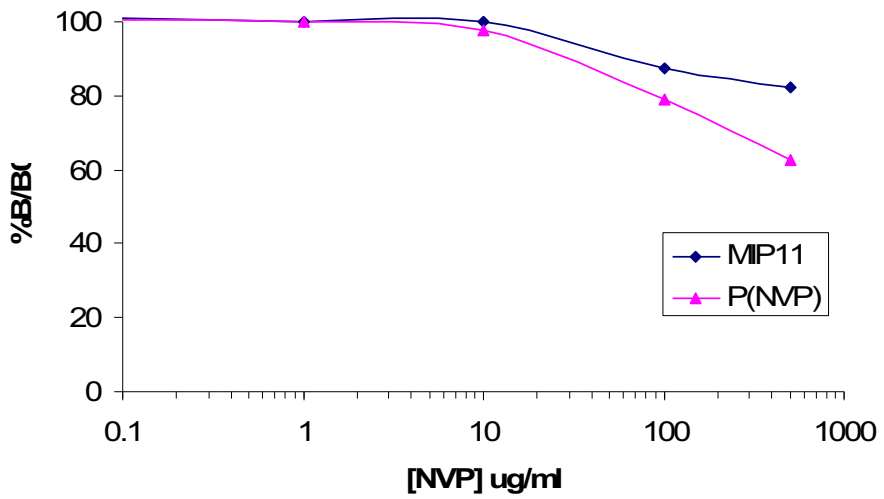
3.4.6.4 การเตรียม molecularly imprinted sorbent assay

ในช่วงแรกของการทดลองได้ใช้วิธีเคลือบพอลิเมอร์ที่หลุม (well) ของ ELISA plate แล้ววัดค่า O.D. ที่ได้หลังจากเติม TMB บนพอลิเมอร์ที่อยู่บน plate โดยตรงตามวิธีที่มีรายงาน แต่พบว่ามีพอลิเมอร์บางส่วนหลุดออกในระหว่างการล้างและ incubate ทำให้ได้ผลที่มีความคลาดเคลื่อนสูงมาก แม้ว่าจะมีการปรับปรุงวิธีการเคลือบพอลิเมอร์แต่ก็ยังให้ผลไม่ดีเท่าที่ควร ดังนั้นจึงได้เปลี่ยนมาใช้วิธีการทำ assay ในหลอด microcentrifuge โดยทำ competition ระหว่าง NVP กับ NVP-HRP conjugate ที่ช่วงความเข้มข้นของ NVP ระหว่าง 0.1-500 μg/ml แล้วทำการวัดการทำงานของเอนไซม์ที่มีเหลืออยู่ในสารละลายหลังจากแยกเอาพอลิเมอร์ออกด้วยเครื่องเหวี่ยงตะกอน ผลที่ได้แสดงดังรูปที่ 3.13 ซึ่งพบว่าเมื่อใช้ MIP11 เป็นตัวจับจะมีช่วงการวิเคราะห์ที่เป็นเส้นตรงที่อยู่ระหว่าง 10-100 μg/ml ส่วน P(NVP) จะมีช่วงการวิเคราะห์ที่เป็นเส้นตรงที่ระหว่าง 10-500 μg/ml ส่วนเมื่อใช้ NIP11 เป็นตัวจับจะไม่มีการแข่งขันระหว่าง enzyme probe กับ NVP เกิดขึ้น จะเห็นว่าความไวของเทคนิคนี้ยังไม่ต่ำพอที่จะใช้ในการหาปริมาณ NVP ในตัวอย่างซึ่งระดับของยา NVP ในพลาสมามักจะพบในช่วงความเข้มข้น 3.0-10.0 μg/ml[30] ทั้งนี้การวัดการทำงานของเอนไซม์ที่มีเหลืออยู่ในสารละลายทำให้มีสัญญาณ background noise สูง ซึ่งอาจจะต้องแก้ไขโดยปรับรูปแบบของการวิเคราะห์ต่อไป



รูปที่ 3.13 Displacement curves with unlabelled NVP as competitor for MIP(NVP) and P(NVP). B/B0 is the ratio of the amounts of NVP–HRP bound in the presence of displacing ligand, B, to the amount bound in absence of displacing, B_0.

3.5 สรุปผลการทดลอง

งานวิจัยนี้ได้สังเคราะห์พอลิเมอร์ลอกแบบโมเลกุลของ NAM และ NVP โดยได้ใช้ NAM ซึ่งเป็นสารที่มีโครงสร้างคล้ายคลึงกับ NVP แต่หาง่ายและราคาถูกกว่ามาเป็นสารต้นแบบสำหรับการคัดสรรเบื้องต้นเพื่อหาสภาวะที่เหมาะสมสำหรับสังเคราะห์พอลิเมอร์ลอกแบบ NVP จากการศึกษาพบว่าพอลิเมอร์ที่สังเคราะห์โดยวิธีพอลิเมอไรเซชันแบบตกตะกอนด้วย template/MAA/TRIM ในอัตราส่วน 1:4:4 สามารถจับกับ NVP ได้ดีในตัวกลางที่เป็นน้ำ เมื่อศึกษาความจำเพาะของพอลิเมอร์ทั้งสองโดยเปรียบเทียบค่า distribution coefficient ของ NVP เทียบกับสารอื่นๆที่มีโครงสร้างใกล้เคียง พบว่า NVP มีค่า Kd ที่สูงมาก ซึ่งแสดงว่าพอลิเมอร์ทั้งสองเลือกจับกับ NVP ได้ดีกว่าสารอื่นๆ โดยที่ P(NVP) มี cross reactivity ที่สูงกับ NAM จาก Scatchard plot analysis พบว่า MIP11 มีค่า Q_{max} และ K_d เท่ากับ 57.92 μg/mg และ 134.27 mg/ml ส่วน P(NVP) มีค่า Q_{max} และ K_d เท่ากับ 95.62 μg/mg และ 166.72 mg/ml เมื่อนำพอลิเมอร์ที่ได้มาใช้แทนแอนติบอดีในการพัฒนาเทคนิค enzyme-linked molecularly imprinted sorbent assay สำหรับการตรวจวิเคราะห์ NVP พบว่าเมื่อใช้ MIP11 จะมีช่วงการวิเคราะห์ที่เป็นเส้นตรงที่อยู่ระหว่าง 10-100 μg/ml ส่วน P(NVP) จะมีช่วงการวิเคราะห์ที่เป็นเส้นตรงที่ระหว่าง 10-500 μg/ml

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Output ที่ได้จากโครงการ

1.สามารถผลิตแอนติบอดีแบบชีวภาพและแบบสังเคราะห์ที่มีประสิทธิภาพดีมีราคาถูกเพื่อเป็นพื้นฐานในการพัฒนาชุดตรวจวิเคราะห์ Nevirapine เพื่อช่วยในการติดตามการใช้ยาในผู้ติดเชื้อ HIV
2.กระบวนการผลิตนี้สามารถนำไปประยุกต์ใช้กับการเตรียมแอนติบอดีต่อยาชนิดอื่นเพื่อนำมาใช้ในงานทางด้านภูมิคุ้มกันวิทยาและสิ่งแวดล้อม
3. เป็นความรู้พื้นฐานที่เป็นประโยชน์ต่อการเรียนการสอน และการทำวิจัยในอนาคต
4. สามารถผลิตผลงานเพื่อตีพิมพ์ในวารสารระดับชาติหรือนานาชาติ

<u>ชื่อผลงานที่ตีพิมพ์ไปแล้ว</u>

Development of a one-step immunochromatographic strip test for the rapid detection of nevirapine (NVP), a commonly used antiretroviral drug for the treatment of HIV/AIDS
Talanta, *Volume 71, Issue 1*, *15 January 2007*, *Pages 462-470*
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Development of a one-step immunochromatographic strip test for the rapid detection of nevirapine (NVP), a commonly used antiretroviral drug for the treatment of HIV/AIDS

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Abstract

Currently, high-performance liquid chromatographic (HPLC) methods are mainly used to measure antiretroviral plasma concentrations in HIV-infected patients. Although the utility of routine therapeutic drug monitoring (TDM) as an additional tool to optimize long-term antiretroviral therapy is unclear, if TDM is to be widely used, the availability of simple, cheap and reliable methods for the measurement of antiretroviral drug levels are needed, particularly in resource-limited settings. In this study, an immunochromatograhic (IC) strip test to detect the presence of nevirapine (NVP) in body fluids has been developed. Antiserum to NVP was first raised in rabbits by immunization against NVP chemically conjugated with bovine serum albumin, and subsequently validated by Western immunoblotting and competitive indirect ELISA. The partially purified anti-NVP antibodies were conjugated with colloidal gold particles. The conjugation of the colloidal gold and polyclonal antibodies was monitored by UV–vis spectroscopy, while transmission electron microscopy images were used to characterize the particle size and shape of the conjugates. The resulting colloidal gold conjugates were used for the production of an IC strip test to detect nevirapine in human plasma. Preliminary assessment suggests no-cross reactivity of the NVP polyclonal antibodies but assessment of plasma samples from HIV-infected patients receiving HAART needs to be conducted. This assay could potentially be used for drug monitoring as part of the clinical care of HIV infected patients.

Keywords: NVP; Immunochromatographic strip test; Colloidal gold; Immunoassay

1. Introduction

Nevirapine (NVP), 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-[3,2-b:2′,3′-e], is a non-nucleoside reverse transcriptase inhibitors (NNRTI) which is widely used as a part of highly active antiretroviral therapy (HAART) for the treatment of HIV/AIDS, and as a single dose prophylactic intervention during labor for the prevention of mother-to-child transmission of HIV (PMTCT) [1,2].

NVP inhibits HIV-1 replication by binding directly to the HIV-l reverse transcriptase enzyme and disrupting its catalytic site. Unfortunately, NVP viral drug resistance can result from a specific single mutation, such as the K103N, in the viral reverse transcriptase gene and these mutations confer cross resistant to the other NNRTIs in the drug class [3]. A relationship between plasma NVP levels and efficacy has been reported [4]; therefore, maintaining sufficient NVP plasma drug levels is critical to reduce the risk for selecting NNRTI resistance viruses. For the majority of antiretroviral drugs, a similar relationship has been demonstrated and it has been proposed that individualization of drug dosing, guided by the measurement of drug levels (i.e., therapeutic drug monitoring (TDM)) could help optimize

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therapeutic outcome and prevent adverse effects [5]. Furthermore, TDM has also been suggested to be an additional tool to the standard methods of questionnaires and pill counts to help monitor drug adherence [6].

For the quantitative analysis of NVP in plasma, high-performance liquid chromatography (HPLC) with UV detection has been primarily used [7]. Recently, LC/MS methods have also been developed, these methods can simultaneously detect up to nine antiretroviral drugs in the same plasma sample [8]. However, since the majority of HIV-infected patients are living in rural areas, simpler and cheaper methods of NVP detection are needed to facilitate the assessment of TDM of antiretrovirals. Indeed, for plasma nevirapine levels, a qualitative thin-layer chromatography (TLC) method as well as a quantitative ELISA method has been reported [9,10].

Immunochromatographic strip tests are simple, rapid and cheap assays which have been developed for the qualitative measurement of many biological markers [11–15]. These assays rely on the transport of a labeled antibody (or antigen) probe to its specific antigen (or antibody) binding partner immobilized on the surface of a membrane. Among the different labeled test systems, colloidal gold appears to be the most attractive as unlike fluorescence or enzyme tags, gold probes are more stable and do not require time-consuming procedures such as incubations, washing steps and enzymatic reactions to generate a signal [16,17]. Due to the accumulation of colloidal gold, a red-purple color can be rapidly observed visually. These characteristics significantly shorten the analysis time and make it a very convenience to assay on-site.

In this study, we described the preparation and characterization of anti-NVP polyclonal antibodies and an antibody–colloidal gold probe. These reagents were used to develop a one-step immunochromatographic (IC) strip test for the rapid detection of NVP.

2. Experimental

2.1. Reagents

All chemicals were purchased from commercial suppliers and used as received. Chloroauric acid ($HAuCl_4{\cdot}3H_2O$), methyl-5-bromovalerate, 4-(dimethylamino)pyridine (DMAP), anhydrous *N,N*-dimethylformamide (DMF), *N*-hydroxy succinimide (NHS), and 1,3-diisopropylcarbodiimide (DIC) were purchased from Fluka, USA. Bovine serum albumin (BSA) was obtained from Sigma, USA. NVP was obtained as a gift from the Government Pharmaceutical Organization (GPO) of Thailand.

2.2. Instruments

Solid-phase EIA was performed in 96-well microtiter plates (Nunc, Denmark), and optical density (O.D.) was measured with an automatic plate reader (Tecan, Austria). NMR spectra were recorded on a Bruker *AVANCE*^{TM} NMR spectrometer (400 MHz for ^{1}H). Chloroform-d ($CDCl_3$) was used as the solvent. Chemical shift values (δ) are reported in ppm relative to internal tetramethylsilane. Coupling constants (J) are expressed in Hz. Transmission electron microscopy (TEM) images were recorded on a transmission electron microscope equipped with energy dispersive spectrometer (EDS) (JEOL JEM-2010, Japan).

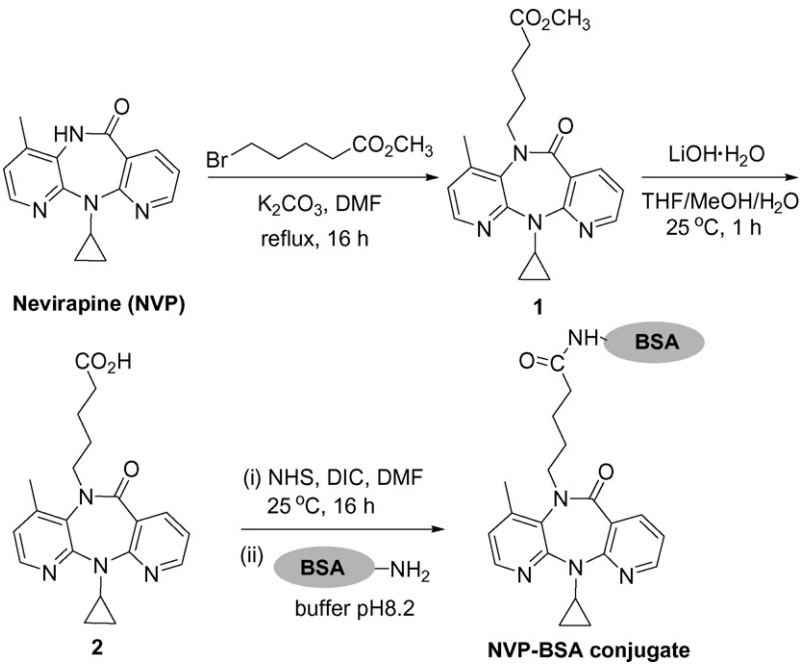
2.3. Preparation of NVP–BSA antigen

The immunogen (NVP–BSA conjugate) was synthesized according to Azoulay et al. [10], except that the carrier protein was changed to BSA instead of keyhole limpet hemocyanin (KLH) because the cost of immunogen production was significantly lower and it was presumed that the immunogenicity would not be affected.

NVP linked methyl ester (compound **1**; see Scheme 1): A solution of methyl 5-bromovalerate (60 μl, 0.36 mmol) in DMF (1.5 ml) was added to a mixture of NVP (100 mg, 0.36 mmol) and potassium carbonate (149.3 mg, 1.08 mmol) in DMF (1.5 ml). The resulting mixture was refluxed for 16 h, and then the solvent removed in vacuo. The crude residue was purified by chromatography on a silica gel (hexane-ethyl acetate 60/40, $R_f = 0.37$) to generate NVP linked methyl ester (**1**) as a yellow oil (78.1 mg, 52%). The following ^{1}H NMR (400 MHz, $CDCl_3$) was produced: δ 8.49 (dd, $J = 2$, 4.8 Hz, 1H), 8.05 (d, $J = 4.8$ Hz, 1H), 7.75 (dd, $J = 2$, 7.6 Hz, 1H), 6.97 (dd, $J = 7.6$, 4.8 Hz, 1H), 6.92 (d, $J = 5.2$, 1H), 4.38 (t, 2H), 3.66 (s, 3H), 3.65 (m, 1H), 2.42 (t, 2H), 2.34 (s, 3H), 1.87 (m, 4H), 0.96 (m, 2H), 0.47 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.7, 162.9, 159.0, 152.8, 151.3, 143.8, 143.5, 137.3, 132.7, 122.0, 119.6, 118.4, 51.5, 33.7, 29.0, 28.2, 21.7, 18.0, 8.7, 8.6.

NVP linked carboxylic acid (compound **2**): Compound **1** (78.1 mg, 0.20 mmol) was dissolved in a solution of THF/MeOH/H_2O 3:2:1 (2 ml). $LiOH{\cdot}H_2O$ (16.7 mg, 0.40 mmol) was then added and the mixture was stirred for 1 h at 25 °C. After concentration of the mixture in vacuo, the crude residue was diluted with water and neutralized with 1 M HCl. The resulting solution was extracted with 2 ml × 2 ml EtOAc, dried with anhydrous Na_2SO_4 and concentrated in vacuo to yield compound **2** as yellow oil (62.3 mg, 85%). The following ^{1}H NMR (400 MHz, $CDCl_3$) was produced: δ 8.49 (dd, $J = 2$, 4.8 Hz, 1H), 8.10(d, $J = 4.8$ Hz, 1H), 7.78 (dd, $J = 2$, 7.6 Hz, 1H), 6.99 (dd, $J = 7.6$, 4.8 Hz, 1H), 6.90 (d, $J = 5.2$ Hz, 1H), 4.40 (t, 2H), 3.66 (m,1H), 2.42 (t, 2H), 2.32 (s, 3H), 1.87 (m, 4H), 0.96 (m, 2H), 0.47 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 162.8, 159.0, 152.6, 151.4, 144.0, 143.7, 137.4, 132.8, 122.0, 119.6, 118.5, 33.6, 29.0, 28.1, 21.0, 18.1, 14.1, 8.9, 8.7.

NVP–BSA conjugate: Compound **2** (33 mg; 0.087 mmol) was reacted with NHS (12.0 mg; 0.1 mmol) in the presence of 1,3-diisopropylcarbodiimide (23.4 mg; 0.11 mmol) in anhydrous DMF (3.6 ml) for 16 h at 25 °C. The mixture was then added dropwise to a stirred solution of BSA in 5 ml of 0.05 M carbonate/bicarbonate buffer pH 8.2 at 0 °C. The solution was stirred for a further hour at 25 °C and then for 24 h at 4 °C. The synthesized immunogen was then dialyzed with 0.1 M phosphate buffer saline (pH 7.4). The resulting solution was kept frozen at −20 °C until it was used.



Scheme 1. Synthesis of the NVP–BSA conjugate.

2.4. Preparation of polycloncal antibodies

An albino New Zealand rabbit was immunized with 1.0 mg of NVP–BSA antigen in complete Freund's adjuvant. The rabbit preimmunized serum was collected before subcutaneous multi-site injections. Booster injections of 1.0 mg of antigen in incomplete Freund's adjuvant were given 1 week post-immunization, followed by three additional boosters at 2-week intervals. The hyperimmune serum obtained 2 weeks after the last immunization was collected and precipitated with saturated $(NH_4)_2$ SO_4 and dialyzed against 5 mM sodium borax buffer pH 9.0.

2.5. Validation of polyclonal antibodies

2.5.1. Western immunoblotting

SDS-PAGE was performed according to the technique described by Laemmli [18]. Reduced forms of NVP–BSA and unconjugated BSA were electrophoretically separated on a 12% polyacrylamide gel. Samples were prepared in 2-mercaptoethanol and SDS under heat denaturizing conditions. The separated polypeptides were transferred to a PVDF membrane as described by Tawbin et al. [19]. The membrane was blocked with 5% skimmed milk in PBS for 1 h. Subsequently, the NVP–BSA immunized rabbit serum (1:2000 with 2% BSA in PBS) was applied to the blocked membrane. After 1 h incubation, excess polyclonal antibodies were washed out and HRP-swine anti-rabbit immunoglobulins conjugate (DAKO, Germany) diluted 1:5000 was added. The TMB/H_2O_2 immunoblotting substrate solution (Fermentas, Lithuania) was used to visualize bound antibodies on the membrane. The molecular size of each reactive band was calculated relative to standard proteins.

2.5.2. Competitive indirect ELISA

A competitive indirect ELISA was used to test the NVP–BSA immunized rabbit serum reactivity with other antiretroviral drugs. The drugs tested were: nucleoside reverse transcriptase inhibitor (NRTI); zidovudine (AZT), lamivudine (3TC), didanosine (ddI), abacavir (ABC) and stavudine (d4T); the non-nucleoside reverse transcriptase inhibitor (NNRTI); efavirenz (EFV); and the protease inhibitors (PIs), indinavir (IDV), saquinavir (SQV), amprenavir (APV), ritonavir (RTV), nelfinavir (NFV), NFV metabolite (M8) and lopinavir (LPV). For antiretroviral drugs, although the therapeutic ranges vary the plasma concentrations achieved during a dosing interval following oral administration are commonly within 0.10–12 μg/ml [20]. For the competitive ELISA high concentrations of 10 μg/ml were used to mimic those concentrations which can be achieve in plasma. NVP–BSA antigen (50 μl) at a concentration of 10 μg/ml in carbonate buffer pH 9.6 was added to polystyrene wells and allowed to passively adsorb for 16 h at 4 °C. Subsequently, 200 μl of 2% skimmed milk in PBS was added to each well and incubated for 2 h at RT. The blocking solution was removed by washing three times with 0.05% TWEEN in PBS. A 1:8000 dilution of rabbit serum was mixed with a fix drug concentration (10 μg/ml) and incubated for 1 h at 37 °C. The mixture (50 μl) was applied into an individual NVP–BSA coated well. After washing the wells, 50 μl of HRP-swine anti-rabbit immunoglobulin conjugate diluted 1:3000 was added, incubated for 1 h and washed. TMB/H_2O_2 substrate solution (100 μl) was added for 15 min. The enzymatic reaction was stopped by adding 100 μl of 1 M HCl. The O.D. was measured at 450 nm. The O.D. value was used to compare the ability of each drug to compete with the anti-NVP polyclonal antibodies in the rabbit hyperimmune serum.

2.6. Synthesis and characterization of colloidal gold

Colloidal gold was synthesized according to the procedure described by Turkevich et al. [21] using a reduced scale. An aqueous solution of chloroauric acid (5 mM $HAuCl_4 \cdot 3H_2O$, 5 ml) was diluted with 90 ml of deionized water. This solution was stirred and heated until boiling and then reduced with 0.5% sodium citrate solution (5 ml). Heating was continued until the solution color changed to a red-purple color.

2.7. Formation of gold conjugate

Polyclonal antibodies were diluted in 5 ml of 5 mM sodium borate buffer pH 9.0 in order to obtain 2.5 mg/ml of immunoglobulins. This solution was then added dropwise to a stirred solution of colloidal gold 0.25 mM, 100 ml. The pH of the colloidal gold solution was pre-adjusted to 7.0 by addition of 0.01 M Na_2CO_3. The mixture was stirred for 30 min and 5 ml of 5% BSA in 5 mM NaCl solution was added. After 5 min, the solution was centrifuged at 4 °C 14,000 rpm for 30 min to remove unconjugated antibodies from the solution. The pellet was subsequently resuspended in Phosphate Gold Diluent buffer (1% BSA in 49 mM Na_2HPO_4) to obtain an antibody–colloidal gold conjugate solution which had an O.D. of 40 at λ 580 nm.

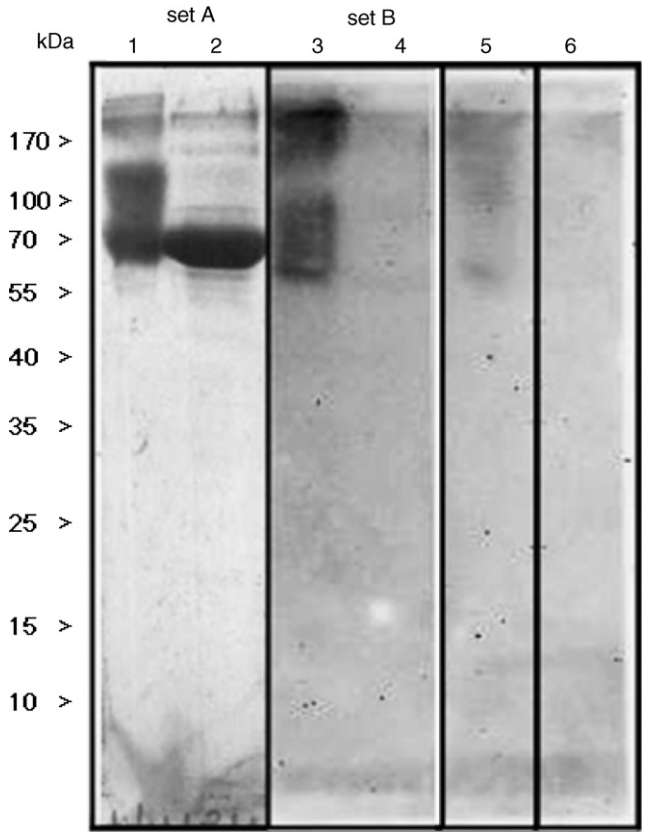


Fig. 1. Western immunoblotting analysis of NVP–BSA and BSA alone using the rabbit hyperimmune serum. Lanes 1, 3, 5 and 6 were NVP–BSA separated under denaturing condition. Lanes 2 and 4 were unconjugated BSA separated under denaturing condition. Set A was stained with Amido black B for protein detection on blotting membrane. Set B was reacted with rabbit serum (dilution 1:2000). Lane 5 was probed with rabbit serum mixed with NVP (10 μg/ml final concentration). No rabbit serum was added to lane 6. The HRP-swine anti-rabbit immunoglobulin conjugate was applied to all lanes. Molecular weight markers (in kilodaltons) are indicated.

2.8. Characterization of colloidal gold conjugates

2.8.1. Transmission electron microscopy (TEM)

The size and shape of colloidal gold with and without conjugated antibodies bound were compared using TEM measurements. The TEM samples were prepared by placing a drop of the colloidal gold in acetone/H_2O mixture onto a carbon-coated TEM copper grid. The resulting film was allowed to dry overnight and then used for TEM imaging. TEM microscope was operated at an acceleration voltage of 120 kV and at a magnification of 80,000. The size distributions of the particles from enlarged photographs of the TEM images were measured using at least 150 counts of the particles. A selected area diffraction (SAD) pattern of a colloidal gold particle containing antibodies was taken directly from the corresponding TEM image and the resulting diffraction pattern was indexed according to a standard procedure [22].

2.8.2. UV–vis spectroscopic studies

The formation of antibody–colloidal gold conjugates was monitored by UV–vis spectroscopy using a double beam spectrophotometer. The UV absorption of the colloidal gold and antibody–colloidal gold solution (0.5 mM in aqueous) were monitored immediately after preparation at λ 200–700 nm.

2.9. Preparation of an immunochromatographic (IC) strip test

An IC test strip was developed using rabbit anti-NVP polyclonal antibodies conjugated with colloidal gold particles (anti-NVP–CGC). A lateral flow test strip was constructed as follows:

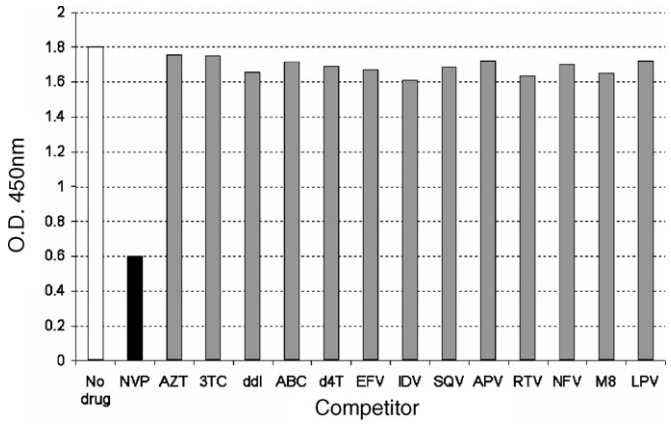


Fig. 2. Assessment of the rabbit anti-NVP polyclonal antibodies by competitive indirect ELISA with various anti-HIV drugs at 10 μg/ml. This experiment was performed in triplicate and a similar absorbance pattern was observed (see Section 2.5.2 for drug names).

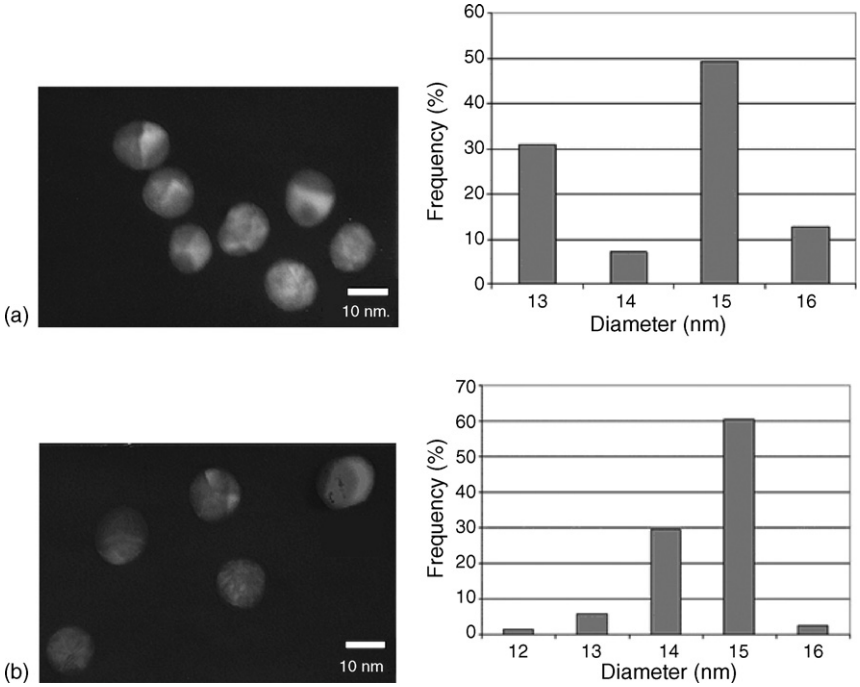


Fig. 3. TEM images and size distribution of (a) unconjugated colloidal gold particles (b) conjugated antibody–colloidal gold particles. For particle size comparison, the 10 nm-scale marker is indicated.

anti-NVP–CGC (O.D. of 40 at λ 580 nm) was jetted onto a glass fiber (conjugate pad) by an isoflow dispenser (Imagene Technology, USA). The nitrocellulose membrane (Scheicher and Schuell, Germany) was laminated on a plastic support by a Precision Laminator (Zeta Corporation, Korea). NVP–BSA at 1.0 mg/ml and goat anti-rabbit IgG (KPL, USA) at 0.5 mg/ml in PBS were jetted onto a laminated nitrocellulose membrane at two separate zones; test line and control line, respectively. Subsequently, the conjugate pad and jetted membrane were incubated for 2 h at 37 °C and then dried in a dessicator at room temperature. After drying, the components of strip, i.e., sample application pad, anti-NVP–CGC sprayed conjugate pad, jetted nitrocellulose membrane and absorbent pad were assembled and then cut into individual strips (3.5 mm/strip), using a strip cutter (INDEX CUTTER, USA).

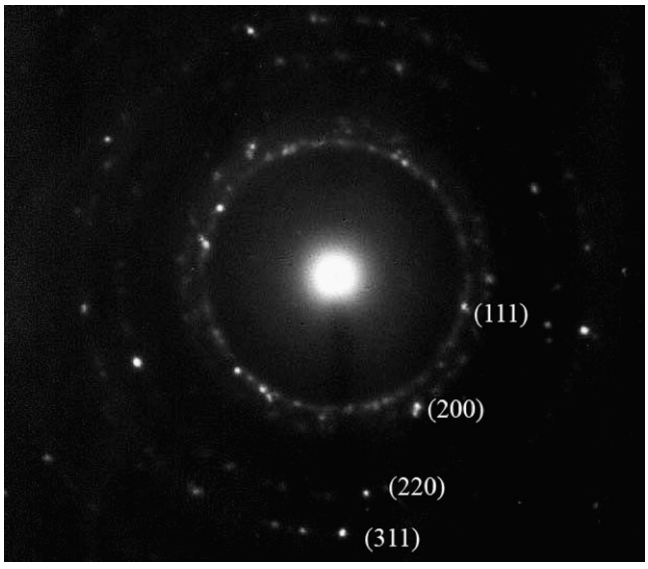


Fig. 4. Selected area diffraction (SAD) pattern derived from a conjugated antibody–colloidal gold particle.

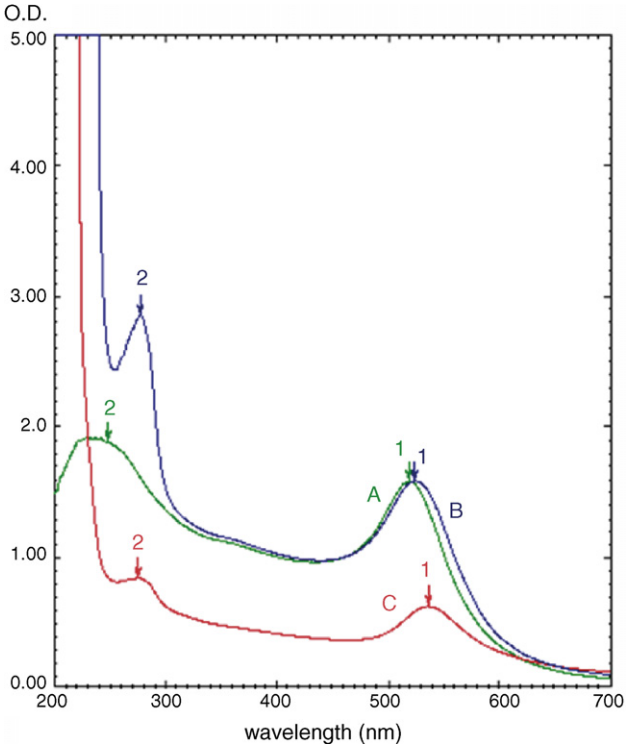


Fig. 5. UV–vis spectra of colloidal gold and antibody–colloidal gold conjugates. Curve A, colloidal gold solution; curve B, anti-NVP–CGC immediately after addition of antibody to gold solution; Curve C, anti-NVP–CGC after centrifugation to remove the unbound antibodies.

2.10. Detection of NVP by the Immunochromatographic strip test

Analysis of NVP by the IC strip test was performed by dipping the strip into standard solutions of NVP in PBS buffer pH 7.4 at 0, 0.1, 0.5, 1.0 and 3.0 μg/ml. The result was interpreted after a red-purple color appeared at the control line. At the test line, the presence of a red-purple color suggested no NVP was present in the sample and vice versa.

2.11. Cross-reactivity of the NVP immunochromatographic test strip

The procedure was performed as described in Section 2.10 excepted that the IC strip test was dipped into NVP and a set of commonly coadministered antiretroviral drugs in PBS buffer pH 7.4. A high drug concentration of 10 μg/ml, which is near the maximum concentration achieved in plasma for many of the antiretroviral drugs, was used. The drugs tested are described in Section 2.5.2. To evaluate the potential influence of the plasma matrix on the strip, human plasma spiked with 5.0 μg/ml of NVP was tested along with blank human plasma.

3. Results and discussion

3.1. Synthesis of NVP–BSA conjugate

NVP–BSA conjugate was synthesized according to Scheme 1 and the structures of the NVP linked methyl ester (compound **1**) and the NVP linked carboxylic acid (compound **2**) were confirmed by NMR (see Section 2.3).

3.2. Validation of polyclonal antibodies

Western immunoblotting in Fig. 1 showed immunoreactive bands of the synthesized NVP–BSA (lanes 1, 3, 5 and 6) in comparison with unconjugated BSA (lanes 2 and 4). NVP–BSA and BSA protein bands were observed in lanes 1 and 2 (set A) by Amido black B staining. The major protein band of BSA was located at 66 kDa. The proteins with higher molecular weight were regarded as impurities from the partially purified BSA fraction. Following incubation with rabbit serum (dilution 1:2000, Set B), the presence of polyclonal anti-NVP antibodies in the serum was demonstrated by the positive signal observed with NVP–BSA (lane 3). No signal was observed with BSA alone (lane 4). This result suggested that anti-BSA antibodies present

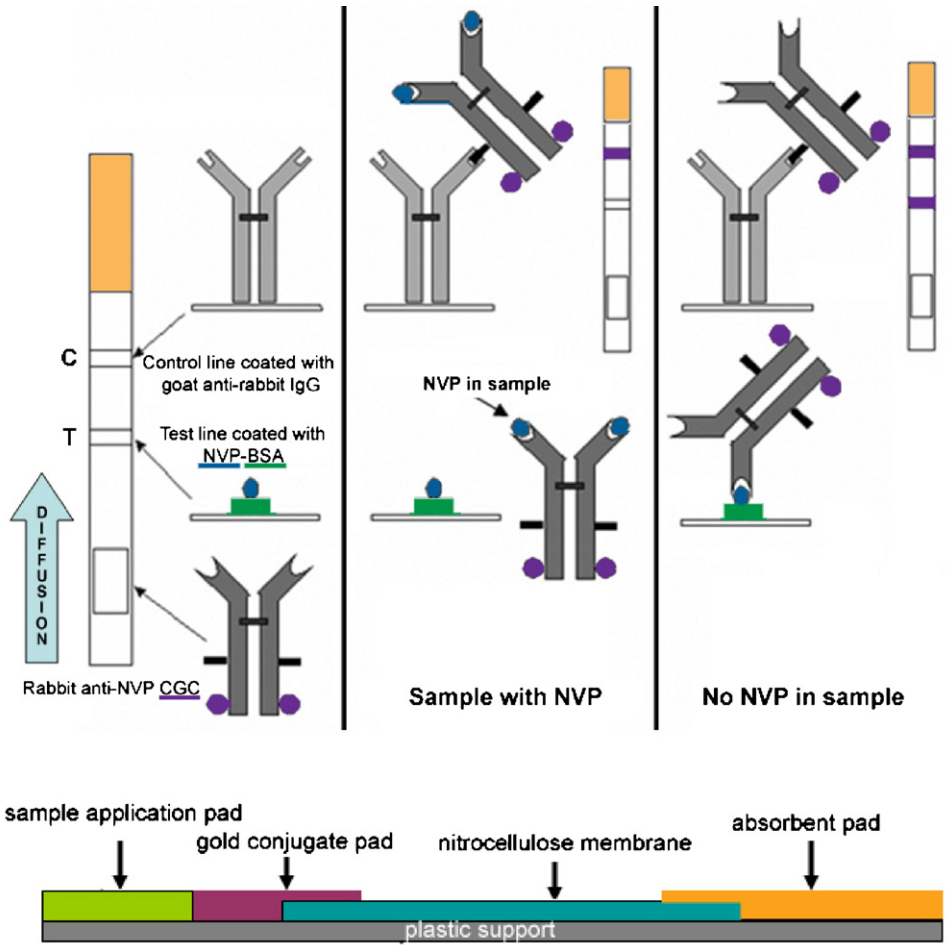


Fig. 6. Configuration of the IC NVP strip test. The schematic diagram shows the areas where rabbit anti-NVP–CGC, NVP–BSA and goat anti-rabbit IgG are immobilized (left panel). The reactions which occur on the IC strip in the presence of NVP (middle panel), and in the absence of NVP (right panel) are shown. A red-purple color appears at the test and/or control lines depending on the presence of NVP.

in the hyperimmune serum were neutralized by BSA in the serum diluting buffer. Furthermore, mixing the hyperimmune serum with 10 μg/ml NVP reduced the intensity of the 66 kDa band and the higher molecular weight proteins (lane 5) confirming the presence of polyclonal anti-NVP antibodies.

The specificity of antibodies was analyzed by competitive indirect ELISA (Fig. 2). Several commonly administered anti-HIV drugs (AZT, 3TC, ddI, ABC, d4T, EFV, IDV, SQV, APV, RTV, NFV (plus NFV metabolite, M8) and LPV) were tested. The O.D. of the well containing NVP as the competitor was significantly lower than that obtained in the control well (no drug). In contrast, the O.D. of the wells with the other antiretroviral-drugs was similar to the control well. This data suggested that the anti-NVP antibodies produced were highly specific to NVP.

3.3. Characterization of the colloidal gold particles and antibody–gold conjugates

3.3.1. TEM imaging

The TEM images of gold colloid and anti-NVP–CGC are shown in Fig. 3(a and b). Both forms of gold particles were homogeneous in size and shape, and there was no marked difference in the size distribution of colloidal gold before and after conjugation. The gold particles obtained showed narrow size distribution with a mean diameter of 15.0 ± 3.05 nm and this is within the typical range of colloidal gold used in IC assays (10–20 nm) [23]. The selected area diffraction (SAD) pattern taken from an antibody gold particle (Fig. 4) shows the central intense direct beam and an array of diffraction spots from different atomic planes. These spots were assigned to the 1 1 1, 2 0 0, 2 2 0 and 3 1 1 planes of a face centered cubic (fcc) lattice of gold, according to JCPDS File No. 4-784 [24]. This result confirms that colloidal gold was successfully produced.

3.3.2. UV–vis spectra

The presence of antibody on the gold surface was characterized using UV–vis spectroscopy. Spectra of the colloidal gold solution were recorded without antibody and immediately after the addition of antibody (Fig. 5a and b). A peak at ~519 nm in curve A was due to surface plasmon resonance of colloidal gold. Immediately following the addition of antibody (curve B), red shift of this band occurred as a result of antibody–colloidal gold interaction and a new band appeared at ~280 nm which corresponded to a protein absorption band. After centrifugation and removal of unbound antibody from antibody–colloidal gold conjugate (curve C) showed two absorption bands at ~510 and 280 nm indicating that antibody was presented on the gold surface. The absorption band at 510 nm was much lower for the antibody–colloidal gold conjugate than for the unbound colloidal gold as some gold particles were lost during the washing step to remove unbound antibodies.

3.4. Construction of Immunochromatographic strip test system using anti-NVP–CGC

A schematic diagram showing the areas where the components are immobilized on the IC-test strip is illustrated in Fig. 6. Dipping the strip into a test solution will cause the liquid to move upward by capillary action and dissolve the dried rabbit anti-NVP–CGC on the conjugate pad. The anti-NVP–CGC complex subsequently migrates up the surface of nitrocellulose membrane. If no NVP is present in the sample, rabbit anti-NVP–CGC will bind to NVP–BSA streaked at the test line (T). The immobilized anti-NVP–CGC generates a red-purple color line caused by colloidal gold particle accumulation. In contrast, if the sample contains NVP, rabbit anti-NVP–CGC will be neutralized by free NVP. Consequently, the intensity of the red-purple color observed will be reduced. For the control line (C), a red-purple color will appear independently of the presence of NVP in the test sample since the amount of rabbit anti-NVP–CGC is in excess. When the rabbit anti-NVP–CGC reaches C, it will be captured by the goat anti-rabbit IgG. The control line is used to monitor whether the anti-NVP–CGC has passed over the test line and the result can be interpreted.

3.5. Analysis of NVP standards with the IC test strips

Results from the IC strip assessing NVP in PBS are shown in Fig. 7. In the absence of NVP, the binding of anti-NVP–CGC with the solid-phase NVP–BSA produced an intense red-purple

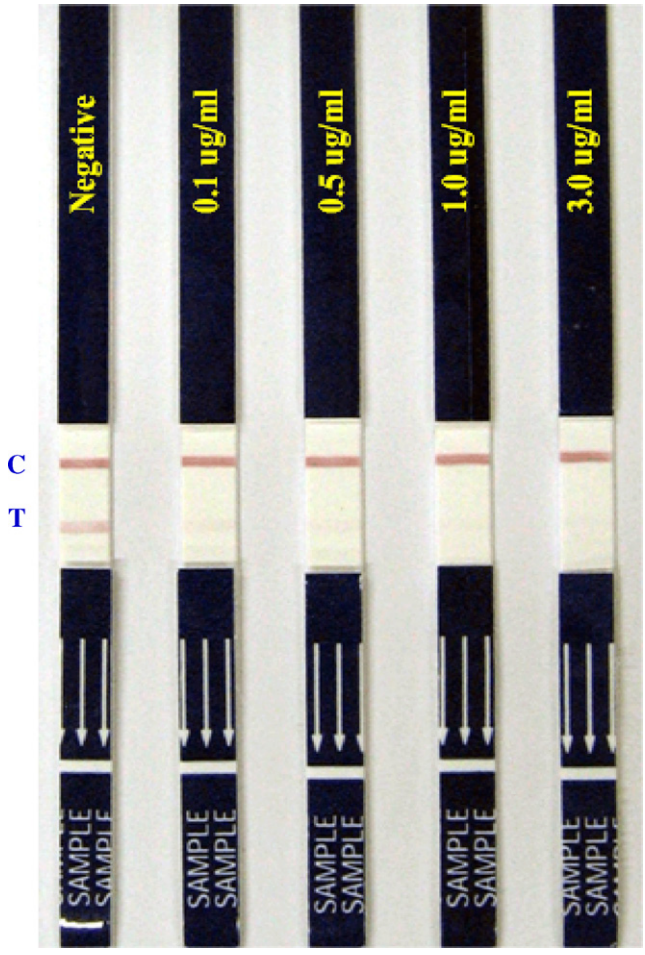


Fig. 7. IC strip test results at NVP concentrations of 0, 0.1, 0.5, 1.0 and 3.0 μg/ml in PBS pH 7.4. C, control line; T, test line.

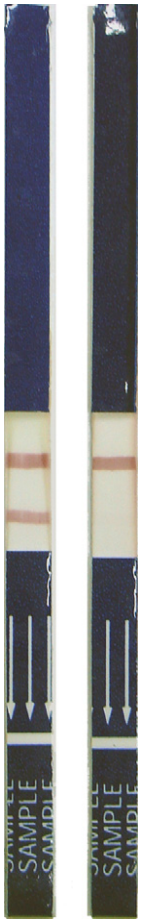


Fig. 8. Evaluation of IC strip with blank human plasma (left strip) and human plasma and spiked with NVP at a concentration of 5.0 μg/ml (right strip).

band at the test line. The intensity of the color faded when the concentrations of NVP were increased. At NVP concentrations of 1.0 and 3.0 μg/ml, no color was observed at the test line. The limit of NVP detection in PBS using the IC strip test was 1.0 μg/ml. The results of the IC strip test could be read within 10 min.

To determine if components in the plasma matrix could interfere with the IC strip test result, human plasma spiked with NVP at a therapeutic drug concentration of 5.0 μg/ml, in addition to blank human plasma were tested. The results showed that NVP could be detected and no interference of the plasma matrix in blank samples was observed (Fig. 8); however, clearly assessment of the strip in plasma samples from HIV-infected patients receiving HAART needs to be conducted.

3.6. Cross reactivity test of the IC strip test

To study the effect of other commonly administered anti-HIV drugs on the IC strip test, twelve other HIV drugs (AZT, 3TC, ddI, ABC, d4T, EFV, IDV, SQV, APV, RTV, NFV and LPV) were tested. A high drug concentration of 10 μg/ml, which is near the maximum concentration achieved in plasma for many of the antiretroviral drugs, was used. The intensity of red-purple color at test line was as the similar to that observed in the absence of NVP (data not shown). This suggested that the presence of these antiretroviral drugs in a test sample will not interfere with the detection of NVP.

4. Conclusion

Herein, we have successfully developed an IC strip test for the rapid detection of NVP. Preliminary results show that it was possible to detect the presence of NVP at a concentration as low as 1.0 μg/ml in PBS, and no cross-reactivity from other commonly administered HIV drugs or components in the human plasma was observed. Following the standard nevirapine 200 mg twice daily dose, plasma nevirapine concentrations range between 3.0 and 10.0 μg/ml, thus the IC test strip should be able to detect NVP concentrations in patients using a NVP based HAART regimen. The established assay is simple and easy to interpret without requirement of any sophisticate instruments. This low cost method could be routinely used in developing countries for drug adherence purposes (potentially including urine specimens) and TDM, and could be extended to other anti-HIV drugs, in particular the protease inhibitors where high interpatient variability has been reported and the use of TDM has been suggested [5]. Overall, this report describes the construction and preliminary assessment of the first immunochromatographic strip test for nevirapine but detailed assessment of this IC strip in plasma samples from HIV-infected patients receiving HAART is needed.

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