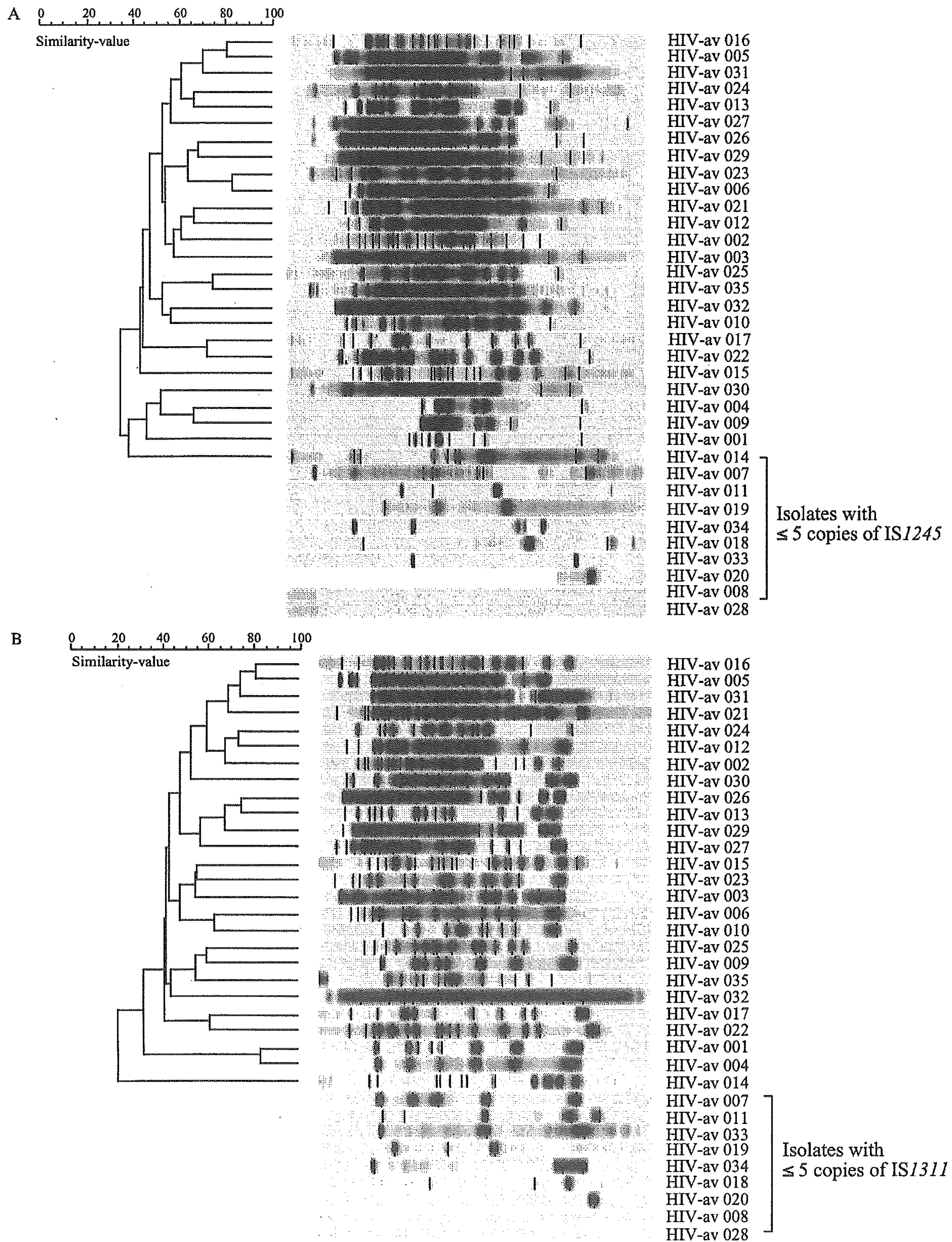

Figure 4 IS*1245*- and IS*1311*-probed DNA fingerprinting patterns of *M. avium* clinical isolates from HIV-seropositive patients in Japan and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*1245* (A) or IS*1311* (B) band is normalized so that the patterns for all strains are comparable. In both the IS*1245*- and IS*1311*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated. The isolates are named as follows: a prefix of 'HIV-av' indicates an HIV-seropositive patient-derived isolate.

The number of IS*6110* bands in the isolates ranged from 0 to 15 (Fig. 2A). Thirty-one different IS*6110* fingerprinting patterns were observed in the isolates. Seven isolates (21.2%) showed 0-5 IS*6110* bands, these isolates were insufficient in band number for cluster analysis. Identical patterns were not found among the isolates (Fig. 2A).

The number of $(CGG)_5$ bands of the copy isolates ranged from 8 to 16 (Fig. 2B). Thirty-three different $(CGG)_5$ fingerprinting patterns were observed in the isolates. Identical patterns were not found among the isolates (Fig. 2B). Three pairs of isolates (HIV-tb 004 and HIV-tb 006, HIV-tb 015 and HIV-tb 017 and HIV-tb 03 and HIV-tb 023) were closely related, with 90% or more similarity. However, the corresponding patients did not show any linkage such as hospital or date of sample isolation.

Comparison of RFLP patterns between HIV-seropositive and -seronegative TB patients

To assess whether the same kinds of mycobacteria prevail in HIV-seropositive and -seronegative patients, RFLP patterns of *M. tuberculosis* clinical isolates were investigated in both groups. In IS*6110*- or $(CGG)_5$-patterns from both groups, the patterns from both HIV-seropositive and -seronegative patients did not consist of apparent clusters and appeared to segregate randomly in the dendrograms (Fig. 3).

Profile of HIV-seropositive patients with *M. avium* infection

The number of HIV-seropositive patients with *M. avium* infection was 36 (Table 1). Mean age was 37.4 ± 9.9 years, ranging from 11 to 56 years. Most of the *M. avium*-infected patients (58.3%) suffered from disseminated infection, and the sputa of 88.9% patients were culture-positive but smear-negative upon preliminary mycobacterial examination. Almost all the *M. avium* isolates were resistant to all anti-TB drugs. Peripheral blood $CD4^+$ cell counts of 34 patients (unknown: 2) at the time of *M. avium* diagnosis ranged from 0 to $202/mm^3$, and the mean $CD4^+$ cell count was $38.6 \pm 60.4/mm^3$. In 26 of 34 patients (76.4%), the $CD4^+$ cell counts were less than $50/mm^3$.

RFLP analysis of *M. avium*

The RFLP patterns of 35 of 36 *M. avium* isolates were investigated (Fig. 4). The number of IS*1245*- and IS*1311*-bands ranged from 0 to 25 and from 0 to 23, respectively, and analysis showed 33 different patterns of each. Nine isolates (25.7%) showed 0-5 bands; these isolates were insufficient for cluster analysis because of few numbers of IS*1245* or IS*1311* bands. Among the isolates, identical patterns were not found. Cluster analysis revealed no clusters. These results indicate that no particular strain of *M. avium* prevailed among HIV-seropositive patients.

Discussion

We analysed mycobacterial isolates obtained from HIV-seropositive patients and found that *M. tuberculosis* and *M. avium* accounted for a large proportion of HIV-associated mycobacterial infection in Japan. Although *Mycobacterium kansasii* is also known to be associated with AIDS,[29,30] it was not isolated in this study. Two isolates of *M. chelonae* were obtained from stool specimens of patients.

It has been suggested that recurrent TB is responsible for most cases of HIV-associated TB, particularly in countries with high-level of transmission.[31] Kanazawa et al.[32] reported that the majority of HIV-positive Japanese patients with TB (83%) were more than 40 years of age and had recurrent TB. In the present study, the age of HIV-seropositive patients shifted to the 30s, suggesting that TB incidence among HIV-positive patients in Japan is transforming from recurrence in older persons to primary infection in younger persons.

With respect to drug resistance, 10.4% of the strains obtained from HIV-seropositive patients showed resistance to one or more anti-TB drugs. Abe et al.[33] reported that 10.3% of *M. tuberculosis* isolates from patients in Japan were resistant to one or more of the four first-line anti-TB drugs: isoniazid, rifampin, streptomycin and ethambutol. A 1996 report noted that the drug resistance rate in New York City was 33%.[34]

We found that both the IS*6110* and $(CGG)_5$ fingerprinting patterns of *M. tuberculosis* isolates from HIV-seropositive patients in Japan differed from those of a TB outbreak in New York City[12,18] and of isolates from the patients in Lima, Peru.[16] Comparing RFLP patterns of *M. tuberculosis* isolates from HIV-seropositive patients with those from HIV-seronegative patients, we found that the DNA fingerprints did not distinguish between these two TB patient groups. These data indicate that TB transmission in Japan occurs via HIV-seronegative TB patients rather than via HIV-seropositive TB patients. The epidemiological studies in Botswana[17] and Tanzania[19] showed no clustering any particular

pattern of DNA fingerprints. These findings are consistent with our present results.

Patients infected with *M. avium* suffer from chronic lung disease. In patients with HIV-associated *M. avium* infection, it is thought that pulmonary symptoms will develop when $CD4^+$ lymphocyte counts fall below $100/mm^3$. The median $CD4^+$ lymphocyte count at *M. avium* diagnosis was $10/mm^3$, and at that time the majority of patients showed disseminated *M. avium* infection. Almost all *M. avium*-infected patients in the present study were in advanced stages of AIDS. *M. avium* organisms can be isolated from environmental sources such as water or soil.[35-37] Because they are capable of causing infection in animals, e.g. birds and pigs, it has been postulated that the source of human infection is either the environment or from animals. Ichiyama et al.[38] searched sources of soil, water and dust in Japan and found *M. avium* isolates in 68.0% of dust samples tested. It is believed that the most frequent mode of *M. avium* infection in humans occurs by inhalation or by deglutition of the agent from environmental sources.[37,39,40] To prevent infection with this agent in HIV-seropositive patients, further studies are needed to identify original sources and to further elucidate infectious routes.

In conclusion, the number of HIV patients in Japan is increasing; according to the latest report,[7] the number is over 10 000. The number of TB patients in Japan remains higher than in other developed countries.[41] However, the number of HIV-infected patients with mycobacterial infection in Japan is limited. With respect to TB, no outbreak among HIV-seropositive patients was found. Further monitoring of mycobacterial infection associated with HIV infection in Japan should be continued.

Acknowledgements

The paper is dedicated to Iwao Ojima, the leader of the International Projects on Anti-Tuberculosis Drug Discovery, in honor of his 60th Birthday, June 5, 2005. We thank M. Nakano (Jichi Medical School, Tochigi Prefecture, Japan) for comments on the manuscript. This study was supported by the Health Sciences Research Grants from the Ministry of Health, Labour and Welfare and by a grant from the Research on Health Sciences focusing on Drug Innovation (KH11008) from the Japan Health Sciences Foundation.

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ORIGINAL RESEARCH

Simultaneous determination of six HIV protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and non-nucleoside reverse transcriptase inhibitor (efavirenz) in human plasma by high-performance liquid chromatography

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ABSTRACT: We report the development of a simple, economical and reliable chromatographic method for the simultaneous determination of six HIV protease inhibitors (PIs; amprenavir, indinavir, lopinavir, nelfinavir, ritonavir and saquinavir), the active metabolite of nelfinavir (M8) and the non-nucleoside reverse transcriptase inhibitor (NNRTI; efavirenz) in human plasma. After extraction from plasma with an ethyl acetate–acetonitrile mixture, the analytes were separated on a phenyl column with a gradient of acetonitrile and phosphate solutions, and detected at three ultraviolet wavelengths. Calibration curves were linear over the range 0.025–15 μg/mL for saquinavir and 0.05–15 μg/mL for the other analytes. The accuracies ranged from −6.9% to +7.6%, and the intra-assay and inter-assay precisions were <9.2 and <11.8%, respectively. Our method, covering most of the PIs and NNRTIs currently used, facilitates ready therapeutic drug monitoring in hospital laboratories. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: protease inhibitors; efavirenz; HIV; therapeutic drug monitoring; high-performance liquid chromatography

INTRODUCTION

Currently, more than 17 anti-human immunodeficiency virus (HIV) drugs have been approved and are in clinical use in industrialized countries. To obtain optimal antiviral efficacy and to prevent viral drug resistance, these drugs are administered to patients in combination regimens, which are referred to as highly active antiretroviral therapy (HAART). Current standard HAART regimens consist of one or two protease inhibitors (PIs) or one non-nucleoside reverse transcriptase inhibitor (NNRTI), together with two nucleoside reverse transcriptase inhibitors (NRTIs; Richman, 2001; British HIV Association Writing Committee, 2003; The Panel on Clinical Practices for Treatment of HIV Infection, 2004; Yeni *et al.*, 2004), but more complex regimens are often needed because of treatment failure. Since the introduction of HAART in the late 1990s, the short-term prognosis of HIV infection has dramatically improved (Palella *et al.*, 1998). However, a large degree of inter-patient variability is observed in the efficacy and toxicity of HAART and still remains to be resolved.

This variability is at least in part attributed to the pharmacokinetics of the drugs, especially PIs and NNRTIs (Barry *et al.*, 1998; Acosta *et al.*, 1999; Marzolini *et al.*, 2001). Cytochrome P450 (CYP), by which PIs and NNRTIs are extensively metabolized, and P-glycoprotein, which plays an important role in transportation of these drugs at tissue and cellular levels, have genetic polymorphisms, leading to the inter-patient variability in pharmacokinetics (Fellay *et al.*, 2002). Furthermore, PIs and NNRTIs are both potent CYP inducers and inhibitors, and as a result, complicated and unpredictable pharmacokinetic interactions with co-administered drugs frequently occur (Dresser *et al.*, 2000; Gerber, 2000). To overcome

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Abbreviations used: APV, amprenavir; CYP, cytochrome P450; EFV, efavirenz; HAART, highly active antiretroviral therapy; IDV, indinavir; LPV, lopinavir; NFV, nelfinavir; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; RTV, ritonavir; SQV, saquinavir; TDM, therapeutic drug monitoring.

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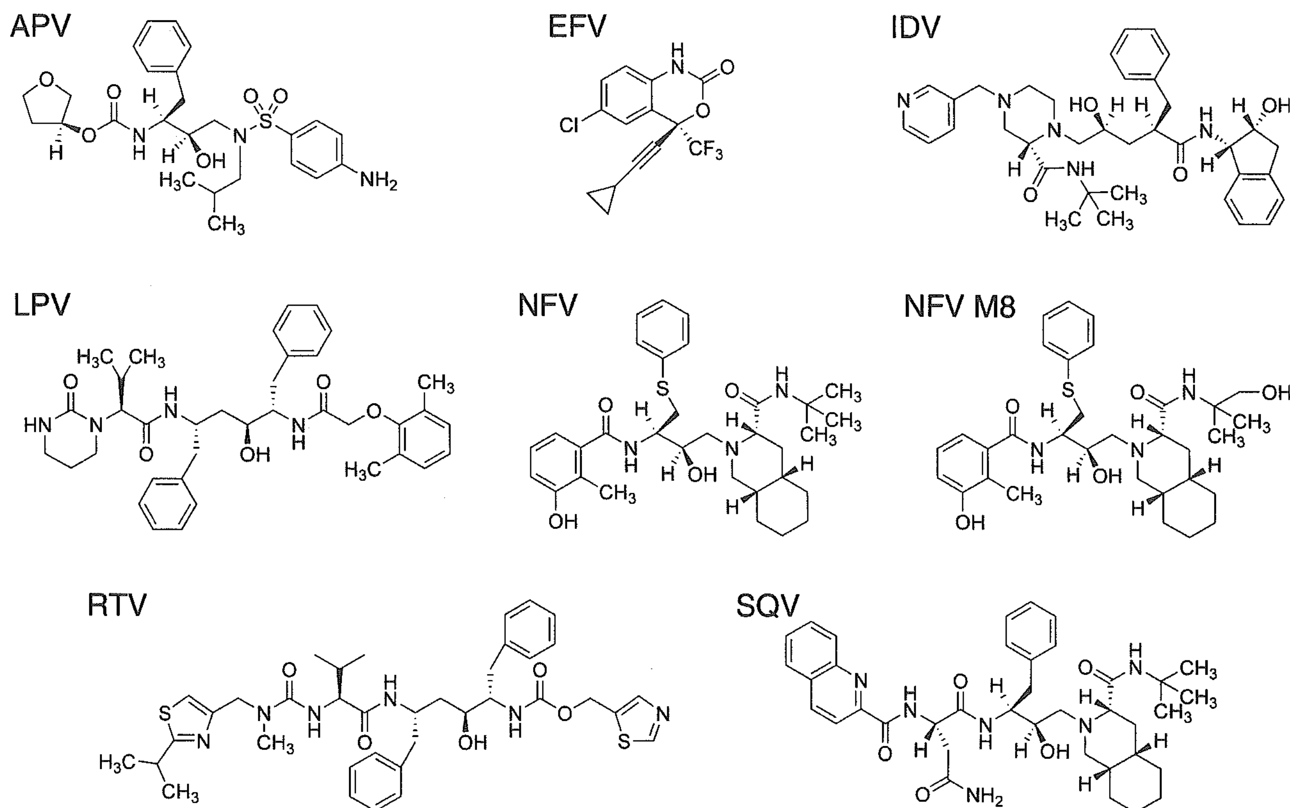

Figure 1. Chemical structures of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV.

the pharmacokinetic variability, therapeutic drug monitoring (TDM) for PIs and NNRTIs has recently been proposed as a practical and potential tool by clinicians (Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002). In addition, TDM may be useful in assessing adherence to treatment, which is another clinical problem in HAART (Hugen *et al.*, 2002).

For the purpose of routine TDM in hospital laboratories, a simple and reliable analytical method that can simultaneously determine plasma concentrations of most PIs and NNRTIs is highly desirable. In this article, we describe a novel chromatographic method for the simultaneous determination of the six widely used PIs [amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV); Fig. 1] and a clinically important active metabolite of NFV, M8, together with efavirenz (EFV), which is the most frequently used NNRTI. Moreover, the usefulness of this method for TDM is discussed.

EXPERIMENTAL

Materials. NFV mesylate and its metabolite M8 were kindly provided by Japan Tobacco Inc. (Tokyo, Japan), IDV sulfate and EFV by Merck & Co. (Rahway, NJ, USA), RTV and LPV by Abbott Laboratories (Abbott Park, IL, USA), APV by Kissei Pharmaceutical Co. (Matsumoto City, Japan) and SQV mesylate by Roche Products (Welwyn Garden City, UK). The compounds tested for possible interference with the analytical method were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), or were extracted from commercial products. Acetonitrile, methanol, distilled water (each of HPLC grade), disodium hydrogenphosphate (Na_2HPO_4), sodium dihydrogenphosphate (NaH_2PO_4) and sodium 1-hexanesulfonate were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ethyl acetate (HPLC grade), *n*-hexane (HPLC grade), ammonium hydroxide and phosphoric acid were obtained from Wako Pure Chemical Industries.

Drug-free blank plasma was collected from eight healthy volunteers in our hospital. Clinical samples were obtained from 10 HIV-infected patients receiving PIs and/or EFV as part of a HAART regimen. The Ethics Committee for the Clinical Study in our hospital approved this study (no. 39) and all participants provided written informed consent. The blood was drawn into heparinized tubes, and plasma was separated by centrifugation at 3000 *g* for 10 min and stored at −30°C.

Separate stock solutions of the eight analytes were prepared by dissolving the compound in methanol to yield a concentration of 1000 μg/mL as free base. Each solution was combined and diluted in 50% (v/v) methanol to give a working solution containing all eight analytes at 100 μg/mL. The working solution was further diluted in blank plasma for the preparation of calibration standards and quality controls.

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Sample pretreatment. An aliquot of 400 μL plasma was transferred to a 5 mL glass tube and alkalinized with 400 μL of 0.1 M ammonium hydroxide adjusted to pH 10.5 with phosphoric acid. After vortexing briefly, 1.8 mL of an ethyl acetate–acetonitrile mixture (9:1, v/v), freshly prepared, was added to each tube. The tubes were vortexed vigorously at room temperature for 5 min and centrifuged at 2000 *g* at 4°C for 10 min. Then, 1.5 mL of the organic phase was transferred to a 2 mL tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. Subsequently, the residue was reconstituted in 150 μL of solvent A (see 'Chromatography'). The resulting solution was washed with 1 mL *n*-hexane by vortexing for 10 s and centrifuging at 2000 *g* for 2 min. After eliminating the upper hexane layer, the non-hexane solution was filtrated through a 0.45 μm filter and transferred to an auto-sampler vial. Aliquots of 50 μL were injected into the HPLC unit.

Chromatography. Chromatography was performed using an integrated high-performance liquid chromatography (HPLC) unit, Integral 100Q (Applied Biosystems, Foster City, CA, USA), which consists of an automatic sample injector, a binary solvent delivery pump and a dual-wavelength ultraviolet detector. The analytical column was a Develosil Ph-UG-3 column (150 × 2.0 mm, 3 μm particle size; Nomura Chemical Co., Seto City, Japan) protected by a Develosil Ph-UG-S precolumn (10 × 1.5 mm; Nomura Chemical Co.). The temperature was maintained at 40°C using a Shodex column heater (Showa Denko Co., Tokyo, Japan). Analytical runs were processed by the Turbochrom software (version 3.01; Applied Biosystems).

The mobile phase consisted of a gradient of solvents A and B. Solvent A was a mixture of 34% (v/v) acetonitrile and 66% (v/v) 25 mM NaH_2PO_4 containing 6 mM sodium 1-hexanesulfonate and exactly adjusted to pH 5.1 with 25 mM Na_2HPO_4. Solvent B was composed of 64% (v/v) acetonitrile and 36% (v/v) 25 mM NaH_2PO_4 containing 6 mM sodium hexanesulfonate and adjusted to pH 5.3. Over the first 14.6 min of the run, 100% solvent A was delivered constantly, and then the proportion of solvent B was increased linearly from 0% to 30% over 2.0 min. From time 16.6 to 35.5 min, a mixture of 70% solvent A and 30% solvent B was used in an isocratic mode. The column was then rinsed with 100% solvent B for 4.5 min and semi-equilibrated with 100% solvent A for 6.8 min. The flow rate was maintained at 200 μL/min. Prior to use, solvents were passed through a 0.45 μm filter and degassed by helium sparging.

Absorbance was monitored at a dual wavelength: channel 1 was set at a wavelength of 212 nm through the run. For channel 2, the wavelength was set at 266 nm for the first 18.3 min, then switched to 239 nm automatically and switched back to 266 nm at the end of the run. APV, SQV and the other six analytes were detected at 266, 239 and 212 nm, respectively.

Method validation. The calibration was established over the range of 0.025–15 μg/mL for SQV and 0.05–15 μg/mL for the other analytes using seven spiked plasma samples. The calibration curve for each analyte was constructed by least-squares linear regression of the observed peak area vs the spiked concentration. Unknown concentrations of quality controls and patient samples were calculated from the linear regression equation of daily calibration curve for each analyte. The data analyses were performed using Turbochrom software (version 3.01; Applied Biosystems) on a Windows personal computer.

The accuracy and precision of the method were determined by assaying plasma samples spiked with the analytes at three different concentrations of 0.3, 2.1 and 12 μg/mL. Accuracy was defined as the percentage of deviation from the nominal concentration. Intra-assay precision was calculated as the relative standard deviation from six replicate quality controls within a single assay. Inter-assay precision was estimated from the analyses of quality controls on five separate days. The lower limits of quantitation (LLQs) were determined with plasma samples spiked with decreasing concentrations of the analytes (0.0125, 0.025, 0.05 and 0.1 μg/mL). The LLQ was defined as the lowest concentration for which the percentage deviation from the nominal concentration and the relative standard deviation were both less than 20%. The LLQ values were used for the lowest concentration of daily calibration curves. The upper limit of quantitation (ULQ) was arbitrarily determined as 15 μg/mL for each analyte.

The efficiency of the extraction procedure was determined with plasma samples spiked at three different concentrations of 0.3, 2.1 and 12 μg/mL in triplicate. The average recovery of each analyte was calculated by comparing the observed peak areas of the processed samples with those of non-processed standard solutions in mobile phase (solvent A). The stability of the analytes in plasma samples was investigated for three different conditions. Plasma was spiked with two concentrations of the analytes (0.3 and 12 μg/mL) and subsequently kept at 60°C for 60 min, 4°C for 7 days and −30°C for 60 days including three freeze–thaw cycles. These samples were analyzed together with freshly prepared samples and the ratios of the observed concentrations were calculated.

RESULTS

Representative chromatograms of plasma spiked with 1.8 μg/mL of each of the six PIs, NFV M8 and EFV are shown in Fig. 2. IDV, NFV M8, EFV, RTV, LPV and NFV were detected at a wavelength of 212 nm [Fig. 2(A)], based on their ultraviolet absorption spectra (data not shown). APV and SQV were monitored at 266 and 239 nm, respectively [Fig. 2(B)], because interfering endogenous peaks were observed near the peaks of these analytes at 212 nm. The retention times were 14.4, 16.2, 21.8, 23.7, 25.8, 30.0, 31.4 and 33.0 min for IDV, APV, NFV M8, EFV, SQV, RTV, LPV and NFV, respectively.

Blank plasma samples from eight healthy individuals showed no endogenous peaks interfering with any analyte. The typical chromatograms are presented in Fig. 3. A total of 35 drugs, which are frequently co-administered to HIV-infected patients, were also examined for possible analytical interference (Table 1). All the drugs but ketoconazole were eluted at retention times of <10 min or were not detected with the method. Ketoconazole had a retention time of 20.2 min,

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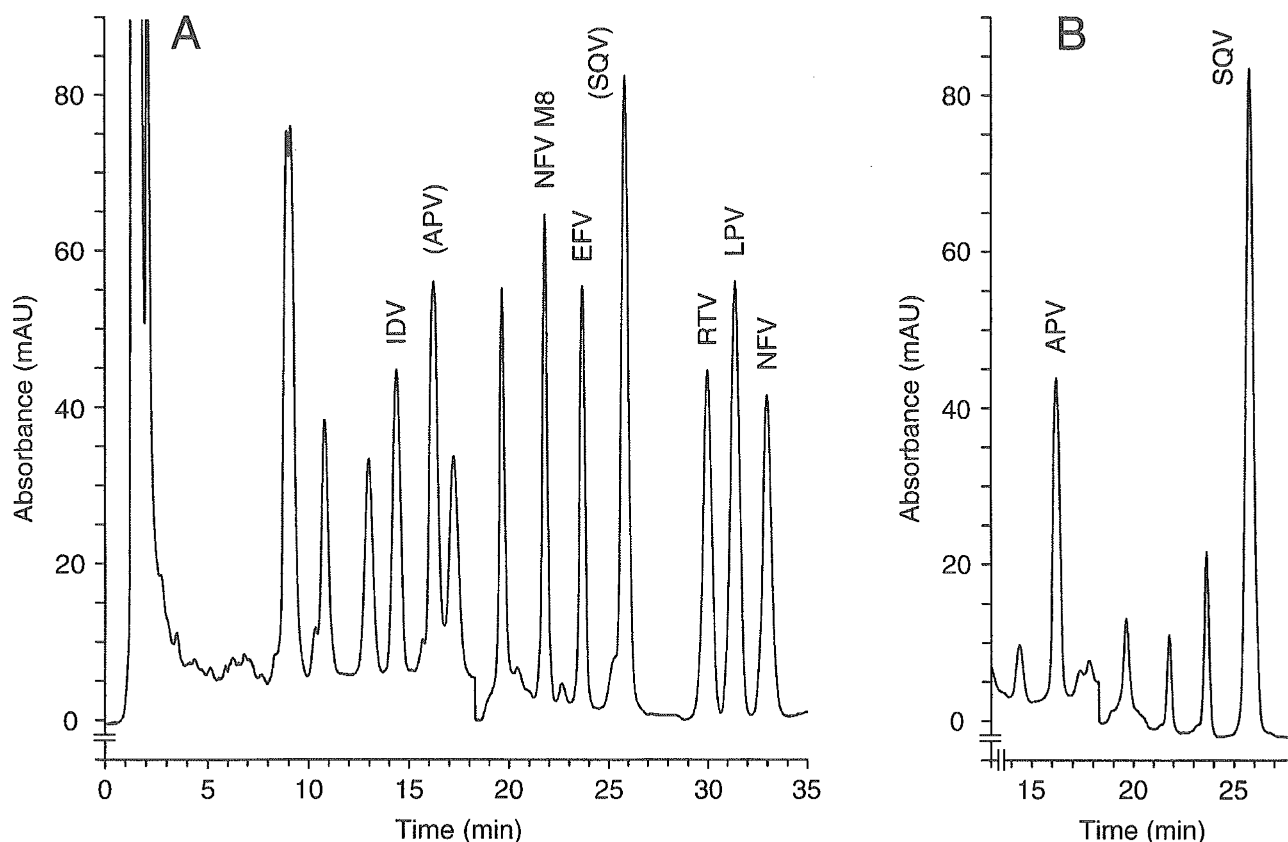

Figure 2. Chromatograms of a plasma sample spiked with 1.8 μg/mL of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

Table 1. Drugs tested for possible analytical interference

Abacavir	Erythromycin	Pentamidine
Acetaminophen	Ethambutol	Prednisolone
Aciclovir	Fluconazole	Pyrazinamide
Amikacin	Foscarnet	Rifampicin
Amoxicillin	Ganciclovir	Stavudine
Amphotericin B	Hydroxyurea	Sulfamethoxazole
Ampicillin	Isoniazid	Trimethoprim
Azithromycin	Kanamycin	Vancomycin
Cefaclor	Ketoconazole	Zalcitabine
Clarithromycin	Lamivudine	Zidovudine
Clindamycin	Metronidazole	Zidovudine glucuronide
Didanosine	Nevirapine	

which was obviously different from that of NFV M8 (21.8 min). Interference with metabolites of PIs and EFV was investigated with clinical samples, because these metabolites except NFV M8 are not available in pure form. No peaks interfering with any analyte were observed in plasma samples from patients receiving PIs and EFV (data not shown).

Over the concentration range 0.025–15 μg/mL for SQV and 0.05–15 μg/mL for the other seven analytes, the calibration curves were constructed by least-squares analysis. The correlation coefficients (r^2) of the curves were 0.995, 0.992, 0.998, 0.998, 0.997, 0.999, 0.998 and 0.999 for APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV, respectively. The results of the accuracy and precision of the method are summarized in Table 2. The accuracies for the analytes at three concentration levels ranged from −6.9 to +7.6%. The intra-assay and inter-assay precisions were <9.2 and <11.8%, respectively. The LLQs were 0.025 μg/mL for SQV and 0.05 μg/mL for the other analytes, as determined with the plasma samples spiked with decreasing concentrations of the analytes. The ULQ was arbitrarily defined as 15 μg/mL for each analyte. The accuracies and precisions at the ULQ were also <20%.

The average extraction recoveries were 84.8, 70.9, 90.6, 88.3, 73.7, 80.0, 93.1 and 95.4% for APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV, respectively. Although the reasons for the relatively lower recoveries of EFV and NFV are unclear, these had no negative effects on the assay performance as described above. The stability of the analytes in plasma samples is shown in Table 3. Under all conditions tested, the analytes proved to be stable with a recovery of >90.6% of the initial concentration.

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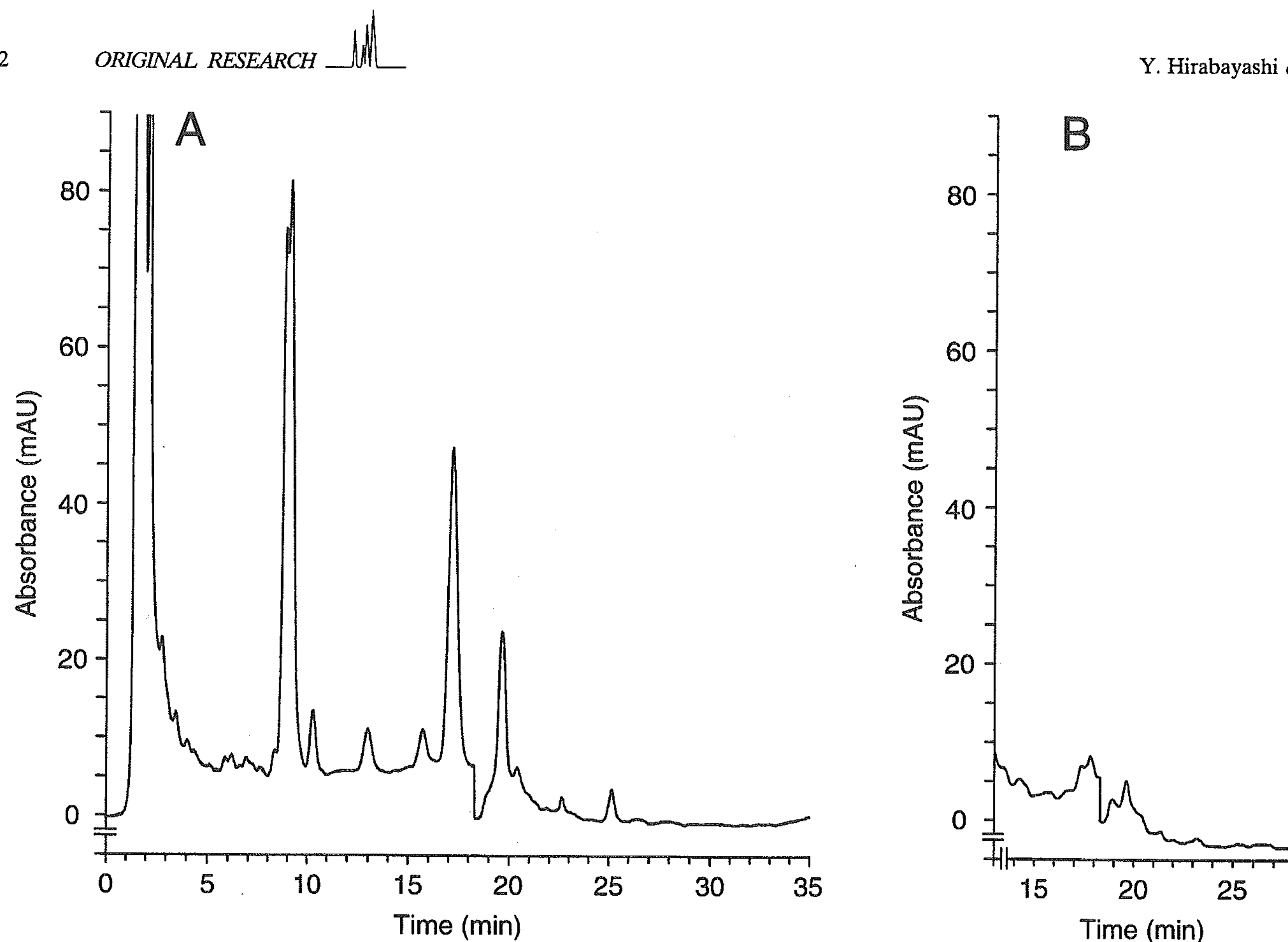

Figure 3. Chromatograms of a blank plasma sample. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

Table 2. Accuracy and precision of the analytical method

Analyte	Concentration (μg/mL)	Accuracy (%)[a] (n = 6)	Precision (%)[b] Intra-assay (n = 6)	Precision (%)[b] Inter-assay (n = 5)
Amprenavir	0.3	+1.3	8.9	7.8
	2.1	−6.9	4.0	5.1
	12.0	+2.0	3.9	6.7
Efavirenz	0.3	+7.6	7.5	11.8
	2.1	−0.3	4.1	8.4
	12.0	+3.5	2.2	5.3
Indinavir	0.3	−2.0	6.1	9.0
	2.1	+1.7	3.1	4.9
	12.0	+5.2	5.4	4.3
Lopinavir	0.3	+4.5	5.6	9.5
	2.1	+2.8	4.0	6.1
	12.0	−0.9	4.3	5.9
Nelfinavir	0.3	+3.1	5.0	5.3
	2.1	+1.9	5.4	4.7
	12.0	−1.6	2.1	3.2
Nelfinavir M8	0.3	+4.4	4.6	6.7
	2.1	−0.3	2.9	4.4
	12.0	−1.6	1.5	3.6
Ritonavir	0.3	−2.0	9.2	7.3
	2.1	+3.2	3.8	5.1
	12.0	−4.1	3.1	5.9
Saquinavir	0.3	+1.7	3.9	2.8
	2.1	−0.2	2.2	3.4
	12.0	+3.1	4.2	4.7

[a] Deviation from nominal concentration.
[b] Relative standard deviation within a single assay or between different assays.

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Table 3. Stability of protease inhibitors and efavirenz in plasma samples

Analyte	Concentration (μg/mL)	Recovery (%)[a]		
		60 min at 60°C (n = 3)	7 days at 4°C (n = 3)	60 days at −30°C with three freeze–thaw cycles (n = 4)
Amprenavir	0.3	94.7	104.8	95.6
	12.0	91.4	103.0	101.7
Efavirenz	0.3	95.8	100.7	98.8
	12.0	90.6	99.1	95.9
Indinavir	0.3	91.5	105.2	94.8
	12.0	93.2	100.8	98.6
Lopinavir	0.3	94.0	104.1	98.0
	12.0	93.3	101.3	100.5
Nelfinavir	0.3	102.4	102.5	99.8
	12.0	96.2	101.8	99.2
Nelfinavir M8	0.3	95.6	98.6	93.3
	12.0	98.1	101.8	97.6
Ritonavir	0.3	96.7	107.3	96.9
	12.0	93.2	103.0	97.2
Saquinavir	0.3	97.3	102.9	97.8
	12.0	92.8	102.6	100.4

[a] Compared with freshly prepared samples.

Table 4. Plasma concentrations of protease inhibitors and efavirenz in 10 patients

Patient no.	Drug	Dose (mg)[a]	Time after dosing (h)	Concentration (μg/mL)
1	Amprenavir	1200, b.i.d.	10.0	0.22
	Efavirenz	600, q.d.	10.0	0.53
2	Amprenavir	1200, b.i.d.	2.0	6.47
	Ritonavir	200, b.i.d.	2.0	0.38
	Efavirenz	600, q.d.	12.0	1.47
3	Indinavir	800, t.i.d.	6.5	0.19
4	Indinavir	400, b.i.d.	2.0	3.53
	Ritonavir	400, b.i.d.	2.0	2.55
5	Lopinavir	400, b.i.d.	3.0	5.94
	Ritonavir	100, b.i.d.	3.0	0.42
6	Lopinavir	400, b.i.d.	12.0	3.06
	Ritonavir	100, b.i.d.	12.0	0.09
7	Nelfinavir	1250, b.i.d.	4.5	1.01
			(nelfinavir M8 0.96)	
8	Nelfinavir	1250, b.i.d.	11.0	0.78
			(nelfinavir M8 0.23)	
9	Saquinavir	400, b.i.d.	3.5	1.49
	Ritonavir	400, b.i.d.	3.5	6.03
10	Saquinavir	400, b.i.d.	11.5	0.56
	Ritonavir	400, b.i.d.	11.5	1.30

[a] b.i.d., twice a day; q.d., once a day; t.i.d., three times a day.

The present method was applied to the determination of drug concentrations in clinical samples (Table 4 and Fig. 4). Plasma samples were randomly collected from 10 patients receiving PIs and EFV. All the samples could be analyzed with no technical difficulties. The observed concentrations were within the concentration ranges reported in literature (Khoo *et al.*, 2001; Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002).

DISCUSSION

In this report, we describe the development, validation and clinical application of a novel chromatographic method for the simultaneous determination of the six widely used PIs and the most frequently used NNRTI, EFV, in plasma. To date, several methods have been reported for the simultaneous determination of different PIs or PIs plus NNRTIs (Leibenguth *et al.*, 2001;

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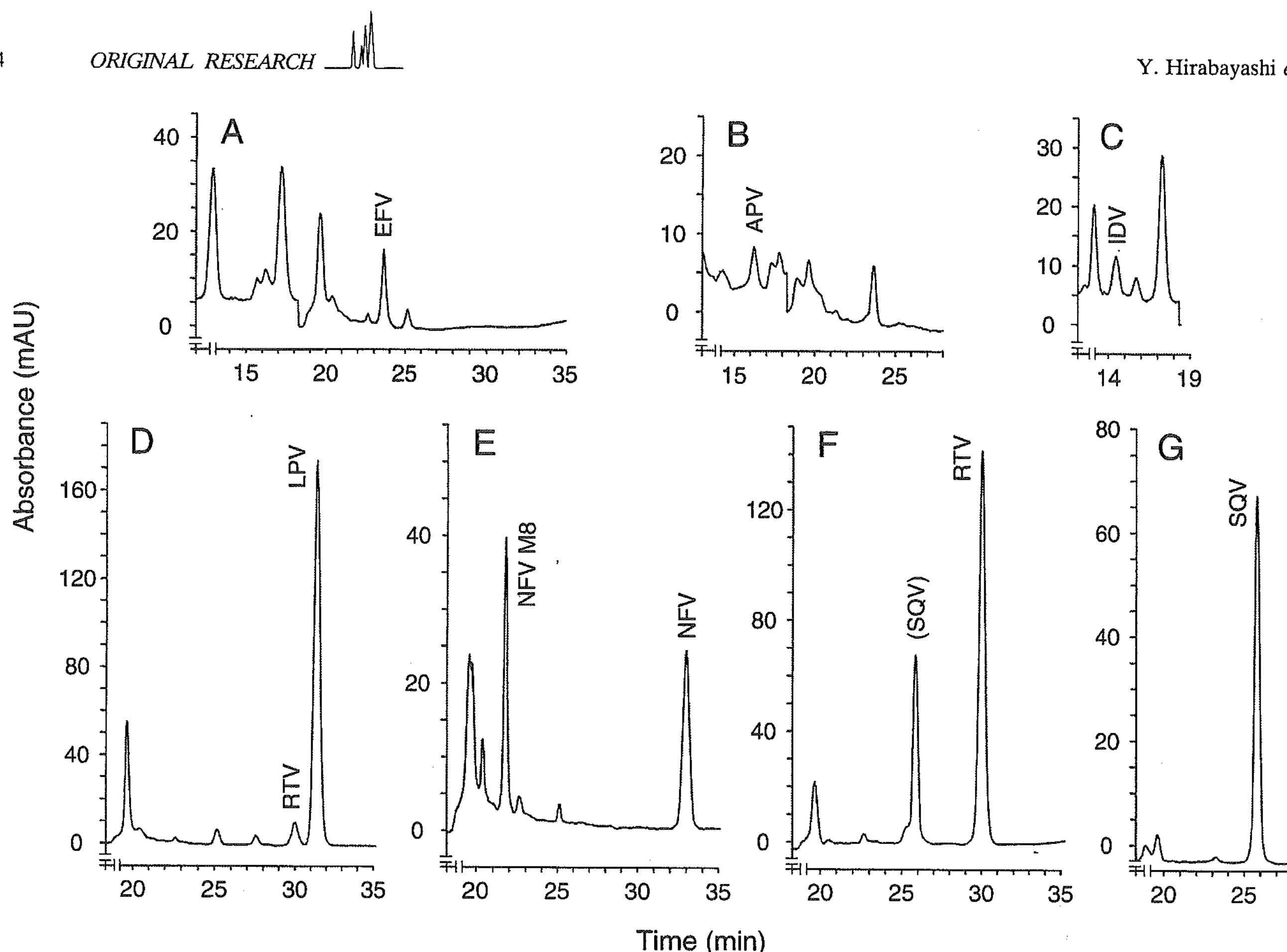

Figure 4. Chromatograms of plasma samples from patient 1 (A and B), patient 3 (C), patient 5 (D), patient 7 (E) and patient 9 (F and G). Absorbance was monitored at 212 nm (A, C, D, E and F) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B and G). For details of the patients, see Table 4.

Poirier *et al.*, 2002; Titier *et al.*, 2002; Tribut *et al.*, 2002; Volosov *et al.*, 2002; Crommentuyn *et al.*, 2003; Frerichs *et al.*, 2003; Rentsch, 2003; Turner *et al.*, 2003; Rezk *et al.*, 2004). However, most of these methods have limitations in clinical application, including insufficient quantitation sensitivity, laborious sample pretreatment with solid-phase extraction, or use of expensive mass spectrometry. A simple, economical and reliable method that is performable with standard hospital laboratory equipment is desirable for routine TDM. The present method proved sufficiently sensitive to be used for TDM, because the LLQ values of the method for individual drugs were lower than the trough concentrations observed with treated patients and the target trough concentrations estimated from clinical and *in vitro* data (Khoo *et al.*, 2001; Acosta *et al.*, 2002; Back *et al.*, 2002; Van Heeswijk, 2002). The method was also accurate and precise over a wide range of drug concentrations as described. Chromatography, which was relatively time-consuming, could be fully automated with no need for technical supervision, since samples were stable for 24 h in the auto-sampler (data not shown). We utilized an ultraviolet detector, commonly used in hospital laboratories, but not expensive and sophisticated mass spectrometry. For the sample pretreatment, we chose liquid–liquid extraction, which is economical compared with solid-phase extraction. To simplify the experimental procedure, an internal standard was not used; nevertheless the method provided satisfactory validation results. Thus, this method would be suitable for routine TDM in conventional hospital laboratory settings.

Moreover, from a clinical point of view, this method is noteworthy for two reasons. First, this method covers most of key drugs currently used other than NRTIs. Since the standard treatment of HIV infection, HAART, consists of various combinations of anti-HIV drugs (Richman, 2001; British HIV Association Writing Committee, 2003; The Panel on Clinical Practices for Treatment of HIV Infection, 2004; Yeni *et al.*, 2004), it would be convenient to use TDM to measure plasma concentrations of several drugs simultaneously with a single method rather than to use each different method for each drug. Although NRTI concentrations cannot be determined with this method, NRTIs are pro-drugs that are converted to their active triphosphate forms within cells, and therefore TDM with plasma is considered less beneficial compared with PIs and NNRTIs,

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which directly exert their antiviral effects (Back *et al.*, 2002). Secondly, this method allows the determination of the M8 metabolite of NFV as well as the parent NFV. NFV M8, which is produced by CYP 2C19 in the liver, is equipotent to the unchanged parent NFV against HIV *in vitro* (Zhang *et al.*, 2001), although almost all the metabolites of PIs and EFV have no obvious antiviral activity. The pharmacokinetics of NFV M8 would be also markedly affected by genetic background, drug interactions and hepatic dysfunction, leading to a wide inter-patient variability (Khaliq *et al.*, 2000). These findings suggest that measurement of the parent NFV plus its M8 metabolite in plasma would be preferable to that of the parent alone for TDM in NFV treatment.

TDM with this method is expected to contribute to the optimization of HIV treatment for individual patients through modification of dosage and assessment of adherence to treatment. This method would be also useful for studying the relationships between drug concentrations and efficacy or toxicity and for analyzing pharmacokinetics and drug interactions in heavily co-medicated patients. Such concentration-oriented approaches and studies are in progress in our hospital.

CONCLUSION

A simple, economical and reliable chromatographic method has been developed for the simultaneous determination of the six PIs, NFV M8 and EFV in plasma. This method would be useful for routine TDM and pharmacokinetic studies in patients receiving PIs and EFV.

Acknowledgments

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PHOTO QUIZ

Philip A. Mackowiak, Section Editor

A Woman Who Excreted a Tape-Like Substance

(See pages 572–4 for the Answer to Photo Quiz)

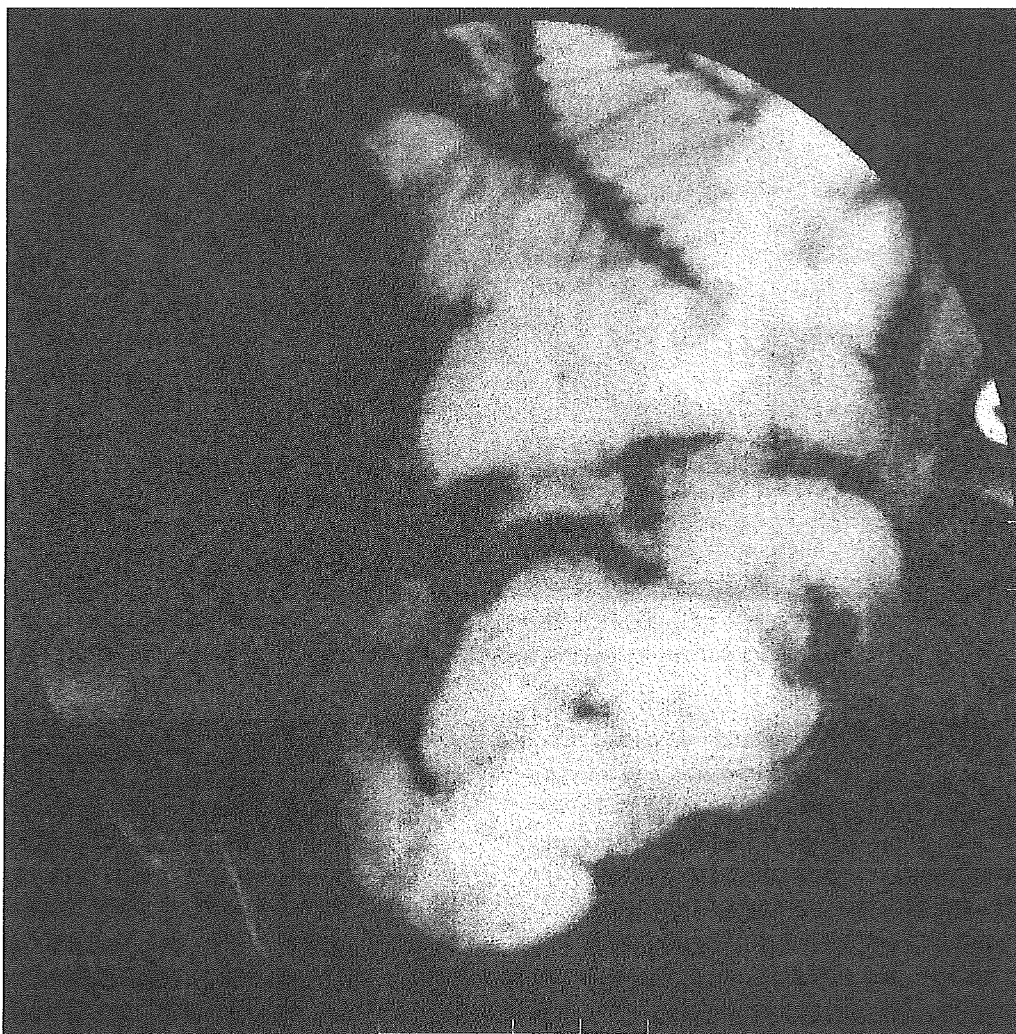

Figure 1. Abdominal radiograph after intraduodenal injection of amidotrizoic acid (Gastrografin; Nihon Shering)

A 26-year-old Japanese woman visited our clinic. One week before the visit, she noticed a tape-like substance ~30-cm long hanging from her anus at defecation, which she dragged out and discarded. She did not have any remarkable past history of illness and looked healthy.

On the next day, the patient fasted in the morning. Ami-

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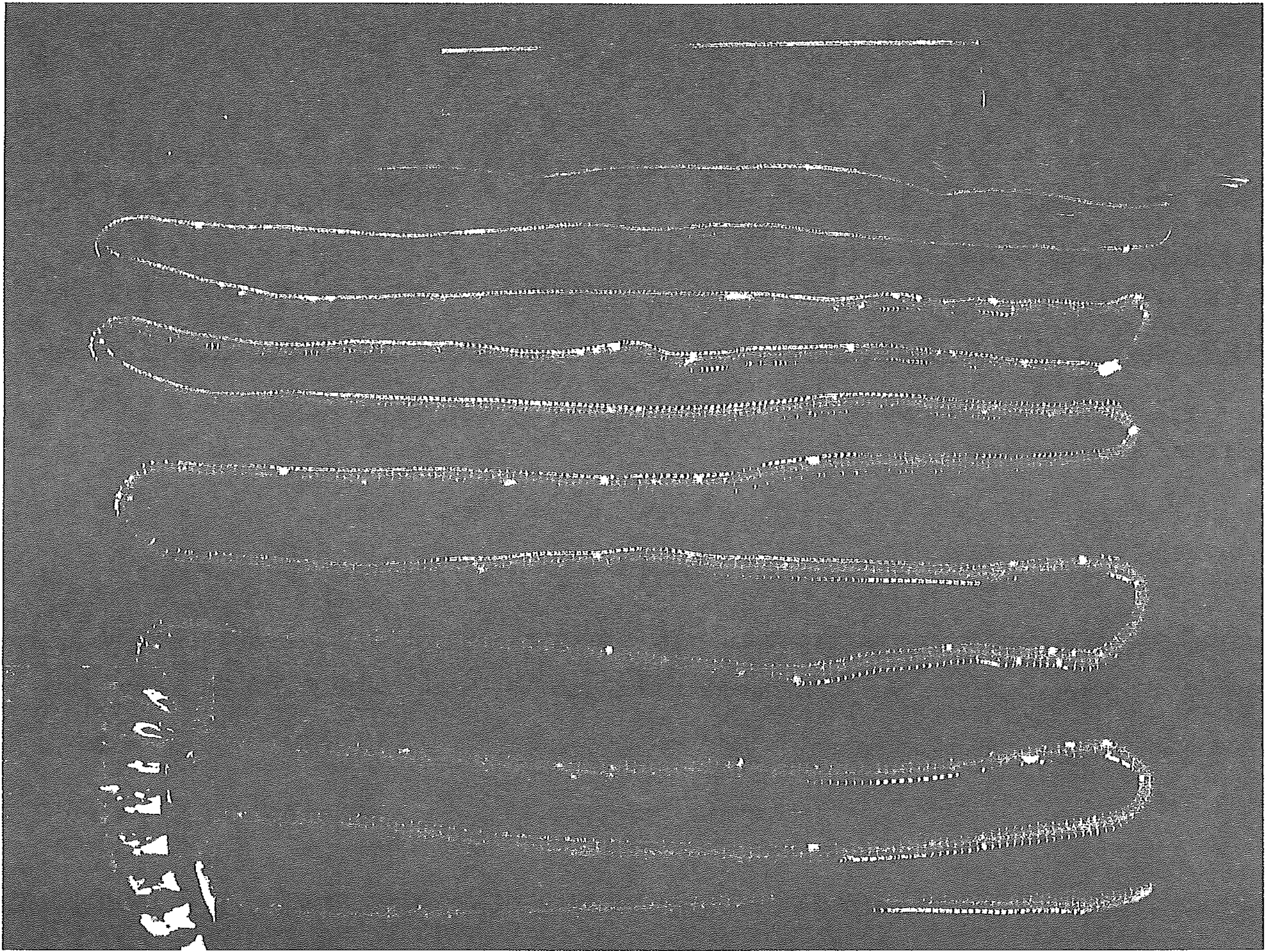

Figure 2. A tape-like substance excreted after Gastrografin injection. *Inset,* enlarged photo of tape-like substance; *top,* 30-cm bar.

dotrizoic acid (Gastrografin; Nihon Shering) was injected into the duodenal tube under fluoroscopic guidance, and a longitudinal filling defect was observed in the ileum (figure 1). Approximately 20 min later, she defecated, and a 7- or 8-m tape-like substance was excreted (figure 2).

What is your diagnosis? What is the excreted organism, and does the patient now need to be treated?

A Woman Who Excreted a Tape-Like Substance

(See pages 516–7 for the Photo Quiz)

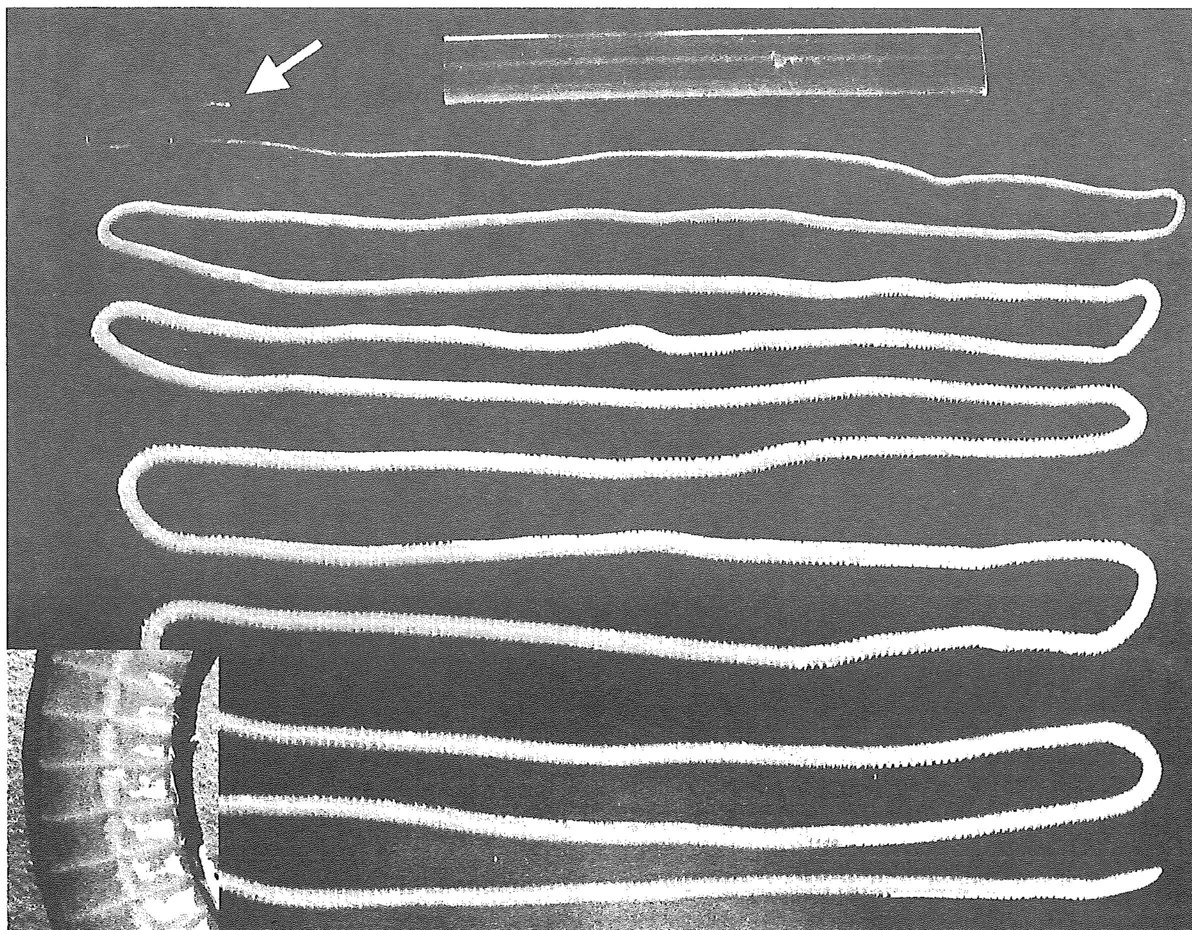

Figure 1. Unfragmented *Diphyllobothrium latum* or *Diphyllobothrium nihonkaiense* excreted after injection of amidotrizoic acid (Gastrografin; Nihon Shering). A dram-stick–shaped scolex was identified *(arrow),* and a characteristic uterus was observed at the center of each proglottid *(inset).*

Diagnosis: Diphyllobothriasis (fish tapeworm infection due to *Diphyllobothrium latum* or *Diphyllobothrium nihonkaiense*).

The excreted organism was composed of a tape-like chain of connected segments (proglottids) with a central uterus (figure 1), which are characteristic of *Diphyllobothrium* species (fish tapeworms). Proglottids of *Diphyllobothrium* organisms are usually wider than they are long. On the other hand, mature proglottids of *Taenia saginata* and *Taenia solium* are longer than they are wide. *D. latum* is the most common *Diphyllobothrium* species in northern Europe (the region including Finland, East Prussia, and Russian Karelia) and in North America [1]. *D. nihonkaiense,* which is common only in Japan, was previously erroneously identified as *D. latum* but thereafter was identified as an independent species [2], although morphological discrimination between these species requires expert assistance. Other *Diphyllobothrium* species are smaller and rarely >1 m long [1]. Therefore, the infecting species in our patient was probably *D. latum* or *D. nihonkaiense.*

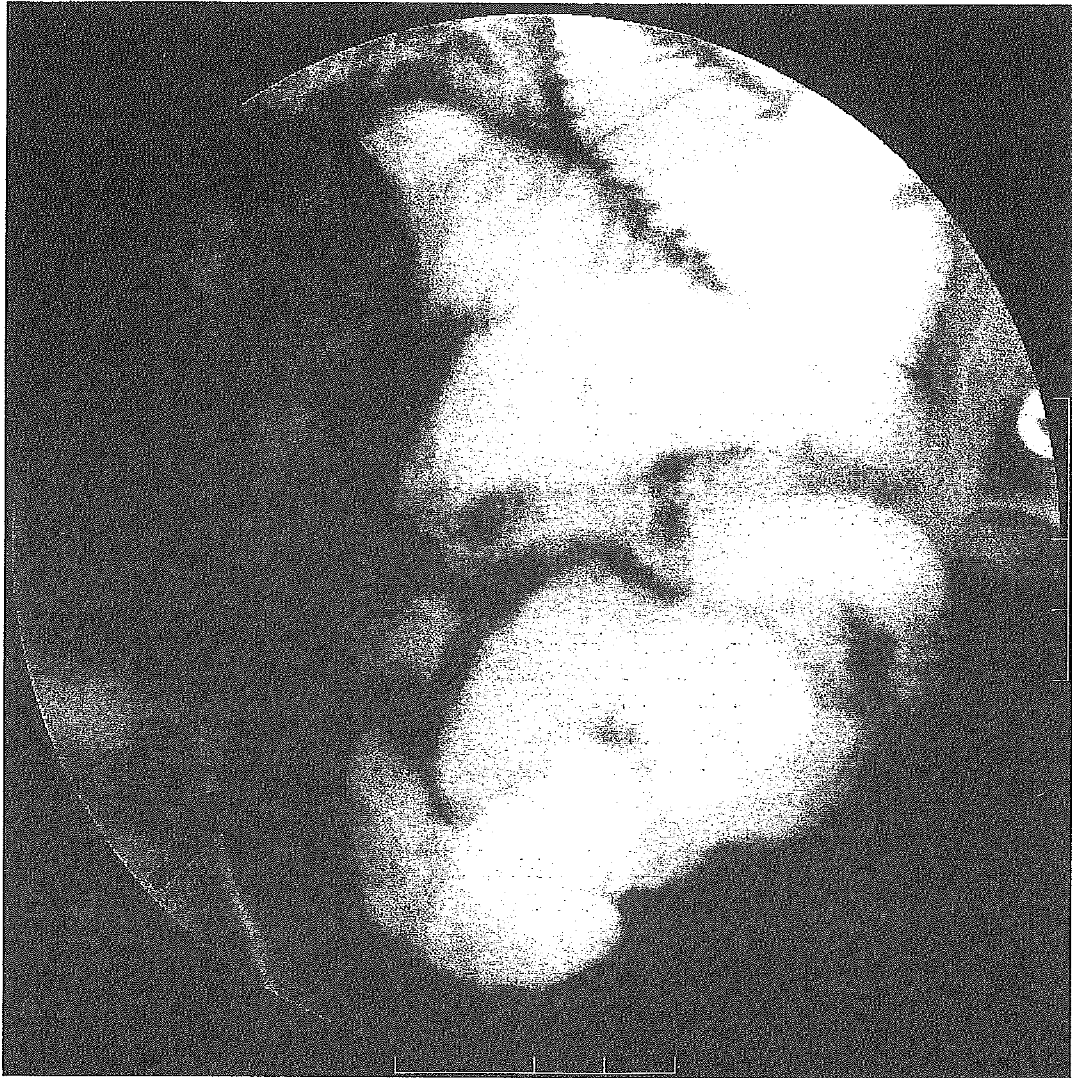

Figure 2. Abdominal radiograph after intraduodenal Gastrografin injection. A longitudinal filling defect was observed in the ileum *(arrows)*.

Humans become infected by ingesting *Diphyllobothrium* plerocercoid larvae in fish (or fish roe) or liver that is raw or incompletely cooked. A variety of freshwater fish, as well as some marine fish, are sources of infection. Among them, salmon and trout are the most common sources [1, 3]. Interview of our patient revealed that she ingested trout sushi 1 month before presentation to the hospital. In Japan and the Scandinavian countries, the strong cultural preferences for eating dishes containing raw fish have been associated with high rates of transmission. In general, the incidence of fish tapeworm infection among humans seems to be decreasing, but in the United States, the increasing popularity of raw fish dishes, such as sushi and sashimi, is placing more consumers at risk for infection.

To treat cestodiasis, many types of antiparasitic agents, including niclosamide and praziquantel, are administered orally, and most of them have the antiparasitic effect of destroying the worm body. However, destroying the worm body makes it difficult to identify an ejected cestode head (or scolex), which is important for confirmation of cure, because the residual scolex in a patient's intestine causes recurrence of cestodiasis. Furthermore, in the case of *T. solium*, the broken proglottids release ova, which may cause dangerous cysticercosis [4]. Since Oi et al. [5] reported the successful expulsion of unfragmented cestodes by intraduodenal injection of amidotrizoic acid (Gastrografin; Nihon Shering), many patients in Japan with cestodiasis have been treated with this agent [6]. Gastrografin injection is now the treatment of choice for cestodiasis in Japan, although the mechanism of the expulsive effect of this agent is not clear.

In this case, radiography after intraduodenal Gastrografin injection revealed the presence of a tapeworm as a longitudinal

filling defect in the ileum (figure 2), and thereafter, an unfragmented tapeworm with a dram-stick shaped scolex (figure 1) was successfully excreted. The patient did not need further treatment.

Acknowledgments

Potential conflicts of interest. All authors: no conflicts.

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分担研究者　滝口　雅文

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tomiyama H, Fujiwara M, Oka S, and **Takiguchi M**.	Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication .	***J Immunol (Cutting Edge)***	174	36-40	2005
Satoh M, Takamiya Y, Oka S, Tokunaga K, and **Takiguchi M**.	Identification and characterization of HIV-1-specific CD8+ T cell epitopes presented by HLA-A*2601.	***Vaccine***	23	3783-3790	2005
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CUTTING EDGE

Cutting Edge: Epitope-Dependent Effect of Nef-Mediated HLA Class I Down-Regulation on Ability of HIV-1-Specific CTLs to Suppress HIV-1 Replication[1]

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***It is believed that Nef-mediated HLA class I down-regulation is one of the mechanisms that allow HIV-1-infected cells to escape from being killed by HIV-1-specific human CTLs. In this study, we show that the effect of Nef-mediated HLA class I down-regulation on the ability of HIV-1-specific CTLs to suppress HIV-1 replication is epitope dependent. The CTLs specific for two Pol epitopes presented by HLA-B*5101, one of the HLA alleles associated with slow progression to AIDS, effectively killed HIV-1-infected CD4⁺ T cells and suppressed HIV-1 replication. In contrast, those specific for the other four epitopes failed to kill HIV-1-infected CD4⁺ T cells and partially or hardly suppressed HIV-1 replication. The difference of the ability between these two types of CTLs may result from the difference of the number of HLA class I epitope complex on the surface of NL-432-infected CD4⁺ T cells. The Journal of Immunology,* 2005, 174: 36–40.**

Human immunodeficiency virus-1 escape from HIV-1-specific $CD8^+$ T cells occurs during acute and chronic phases of HIV-1 infections, although the mechanisms of the HIV-1 escape still remain unclear. Previously, various investigators proposed several hypotheses concerning mechanisms of HIV-1 escape from the host immune system such as mutations of immunodominant epitopes (1), reduction in the number of HIV-1-specific CTLs by apoptosis of $CD8^+$ T cells via Fas and TNF (2), skewed maturation of HIV-1-specific $CD8^+$ T cells (3), and impaired cytolytic activity of HIV-1-specific CTL toward HIV-1-infected $CD4^+$ T cells by Nef-mediated down-regulation of HLA class I molecules (4).

Nef down-regulates the surface expression of both HLA-A and -B molecules in HIV-1-infected cells because of internalization of these molecules from the cell surface by endocytosis in the presence of Nef (5). A previous study showed that the expression of HLA-A2 molecules on Nef-positive (Nef^+) HIV-1-infected primary $CD4^+$ T cells was 200- to 300-fold lower than that on Nef-defective (Nef^-) HIV-1-infected ones (6). These observations suggested that the Nef-mediated HLA class I down-regulation may decrease the recognition of HIV-infected cells by HIV-1-specific CTLs. In fact, it was shown that HLA-A*0201-restricted HIV-1-specific CTLs failed to kill Nef^+ HIV-1-infected $CD4^+$ T cells but effectively killed Nef^- HIV-1-infected ones (4). This was further confirmed by a study using two HLA-B*3501-restricted, HIV-1-specific CTL clones (7). The ability of HIV-1-specific CTLs to suppress HIV-1 replication was also impaired by Nef-mediated HLA class I down-regulation (7, 8). These studies strongly suggest that Nef-mediated HLA class I down-regulation is one of the major mechanisms by which HIV-1 escapes from HIV-1-specific CTLs. However, because only a very restricted number of HIV-1-specific CTLs has been tested for their abilities to kill Nef^+ and Nef^- HIV-1-infected $CD4^+$ T cells and to suppress the HIV-1 replication, it still remains uncertain whether Nef-mediated HLA class I down-regulation affects the killing ability of all HIV-1-specific CTLs.

HLA-B57, -B51, and -B27 alleles are associated with slow progression to AIDS (9). It has been speculated that long-term nonprogressors (LTNPs)[3] and slow progressors carry CTLs specific for conserved and dominant epitopes whose recognition is not affected by Nef-mediated HLA class I down-regulation. However, no study has yet investigated this hypothesis. To clarify the effect of Nef-mediated HLA class I down-regulation on CTLs specific for HIV-1 epitopes presented by HLA alleles that are associated with or not associated with slow progression to AIDS, we investigated the ability of both HLA-B*5101-restricted and HLA-A*3303-restricted HIV-1-specific CTLs to recognize HIV-1-infected $CD4^+$ T cells. In this study, we show that HIV-1-specific $CD8^+$ T cells have various ranges of ability to kill HIV-1-infected $CD4^+$ T cells and to suppress the replication of HIV-1.

Materials and Methods

HIV-1-specific CTL clones and lines

HIV-1-specific CTL clones and CTL lines were generated previously (10–12). All CTLs were cultured in R10 medium supplemented with 200 U/ml recombinant human IL-2 and stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

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[3] Abbreviation used in this paper: LTNP, long-term nonprogressor.

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