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Journal of Chromatography B, 847 (2007) 38–44
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Validation of an HIV-1 inactivation protocol that is compatible with intracellular drug analysis by mass spectrometry[☆]

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Received 19 June 2006; accepted 1 October 2006
Available online 20 October 2006

Abstract

Mass spectrometry is a powerful tool for studying the intracellular pharmacokinetics of antiretroviral drugs. However, the biohazard of HIV-1 calls for a safety protocol for such analyses. To this end, we extracted HIV-1 producing cells with methanol or ethanol at 4 °C. After extraction, no viral infectivity was detected, as shown by a reduction in infectious titers of more than 6 log. In addition, this protocol is compatible with the quantitative analysis of antiretroviral drugs in cell extracts using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. Thus, using this protocol, infectious HIV-1 is inactivated and antiretroviral drugs are extracted from cells in a single step.
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Keywords: HIV; Infectious; Antiviral; Inactivation; MALDI; Intracellular

1. Introduction

The majority of antiretroviral drugs exert their action in target cells for HIV infection, i.e. cells that express CD4 in combination with CXCR4 and/or CCR5. The field of intracellular pharmacokinetics of antiretroviral drugs is therefore an active area of research, which is often approached through mass spectrometry [1–7]. HIV-1 represents a biohazard, so preparation of HIV-1 infected samples must be carried out in laboratories with biosafety level (BSL) 2 or 3. Since BSL 2 or 3 laboratories require specific staff training and since the necessary safety procedures involve more laborious sample preparation, it is highly desirable to minimize the sample preparation steps in such laboratories. A procedure that inactivates infectious HIV-1 in freshly isolated cells, but which does not affect drug levels, is thus warranted. Such a safety procedure should not interfere with sample analysis, which is normally performed by an HPLC system coupled online to an electrospray ionization triple quadrupole mass spectrometer. The antiretroviral drugs can be extracted from the intracellular compartment by lysing the cells with solutions containing a high percentage of organic solvents. For this purpose, methanol and ethanol are suitable candidates, which can also be used to inactivate a variety of viruses. Therefore, procedures to extract antiretroviral drugs from cells could simultaneously inactivate infectious HIV-1.

Here, we describe a dual-purpose procedure whereby antiretroviral drugs can be extracted from cells and by which infectious HIV-1 is simultaneously inactivated. To achieve this, we treated HIV-1 infected cells using phosphate buffered solution containing 40–100% methanol or ethanol. The supernatants of the extracted cells were diluted in culture medium and tested for infectious virus on susceptible cells. Finally, we show that this drug extraction/HIV-1 inactivation procedure is compatible with accurate and precise quantification of ritonavir in extracts of peripheral blood mononuclear cells (PBMCs) using

[☆] This paper was presented at Biomarker Discovery by Mass Spectrometry, Amsterdam, The Netherlands, 18–19 May 2006.

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doi:10.1016/j.jchromb.2006.10.001

a recently developed method based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry [7].

2. Material and methods

2.1. Virus

HIV-I_{IIIB} was obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) [8–10].

2.2. H9 cells

H9 cells were obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) [8,9,11]. H9 cells were cultured in RPMI-1640 (Cambrex Bioscience, Belgium) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/mL L-glutamine (all from Cambrex Bioscience, Belgium) (R10F). Cells were cultured at 37 °C and 5% CO_2. Pellets of 4×10^6 uninfected H9 cells were resuspended in 15 mL tubes with 3 mL virus dilution, methanol/ethanol solution, or reconstituted inactivated supernatants, and were incubated for 2 h (see specific sections). Subsequently, cells were washed three times with R10F, resuspended in 5 mL R10F, and cultured for 10 days for the sensitivity experiments and validation experiments, or 4 days for the methanol/ethanol titration experiments.

2.3. GHOST cells

GHOST (3) CXCR4 cells (GHOST cells) were obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) [12]. GHOST cells were cultured in 24-well plates with 1 mL R10F supplemented with 500 μg/mL geneticin (GIBCO, USA), 2 μg/mL puromycin (Cayla, France), and 100 μg/mL hygromycin (Cayla). GHOST cells were cultured at a density of 3.6×10^4 per well for 24 h. The next day, wells were washed with phosphate buffered saline (PBS). To each well, 500 μL virus dilution, or methanol/ethanol solution, or reconstituted inactivated supernatants was added, and cells were incubated for 4 h (see specific sections). Next, the incubation solutions were discarded, and cells were cultured with 1 mL culture medium supplemented with 4 μg/mL polybrene (Sigma–Aldrich, Germany) for 4 days. At day 4, cells were harvested with trypsin (trypsin-EDTA 1x, GIBCO, USA), and cell pellets were resuspended in an appropriate medium (see specific sections) for analysis by flow cytometry. Cells were cultured at 37 °C and 5% CO_2.

2.4. Flow cytometry

Measurements were carried out on a FACSCalibur flow cytometer (Becton Dickinson, USA). For quantitative comparison of samples, running mode and measure time were kept constant from sample to sample. Flow cytometric data were analyzed using WinMDI version 2.8.

2.5. p24 ELISA

Qualitative and quantitative p24 determinations were obtained with the enzyme-linked immuno-sorbent assay (ELISA) using a Genetic Systems HIV-1 Ag EIA (Bio-Rad, France) and an HIV-1 antigen standard (Bio-Rad). All experiments were performed and interpreted according to the manufacturer's guidelines.

2.6. Sensitivity of HIV-1 detection assays

2.6.1. Virus dilutions

The supernatant of an HIV-1 infected H9 culture (p24 concentration 200 ng/mL) was diluted 10^2 to 10^8 times (in steps of 10) in R10F supplemented with 4 μg/mL polybrene. R10F with 4 μg/mL polybrene but without supernatant was used as negative control.

2.6.2. GHOST assay

GHOST cell pellets were resuspended in 300 μL PBS with 10% FBS and analyzed with flow cytometry. Side scatter (SSC) was plotted against the forward scatter (FSC), and debris and dead cells were gated out. Subsequently, fluorescent channel 1 (FL1) was plotted against the FSC and the percentage of green fluorescent protein (GFP)-positive cells was determined. The cut-off value for a HIV-1 positive GHOST culture was calculated as follows: mean percentage of positive cells in the negative control samples +3 times the standard deviation. Experiments were performed in triplicate.

2.6.3. p24 ELISA assay

After 10 days, culture media were collected and cell-free supernatants were frozen at −20 °C until analysis by p24 ELISA. Three replicate inoculations were performed for each virus dilution. One p24 determination was assessed per inoculation.

2.6.4. Statistics

The virus dilution that results in infection in 50% of the replicate inoculations, i.e. 50% tissue culture infectious dose ($TCID_{50}$), was calculated according to the Reed–Muench method [13].

2.7. Methanol/ethanol titration on H9 and GHOST cells

2.7.1. Methanol and ethanol solutions

R10F was supplemented with 1% methanol (MeOH; Sigma–Aldrich, Germany) or 1% ethanol (EtOH; Sigma–Aldrich) and 4 μg/mL polybrene. R10F supplemented with 4 μg/mL polybrene but without methanol or ethanol was used as negative control.

2.7.2. GHOST cells

GHOST cell pellets were resuspended in 300 μL PBS with 2% FBS, 25 nM TO-PRO-3 iodide (Molecular Probes, USA),

and 2.5 mM EDTA. Cell suspensions were incubated for 20 min and subsequently analyzed with flow cytometry. FSC was plotted against the FL4 and viable cells were counted. Experiments were performed in triplicate.

2.7.3. H9 cells

After 4 days, cell pellets were resuspended in 300 μL PBS with 2% FBS, 25 nM TO-PRO-3 iodide and 2.5 mM EDTA and incubated for 20 min. Subsequently, cell suspensions were diluted 10 times in PBS with 2% FBS, 25 nM TO-PRO-3 iodide and 2.5 mM EDTA, and analyzed with flow cytometry as described above. Experiments were performed in triplicate.

2.7.4. Statistics

Two-tailed two-sample *t*-tests assuming equal variances were performed to test whether the viable cell count differed significantly between titrated and non-titrated cells. The results were considered to be significant if the *p*-value was $\leq$0.05.

2.8. Validation of HIV-1 inactivation protocols

2.8.1. Virus culture

Ten pellets of 10×10^6 uninfected H9 cells were resuspended in 15 mL tubes with 300 μL virus supernatant supplemented with 4 μg/mL polybrene, and incubated for 1 h. Subsequently, cells were washed with R10F, pooled in one 162 cm^2 cell culture flask, and cultured for 6 days. At day 6, 5 mL virus culture was transferred to a 25 cm^2 cell culture flask and cultured for another day to assess whether p24 was still being actively produced. The 95 mL virus culture remaining at day 6 was washed four times with R10F, counted using trypan blue dye exclusion, and used to test the inactivation protocols.

2.8.2. Percentage of HIV-1 infected cells

Pellets of 1×10^6 infected H9 cells and 1×10^6 uninfected H9 cells were fixed with Cytofix/Cytoperm (BD Biosciences, USA) and colored with KC57-RD1 (BD Biosciences) according to the manufacturer's protocol. Fixed cells were resuspended in 300 μL PBS with 2% FBS, and analyzed with flow cytometry. FSC was plotted against the SSC, and non-viable cells were gated out. Subsequently, FL2 was plotted against FSC and the percentage of KC57-RD1 positive cells was determined.

2.8.3. Inactivation solutions

Solutions were made of 40, 60, 80 and 100% EtOH or MeOH in PBS. Deionized water was used as positive control.

2.8.4. Inactivation protocols

Pellets of 1.6×10^6 infected H9 cells and 1.6×10^6 uninfected H9 cells (negative control) were resuspended in 1.5 mL tubes (Biopur; Eppendorf, Germany) with 100 μL inactivation solution, and incubated at 4 °C for 1 h. Subsequently, samples were centrifuged and supernatants were collected. Experiments were performed in triplicate.

2.8.5. p24 ELISA read-out

Supernatants (30 μL) were mixed with 2967 μL R10F and 3 μL polybrene (4 mg/mL). Pellets of uninfected H9 cells were resuspended with reconstituted inactivated supernatants as described in section 2.2. Supernatant of a virus culture on which no inactivation protocol had been performed was used as absolute positive control. At day 10, culture media were collected and cell-free supernatants of each cell culture were stored at −20 °C until analysis by p24 ELISA.

2.8.6. GHOST assay read-out

Supernatants (5 μL) were mixed with 490 μL R10F and 5 μL polybrene (400 μg/mL). GHOST cells were incubated with the reconstituted inactivated supernatant as described in section 2.3. At day 4, GHOST cells were harvested, resuspended in 300 μL PBS with 2% FBS, and analyzed with flow cytometry. SSC was plotted against the FSC and non-viable cells were gated out. Subsequently, FL1 was plotted against FSC and the percentage of GFP-positive cells was determined. Two-tailed two-sample *t*-tests assuming equal variances were performed to test whether the percentage of GFP-positive cells differed significantly between the negative controls and inactivated samples. The results were considered to be significant if the *p*-value was $\leq$0.01.

2.9. Mass spectrometry

2.9.1. Matrix preparation and spotting

The matrix solution contained 20 mg/mL meso-Tetrakis (pentafluorophenyl)porhyrin (F20TPP; TCI Europe, Belgium) and 20 mM lithium iodide (Sigma–Aldrich, Germany) in 100% acetone. The matrix solution was spotted onto an 800 μm AnchorChip™ (Bruker Daltonics) using the brushing spotting technique, whereby a 10 μL pipette tip was filled with matrix solution, and the pipette was positioned such that the tip just touched the target plate above the first spot. Next, the plunger was slightly pressed and at the same time the pipette was quickly dragged downwards over the target spots.

2.9.2. Sample preparation

Standards of ritonavir (kindly provided by Abbott Laboratories) were prepared in methanol/water (1:1); nelfinavir (kindly provided by Pfizer Inc.) was used as internal standard. Peripheral blood mononuclear cells were isolated from a buffy coat (Sanquin, The Netherlands) using a Ficoll density gradient. PBMCs were extracted overnight in methanol/water (1:1) (100 μL per 10^6 PBMCs) at 4 °C. Subsequently, cell debris was spun down and the supernatants of 1×10^6 PBMCs (100 μL) were supplemented with 100 μL ritonavir standard, 100 μL methanol/water (1:1), and 300 μL HPLC grade water (Sigma–Aldrich, Germany). Subsequently, extracts were cleaned up using a 96-well solid phase extraction plate (Oasis HLB μelution plate, Waters, USA). The loaded samples were washed twice with 200 μL methanol/water (1:19) and once with 200 μL methanol/water (1:1). Samples were eluted from the solid phase extraction plate using 100 μL acetone. Eluents

Table 1
Calculation of $TCID_{50}$ of the H9/p24 assay and the GHOST assay

	log virus dilution	HIV positive replicates	Cumulative HIV positive (A)	Cumulative HIV negative (B)	$A/(A+B)$	Percent infected replicates
p24 ELISA assay	−2	3/3	15	0	15/15	100
	−3	3/3	12	0	12/12	100
	−4	3/3	9	0	9/9	100
	−5	2/3	6	1	6/7	85.7
	−6	2/3	4	2	4/6	66.7
	−7	2/3	2	3	2/5	40
	−8	0/3	0	6	0/6	0
GHOST assay	−2	3/3	6	0	6/6	100
	−3	3/3	3	0	3/3	100
	−4	0/3	0	3	0/3	0
	−5	0/3	0	6	0/6	0
	−6	0/3	0	9	0/9	0
	−7	0/3	0	12	0/12	0
	−8	0/3	0	15	0/15	0

Proportionate distance = (% positive above 50% − 50%)/(% positive above 50% − % positive below 50%). $TCID_{50}$ = log dilution above 50% + (proportionate distance × log dilution factor). For the H9/p24 assay, the proportionate distance = 0.6, the $TCID_{50} = -6.6$ log, the $TCID_{50}$ per mL is −6.1 log = 7.9×10^{-7}. For the GHOST assay, the proportionate distance = 0.5, the $TCID_{50} = -3.5$ log, the $TCID_{50}$ per mL is −3.8 log = 1.6×10^{-4}.

were dried using a SpeedVac (Savant, USA), and reconstituted in 25 μL ethanol/water (1:1). The reconstituted eluents were spotted in 4-fold on top of the matrix crystals. The theoretical concentrations of ritonavir in the reconstituted eluents were 1000, 500, 250, 125, 62.5, 31.25, 15.625 femtomole per μL, and the theoretical concentration of nelfinavir was 500 femtomole per μL.

2.9.3. Sample measurement

All experiments were performed on an Ultraflex™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a 50 Hz nitrogen laser (337 nm). Mass spectra were recorded in the positive ion reflectron mode. FlexControl™ version 2.4 software (Bruker Daltonics) was used to operate the mass spectrometer. A sample spot containing matrix only was used to manually set the laser power approximately 5% above the threshold for ionization. The samples containing the analytes were measured automatically using the AutoXecute part of the FlexControl™ software. Mass spectra were recorded by accumulating 50 shots on 20 different positions on each sample spot. No spectra were rejected (i.e., fuzzy control for spectra accumulation was not used).

2.9.4. Data analysis

The areas under the monoisotopic peaks of the lithiated ritonavir and nelfinavir were calculated using FlexAnalysis™ software (version 2.4, Bruker Daltonics). Precisions (expressed as relative standard deviation) of the ratio of the ritonavir–to–nelfinavir peak area (RTV/NFV) were calculated for the four replicate analyses of each calibrator. Subsequently, the mean RTV/NFV of each calibrator was plotted against the ritonavir concentrations, and accuracies (expressed as percent deviation from the theoretical concentration) were calculated using a $1/x^2$ weighed quadratic curve fitting method.

3. Results and discussion

van Bueren et al. have reviewed the literature on alcohol inactivation of HIV in suspensions in addition to presenting their own data [14]. They showed that, depending on the procedure followed, reductions of infectious virus titers varied from 10^2 to 10^7. Our procedure differs in that we inactivate HIV at 4 °C, whereas published protocols were executed at room temperature or at 37 °C. Antiretroviral drugs are normally extracted from cells at 4 °C. In addition, our inactivation protocol is compatible with intracellular drug analysis by MALDI mass spectrometry (vide infra).

To validate our HIV-1 inactivation protocol, we first tested two assays to quantify the infectious titers of HIV-1. For this, we used (1) A rapid direct assay (GHOST assay), which detects HIV replication in susceptible GHOST cells via a GFP reporter gene that is transcribed only in the presence of the HIV protein Tat. GFP-positive GHOST cells are distinguished from GFP-negative GHOST cells by flow cytometry; (2) a time-consuming indirect assay (H9/p24 assay), which detects the viral core protein p24 by ELISA after the virus has been amplified in H9 cells.

First, we tested which of the two assays provided the best sensitivity for detecting infectious HIV-1. Supernatant of an HIV-1 infected H9 culture (p24 concentration 200 ng/mL) was diluted 10^2–10^8 times and used to inoculate non-infected GHOST cells and H9 cells. The $TCID_{50}$ was calculated for both assays according to the Reed–Muench method (see Table 1): The $TCID_{50}$ per milliliter was 7.9×10^{-7} for the H9/p24 assay and 1.6×10^{-4} for the GHOST assay. Thus, for safety reasons the H9/p24 assay

Table 2
Validation of the HIV-1 inactivation protocol

	Loss of infectivity
No extraction procedure	–
Deionized water	–
40–100% methanol	+
40–100% ethanol	+

is preferred to validate the HIV-1 inactivation protocol, given its superior sensitivity.

The extraction of antiretroviral drugs from the intracellular compartment is normally performed by lysing the cells in 40–100% methanol or ethanol. To test whether infectious virus was still present in the supernatant, we inoculated non-infected cells with the supernatants of such lysates (vide infra).

To avoid the cytotoxicity caused by the organic solvents, the supernatants of the lysates were diluted in culture medium to an organic solvent concentration of ≤1%. We verified whether 1% methanol or ethanol had affected the viability of GHOST cells or H9 cells, and found no significant difference in the total number of viable H9 or GHOST cells between the negative controls and the cultures containing 1% methanol or ethanol (data not shown).

The percentage of HIV infected cells and the number of infectious HIV particles in *in vitro* cultures are much greater than those in clinical samples of HIV-1 infected patients. We therefore, chose to validate the HIV-1 inactivation protocols on *in vitro* cultures of HIV-1 infected H9 cells. Immediately, before the start of the inactivation protocols, the p24 concentration of the HIV-1 infected H9 culture was 440 ng/mL, and 12% of the cells expressed HIV-1 proteins. Part of the HIV-1 infected H9 cells were cultured for another day, in which the p24 concentration rose to 620 ng/mL, showing active viral replication on the day the inactivation protocols were tested. The inactivation solutions were prepared in phosphate buffered solution to ensure that the inactivation of infectious HIV-1 was caused only by the methanol or ethanol and not by the handling of the sample. Whereas no infectious HIV-1 was detected by either assay after HIV-1 infected H9 cells had been incubated with 40, 60, 80 or 100% methanol or ethanol at 4 °C for 1 h, infectious virus was recovered after cell extraction with de-ionized water (see Table 2). This indicates that the organic solvent was indeed the inactivating factor in the protocol. To eliminate spillover of p24 from the inoculum, HIV-1 infected cells were washed four times as to remove p24 that might contaminate the H9 read-out culture of the H9/p24 assay. Despite these wash steps, some p24 was still present in the positive control samples before start of the HIV-1 amplification. The OD_{450} of the positive control samples ranged between 0.067 and 0.144 after washing and before start of the HIV-1 amplification. These titers rose to ≥3.5 after the amplification in H9 cells, indicating that active virus replication was only ongoing in the positive control and in the H_2O extracted cells.

Finally, we tested whether this inactivation protocol was compatible with quantitative analysis of antiretroviral drugs by mass spectrometry. To this end, we used a recently developed method for quantitative analysis of lopinavir in PBMC extracts by MALDI-TOF mass spectrometry [7]. Normally, quantitative analysis of drugs by MALDI-TOF mass spectrometry is hampered by matrix-derived chemical interference in the low mass range and poor reproducibility of signal abundances. Our experimental method was specifically designed to enhance the precision of the MALDI-TOF response and to decrease matrix-derived chemical noise in the low mass range. The use of a high molecular weight matrix, i.e. meso-Tetrakis(pentafluorophenyl)porphyrin (F20TPP), significantly decreased the matrix-derived chemical noise in the low mass range. To increase reproducibility (precision), we developed a fast evaporation protocol for the matrix F20TPP using pre-structured target plates (AnchorChip^{TM}). To further increase reproducibility, instrument response variation was minimized by using an internal standard, by averaging out 1000 spectra per sample, and by measuring each sample in 4-fold. An interesting observation for F20TPP is that this matrix is a poor proton donor and so analyte molecules do not become protonated. However, mixing the F20TPP with alkali salts, in particular with lithium salts, results in intense signals for the cationized drugs. In this study, we have extracted PBMCs in methanol/water (1:1) and spiked the supernatants with various concentrations of ritonavir and nelfinavir (see Fig. 1). The supernatants of the extracted PBMCs were cleaned up using a solid phase extraction plate. The eluents (100 μL) were dried and subsequently reconstituted in a smaller volume of 25 μL to concentrate the sample. Acetone was used as eluents to enhance the drying speed of the samples. Fig. 2 shows the mass spectra of the F20TPP matrix, PBMC extract, and PBMC extracts spiked with nelfinavir and/or ritonavir. The mass spectrum of the F20TPP matrix shows that there are only a few matrix-derived interferences in the low mass range (see Fig. 2 panel A).

The mass spectrum of the PBMC extract spiked with nelfinavir shows a peak at *m/z* 574 for nelfinavir cationized by Li^+ (see Fig. 2 panel C). In addition, there is a peak at *m/z* 467, which

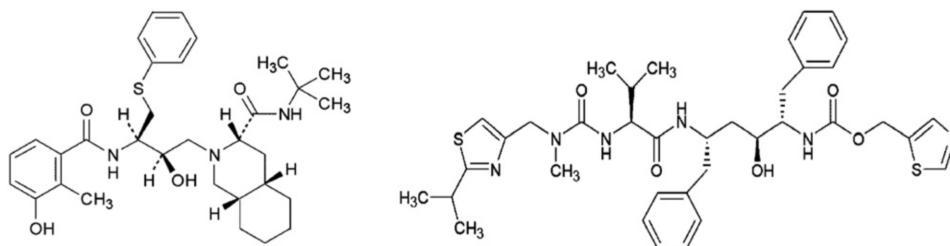

Fig. 1. Structures of nelfinavir and ritonavir. The left hand shows the structure of nelfinavir (internal standard; $C_{32}H_{45}N_3O_4S$; MW 567.3). The right hand shows the structure of ritonavir ($C_{37}H_{48}N_6O_5S_2$; MW 720.3).

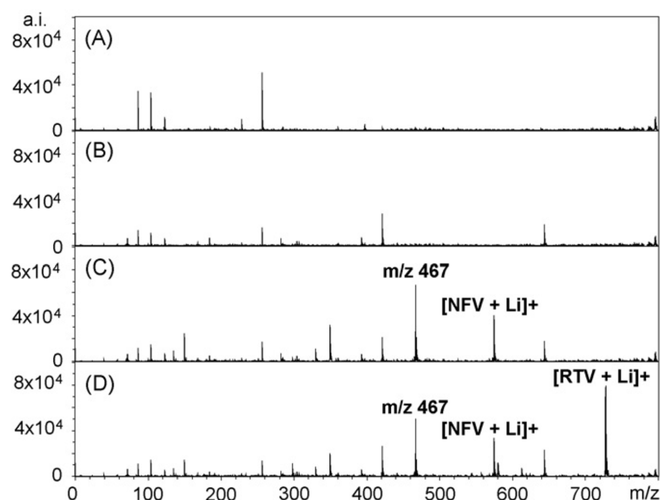

Fig. 2. Mass spectra of the F20TPP matrix, PBMC extract, and PBMC extract spiked with nelfinavir and/or ritonavir. NFV = nelfinavir; RTV = ritonavir. Panel A, mass spectrum of the F20TPP matrix (20 mg/mL F20TPP + 20 mM LiI); Panel B, mass spectrum of PBMC extract cleaned with solid phase extraction; Panel C, mass spectrum of PBMC extract spiked with 500 femtomole nelfinavir; Panel D, mass spectrum of PBMC extract spiked with 500 femtomole nelfinavir and 500 femtomole ritonavir.

does not contain lithium (no $^{6}Li^{+}$ adduct is present). This peak at *m/z* 467 is not observed in the mass spectrum of the non-spiked PBMC extract. This peak was not used for the quantitative analysis of ritonavir. The concentration-response relationship showed nonetheless a good linearity with an r^2 of 0.99992 (see Fig. 3). Mean precision was 5.6% (sd 3.7) and mean accuracy was 2.4% (sd 2.2). Table 3 shows the precisions and accuracies for each ritonavir standard. All values meet the FDA ±20/15 criteria for precision and accuracy [15].

We have previously shown that pure lopinavir, ritonavir, nelfinavir, indinavir, saquinavir, amprenavir, and tipranavir could be detected between 5 femtomole and 40 femtomole per μL using MALDI-TOF mass spectrometry [7]. In addition, the limit of quantification for lopinavir in PBMC extracts was 25 femtomole per μL. In the present study, we show that the limit of quantification for ritonavir in PBMC extracts is 15 femtomole

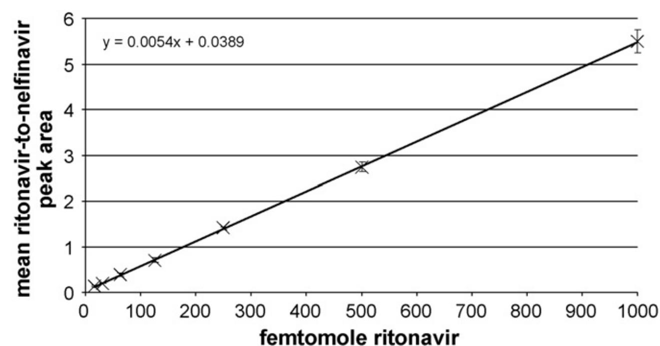

Fig. 3. Concentration-response relationship for ritonavir in PBMC extracts. Concentration-response relationship for ritonavir in extracts of 1×10^6 PBMCs using nelfinavir as internal standard. Each ritonavir calibrator was analyzed in 4-fold. Error bars indicate the standard deviation of plus one and minus one (4 replicates per calibrator). The lower limit of quantification was 15.625 femtomole.

Table 3
Precision and accuracy of quantitative analysis of ritonavir in PBMC extracts using MALDI-TOF mass spectrometry

Femtomole ritonavir	Precision	Accuracy
1000	4.6	−0.7
500	4.2	0.2
250	2.1	3.0
125	7.2	−0.8
62.5	12.0	2.4
31.25	1.3	−6.8
15.625	8.0	2.7

Precision is expressed as relative standard deviations. Accuracy is expressed as the percent deviation of the measured concentration from the theoretical concentrations. The calibration curve was constructed using a $1/x^2$ quadratic curve fitting method. Each calibrator was measured in 4-fold.

per μL. Notari et al. used the tandem mass spectrometry mode (MS/MS) on a MALDI-TOF/TOF mass spectrometer for quantitative analysis of anti-HIV drugs in human plasma [5]. The limit of quantification for lopinavir and ritonavir was 2.5 femtomole per μL. These better limits of quantification can be attributed to the use of MS/MS, which leads to an increased selectivity and higher signal-to-noise ratios. Time-of-flight mass analyzers are normally not the first choice for quantitative analysis of drugs; the dynamic range is relatively small and most time-of-flight mass analyzers are not designed for tandem mass experiments. The development of new types of MALDI mass spectrometers, such as MALDI-triple quadrupole mass spectrometers, is therefore of special interest for quantitative analysis of drugs [16–20].

Consideration of MALDI for quantitative analysis of antiretroviral drugs leads to questions on how this technique performs compared to HPLC-ESI-triple quadrupole mass spectrometry (LC–MS/MS). LC–MS/MS is currently the gold standard for quantitative analysis of drugs due to its selectivity, sensitivity and robustness. To our best knowledge, three studies have been published that give an in-depth description of the development and validation of an LC–MS/MS method for quantitative analysis of protease inhibitors in PBMCs [2,21,22]. Jemal et al. developed an LC–MS/MS method for quantitative analysis of atazanavir in PBMCs [22]. The limit of quantification was 5 femtomole per 10^6 PBMCs. Rouzes et al. obtained a limit of quantification of 3 picomol per 3×10^6 PBMC for lopinavir and 1 picomol per 3×10^6 PBMC for ritonavir using an LC–MS/MS method for quantitative analysis of four protease inhibitors and one non nucleoside reverse transcriptase inhibitor (NNRTI) [21]. Colombo et al. developed an LC–MS/MS method for quantitative analysis of seven protease inhibitors and two NNRTIs in PBMCs [2]. They obtained limits of quantification of 5 femtomole for ritonavir and 6 femtomole for lopinavir (minimum quantifiable drug on column). Our obtained limits of quantification for lopinavir and ritonavir in PBMC extracts using MALDI-TOF mass spectrometry are comparable to those obtained by LC–MS/MS. One further aspect concerns the sample analysis time, which is considerably shorter for MALDI than for LC–MS/MS. Sample analysis time of the LC–MS/MS methods discussed above range from 4 to 20 min. Time to analyze one sample in 4-fold using our MALDI-TOF method takes

2 min and 20 s. Following the development of high repetition rate lasers (1000 Hz), sample analysis times have decreased to 15 s and less using a MALDI-triple quadrupole mass spectrometer [17,18]. More studies are needed to delineate the future role of MALDI mass spectrometry for bioanalysis of drugs. However, it already appears that the quantitative analysis of drugs is a new and promising application of MALDI mass spectrometry.

In conclusion, we have shown that extraction of HIV-1 infected cells with 40–100% methanol or ethanol for 1 h at 4 °C results in a loss of HIV infectivity of at least 6.1 log. Because this level of reduction shows that, under physiological conditions, all infectious virus particles are destroyed, it guarantees that samples can be handled safely outside special facilities. The validated HIV-1 inactivation procedure provides an opportunity to optimize the drug extraction process by adjusting extraction time and/or the percentage of methanol or ethanol without the need to re-test whether the drug extraction procedure inactivates infectious HIV-1. Furthermore, the protocol is compatible with accurate and precise quantitative analysis of ritonavir in PBMC extracts using MALDI-TOF mass spectrometry.

Acknowledgements

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: GHOST (3) CXCR4 cells from Drs. Kewalramani and Littman [12], H9 and HTLV-III_B/H9 from Dr. Robert Gallo [8,9,11]. We would like to thank Aids Fonds, The Netherlands (project numbers 2004051 JvK, 2005038 RG) and VIRGO for financial support. We would also like to thank Hetty Blaak and Carel van Baalen for their helpful discussions on the methodology. Ritonavir and nelfinavir were kindly provided by Abbott Laboratories and Pfizer Inc., respectively.

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