: efavirenz (E), lamivudine (L), zidovudine (Z) (purity >99.9%, Filaxis, Córdoba, Argentina), tenofovir (T), stavudine (S) (purity >99.9% Bristol Myers Squibb, New York, NY, USA). The antiretroviral were studied grouped in sets as follows: mixture 1 = L + Z + E; mixture 2 = L + S + E; and mixture 3 = L + T + E.

2.2 Equipment and chromatographic conditions

A Metter-Toledo analytical balance (Greifensee, Switzerland) was used to weigh the analyte. The pH was measured with a Crison potentiometer (Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. An ultrasonic bath was used to dissolve the standards (model Ultrasons-H, Selecta, Abrera, Spain).

Chromatographic separations were performed using an Agilent Technologies Series 1100 system (Palo Alto, CA, USA) equipped with an isocratic pump, a degasser, an autosampler and an absorbance diode array detector (DAD). The stationary phase was in a Kromasil C18 column with the following characteristics: pore size 100 Å, length 15 cm, internal diameter 4.6 mm, particle size 5 μm. Several mobile phases were tested by varying the SDS concentration, the amount of 1-propanol and 1-pentanol, and the pH. The optimal mobile phase composition for each antiretroviral and its corresponding retention time are shown in Table 4.1. In all cases, the pH was fixed to 7 using 0.01 M phosphate buffer.

Mobile phases run under the isocratic mode at 1 mL min^{-1} without controlling the temperature. The injection volume was 20 μL and detection were set at 214 nm. The solutions and the mobile phases were filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA). Special care was taken for the chromatographic system, due to the use of micellar solutions [8].

2.3 Blood collection

The blood samples used for spiking were taken from healthy, AIDS-infected and heroin addict AIDS-infected volunteers. These samples were collected with DB SST Tubes (BD Vacutainer Systems, Plymouth, UK), centrifuged at 3000 rpm for 10 min and the serum was finally separated and either used immediately or frozen and stored at - 20ºC. Blood samples were provided by the La Plana Hospital in Vilarreal and the General Hospital in Castelló, Spain, after consent had been obtained from the Ethical Committee and from patients.

2.4 Solution and sample preparation

Mobile phases were prepared by dissolving the appropriate amount of SDS and $NaH_2PO_4.H_2O$ in ultrapure water. Then the pH was adjusted by adding drops of HCl or NaOH solutions to reach the desired value. Furthermore, the appropriate volume of organic solvent (1-propanol or 1-pentanol) was added and the solution was adjusted to the desired volume with ultrapure water, ultrasonicated and filtered.

Stock solutions of each antiretroviral were prepared in methanol-water 5 : 95 (v/v). Working solutions were prepared by diluting these stock solutions in ultrapure water. All stock and working solutions stored at +4 ºC were stable for at least 3 months [6].

For optimization and validation studies, spiked serum samples were prepared by adding the appropriate volume of stock or working solution to 0.5 mL of serum and then

adjusting the final volume to 5 mL with a 0.05 M SDS aqueous solution buffered at pH 7. The sample was vigorously shaken to favor homogenization and stored for one day in the fridge at 5 ºC to favor the contact between analytes and the sample, and also solvent evaporation [20,21]. Then the serum was 1/10 diluted.

2.5 Statistical treatment of the data

In order to perform the optimization, the selected chromatographic parameters were studied: retention time (t_R: time at maximal value of absorbance signal; min); efficiency (N; number of theoretical plates); asymmetry (B/A; B = distance between t_R and the time at 0.1 x signal height at the end of the peak; A = distance between t_R and the time at 0.1 x signal height at the beginning of the peak). These parameters were calculated for each peak from chromatograms using Michrom software [22].

A chromatographic peak is considered as satisfactory if the retention time is low, but higher than 2 min (far enough from dead time), the efficiency is high and the asymmetry is close to 1. Optimization was made by the criterion of obtaining more satisfactory chromatographic peaks (see above) without overlapping [8].

3. Results and discussion

3.1. Optimization of the separation conditions

The separation conditions were changed from those proposed in ref. [6] in order to improve the chromatographic parameters (efficiency, asymmetry and retention time) by focusing the study in the three compounds of each HAART mixture potentially administrated to a patient, instead of a wide number of independent antiretroviral. The study was limited to isocratic running mobile phases, because no stabilization time is required between two injections, increasing the robustness and the number of analyzed samples per day. The

optimization was performed using standards 1/10-diluted in 0.05 M SDS at pH 7.

The stationary phase, the pH of the mobile phase and the detection wavelength were set as C18, 7 and 214 nm, respectively, because it was proven in [6] that these conditions provide an adequate separation and sensitivity. Moreover, the selected pH is inside the working pH of the column (1.5–9.5). The improvement of the chromatographic parameters was made by varying the mobile phase composition (SDS and organic modifier amount).

Lamivudine, zidovudine, stavudine and tenofovir are quite polar compounds, according to their low log Po/w. That means that using a C18 column and pure mobile phase would provide adequate resolution and retention time. However, a short chain alcohol (as 1-propanol) can be added in order to improve the chromatographic parameters. On the other hand, efavirenz has a high log Po/w, indicating that it is more hydrophobic, and would show a high retention time. In this case, an organic modifier (1-butanol or 1-pentanol) should be added to the SDS aqueous mobile phase to obtain useful chromatographic parameters. Then optimization of mobile phases was performed by testing a pure SDS aqueous mobile phase and adding 1-propanol, 1-butanol and 1-pentanol.

In order to find the most adequate mobile phase composition, each antiretroviral (20 μg/mL standard) was analyzed the following mobile phases, containing SDS mM/modifier %: 0.05/0; 0.10/0; 0.15/0; 0.05/1-propanol 2.5; 0.05/1-propanol 12.5; 0.1/1-propanol 7.5; 0.15/1-propanol 2.5; 0.15/1-propanol 12.5; 0.05/1-butanol 1; 0.05/1-butanol 7; 0.1/1-butanol 4; 0.15/1-butanol 1; 0.15/1-butanol 7; 0.05/1-pentanol 2; 0.05/1-pentanol 6; 0.1/1-pentanol 4;0.15/1-pentanol 2 and 0.15/1-pentanol 6. The obtained chromatographic peaks were used for optimization as indicated in Section 2.5.

The optimized mobile phases are indicated in Table 4.1, and the chromatograms obtained by the analysis of the standards under the selected chromatographic conditions are shown in Fig. 4.1.

Table 4.1. Composition of the optimum mobile phase and chromatographic parameters to separate the

antiretroviral belonging to each HAART set

Mixture	Antiretroviral	t_R[a], min	[SDS], M	Alcohol (amount, %)	N	B/A
1	Lamivudine	2.05 ± 0.06	0.05	1-propanol (2.5%)	2046	1.286
	Zidovudine	2.58 ± 0.05			2694	1.178
	Efavirenz	14.20 ± 0.15	0.05	1-pentanol (6%)	2569	1.062
2	Stavudine	2.65 ± 0.07	0.05	--	2462	1.202
	Lamivudine	4.96 ± 0.09			2172	1.287
	Efavirenz	14.20 ± 0.15	0.05	1-pentanol (6%)	2569	1.062
3	Lamivudine	2.69 ± 0.05	0.15	--	2009	1.103
	Tenofovir	4.01 ± 0.04	0.05	1-pentanol (6%)	2060	1.172
	Efavirenz	14.20 ± 0.15			2569	1.062

[a]n = 5

3.1.1 Separation conditions for lamivudine and efavirenz

As these two compounds are in the three mixtures, their results are firstly discussed.

Lamivudine shows a low retention time in all cases (according to its polarity). Mobile phases providing a retention time lower than 2.00 min were not considered. In this case, it elutes too close to the dead time, and the probability of overlapping with matrix compounds is high. Only a pure aqueous mobile phase, 0.05 M SDS/1-propanol 2.5% and 0.05 M SDS/1-butanol 1% were able to elute lamivudine with a retention time of more than 2 min.

Efavirenz provides a high retention time in all cases (in agreement with its low polarity). Mobile phases eluting the analyte with a retention time of more than 20 min were not considered. Indeed, a greater retention time would lower the efficiency and is not suitable for routine analysis, where a high amount of samples should usually be analyzed. Following this criterion, only 0.15 M/1-propanol 12.5%, 0.15 M/1-butanol 7%, 0.05 M SDS/1-pentanol 6%, 0.1 M SDS/1-pentanol 4% and 0.15 M SDS/1-pentanol 6% were considered.

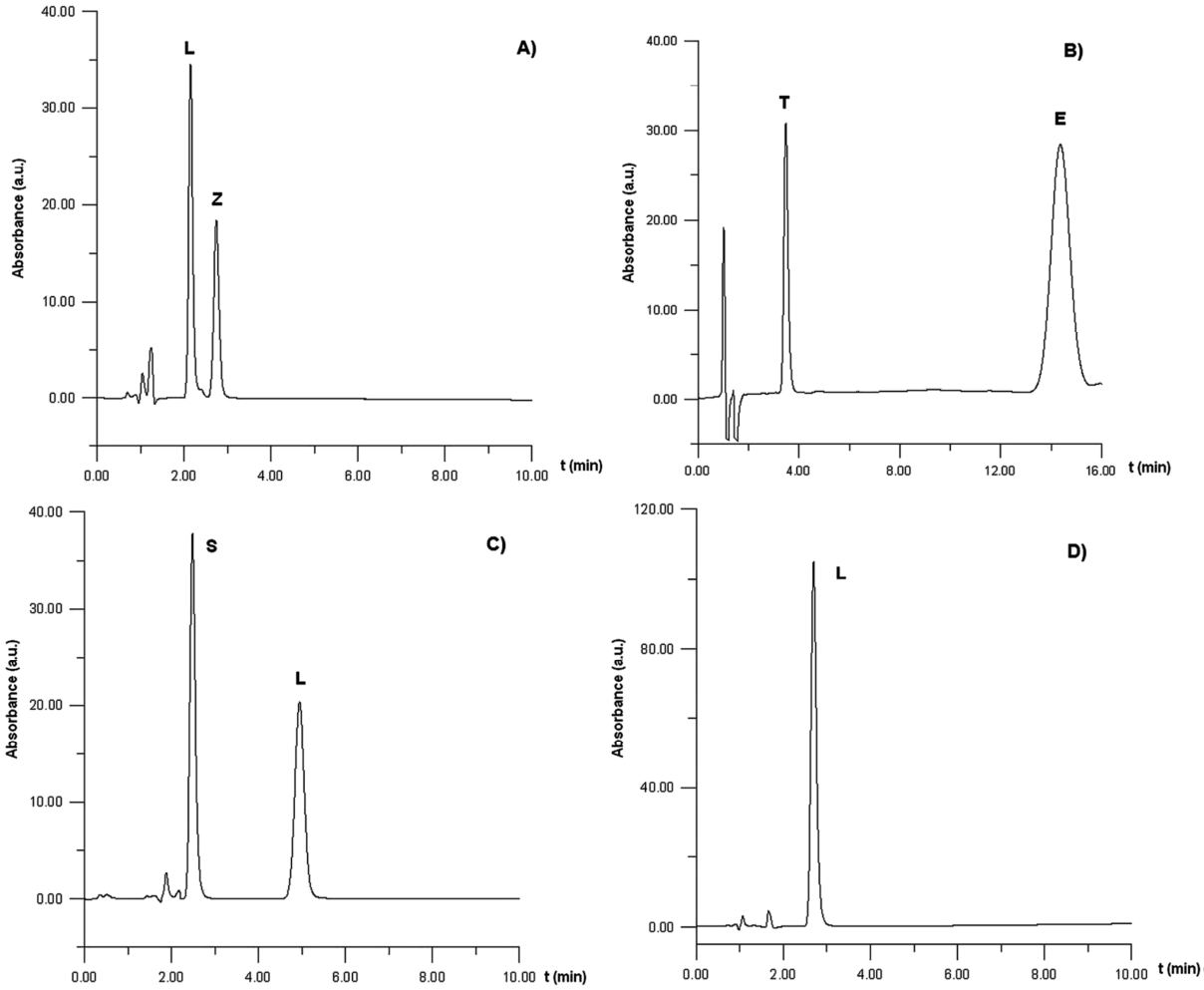

Figure 4.1. Chromatograms obtained by analysis of antiretroviral standard (20 μg/mL) using for each one its optimized mobile phase: (A) 0.05 M SDS/2.5% 1-propanol for lamivudine and zidovudine in mixture 1; (B) 0.05 M SDS/6% 1-pentanol for tenofovir (in mixture 3) and efavirenz (in all mixtures); (C) 0.05 M SDS for stavudine and lamivudine in mixture 2, and (D) 0.15 M SDS for lamivudine in mixture 3.

As seen, the resolution of a mixture of lamivudine and efavirenz is not possible in a pure mobile phase, because of the strong difference of hydrophobicities. Therefore, for each mixture two mobile phases would be needed. The final selection of the optimum mobile phase was made separately for each HAART mixture, depending on the chromatographic parameters of the third compound (zidovudine, stavudine and tenofovir). The chromatographic parameters for each analyte in its optimized mobile phase are shown in Table 4.1.

3.1.2 Mixture 1: lamivudine + zidovudine + efavirenz

In all tested mobile phases, lamivudine and zidovudine are eluted at similar retention times. In fact, they overlap in all mobile phases except in 0.15 M SDS and 0.05 M SDS/1-propanol 2.5%, where an adequate separation is observed. Finally, 0.05 M SDS/1-propanol 2.5% was selected as it provides a lower retention timeand maximum separation between the analytes.

Efavirenz was analyzed using other mobile phase. Among those indicated in Section 3.1.1, 0.05 M SDS/1-pentanol 6% was considered as optimal because it shows the maximal efficiency (N = 2569) and asymmetry (B/A = 1.062).

3.1.3 Mixture 2: lamivudine + stavudine + efavirenz

Lamivudine and stavudine show a similar behaviour in the studied mobile phases; in fact, they are resolved only in aqueous mobile phases and they co-elute when an organic modifier is added. The mobile phase 0.05 M SDS was considered as optimal as it provides the maximal separation between the two analytes, maintaining a low retention time (less than 5 min). For efavirenz, the same mobile phase as selected in Section 3.1.1 was taken.

3.1.4 Mixture 3: lamivudine + tenofovir + efavirenz

Tenofovir cannot be eluted using aqueous mobile phases, as it shows too high a retention time. Lamivudine and tenofovir can be analyzed together only in 0.05 M/1-propanol 2.5%. However, the retention time was found to be high (≈ 12.46 min) and the efficiency was N = 1889. Finally, tenofovir was analyzed using the optimal mobile phase deduced in Sections 3.1.1 and 3.1.2 for efavirenz (0.05 M SDS/1-pentanol 6%), where it provides a more useful retention time (≈ 4.01 min) and higher efficiency (N = 2060). Then this mobile phase

was taken as optimal for the quantification of tenofovir and efavirenz.

In this case, lamivudine was identified under other chromatographic conditions. The selected mobile phase was aqueous 0.15 M SDS without an organic modifier, providing the elution of the analyte at a low time (≈ 2.69 min) but with enough separation from the dead time and with adequate efficiency (N = 2009).

3.1.5 Separation of standard and matrix compounds

Blank serum samples (free of antiretroviral) were studied in order to assess if the analytes overlap with other compounds of the matrix. In order to ensure that the tested blank serum samples do not contain any antiretroviral, these samples were taken from ten healthy volunteers (five males and five females) who have never taken any of the studied drugs. Results (not shown) were found to be similar for all the blanks. Despite the complexity of the serum matrices, no peaks were detected with a retention time of > 2.00 min, but the front of the chromatograms showed a very high absorbance. This can be explained by the use of micellar media. Serum hydrophobic compounds are solubilized by their introduction into the micelles (hydrophobic environment). When the blank serum/micellar dilution sample is injected into the chromatographic system, these interactions remain in the micellar mobile phase, strongly decreasing the interaction between these compounds and the stationary phase, and then shortening the elution time. These results have already been found in previous studies on the analysis of serum samples using micellar mobile phases [13–15]. Therefore, there are no compounds that could overlap with the studied antiretroviral because their retention times were higher than 2.00 min, far enough from the front of the chromatogram. Then in the spiked samples, the chromatographic peaks corresponding to the antiretroviral may be observed as sufficiently separated between them and the other peaks, thus avoiding overlapping. This proves the selectivity of the method.

3.2. Method validation

Validation was carried out following the guidelines of the FDA [19]. The evaluated parameters were: linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness. As lamivudine is resolved using three different separation conditions depending on the HAART mixture, and its validation was performed for each mobile phase. The whole calibration was performed using a spiked sample serum (initially free of antiretroviral) from a healthy volunteer. In all cases, the preparation of the spiked serum samples has been performed as explained in Section 2.4.

3.2.1 Linearity and sensitivity

For calibration purposes, a blank serum sample was spiked at nine concentration levels between 0.5 and 50 μg/mL. The calibration was performed separately for each antiretroviral.

The slope, y-intercept and determination coefficient (r^2) of the calibration curves were obtained by linear correlation between the areas of the chromatographic peaks of each analyte *vs.* the concentration by unweighted least-square linear regression. Each calibration level was repeated six times. Calibration was repeated five times (preparing the samples on each occasion) on different days over a period of two months. The regression curve of each analyte, taken as the average of the five measurements is shown in Table 4.2. The considered absorbance was in arbitrary units and concentration was in μg/mL.

The LOD is defined as the lowest concentration in the serum which provides a significant signal above the baseline noise. It is taken as 3 times the standard deviation of the blank (LOD, 3s criterion). The LOQ is defined as the lower amount of antiretroviral which can be reliably quantified and is set as the lowest concentration reached in the calibration curve [19]. The LOD value calculated for each analyte is shown in Table 4.2. The LOQ was 0.5 μg/mL for the studied antiretroviral.

Table 4.2. Regression curve parameters (slope, y-intercept and determination coefficient) and sensitivity values (LOD and LOQ) calculated for each studied antiretroviral (Concentration in μg/mL).

Mixture	Compound	Slope	Y-intercept	r^2	LOD
1	Lamivudine	37.5 ± 0.2	5.1 ± 1.9	0.9999	0.18
1	Zidovudine	43.5 ± 0.3	- 5 ± 8	0.9998	0.15
1/2/3	Efavirenz	58.3 ± 0.4	- 3 ± 4	0.9997	0.10
2	Stavudine	52.7 ± 0.3	- 5 ± 9	0.9995	0.15
2	Lamivudine	35.7 ± 0.3	4 ± 2	0.9999	0.21
3	Lamivudine	36.5 ± 0.2	4 ± 3	0.9996	0.20
3	Tenofovir	44.8 ± 0.3	2 ± 3	0.9997	0.25

3.2.2 Precision and accuracy

The intra- and inter-day precision and accuracy of the proposed methodology were calculated for each antiretroviral and HAART set. These parameters were determined at 0.5, 5 and 20 μg/mL using spiked serum samples.

The precision was determined as the standard deviation of the provided signal (RSD, %), whereas the accuracy was calculated as the recovery, *i.e.* the ratio between the detected and the added concentration. The intra-day analysis was determined by injecting aliquots of these samples six times on the same day, while the inter-day analyses correspond to the average of five measurements of the intra-day values taken over a two-month period. The results are shown in Table 4.3. For the quantification of the five studied antiretrovirals, intra- and inter-day precision were <7.1% and <5.2%, respectively, whereas intra- and inter-day accuracy were between 88.5 and 105.3% and between 93.5 and 101.3%, respectively. These values are within the limits proposed by the FDA guidelines, which accepts a maximal RSD of 15% for precision and a recovery of between 80 and 120% [19].

Table 4.3. Intra- and inter-day precision and accuracy for the detection of each studied antiretroviral and HAART set obtained by applying the analytical methodology (Concentration in μg/mL).

			Intraday[a]		Interday[b]	
Mixture	Antiretroviral	Spiked concentration	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
1	Lamivudine	0.5	105.3	4.4	99.1	2.9
		5.0	102.3	3.3	101.2	3.0
		20	95.5	2.5	99.8	1.5
1	Zidovudine	0.5	89.8	5.2	95.2	4.0
		5.0	95.5	4.8	94.7	3.5
		20	99.1	1.1	97.0	3.0
1/2/3	Efavirenz	0.5	92.1	6.1	97.5	4.2
		5.0	98.5	5.2	95.5	1.8
		20	4.8	97.5	97.5	2.2
2	Stavudine	0.5	89.5	6.2	96.5	5.2
		5.0	92.5	4.2	98.5	3.5
		20	98.2	3.1	97.5	2.4
2	Lamivudine	0.5	103.3	4.9	97.1	1.9
		5.0	101.3	1.7	98.2	2.0
		20	97.5	3.5	99.8	0.9
3	Lamivudine	0.5	102.5	3.5	98.5	3.8
		5.0	101.3	1.9	99.5	0.5
		20	98.5	2.5	97.5	1.2
3	Tenofovir	0.5	88.5	7.1	93.5	4.2
		5.0	92.5	4.2	95.6	2.8
		20	98.5	2.8	99.6	1.2

[a]n = 6; [b]n = 5

3.2.3 Robustness

The robustness of the method was performed by analyzing serum samples (n = 3) spiked with 20 μg/mL of each studied antiretroviral, by making slight changes to the following chromatographic conditions (with the others remaining constant each time): SDS amount (average value ± 0.05 M), quantity of organic modifier (average value ± 0.1 %), pH (6.9 - 7.1) and flow rate (0.95 - 1.05 mL min^{-1}). The variation of the retention time and the peak area were studied. The robustness was examined for each antiretroviral and each optimized mobile phase. Insignificant differences in peak areas and less variability in retention time were observed (see Table 4.4). Results indicate that the selected factors remain unaffected by small variations in these parameters; the RSD was below 6.5%.

3.3 Analysis of real samples

The proposed method was applied to determine the levels of antiretroviral in serum samples from several AIDS patients from a local Hospital. Only one kind of HAART regimen has been prescribed to each patient. For confidentiality reasons, no more information about this can be provided. Results have been organized depending on the set of antiretrovirals that they take, as can be seen in Table 4.5. Results have been shown considering the 1/10-dilution performed in the experimental procedure (Section 2.4). In the case of the measured amount of an antiretroviral upper to 50 μg/mL, an extra 1/10-dilution was carried out. Fig. 4.2 shows the chromatogram of a patient to whom the mixture 1 HAART (lamivudine, zidovudine and efavirenz) has been prescribed. Antiretrovirals were detected and quantified without interferences. According to the results, the patient effectively takes the medication.

Table 4.4. Evaluation of the robustness of the micellar liquid chromatography method (all data n = 6).

Mixture	Antiretroviral	Parameter	Retention time, min (RSD, %)	Area (arbitrary unit) (RSD, %)
1	Lamivudine	[SDS]	2.11 ± 0.10 (4.7)	778 ± 23 (3.0)
		pH	2.06 ± 0.09 (4.4)	762 ± 25 (3.3)
		1-propanol amount	2.04 ± 0.11 (5.4)	758 ± 30 (4.0)
		Flow rate	2.05 ± 0.13 (6.3)	750 ± 17 (2.3)
1	Zidovudine	[SDS]	2.60 ± 0.09 (3.5)	870 ± 19 (2.2)
		pH	2.55 ± 0.11 (4.3)	861 ± 21 (2.4)
		1-propanol amount	2.61 ± 0.12 (4.6)	880 ± 30 (3.4)
		Flow rate	2.52 ± 0.10 (4.0)	851 ± 14 (1.6)
1/2/3	Efavirenz	[SDS]	14.4 ± 0.5 (3.5)	1200 ± 50 (4.2)
		pH	14.1 ± 0.4 (2.8)	1130 ± 30 (2.7)
		1-pentanol amount	14.5 ± 0.5 (3.4)	1150 ± 40 (3.5)
		Flow rate	14.2 ± 0.6 (4.2)	1190 ± 30 (2.5)
2	Stavudine	[SDS]	2.60 ± 0.17 (6.5)	1070 ± 40 (3.7)
		pH	2.68 ± 0.1 (4.1)	1050 ± 20 (1.9)
		Flow rate	2.62 ± 0.23 (5.0)	1030 ± 30 (2.9)
2	Lamivudine	[SDS]	5.01 ± 0.22 (4.4)	724 ± 24 (3.3)
		pH	4.92 ± 0.15 (3.0)	720 ± 15 (2.1)
		Flow rate	4.98 ± 0.19 (3.8)	710 ± 20 (2.8)
3	Lamivudine	[SDS]	2.72 ± 0.11 (4.0)	750 ± 24 (3.2)
		pH	2.67 ± 0.08 (3.0)	731 ± 19 (2.6)
		Flow rate	2.70 ± 0.09 (3.3)	742 ± 16 (2.2)
3	Tenofovir	[SDS]	4.06 ± 0.13 (3.2)	870 ± 30 (3.4)
		pH	4.04 ± 0.09 (2.2)	910 ± 19 (2.1)
		1-pentanol amount	3.95 ± 0.13 (3.0)	890 ± 24 (2.7)
		Flow rate	4.00 ± 0.17 (4.3)	900 ± 15 (1.7)

Table 4.5. Amount of antiretroviral found (μg/mL) in AIDS patient serum samples (n = 5).

HAART regimen prescribed = mixture 1			
Patient	Lamivudine	Zidovudine	Efavirenz
576704	5.6 ± 0.4	27.7 ± 1.1	125 ± 8
505933	13.7 ± 0.9	36 ± 3	23 ± 3
519824	179.5 ± 1.5	12.8 ± 1.8	Under LOD
521320	5.3 ± 1.5	32.8 ± 0.8	393 ± 9
520287	12 ± 4	29.0 ± 0.3	84 ± 6
516064	7.0 ± 0.9	45.4 ± 1.8	Under LOD
505459	104 ±8	89 ± 5	122 ± 7
HAART regimen prescribed = mixture 2			
Patient	Lamivudine	Stavudine	Efavirenz
521787	35 ± 4	130 ± 15	510 ± 40
521321	31 ±5	72 ± 9	183 ± 22
HAART regimen prescribed = mixture 3			
Patient	Lamivudine	Tenofovir	Efavirenz
532374	16.6 ± 1.6	Under LOD	91 ± 6
532378	34.3 ± 1.6	Under LOD	305 ± 13
579254	26.7 ± 0.6	Under LOD	1010 ± 50
579253	14.2 ± 0.6	Under LOD	660 ± 40
519821	34.9 ± 1.0	Under LOD	38 ± 4
516060	20.6 ± 2.1	Under LOD	365 ± 21
505948	49 ± 3	Under LOD	150 ± 12
514486	30.7 ± 1.2	Under LOD	24 ± 3
505050	29.3 ± 2.0	Under LOD	13.1 ± 0.4
505045	41.3 ± 1.4	Under LOD	160 ± 7
520289	24 ± 3	Under LOD	159 ± 9

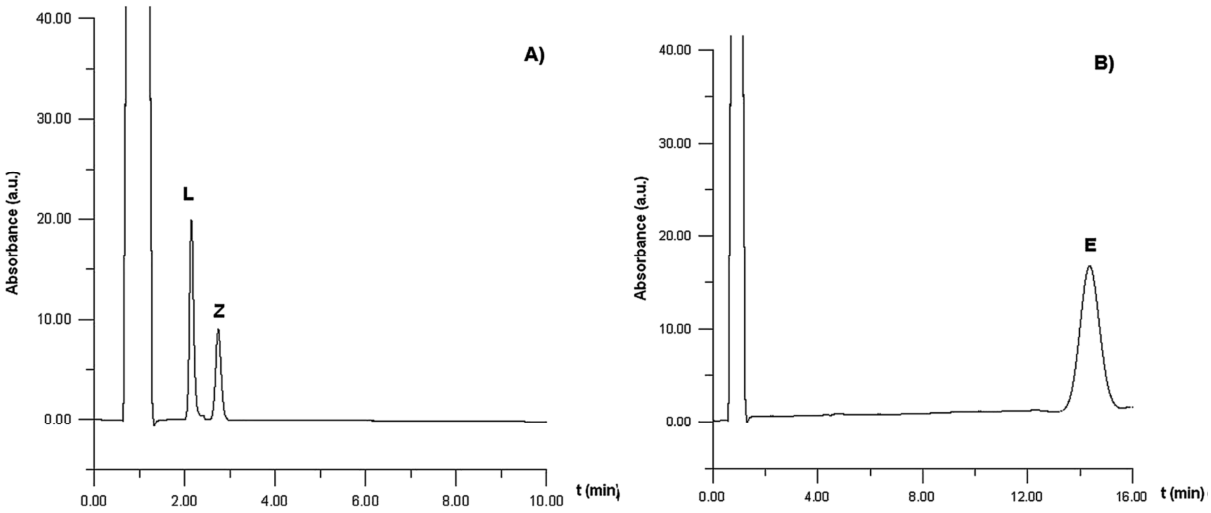

Fig. 4.2. Chromatograms obtained by the analysis of a serum sample of the AIDS patient n^{o}. 505459, who takes the HAART regimen composed of lamivudine, zidovudine and efavirenz (see Table 4.5 for quantitative values).

4. Conclusions

MLC has been proven as a valuable technique to monitor the antiretroviral drugs belonging to the three HAART regimens mainly prescribed by doctors for AIDS patients. Analysis was performed without long and tedious extractions and/or sample cleaning, by the direct injection of the sample after a simple dilution and filtration of the matrix. The suggested methodology allows the separation of the analytes in less than 15 min, with adequate efficiency and asymmetry. Moreover, validation was performed according to FDA guidelines with satisfactory results in selectivity, linearity, sensitivity, precision, accuracy and robustness. As low amount of organic solvent and biodegradable salts were used, and the method can be considered as environmentally friendly. Besides, the instrumentation is relatively inexpensive and a high amount of samples can be successively analyzed using an autosampler, making it more attractive for routine analysis in a clinical laboratory.

5. Acknowledgements

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Chapter 5

A micellar liquid chromatography method for the quantification of abacavir, lamivudine and raltegravir in plasma

Abstract

An analytical methodology based on MLC has been developed to quantify abacavir, lamivudine and raltegravir in plasma. These three antiretroviral drugs are prescribed as a set in HAART for AIDS patients. The experimental procedure consists in the dilution of the sample in micellar media, followed by filtration and, without cleanup step. The analytes were resolved in less than 30 min using a mobile phase of 0.05 M SDS at pH 7, running at 1 mL min^{-1} under isocratic mode at room temperature through a C18 column (125 × 4.6 mm, 5 μm particle size). The UV detection wavelength was set at 260 nm. The method was successfully validated following the requirements of ICH guidelines in terms of: linear range (0.25–2.5 μg mL^{-1}), linearity (r^2 > 0.990), intra- and interday precision (<6.8%) and accuracy (92.3–104.2%), and robustness (<7.1%). To the extent of our knowledge, this is the first published method to quantify these three drugs in plasma. Several blood samples from AIDS patients taking this HAART set provided by a local hospital were analyzed with satisfactory results.

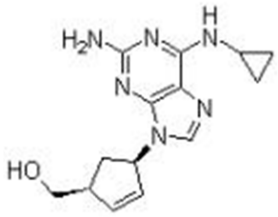
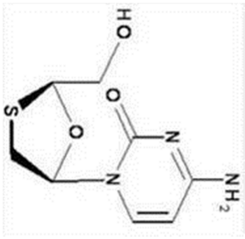
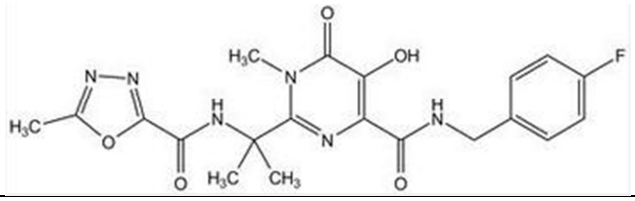
1. Introduction

Since its discovery in 1981, acquired immunodeficiency syndrome (AIDS) has caused nearly 30 million deaths and approximately 34 million people are globally infected with human immunodeficiency virus 1 (HIV-1) [1–3]. Different attempts have been undertaken to find a cure for the disease, but with limited success. In 1996, HAART was introduced, with impressive clinical results in suppressing the activity of HIV. Generally, HAART regimen combines three or four different antiretroviral (ARV), which acts together against HIV. But this therapy is complex, has many unwanted effects, is difficult to adhere to and has to be lifelong because it does not get rid of HIV [4].

Recently, a new HAART combination (Table 5.1) [5], containing abacavir (Ziagen ®), lamivudine (Epivir ®) and raltegravir (Isentress ®), has been introduced in the Spanish market, for patients in which fails the normal treatment with other mixtures [6]. Abacavir and lamivudine are reverse transcriptase inhibitors, which interacts with an HIV viral enzyme, used to generate new virus. Inhibition of this enzyme prevents the virus completes this reproductive cycle [7]. Raltegravir is an integrase inhibitor. It inhibits the integrating the viral genetic material into human chromosomes [8].

Therapeutic drug monitoring (TDM) consists in the quantification of drugs in physiologic matrices at several times after the consumption of the formulation. This information can be used to establish its pharmacokinetics and explain the therapeutic and adverse effects of the drug. This information can be used to determine the proper dose of a drug for a specific patient. This is important because the presence of subtherapeutic levels of antiretroviral will result in the appearance of drug resistance mutations that can endanger drug treatment options [9]. On the other hand, the lack of compliance to HAART is the first cause of therapeutic failure and should be evaluated. A treatment compliance level above 95% is necessary [10]. In order to optimize the response of the patient to this new HAART regimen, clinicians need analytical methods to quantify abacavir, lamivudine and raltegravir in plasma.

Table 5.1. Structure, pKa and log Po/w.

Compound	Structure	pKa	log Po/w
Abacavir		5.01	1.2
Lamivudine		Not found	- 0.952
Raltegravir		6.7	- 1.055

Prominent among the techniques used for TDM, chromatographic methods such a thin layer chromatography (TLC), gas chromatography (GC) and HPLC are widely used to analyze antiretroviral [11]. Abacavir, lamivudine and raltegravir have been analyzed in several ways, such as HPLC-UV [12,13] and LC–MS/MS [14–16]. However, to the best of our knowledge, a method to simultaneously analyze these three drugs in plasma has not been previously issued. Mass spectrometry is an easy-to-contaminate instrumentation and quite expensive. Besides, chromatographic methods usually require tedious and time-consuming extraction and cleanup steps prior to the chromatographic resolution.

Authors have proven that plasma samples can be studied avoiding these problems using MLC [17]. Previously published papers have detailed the development of MLC-based methodologies, using SDS as surfactant, for the screening of thirteen ARV [4] and the quantification of 3 HAART mixtures by the use of only three mobile phases [3]. These methods can be adapted to the analysis of abacavir, lamivudine and raltegravir, to maintain the benefits of MLC, as direct injection (after dilution and filtration), use of isocratic mobile

phases, versatility and the use of low amount of pollutant chemicals, reducing the analysis time and cost [18].

The aim of this work was to develop a sensitive and reliable MLC-based method to determine three ARV included in a new HAART combination: abacavir, lamivudine and raltegravir, in plasma samples of AIDS patients. The method must be simple, inexpensive, environmentally-friendly and with a reduced experimental protocol. The developed method was validated according the ICH Guidelines [19] in terms of linear interval, calibration parameters, sensitivity expressed as limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness. Finally, the developed analytical method was used to quantify the ARV in human plasma from AIDS patients. Samples were supplied by the Hospital La Plana of Vila-real.

2. Materials and Methods

2.1 Reagents and instrumentation

The following ARV standards (purity > 99.5%) were taken for the analysis: abacavir, lamivudine (GlaxoSmithKline, Brentford, UK) and raltegravir (Merck, MSD, Darmstadt, Germany).

Selected surfactant was SDS (purity > 99%, Merck, Darmstadt, Germany). HCl, NaOH and $NaH_2PO_4.H_2O$ (reagent quality) were purchased from Scharlab (Barcelona, Spain). Ultrapure water was prepared by purification of deionized water using an ultrapure water generator device (Millipore S.A.S., Molsheim, France) and used for preparing all mobile phases and solutions.

The instrumentation used in the study and the treatment of the data is described in [3].

2.2 Solutions and mobile phases

Mobile phases and solution of ARV were prepared and stored as reported in [3]. The concentrations of the stock solutions 100 μg mL^{-1} for abacavir and lamivudine, and 250 μg mL^{-1} for raltegravir.

2.3 Sample treatment

The blood collection and extraction of plasma was performed as reported in [3]. Afterwards, plasma samples were frozen and stored at −20 ºC until its analysis, it was then thawed just before use. The samples were obtained after consent from the patients.

For validation and analysis purpose, 1 mL volume of plasma samples was introduced into a vial and the volume was adjusted to 5 mL with 0.05 M SDS at pH 7 (1/5 dilution). In the case of spiked samples, the appropriate amount of the ARV standard solution was added before filling. The diluted sample was vigorously shaken to favor homogenization and stored for one day in the fridge at 5 ºC to favor the contact between antiretroviral and the sample [20]. These samples were filtered using 0.45-μm nylon membranes (Micron Separations, Westboro, MA, USA) and injected in the chromatographic system.

2.4 Chromatographic conditions

Several mobile phases were tested in order to study the chromatographic behavior or the analytes by varying SDS amount in the 0.05–0.15 M range.

The analysis were performed using an 0.05 M SDS-pH 7 mobile phase running through a Kromasil C18 (Scharlab) column (150 × 4.6 mm; particle size 5 μm: pore size 100 Å, working pH range 1.5–7.5) under isocratic mode at 1 mL min^{-1} at room temperature. The injection volume was 20 μL and detection absorbance wavelength was set at 260 nm. The

special care needed when using with micellar media in liquid chromatography instrumentation is described in [21]. Under these conditions, the column was a lifespan of nearly 1000 injections.

3. Results and Discussion

3.1 Optimization of the chromatographic conditions

The main chromatographic conditions (stationary phase, surfactant, pH, wavelength detection) were taken from a published paper. This previous issue shows the analysis of, among others, lamivudine and abacavir in serum [4]. The dilution ratio was taken from a paper about the analysis of other drugs in plasma. It was proven that a 1/5 value is enough to protect stationary phase from harmful compounds of the matrix, without strongly reducing the sensitivity [17].

The composition of the mobile phase was optimized focusing on the simultaneous analysis of the three studied ARV: abacavir, lamivudine and raltegravir.

3.1.1 Selection of the absorbance wavelength detection

The maximum absorbance wavelength for lamivudine and abacavir solved in micellar solution was 260 nm [4]. The UV–Vis spectrum of raltegravir was obtained in a micellar environment, by analyzing a 5 μg mL^{-1} solution using the optimal mobile phase. A strong absorbance was found at 260 nm, therefore this wavelength was chosen for the analysis. Thus, the chromatograms were registered without changing the wavelength detection.

3.1.2 Optimization of the micellar mobile phase composition

Lamivudine is already eluted very early (before 4 min) in a pure SDS micellar mobile phase. If an organic modifier is added, lamivudine overlaps with the protein band [3]. Thus, the study was restricted to pure aqueous micellar mobile phase.

The optimal mobile phase was selected following the maximum resolution-minimum analysis time criteria, using an interpretative optimization strategy using Michrom [22] as described in [23].

The optimum mobile phase selected for the analysis of abacavir, lamivudine and raltegravir, was 0.05 M SDS-pH 7. Fig. 5.1 shows the chromatogram obtained by analysis of a 2.5-μg mL^{-1} mixture of each ARV. Under these conditions, the global resolution was 0.9997. The individual values of the chromatographic parameters were: abacavir (t_R= 28.2 min; N = 2567; B/A = 1.31); lamivudine (t_R= 3.9; N = 7529; B/A = 1.45) and raltegravir (t_R= 21.4; N = 1585; B/A = 1.14). Errors <3.1% were obtained for the prediction of retention times.

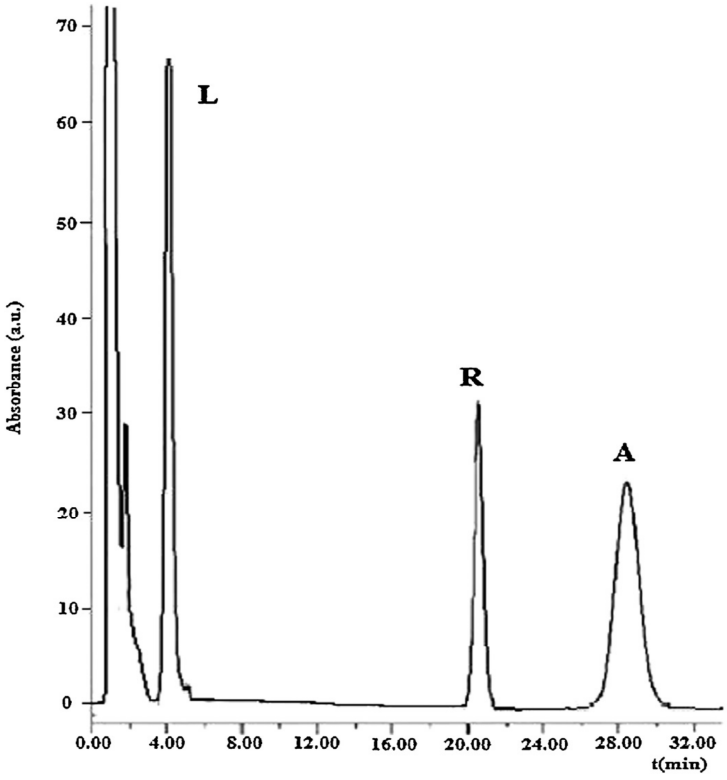


Fig. 5.1. Chromatogram by analysis of a blank plasma spiked with 2.5 μg mL^{-1} of abacavir (A), lamivudine (L) and raltegravir (R) analyzed through the developed method.

3.2 Method validation

The optimized method was validated following the ICH Guidelines [19]. The whole validation was performed using a spiked blank plasma sample. The numerical values refer to the concentration of antiretroviral in the original plasma sample, not in injected aliquot.

To assess the selectivity of the method, free-ARV plasma samples were analyzed, blank and spiked with 2.5 μg mL^{-1} of each antiretroviral. The protein band appears at the dead time and is totally eluted in <2 min. No peaks were eluted near the retention times of the studied ARV, and then no interferon was detected (Fig. 5.1). Thus, the three analytes can unambiguously be identified and quantified without overlapping.

Calibration curves were constructed using the areas of the chromatographic peaks, after triplicate injections, obtained at seven different concentrations, in the 0.25–25 μg mL^{-1} range. The slope, y-intercept and determination coefficient (r^2> 0.990) were obtained by least-square linear regression. The presented calibration parameters were calculated from five intraday calibration curves constructed over a 3-months period. Results are shown in Table 5.2.

Table 5.2. Calibration parameters and sensitivity (concentration in μg mL^{-1}).

Compound	Slope	Y-intercept	r^2	LOD	LOQ
Abacavir	64.8 ± 0.6	4 ± 6	0.9998	0.090	0.250
Lamivudine	56 ± 1.8	9 ± 5	0.9979	0.070	0.200
Raltegravir	42 ± 3	−5 ± 4	0.9902	0.090	0.250

n = 5

The detection limit (LOD) of a method, is the lowest ARV concentration in plasma, that produces a response that is detectable above the noise level of the system, whereas the quantification limit (LOQ) of a method, is the lowest antiretroviral concentration in plasma that can be reliably measured. LOD and LOQ were calculated using the 3.3s and 10s criterion, respectively [19], and are shown in Table 5.2. These results indicate that the developed method is sensitive enough to detect the three ARV in plasma of patients taken

these HAART regimens for clinical purposes.

The intra- and inter-day precision and accuracy of the methodology were measured as indicated in [3] at three concentration levels (0.5; 5 and 15 μg mL^{-1}). Recoveries were 92.3–104.2% and signal variability RSD values were <6.8% (Table 5.3). These results indicate that the method is useful for the routine analyses of abacavir, lamivudine and raltegravir.

Table 5.3. Intra- and inter-day accuracy and precision

ARV	Concentration (μg mL^{-1})	Intra-day[a]		Inter-day[b]	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Abacavir	0.5	94.6	6.2	96.8	3.3
	5	97.8	4.6	98.6	3.2
	15	98.8	2.3	99.7	0.7
Lamivudine	0.5	94.3	4.7	97.5	6.2
	5	98.6	4.8	101.3	2.6
	15	98.2	2.3	96.8	1.7
Raltegravir	0.5	92.3	6.8	104.2	5.2
	5	96.7	4.2	97.6	2.1
	15	98.4	2.2	98.8	1.4

[a]n = 6; [b]n = 5

The robustness of the method was examined by replicate injections (n = 3) of a standard solution at 5 μg mL^{-1} with slight modifications to the following chromatographic parameters (range): SDS concentration (0.049–0.051 M), pH (6.9–7.1), flow rate (0.95–1.05 mL min^{-1}), absorbance wavelength detection (255–265 nm) and injection volume (19.5–20.5 μL). Variations of the main chromatographic conditions had no significant effect on retention time (<7.1%) and peak area (<6.2%), and then the method can be considered as enough robust to be used in routine analysis.

3.3 Analysis of plasma samples of HIV patients

Some plasma samples from patients being treated with these substances were analyzed. Results are shown in Table 5.4. Fig. 5.2 shows the chromatogram obtained by analyzing the sample from patient P1. The three ARV could be clearly quantified without interferences, including the band of proteins that elutes before 2 min. The chromatograms obtained from the analysis of the other samples show similar shape.

Table 5.4. Quantification of the ARV (μg mL^{-1}) in plasma of HIV patients (n = 3)

Patient	Abacavir	Lamivudine	Raltegravir
P1	1.43 ± 0.03	0.78 ± 0.05	1.02 ± 0.03
P2	1.10 ± 0.08	1.06 ± 0.05	0.67 ± 0.04
P3	1.32 ± 0.07	0.85 ± 0.06	0.90 ± 0.08
P4	1.64 ± 0.05	1.15 ± 0.09	1.36 ± 0.05
P5	1.45 ± 0.07	0.80 ± 0.05	1.08 ± 0.04
P6	1.08 ± 0.09	0.90 ± 0.08	1.24 ± 0.09
P7	0.85 ± 0.07	0.68 ± 0.04	0.85 ± 0.08
P8	0.59 ± 0.05	0.87 ± 0.06	0.57 ± 0.07
P9	1.46 ± 0.10	1.30 ± 0.11	1.18 ± 0.14
P10	0.79 ± 0.12	0.96 ± 0.07	0.96 ± 0.11

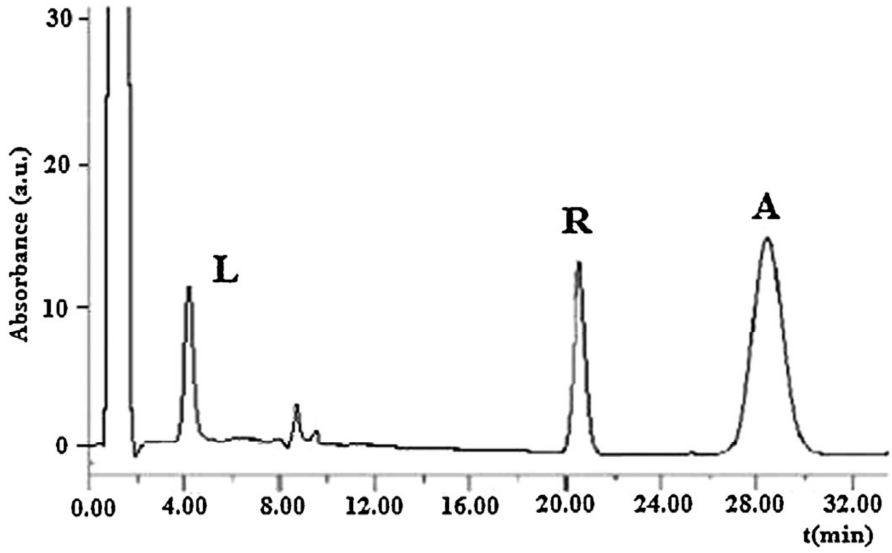


Fig. 5.2. Chromatogram corresponding to P1, obtained using the optimum mobile phase (SDS 0.05 M-pH 7), marks as in Fig. 5.1.

4. Conclusions

MLC has proved to be a suitable technique to analyze abacavir, lamivudine and raltegravir in plasma samples of AIDS patients. As far as we know, this is the first developed method showing the simultaneous determination of these drugs in plasma. This method does not require complex procedures, such as sample extraction and/or sample cleaning because it allows the direct injection of plasma sample in the chromatographic system without any pretreatment, except filtration. The time required for the determination of three antiretroviral plasma samples was only 30 min. The chromatographic procedure provides adequate results for the determination of abacavir, lamivudine and raltegravir in plasma samples in terms of selectivity, linearity, accuracy, robustness and sensitivity at the ng mL^{-1} level. This method has a low environmental impact as it uses a smaller amount of toxic organic solvents. In addition, it is relatively inexpensive and only needs a simple chromatographic apparatus that is available in any hospital laboratory.

5. Conflict of interest disclosure

The authors declare that they do not have any financial/commercial conflict of interest.

6. Acknowledgements

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Chapter 6

Use of micellar liquid chromatography to analyze darunavir, ritonavir, emtricitabine and tenofovir in plasma

Abstract

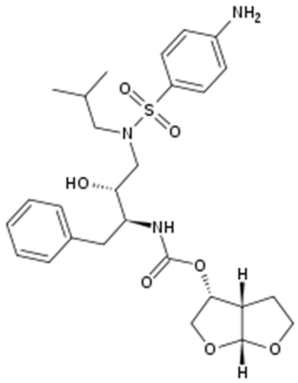
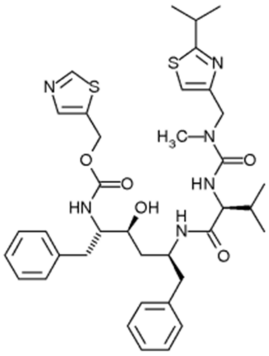
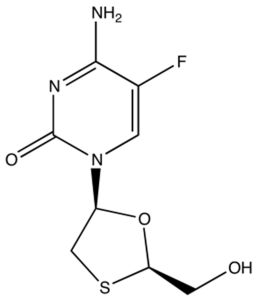
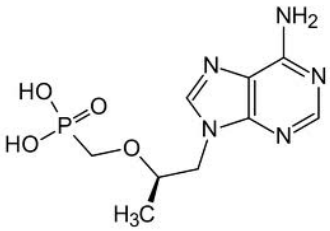
Danuravir, ritonavir, emtricitabine and tenofovir are together prescribed against AIDS, as a HAART regimen. Micellar liquid chromatography has been applied to determine these four antiretroviral drugs in plasma. The sample preparation is shortened to the dilution of the sample in a micellar solution, filtration and injection. Clean-up steps are avoided, due to the solubilization of plasma matrix in micellar media. The drugs were analyzed in < 20 min using a mobile phase of 0.06 M SDS - 2.5 % 1-pentanol - pH 7 running under isocratic mode through a C18 column at 1 mL min^{-1} at room temperature. Absorbance wavelength detection was set at 214 nm. The method was successfully validated following the ICH Harmonized Tripartite Guideline in terms of: selectivity, limit of detection (0.080 - 0.110 μg mL^{-1}), limit of quantification (0.240 - 0.270 μg mL^{-1}), linearity between 0.25 and 25 μg mL^{-1} (r^2 > 0.995), accuracy (89.3 - 103.2 %), precision (<8.2 %) and robustness (<7.5 %). Real plasma sample from patients taking this therapy were analyzed. This is the first paper showing the simultaneous detection of these four drugs. Therefore, the methodology was proven useful for the routine analysis of these samples in a laboratory Hospital for clinical purposes.

1. Introduction

HAART (highly active antiretroviral therapy) was introduced in 1996 to treat patients with acquired immunodeficiency syndrome (AIDS) with strong clinical results [1]. It consists in the prescription of a set (named HAART regimen) of antiretroviral (ARV) which prevents the activity of human immunodeficiency virus 1 (HIV-1). Generally, a HAART regimen combines three or four different ARV [2]. These ARV mixtures reduce the amount of active virus, even until it is undetectable by current blood testing techniques [3]. However, it does not reach a definitive cure of the disease, so that the treatment has to be lifelong, complicating the compliance and favoring the appearance of drug resistance mutations. Moreover, the pharmacokinetics of the drugs is sometimes unpredictable, and they show many side effects [4]. Continuously, clinicians propose new combinations looking for higher therapeutic activity and less adverse effects for a specific patient than previous regimen [5].

Recently, a new HAART combination, containing darunavir (Prezista ®), ritonavir (Norvir ®), tenofovir and emtricitabine (Truvada ®), has been introduced in the Spanish market for naïve-patients, those do not have previously taken other HAART regimen. The structures and main physico-chemical characteristics can be seen in Table 6.1. The initially prescribed dose, be taken one time per day, is: one tablet of Prezista ® + Norvir ® (400 mg darunavir + 100 mg ritonavir) and one tablet of Truvada ® (300 mg tenofivir + 200 mg emtricitabine). Darunavir and ritonavir are protease inhibitors (PI). They act by blocking HIV aspartyl protease which the virus needs to cleave the HIV polyprotein into its functional fragments, preventing the completion of the reproductive cycle of the virus. Tenofovir and emtricitabine are nucleotide and nucleoside reverse transcriptase inhibitor (NtRTIs/NRTIs), respectively. These drugs work by inhibiting reverse transcriptase, the enzyme that copies HIV RNA into new viral DNA, and foiling the generation of new virus [6,7].

Table 6.1. Structure, pKa and log Po/w of the studied antiretroviral.

Compound	Structure	pKa (protonated/neutral)	Charge at pH = 7	log*Po/w*
Darunavir		(Darunavir+H^+) 2.10	0	1.80
Ritonavir		(Ritonavir-H^+) 2.84	0	3.90
Emtricitabine		(Emtricitabine-H^+) 2.65	0	-1.4
Tenofovir		3.75	-1	1.25

The simultaneous quantification of darunavir, ritonavir, emtricitabine and tenofovir in blood from a patient treated with this HAART regimen can be useful to study their biological behavior and to explain therapeutic and adverse effects, including failure [8]. This information would be useful to personalize the prescription timeline of the HAART regimen for each patient, improving the probability of success [9]. The quantification of ARV can also be performed to check the compliance level with the treatment, which must be above 95 % [10], and to detect sub-therapeutic levels of ARV in blood, that may provoke the virus to develop resistance [11]. However, at our knowledge, a method to simultaneously determine these four drugs in blood has not been previously issued.

Reverse-phase high performance liquid chromatography (RP-HPLC), using mixtures of organic solvent and water as mobile phases, is the preferred technique for the analysis antiretroviral in plasma [12]. HPLC coupled to mass spectrometry (MS) has been used to detect ritonavir, darunavir [13], tenofovir [13,14] and emtricitabine [14,15]. However, LC-MS has serious limitations, as high cost and expensive maintenance. Emtricitabine can be analyzed using HPLC coupled with fluorescence detection (HPLC-FLD) [16], but the other studied drugs are not fluorescent. Several methods based on HPLC coupled with UV-Visible absorbance (HPLC-UV) have been developed to measure the concentration of darunavir [17], ritonavir mixed with other ARV [18-20] and darunavir/ritonavir mixtures [21,22]. HPLC-UV has been also applied to simultaneously determine ARV mixtures containing emtricitabine [20] and tenofovir [23], and to quantify a combination of emtricitabine and tenofovir [24]. These chromatographic methods involve tedious and time-consuming cleanup steps as liquid/liquid [19,21,22] solid/liquid [17,20,23,24] extraction and deproteneization by precipitation [18], to avoid the introduction of harmful compounds in the chromatographic system.

Micellar liquid chromatography using sodium dodecyl sulfate (SDS) as surfactant is an excellent choice as analytical method when dealing with plasma analysis [25]. Proteins are denatured and, together with other hydrophobic compounds, solubilized in micellar media, and eluted at the front of the chromatogram. Therefore, plasma samples can be directly injected, after simple dilution and filtration, into the chromatographic system without risk of precipitation [26]. SDS monomers modify the nature of the stationary phase, and the apolar

inner core of the micelle can interact with analytes, introducing a new environment in the retention mechanism. A chemometric approach provides a simulation of the chromatographic behavior of the ARV from the data obtained in few mobile phases following an experimental design, expediting the optimization of the mobile phase composition [27]. The use of MLC also reduces the cost and the environmental impact of the analysis, as micellar mobile phases usually contain non-pollutant inorganic reagents and organic solvents only up to 12.5 % [28]. MLC-based methods have been developed to analyze antiretroviral mixtures in plasma, including ritonavir [29] and tenofovir [30].

The aim of the work was to develop an analytical procedure based on MLC using SDS as surfactant for the simultaneous quantification of four antiretroviral forming a HAART regimen (darunavir, ritonavir, emtricitabine and tenofovir) in plasma. The method should be able to resolve the mixture of the four drugs in low analysis time with high sensitivity, reliable, simple, inexpensive and environmentally-friendly. The method was validated following the requirements a validation guide proposed by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use: ICH Harmonized Tripartite Guideline. The evaluated parameters were: selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy and robustness [31]. The results can be used to study the pharmacokinetics of these drugs in AIDS patients taking this regimen, and evaluate the compliance with the treatment.

2. Materials and methods

2.1 Chemical and reagents

The drug standards (purity > 99.5 %) were: darunavir (Janssen Pharmaceuticals Titusville, NJ, USA), ritonavir (Abbot Laboratories, North Chicago, IL, USA), emtricitabine (Gilead Sciences, Foster City, CA, USA) and tenofovir (Bristol Myers Squibb, New York, NY, USA). The main physicochemical parameters (pKa; log Po/w) of the studied ARV are:

darunavir (2.1; 1.80), ritonavir (2.84; 3.90), emtricitabine (2.65; -1.4) and tenofovir (3.75; 1.25). The structures can be found in [6].

SDS (purity > 99%) was purchased from Merck (Darmstadt, Germany). HCl, NaOH, $NaH_2PO_4.H_2O$ (reagent quality), methanol and 1-pentanol (HPLC grade) were supplied by Scharlab (Barcelona, Spain). An ultrapure water device (Millipore S.A.S., Molsheim, France) was used to generate ultrapure water from deionized water. Ultrapure water was used for all aqueous solutions.

2.2 Instrumentation

Solids were weighted using a Mettler-Toledo analytical balance (Greifensee, Switerland). The pH measurements were performed using a GLP 22 potentiometer (Crison, Barcelona, Spain) equipped with a combined Ag/AgCl/glass electrode. An ultrasonic bath (model Ultrasons-H, Selecta, Abrera, Spain) used to dissolve the standards and mobile phases.

The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA). It was equipped with an isocratic pump, a degasser, an autosampler and UV-Visible diode array detector (DAD). The signal was acquired by a personal computer connected to the chromatograph by means of an Agilent Chemstation version B.01.01. Chromatograms were treated using Michrom software (Marcel Dekker, New York, NY, USA) to extract the chromatographic parameters: retention time (t_R), retention factor (k), dead time (t_0), efficiency (N), asymmetry (B/A) and peak area (A) [30,32].

All solutions and mobile phases were filtered before injection in the chromatographic system, through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA).

2.3 Standard solutions and mobile phase preparation

Antiretroviral standard stock solutions were prepared by weighting the appropriate amount to obtain 100 μg mL^{-1}, solving it in few mL of methanol and diluting with water up to 10 mL. Working solutions were prepared by dilution of the stock solution in water. The standard solutions were kept at 4ºC in amber flask. No degradation was detected after 3 months of use.

Mobile phases were prepared by dissolving the appropriate amount of SDS and sodium dihydrogenphosphate in water. The pH was adjusted to the desired value by adding drops of HCl and NaOH solutions, and the suitable volume of 1-pentanol was added. At last, ultrapure water was added to reach the final volume, ultrasonicated and filtered.

2.4 Sample treatment

Blood samples were provided for Hospital La Plana of Vila-real (after consent by the patients) and collected from AIDS patients taking the studied HAART regimen and healthy volunteers, as control samples. The samples were collected with DB SST tubes (SBD Vacutainer Systems, Plymouth, UK), centrifuged at 3000 rpm for 10 min (3000 g) to obtain the non-cellular fraction. Obtained plasma was frozen and stored at -20ºC. Samples were thawed one day before the analysis and kept at +4ºC in amber flasks.

One mL of plasma was introduced in a vial and filled up to 5 mL with a 0.05 M SDS solution at pH 7 (1/5 dilution). Then, these samples were then injected into the chromatographic system without another treatment than filtration.

Optimization and validation of the method were performed using spiked plasma blank (drug-free) samples from healthy volunteers. The spiking was performed by adding the adequate volume of the antiretroviral working standard solution prior to filling. ARV concentration taken for calculations were those the equivalent in the original plasma sample (considering the dilution factor), instead of the concentration of the injected aliquot.

2.5 Chromatographic conditions

The analysis were performed using a mobile phase of 0.060 M SDS - 2.5 % 1-pentanol fixed at pH 7 with 0.01 M phosphate buffer, running at 1 mL min^{-1} under isocratic mode through a Kromasil C18 (Scharlab) column (150 x 4.6 mm; particle size 5 μm: pore size 100 Å, working pH range 1.5 - 7.5) at room temperature. The injection volume was 20 μL and detection absorbance wavelength was set at 214 nm. The special care applied to chromatographic instrumentation when dealing micellar mobile phases are detailed in [33]. The needle was cleaned by injecting water between two injections. Under these conditions, the column was a life span of nearly 1000 injections.

3. Results and discussion

3.1 Optimization of the chromatographic conditions

Several chromatographic conditions (stationary phase, surfactant, pH, injection volume, temperature) were taken from previously published papers [29,30]. The detection conditions and the composition of the mobile phase were optimized for the analysis of a mixture of darunavir, ritonavir, emtricitabine and tenofovir in plasma.

3.1.1 Optimization of the absorbance wavelength detection

The optimum absorbance wavelength detection for ritonavir [29] and tenofovir [30] in micellar medium is 214 nm. Darunavir [17,21] and emtricitabine [20,24] show absorbance at < 280 nm in hydroorganic solvents. However, the new organization of the analytes in micellar media can provoke the shifting of the maximal absorption wavelength and the change of the absorbance intensity. Thus, UV-Visible spectra of darunavir and emtricitabine

were taken by analyzing a plasma sample spiked with 5 μg mL^{-1} of these two drugs, using the optimal micellar mobile phase. As a strong absorbance was found at 214 nm, this value was selected for the analysis. Therefore, the whole chromatogram was registered using the same wavelength detection.

3.1.2 Optimization of the micellar mobile phase composition

The composition of the mobile phase was optimized focusing on the separation of the peaks corresponding to the four studied ARV. Ritonavir [29] and tenofovir [30] are strongly retained on C18 columns, and need a mobile phase with high elution ability. Thus, the study was restricted to aqueous micellar mobile phase with 1-pentanol.

The optimal SDS and 1-pentanol concentration were simultaneously optimized using an interpretative strategy. A mixture of 5 μg mL^{-1} of darunavir, ritonavir, emtricitabine and tenofovir were analyzed by five mobile phases containing the following SDS (M)/1-pentanol (%) values: 0.05/2; 0.05/6; 0.10/4; 0.15/2 and 0.15/6. In all the tested mobile phase, the protein band elutes at < 2.5 min and does not overlap with any drug. Indicative parameters of the chromatographic behavior, as *k*; t_R; N; B/A and t_0, were measured for each ARV and mobile phase using Michrom software [32].

These data were processed using a statistical model based on chemometrics. It is able to relate the chromatographic behavior of the analytes with the composition of the mobile phase. Equation 6.1 provides the relationship between retention factor and SDS/1-pentanol concentration [34]:

$$k = \frac{K'_{AS}/(1+K_{AD}\varphi)}{1+[M](K_{AM}(1+K_{MD}\varphi)/(1+K_{AD}\varphi))} \quad (6.1)$$

where [M] is the SDS concentration (M) and φ is the amount of 1-pentanol (%). The meaning of the constants can be found in [33].

The peak shape, which depends on N and B/A, is modeled through the equation 6.2,

which provides the predicted h(t) (absorbance signal provided by the detector) at each elution time (t) [34]:

$$h(t) = H_0 e^{-0.5\left(\frac{t-t_R}{s_0+s_1(t-t_R)+s_2(t-t_R)^2+\ldots}\right)^2} \quad (6.2)$$

s_i are constants depending on t_R; N and B/A. They remain ideally invariant for each ARV and mobile phase. H_0 (peak height) depends on the concentration of the ARV.

The data obtained in the tested mobile phases (k; N and B/A) are used by the Michrom software as "calibration levels" to calculate the constant parameters of the two equations. Once known, the statistical model allows the prediction of the chromatographic conditions of the analytes and the drawing of simulated chromatograms, at intermediate values of SDS/1-pentanol. The global resolution (R) is considered as the resolution (r_{ij}) of the least resolved peak pair, calculated by the valley-peak criterion.

The selected mobile phase was 0.060 M SDS - 2.5 % 1-pentanol at pH 7. It provides the maximal global resolution (R = 0.9998) in the minimum analysis time. A plasma sample spiked with a mixture of darunavir, ritonavir, emtricitabine and tenofovir at 5 μg mL^{-1} was analyzed using the optimal conditions (Figure 6.1B). The experimental chromatographic parameters (t_R; N; B/A) were: darunavir (8.2 min; 3958 theoretical plates; 0.97), ritonavir (18.4; 3201; 1.07), emtricitabine (3.6; 4682; 1.11) and tenofovir (5.5; 4501; 1.20). The errors of the predicted retention times were < 5 %.

The possibility to simultaneously analyze these four drugs using a mobile phase running under isocratic mode is highly interesting. This reduces the total analysis time because the stabilization time between two successive injections is not needed, facilitating the analysis of a large amount of samples. Moreover, the optimized micellar mobile phase uses a less amount of toxic organic solvent, than typically employed in hydroorganic mobile phases.

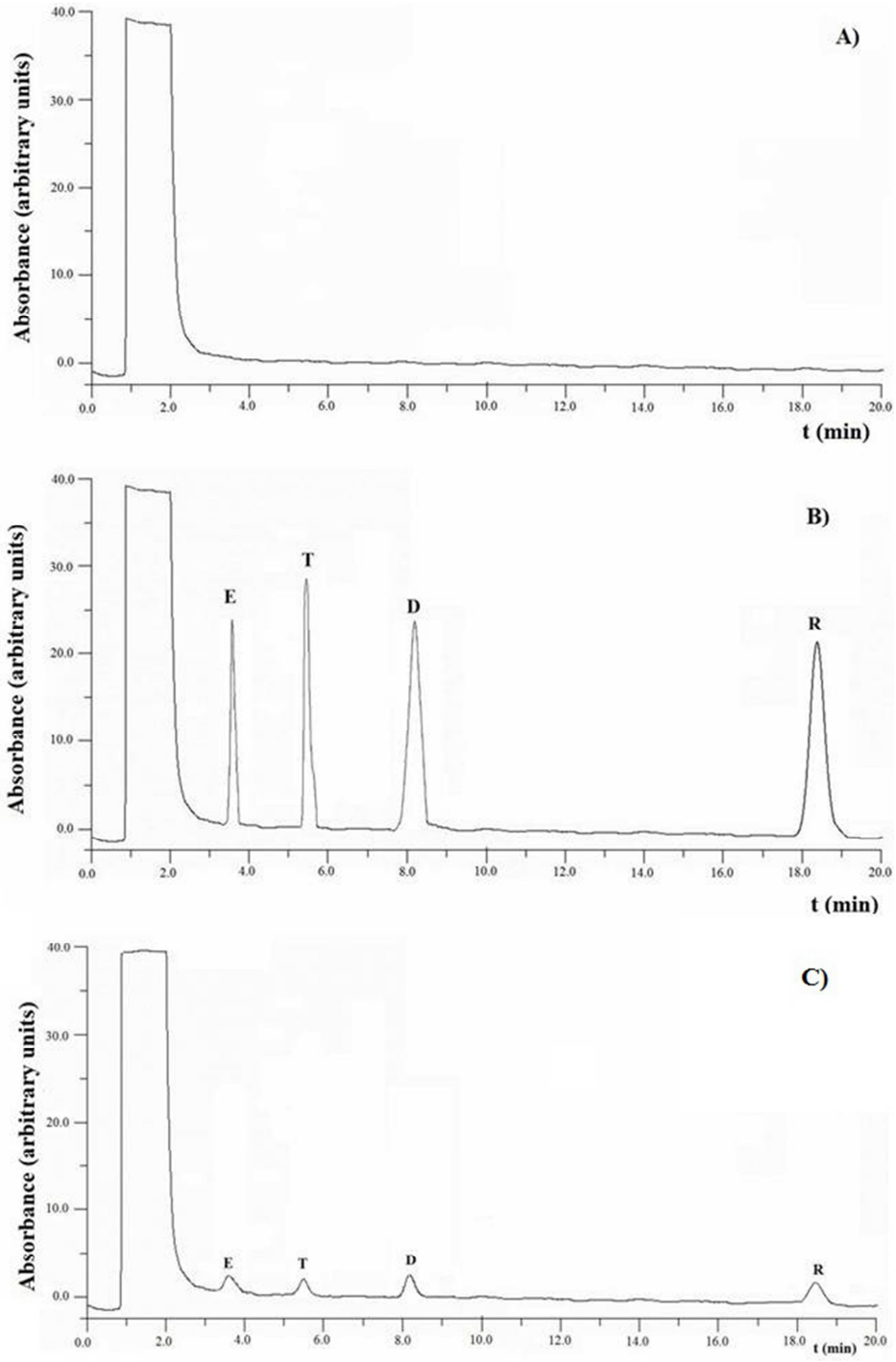


Fig. 6.1. Chromatogram by analysis of a plasma sample: A) blank, B) spiked with 5 μg mL^{-1} of darunavir (D), ritonavir (R), emtricitabine (E) and tenofovir (T) and C) spiked at the LOQ level for each ARV.

3.2. Method validation

The validation was performed following the requirements of the ICH Harmonized Tripartite Guideline, especially devoted to the analysis of drugs in any kind of samples worldwide [31]. The evaluated parameters were: specificity, calibration range, linearity, sensitivity (LOD and LOQ), inter- and intraday accuracy, repeatability, intermediate precision and robustness. The method was validated using spiked blank plasma samples, collected from healthy volunteers and originally free of ARV.

3.2.1 Specificity

Ten different blank plasma samples (from five males and five females) were analyzed using the optimized conditions (Figure 6.1A). In all cases, the chromatograms only show a high front and totally eluted at < 2.5 min, corresponding to the protein band. No peaks were detected at higher retention times. As the four studied ARV elutes at > 2.5 min, there are no potential interfering substances. The obtained chromatograms are clean, despite the complexity of plasma and the absence of cleanup procedure. The proteins and the endogenous compounds of plasma strongly interacts with the micelles in the mobile phase, provoking their fast elution. This result is habitual when analyzing plasma with MLC, expediting the analysis of drugs in plasma [30].

The blank plasma samples were spiked with 5 μg mL^{-1} of darunavir, ritonavir, emtricitabine and tenofovir, and analyzed. Results are shown in Figure 6.1B. The analytes are eluted enough separate between them and the protein band, and no coeluting endogenous compounds were detected, thus avoiding overlapping. Therefore, the ARV can be unambiguously identified and their area measured without error.

The possible interference of other drugs also administered to HAART patients was studied by analyzing a 5 μg mL^{-1} solution of each one (log Po/w, [6]): acebutolol (1.71), N-acetylprocainamide (0.99 [35]), acetaminophen (0.46), acetylsalycilic acid (1.19), amiodarone (7.57), amoxicillin (0.87), ampicillin (1.35), atenolol (0.16), azithromycin (4.02), bromazepam (2.54), caffeine (-0.07), captopril (1.02), carbamazepine (2.1), ciprofloxacin (-

0.57), clonazepam (2.76), chloramphenicol (1.14), cocaine (2.30), codeine (1.19), corticosterone (1.99 [35]), desipramine (4.90), dexamethasone (1.83), diazepam (2.82), diltiazem (2.8), doxycycline (-0.72), ephedrine (1.00), flunitrazepam (2.2), furosemide (2.03), gentamicin (-1.6), hydrocortisone (1.79), imipramine (4.80), levofloxacin (2.1), lidocaine (2.44), loratadine (4.8), medazepam (4.43 [35]), methotrexate (-1.85), nicotine (1.17), nifedipine (2.49), norfloxacin (-0.47), oxazepam (2.01), phenylephrine (-0.69), propranolol (3.48), quinidine (3.44), sulphametoxazole (0.79), sulpfthiazole (0.88), tetracycline (-1.30), theophylline (-0.02) and valproic acid (2.75) [29]. None of them eluted near the studied ARV under the optimized conditions.

3.2.2 Carry over effect

In order to evaluate the carry over effect, a plasma sample spiked with 25 μg mL^{-1} of darunavir, ritonavir, emtricitabine and tenofovir was analyzed under the optimal conditions. There concentration is over the expected in plasma patient. Immediately after, a blank plasma sample was analyzed, and no peak was observed near the retention time of the studied ARV. Therefore, there is not significant carry over effect at ARV concentrations under 25 μg mL^{-1}.

3.2.3 Calibration and sensitivity

Nine increasing concentrations ranging from 0.25 to 25 μg mL^{-1} for each ARV were analyzed by triplicate to construct a calibration curve. All the analyses were performed the same day. The slope, y-intercept and determination coefficient (r^2) were calculated by correlating the chromatographic areas (average value of the three replicates) with the corresponding concentration by least-square linear regression.

The regression curve for each ARV was calculated as the average of five calibration curves built over a 2-months period, by preparing all the spiked samples each time. The results are shown in Table 6.2.

The limit of detection is the minimal ARV concentration producing a response significantly different from the noise of the baseline. It is calculated by the 3.3s criteria: 3.3 times the standard deviation of the signal obtained by analysis of blank samples, divided by the sensitivity (slope of the calibration curve). The limit of quantification (LOQ) is the lowest concentration of ARV that can be quantified with acceptable accuracy and precision, and was taking following the 10s criteria. Results are presented in Table 6.2. A chromatogram of a blank plasma sample spiked with the LOQ for each ARV is shown in Figure 6.1C. The analytes can be detected enough above the noise level.

Excellent linearity was found in the studied interval, and the sensitivity was enough to detect the ARV in plasma from patients taking this HAART for pharmacokinetics purposes.

Table 6.2. Calibration curve parameters and sensitivity ([ARV] in μg mL^{-1})

Compound	Slope	Y-intercept	r^2	LOD	LOQ)
Darunavir	3.8 ± 0.2	0.4 ± 0.8	0.9996	0.090	0.250
Ritonavir	4.6 ± 0.3	- 0.7 ± 0.9	0.9991	0.080	0.240
Entricitabine	2.41 ± 0.05	0.3 ± 0.5	0.998	0.110	0.270
Tenofovir	2.88 ± 0.03	-0.5 ± 0.2	0.995	0.100	0.260

n = 5

3.2.4 Accuracy and precision

Intraday accuracy and repeatability was measured for darunavir, ritonavir, emtricitabine and tenofovir at 0.5; 2.5 and 5 μg mL^{-1}. The solutions were different that those prepared for the calibration studies. The analyses were 6-fold performed within the same day. The accuracy was the closeness between the average value of ARV concentrations provided by the method and the true spiked value (recovery), whereas the repeatability was calculated as the relative standard deviation (RSD) of the signal. Interday accuracy and intermediate precision were determined as the average values of five intraday measurements taken over a

3-months period. Results are shown in Table 6.3. Adequate recoveries (89.3 - 103.2 %) and high precision (<8.2 %) of the signal were found, thus indicating the reliability of the ARV concentrations in plasma obtained by the analytical method.

Table 6.3. Intra- and inter-day accuracy and precision.

		Intra-day[a]		Inter-day[b]	
Antiretroviral	[ARV] (μg mL^{-1})	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Darunavir	0.5	93.6	8.2	96.8	6.0
	2.5	102.5	4.6	100.5	4.2
	5	98.4	2.9	97.7	1.3
Ritonavir	0.5	89.3	5.7	93.5	6.5
	2.5	94.6	3.3	97.4	5.0
	5	103.2	4.0	99.4	3.5
Emtricitabine	0.5	92.4	7.0	91.2	6.3
	2.5	96.2	6.2	95.6	5.2
	5	97.5	4.0	98.4	2.4
Tenofovir	0.25	101.1	7.2	98.9	5.0
	2.5	94.8	4.8	96.5	3.9
	5	98.2	1.1	100.6	2.5

[a]n=6; [b]n = 5

3.2.5 Robustness

The variability of the elution strength and sensitivity was studied at slight variations of the following chromatographic conditions (range): SDS concentration (0.055 - 0.065 M), 1-pentanol amount (2.4 - 2.6 %), pH (6.9 - 7.1) and flow rate (0.95 - 1.05 mL min^{-1}). Thus, a plasma sample spiked with 5 μg mL^{-1} of each ARV was analyzed (n = 3), at three levels:

minimum, optimum and maximum value for each condition. The relative standard deviation of the three obtained values for retention time and peak area was calculated.

As shown in Table 6.4, the possible experimental oscillations of the chromatographic conditions would have a minimal effect in the retention time (<7.5 %) and peak area (<5.4 %).

Table 6.4. Evaluation of the robustness of the MLC method.

Compound	Parameter (Studied range)	Retention time (RSD, %)	Peak area (RSD, %)
Darunavir	SDS (0.055 - 0.065 M)	3.5	2.6
	1-pentanol (2.4 - 2.6 %)	4.8	3.0
	pH (6.9 - 7.1)	2.3	1.9
	Flow rate (0.95 - 1.05 mL min^{-1})	5.6	3.2
Ritonavir	SDS	5.2	2.8
	1-pentanol	6.2	5.4
	pH	4.5	3.1
	Flow-rate	7.5	1.5
Emtricitabine	SDS	3.5	4.7
	1-pentanol	6.2	1.8
	pH	2.6	4.2
	Flow-rate	5.9	2.5
Tenofovir	SDS	4.8	3.4
	1-pentanol	5.7	2.8
	pH	3.8	1.9
	Flow rate	5.8	3.1

3.2.6 Stability

For stability study purpose, a blank sample plasma was spiked with 5 μg mL^{-1} of each ARV and kept in a fridge (-20ºC in absence of light). Each day, this sample was thawed, analyzed and put another time in the fridge, during a 3-months period. No significant diminution of the peak area was detected for the analytes in this period. As the normal storage time in hospitals is about ten days, the analytical method does not show degradation problems.

3.3. Analysis of plasma samples of AIDS patients

Several plasma samples from AIDS patients whose the doctors have prescribed this HAART regimen were studied to measure the concentration of darunavir, ritonavir, emtricitabine and tenofovir. The samples were taken 6 hours after the ingestion of the tablets. For confidentiality reasons, no more information about this can be provided.

In order to assure the quality of the results, blank sample plasma was analyzed at the beginning of the analytical run and each 5 analysis. The quality control samples (blank sample spiked with 1 and 5 μg mL^{-1} of ARV) were analyzed at the end of the run. The acceptance criteria were: blank runs, no detection of ARV; and quality control (QC) samples, a bias and variability < 15 %. Results are shown in Table 6.5 and meet the acceptance criteria. According to the obtained values, the patients effectively take their medications.

The chromatogram obtained by the analysis of the plasma sample from the patient P1 can be seen in Figure 6.2. In all cases, the four ARV were clearly detected and quantified without interferences. No other peaks were detected in the chromatogram. The chromatograms obtained by the analysis of the other patients showed similar shape.

Table 6.5. Quantification of the antiretroviral (μg mL^{-1}) in plasma of HIV patients (n = 3)

Patient	Darunavir	Ritonavir	Emtricitabine	Tenofovir
P1	0.65 ± 0.04	1.78 ± 0.08	3.1 ±0.05	under LOD

P2	1.05 ± 0.09	0.85 ± 0.04	5.1 ± 0.4	0.45 ± 0.05
P3	0.85 ± 0.04	3.14 ± 0.9	0.75 ± 0.06	under LOD
P4	4.2 ± 0.3	3.7 ± 0.4	6.7 ± 0.5	0.82 ± 0.06
P5	3.4 ± 0.2	2.8 ± 0.4	0.95 ± 0.08	under LOD
P6	3.8 ± 0.3	3.0 ±0.4	1.8 ± 0.2	0.56 ± 0.04
P7	2.4 ± 0.2	4.5 ± 0.5	3.7 ± 0.3	1.05 ± 0.09
P8	5.1 ± 0.3	1.7 ± 0.3	4.2 ± 0.7	under LOD
QC 1 μg mL^{-1}	0.94 ± 0.21	1.01 ± 0.10	0.95 ± 0.09	0.98 ± 0.15
QC 5 μg mL^{-1}	4.8 ± 0.2	4.7 ± 0.3	5.0 ± 0.3	4.8 ± 0.4

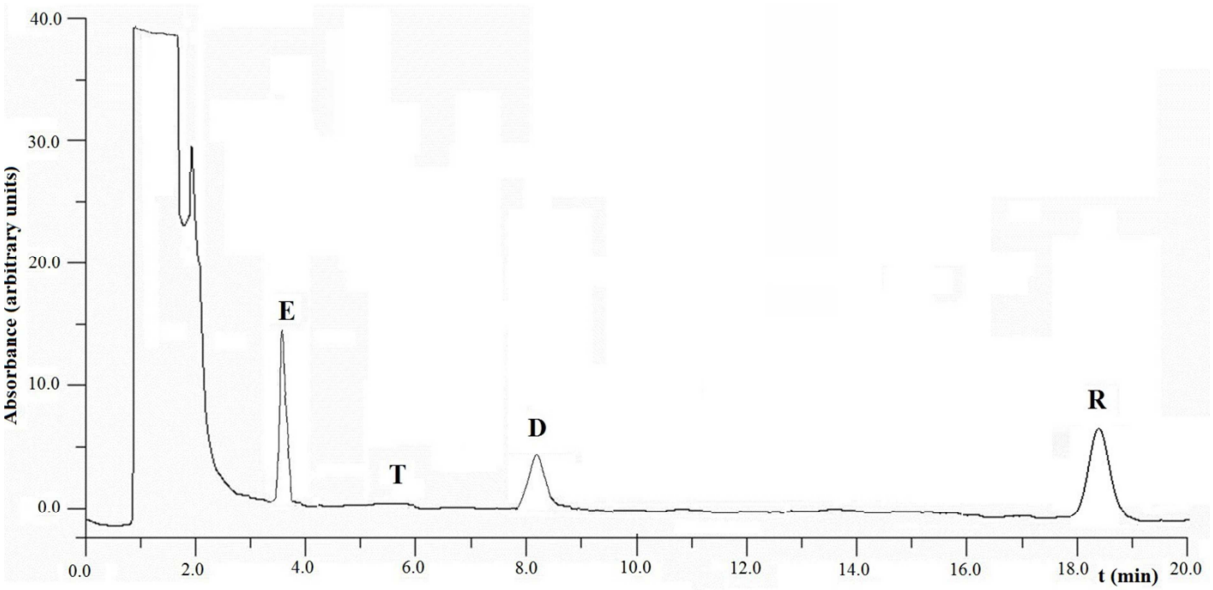


Figure 6.2. Chromatogram corresponding to P1, obtained using the optimal analysis conditions (Marks are as in Fig. 6.1)

4. Concluding remarks

MLC has been proven as a valuable methodology to analyze four ARV forming a HAART regimen (darunavir, ritonavir, emtricitabine and tenofovir) in plasma of AIDS patients. This is the first paper showing the determination of these four antiretroviral in plasma. The drug mixture is effectively resolved with good peak shape using an isocratic-running mobile phase in less than 20 min. The use of an interpretative strategy based on a chemometric tool has allowed the optimization of SDS and 1-pentanol amount by testing only five the mobile phase.

The main advantage of this technique is the shortening and simplification of the preparation of the sample, despite the complexity of the matrix. The method was successfully validated following the ICH Harmonized Tripartite Guideline, assessing the reliability of the quantitative data. The method can be considered environmental friendly, as it uses low amounts of toxic organic solvents and biodegradable reagents. Moreover, the analysis can be performed at a low cost, because it only needs inexpensive reagents and instrumentation, the reduction of the global analysis time and the possibility to successively analyze a high amount of samples. All these characteristics make it highly attractive in routine analysis of this HAART regimen in plasma from AIDS-patients for clinical purposes.

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6. Conflict of interest disclosure

The authors declare that they do not hold any financial/commercial conflict of interest.

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Chapter 7

General Conclusions

En la present memòria es presenten diferents estratègies analítiques basades en MLC per a la determinació de antirretrovirals i antivirals usats en la terapia HAART (prescrita contra la SIDA) en mostres de plasma i sèrum. En primer lloc, es proposà un mètode general capaç de detectar en sèrum els tretze farmacs més habituals en HAART: aciclovir, didanosina, ganciclovir, estavudina, zidovudina, abacavir, lamivudina, nevirapina, valaciclovir, zalcitabina, efavirenz, indinavir i ritonavir. Així doncs, es pot aplicar com a mètode de cribat per a identificar els medicaments que ha ingerit un determinat pacient. Posteriorment, es desenvoluparen cinc mètodes analítics, centrant-se en la quantificació simultànea dels tres o quatre antirretrovirals que formen cada un dels cinc tractaments HAART més prescrits: HAART1 (lamivudina + zidovudina + efavirenz), HAART2 (estavudina + lamivudina + efavirenz), HAART3 (lamivudina + tenofovir + efavirenz) en sèrum, HAART4 (abacavir + lamivudina + raltegravir) i HAART5 (darunavir + ritonavir + emtricitabine + tenofovir), en plasma. En tots els cassos s'utilitzen columnes apolars C18 com a fases estacionàries i dissolucions del tensiactiu docecil sulfat sòdic (SDS) per damunt de la concentració micel·lar crítica, tamponades amb fosfat, i circulant en mode isocràtic a 1 mL/min, com a fase mòbil. Tanmateix, a tots els cassos la detecció es va realitzar per absorció UV-Visible.

La preparació de la mostra es va simplificar a causa de la habilitat de les dissolucions micel·lars de solubilitzar matrius complexes. Per a totes les metodologies, el protocol experimental va consistir en la dilució de la mostra de sèrum o plasma en una dissolució 0.05 M SDS a pH 7, filtració i injecció. Per tant, l'aliquota va ser quantitativament injectada. El ratio de dilució es va optimitzar considerant la necessitat de evitar un bloqueix prematur del filtre per a obtindre una aliquota representativa de la mostra en quantitat suficient, i de no disminuir excessivament la sensitivitat. Finalment, el factor de dilució va ser de 1/10 per al sèrum i 1/5 per al plasma. Com que la fase mòbil també és una dissolució micel·lar, les proteïnes i els altres compostos hidròfobs hi romangueren solubilitzats i no representaren un perill per a la columna. Per tant, la introdució de etapes de extracció i neteja de la mostra no foren necessaries, al contrari que en la HPLC aquoorgànica, ón es deu separar els analits de la matriu i evitar la injecció de aquestos compostos potencialment precipitables als conductes cromatogràfics. La menor manipulació de la mostra i dels reactius i la disminució del nombre d'etapes d'anàlisi va permetre minimitzar la intervenció del analista i un major grau

d'automatització, reduïnt les fonts d'errors i la possibilitat de la pèrdua de l'analit, incrementant la reproduïbilitat dels mètodes. Els procediments experimentals resultants son senzills, ràpids, efectius i no requerixen l'ús de reactius tòxics o d'instrumentació específica.

La composició de la fase mòbil (SDS i alcohol) es va optimitzar fins a aconseguir la elució de cada analit sense interferències del altres fàrmacs i dels compostos de la matriu, en un temps raonablement breu. Com que el pH no va influir significativament en la retenció dels analits, es va fixar a 7, per a obtindre un entorn menys agressiu per a la columna. Es va fer un estudi complet del comportament cromatogràfic dels antirretrovirals i antivirals, i de les matrius en fases mòbils de SDS pur (0.05 - 0.20 M), i fases mòbils híbrides de SDS (0.05 - 0.15 M) amb 1-propanol (2.5 - 12.5 %); 1-butanol (1 - 7 %) i 1-pentanol (2 - 6 %). Les concentracions es seleccionaren seguint un diseny experimental que inclou quatre punts amb la combinació dels valors màxims i mínims tradicionalment recomanats per a SDS i l'alcohol a l'MLC, i un punt central, considerant els valor intermedis. Posteriorment, les dades obteses es processaren estadísticament per a ajustar equacions matemàtiques capaces de modelitzar els paràmetres cromatogràfics dels analits (temps de retenció, eficacia, asimetria) segons la composició de la fase mòbil, gràcies a forta estabilitat i reproduïbilitat dels processos de repartiment en l'MLC. Es poden usar per a prediure el seu valor a concentracions intermèdies de SDS i alcohol. Combinant les dades de un grup de substàncies, també es va modelitzar la resolució per parells i global, i inclús visualitzar els resultats mitjançant la construcció de cromatogrames simulats. Aquesta eina matemàtica va permetre usar una estratègia interpretativa per a la optimització simultànea de la concentració de SDS i alcohol, amb l'estudi de només cinc fases mòbils. Es va reduïr el esforç necessari per a realitzar aquesta etapa, al requerir únicament l'anàlisi per cinc fases mòbils per cada alcohol.

El comportament de la matriu es va estudiar mitjançant l'anàlisi de mostres de plasma i sèrum lliure de fàrmacs. En totes les fases mòbils estudiades, les proteïnes, macromolècules i altes compostos de la matriu foren eluits entre 1 i 2 min, formant una intensa banda al front del cromatograma. Els compostos de la matriu mantenen interaccions preferents amb les micel·les i gairebé no es retenen a la columna. Per tant, en descartar les fases mòbils que eluixen els analits a temps menors a 2 min, es varen eliminar els problemes d'interferència de la matriu, malgrat la gran diversitat de compostos presents al plasma i al sèrum. Per tant, l'ùs de MLC va ser clau per a facilitar la etapa d'optimització de la fase mòbil.

En el cas del mètode de cribat, a causa de la semblança entre alguns fàrmacs, no es va obtindre una fase mòbil capaç de resoldre els tretze analits. Es va dividir en tres grups, i cadascú es va analitzar amb una fase mòbil òptima: el grup A (acyclovir, didanosina, ganciclovir, estavudina, zidovudina) es va eluir en menys de 10 min amb 0.05 M SDS; el grup B (abacavir, lamivudina, nevirapina, valaciclovir i zalcitabina) en < 10 min amb SDS 120 mM/4.5% 1-propanol, i el grup C (efavirenz, indinavir i ritonavir) en < 18 min, a SDS 150 mM/5% 1-pentanol. No es va obtindre una fase mòbil capaç de resoldre adequadament els tres fàrmacs inclosos a HAART1, HAART2 i HAART3, a causa de la gran diferencia de hidrofibicitat de lamivudina i efavirenz. Així doncs, aquestos dos fàrmacs s'analitzaren usant una fase mòbil per a cada un d'ells, mentre que el tercer antirretroviral es va analizar conjuntament amb l'ún o l'altre. Per a HAART1, lamivudina i zidovudina s'eluiren amb 0.05 M/2.5 % 1-propanol en 4 min, i l'efavirenz en 16 min amb 0.05 M SDS/6 % 1-pentanol. En HAART2, lamivudina i estavudina foren detectats en < 6 min amb 0.05 M SDS i per a l'efavirenz es va fer servir el la mateixa fase mòbil que a HAART1. En HAART3, lamivudina es va eluir amb 0.15 M SDS en 4 min, mentre que tenofovir i efavirenz foren eluits en 16 min amb 0.05 M SDS/6 % 1-pentanol. Per a HAART4, la fase mòbil óptima ve ser de 0.05 M (elució en < 32 min). Finalment, l'anàlisi cromatogràfic de HAART5 es completà en 0.06 M SDS - 2.5 % 1-pentanol en < 20 min. En tots els cassos, els pics cromatogràfics presentraren una forma aproximadament gaussiana, amb una elevada eficacia (1585 - 11150 plats teòrics) i factors d'assimetria al voltant de 1 (0.71 - 1.45).

Aquestos resultats mostren que l'MLC és aplicable a la detecció dels antirretrovirals i antivirals més prescrits, en temps breu, considerant la similaritat de propietats i el ample rang de hidrofobicitat. La elevada versatilitat de l'MLC respecte de la HPLC es deu a la varietat de interaccions en la fase mòbil (fase estacionària - fase aquosa - entorn lipofílic a l'interior de la micel·la - entorn polar i aniònic a l'exterior de la micel·la). L'addició d'un alcohol de cadena curta augmenta el poder eluent i la eficacia de la fase mòbil. Les fases mòbils micel·lars usen només l'SDS i tampó fosfat: reactius barats, biodegradables i fàcils d'eliminar del sistema cromatogràfic a causa de la seua baixa viscositat. A més a més, contenen una quantitat reduïda de dissolvent orgànic (fins a 12.5 %, mentre que a l'HPLC hidroorgànica pot ser fins a 100 %). Aquestes característiques fan que l'MLC siga més segura (per la menor inflamabilitat i toxicitat de les dissolucions), més barata (usa reactius i instrumentació

senzilla) i tinga un menor impacte mediambiental (ús de un menor volum de reactius tòxics). El fet que les fases mòbils circulen en mode isocratic, unit a la simplicitat del procediment experimental, permeten el processament successiu i automatitzat de una gran quantitat de mostres per dia, baixant el cost de l'anàlisi.

La detecció es va realitzar per absorció UV-Visible (el detector més comú i barat), ja que tots els fàrmacs estudiats son cromògens. La longitut d'ona de detecció de es va triar en base a les longituts d'ona màximes de cada analit en medi micel·lar. Per a cada grup resolt conjuntament, es va triar una longitud d'ona a la qual tots els analits presentaren una forta absortivitat, per a evitar canvis de longitud d'ona dins de una carrera cromatogràfica. Així doncs, el analits inclosos als grups A i B del mètode de cribat i els antirretrovirals del tractament HAART4 es detectaren a 260 nm, mentre que la resta d'analits es detectaren a 214 nm. Sota aquestes condicions, per al mètode d'anàlisi dels antirretrovirals i antivirals més comuns s'obtingueren uns LOD de 55 a 165 ng/mL i uns LOQ de 150-450 ng/mL, que permeten realitzar un cribat de fàrmacs eficaç en mostres de sèrum. Aplicant els mètodes de quantificació dels antirretorivirals inclosos als tractaments HAART més habituals, es va arrivar a LODs de 70 - 250 ng/mL i LOQs de 200-500 ng/mL, suficients per a realizar estudis de farmacocinètica, detectar quantitats anormals de fàrmacs en sang i comprovar d'adherència del pacient al tractament. Les condicions experimentals (dilució i longitut d'ona de detecció) permitiren obtindre una sensibilitat suficient per a assolir els objectius prevists, ja que la concentració de aquestos fàrmacs sol oscil·lar entre 0.5 i 5 μg/mL, sense la necessitat de una etapa de preconcentració.

Els mètodes es validaren seguint les requeriments de dos Guíes Oficials de Validació. Es va seleccionar la *Guidance for Industry: Bioanalytical Method Validation* de la US Food and Drug Administration i la *ICH Harmonized Tripartite Guideline* de la International Conference of Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, especialment proposades per al estudi de fàrmacs. La validació es va fer en matriu, per a donar més rigorositat a l'estudi. Els parametres de validació i els resultats obtesos per a tots els analits es descriuen a continuació: selectivitat (tots els analits son detectats sense interferències entre ells ni amb altres compostos de la matriu), intèrval de calibratge (del LOQ a 10 μg/L), linealitat (r^2 > 0.995), exactitut (84.5 - 105.3 %), precisió (<8.2 %), robustessa (< 7.5 %) i estabilitat (els analits es mantenen sense degradació

significativa tres messos enmagatzemats en la foscor a -20ºC). Aquestos resultats estan dins dels nivells acceptats per les guíes de validació, demostrant que les concentracions de antirretrovirals i antivirals mesurades en mostres reals de plasma i sèrum estaran pròximes al valor correcte. Finalment, els mètodes s'aplicaren amb èxit a l'estudi de mostres extretes de sang de pacients de SIDA tractats amb la terapia HAART, procedents del Hospital La Plana de Vil·la-real. Per tant, emprant aquestos mètodes, els metges estaran correctament assessorats per a extraure conclusions clíniques a partir de les concentracions de antirretrovirals i antivirals en plasma i sèrum.

Les principals característiques dels mètodes desenvolupats: senzillesa del procediment experimental, millora de la seguretat al laboratori, alt grau d'automatització, reducció del preu de l'anàlisi, baixa polució, sensibilitat adecuada, fiabilitat del resultats i la possibilitat de processar consecutivalement una gran cantitat de mostres per dia, els converteixen en una eina molt útil en laboratoris dedicats a l'anàlisi clínic que estudien mostres de plasma i sèrum de malats de SIDA tractats amb HAART.

Annex 1. Aportacions originals

Els estudis descrits han sigut realitzats gràcies al finançament rebut pel Ministeri d'Educació i Ciència (CTQ 2007 764473/BQU), Fundació Caixa Castelló-Bancaixa (P1-1B2006-12) i del Plà per a la Promoció de l'Investigació de la Universitat Jaume I (P1.1B2012-36), organismes que consideraren que la seua importància i la rellevancia social de la recerca mereixia el seu suport. El treball realitzat ha permés la publicació de les següents aportacions originals en revistes i reunions científiques de primer nivell:

Articles en revistes de recerca científica

1. M.A. Raviolo, I. Casas Breva, J. Esteve-Romero, Screening and monitoring antiretrovirals and antivirals in the serum of acquired immunodeficiency syndrome patients by micellar liquid chromatography. *J. Chromatogr. A* 1216 (2009) 3546–3552

2. I. Casas-Breva, J. Peris-Vicente, M. Rambla-Alegre, S. Carda-Broch, J. Esteve-Romero, Monitoring of HAART regime antiretrovirals in serum of acquired immunodeficiency syndrome patients by micellar liquid chromatography. *Analyst* 137 (2012) 4327-4334

3. J. Peris-Vicente, M. Villareal-Traver, I. Casas-Breva, S. Carda-Broch, J. Esteve-Romero, A micellar liquid chromatography method for the quantification of abacavir, lamivudine and raltegravir in plasma. *J. Pharm. Biomed. Anal.* 98 (2014) 351–355

4. J. Peris-Vicente, M. Villarreal-Traver, I. Casas-Breva, S. Carda-Broch, J. Esteve-Romero, Use of micellar liquid chromatography to analyze darunavir, ritonavir, emtricitabine and tenofovir in plasma. *J. Sep. Sci.* 37 (2014) 2825–2832.

Ponències en congressos internacionals de recerca científica

31st International Symposium on High Performance Liquid Phase Separation and Related Techniques (HPLC 2007). 17-22 de Junio de 2007; Ghent, Bélgica.

1) "Screening of antiviretrovirals in human serum" (P18-08) J. Esteve-Romero, M.A. Raviolo, I. Casas Breva, M. Rambla-Alegre, S. Carda-Broch, M.E. Capella-Peiró, J. Clausell-Tormos

2) "Hospitalary drug monitorization by micellar liquid chromatography" (P18-111) J. Esteve-Romero, A. Martinavarro-Domínguez, I. Casas Breva, D. Bose, S. Carda-Broch, L. Monferrer-Pons, L. Álvarez-Rodríguez, M.E. Capella-Peiró, M. Rambla-Alegre, M.L. Chin-Chen

3) "Determination of tamoxifen in serum samples" (P18-112) J. Esteve-Romero, D. Bose, Abhilasha Durgbanshi, Adrià Martinavarro-Domínguez, E. Ochoa-Aranda, S. Carda-Broch, L. Àlvarez-Rodríguez, I. Casas Breva

32nd International Symposium on High Performance Liquid Phase Separation and Related Techniques (HPLC 2008). 10-16 de Mayo de 2008; Baltimore, MD, USA

4) "Monitorization of acyclovir, didanosine, ganciclovir, stavudine and zidovudine in serum samples of HIV patients" (P-1809-W) J. Esteve-Romero, I. Casas-Breva, M.A. Raviolo, E. Ochoa-Aranda, A. Martinavarro-Domínguez, M. Rambla-Alegre, S. Carda-Broch

5) "Determination of tamoxifen and its metabolite endotamoxifen in serum samples of breast cancer patients" (P-1810-Th) J. Esteve-Romero, E. Ochoa-Aranda, D. Bose, A. Durgbanshi, A. Martinavarro-Domínguez, S. Carda-Broch, I. Casas-Breva

33nd International Symposium on High Performance Liquid Phase Separation and Related Techniques (HPLC 2009). 29 de Junio y 2 de Julio de 2009; Dresden, Alemania.

6) "Chromatographic determination of disopyramide, lidocaine and quinidine" (PAQ15-We) J. Esteve-Romero, A. Martinavarro-Domínguez, M.E. Capella-Peiró, S. Carda-Broch, M.

Rambla-Alegre, M.L. Chin-Chen, E. Ochoa-Aranda, I. Casas-Breva, D. Bose, A. Durgbanshi

36th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2011). 19-23 de Junio de 2011; Budapest, Hungría

7) "Study of the spoilage of fish sauce by the evaluation of biogenic amines amount using a micellar liquid chromatography-based method after derivatization with 3,5-dinitrobenzoyl chloride" (P2-G-627-WE) M.L. Chin-Chen, S. Carda-Broch, J. Peris-Vicente, M. Rambla-Alegre, J. Esteve-Romero, B. Beltrán-Martinavarro, S. Marco-Peiró, I. Casas-Breva

8) "Analysis of Antibiotics in Biological Fluids using Surfactant Mediated Mobile Phases" (P1-S-350-TU) M. Rambla-Alegre, J. Paños-Pérez, J. Peris-Vicente, I. Casas-Breva, D. Bose, N. Agrawal, J. Esteve-Romero, S. Carda-Broch

9) "Micellar liquid cromatography in bioanalytical chemistry" (P1-S-396-TU) J. Esteve-Romero, S. Carda-Broch, M. Rambla-Alegre, M.L. Chin-Chen, M.A. Raviolo, D. Bose, A. Durgbanshi, I. Casas-Breva

38th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2012). 16-21 de Junio de 2012; Anaheim, CA, USA

10) “Development of A Micellar Liquid Chromatography-Based Methodology to Monitor Antiretroviral Serum of Acquired inmunodeficiency Syndrome Patients” (P-330-Wed) I. Casas-Breva, J. Peris-Vicente, S. Carda-Broch, M.A. Raviolo, J. Esteve-Romero

11) "Method Validation in Micellar Liquid Chromatography" (P-451-Tue) M. Rambla-Alegre, B. Beltrán-Martinavarro, S. Marco-Peiró, I. Casas-Breva, D. Bose, S. Carda-Broch, J. Peris-Vicente, J. Esteve-Romero

12) "Analysis of Antibiotics in Fish, Egg and Milk Samples by Micellar Liquid Chromatography" (P-148-Tue) M. Rambla-Alegre, S. Carda-Broch, M.A. Raviolo, I. Casas-Breva, J. Peris-Vicente, J.V. Gimeno-Adelantado, J. Esteve-Romero

39th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2013). 16-20 de Junio de 2013; Amsterdam, The Netherlands

13) "Hospitalary Monitorization in Blood of an Highly Active Antiretroviral Therapy Regime" (BIOM25_MO) J. Peris-Vicente, I. Casas-Breva, S. Carda-Broch, M. Villarreal-Traver, D. Fabregat-Safont, N. Fuentes-Navarro, E. Peris-García, J. Esteve-Romero

40th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2013 - Hobart). 18-21 de Noviembre de 2013; Hobart, Australia

14) "Micellar liquid chromatographic determination of antiretroviral prescribed for naïve-aids-patients in human plasma" (140) J. Esteve-Romero, J. Peris-Vicente, I. Casas-Breva, M. Villarreal-Traver, S. Carda-Broch, D. Fabregat-Safont, M.A. Raviolo, E. Ochoa-Aranda

Annex 2. Futures línees de recerca

El grup de Química Bioanalítica té com a principal línia de recerca el desenvolupament de mètodes ràpids, simples, econòmics, segurs, fiables i aplicables al anàlisi de fàrmacs i continuaré participant dins d'aquesta línia.

En un futur tinc previst ampliar el estudi detallat en aquesta memòria a altres antirretrovirals i antivirals, i a diferents famílies de fàrmacs, com antibiòtics, antitumorals, antidepresants, antihistamínics i altres, segons les necessitats dels Hospitals. Així doncs, disposaré de una col·lecció de nous mètodes que es podran proposaran per a la seua implantació en anàlisi clínic. També participaré en treballs d'optimització i validació de mètodes per a la detecció de fàrmacs en altres matrius, com a formulacions farmacèutiques, per a aplicar a control de qualitat, i a altres mostres biològiques, com orina, saliva, òrgans i grasses corporals, els quals es podran emprar per a fer un estudi més complet de seua farmacocinètica e interacció en el cos humà.

En un futur, treballaré en projectes de recerca amb el grup de Química Bioanalítica de l'UJI, sempre que estiguen relacionats amb l'àmbit de la salut i de l'anàlisi clínic.

Annex 3. Acceptació dels coautors de les publicacions que integren la tesis, de que el doctorand presenta el treball com a tesi i renúncia expressa d'aquests a presentar-ho com a part d'una altra tesi doctoral (segons Art. 23 de la NORMATIVA DELS ESTUDIS DE DOCTORAT, REGULATS PEL RD 99/2011, EN LA UNIVERSITAT JAUME I, Aprovada pel Consell de Govern núm. 19 de 26 de gener de 2012)

Josep Esteve Romero, director de la present tesi, declara que els coautors de les publicacions que es presenten en aquesta tesi, i que pase a enumerar: Samuel Carda Broch, Juan Peris Vicente, Maria Rambla Alegre, Mónica Ana Raviolo i Mónica Villarreal Traver no utilitzarem el material que aci es presenta per a formar part d'altres tesis. I per a que conste on convinga, signe la present.

Josep Esteve Romero

Juan Peris Vicente

Samuel Carda Broch

Mónica Villarreal Traver

Universitat Jaume I, 15 de Maig de 2015

Annex 4. Abreviatures i acrònims

antirretrovirals (ARV):

FI: *Fusion inhibitor*

ISTI: *integrase strand transfer inhibitor*

NRTI: *Nucleoside reverse transcriptase inhibitor*

- 3TC: *lamivudine*
- ABC: *abacavir*
- AZT: *zidovudine*
- d4T: *stavudine*
- ddC: *zalcitabine*
- ddl: *didanosine*
- FTC: *emtricitabine*

NtRTI: *Nucleotide reverse transcriptase inhibitor*

NNRTI: *Non-nucleoside reverse transcriptase inhibitor*

- DLV: *delavirdine*
- EFV: *efavirenz*
- NVP: *nevirapine*

PI: *Protease inhibitor*

- APV: *amprenavir*
- ATV: *atazanavir*
- IDV: *indinavir*
- LPV: *lopinavir*

NFV: *nelfinavir*

RTV: *ritonavir*

SQV: *saquinavir*

Reactius de laboratori:

$NaH_2PO_4.H_2O$: Dihidrogenfosfat de sodi monohidratat

Na_2HPO_4: Hidrogenofosfat de disodi

NaOH: Hidròxid de sodi

HCl: Àcid clorhídric

Tensioactius:

Brij-35: polioxietilè (23) lauril èter (*polyoxyethylene (23) lauryl ether*)

CTAB: bromur de cetiltrimetilamoni (*Cetyl Trimethyl Ammonium Bromide)*

SDS: dodecilsulfat sòdic *(Sodium Dodecyl Sulfate)*

Termes biològics:

CD4: cúmul de diferenciació 4 (*cluster of differentiation 4*)

DNA: *Desoxirribonucleic acid*

HAART: *Highly active antiretrovial therapy*

RNA: *Ribonucleic acid*

SIDA (*AIDS*): síndrome de inmunodeficiencia adquirida (*acquired immunodeficiency syndrome*)

TDM: Monitorització terapèutica de fàrmacs (*Therapeutic drug monitoring*)

VIH (*HIV*): virus de inmunodeficiencia humana (*human immunodeficiency virus*)

Tècniques analítiques

GC: cromatografia de gasos (*Gas Chromatography*)

HPLC, RPLC o RP-HPLC: cromatografia líquida en fase inversa (*High Performance Liquid Chromatography- Reversed Phase)*

HPLC-DAD: HPLC acoblada a un detector per matriu de diodes (*Diode Array Detection)*

HPLC-FLD: HPLC acoblada un detector de fluorescència (*Fluorescence Detector*)

HPLC-MS o LC/MS: HPLC acoblada a un espectròmetre de masses (*Mass Spectrometry*)

HPLC-MS/MS o LC-MS-MS: HPLC acoblada a un espectròmetre de masses en tàndem

HPLC-UV: HPLC acoblada a un detector d'absorbància UV-visible d'onda variable

IPC: cromatografia per parells d'ions (*Ion-pair chromatography*)

MLC: cromatografia líquida micel·lar (*Micellar Liquid Chromatography*)

TLC: cromatografia en capa fina (*Thin layer chromatography*)

UV: ultraviolat

Associacions i Organismes

CDC: *Centers for Disease Control and Prevention*

FDA: *Food and Drug Administration*

FPU: Formación del Profesorado Universitario

ICH: Conferència Internacional d'Armonització (*Internacional Conference on Harmonization*)

MEC: Ministerio de Educación y Cultura

OCIT: Oficina de Cooperació en Investigació i Desenvolupament Tecnològic

UV: Universitat de València

Paràmetres cromatogràfics

B/A: Factor d'asimetria

k: factor de retenció o factor de capacitat

N: nombre de plats teòrics (eficiència)

t_0: temps mort

t_R: temps de retenció

K_{AS} o PWS: Equilibri de repartiment del analit entre l'aigua pura i la fase estacionaria

K_{AM} o PWM: Equilibri de repartiment del analit entre l'aigua pura i la micel·la

K_{AD}: mesura la variació de concentració de analit en la fase aquosa per l'introducció del dissolvent orgànic.

K_{MD}: mesura la variació en la concentració de l'analit en la micel·la a causa de l'introducció del dissolvent orgànic.

PMS: Equilibri de repartiment del analit entre la micel·la i la fase estacionària

Paràmetres químics

CMC: concentració micel·lar crítica (*critical micellar concentration)*

Ka: constant de desprotonació d'un àcid

Po/w : coeficient de repartiment octanol-aigua *(octanol-water partition coefficient)*

rpm: revol·lucions per minut

Paràmetres de validació

QC: control de qualitat (*Quality control*)

LOD: límit de detecció (*Limit of Detection*)

LOQ: límit de quantificació (*Limit of Quantitation)*

r^2: coeficient de determinació (*determination coefficient*)

RSD: desviació estàndard relativa (*Relative Standard Derivation)*