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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

2.2. MALDI-TOF/TOF system

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

Table 1
MALDI-TOF/TOF calibration standards

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

2.3. Stock, working, and plasma solution

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

2.4. Sample preparation

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

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

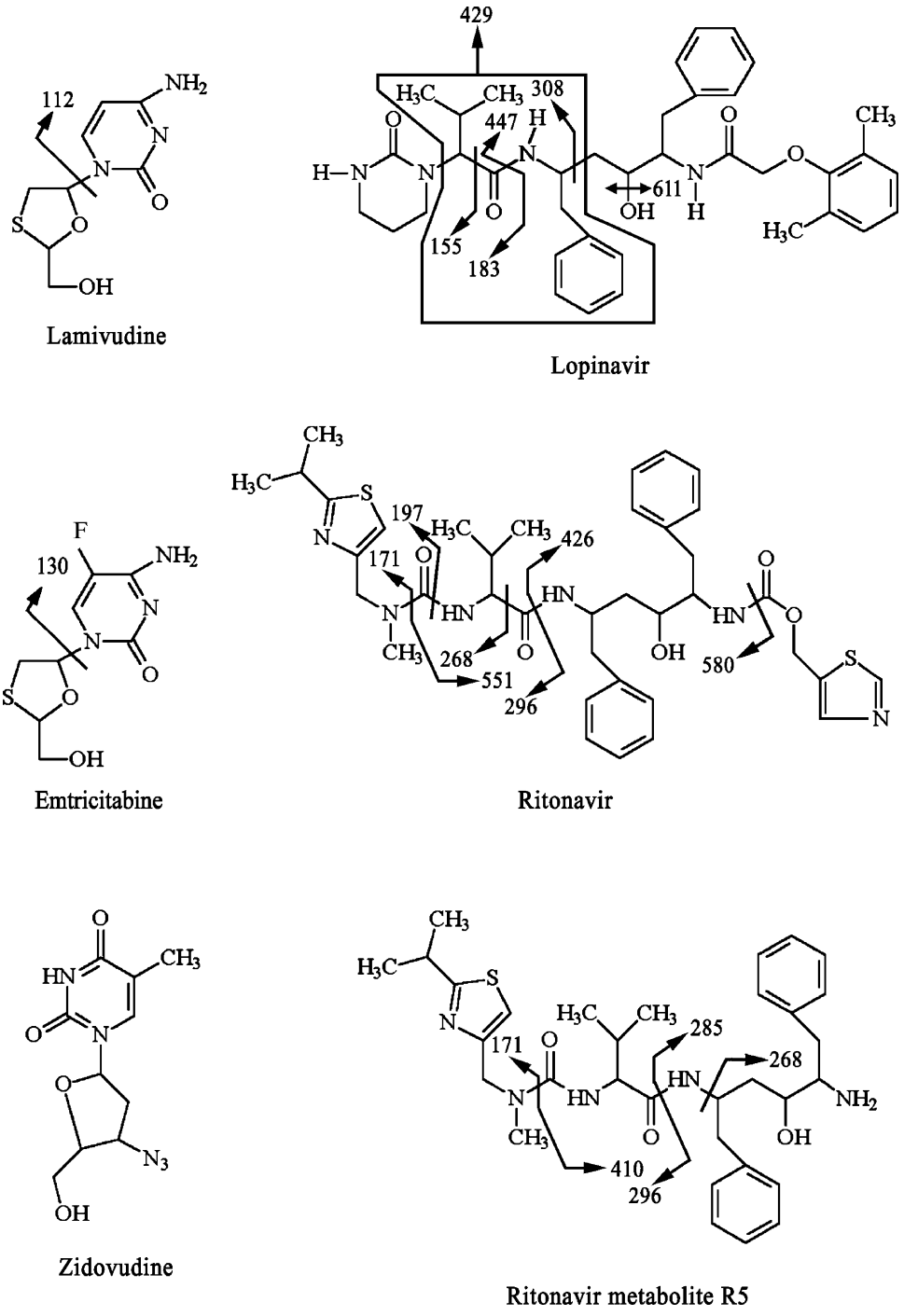

Fig. 1. Chemical structures of emtricitabine, lamivudine, lopinavir, ritonavir, ritonavir metabolite R5, and zidovudine. Fragment ions observed under MS/MS conditions are shown. For details, see text, Figs. 2 and 3 as well as Table 2.

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

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

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

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

2.6. Recovery

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

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

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

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

3. Results and discussion

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

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

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

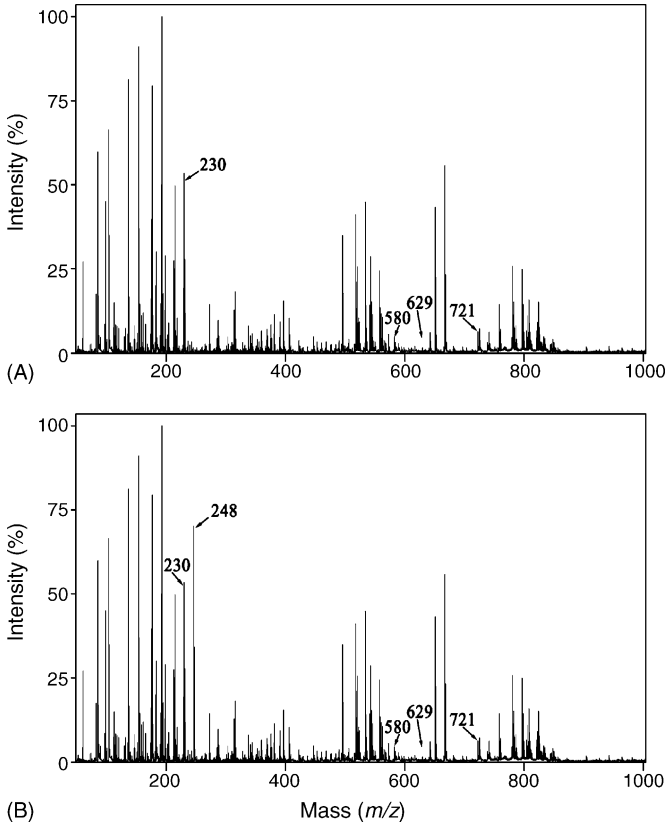

Fig. 2. MALDI-TOF/TOF MS spectrum of the plasma of a HIV-infected patient under therapeutical treatment with lamivudine, lopinavir, ritonavir, and zidovudine in the absence (panel A) and presence (panel B) of the internal standard emtricitabine. Zidovudine was undetectable. The full scan mass spectral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

Table 2
Analyte fingerprints[a]

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

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

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

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

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

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

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

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

Table 3
Anti-HIV drug parameters

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

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

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

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

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

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

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

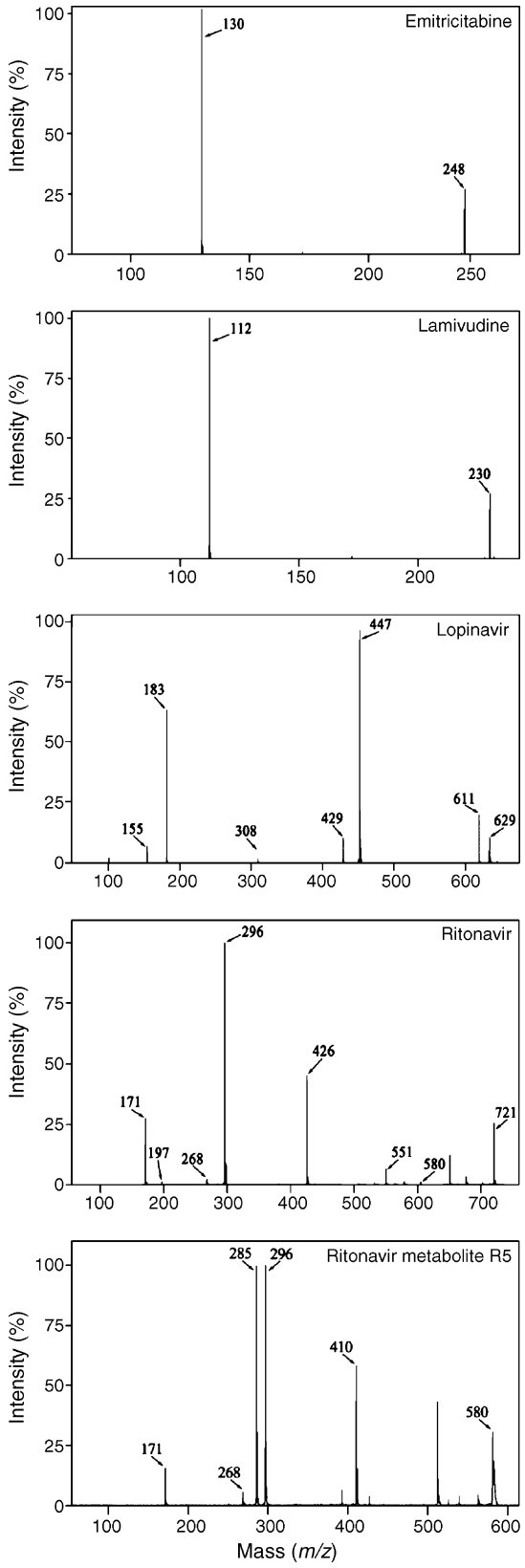

Fig. 3. MALDI-TOF/TOF MS/MS spectra of emtricitabine, lamivudine, lopinavir, ritonavir, and ritonavir metabolite R5 from the plasma of a HIV-infected patient spiked with emtricitabine. For details, see text, Figs. 1 and 2 as well as Table 2.

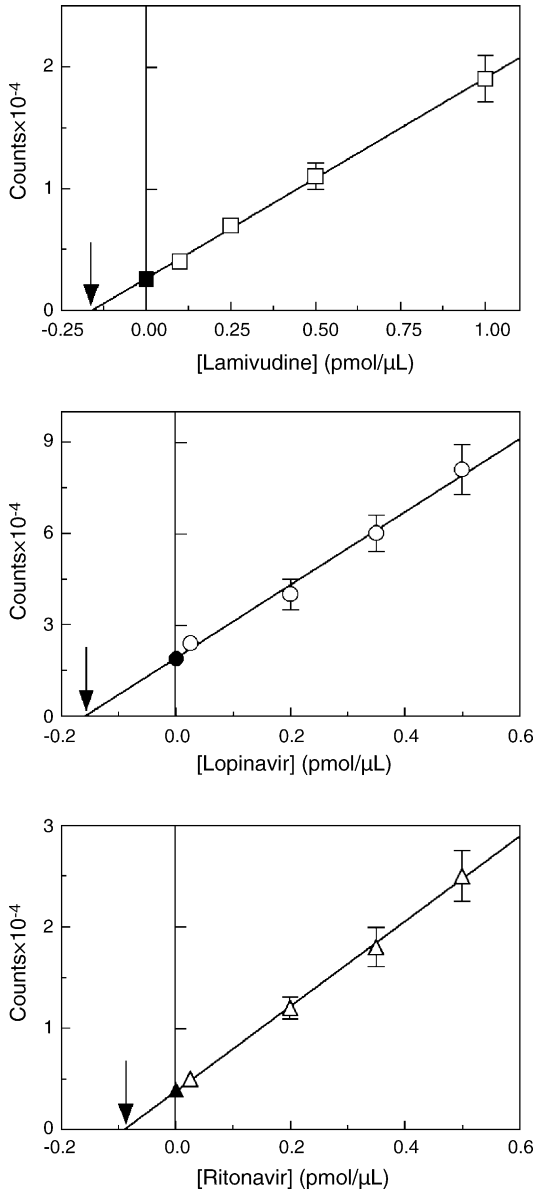

Fig. 4. Linear regression of standard additions of lamivudine, lopinavir, and ritonavir. Filled symbols on the *y*-axis indicate counts corresponding to the unknown anti-HIV drug concentration present in the plasma of the HIV-infected patient. The *x*-axis intercept of the regression straight line (arrow)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

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

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

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

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

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

[a] Results are the mean of four experiments.

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

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

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

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

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

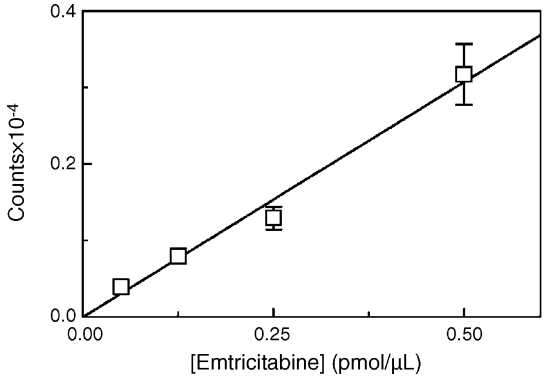

Fig. 5. Calibration curve for the determination of lamivudine concentration based on the fluorinated analog emtricitabine as the internal standard. Averages and error bars were obtained from at least four repeats. Where error bars are not seen, they are smaller than the data point symbols. For details,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

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