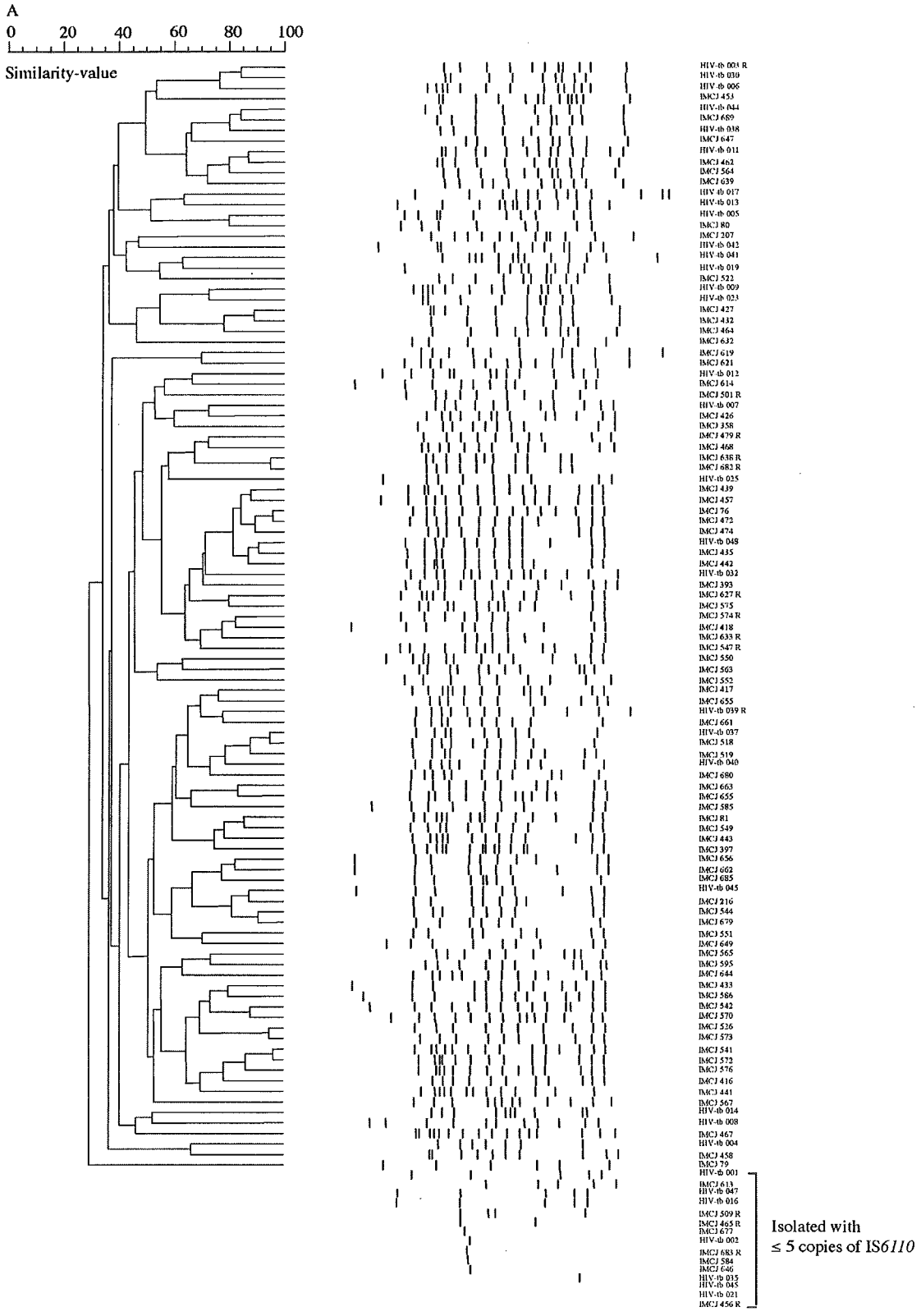

Figure 3. IS*6110*- and $(CGG)_5$-probed DNA fingerprinting patterns of *M. tuberculosis* clinical isolates from HIV-seropositive and HIV-seronegative patients and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of each IS*6110* (A) or $(CGG)_5$ (B) band is normalized so that the patterns for all strains are comparable. In the IS*6110*-probed DNA fingerprint patterns, isolates with five or fewer copies are indicated in Panel A. The isolates are named as follows: a prefix of 'HIV-tb' indicates an HIV-seropositive patient-derived isolate, a prefix of 'IMCJ' indicates an HIV-seronegative patient-derived isolate, and a suffix of 'R' indicates a drug-resistant isolate. For example, IMCJ 627 R is an HIV-seronegative patient-derived isolate.

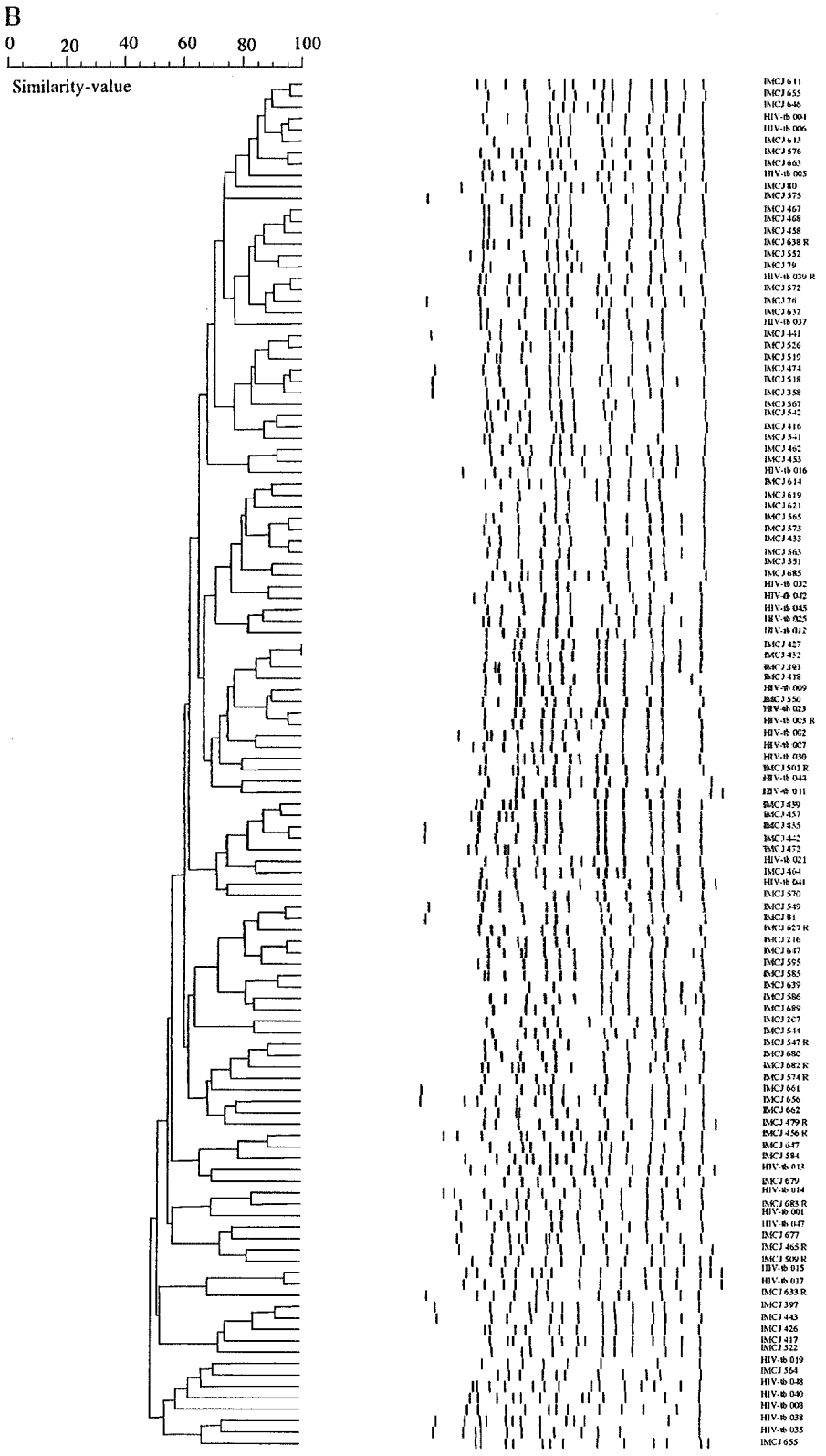

Figure 3 (*continued*)

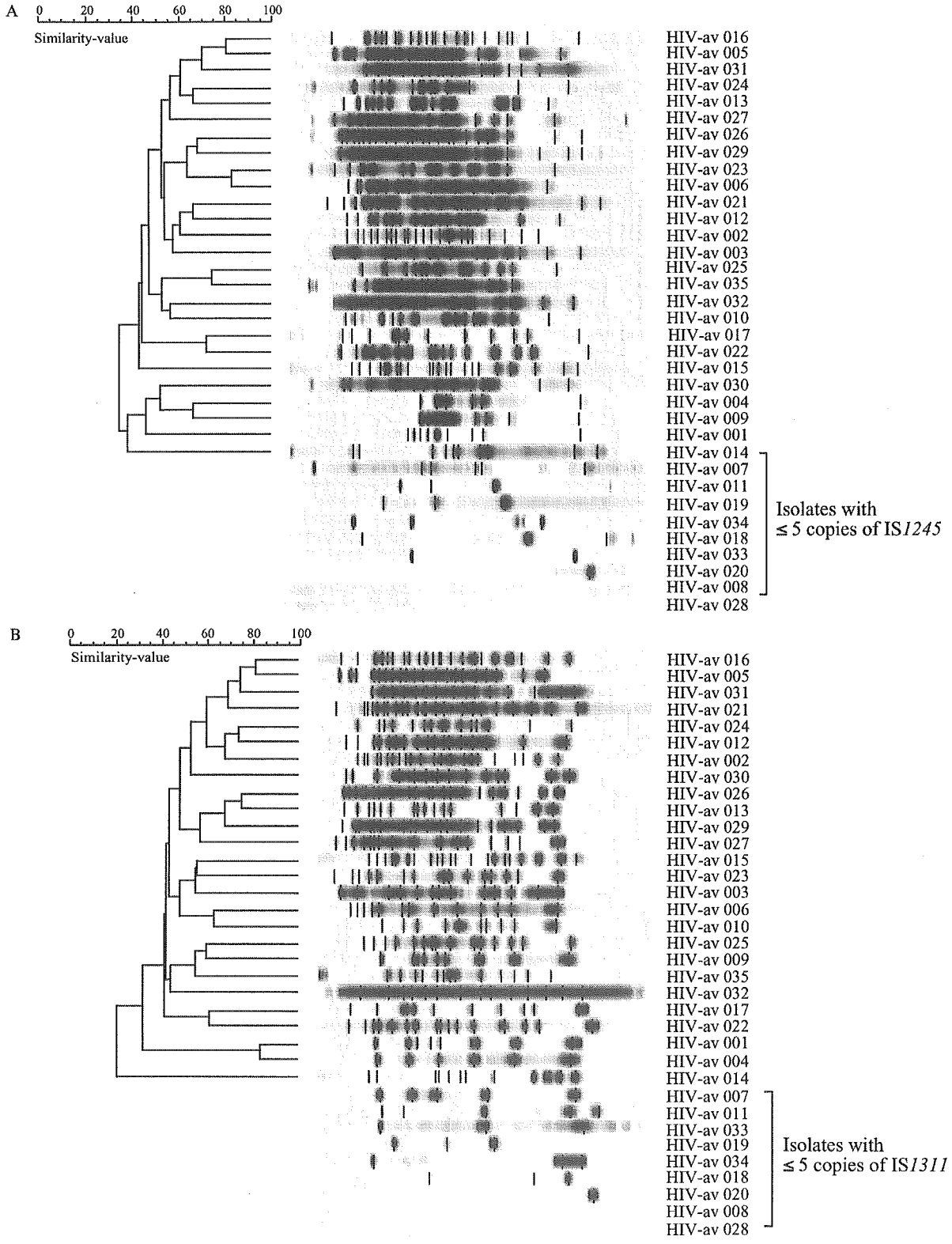

Figure 4 IS*1245*- and IS*1311*-probed DNA fingerprinting patterns of *M. avium* clinical isolates from HIV-seropositive patients in Japan and corresponding dendrograms. The fingerprint patterns are ordered by similarity. The corresponding dendrograms are to the left of the patterns. The position of 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 ± 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.

References

1. Rosenberg PS. Scope of the AIDS epidemic in the United States. *Science* 1995;**270**:1372-5.
2. Centers for Disease Control and Prevention. HIV/AIDS surveillance report 1997; **9**: 1-43.
3. Centers for Disease Control and Prevention. Update: trends in AIDS incidence, deaths, and prevalence–United States, 1996. *MMWR Morb Mortal Wkly Rep* 1997;**46**:165-73.
4. Centers for Disease Control and Prevention. Diagnosis and reporting of HIV and AIDS in states with integrated HIV and AIDS surveillance–United States, January 1994-June 1997. *MMWR Morb Mortal Wkly Rep* 1998;**47**:309-14.
5. European centre for the epidemiological monitoring of AIDS. HIV/AIDS surveillance in Europe. Quarterly report 1998; **57**.
6. Monitoring the AIDS pandemic (MAP) network. The status and trends of the HIV/AIDS epidemic in the world. Provisional report. Geneva, Switzerland; 1998.
7. Nemoto T. HIV/AIDS surveillance and prevention studies in Japan: summary and recommendations. *AIDS Educ Prev* 2004;**16**:27-42.
8. Pape JW, Liautaud B, Thomas F, Mathurin JR, St Amand MM, Boncy M, et al. Characteristics of the acquired immunodeficiency syndrome (AIDS) in Haiti. *N Engl J Med* 1983;**309**: 945-50.
9. Van de Perre P, Rouvroy D, Lepage P, Bogaerts J, Kestelyn P, Kayihigi J, et al. Acquired immunodeficiency syndrome in Rwanda. *Lancet* 1984;**2**:62-5.
10. Piot P, Quinn TC, Taelman H, Feinsod FM, Minlangu KB, Wobin O, et al. Acquired immunodeficiency syndrome in a heterosexual population in Zaire. *Lancet* 1984;**2**:65-9.
11. Dooley SW, Villarino ME, Lawrence M, Salinas L, Amil S, Rullan JV, et al. Nosocomial transmission of tuberculosis in a hospital unit for HIV-infected patients. *JAMA* 1992;**267**: 2632-4.
12. Fischl MA, Uttamchandani RB, Daikos GL, Poblete RB, Moreno JN, Reyes RR, et al. An outbreak of tuberculosis caused by multiple-drug-resistant tubercle bacilli among patients with HIV infection. *Ann Intern Med* 1992;**117**: 177-83.
13. De Cock KM, Soro B, Coulibaly IM, Lucas SB. Tuberculosis and HIV infection in sub-Saharan Africa. *JAMA* 1992;**268**:1581-7.
14. Onorato IM, McCray E. Prevalence of human immunodeficiency virus infection among patients attending tuberculosis clinics in the United States. *J Infect Dis* 1992;**165**:87-92.
15. Sudre P, ten Dam G, Kochi A. Tuberculosis: a global overview of the situation today. *Bull WHO* 1992;**70**:149-59.
16. Ahmed N, Caviedes L, Alam M, Rao KR, Sangal V, Sheen P, et al. Distinctiveness of *Mycobacterium tuberculosis* genotypes from human immunodeficiency virus type 1-seropositive and -seronegative patients in Lima Peru. *J Clin Microbiol* 2003;**41**:1712-6.
17. Lockman S, Sheppard JD, Braden CR, Mwasekaga MJ, Woodley CL, Kenyon TA, et al. Molecular and conventional epidemiology of *Mycobacterium tuberculosis* in Botswana: a population-based prospective study of 301 pulmonary tuberculosis patients. *J Clin Microbiol* 2001;**39**:1042-7.
18. Edlin BR, Tokars JI, Grieco MH, Crawford JT, Williams J, Sordillo EM, et al. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. *N Engl J Med* 1992;**326**: 1514-21.
19. Yang ZH, Mtoni I, Chonde M, Mwasekaga M, Fuursted K, Askgard DS, et al. DNA fingerprinting and phenotyping of

Mycobacterium tuberculosis isolates from human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients in Tanzania. *J Clin Microbiol* 1995;**33**:1064-9.
20. Dautzenberg B. Rifabutin in the treatment of *Mycobacterium avium* complex infection: experience in Europe. *Clin Infect Dis* 1996;**22**:S33-S6.
21. Falkinham III JO. Epidemiology of infection by nontuberculous mycobacteria. *Clin Microbiol Rev* 1996;**9**:177-215.
22. Inoue Y, Yamazaki Y, Seki Y, Wakabayashi C, Kihara M. Sexual activities and social relationships of people with HIV in Japan. *AIDS Care* 2004;**16**:349-62.
23. NCCLS. *Susceptibility testing of mycobacteria, nocardia and other aerobic actinomycetes; tentative standard, NCCLS document M24-T2*. 2nd ed. Wayne, PE: NCCLS; 2002.
24. Niemann S, Rusch-Gerdes S, Richter E. IS*6110* fingerprinting of drug-resistant *Mycobacterium tuberculosis* strains isolated in Germany during 1995. *J Clin Microbiol* 1997;**35**: 3015-20.
25. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993;**31**:406-9.
26. Otsuka Y, Parniewski P, Zwolska Z, Kai M, Fujino T, Toyata F, et al. Characterization of a trinucleotide repeat sequence $(CGG)_5$ and potential use in restriction fragment length polymorphism typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2004;**42**:3538-48.
27. Guerrero C, Bernasconi C, Burki D, Bodmer T, Telenti A. A novel insertion element from *Mycobacterium avium*, IS*1245*, is a specific target for analysis of strain relatedness. *J Clin Microbiol* 1995;**33**:304-7.
28. Roiz MP, Palenque E, Guerrero C, Garcia MJ. Use of restriction fragment length polymorphism as a genetic marker for typing *Mycobacterium avium* strains. *J Clin Microbiol* 1995;**33**:1389-91.
29. Fujita J, Nanki N, Negayama K, Tsutsui S, Taminato T, Ishida T, et al. Nosocomial contamination by *Mycobacterium gordonae* in hospital water supply and super-oxidized water. Pulmonary diseases due to *Mycobacterium szulgai* in Japan. Large-restriction-fragment analysis of *Mycobacterium kansasii* genomic DNA and its application in molecular typing. *J Hosp Infect* 2002;**51**:65-8.
30. Tsukamura M, Shimoide H, Kita N, Kawakami K, Ito T, Nakajima N, et al. Epidemiologic studies of lung disease due to mycobacteria other than *Mycobacterium tuberculosis* in Japan. *Rev Infect Dis* 1981;**3**:997-1007.
31. Selwyn PA, Hartel D, Lewis VA, Schoenbaum EE, Vermund SH, Klein RS, et al. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *N Engl J Med* 1989;**320**:545-50.
32. Kanazawa M, Fujita A, Toyoda T, Takasugi T, Osumi M, Nishio K, et al. Clinical presentation of pulmonary tuberculosis associated with acquired immunodeficiency syndrome in metropolitan Tokyo. *Intern Med* 1996;**35**: 946-52.
33. Abe C, Hirano K, Wada M, Aoyagi T. Resistance of *Mycobacterium tuberculosis* to four first-line anti-tuberculosis drugs in Japan, 1997. *Int J Tuberc Lung Dis* 2001;**5**: 46-52.
34. Frieden TR, Sterling T, Pablos-Mendez A, Kilburn JO, Cauthen GM, Dooley SW. The emergence of drug-resistant tuberculosis in New York City. *N Engl J Med* 1993;**328**: 521-6.
35. von Reyn CF, Waddell RD, Eaton T, Arbeit RD, Maslow JN, Barber TW, et al. Isolation of *Mycobacterium avium* complex from water in the United States, Finland, Zaire, and Kenya. *J Clin Microbiol* 1993;**31**:3227-30.
36. von Reyn CF, Maslow JN, Barber TW, Falkinham III JO, Arbeit RD. Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet* 1994;**343**:1137-41.
37. Yajko DM, Chin DP, Gonzalez PC, Nassos PS, Hopewell PC, Reingold AL, et al. *Mycobacterium avium* complex in water, food, and soil samples collected from the environment of HIV-infected individuals. *J Acquir Immune Defic Syndr Hum Retrovirol* 1995;**9**:176-82.
38. Ichiyama S, Shimokata K, Tsukamura M. The isolation of *Mycobacterium avium* complex from soil, water, and dusts. *Microbiol Immunol* 1988;**32**:733-9.
39. Chin DP, Hopewell PC, Yajko DM, Vittinghoff E, Horsburgh Jr CR, Hadley WK, et al. *Mycobacterium avium* complex in the respiratory or gastrointestinal tract and the risk of *M. avium* complex bacteremia in patients with human immunodeficiency virus infection. *J Infect Dis* 1994;**169**: 289-95.
40. Damsker B, Bottone EJ. *Mycobacterium avium-Mycobacterium intracellulare* from the intestinal tracts of patients with the acquired immunodeficiency syndrome: concepts regarding acquisition and pathogenesis. *J Infect Dis* 1985;**151**: 179-81.
41. Japan Anti-Tuberculosis Association. *Tuberculosis year book*. Available at: http://www.jata.or.jp/eindex.htm/.

J Infect Chemother (2005) 11:231–233
DOI 10.1007/s10156-005-0396-9

ORIGINAL ARTICLE

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Unselective use of intranasal mupirocin ointment for controlling propagation of methicillin-resistant *Staphylococcus aureus* in a thoracic surgery ward

Received: January 26, 2005 / Accepted: June 17, 2005

Abstract We executed a blanket-use program of intranasal application of mupirocin ointment to control the propagation of methicillin-resistant *Staphylococcus aureus* (MRSA) that occurred in a thoracic surgery ward of a university hospital. During an intervention of 14 weeks, all patients admitted to the ward for scheduled surgery received the ointment to their nares three times daily for 3 days before surgery, once on return to the ward, and three times weekly for the following 2 weeks. None of 84 patients was newly colonized with MRSA, and the daily rates of patients with MRSA in a recovery room in the ward significantly decreased in the period. We consider that the unselective application of mupirocin ointment is one of the effective measures to control MRSA propagation in a thoracic surgery unit.

Key words Methicillin-resistant *Staphylococcus aureus* (MRSA) · Mupirocin · Thoracic surgery · Infection control

Introduction

Staphylococcus aureus is one of the major causative agents of surgical-site infections.[1] Especially, methicillin-resistant *S. aureus* (MRSA) is an important pathogen because the organism is susceptible to only a few antimicrobial agents.[2] In addition, *S. aureus* may spread nosocomially via the hands of personnel.[3] Therefore, all healthcare workers should pay attention to minimize staphylococcal infection after surgical operations.

The major reservoir of *S. aureus*, including MRSA, is the anterior nares.[3] Nasal carriage of *S. aureus* is an important factor in the development of staphylococcal infection.[4,5] Mupirocin ointment is an effective agent to eliminate MRSA colonization in the nasal cavities.[6] We previously succeeded in controlling MRSA propagation in a neonatal intensive care unit with unselective use of the ointment.[7] However, this strategy, called a mupirocin blanket-use program, has been applied and evaluated in only limited settings in Japan.[7,8] Therefore, we applied the strategy to control MRSA propagation in a thoracic surgery word, which occurred after the success in the neonatal intensive care unit.

Patients, materials, and methods

Mupirocin blanket-use program

We conducted a 28-week investigation, between March 16 and September 27, 1998, in a thoracic surgery ward of a university hospital in Tokyo. The ward consisted of 36 beds (including 5 in a recovery room), in which about 350 patients per year with respiratory and/or cardiovascular diseases were hospitalized for the purpose of surgery. Patients admitted to the ward for scheduled surgery in the first and second 14-week periods of our investigation were classified as the controls and the intervention group, respectively. Those without scheduled surgery, including those admitted for pre- or post-surgery examinations, were excluded from this program. In the intervention group, mupirocin ointment was administered to the nasal cavities three times daily for 3 days before surgery, once on return to the ward, and three times weekly for the following 2 weeks. Informed

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Table 1. Descriptions of attributes in each group

	Control group	Intervention group	*P* values
Date	March 16–June 21, 1998	June 22–September 27, 1998	
No. of patients	79	84	
Sex (male/female)	49/30	51/33	0.86
Age (years)	47.2 ± 25.7 (0–81)[a]	49.3 ± 26.6 (0–81)[a]	0.63
Duration of operation (min)	290.2 ± 195.2 (20–955)[a]	295.0 ± 203.7 (40–870)[a]	0.88
Site of operation (heart/lung)	45/34	53/32[b]	0.48
Days of hospitalization	34.7 ± 33.0 (2–192)[a]	27.5 ± 22.6 (2–123)[a]	0.10
Days between surgery and hospital discharge	25.8 ± 30.2 (1–190)[a]	20.1 ± 20.2 (1–120)[a]	0.15

[a] Average ± SD (range)
[b] Including one involving both heart and lung

Table 2. Descriptions of patients from whom MRSA was newly isolated after surgery

Group	Patient	Sex	Age (years)	Material of isolation	No. of days after operation when MRSA was isolated	Status
Control group	a	Male	73	Sputum	14	Infection
	b	Male	58	Wound swab	11	Colonization
	c	Male	78	Sputum	13	Infection
	d	Male	53	Sputum	3	Infection
	e	Female	65	Sputum	5	Infection
Intervention group	None	–	–	–	–	–

MRSA, methicillin-resistant *Staphylococcus aureus*

consent to participate in the present study was given by the patients themselves or patients' guardians in the intervention group. Ordinary infection control measures, including hand washing and disinfection, the wearing of disposable gloves and gowns if necessary, and appropriate disinfection and sterilization of medical instruments, were practiced throughout the program. Antimicrobial agents other than mupirocin were administered according to doctors' decisions.

We evaluated the efficacy of this program in two ways: the numbers of patients with new MRSA isolation within 2 weeks after surgery, and the daily rates of patients with MRSA colonization (including those proved to be colonized before surgery) in the recovery room. The findings were statistically analyzed with Fisher's exact test and Student's *t*-test, respectively.

Bacterial examination

Specimens for bacterial examination, including sputum, nasal swabs, and discharges from surgical sites, were submitted according to doctors' decisions. In addition, to detect MRSA colonization more sensitively, nasal swabs of patients in the intervention group were obtained once within 7 days before and 1 and 2 weeks after surgery. MRSA in the specimens was isolated and identified as described previously.[7]

Results

The control and the intervention groups comprised 81 and 91 patients, respectively. Because 2 and 7 of the patients were proven to be already colonized with MRSA before surgery, 79 and 84 patients, respectively, in these groups were considered to be susceptible to new MRSA colonization. There was no significant difference between the attributes of the two groups (Table 1).

In the control group, MRSA was newly isolated from 5 of the 79 susceptible patients within 2 weeks after surgery (Table 2). All of the 5 patients had received cardiovascular surgery. Four of them were considered to have developed infections caused by MRSA. In contrast, MRSA was not isolated from any susceptible patients in the intervention group. The decrease of new MRSA isolations was statistically significant ($P = 0.025$). MRSA propagation in the ward had finally ceased after the program.

The daily rates of MRSA-colonized patients in the recovery room in the control and the intervention group were on average 0.32 and 0.23, respectively. The rate in the intervention group was significantly lower compared with that in the control group ($P = 0.0059$; Fig. 1).

Discussion

The mupirocin blanket-use program is a strategy that reduces the numbers of both colonized and susceptible patients and prevents the propagation of MRSA in certain populations. In the present study, we applied this program in a thoracic surgery ward where new isolation of MRSA could have caused an outbreak of severe surgical-site infection, and found that both the numbers of patients newly colonized within 2 weeks after surgery and the daily rates of MRSA-colonized patients in the recovery room decreased markedly. We finally succeeded in terminating

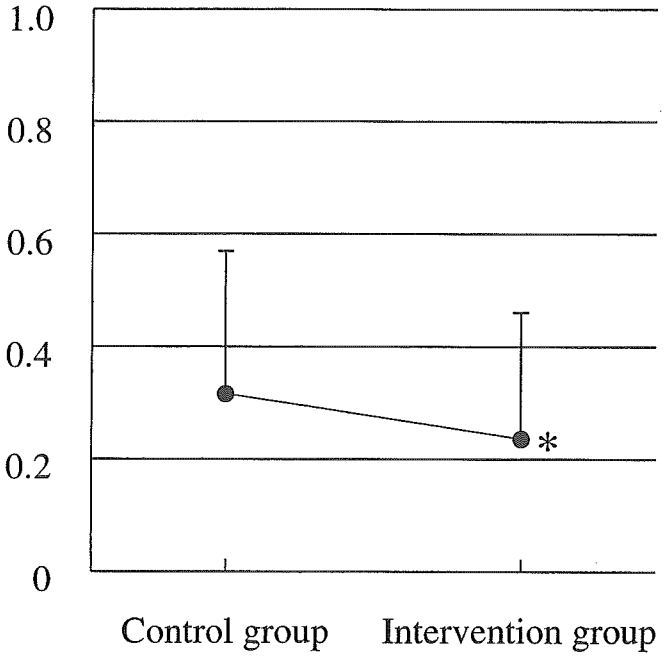

Fig. 1. Daily rates of patients with methicillin-resistant *Staphylococcus aureus* (MRSA) colonization in the recovery room. *Dots* and *horizontal bars* indicate averages and SE of each group. *$P = 0.0059$

the consecutive propagation of MRSA in the ward with the program.

Elimination of the nasal carriage of *S. aureus* with mupirocin ointment reduces surgical-site infection in cardiothoracic surgery.[9] In the present study, the decrease in new MRSA infections (four to zero) was statistically significant ($P = 0.025$). We did not execute the program for a longer period because we principally aimed to terminate the consecutive colonization of MRSA, and expanded use of the ointment would have induced mupirocin-resistant strains. Because we succeeded in reducing the prevalence of MRSA carriage in two critical groups of patients, those immediately after surgery and those admitted to the recovery room and provided with more intensive care, we speculated that this program would be also effective to reduce MRSA infection in the thoracic ward.

In Japan, mupirocin ointment is approved only for the eradication of MRSA in the nares of patients with a high risk of MRSA infection. We think that the administration is only temporarily effective for preventing nosocomial propagation of MRSA, because MRSA is prevalent in both hospitals and communities today[2] and new MRSA carriers will be hospitalized frequently. However, we do not recommend the unrestricted and long-term use of the ointment, because mupirocin-resistant MRSA strains, which have already emerged in Japan,[10,11] will be selected. We consider that strict adherence to contact precautions is the primary strategy to reduce MRSA propagation. Only in limited situations, including outbreaks involving highly immunocompromised patients or outbreaks in which MRSA propagation is not controllable by adherence to standard and contact precautions, unselective use of the ointment should be designed prudently and practiced under the supervision of experts.

References

1. Mangram AJ, Horan TC, Pearson ML, Silver LC, Jarvis WR. Guideline for prevention of surgical site infection, 1999. Am J Infect Control 1999;27:97–132.
2. Lowy FD. *Staphylococcus aureus* infections. N Engl J Med 1998;339:520–32.
3. John JF, Barg NL. *Staphylococcus aureus.* In: Mayhall CG, editor. Hospital epidemiology and infection control. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 1999. p. 325–645.
4. Kluytmans JAJW, Mouton JW, Ijzerman EPF, Vandenbroucke-Grauls CMJE, Maat AWPM, Wagenvoort JHTW, et al. Nasal carriage of *Staphylococcus aureus* as a major risk factor for wound infections after cardiac surgery. J Infect Dis 1995;171:216–9.
5. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. N Engl J Med 2001;344:11–6.
6. Hudson IRB. The efficacy of intranasal mupirocin in the prevention of staphylococcal infections: a review of recent experience. J Hosp Infect 1994;27:81–98.
7. Hitomi S, Kubota M, Mori N, Yano H, Okuzumi K, Kimura S, et al. Control of a methicillin-resistant *Staphylococcus aureus* outbreak in a neonatal intensive care unit by unselective use of nasal mupirocin ointment. J Hosp Infect 2000;46:123–9.
8. Amano H, Ohama J, Suda K, Hori M. Management of an outbreak of methicillin-resistant *Staphylococcus aureus* in an emergency ward with blanket use of intranasal mupirocin and long-term prophylaxis (in Japanese). Jpn J Chemother 1999;47:558–65.
9. Kluytmans JAJW, Mouton JW, VandenBergh MFQ, Manders M-JAAJ, Maat APWM, Wagenvoort JHT, et al. Reduction of surgical-site infections in cardiothoracic surgery by elimination of nasal carriage of *Staphylococcus aureus.* Infect Control Hosp Epidemiol 1996;17:780–5.
10. Watanabe H, Masaki H, Asoh N, Watanabe K, Oishi K, Furumoto A, et al. Emergence and spread of low-level mupirocin resistance in methicillin-resistant *Staphylococcus aureus* isolate from a community hospital in Japan. J Hosp Infect 2001;47:294–300.
11. Kikuchi K. Mupirocin resistant MRSA in Japan (in Japanese). Nippon Rinsho 2001;59:724–7.

BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **20**: 28–36 (2006)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.521

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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Received 9 December 2004; revised 10 March 2005; accepted 15 March 2005

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.

Contract/grant sponsor: Ministry of Health, Labour and Welfare of Japan; Contract/grant number: H15-15100101.
Contract/grant sponsor: Japanese Foundation for AIDS Prevention.
Contract/grant sponsor: Organization of Pharmaceutical Safety and Research; Contract/grant number: 01-4.

Published online 22 June 2005

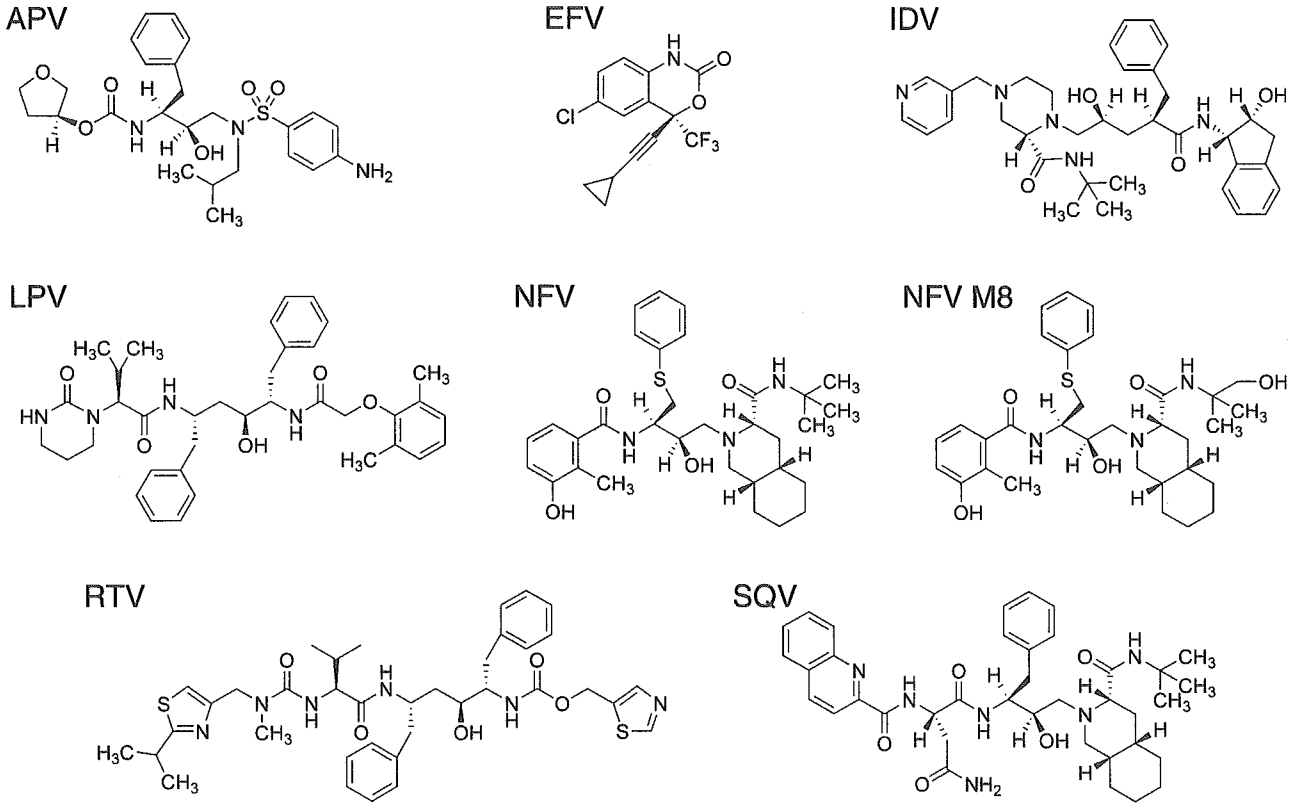

Figure 1. Chemical structures of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV.

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.

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,

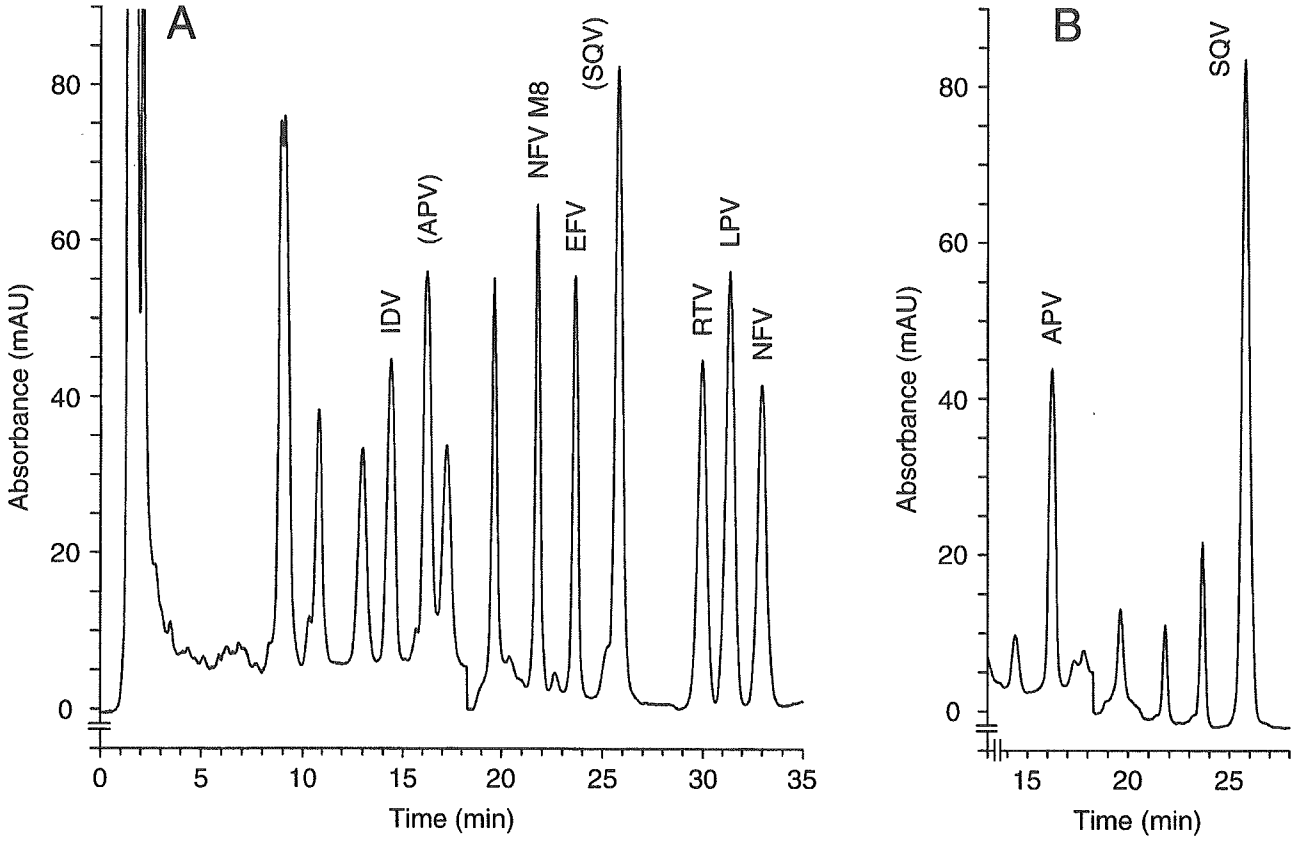

Figure 2. Chromatograms of a plasma sample spiked with 1.8 μg/mL of APV, EFV, IDV, LPV, NFV, NFV M8, RTV and SQV. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

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.

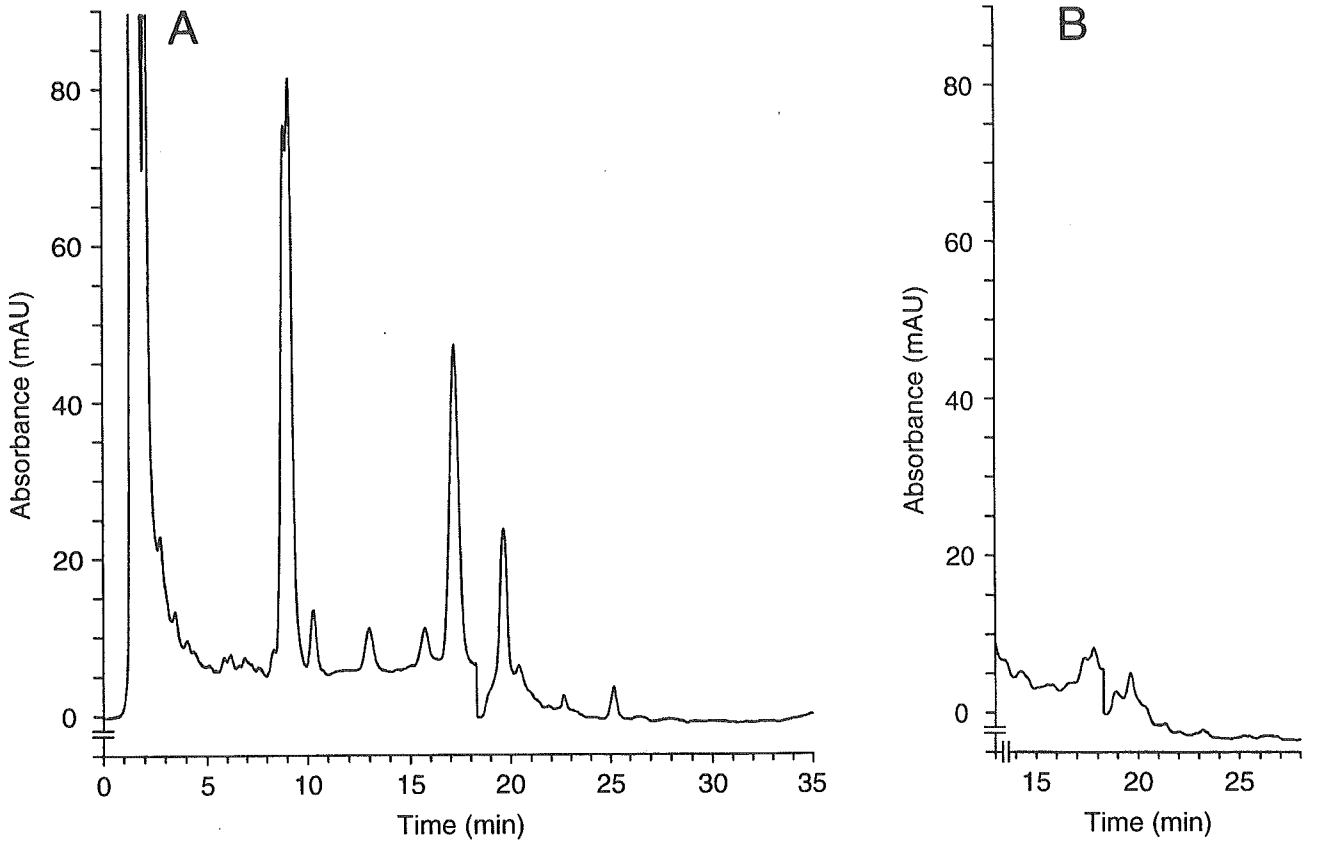

Figure 3. Chromatograms of a blank plasma sample. Absorbance was monitored at 212 nm (A) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B).

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.

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;

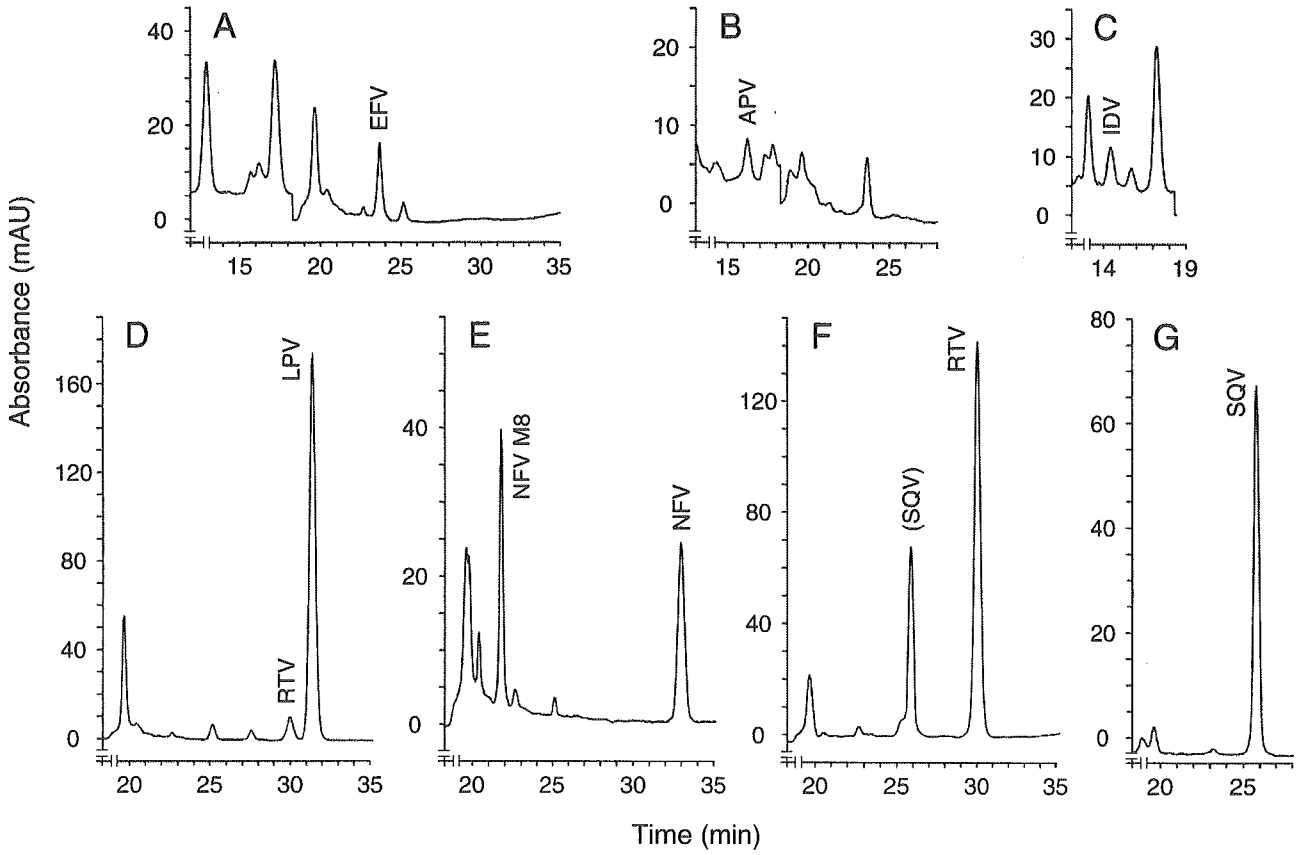

Figure 4. Chromatograms of plasma samples from patient 1 (A and B), patient 3 (C), patient 5 (D), patient 7 (E) and patient 9 (F and G). Absorbance was monitored at 212 nm (A, C, D, E and F) and 266/239 nm (switched from 266 to 239 nm at 18.3 min) (B and G). For details of the patients, see Table 4.

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,

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 comedicated 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

The authors thank Hiroyuki Fukuda of Applied Biosystems Japan Ltd for helpful suggestions. The authors also thank Abbott Laboratories, Japan Tobacco Inc., Kissei Pharmaceutical Co., Merck & Co. and Roche Products for providing the reference compounds. This study was supported in part by Grants-in-Aid for AIDS research from the Ministry of Health, Labour and Welfare of Japan (H15-15100101), by the Japanese Foundation for AIDS Prevention, and by the Organization of Pharmaceutical Safety and Research (01-4).

REFERENCES

Acosta EP, Henry K, Baken L, Page LM and Fletcher CV. Indinavir concentrations and antiviral effect. *Pharmacotherapy* 1999; **19**: 708.

Acosta EP, Gerber JG and The adult pharmacology committee of the AIDS clinical trials group. Position paper on therapeutic drug monitoring of antiretroviral agents. *AIDS Research and Human Retroviruses* 2002; **18**: 825.

Back D, Gatti G, Fletcher C, Garaffo R, Haubrich R, Hoetelmans R, Kurowski M, Luber A, Merry C and Perno CF. Therapeutic drug monitoring in HIV infection: current status and future directions. *AIDS* 2002; **16**: S5.

Barry MG, Merry C, Lloyd J, Halifax K, Carey P, Mulcahy F and Back DJ. Variability in trough plasma saquinavir concentrations in HIV patients: a case for therapeutic drug monitoring. *British Journal of Clinical Pharmacology* 1998; **45**: 501.

British HIV Association Writing Committee. BHIVA guidelines for the treatment of HIV-infected adults with antiretroviral therapy: July 2003; www.bhiva.org (access date: 18 October 2004).

Crommentuyn KML, Rosing H, Nan-Offeringa LGAH, Hillebrand MJX, Huitema ADR and Beijnen JH. Rapid quantification of HIV protease inhibitors in human plasma by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Journal of Mass Spectrometry* 2003; **38**: 157.

Dresser GK, Spence JD and Bailey DG. Pharmacokinetic–pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clinical Pharmacokinetics* 2000; **38**: 41.

Fellay J, Marzolini C, Meaden ER, Back DJ, Buclin T, Chave JP, Decosterd LA, Furrer H, Opravil M, Pantaleo G, Retelska D, Ruiz L, Schinkel AH, Vernazza P, Eap CB and Telenti A. Response to antiretroviral treatment in HIV-1-infected individuals with allelic variants of the multidrug resistance transporter 1: a pharmacogenetics study. *Lancet* 2002; **359**: 30.

Frerichs VA, DiFrancesco R and Morse GD. Determination of protease inhibitors using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography B* 2003; **787**: 393.

Gerber JG. Using pharmacokinetics to optimize antiretroviral drug-drug interactions in the treatment of human immunodeficiency virus infection. *Clinical Infectious Diseases* 2000; **30**: S123.

Hugen PW, Langebeek N, Burger DM, Zomer B, van Leusen R, Schuurman R, Koopmans PP and Hekster YA. Assessment of adherence to HIV protease inhibitors: comparison and combination of various methods, including MEMS (electronic monitoring), patient and nurse report, and therapeutic drug monitoring. *Journal of AIDS* 2002; **30**: 324.

Khaliq Y, Gallicano K, Seguin I, Fyke K, Carignan G, Bulman D, Badley A and Cameron DW. Single and multiple dose pharmacokinetics of nelfinavir and CYP2C19 activity in human immunodeficiency virus-infected patients with chronic liver disease. *British Journal of Clinical Pharmacology* 2000; **50**: 108.

Khoo SH, Gibbons SE and Back DJ. Therapeutic drug monitoring as a tool in treating HIV infection. *AIDS* 2001; **15**: S171.

Leibenguth P, Le Guellec C, Besnier JM, Bastides F, Mace M, Gaudet ML, Autret-Leca E and Paintaud G. Therapeutic drug monitoring of HIV protease inhibitors using high-performance liquid chromatography with ultraviolet or photodiode array detection. *Therapeutic Drug Monitoring* 2001; **23**: 679.

Marzolini C, Telenti A, Decosterd LA, Greub G, Biollaz J and Buclin T. Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. *AIDS* 2001; **15**: 71.

Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ and Holmberg SD. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *New England Journal of Medicine* 1998; **338**: 853.

Poirier JM, Robidou P and Jaillon P. Simultaneous determination of the six HIV protease inhibitors (amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, and saquinavir) plus M8 nelfinavir metabolite and the nonnucleoside reverse transcription inhibitor efavirenz in human plasma by solid-phase extraction and column liquid chromatography. *Therapeutic Drug Monitoring* 2002; **24**: 302.

Rentsch KM. Sensitive and specific determination of eight antiretroviral agents in plasma by high-performance liquid chromatography-mass spectrometry. *Journal of Chromatography B* 2003; **788**: 339.

Rezk NL, Tidwell RR and Kashuba AD. High-performance liquid chromatography assay for the quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma. *Journal of Chromatography B* 2004; **805**: 241.

Richman DD. HIV chemotherapy. *Nature* 2001; **410**: 995.

The Panel on Clinical Practices for Treatment of HIV Infection. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents: March 23, 2004. AIDSinfo.nih.gov (access date: 18 October 2004).

Titier K, Lagrange F, Pehourcq F, Edno-Mcheik L, Moore N and Molimard M. High-performance liquid chromatographic method for the simultaneous determination of the six HIV-protease inhibitors and two non-nucleoside reverse transcriptase inhibitors in human plasma. *Therapeutic Drug Monitoring* 2002; **24**: 417.

Tribut O, Arvieux C, Michelet C, Chapplain JM, Allain H and Bentue-Ferrer D. Simultaneous quantitative assay of six HIV protease inhibitors, one metabolite, and two non-nucleoside reverse transcriptase inhibitors in human plasma by isocratic reversed-phase liquid chromatography. *Therapeutic Drug Monitoring* 2002; **24**: 554.

Turner ML, Reed-Walker K, King JR and Acosta EP. Simultaneous determination of nine antiretroviral compounds in human plasma using liquid chromatography. *Journal of Chromatography B* 2003; **784**: 331.

Van Heeswijk RP. Critical issues in therapeutic drug monitoring of antiretroviral drugs. *Therapeutic Drug Monitoring* 2002; **24**: 323.

Volosov A, Alexander C, Ting L and Soldin SJ. Simple rapid method for quantification of antiretrovirals by liquid chromatography–tandem mass spectrometry. *Clin. Biochem.* 2002; **35**: 99.

Yeni PG, Hammer SM, Hirsch MS, Saag MS, Schechter M, Carpenter CC, Fischl MA, Gatell JM, Gazzard BG, Jacobsen DM, Katzenstein DA, Montaner JS, Richman DD, Schooley RT, Thompson MA, Vella S and Volberding PA. Treatment for adult HIV infection: 2004 recommendations of the International AIDS Society—USA panel. *Journal of the American Medical Association* 2004; **292**: 251.

Zhang KE, Wu E, Patick AK, Kerr B, Zorbas M, Lankford A, Kobayashi T, Maeda Y, Shetty B and Webber S. Circulating metabolites of the human immunodeficiency virus protease inhibitor nelfinavir in humans: structural identification, levels in plasma, and antiviral activities. *Antimicrobial Agents and Chemotherapy* 2001; **45**: 1086.

Antiviral Chemistry & Chemotherapy 16:363–373

A novel small molecular weight compound with a carbazole structure that demonstrates potent human immunodeficiency virus type-1 integrase inhibitory activity

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The integration of reverse transcribed proviral DNA into a host genome is an essential event in the human immunodeficiency virus type 1 (HIV-1) replication life cycle. Therefore, the viral enzyme integrase (IN), which plays a crucial role in the integration event, has been an attractive target of anti-retroviral drugs. Several IN inhibitory compounds have been reported previously, yet none has been successful in clinical use. To find a new, more successful IN inhibitor, we screened a diverse library of 12 000 small molecular weight compounds randomly by *in vitro* strand-transfer assay. We identified a series of substituted carbazoles that exhibit strand-transfer inhibitory activity at low micromolar concentrations. Of these, the most potent compound exhibited an IC_{50} of 5.00 ±3.31 μM (CA-0). To analyse the structural determinants of strand-transfer inhibitory activity of the carbazole derivatives, we selected 23 such derivatives from our compound library and performed further analyses. Of these 23 compounds, six showed strong strand-transfer inhibition. The inhibition kinetics analyses and ethidium bromide displacement assays indicated that the carbazole derivatives are competitive inhibitors and not intercalators. An HeLa4.5/LTR-nEGFP cell line was employed to evaluate *in vitro* virus replication inhibition of the carbazole derivatives, and IC_{50} levels ranged from 0.48–1.52 μM. Thus, it is possible that carbazole derivatives, which possess structures different from previously-reported IN inhibitors, may become novel lead compounds in the development of IN inhibitors.

Keywords: integrase inhibitor, carbazole, HIV-1, antiretroviral drug

Introduction

Human immunodeficiency virus type 1 (HIV-1), causative agent of acquired immunodeficiency syndrome (AIDS), possesses three critical enzymes for replication. These are protease (PR), reverse transcriptase (RT), and integrase (IN) (Ruscetti, 1985; Kohl *et al.*, 1988; LaFemina *et al.*, 1992). As inactivating any of these enzymes may negate the infectivity of HIV-1, the enzymes have been targets of anti-retroviral drug development. Indeed, great progress in anti-retroviral drug discovery has been achieved in recent decades, and today 10 RT inhibitors and eight PR inhibitors (De Clercq, 1992; Tronchet & Seman, 2003; Balzarini, 2004; Imamichi, 2004) are available for anti-retroviral treatments. The third enzyme, IN, has also been a major target of inhibitor development. L-708,906 and L-731,988, which possess diketo acid moieties within their structures, were the first IN-specific inhibitors discovered (Pommier *et al.*, 2000; Dayam & Neamati, 2003; Pluymers *et al.*, 2002; Hazuda *et al.*, 2000). S-1360 and L-870,810, which also have diketo acid moieties, are IN inhibitors that have reached clinical Phase I/II trials for the first time (Johnson *et al.*, 2004; Hazuda *et al.*, 2004). However, although there have been large advances in the development of IN inhibitors, further research and analysis is required to develop clinically usable compounds.

Integrase (IN), the leading target of novel anti-retroviral inhibitor development, is the enzyme responsible for integration, wherein reverse transcribed HIV-DNA is inserted into a host genome, and is critical for viral replication, which in turn establishes latency and chronic infection (Chun *et al.*, 1995). IN is composed of three distinct

domains – the N-terminal domain (amino acids 1–50) with a zinc-binding motif (Schauer & Billich, 1992; Burke *et al.*, 1992), the catalytic core domain (amino acids 50–212) with polynucleotidyl transfer activity and sequence-specific endonuclease activity (Engelman & Craigie, 1992; Engelman *et al.*, 1994) and the C-terminal domain (amino acids 212–288), which has been thought to relate to nonspecific DNA binding (Khan *et al.*, 1991; Woerner & Marcus-Sekura, 1993).

At present, the function and structure of each domain has not been fully understood. The most well-analysed domain is the catalytic core domain, and its active site has highly conserved amino acidic residues Asp64, Asp116 and Glu152, which are critical for polynucleotidyl transfer activity (LaFemina *et al.*, 1992; Engelman *et al.*, 1995). Previously reported potent IN inhibitors L-708,906, L-731,988, L-801,810, S-1360 and 5-CITEP are all targeted to this domain. These inhibitors bind to the active site, displace divalent metal ion Mg^{2+} from the active site and inactivate the catalytic activity of IN (Grobler *et al.*, 2002; Dayam & Neamati, 2003; Goldgur *et al.*, 1999; Johnson *et al.*, 2004). No specific inhibitors have been reported for the N-terminal and C-terminal domains.

In the present study we attempted to identify novel IN inhibitory compounds, and therefore we conducted a random screening of a library of small molecular weight compounds. As a result, we discovered a series of novel IN inhibitory compounds with carbazole structures, that are quite different from previously reported inhibitory compounds.

Materials and methods

Preparation of integrase

The sequence coding the NL4-3 integrase (IN) was cloned into pET28b(+) (Novagen, Madison, WI, USA), generating pET-IN that codes NL4-3 IN with a hexa-histidine tag at the N-terminus. *Escherichia coli* strain Rosetta (DE3) (Novagen) transformed with pET-IN was grown in 1 l of Super Broth (Biofluids, Camarillo, CA, USA) containing 100 μg/ml kanamycin at 30°C until the optical density of the culture had reached between 0.5 and 0.7 at 600 nm. The recombinant protein expression was induced by isopropyl-1-thio-D-galactopyranoside. After incubation for 3 h, the cells were harvested and resuspended in 100 ml of preparation buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl) and disrupted by sonication. Following high-speed centrifugation at 40 000×g for 45 min at 4°C, the pellet was homogenized in GBB buffer (50 mM Tris-HCl, pH 8.0, 6 M Guanidine HCl and 2 mM 2-ME). The residual pellet was again sonicated and centrifuged at 40 000×g for 30 min at 4°C.

The supernatant was filtered through a 0.22 μm filter and mixed with 1 ml of nickel-affinity resin (Sigma, St. Louis, MO, USA), and incubated overnight at 4°C. The resin was washed twice by mixing with 20 ml of GBB containing 5 mM imidazole (Sigma). The protein was eluted with GBB containing 1 M imidazole. The fractions containing integrase were pooled and 0.5 M EDTA was added to a final concentration of 5 mM. This eluted protein was then sequentially dialysed against (i) 6 M guanidine HCl, 50 mM Tris-HCl (pH 8.0), 2 mM 2-ME, 1 mM EDTA for 2 h at room temperature, (ii) 6 M guanidine HCl, 50 mM Tris-HCl (pH 8.0), 10 mM DTT, 1 mM EDTA for 16 h at room temperature, (iii) 4 M urea, 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM DTT, 0.1 mM EDTA for 16 h at 4°C, (iv) 2 M urea, 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1 mM DTT, 0.1 mM EDTA, 20% (w/v) glycerol for 16 h at 4°C, (v) 1 M urea, 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM DTT, 0.1 mM EDTA, 15 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20% (w/v) glycerol for 16 h at 4°C, and (vi) 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1 mM DTT, 0.1 mM EDTA, 15 mM CHAPS, 20% (w/v) glycerol for 16 h at 4°C. The final preparation was stored at −80°C.

The purified enzyme activity was confirmed and evaluated by strand-transfer assay using M8 apparatus (IGEN, Gaithersburg, MD, USA).

Preparation of test compounds

A diverse library of 12 000 small-molecule compounds was supplied by Toyama Chemicals Co. Ltd. (Toyama, Japan). All test compounds were dissolved in DMSO and adjusted to 2 mM concentration. S-1360 was synthesized as positive control for strand transfer assay.

Construction of strand-transfer assay

Two different strand-transfer assay systems were employed in the IN inhibitor screening trial. For the first screening step, an M8 apparatus and strand-transfer assay kit, ORIGEN HIV integrase assay (IGEN), was used. In brief, magnetic beads coated with 29 mer donor double-stranded DNA (dsDNA) were mixed with purified IN (15 pmol), followed by adding the test compound and 20 mer target dsDNA tagged with ruthenium, conducting electronically inducible fluorescence chemistry, and incubating for 1 h at 37°C. Subsequently, the entire reaction solution was applied to the M8 apparatus, and then strand-transfer products were captured by a magnet in the flow-circuit of the equipment. The amount of the strand-transfer product was measured by ruthenium fluorescence activity. For the second and later screening steps, in-house strand-transfer assay was employed. The in-house assay was designed in 96-well plate format to achieve high-throughput screening.