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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)
method to provide a simple and economical tool for semiquantitative 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 routine visit to the clinic, unannounced paired blood and saliva samples were collected within 5 min of each other. Stimulated

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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 (≥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 $k$ [7]) and 2 different batches of TLC sheets (Cohen’s $k$ [8]) was evaluated in these 25 saliva samples.

The stability of the stock solution (nevirapine in DMSO) was tested after storage at −40°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°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 (≥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 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 \( >1 \) (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 specificity of the TLC method was 100% (table 1). Three of the 263 saliva samples found to have therapeutic nevirapine concentrations (≥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 sensitivity 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...
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.

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