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# Therapeutic Drug Monitoring of Nevirapine in Resource-Limited Settings

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**Background.** We developed a simple and inexpensive thin-layer chromatography (TLC) assay for semiquantitative detection of saliva concentrations of nevirapine in resource-limited settings. The method was validated in an African target population.

**Methods.** Paired plasma and saliva nevirapine concentrations were assayed by high-performance liquid chromatography (HPLC); saliva concentrations of nevirapine were also assayed by TLC. The rate of false-positive results was the proportion of subtherapeutic nevirapine saliva and plasma concentrations determined by HPLC that were judged to be therapeutic in saliva specimens by TLC. The rate of false-negative results was the proportion of therapeutic nevirapine saliva and plasma concentrations determined by HPLC that were judged to be subtherapeutic in saliva specimens by TLC. The extent of agreement in TLC readings between 5 technicians and 2 batches of TLC sheets was evaluated.

**Results.** Twenty-five (9%) of 286 African adults had a subtherapeutic plasma nevirapine concentration. The median ratio of nevirapine concentrations in saliva to those in plasma was 0.51:1. The rate of false-positive results for TLC was 0% (0 of 23 specimens) when TLC results were compared with HPLC results for saliva specimens and 8% (2 of 25 specimens) when TLC results were compared with HPLC results for plasma specimens. The rate of false-negative results for TLC was 1% (3 of 263 specimens) when TLC results were compared with HPLC results for saliva specimens and 1% (3 of 261 specimens) when TLC results were compared with HPLC results for plasma specimens. The extent of agreement of TLC results was substantial for the 5 technicians (Fleiss's  $\kappa = 0.77$ ) and for the 2 batches of sheets (Cohen's  $\kappa = 0.80$ ).

**Conclusions.** The TLC assay was found to be sensitive, specific, and robust in the detection of subtherapeutic nevirapine concentrations in saliva specimens obtained from African HIV-infected adults. It is an attractive alternative to HPLC for therapeutic drug monitoring of nevirapine in resource-limited settings.

Nevirapine is widely prescribed in combination with nucleoside reverse-transcriptase inhibitors for the treatment of HIV infection in resource-limited countries. Adequate plasma concentrations of nevirapine are required to achieve a successful response, whereas subtherapeutic concentrations (defined as concentrations  $<3.0$  mg/L) are related to development of mutations and virological failure [1–3]. Even in the case of perfect

adherence to regimens at standard doses, some patients will remain at risk for underdosing because of interpatient variability in nevirapine exposure or drug-drug interactions.

In the developed world, therapeutic drug monitoring (TDM) [3] is a well-known tool for the optimization of nevirapine dosing in HIV-infected patients. As a result of a lack of simple and affordable methods to determine the level of exposure to nevirapine, TDM is hardly ever performed in resource-limited settings. Previous studies have suggested that saliva specimens may be used as an alternative body fluid sample for TDM of nevirapine [4, 5]. Saliva concentrations of nevirapine in HIV-infected [4] and healthy [5] white persons are approximately one-half of the values observed in plasma specimens obtained from such persons. We recently developed a thin-layer chromatography (TLC)

Received 29 April 2008; accepted 30 July 2008; electronically published 9 October 2008.

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**Clinical Infectious Diseases** 2008;47:1339–44

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1058-4838/2008/4710-0015\$15.00

DOI: 10.1086/592694

method to provide a simple and economical tool for semi-quantitative measurement of the saliva concentration of nevirapine.

The primary objective of this study was to validate our newly developed TLC method for TDM of nevirapine concentrations in saliva samples obtained from HIV-infected Africans. Secondary objectives were to determine the relationship between saliva and plasma nevirapine concentrations and the proportion of subtherapeutic nevirapine concentrations in an African population.

## METHODS

**Study population.** Three hundred HIV-infected adults who had been receiving a regimen that contained nevirapine for at least 4 weeks were eligible for enrollment at a routine visit to the adult HIV clinic of the Kilimanjaro Christian Medical Centre (Moshi, Tanzania). Patients who had oral lesions or ulcers and those who were unable to self-report the date and time of the most recent ingestion of nevirapine were excluded from the study. The protocol was reviewed and approved by the Ethics Committee of Tumaini University (Moshi). Written informed consent was obtained from all subjects before enrollment.

**Sample collection and drug concentration assays.** At a routine visit to the clinic, unannounced paired blood and saliva samples were collected within 5 min of each other. Stimulated saliva specimens were obtained with a salivette (Sartstedt; Etten-Leur) using a dental cotton roll impregnated with citric acid (20 mg), which stimulates the salivary flow. Study subjects were asked to chew on the roll for ~1 min. Blood and saliva samples were stored at 2°C–8°C for a maximum of 8 h. Plasma was separated and stored at –80°C until transportation to The Netherlands on dry ice. Saliva was obtained by centrifugation of the cotton roll at 800 g for 10 min. Two aliquots were stored at –80°C: one for analysis by TLC in Tanzania, and one for transportation to The Netherlands and analysis by high-performance liquid chromatography (HPLC).

Saliva and plasma concentrations of nevirapine were determined at the Department of Clinical Pharmacy of the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands) using validated HPLC assays with UV detection, as modified from a method described by Hollanders et al. [6]. Samples were pretreated as described by Hollanders and colleagues, and preparation of saliva samples did not differ from preparation of plasma samples. In brief, 150 µL of saliva or plasma was mixed with 150 µL of perchloric acid, vortexed for 20 s, and centrifuged for 5 min at 12,175 g. Subsequently, 200 µL of the clear supernatant was transferred to insert vials and placed in the autosampler. The lower and upper limits of quantification of the modified assays were 0.167 and 16.7 mg/L, respectively, for plasma and 0.158 and 15.8 mg/L, respectively, for saliva. The intraday precision of the assays ranged from 0.4% to 3.2%

for plasma and from 1.3% to 4.1% for saliva. Additional variation as a result of performing the assays on different days ranged from 0.0% to 0.4% for plasma and from 0.8% to 2.2% for saliva. The accuracy of the assays ranged from 102% to 105% for plasma and from 99% to 102% for saliva. Ratios of saliva nevirapine concentration to plasma nevirapine concentration and the proportion of subjects with a subtherapeutic nevirapine plasma concentration (i.e., <3.0 mg/L) [3] were determined. In addition, saliva concentrations of nevirapine were semiquantitatively analyzed at the Biotechnology Laboratory of Kilimanjaro Christian Medical Centre using a newly developed TLC method.

**Experimental TLC method.** A reference solution of nevirapine (Viramune; Boehringer Ingelheim), 1.75 mg/L, was obtained by dilution of a stock solution (nevirapine, 0.875 mg/mL, in dimethylsulfoxide [DMSO]; Merck) in blank saliva (i.e., saliva without nevirapine or any other drug). Both were kept at –80°C.

The reference solution and saliva samples were thawed at room temperature, mixed for 1 min, and centrifuged at 9000 g for 1 min. Of the reference and samples, 1.0 mL was transferred into a 10-mL glass test tube. After addition of 0.5 mL of 0.2 M ammonia (diluted from 25% ammonia; Labopharma) and 5 mL tert-butylmethylether (Riedel–de Haën), tubes were closed, mixed for 1 min, centrifuged at 1150 g for 5 min, and stored at –80°C until the lower layer was frozen completely (20 min). The organic layer was poured into a 10-mL glass test tube and was dried in 2 days.

An eluent (or mobile phase) was prepared (ratio of toluene to ethyl acetate, 1:1; ACME Chemicals and Unilab, respectively) and poured into a TLC container. The container was closed and left for 1 h. After addition of 50 µL of methanol to the dried test tubes, they were closed and mixed for 1 min. One µL of the reference and of the samples was slowly pipetted in small, dense dots at least 1.5 cm from the side and 2.0 cm from the bottom of a silica gel TLC sheet (height, 10.0 cm; width, 20.0 cm; Merck), as shown in figure 1. After drying, spots were checked for similarity of size and shape under UV light (254 nm; CAMAG 022.9120). The TLC sheet was placed in the container with eluent for ~12 min (figure 2). It was marked for the distance that the eluent had moved and was dried in the air. The intensity of the spots was determined under UV light and compared with the reference spot (figure 3). A spot that was less intense than the reference was considered to be subtherapeutic. A spot that was comparable to or more intense than the reference spot was considered to be therapeutic.

**Technical validation of TLC method.** All saliva samples were used to determine the technical sensitivity and specificity of the TLC method. The readings were performed by 1 technician. Saliva nevirapine concentrations determined by HPLC were defined as subtherapeutic if they were <1.5 mg/L, which



**Figure 1.** Preparation of thin-layer chromatography sheet with saliva samples.

is one-half of the cutoff value for plasma specimens (3.0 mg/L) [3]. The rate of false-positive results was defined as the proportion of subtherapeutic saliva concentrations of nevirapine (i.e., <1.5 mg/L), as determined by HPLC [6], that were reported to be therapeutic on the basis of TLC of saliva specimens. The rate of false-negative results was defined as the proportion of therapeutic saliva concentrations of nevirapine ( $\geq 1.5$  mg/L), as determined by HPLC [6], that were reported to be subtherapeutic on the basis of TLC of saliva specimens.

Twenty-five saliva samples with nevirapine concentrations determined by HPLC [6] closest to the TLC reference value of 1.75 mg/L were selected to test the robustness of the TLC method. The extent of agreement in TLC result between 5 different technicians (Fleiss's  $\kappa$  [7]) and 2 different batches of TLC sheets (Cohen's  $\kappa$  [8]) was evaluated in these 25 saliva samples.

The stability of the stock solution (nevirapine in DMSO) was tested after storage at  $-40^{\circ}\text{C}$  for 50 months. The stability of the reference solution (nevirapine in blank saliva) was tested after storage at room temperature for 17 h, after storage at  $-40^{\circ}\text{C}$  for 2 months, and after freezing and thawing twice. Interference of possible concurrently used medications was evaluated by comparison of the retention factor (i.e., the distance travelled by compound divided by distance travelled by eluent front) of extractable and detectable (UV light; 254 nm) compounds with the retention factor value of nevirapine. In addition, blank saliva samples obtained from at least 6 subjects who had not been taking nevirapine were tested for interference by endogenous substances.

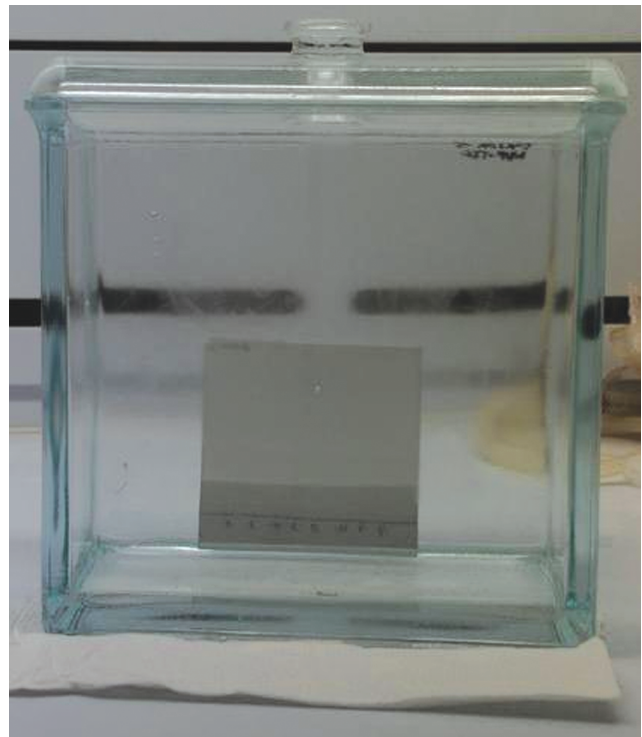
**Biological validation of TLC method.** All plasma and saliva samples were used to determine the biological sensitivity and specificity of the TLC method. The rate of false-positive results

was defined as the proportion of subtherapeutic nevirapine plasma concentrations (<3.0 mg/L [3]) determined by HPLC [6] that were reported to be therapeutic on the basis of TLC of saliva specimens. The rate of false-negative results was defined as the proportion of therapeutic nevirapine plasma concentrations ( $\geq 3.0$  mg/L [3]) determined by HPLC [6] that were reported to be subtherapeutic on the basis of TLC of saliva specimens.

## RESULTS

Of the 300 subjects enrolled in the study, 14 were excluded because of problems with labelling, because the saliva volume was too small, or because the saliva specimen was lost. The mean age of the remaining 286 African subjects (200 of whom were women) was 41 years (range, 17–71 years). All subjects were treated with a nevirapine dosage of 200 mg twice per day, in combination with lamivudine and either stavudine or zidovudine.

The mean plasma concentration of nevirapine, as determined by HPLC, was 7.35 mg/L (range, <0.167 to 28.59 mg/L). Twenty-five (9%) of 286 subjects had a subtherapeutic plasma nevirapine concentration (i.e., <3.0 mg/L); 18 of 25 had a concentration that was less than the detection limit of the HPLC assay (i.e., <0.15 mg/L). The median ratio of saliva nevirapine concentration to plasma nevirapine concentration, as determined by HPLC, was 0.51:1 (interquartile range, 0.47:1–0.54-



**Figure 2.** Thin-layer chromatography sheet in container with eluent



**Figure 3.** Determination of spot intensity under UV light

:1), and the median time from the last ingestion of medication and to sample collection was 3.40 h (interquartile range, 2.52–4.37 h). Six subjects had a ratio of saliva nevirapine concentration to plasma nevirapine concentration  $>1.00$  (range, 1.25–15.68); the median time from last ingestion of medication to sampling for these 6 subjects was 1.19 h (interquartile range, 0.85–1.74 h).

None of the 23 saliva specimens that were found to contain subtherapeutic concentrations of nevirapine (i.e.,  $<1.5$  mg/L) by HPLC were reported to have therapeutic values by TLC (rate of false-positive results, 0%); the technical sensitivity of the TLC method was 100% (table 1). Three of the 263 saliva specimens that were found to have therapeutic concentrations of nevirapine (i.e.,  $\geq 1.5$  mg/L; 1.62, 1.74, and 1.96 mg/L) by HPLC were reported to have subtherapeutic values by TLC (rate of false-negative results, 1%); the technical specificity of the TLC method was 99% (table 1).

The extent of agreement among TLC results (i.e., subtherapeutic or therapeutic nevirapine saliva concentrations) for 25 selected samples with a concentration closest to the reference sample was substantial for the 5 technicians (Fleiss's  $\kappa$ , 0.77) and 2 batches of TLC sheets (Cohen's  $\kappa$ , 0.80).

The stock solution for the TLC method (nevirapine in DMSO) was stable at  $-40^{\circ}\text{C}$  for at least 50 months (mean rate of recovery  $\pm$  SD,  $99.7\% \pm 0.3\%$ ). The reference solution (nevirapine in blank saliva) was stable at room temperature for at

least 17 h (mean rate of recovery  $\pm$  SD,  $107.6\% \pm 5.8\%$ ) and at  $-40^{\circ}\text{C}$  for at least 2 months (mean rate of recovery  $\pm$  SD,  $94.3\% \pm 3.2\%$ ). Freezing and thawing twice had no effect on the stability of nevirapine in blank saliva (mean rate of recovery  $\pm$  SD,  $95.1\% \pm 1.1\%$ ). The mean nevirapine retention factor value was 0.29; none of the possible comedications that were extractable and detectable had a similar retention factor value. Also, no interference by endogenous substances was observed in the blank saliva samples obtained from subjects not taking nevirapine.

Two of the 25 plasma samples found to have subtherapeutic nevirapine concentrations ( $<3.0$  mg/L; 2.61 and 2.91 mg/L) by HPLC were reported to have therapeutic concentrations by TLC (rate of false-positive results, 8%); the biological sensitivity of the TLC method was 92% (table 1). Three of the 261 plasma samples found to have therapeutic nevirapine concentrations ( $\geq 3.0$  mg/L; 3.41, 3.52, and 4.28 mg/L) by HPLC were reported to have subtherapeutic concentrations by TLC (rate of false-negative results, 1%); the biological specificity of the TLC method was 99% (table 1).

## DISCUSSION

Twenty-five (9%) of 286 HIV-infected African adults had a subtherapeutic plasma concentration of nevirapine (i.e.,  $<3.0$  mg/L) [3]. Saliva nevirapine concentrations were approximately one-half of the values observed in plasma specimens. A TLC method for semiquantitative detection of nevirapine in saliva specimens was found to be sensitive, specific, and robust. This simple and economical tool is a good option for TDM of nevirapine in resource-limited settings.

Use of saliva specimens instead of plasma specimens for TDM of nevirapine implies painless and noninvasive sampling, a diminished risk of HIV transmission to health care workers, and a lower cost [4]. TLC is a relatively inexpensive assay technique, compared with HPLC [9], which is commonly used in the developed world for TDM of nevirapine in plasma samples. An HPLC system for TDM of nevirapine costs approximately €40,000, and the costs for consumables per sample are estimated to be €15. In contrast, the estimated initial setup cost of a new TLC method for semiquantitative measurement of nevirapine in saliva is €800, and the cost of consumables per sample is approximately €1.60. Because this simple and inexpensive method was developed to perform TDM of nevirapine in resource-limited settings, it was validated in an African target population in our study.

The percentage of HIV-infected adults in our Tanzanian population (9%) with a subtherapeutic plasma concentration of nevirapine [3], as determined by HPLC [6], was lower than the percentage in a previous report from Malawi (16%) [10]. This may be associated with the unreliable drug supply during the time of the Malawian study (2003), which explained more

**Table 1. Number of patients with (sub)therapeutic nevirapine concentrations according to thin-layer chromatography (TLC), compared with according to high-performance liquid chromatography (HPLC), and sensitivity and specificity of the TLC assay.**

Specimen, concentration determined by HPLC	TLC of saliva specimens			
	No. of results		Sensitivity, %	Specificity, %
	Subtherapeutic concentration	Therapeutic concentration		
<b>Saliva</b>				
Subtherapeutic concentration	23	0	100	...
Therapeutic concentration	3	260	...	99
<b>Plasma</b>				
Subtherapeutic concentration	23	2	92	...
Therapeutic concentration	3	258	...	99

**NOTE.** Subtherapeutic concentrations of nevirapine were defined as <1.75 mg/L for TLC of saliva specimens, <1.5 mg/L for HPLC of saliva specimens, and <3.0 mg/L for HPLC of plasma specimens. Therapeutic concentrations were defined as values greater than these cutoff values.

than one-third of the total reported nonadherence to treatment [10]. The fact that 18 of the 25 subtherapeutic nevirapine plasma concentrations in our Tanzanian population were undetectable indicates nonadherence to treatment, because nevirapine is generally detected up to weeks after termination of drug intake because of its long elimination half-life [11].

The median ratio of saliva nevirapine concentration to plasma nevirapine concentration of 0.51:1, as determined by HPLC [6], in our population of HIV-infected Africans was comparable to the ratios observed in HIV-infected [4] and healthy [5] white persons. It has been suggested that thorough rinsing of the mouth is required before saliva sampling, because remnants of orally administered medicines may contaminate saliva specimens and yield spuriously high values [12]. Indeed, 6 of 286 subjects in our study had an unexpectedly high ratio of saliva to plasma nevirapine concentration of >1:1 [4, 12]; this may be because the subjects had chewed tablets without rinsing the mouth before samples were obtained. This explanation is supported by our observation that the time between last ingestion and sampling was shorter for subjects with unexpectedly high nevirapine concentrations in saliva specimens, compared with concentrations in plasma specimens.

Because the saliva nevirapine concentrations determined by HPLC were approximately one-half of the values observed in plasma specimens, they were defined as subtherapeutic if they were <1.5 mg/L rather than if they were less than the cutoff value for plasma (i.e., 3.0 mg/L) [3]. The main requirement of our TLC method for semiquantitative detection of nevirapine in saliva specimens was appropriate sensitivity, meaning detection of nevirapine concentrations that were found to be subtherapeutic by HPLC, to pick out subjects at risk for nevirapine resistance. During the development of our TLC method, subtherapeutic saliva concentrations slightly less than 1.5 mg/L were sufficiently distinguishable from a TLC reference

value of 1.75 mg/L but not from a reference value of 1.5 mg/L. Therefore, for the validation of our TLC method, we chose a reference value of 1.75 mg/L, which led to excellent technical sensitivity (100%) and specificity (99%). None of the subtherapeutic nevirapine saliva concentrations found by HPLC were reported to be therapeutic by TLC, and the few therapeutic saliva concentrations that were reported to be subtherapeutic by TLC were all close to the cutoff value for saliva (1.5 mg/L).

Biological validation compared saliva concentrations of nevirapine determined by TLC with plasma concentrations determined by HPLC (either subtherapeutic or therapeutic levels), finding acceptable sensitivity (92%) and excellent specificity (99%). False-positive and false-negative results were all close to the cutoff value for plasma (3.0 mg/L). The somewhat lower biological sensitivity can be explained by the fact that some subjects with a subtherapeutic plasma concentration determined by HPLC had a therapeutic saliva concentration according to both HPLC and TLC. Although these subjects had a higher ratio of saliva to plasma nevirapine concentration than average, none of them had a ratio >1:1.

As expected, it was found to be most difficult to interpret nevirapine saliva concentrations that were closest to the TLC reference value. To test the worst-case scenario robustness of the TLC method, we selected 25 samples closest to the reference value. The fact that the extent of agreement in TLC results between 5 technicians and 2 batches of TLC sheets was substantial provides an indication that the outcome of our TLC method is reliable during normal use.

We are planning to roll out the TLC method in various African countries and in Indonesia. This will make it possible to continue to evaluate the method's robustness and its practical application to adherence monitoring. Potentially, the assay could be further developed commercially.

In conclusion, we developed a simple and economical TLC

method for semiquantitative determination of saliva concentrations of nevirapine. The assay was found to be sensitive, specific, and robust for detection of subtherapeutic nevirapine concentrations in an African population. It is an attractive alternative to HPLC for TDM of nevirapine in resource-limited settings.

### Acknowledgments

We thank the patients for their participation in this study. Laizer Leshoki Saitory of the Kilimanjaro Christian Medical Centre is kindly acknowledged for the screening of study subjects. The study nurses at Kilimanjaro Christian Medical Centre are thanked for the collection of study samples.

**Financial support.** Poverty Related Infection Oriented Research, a research network sponsored by The Netherlands Foundation for the Advancement of Tropical Research (NWO-WOTRO) and The Netherlands Foundation for Health Research and Development (ZonMw).

**Potential conflicts of interest.** All authors: no conflicts.

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