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Screening and monitoring antiretrovirals and antivirals in the serum of acquired immunodeficiency syndrome patients by micellar liquid chromatography

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ABSTRACT

Thirteen different antiretrovirals 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 sulphate 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% propanol) is used for the group containing abacavir, lamivudine, nevirapine, valaciclovir and zalcitabine, whereas the third mobile phase (SDS 150 mM/5% 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 $\mu g\,mL^{-1}$. 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 inter-day 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 antiretrovirals and antivirals.

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 regimes (Fig. 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 how people break down (metabolise) 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 from 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].

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 to a

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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, fulfilment 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 sulphate (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 antiretrovirals and antivirals used in the treatment of patients with AIDS, using three mobile phases that contained sodium dodecyl sulphate alone or with propanol, butanol or pentanol and with direct injection of human serum.

2. Experimental

2.1. Chemicals and reagents

The antivirals studied were: abacavir sulphate, valaciclovir hydrochloride (Glaxo Smith Kline, Brentford, UK), acyclovir sodium, ganciclovir sodium (Roche Farma, Barcelona, Spain), didanosine, efavirenz, indinavir sulphate, 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). Sodium dodecyl sulphate, or SDS, 1-propanol, 1-butanol, 1-pentanol, disodium hydrogenphosphate, sodium dihydrogenphosphate, hydrochloric acid and sodium hydroxide 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 °C. The antivirals, the antiretrovirals and the blood samples were provided by the La Plana Hospital in Vila-real 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 optimisation 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 2 mL 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 1 mL 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 optimisation 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 mobile phase. All stock and working solutions stored at +4 °C 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 hydrogenphosphate/sodium dihydrogenphosphate 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. Optimisation strategy and mobile phase selection

In order to find the best mobile phase composition that allows the 13 antiretrovirals and antivirals considered in this study to be analysed simultaneously, each of them was injected in mobile phases at pH 7 containing SDS (mM)/modifier (%): SDS/pentanol (50/1, 50/5, 75/3, 100/3, 125/3, 150/1, 150/5); SDS/butanol (50/1, 50/7, 75/4.5, 100/4, 125/4.5, 150/1, 150/7); SDS/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