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

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Abstract

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

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

1. Introduction

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

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

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

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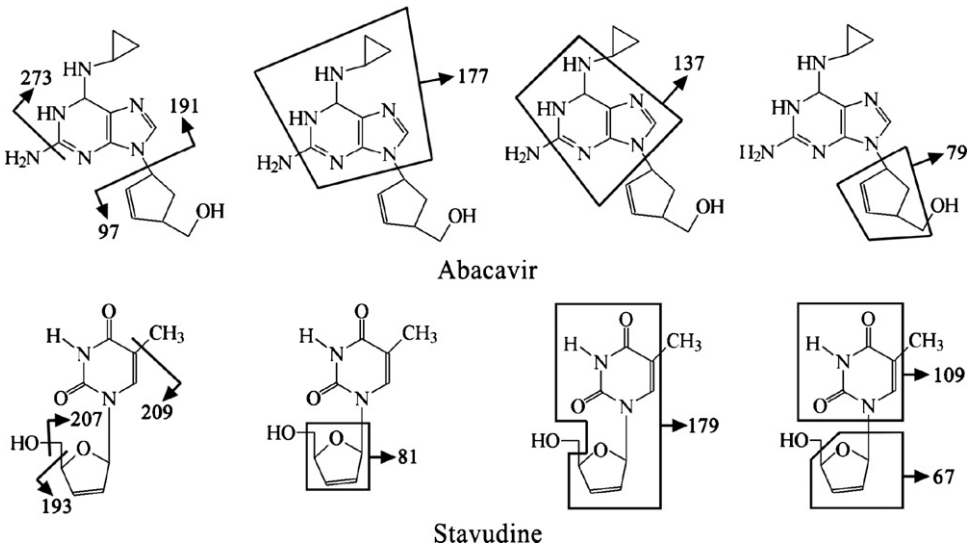

Fig. 1. Chemical structures of the HIV NRTIs abacavir and stavudine. Fragment ions observed under MS/MS conditions are shown. The mass of MS/MS fragments is expressed as *m/z*. For details see text.

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

2. Materials and methods

2.1. Chemicals

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

Stock solutions of abacavir, amprenavir, didanosine, efavirenz, lamivudine, lopinavir, nevirapine, ritonavir, and stavudine were prepared by dissolving 5.0 mg of each anti-HIV drug in 5.0 mL of methanol. Stock solutions were diluted with methanol to a final concentration ranging between 2.00×10^{-2} pmol/μL and 2.00 pmol/μL.[1] The HBA saturated solution (≥2.0 g/L) was

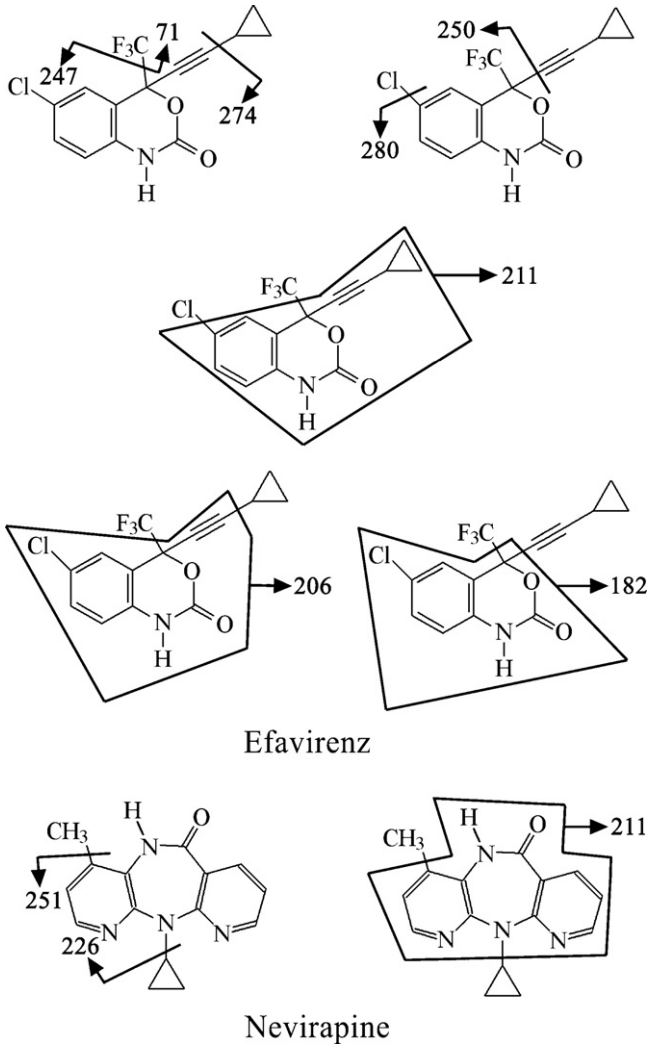

Fig. 2. Chemical structures of the HIV NNRTIs efavirenz and nevirapine. Fragment ions observed under MS/MS conditions are shown. The mass of MS/MS fragments is expressed as *m/z*. For details see text.

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

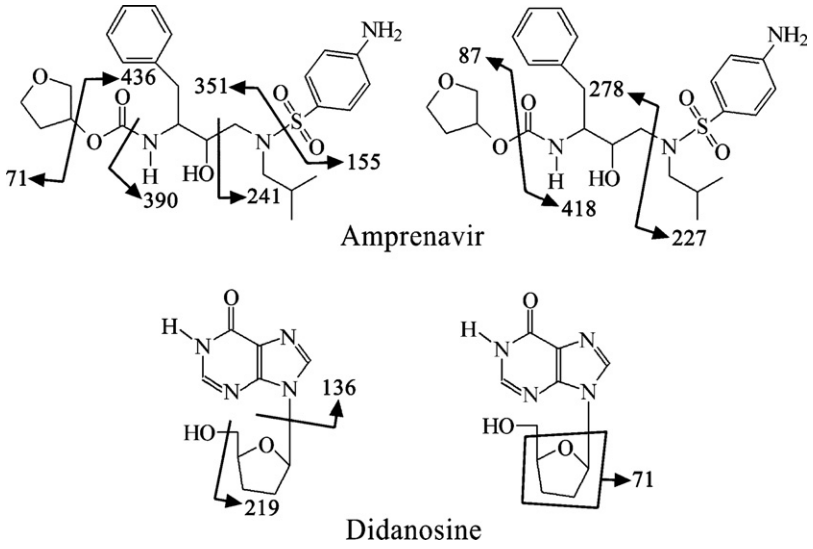

Fig. 3. Chemical structures of the HIV PIs amprenavir and didanosine. Fragment ions observed under MS/MS conditions are shown. The mass of MS/MS fragments is expressed as *m/z*. For details see text.

prepared by dissolving HBA in 50% acetonitrile–0.1% trifluoracetic acid.

2.2. Sample preparation

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

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

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

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

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

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

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

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

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

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

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

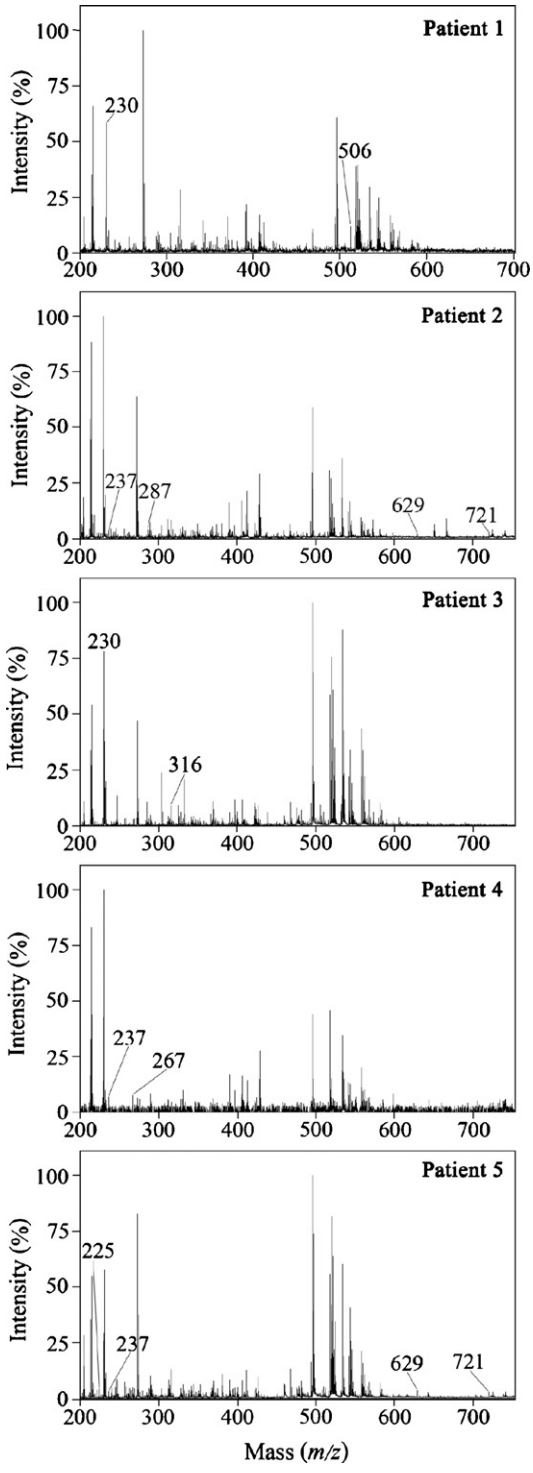

Fig. 4. MALDI-TOF/TOF MS spectra of the plasma of five HIV-infected patients under therapeutical treatment with amprenavir and lamivudine (Patient 1, see Table 2), abacavir, didanosine, lopinavir, and ritonavir (Patient 2, see Table 2), efavirenz and lamivudine (Patient 3, see Table 2), didanosine and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

2.5. Recovery

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

2.6. Data analysis

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

3. Results and discussion

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

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

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

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

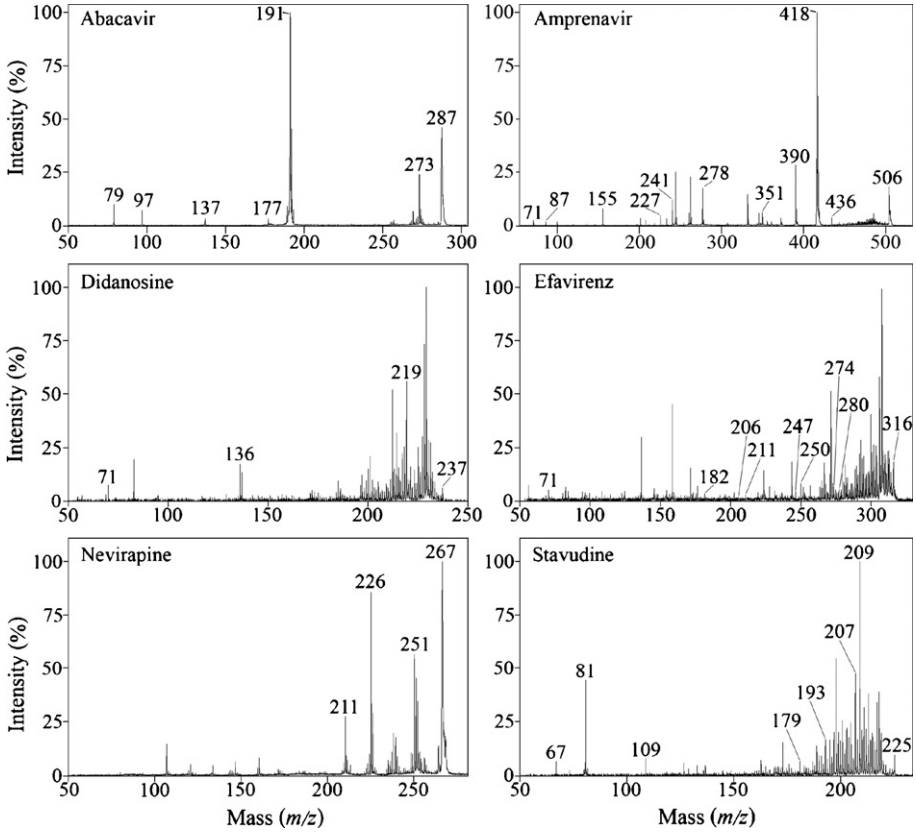

Fig. 5. MALDI-TOF/TOF MS/MS spectra of abacavir (Patient 2, see Table 2), amprenavir, (Patient 1, see Table 2) didanosine (Patient 2, see Table 2), efavirenz (Patient 3, see Table 2), nevirapine, (Patient 4, see Table 2), and stavudine (Patient 5, see Table 2) from the plasma of HIV-infected patients.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

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

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

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

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

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

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

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

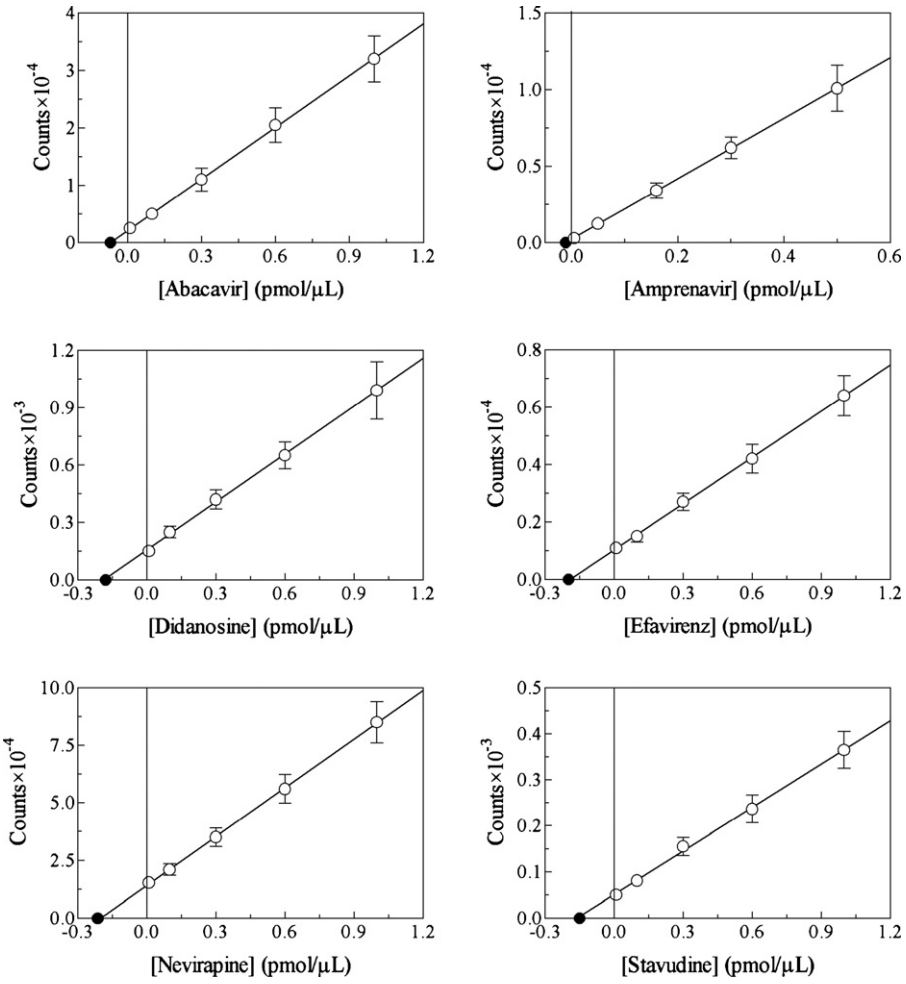

Fig. 6. Linear regression of standard additions of abacavir (Patient 2, see Table 2), amprenavir (Patient 1, see Table 2), didanosine (Patient 2, see Table 2), efavirenz (Patient 3, see Table 2), nevirapine (Patient 4, see Table 2), and stavudine (Patient 5, see Table 2). Filled symbols on the *x*-axis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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

[a] Results are the mean of four experiments.

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References

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