個室を使用し，情報の取り扱いには十分に注意する．初期対応を行う部署の担当者や責任者にも，プライバシーの保護についてあらかじめ理解してもらうことが必要である．

また，曝露後，不幸にも感染が成立した場合には，その個人の不利益にならないように，プライバシーへの配慮はもちろんのこと，擁護する存在となることも感染管理担当者の役割である．

報告システムにおける個人情報の取り扱い

多くの施設で,針刺し・切創が起こった場合の，報告書式や報告ルート，書式の活用方法や保管方法が決められていると思われる．個人情報保護法が施行された今，針刺し・切創の報告システムにおいても個人情報の取り扱いについて再考する必要がある．

「施設の現行の報告ルートには本来不必要と思われるルートはないだろうか」「情報の管理方法は適切だろうか」……．病院管理者も含め，感染管理担当者や職員の健康管理担当者と十分な吟味を行い，報告ルートを整理し，情報の管理方法についてもルールを決めておくことが望ましい．

よりよい医療を提供するため，医療従事者を感染から守る

針刺し・切創時の倫理的な対応について述べてきたが，筆者も，感染管理担当者として自分自身がこれまでどうであったかを振り返る機会となった．医療従事者が安全で安心して医療を提供できるよう，「倫理的な配慮」を常に考えながら職業感染防止に努めていきたい．

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9

PNA–*In Situ* Hybridization Method for Detection of HIV-1 DNA in Virus-Infected Cells and Subsequent Detection of Cellular and Viral Proteins

Tomoko Hagiwara, Junko Hattori, and Tsuguhiro Kaneda

Summary

We describe *in situ* hybridization protocols using peptide nucleic acid (PNA) as a probe for detecting HIV-1 DNA in virus-infected cells and the subsequent detection of cellular and/or viral proteins. Because a PNA probe of approx 20 bases was sufficiently long to detect a specific target sequence, a conserved sequence of such a short length was easily identified. Therefore, this probe is valuable even to identify quasi-species of HIV-1. In addition, we adopted a catalyzed signal amplification method to amplify weak viral DNA signals; thus, stringent washing was crucial for eliminating false-positive signals. Our double-staining method using PNA–*in situ* hybridization and subsequent immunostaining enabled the active and inactive proviruses to be distinguished.

Key Words: *In situ* hybridization; peptide nucleic acid; catalyzed signal amplification; HIV-1 provirus; CD4-positive T lymphocytes; p24; HLA-DR.

1. Introduction

In situ hybridization (ISH) is now popularly used in cytogenetic studies to determine the localization of a specific gene on a chromosome and to detect mRNA expression and viral infection within cells using DNA or RNA probes. Nonradioactive ISH methods using fluorescence or visible light to visualize signals generally are used for the detection of target nucleic acid sequences. In general, probes of more than 500 bp in length are required in such studies *(1)*.

Until recently, the detection of HIV-1 has been performed by using autoradiography, using probes labeled with radioisotopes, such as ^{35}S *(2–4)* and ^{125}I *(2,5)*. Although the use of radioactive ISH for the detection of HIV-1 is time consuming and not very convenient, the small copy number of HIV-1 in

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infected cells has hindered the development of a conventional nonradioactive detection system. In addition, designing a suitable proper probe with a length of more than 150 bp is very difficult because of the general lack of long-conserved DNA sequences in viruses, such as HIV-1, that have error-prone reverse transcriptase without any repair activity. To bypass this problem, we developed a peptide nucleic acid (PNA) that mimics the DNA configuration ***(6–8)***. PNA, being electrically neutral, can penetrate into cells more easily than DNA and, in addition, PNA can more strongly hybridize with DNA than DNA ***(9,10)***. For these reasons, a PNA probe of approx 20 bases in length is long enough to detect a specific target sequence ***(11)***.

To overcome the weak viral DNA signal, one of the following methods for signal amplification can be used: one is *in situ* polymerase chain reaction (PCR *[12]*), and the other is a catalyzed signal amplification (CSA) using biotinyl tyramide ***(13)***. *In situ* PCR is quite powerful for amplification of rare target DNA within cells; therefore, *in situ* PCR-driven ISH would be suitable for detecting low copy number DNA sequences. However, this method carries the risk of amplicons synthesized *in situ* diffusing and resulting in false-positives ***(14)***. On the other hand, a single copy of the HPV-16 virus was detected successfully using the CSA method ***(14)***. Therefore, we adopted the CSA method for detecting HIV-1 DNA ***(15)*** and incorporated a crucial stringent washing step to eliminate nonspecific signals that arise from the CSA procedure.

Here, we describe the procedures for detecting HIV-1 DNA in infected CD4-positive T lymphocytes, and the phenotypic determination of HIV-1 DNA-positive cells by a double staining method.

2. Materials

2.1. Cell Lines

1. ACH2: positive control. Human lymphoid cell line latently infected with HIV-1.
2. MOLT4-IIIB: positive control. Human leukemic cell line persistently infected with HIV-1 strain IIIB.
3. MOLT4: negative control.

2.2. Sample Preparation

2.2.1. CD4-Positive T-Lymphocytes Smears

1. StemSep column chromatography (Stem Cell Technologies, Inc., Vancouver, BC, Canada; cat. no. STS-14032).
2. Phosphate-buffered saline (PBS) with 3% fetal bovine serum.
3. 4% paraformaldehyde (PFA) containing 0.1 *M* sodium phosphate buffer, pH 7.4.
4. Ethanol.
5. Silane-coated slides.

6. Wax pen (DakoCytomation A/S, Glostrup, Denmark).
7. Staining racks and containers.
8. Dryer.

2.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Tissue blocks of 20% formalin-fixed or 4% PFA-fixed, paraffin-embedded samples.
2. Silane-coated slides.
3. Staining racks and containers.
4. Xylene.
5. Rehydration series: 100, 95, 90, and 70% ethanol.

2.3. Pretreatment

2.3.1. CD4-Positive T Lymphocytes Smears

1. Tris-buffered saline containing Tween-20 (TBST): 50 m*M* Tris-HCl, 300 m*M* NaCl, 0.1% Tween-20, pH 7.6.
2. Target retrieval solution (DakoCytomation A/S; cat. no. S1700).
3. Methanol containing 0.3% H_2O_2.
4. Water bath.
5. Staining racks and plastic containers.

2.3.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. The same as **Subheading 2.3.1., items 1–5**.
2. Proteinase K (DakoCytomation A/S, S3004).

2.4. Preparation of PNA Probe

1. PNA probe: the structure of the probe is as follows: FITC·HN-GCAGCTTCCT-CATTGATGG-$CONH_2$ (FASMAC Co. Ltd., Kanagawa, Japan; *see* **Note 1**).
2. DNA ISH solution (DakoCytomation A/S; cat. no. S3305).
3. Cover slips.

2.5. Heat Denaturation

1. Hotplate.

2.6. Hybridization

1. Stringent wash solution (DakoCytomation A/S; cat. no. K5201).
2. TBST.
3. Incubator.
4. Plastic containers.
5. Water bath.
6. Moist chamber.

2.7. PNA Probe Detection by CSA

1. Horseradish peroxidase (HRP)-conjugated anti-FITC antibody (DakoCytomation A/S; cat. no. P5100).
2. Biotinyl tyramide solution (DakoCytomation A/S, GenPoint kit K0620).
3. HRP-conjugated streptavidin (DakoCytomation A/S, GenPoint kit K0620).
4. Alexa Fluor 488-labeled streptavidin (Invitogen, Carlsbad, CA).
5. 4,6-diamino-2-phenylindole (DAPI) II (Vysis, Inc., Downers Grove, IL).
6. TBST.
7. Staining racks and containers.
8. Cover slips.

2.8. PNA–ISH and Immunohistochemistry (IHC) (Indirect Method)

1. Mouse anti-human CD4 monoclonal antibody (Novocastra Laboratories, Ltd., Newcastle, UK; cat. no. NCL-CD4-1F6) or mouse anti-human HLA-DR monoclonal antibody (DakoCytomation A/S; cat. no. M0746).
2. Alexa Fluor 594-labeled goat anti-mouse IgG antibody (Invitrogen; cat. no. A-11005).
3. DAPI II (Vysis, Inc.).
4. Cover slips.

2.9. PNA–ISH and IHC (Labeled Streptavidin-Biotin Method)

1. Avidin solution (DakoCytomation A/S; cat. no. X0590).
2. Biotin solution (DakoCytomation A/S; cat. no. X0590).
3. Mouse monoclonal anti-HIV-1 p24 antibody (DakoCytomation A/S; cat. no. M0857).
4. Biotinylated goat anti-mouse Ig antibody (DakoCytomation A/S; cat. no. E0433).
5. Alexa Fluor 594-labeled streptavidin (Molecular Probes, Inc.; cat. no. S-11227).
6. DAPI II (Vysis, Inc.).
7. Cover slips.

3. Methods

3.1. Sample Preparation (see Note 2)

3.1.1. CD4-Positive T Lymphocytes Smears

1. Negatively select and purify CD4-positive T lymphocytes by StemSep column chromatography according to the manufacturer's instructions.
2. Spin down the collected cells at 250*g* for 5 min.
3. Discard supernatant and resuspend with PBS.
4. Mark the area for sample-mount on a silane-coated slide with a wax pen. Drop 5 µL of the cell suspension onto the slide and spread it out gently using the pipet tip.
   The area of the sample-mount (15 × 15 mm).
5. Dry the slides using a dryer at a cool setting.
6. Fix the slides with 4% PFA containing 0.1 mol/L sodium phosphate buffer, pH 7.4, at room temperature for 60 min or at 4°C overnight.

7. Rinse the slides in PBS (3 min, three times).
8. Dehydrate the slides in absolute ethanol and then store at –20°C until use.

3.1.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Place a 4- to 5-μm section onto a slide. Heat the slide to melt the paraffin in a 60°C oven for 15 min and dry at 37°C overnight.
2. Deparaffinize sections in fresh xylene (3 min, three times) and rehydrate in graded (100, 100, 95, 90, and 70%) ethanols and autoclaved water.

3.2. Pretreatment

3.2.1. CD4-Positive T-Lymphocyte Smears

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C, and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Immerse the slides in methanol containing 0.3% H_2O_2 for 20 min.
5. Wash the slides in autoclaved water for 1 min.
6. Briefly immerse slides in 95% ethanol and allow to air dry.

3.2.2. Formalin-Fixed, Paraffin-Embedded Tissue Sections

1. Immerse the slides in autoclaved water for 5 min.
2. Immerse the slides in preheated target retrieval solution for 40 min at 95°C and allow to cool for 20 min.
3. Wash the slides in autoclaved water (1 min, three times).
4. Digest sections with proteinase K for 10 min at room temperature (*see* **Note 3**).
5. Wash the slides in autoclaved water (1 min, three times).
6. Treat the slides with methanol containing 0.3% H_2O_2 for 20 min.
7. Wash the slides in autoclaved water for 1 min.
8. Briefly immerse the slides in 95% ethanol and allow to air-dry.
9. Mark the area of the section with a wax pen.

3.3. Preparation of PNA Probe

1. Dilute FITC-conjugated PNA probe in hybridization solution to a final concentration of between 0.2 and 0.5 μg/mL.
2. Apply 25 μL of hybridization solution containing PNA probe to the marked area of the slide.
3. Carefully apply the cover slip, avoiding the introduction of air bubbles.

3.4. Heat Denaturation

1. Heat the slides at 93°C for 5 min on a hotplate to denature the double-stranded DNA.

3.5. Hybridization

1. Incubate the slides with the PNA probe at 45°C for 60–90 min in a moist chamber.
2. After hybridization, immerse the slides in TBST and gently remove the cover slips.

3. Wash the slides in prewarmed stringent wash solution at 57°C (20 min, twice).
4. Immerse the slides in TBST at room temperature for 5 min.

3.6. PNA Probe Detection by CSA (see Notes 4 and 5; Fig. 1)

1. Incubate HRP-conjugated rabbit anti-FITC antibody (1:500 dilution) for 60 min (*see* **Note 6**).
2. Wash the slides in TBST (3 min, three times).
3. Incubate biotinyl-tyramide for 15 min.
4. Wash the slides in TBST (3 min, three times).
5. Incubate HRP-conjugated streptavidin (1:600–800 dilution) for 15 min.
6. Wash the slides in TBST (3 min, three times).
7. Incubate biotinyl-tyramide for 15 min.
8. Wash the slides in TBST (3 min, three times).
9. Incubate 0.5 μg/mL Alexa Fluor 488-labeled streptavidin for 15 min in the absence of light.
10. Wash the slides in TBST in the absence of light (3 min, three times).
11. Immerse the slides in distilled water.
12. Apply DAPI II and mount cover slip.

3.7. Fluorescence Microscopy

The slides were examined under a fluorescence microscope (BX50 and BX-FLA, Olympus Corp., Tokyo, Japan) equipped with appropriate filter sets (61002, Chroma Technology Corp., Rockingham, VT). Photographic images of the fluorescent signals were taken with a CCD camera (SenSys 0400, Photometrics Ltd., Tucson, AZ) and were uploaded to a microcomputer using IPLab software (Scanalytics, Inc., Fairfax, VA). The stored images were merged to reveal various aspects (*see* **Note 7**; **Fig. 2**).

3.8. Subsequent Determination of Phenotypes After PNA–ISH Treatment

We describe here a double staining method by PNA–ISH and IHC methods for the detection of both HIV-1 DNA and a protein in the same cell, respectively. Proteins can be detected with one of two methods with IHC: an indirect method and a labeled streptavidin-biotin (LSAB) method. For the detection of CD4 or HLA-DR molecules, the conventional indirect method was used. On the other hand, the LSAB method that has higher sensitivity than the indirect method was performed to detect the p24 HIV-1 capsid protein.

Fig. 1. (*opposite page*) Schematic representation of the procedures for using the PNA-probe in the ISH method. The hybridized probe was detected by sequential reactions of the following antibodies and reagents: HRP-conjugated anti-FITC antibody, biotinylated tyramide (first amplification), HRP-labeled streptavidin, biotinylated tyramide (second amplification), and streptavidin-conjugated Alexa 488.

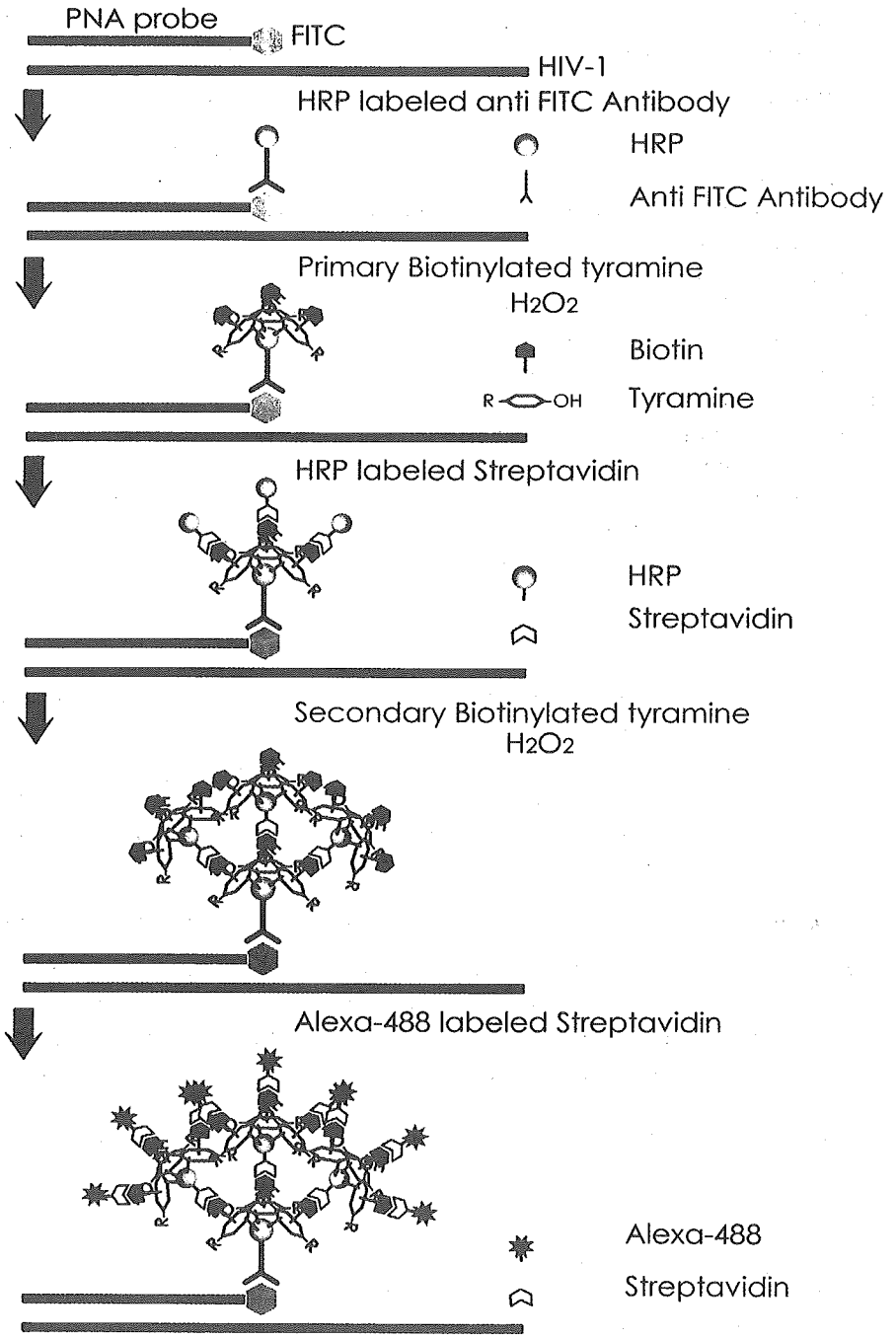
PNA probe
FITC
HIV-1
HRP labeled anti FITC Antibody
HRP
Anti FITC Antibody
Primary Biotinylated tyramine
H2O2
Biotin
R
OH
Tyramine
HRP labeled Streptavidin
HRP
Streptavidin
Secondary Biotinylated tyramine
H2O2
Alexa-488 labeled Streptavidin
Alexa-488
Streptavidin

Fig. 1

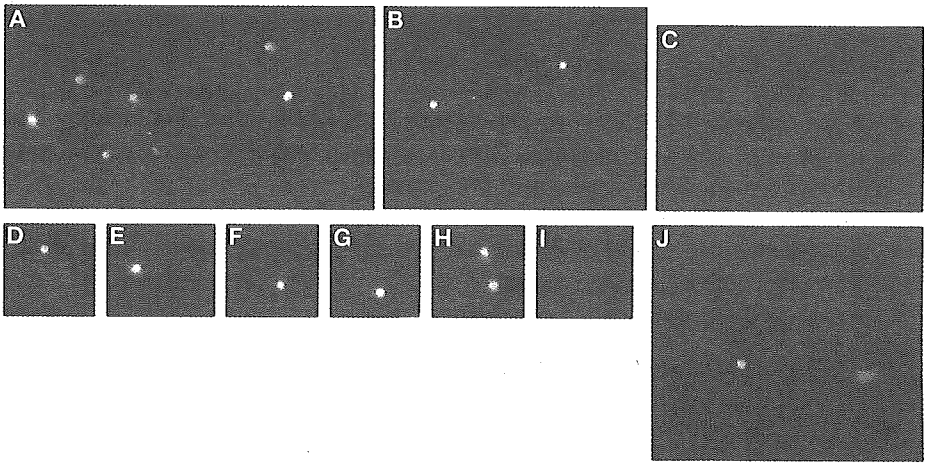

Fig. 2. Detection of HIV-1 provirus by PNA–ISH. Signals detecting HIV-1 proviruses were observed as green dots in a nucleus stained blue by DAPI. (**A**) MOLT4-IIIB. One to four proviruses per cell. (**B**) ACH2. One provirus per cell. (**C**) Negative control (MOLT4). No provirus. (**D–H**) CD4-positive T lymphocytes from HIV-1-infected patients. Most provirus-positive cells contained one provirus. Positive cells rarely contained more than two proviruses. (**I**) Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). No provirus. (**J**) Paraffin-embedded section of bone marrow from an AIDS patient. Positive cells contained one provirus in a nucleus.

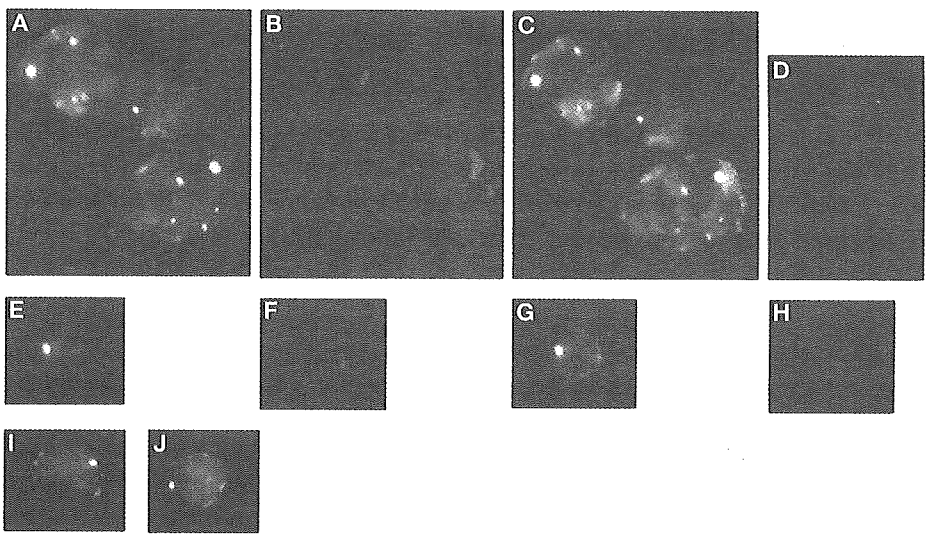

Fig. 3. Subsequent determination of phenotypes after PNA–ISH treatment. Proteins were stained red and localization of the proteins can be observed. (**A–C**) p24/HIV-1 provirus of MOLT4-IIIB. (**D**) Negative control (MOLT4). (**E–G**) p24/provirus

3.8.1. PNA–ISH and IHC (Indirect Method)

1. Follow **steps 1–10** inclusive for PNA Probe Detection by CSA (**Subheading 3.6.**).
2. Incubate with mouse anti-human CD4 monoclonal antibody or mouse anti-human HLA-DR monoclonal antibody at 4°C overnight.
3. Wash in TBST (3 min, three times).
4. Incubate with Alexa Fluor 594-labeled goat anti-mouse IgG antibody for 30 min.
5. Wash in TBST (3 min, three times).
6. Apply DAPI II and mount a cover slip.

3.8.2. PNA–ISH and IHC (LSAB Method)

Biotin blocking of biotinyl-tyramide after PNA–ISH staining is recommended before the reaction with primary antibody for the target protein.

1. Follow **steps 1–10** inclusive for PNA Probe Detection by CSA (**Subheading 3.6.**).
2. Incubate with avidin for 10 min.
3. Wash in TBST (3 min, three times).
4. Incubate with biotin for 10 min.
5. Wash in TBST (3 min, three times).
6. Incubate with mouse monoclonal anti-HIV-1 p24 antibody at 4°C overnight.
7. Wash in TBST (3 min, three times).
8. Incubate with biotinylated goat anti-mouse Ig antibody for 30 min.
9. Wash in TBST (3 min, three times).
10. Incubate with Alexa Fluor 594-labeled streptavidin for 15 min.
11. Wash in TBST (3 min, three times).
12. Apply DAPI II and mount a cover slip.

3.8.3. Fluorescence Microscopy of Double-Stained Sample

The slides were examined under a fluorescence microscope with appropriate filter sets. Photographic images of the fluorescent signals were taken with a CCD camera, and were uploaded to a microcomputer using IPLab software. The stored images were merged to reveal various aspects (**Fig. 3**).

Fig. 3. *(continued from opposite page)* of CD4-positive T lymphocytes from HIV-1-infected patients. (**H**) Negative control (CD4-positive T lymphocytes from a HIV-1-negative volunteer). (**A**,**E**) provirus (green) and nucleus (blue) were merged. (**B**,**F**) p24 (red). p24 HIV-1 capsid proteins were observed in the cytoplasm of HIV-1 provirus positive cells. (**C**,**G**) provirus (green), p24 (red) and nucleus (blue) were merged. (**I**,**J**) CD4-positive T lymphocytes from a HIV-1-infected patient; HIV-1 provirus (green) was seen in the cell nucleus. In contrast, CD4 molecules (red) were seen in the outer-membrane (**I**) Membrane-bound or cytoplasmic HLA-DR molecules (red) were detected in HIV-1 provirus positive cells (**J**).

4. Notes

1. The base sequence corresponds to the region from 1379 to 1397 of the HIV-1 gag gene. As the melting temperature of the anti-parallel probe is higher than that of the parallel, the use of anti-parallel type is recommended. PNA probe can be dissolved with DEPC-treated water to a concentration of 100 µg/mL, aliquotted, and stored at –20°C. A 0.01% trifluoroacetic acid can be replaced instead of water.
2. Wear gloves throughout the steps until the hybridization step is completed. Use the dry-sterilized glassware equipment and those made by stainless steel (200ºC, 2 h). Use autoclaved water (121ºC, 15 min) through the hybridization step.
3. To obtain an optimal concentration of proteinase K, treat fixed specimens with three different concentrations between 2 and 7 µg/mL of the enzyme.
4. This amplification method is based on the binding reaction of biotinylated tyramine to a phenol derivatives of a protein by peroxidase. This step sometimes gives nonspecific signals, therefore thorough pretreatment of specimens with methanol containing 0.3% H_2O_2 is essential to diminish the endogenous peroxidase activity. Also, it is important to stain two kinds of negative control to allow the identification of nonspecific signals: (1) HIV-1 DNA negative-specimen with a PNA probe and (2) HIV-1 DNA positive-specimen without a PNA probe (**Fig. 1**).
5. A single amplification method was successfully applied for HIV-1 RNA detection ***(16)***.
6. Considerable amounts of endogenous biotin is contained in liver, kidney, mucosa of digestive tract, and brain. Even in other organs, endogenous biotin becomes exposed through an activation step (*see* **Subheading 3.2.2., steps 2** and **4**). A biotin blocking of the endogenous biotin is recommended between the steps of stringent wash and HRP-FITC antibody reaction. Biotin blocking system (DakoCytomation A/S, X0590): (1) Incubate with avidin solution for 10 min, (2) wash in TBST three times for 3 min, (3) Incubate with biotin solution for 10 min, (4) wash in TBST three times for 3 min.
7. To measure the positivity of HIV-1 provirus in the CD4-positive T lymphocytes, we count 500 cells and calculate. The positivity of the HIV-1 provirus among 62 HIV-1-infected patients ranged between 0.3% and 7.9% (average of 2.7).

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Conventional HPLC Method Used for Simultaneous Determination of the Seven HIV Protease Inhibitors and Nonnucleoside Reverse Transcription Inhibitor Efavirenz in Human Plasma

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We developed a simple HPLC method for the simultaneous quantitative determination of seven HIV protease inhibitors: amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and a nonnucleoside reverse transcription inhibitor, efavirenz (EFV). This method involves a rapid liquid–liquid drug extraction from plasma, the use of an isocratic elution on a reversed-phase C_{18} column, and an ultraviolet detection at a single wavelength (205 nm). The mobile phase consisted of 39% 50 mM phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. Forty-eight samples could be measured in one day since the runtime of one sample is 30 min. The assay has been validated over a concentration range of 0.05 to 12.20 μg/ml for APV, 0.09 to 12.05 μg/ml for ATV, 0.05 to 12.01 μg/ml for IDV, 0.12 to 12.36 μg/ml for LPV, 0.18 to 12.20 μg/ml for NFV, 0.12 to 12.33 μg/ml for RTV, 0.12 to 12.06 μg/ml for SQV, and 0.05 to 12.17 μg/ml for EFV. Calibration curves were linear in the described concentration ranges. The average accuracy ranged from 97.2 to 106.8%. Both the interday and intraday coefficients of variation for all drugs tested were less than 8.5%. This method provides a simple, accurate, and precise method for the therapeutic drug monitoring of the seven protease inhibitors and EFV in clinical routine use.

Key words HPCL; therapeutic drug monitoring; HIV protease inhibitor; human immunodeficiency virus (HIV)-1

The clinical treatment of patients with human immunodeficiency virus (HIV)-1 infection has been advanced by the development of 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 concentrations. Therefore, monitoring plasma drug concentrations is essential to ensure optimal drug efficacy, to prevent viral resistance, to manage drug interactions, to avoid adverse effects, and to assess nonadherence.

In recent years several HPLC methods for simultaneous determination of antiretroviral drugs in plasma have been published.[1—15] However, to popularize the simultaneous determination method, a simplified technique is necessary because the reported techniques require a solid-phase extraction,[4—8] and/or use of a gradient elution,[6—13] and/or an ultraviolet detection at multi wavelengths,[4,5,7—9,11,13—15] all of which are not routinely available in conventional hospital laboratories. Therefore, we aimed to develop a simple procedure for simultaneous quantitative determination of seven protease inhibitors (PI): amprenavir (APV), atazanavir (ATV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), and the nonnucleoside reverse transcription inhibitor, efavirenz (EFV), in human plasma. Our technique involves rapid liquid–liquid drug extraction from plasma, the use of an isocratic elution, as well as an ultraviolet detection at a single wavelength. This assay is based on our previously published HPLC method.[16]

MATERIALS AND METHODS

Chemicals and Reagents APV was kindly provided by Glaxosmithkline Research and Development, Ltd. (Park Road, Ware, U.K.), ATV was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, U.S.A.), and IDV and EFV were provided by Merck & Co., Inc. (Rahway, NJ, U.S.A.). LPV, RTV and the internal standard (IS), (5*S*,8*S*,10*S*,11*S*)-9-hydroxy-2-cyclopropyl-5-(1-methylethyl-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester, were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A.). NFV was provided by Agouron Pharmaceuticals (La Jolla, CA, U.S.A.) and SQV was provided by Roche Products (Welwyn Garden City, U.K.). Acetonitrile, methanol, ethyl acetate and *n*-hexane (Kanto Chemical, Tokyo, Japan) were HPLC grade. Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q® system (Millipore, Bedford, MA, U.S.A.).

Chromatography The HPLC system consisted of a Waters pump (model 515), WISP 712 auto sample processor, and a 2487 dual absorbance detector coupled to the Millenium[32] version 3.21 software (Waters, Milford MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C_{18} column (4 μm, 8×100 mm, Waters) protected by Guard-Pak Inserts Nova-Pak C_{18} precolumn. Absorbance was measured at 205 nm and separations were performed at 30 °C. The mobile phase consisted of 39% 50 mM phosphate buffer (pH 5.9), 22% methanol and 39% acetonitrile. The assay run time was 30 min with a flow rate of 1.8 ml/min. Drugs were quantified by measuring the peak areas under the chromatograms.

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Standard Solutions Stock solutions of all seven protease inhibitors and EFV were prepared by dissolving accurately weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 106.1 μg/ml for APV, 502.0 μg/ml for ATV, 100.1 μg/ml for IDV, 95.1 μg/ml for LPV, 305.0 μg/ml for NFV, 425.0 μg/ml for RTV, 67.0 μg/ml for SQV, and 93.6 μg/ml for EFV. These stock solutions were stored at −80 °C and thawed on the day of analysis. Each stock solution was diluted in drug-free plasma to yield concentrations of 0.05, 0.85, 2.12, 7.43 and 12.20 μg/ml for APV, 0.09, 0.88, 2.20, 6.02 and 12.05 μg/ml for ATV, 0.05, 0.80, 2.00, 6.01 and 12.01 μg/ml for IDV, 0.12, 0.95, 1.90, 6.18 and 12.36 μg/ml for LPV, 0.18, 0.92, 3.05, 6.10 and 12.20 μg/ml for NFV, 0.12, 0.86, 1.71, 6.16 and 12.33 μg/ml for RTV, 0.12, 0.92, 3.05, 6.10 and 12.06 μg/ml for SQV, and 0.05, 0.84, 1.87, 6.08 and 12.17 μg/ml for EFV.

Sample Preparation Two milliliters of ethyl acetate/*n*-hexane (50 : 50, v/v) containing the IS (2.02 μg/ml) and 1 ml of 0.5 M sodium carbonate were added to a 500 μl plasma sample. The mixture was vortexed and then centrifuged at 3500×*g* for 5 min. The organic layer was separated and evaporated dry. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at 13000×*g* for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column. Before taking peripheral blood, written informed consent was obtained from all patients and a healthy volunteer.

Validation Intraday and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV five times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of the nominal concentration. Drug recovery from plasma was evaluated by analyzing triplicate samples with or without extraction.

RESULTS

Chromatograms of Plasma Sample Figure 1A shows the chromatogram of a plasma sample containing 2.12 μg/ml of APV, 2.20 μg/ml of ATV, 2.00 μg/ml of IDV, 1.90 μg/ml of LPV, 3.05 μg/ml of NFV, 1.71 μg/ml of RTV, 3.05 μg/ml of

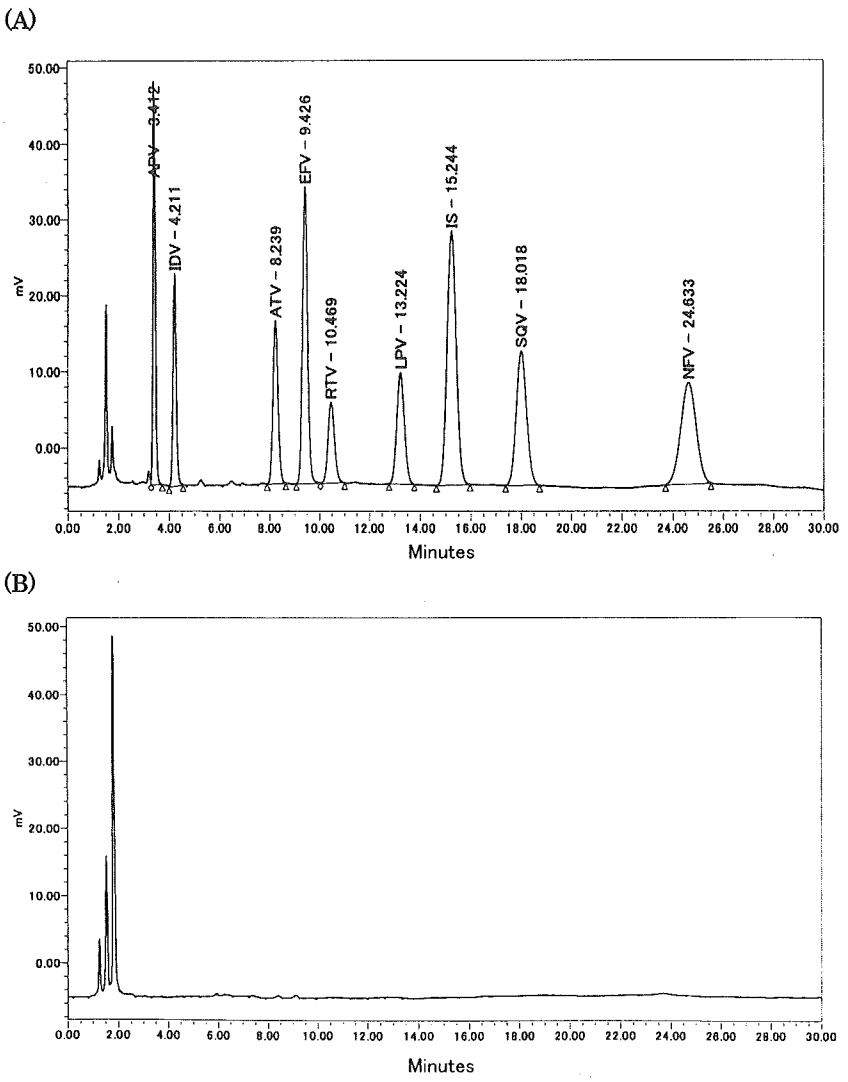

Fig. 1. Chromatograms Obtained after Extraction of (A) Spiked Plasma Sample Containing 2.12 μg/ml of APV, 2.20 μg/ml of ATV, 2.00 μg/ml of IDV, 1.90 μg/ml of LPV, 3.05 μg/ml of NFV, 1.71 μg/ml of RTV, 3.05 μg/ml of SQV and 1.87 μg/ml of EFV and (B) Drug-Free Human Plasma Sample from a Healthy Volunteer.

Table 1. Intraday and Interday Precision and Accuracy for Seven PIs and EFV

	Expected (μg/ml)	Intraday (n=5)		Interday (n=15)		Accuracy (%)	Recovery (%)
		Measured (μg/ml)	CV (%)	Measured (μg/ml)	CV (%)		
APV	0.05	0.05±0.01	6.5	0.05±0.01	5.2	97.8±5.1	98.4±3.3
	0.85	0.85±0.02	2.7	0.85±0.03	3.0	99.7±3.0	90.9±4.4
	2.12	2.14±0.03	1.4	2.12±0.04	2.0	100.1±2.0	91.1±2.6
	7.43	7.48±0.12	1.6	7.45±0.18	2.4	100.3±2.4	90.1±1.0
	12.20	12.27±0.32	2.6	12.25±0.28	2.3	100.4±2.3	94.0±2.6
ATV	0.09	0.09±0.01	4.8	0.09±0.01	4.1	98.4±4.0	92.2±1.7
	0.88	0.88±0.05	5.6	0.86±0.04	5.0	98.1±4.9	93.4±3.3
	2.20	2.26±0.03	1.2	2.22±0.06	2.6	100.9±2.6	94.4±2.5
	6.02	6.04±0.12	1.9	5.95±0.15	2.6	98.8±2.5	91.1±1.0
	12.05	12.08±0.26	2.1	12.29±0.25	2.0	102.0±2.1	95.0±2.3
IDV	0.05	0.05±0.01	5.4	0.05±0.01	7.5	101.8±7.7	83.4±4.4
	0.80	0.79±0.02	2.8	0.82±0.05	5.7	102.5±5.8	96.0±5.0
	2.00	2.01±0.04	1.8	2.00±0.03	1.4	100.1±1.4	82.6±2.8
	6.01	5.93±0.11	1.8	5.93±0.13	2.2	98.8±2.2	81.2±2.3
	12.01	11.96±0.29	2.5	12.15±0.26	2.2	101.2±2.2	85.6±1.4
LPV	0.12	0.13±0.01	6.6	0.13±0.01	7.0	102.7±7.2	97.5±8.5
	0.95	0.97±0.02	2.2	0.95±0.03	3.4	99.7±3.4	93.8±5.7
	1.90	1.98±0.02	1.2	1.95±0.04	2.3	102.3±2.3	93.9±1.1
	6.18	6.20±0.09	1.4	6.09±0.15	2.5	98.5±2.5	92.0±1.1
	12.36	12.62±0.36	2.9	13.14±0.49	3.8	106.3±4.0	95.2±2.4
NFV	0.18	0.18±0.02	8.5	0.18±0.01	8.4	97.2±8.2	96.4±8.3
	0.92	0.94±0.03	3.1	0.93±0.06	6.3	101.2±6.4	92.7±5.7
	3.05	3.10±0.02	0.6	3.09±0.07	2.2	101.4±2.3	88.6±2.1
	6.10	6.12±0.16	2.7	6.02±0.22	3.7	98.7±3.7	89.6±2.0
	12.20	12.41±0.46	3.7	12.58±0.35	2.8	103.1±2.9	92.8±2.2
RTV	0.12	0.11±0.01	7.8	0.12±0.01	7.5	98.7±7.4	94.2±9.9
	0.86	0.85±0.02	2.5	0.89±0.04	4.9	103.8±5.0	90.4±1.8
	1.71	1.72±0.06	3.2	1.75±0.06	3.4	102.2±3.5	90.4±3.3
	6.16	6.15±0.21	3.4	6.12±0.14	2.3	99.3±2.3	90.4±2.1
	12.33	12.27±0.32	2.6	12.55±0.31	2.5	101.8±2.5	94.4±1.9
SQV	0.12	0.13±0.01	6.4	0.12±0.01	6.4	101.4±6.5	91.0±5.4
	0.92	0.89±0.02	1.9	0.92±0.03	3.6	100.4±3.6	89.9±3.4
	3.05	3.11±0.01	0.4	3.08±0.08	2.7	101.0±2.7	91.2±2.1
	6.10	6.05±0.16	2.7	5.99±0.17	2.8	98.3±2.7	90.2±1.5
	12.06	12.46±0.55	4.4	12.88±0.53	4.1	106.8±4.4	94.1±2.3
EFV	0.05	0.05±0.01	3.8	0.05±0.01	3.5	101.8±3.6	93.0±6.1
	0.84	0.87±0.02	1.8	0.85±0.06	6.5	101.5±6.6	95.2±3.4
	1.87	1.89±0.04	2.0	1.90±0.05	2.6	101.4±2.6	92.0±2.4
	6.08	6.24±0.24	3.8	6.10±0.20	3.3	100.2±3.4	91.1±1.1
	12.17	12.19±0.27	2.2	12.49±0.33	2.7	102.6±2.7	95.2±2.2

SQV, and 1.87 μg/ml of EFV. Under the described chromatographic conditions, retention times were 3.4, 4.2, 8.2, 10.5, 13.2, 15.2, 18.0, 24.6, and 9.4 min for APV, IDV, ATV, RTV, LPV, IS, SQV, NFV, and EFV, respectively. At a detection wavelength of 205 nm, assays performed on drug-free human plasma failed to show the presence of any interfering peaks during the interested intervals of the retention times (Fig. 1B). These results indicated that the established conditions were valid when measuring these drugs simultaneously.

Validation: Linearity, Precision, Accuracy and Recovery Calibration curves of APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV appeared linear in the concentration range of 0.05 to 12.20 μg/ml for APV, 0.09 to 12.05 μg/ml for ATV, 0.05 to 12.01 μg/ml for IDV, 0.12 to 12.36 μg/ml for LPV, 0.18 to 12.20 μg/ml for NFV, 0.12 to 12.33 μg/ml for RTV, 0.12 to 12.06 μg/ml for SQV and 0.05 to 12.17 μg/ml for EFV, with correlations of 1.000, 0.9999, 0.9999, 0.9993, 0.9997, 0.9999, 0.9991, and 0.9999, respectively.

Precision, accuracy, and extraction recovery of our HPLC method are shown in Table 1. The selected concentration of each drug covers the expected plasma concentrations found in the patients.

The CVs calculated for APV in the intraday and interday assays ranged from 1.4 to 6.5% and 2.0 to 5.2%, respectively. In ATV CVs ranged from 1.2 to 5.6% and 2.0 to 5.0%, and in IDV, CVs ranged from 1.8 to 5.4% and 1.4 to 7.5%. For LPV, CVs ranged from 1.4 to 6.6% and 2.3 to 7.0%, NFV CVs ranged from 0.6 to 8.5% and 2.2 to 8.4%, RTV CVs ranged from 2.5 to 7.8% and 2.3 to 7.5%, SQV CVs ranged from 0.4 to 6.4% and 2.7 to 6.4%, while for EFV the CVs ranged from 2.0 to 3.8% and 2.6 to 6.5%. Accuracies ranged from 97.8 to 100.4%, 98.1 to 102.0%, 98.8 to 102.5%, 98.5 to 106.3%, 97.2 to 103.1%, 98.7 to 103.8%, 98.3 to 106.8% and 100.2 to 102.6% for APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV, respectively.

Drug recoveries from plasma ranged from 90.1 to 98.4%, 91.1 to 95.0%, 81.2 to 96.0%, 92.0 to 97.5%, 88.6 to 96.4%, 90.4 to 94.4%, 89.9 to 94.1% and 91.1 to 95.2% for APV, ATV, IDV, LPV, NFV, RTV, SQV and EFV, respectively. Extraction recovery of the IS was 100%.

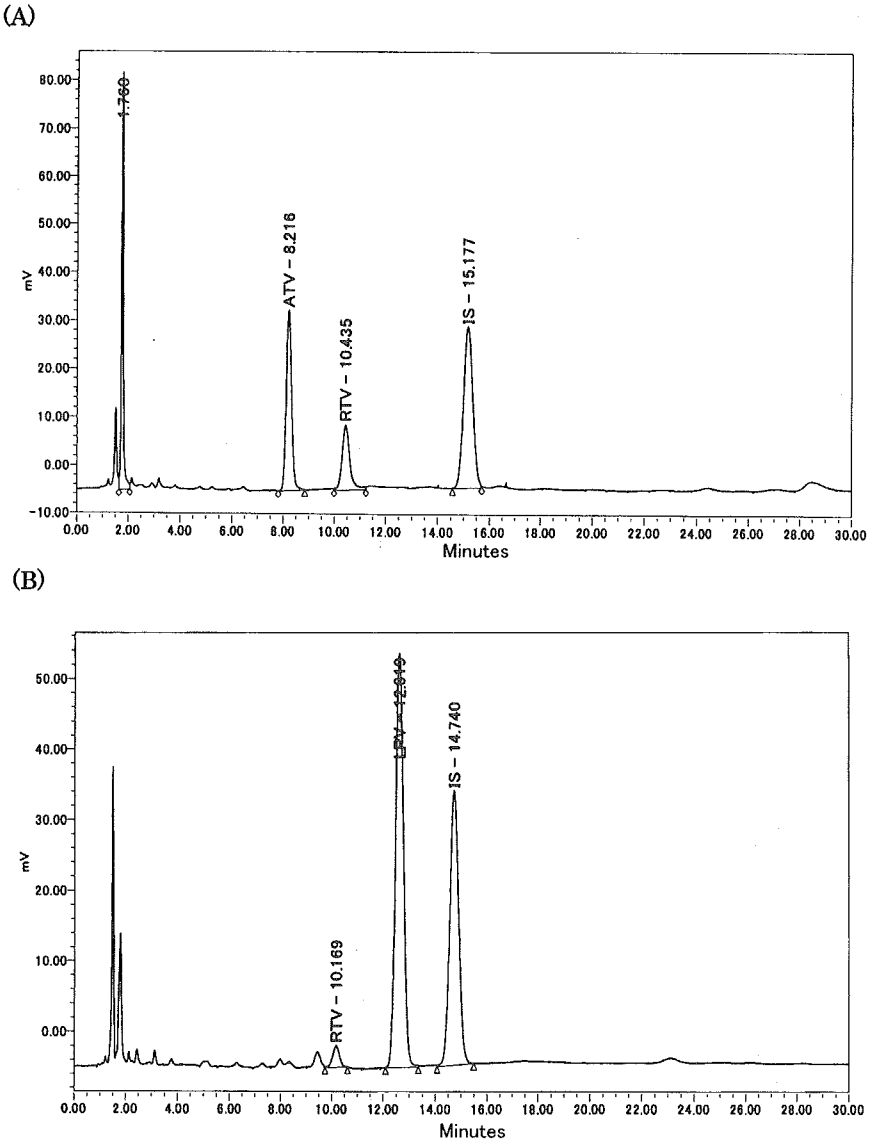

Fig 2. (A) Chromatogram of a Plasma Sample from an HIV-1-Infected Patient Treated with ATV and RTV and (B) Chromatogram of a Plasma Sample from an HIV-1-Infected Patient Treated with LPV and RTV

Chromatograms of Patient Samples Figure 2A shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with ATV and RTV. Fig. 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with LPV and RTV. There is no significant drift from the baseline and no interfering peaks affecting quantification of ATV, LPV and RTV in this chromatogram. These results were validated by peak testing and library matching performed with Millenium[32] software.

DISCUSSION

There have been some recent reports of HPLC methods for simultaneous determination of antiretroviral drugs.[1—15] However, these methods have several disadvantages in terms of cost performance, time consumption and necessary equipment; for example, the use of expensive disposable cartridges at the solid-phase drug extraction, gradient elution control by a gradient HPLC pump system, and the ultraviolet detection at multiple wavelengths.

We describe the development, validation, and application of a simple HPLC method for simultaneous quantitative determination of seven PIs currently on the market as well as EFV. The principal advantages of our method are a rapid liquid–liquid drug extraction from plasma, an isocratic elution on a reversed-phase C_{18} column, and ultraviolet detection at a single wavelength (205 nm).

Generally, the retention time of antiretroviral drugs are dependent on pH of the mobile phase buffer.[14] In fact, some previously reported assays used the mobile phase buffer at a variety of pH values. We sought the optimum pH of the mobile phase buffer by changing pH every 0.5 from pH 2 to pH 11. Finally, we determined pH 5.9 phosphate buffer for the mobile phase to separate each drug. A mobile phase with a pH 9.4 phosphate buffer also enabled excellent drug separation. The overlapping profile of some peaks was not excluded in the pH condition except about 6 and 9.5. However, we recommend pH 5.9 phosphate buffer, because alkaline buffer rapidly degrades the C_{18} reversed-phase column and causes a baseline drift.

In this study, calibration curves of all the drugs used were linear in the described concentration ranges and the average accuracy ranged from 97.2 to 106.8%. Both interday and intraday CVs for all drugs were less than 8.5%, which is similar to or much lower than previously reported values.[1—15] Mean extraction recoveries varied from 81.2% (IDV) to 98.4% (APV). These results indicate that the method developed here achieves a high degree of reproducibility and accuracy.

When antiretroviral drugs are administered at the recommended dose, plasma concentrations are expected in the 0.3 to 8.2 μg/ml range for APV,[12] the 0.2 to 10 μg/ml range for ATV,[17] the 0.1 to 7.7 μg/ml range for IDV,[18,19] the 5.5 to 9.6 μg/ml range for LPV,[12] the 0.1 to 4.0 μg/ml range for NFV,[18,20] the 0.1 to 11.2 μg/ml range for RTV,[18,20] the 0 to 0.2 μg/ml range for SQV,[18] and the 1 to 4 μg/ml range for EFV.[21] Our method successfully covers these regions with good precision and accuracy.

Figure 1 illustrates the chromatogram of a plasma sample containing IS, APV, ATV, IDV, LPV, NFV, RTV, SQV, and EFV. Our isocratic elution yields sharp peaks for all the drugs tested and gives an excellent separation for each. The chromatogram of the blank plasma sample shows that there was no significant drift from the baseline and no interfering peaks affecting the quantification of all the drugs. Furthermore, it turns out that no analytical interference was encountered from endogenous substances or other co-administered drugs at the retention time for each drug, even in the chromatogram of a plasma sample from an HIV-1-infected patient treated with either ATV/RTV or LPV/RTV. Therefore, our method can be made available for optimal follow-up of HIV-infected patients through therapeutic drug monitoring.

In conclusion, this simple HPLC method can be conveniently used as a routine clinical application and enables study of the drug pharmacokinetics in conventional hospital laboratories. This method can also offer continuous measurement of 48 samples in one day.

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New estimation method for highly sensitive quantitation of human immunodeficiency virus type 1 DNA and its application

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Abstract

A new estimation method for quantitation of HIV-1 DNA was established by introducing a pre-quantitation polymerase chain reaction (PCR) before conventional real-time PCR. Two alternative methods for estimating the copy number can be used: the first method utilizes the rate of β2-microglobulin (β2M) gene amplification during the pre-quantitation PCR, and the second utilizes a calibration curve of the crossing point of real-time PCR versus the standard HIV-1-plasmid concentration. These methods could be used to reproducibly and accurately detect a provirus density down to five copies/10^6 cells (for methods 1 and 2, inter-assay CV = 17 and 16% and accuracy = 81 and 92%, respectively). The levels of HIV-1 DNA could be measurable using as little as 100 μl of whole blood or buffy coat cells. Using a combination of a conventional and highly sensitive methods, we found that the amount of HIV-1 DNA ranged from 2 to 5960 copies/10^6 cells (median of 830 copies/10^6 cells) in CD4-positive T lymphocytes isolated from 30 patients responding well to highly active antiretroviral therapy (HAART). Thus, the highly sensitive method developed in this study allows estimation of the HIV-1 reservoirs in peripheral CD4-positive T lymphocytes of patients responding well to HAART.

Keywords: Highly sensitive quantitation; HIV-1 DNA; real-time PCR; HAART

1. Introduction

Due to the recent development of highly active antiretroviral therapy (HAART), it has been possible to effectively suppress the progression of HIV-1 infectious disease to AIDS. In many cases, the treatment of HIV-1-infected patients with HAART, which uses a combination of reverse transcriptase and protease inhibitors, can rapidly suppress plasma HIV-1 RNA levels below the detectable level (Mellors et al., 1996; Perelson et al., 1997). Therefore, the effectiveness of the treatment has been monitored by measuring plasma viral load.

Given their mode of action, it is clear that reverse transcriptase and protease inhibitors do not eliminate the HIV-1 infected cells. Consequently, quantitation of HIV-1 DNA in virus-infected cells has become important for estimating the residual viral reservoir in patients receiving HAART (Chun

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; PCR, polymerase chain reaction; β2M, β2-microglobulin; WBC, white blood cells; Cp, crossing point; CV, coefficient of variation; VL, viral load

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et al., 1997; Désiré et al., 2001; Ibanez et al., 1999; Izopet et al., 1998; Riva et al., 2001, 2003; Sharkey et al., 2000; Yerly et al., 2000). A recently developed technique, real-time polymerase chain reaction (PCR), has made this possible because it can rapidly and specifically quantify a target DNA without complex manipulations (Damond et al., 2001; Désiré et al., 2001; Izopet et al., 1998; Riva et al., 2001; Wada et al., 2004; Walker, 2002; Zhao et al., 2002).

Recently, we demonstrated the clinical significance of HIV-1 DNA quantitation by real-time PCR as measured with the LightCycler system with a detection limit of 500 copies/10^6 cells (Wada et al., 2004). However, the HIV-1 DNA levels in 30% of therapy-naïve or HAART-receiving patients could not be quantified with the conventional method because of a relatively high detection limit. Therefore, a more sensitive method must be developed to quantify much lower levels of HIV-1 DNA, which is particularly important for following patients responding well to HAART.

2. Materials and methods

2.1. Quantitation of HIV-DNA

The two methods established in this study for quantifying HIV-1 DNA are shown in Figs. 1 and 2. Method 1 was based on an estimation in which the HIV-1 DNA copy numbers were calculated by dividing the amount of amplified HIV-1 DNA by the rate of human β2-microglobulin (β2M) gene amplification (Fig. 1). First, both HIV-1 DNA and β2M genes were simultaneously amplified in the same tube. Second, the copy numbers of amplified HIV-1 DNA was separately quantified by real-time PCR. A standard curve was then generated by plotting the crossing point (Cp) from real-time PCR as a function of the concentration (copy number) of HIV-1 standard plasmid (serial 10-fold dilutions from 10^{10} to 10 copies). The Cp is the PCR cycle number where the amount of PCR product is the same in all curves (Rasmussen, 2001). As shown

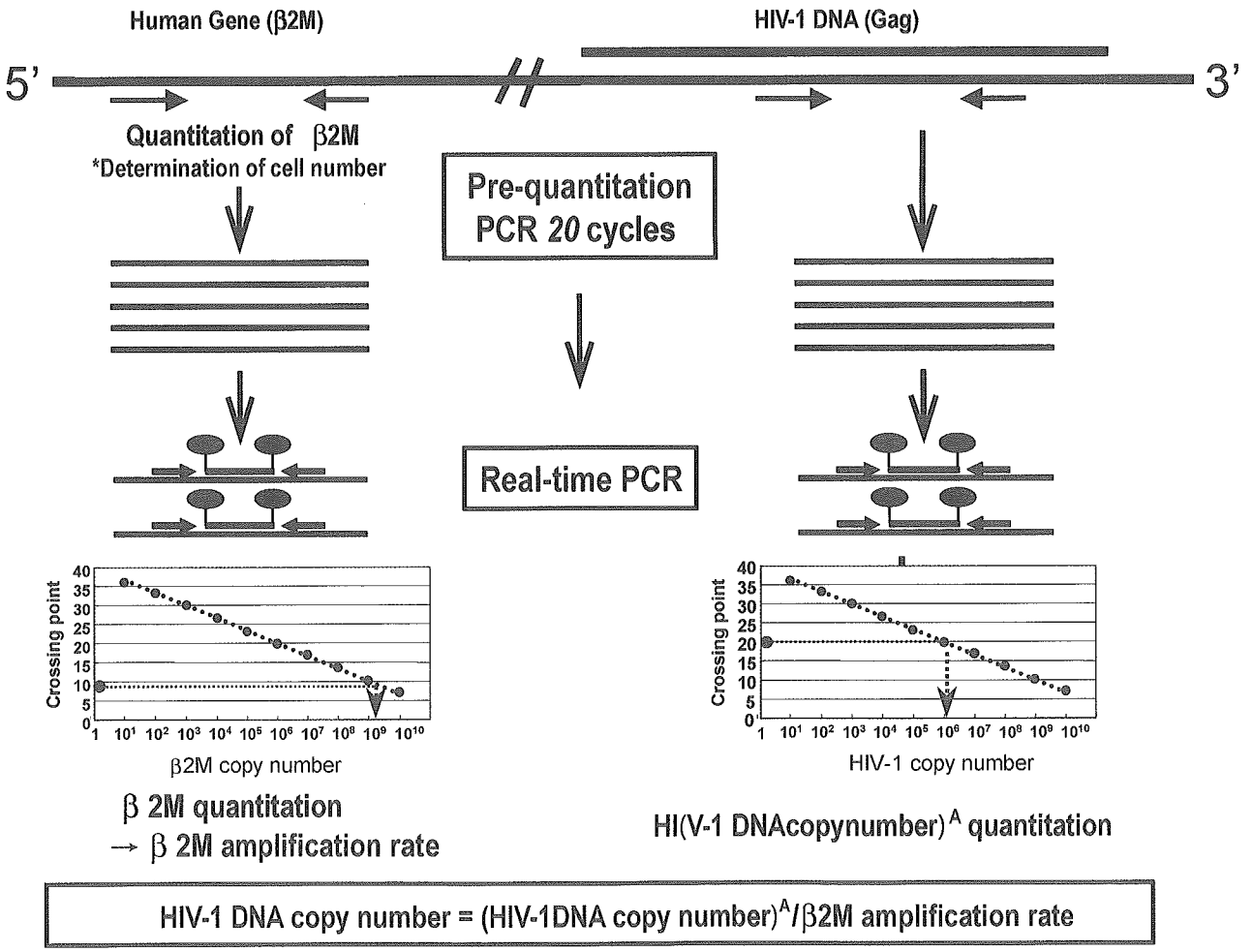

Fig. 1. Method 1: first, both HIV-1 DNA and β2M genes are simultaneously amplified in one tube (pre-quantitation PCR). Second, the copy numbers of amplified HV-1 DNA, (HIV-1 DNA copy numbers)^A and β2M genes are separately quantified by real-time PCR. The amplification rate of β2M genes are determined by dividing the PCR-amplified copy numbers by the original copy numbers before pre-quantitation PCR. Finally, HIV-1 copy numbers are calculated as amplified HIV-1 DNA copy numbers/β2M gene amplification rate. Using the standard curve (blue dotted line), the amount of HIV-1 (red arrow head) is estimated from the measured Cp (red circle).

in Fig. 1, by using the standard curve (blue dotted line), the amount of HIV-1 (red arrow head) was estimated from the measured Cp (red circle). Next, the amplification rate of β2M genes was determined by dividing the pre-quantitation PCR-amplified copy numbers by the copy numbers obtained before the PCR. Finally, the HIV-1 copy number was calculated as the amplified HIV-1 DNA copy number divided by the β2M gene amplification rate.

In method 2, HIV-1 copy numbers were calculated according to a standard curve obtained from the amplified HIV-1 standard plasmids (Fig. 2). First, pre-quantitation PCR was performed as described for Method 1. HIV-1 DNA samples and serially diluted pUC-IIIB standard plasmids (1000, 100, 50, 10, or 5 copies/assay) were amplified at the same time in different tubes. Second, the Cp of each sample was determined by real-time PCR using aliquots of amplified HIV-1 DNA and pUC-IIIB standard plasmids. The quantity of HIV-1 DNA was determined using a standard curve of the Cp obtained in real-time PCR versus the logarithm of the standard plasmid concentration in pre-quantitation PCR. Finally, the copy numbers of HIV-1 DNA were determined using the Cp of the HIV-1 sample obtained in real-time PCR.

Intra- and inter-day precision values for the methods were estimated by quantifying four different copy numbers of HIV-1 five times on the same day and on three separate days, respectively, to obtain coefficient of variation (CV), which is the standard deviation divided by the average.

Accuracy was determined as the percentage of the nominal HIV-1 copy number.

2.1.1. *Preparation of pre-quantitation PCR primers*

To obtain the highly conserved nucleotide sequences for creation of pre-quantitation PCR primers, we determined the DNA sequences of the HIV-1 gag p17 gene flanking the conventional real-time PCR primers. DNA was amplified by nested-PCR with Pyrobest DNA polymerase (TaKaRa, Shiga, Japan). External primers, ExF (5′-tacataatacagtagcagtcctctattgtgtgca-3′) and ExR (5′-tctaaagggttcctttggtcct-3′), and the internal primers, InF (5′-agtcctctattgtgtgcatcaaagga-3′) and InR

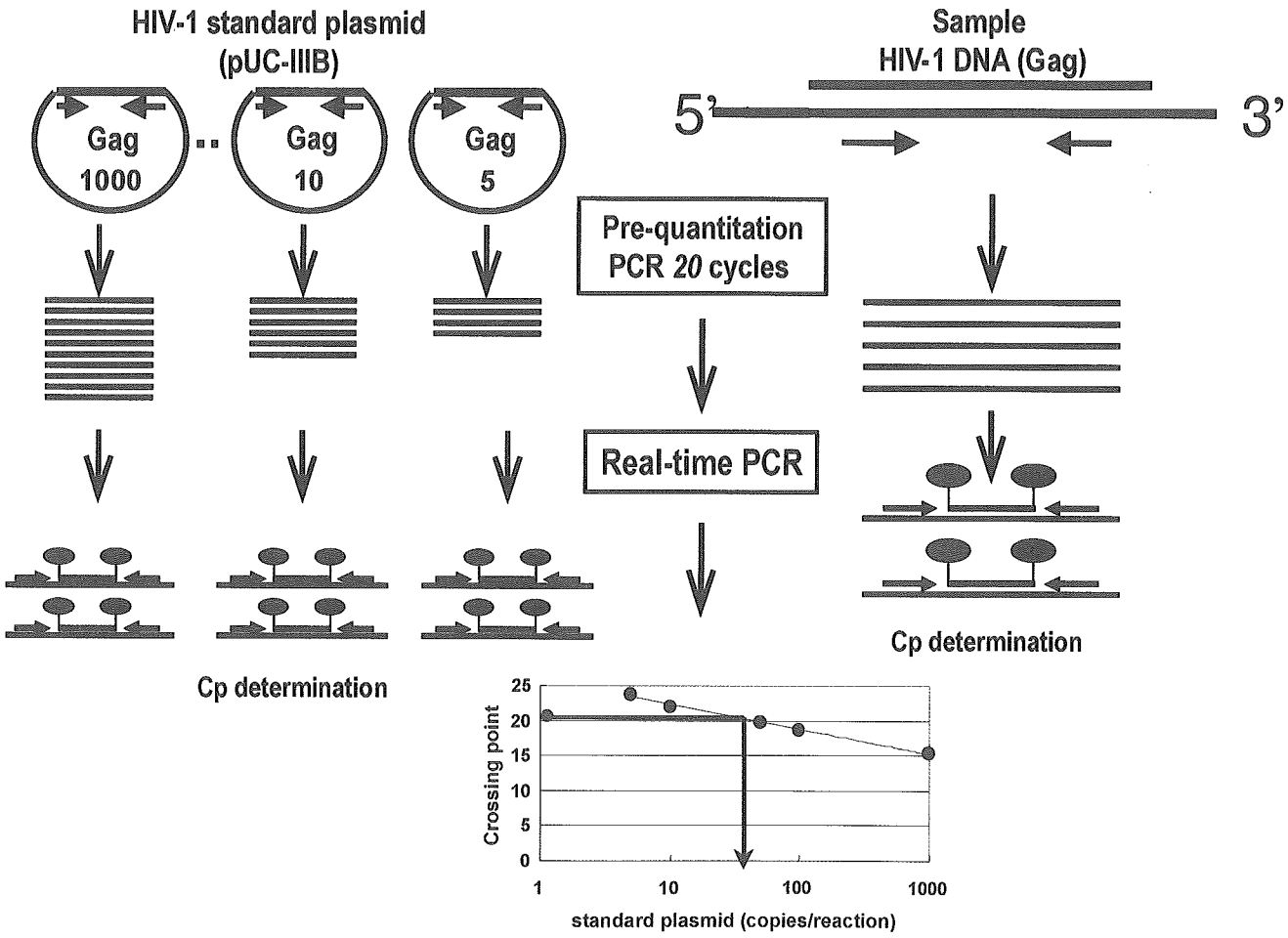

Fig. 2. Method 2: first, pre-quantitation PCR is performed as is performed in method 1. HIV-1 DNA samples and serial-diluted pUC-IIIB standard plasmid (1000, 100, 50, 10, or 5 copies/assay) are separately amplified at the same time in different tubes. Second, the Cp of each sample is determined by real-time PCR where aliquots of the amplified HIV-1 DNA and pUC-IIIB standard plasmid are used. Quantitation is based on a standard curve of the Cp obtained in real-time PCR vs. the log standard plasmid concentrations in pre-quantitation PCR. Finally, the copy numbers of HIV-1 DNA are determined using the Cp of the HIV-1 sample obtained in real-time PCR.