DRUG FORMULATIONS AND CLINICAL METHODS

# An Interlaboratory Investigation on the Use of High-Performance Thin Layer Chromatography to Perform Assays of Lamivudine–Zidovudine, Metronidazole, Nevirapine, and Quinine Composite Samples

ELIANGIRINGA KAALE and PETER RISHA
Muhimbili University of Health and Allied Sciences, School of Pharmacy, Laboratory for Pharmaceutical Analysis, PO Box 65013, Dar es Salaam, Tanzania
EIKE REICH
CAMAG-Laboratory, Sonnenmattstrasse 11, Muttenz, Switzerland
THOMAS P. LAYLOFF
Management Sciences for Health, 4301 N. Fairfax Dr, Suite 400, Arlington, VA

**Two laboratories extensively investigated the use of HPTLC to perform assays on lamivudine–zidovudine, metronidazole, nevirapine, and quinine composite samples. To minimize the effects of differences in analysts' technique, the laboratories conducted the study with automatic sample application devices in conjunction with variable-wavelength scanning densitometers to evaluate the plates. The HPTLC procedures used relatively innocuous, inexpensive, and readily available chromatography solvents used in the Kenyon or the Global Pharma Health Fund Minilabs® TLC methods. The use of automatic sample applications in conjunction with variable- wavelength scanning densitometry demonstrated an average repeatability or within-laboratory RSD of 1.90%, with 73% less than 2% and 97% at 2.60% or less, and an average reproducibility or among-laboratory RSD of 2.74%.**

The HPTLC technology with the automatic sample application devices and variable-wavelength scanning densitometers provide a pharmaceutical analytical assessment capability similar to that obtained with HPLC, but with much simpler and more robust chromatography technology. These HPTLC procedures should be amenable for use in enforcing product quality standards.

Reports have previously described the use of TLC methods with manual sample application and visual comparisons to authenticate materials and to perform pharmaceutical qualitative and quantitative screening assessments (1–5). The RSD values observed with these assessments are generally greater than 10% because of differences in analysts' manual sample spotting techniques and in individual visual acuity in assessing the amounts contained in the developed sample spots (6).

In 1997, the Global Pharma Health Fund (Frankfurt am Main, Germany) introduced the Minilab®, which incorporates this TLC technology platform into a very facile, robust, inexpensive, convenient, and secure system that can be used to detect substandard and counterfeit pharmaceutical products (7). In addition, the Minilab features excellent manuals and presentations, which make it convenient for users to identify needed supplies and to refresh their training. The Minilab platform currently includes assessment methods for over 40 products; the majority of the methods are for products in the World Health Organization essential medicines list. The Minilabs have been implemented successfully not only by laboratory analysts, but also by inspectors specially trained to perform screening tests in areas where laboratory resources are not adequate for monograph testing and trained laboratory analysts are not available. Since its introduction, 70 countries, primarily in Africa and Asia, have put over 300 units into use (7). The wide dissemination of this technology has created a cadre of hundreds of persons who can successfully perform TLC experiments.

Despite the simplicity and potential usefulness of the Minilab technology and the Kenyon methods in identifying wrongly labeled, grossly substandard medicines or counterfeit medicines in resource-poor settings, these procedures cannot be used to support a regulatory compliance action due to inherent limitations in the technology related to the operator's visual acuity and/or proficiency in applying samples on the chromatographic plates (6).

Recently, there have been improvements in technology whereby automatic application devices have made it possible to greatly minimize the variations associated with manual sample application while retaining the advantages associated with TLC: high throughput and simplicity. Furthermore, the skill level required to perform the HPTLC analysis is

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Corresponding author's e-mail: elia.kaale@muhas.ac.tz or elia.kaale@lycos.com

**Table 1. Developing solvent mixture ratios**

| Pharmaceutical product | Solvent mixture ratios | | | | |
|---|---|---|---|---|---|
| | Ethyl acetate | Toluene | Methanol | Ammonia | Acetone |
| Lamivudine–zidovudine | 12 | 5 | 3 | — | — |
| Metronidazole | 50 | — | — | 1 | — |
| Nevirapine | 3 | 1 | | | |
| Quinine | 22 | 3 | — | — | 5 |

essentially the same as that required to perform TLC experiments, so the individuals trained to use Minilabs can, with some minimal additional training, successfully perform HPTLC experiments, potentially a cost benefit for countries where Minilab technology has already been introduced. By using variable-wavelength scanning densitometers instead of visual detection, the precision of the HPTLC-based methods has greatly increased, and individuals skilled in Minilab technology could reliably perform selected pharmaceutical product quality attribute assessments.

HPTLC has increasingly been shown to be a simple and reliable method for determination of pharmaceutical products, both in the bulk as well as in single- or fixed-dose combination dosage forms (8–14). The precision levels achieved by the HPTLC procedures with the improved technologies can approach those levels generally attained with HPLC (15). It should be noted that HPLC procedures may be capable of resolving hundreds of compounds in a single experiment, and this is not achievable with HPTLC. However, both technologies are necessary; the choice between them should be based on the consideration of performance needs and costs.

Recently, simple and precise HPTLC densitometric methods for determination of metronidazole (16), nevirapine (17), a fixed-dose combination of lamivudine and zidovudine (18), and quinine (19) in pharmaceutical dosage forms were developed and extensively validated via single-laboratory validation (SLV) experiments in the Laboratory of Pharmaceutical Analysis (LPA), Muhimbili University of Health and Allied Sciences (MUHAS), Dar es Salaam, Tanzania. These methods were performed according to International Conference on Harmonization (ICH) guidelines for analytical method validation Q2R1.

**Table 2. Detection wavelength by pharmaceutical product**

| Pharmaceutical product | Detection wavelength, nm |
|---|---|
| Lamivudine–zidovudine | 289 |
| Metronidazole | 313 |
| Nevirapine | 289 |
| Quinine | 327 |

To bring the HPTLC technology into wider routine use in analysis, it is necessary to demonstrate that the achievable separation and detection precision in one laboratory can be reliably replicated in another. This work presents the results of an interlaboratory collaboration study using the above-cited methods for analysis of four composite samples of pharmaceutical products, one of them being a fixed-dose combination.

## Experimental

### *Solvents, Standards, and Chemicals*

All solvents used were reagent grade and independently purchased from several suppliers: ethyl acetate, ammonia, acetone, methanol 99%, and toluene (Merck, Darmstadt, Germany). Quinine standard was from BDH Chemicals Ltd (Poole, UK) and metronidazole standard from the *European Pharmacopoeia*, BP907–F67029 (Strasbourg Cedex, France). Nevirapine anhydrous 99.8%, lamivudine, and zidovudine bulk chemicals were donated by local pharmaceutical industries. The water used was either deionized or distilled.

### *Equipment*

The Adventurer analytical balance was purchased from Ohaus Corp. (Pine Brook, NJ). Chromatographic separation was achieved using 20 × 10 cm 200 m silica gel $F_{254}$ 60 HPTLC plates (Merck). Sample solutions were applied on the HPTLC plates using a Linomat 5 applicator (CAMAG, Muttenz, Switzerland). After chromatographic development of the plates, detection was done using a variable-wavelength CAMAG TLC Scanner 3. Data acquisition and calculations were performed using winCATS 1.4.3-Planar Chromatography Manager software (CAMAG).

### *Preparation of Stock and Standard Solutions*

The assay procedures were performed on "unknowns" prepared from weighed portions of ground composites of tablets containing each of the drugs. The lead collaborating laboratory, the LPA in Dar es Salaam, developed and extensively tested the HPTLC method for metronidazole to

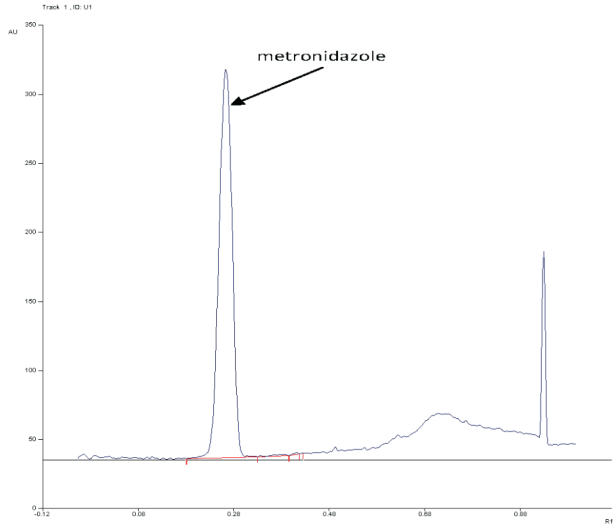


**Figure 1. Typical densitogram of a 5 L application of 0.075 g/ L metronidazole solution; mobile phase is ethyl acetate–ammonia (25 + 0.5, v/v); UV detection at 313 nm.**

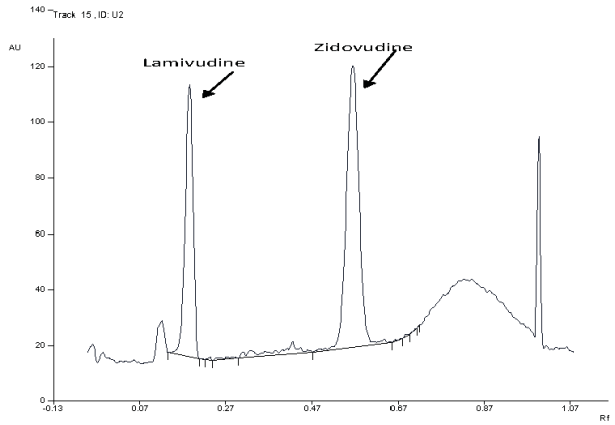


**Figure 2. Typical densitogram of a 5 L application of 0.135 g/ L lamivudine–zidovudine solution; mobile phase is ethyl acetate–toluene–methanol (12 + 5 + 3, v/v/v); UV detection at 289 nm.**

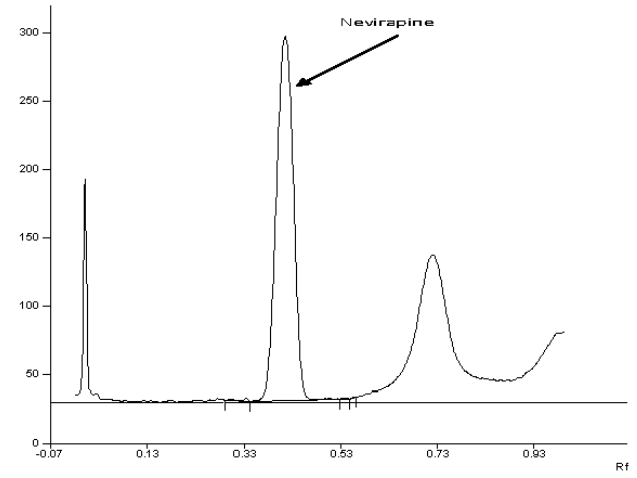


**Figure 3. Typical densitogram of a 5 L application of 0.1 g/ L nevirapine solution; mobile phase is ethyl acetate–toluene (7.5 + 2.5, v/v); UV detection at 289 nm.**

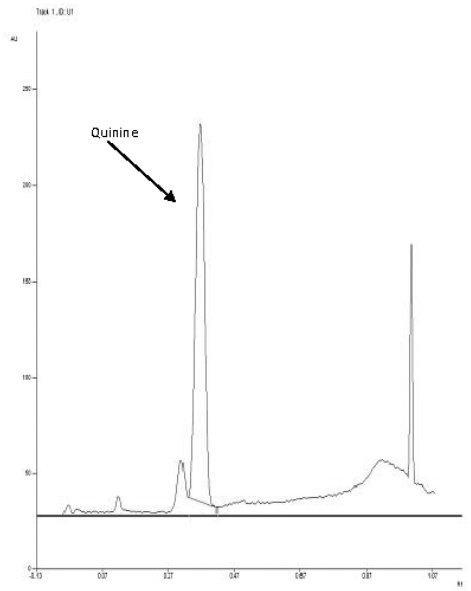


**Figure 4. Typical densitogram of a 5 L application of 0.25 g/ L quinine solution; mobile phase is ethyl acetate–acetone–ammonia (73 + 17 + 3, v/v/v); UV detection at 327 nm.**

work through the experimental protocols that are available upon request from the corresponding author. The method development and extensive SLV experiments were performed according to ICH guidelines for analytical method validation Q2R1, and are described elsewhere (17–20). For all products, the amount of sample loading on the plate was established by setting the 10% assessment level approximately at the LOQ to assure that the amounts of sample over the 50–150% range were within the plate and densitometer performance ranges.

Based on the established working ranges, stock solutions were prepared from accurately weighed portions of each reference material dissolved in methanol. The other standard solutions were prepared from these stock solutions. The stock solutions were prepared by accurately weighing the approximate amounts cited below. The concentrations of the stock solutions and dilutions to the 100% estimate level were 15 mg lamivudine and 30 mg zidovudine diluted to a stock solution concentration of 2.25 mg/mL (100% dilution 0.1125 mg/mL),

**Table 3. Mean $R_f$ values for pharmaceutical products by laboratory**

| | Mean $R_f$ value | | | | |
|---|---|---|---|---|---|
| Laboratory | Lamivudine | Metronidazole | Nevirapine | Quinine | Zidovudine |
| CAMAG | 0.19 | 0.28 | 0.34 | 0.33 | 0.57 |
| LPA | 0.21 | 0.21 | 0.29 | 0.33 | 0.60 |

30 mg metronidazole diluted to a stock solution concentration of 0.75 mg/mL (100% dilution 0.075 mg/mL), 10 mg nevirapine diluted to a stock solution of 1 mg/mL (100% dilution 0.1 mg/mL), and 50 mg quinine diluted to a stock solution concentration of 5 mg/mL (100% dilution 0.25 mg/mL).

Each HPTLC test series was performed on four unknown solutions, each prepared by independently weighing the composite material separately in each of the laboratories. To help assure that the assay standard provided an envelope around the unknown assay value, and to assess the system linearity for each analyte, standard solutions at the 50, 80, 100, 120, and 150% levels anticipated for the unknown value were prepared. These values also spanned the usual 75–125% required for solid pharmaceutical dosage form uniformity assessments. All HPTLC test series were performed on three separate HPTLC plates using the same application format pattern of unknowns and standards.

### *Chromatography*

On each of the HPTLC plates used for chromatographic separation, a development target line was placed 7 cm from the bottom of the plate before adding test solutions. Five L of each test solution were applied as 8 mm bands, 8 mm from the bottom of the plate, using the Linomat 5 applicator. The application of test solutions was initiated 15 mm from the left edge of the plate.

On each of the three plates for each active pharmaceutical ingredient, assay sets were applied in the sequence U1-U2-U3-U4-S1-S2-U1-U2-S3-U3-U4-S4-S5-U1-U2-U3-U4, where S1 is a standard solution at the 50% anticipated level, S2 at the 80% level, S3 at the 100% level, S4 at the 120% level, and S5 at the 150% level, and the U1, U2, U3, and U4 are solutions prepared from separate individual weighings of the composite at the LPA—and in the sequence U5-U6-U7-U8-S6-S7-U5-U6-S8-U7-U8-S9-S10-U5-U6-U7-U8, where S6 is a standard solution at the 50% anticipated level,

**Table 4. Polynomial correlations: 50–80–100–120–150%**

| | | LPA | | CAMAG | |
|---|---|---|---|---|---|
| Product | Plate | Correlation coefficient | RSD, % | Correlation coefficient | RSD, % |
| Lamivudine | 1 | 0.9989 | 2.17 | 0.9992 | 1.95 |
| | 2 | 0.9999 | 0.64 | 0.9998 | 1.10 |
| | 3 | 0.9987 | 2.36 | 0.9999 | 0.72 |
| Metronidazole | 1 | 0.9997 | 0.90 | 0.9999 | 1.65 |
| | 2 | 0.9999 | 0.23 | 0.9998 | 1.66 |
| | 3 | 0.9995 | 1.18 | 0.9997 | 0.99 |
| Nevirapine | 1 | 0.9996 | 1.26 | 0.9999 | 0.63 |
| | 2 | 0.9994 | 1.50 | 0.9997 | 1.16 |
| | 3 | 0.9998 | 0.93 | 1.0000 | 0.14 |
| Quinine | 1 | 0.9992 | 1.50 | 0.9999 | 0.83 |
| | 2 | 0.9999 | 0.43 | 0.9999 | 0.53 |
| | 3 | 0.9997 | 1.26 | 0.9998 | 0.88 |
| Zidovudine | 1 | 0.9996 | 1.31 | 0.9995 | 1.84 |
| | 2 | 0.9995 | 1.40 | 0.9999 | 0.76 |
| | 3 | 0.9996 | 1.27 | 0.9996 | 1.43 |
| Overall average | | 0.9992 | 1.20 | 0.9997 | 1.02 |

**Table 5. Lamivudine–zidovudine assay**

| | Found, % | | | Found, % | |
|---|---|---|---|---|---|
| | Lamivudine | Zidovudine | | Lamivudine | Zidovudine |
| | LPA Plate 1 | | | CAMAG Plate 1 | |
| U1 | 106.14 | 109.76 | U5 | 100.39 | 97.03 |
| U2 | 103.66 | 107.07 | U6 | 99.90 | 103.79 |
| U3 | 102.83 | 108.21 | U7 | 103.61 | 106.57 |
| U4 | 101.5 | 103.56 | U8 | 102.89 | 109.27 |
| Average, % | 103.53 | 107.15 | | 101.69 | 104.16 |
| RSD, % | 1.88 | 2.45 | | 1.80 | 5.08 |
| | LPA Plate 2 | | | CAMAG Plate 2 | |
| U1 | 105.87 | 104.92 | U5 | 97.06 | 106.01 |
| U2 | 101.35 | 106.01 | U6 | 99.08 | 109.08 |
| U3 | 104.79 | 108.09 | U7 | 100.79 | 109.29 |
| U4 | 100.41 | 103.72 | U8 | 100.52 | 109.44 |
| Average, % | 103.10 | 105.68 | | 99.36 | 108.46 |
| RSD, % | 2.55 | 1.75 | | 1.72 | 1.51 |
| | LPA Plate 3 | | | CAMAG Plate 3 | |
| U1 | 108.58 | 109.73 | U5 | 100.97 | 104.24 |
| U2 | 107.22 | 108.03 | U6 | 100.75 | 106.19 |
| U3 | 107.66 | 107.87 | U7 | 103.74 | 106.59 |
| U4 | 102.97 | 104.55 | U8 | 102.55 | 106.83 |
| Average, % | 106.60 | 107.55 | | 102.00 | 105.96 |
| RSD, % | 2.33 | 2.17 | | 1.38 | 1.11 |

| LPA | | CAMAG | |
|---|---|---|---|
| Lamivudine average, % | 104.4 | Lamivudine average, % | 101.0 |
| Lamivudine RSD, % | 2.58 | Lamivudine RSD, % | 1.92 |
| Zidovudine average, % | 106.8 | Zidovudine average, % | 106.2 |
| Zidovudine RSD, % | 2.05 | Zidovudine RSD, % | 3.26 |

| Interlaboratory summary | | | |
|---|---|---|---|
| Lamivudine average, % | 102.7 | Zidovudine average, % | 98.92 |
| Lamivudine RSD, % | 2.88 | Zidovudine RSD, % | 3.21 |

S7 at the 80% level, S8 at the 100% level, S9 at the 120% level, and S10 at the 150% level, and the U5, U6, U7, and U8 are solutions prepared from separate individual weighings of the composite at CAMAG. All standard solutions were prepared by dilutions from a single stock solution of a highly purified portion or reference standard of the active pharmaceutical ingredient.

A CAMAG twin-trough chamber for 20 × 10 cm plates was used for development. Prior to development, the chambers were saturated with developing solvent and a filter paper, which was placed in the rear trough. The filter paper was wetted homogenously with 20 mL developing solvent (mobile phase). The liquid was distributed equally between both troughs by tilting the chamber. After 20 min saturation, the plates were developed by placing them upright into the front trough of the chamber so that the stationary phase faced the inside of the chamber. The compositions of the developing solvents are presented in Table 1.

### *Chromatographic Detection*

After development to the target mark, the plates were removed and allowed to dry. After drying, the plates were

**Table 6. Metronidazole assay**

| Found, % | | | |
|---|---|---|---|
| LPA Plate 1 | | CAMAG Plate 1 | |
| U1 | 97.95 | U5 | 96.97 |
| U2 | 99.92 | U6 | 97.41 |
| U3 | 99.46 | U7 | 100.89 |
| U4 | 103.35 | U8 | 99.39 |
| Average, % | 100.17 | | 98.67 |
| RSD, % | 2.28 | | 1.84 |
| LPA Plate 2 | | CAMAG Plate 2 | |
| U1 | 97.38 | U5 | 95.21 |
| U2 | 98.65 | U6 | 96.58 |
| U3 | 99.28 | U7 | 98.49 |
| U4 | 101.28 | U8 | 99.38 |
| Average, % | 99.15 | | 97.41 |
| RSD, % | 1.64 | | 1.93 |
| LPA Plate 3 | | CAMAG Plate 3 | |
| U1 | 96.78 | U5 | 95.69 |
| U2 | 98.42 | U6 | 95.70 |
| U3 | 97.83 | U7 | 98.86 |
| U4 | 102.64 | U8 | 99.16 |
| Average, % | 98.92 | | 97.35 |
| RSD, % | 2.60 | | 1.97 |
| Summary | | | |
| LPA | | CAMAG | |
| Average, % | 99.41 | Average, % | 97.81 |
| RSD, % | 2.08 | RSD, % | 1.81 |
| Interlaboratory summary | | | |
| Average, % | 98.61 | RSD, % | 2.09 |

**Table 7. Nevirapine assay**

| Found, % | | | |
|---|---|---|---|
| LPA Plate 1 | | CAMAG Plate 1 | |
| U1 | 102.28 | U5 | 96.01 |
| U2 | 103.99 | U6 | 98.33 |
| U3 | 101.17 | U7 | 94.55 |
| U4 | 99.51 | U8 | 96.56 |
| Average, % | 101.7 | | 96.36 |
| RSD, % | 1.85 | | 1.62 |
| LPA Plate 2 | | CAMAG Plate 2 | |
| U1 | 101.77 | U5 | 95.57 |
| U2 | 104.20 | U6 | 97.27 |
| U3 | 100.54 | U7 | 95.00 |
| U4 | 100.35 | U8 | 95.03 |
| Average, % | 101.7 | | 95.72 |
| RSD, % | 1.74 | | 1.11 |
| LPA Plate 3 | | CAMAG Plate 3 | |
| U1 | 102.23 | U5 | 95.96 |
| U2 | 102.69 | U6 | 98.33 |
| U3 | 100.95 | U7 | 95.01 |
| U4 | 101.32 | U8 | 95.38 |
| Average, % | 101.79 | | 96.17 |
| RSD, % | 0.78 | | 1.55 |
| Summary | | | |
| LPA | | CAMAG | |
| Average, % | 101.75 | Average, % | 96.08 |
| RSD, % | 1.39 | RSD, % | 1.34 |
| Interlaboratory summary | | | |
| Average, % | 98.92 | RSD, % | 3.21 |

scanned using the indicated wavelength for each compound using a CAMAG TLC Scanner 3 (Table 2). The detector slit parameters were set at 6 × 0.45 mm, scanning speed at 20 mm/s, and data resolution at 100 m/step. The data capture and calculations obtained from the scanned plates were performed with the CAMAG winCATS 1.4.3-Planar Chromatography Manager software.

## Results and Discussion

The chromatograms obtained for each drug are shown in Figures 1 to 4, and the $R_f$ values observed with these developing solvent systems are given in Table 3. The standard data were fitted to linear and polynomial correlation models using the LPA data, and the polynomial model was selected to perform the calculations because the correlation coefficient and SD of the fit were better. The average linear correlation coefficient was 0.9974 versus 0.9992 for the polynomial correlation coefficient, and the average RSD was 2.26% for the linear and 1.20% for the polynomial fits. The polynomial correlation coefficients and RSDs are presented in Table 4.

The percentage assay and SD data determined for the composite samples are summarized by sample in Tables 5–8.

**Table 8. Quinine assay**

| Found, % | | | |
|---|---|---|---|
| LPA Plate 1 | | CAMAG Plate 1 | |
| U1 | 95.3 | U5 | 98.95 |
| U2 | 92.18 | U6 | 98.92 |
| U3 | 94.35 | U7 | 101.33 |
| U4 | 95.25 | U8 | 100.45 |
| Average, % | 94.27 | | 99.91 |
| RSD, % | 1.54 | | 1.19 |
| LPA Plate 2 | | CAMAG Plate 2 | |
| U1 | 98.33 | U5 | 96.40 |
| U2 | 94.39 | U6 | 95.81 |
| U3 | 94.56 | U7 | 98.00 |
| U4 | 96.02 | U8 | 99.49 |
| Average, % | 95.82 | | 97.42 |
| RSD, % | 1.90 | | 1.70 |
| LPA Plate 3 | | CAMAG Plate 3 | |
| U1 | 100.07 | U5 | 96.72 |
| U2 | 94.65 | U6 | 96.21 |
| U3 | 97.43 | U7 | 99.11 |
| U4 | 95.49 | U8 | 98.69 |
| Average, % | 96.91 | | 97.68 |
| RSD, % | 2.48 | | 1.47 |
| Summary | | | |
| LPA | | CAMAG | |
| Average, % | 95.67 | Average, % | 98.34 |
| RSD, % | 2.17 | RSD, % | 1.78 |
| Interlaboratory summary | | | |
| Average, % | 97.00 | RSD, % | 2.39 |

The results were calculated using the CAMAG winCATS 1.4.3-Planar Chromatography Manager software.

Two laboratories collaborated on this study—the very experienced CAMAG laboratory in Muttenz, Switzerland, and the newly formed LPA at the Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania. The LPA developed the study in conjunction with CAMAG, prepared the composite samples used in the study, and furnished all of the reference materials. The laboratories used HPTLC plates from the same supplier and solvents and development chambers from their usual laboratory stocks.

The within-laboratory RSD for the four separate composite weighings for each development plate calculated from the polynomial correlation obtained from the five standard levels ranged from 0.98–5.08% with 73% of the RSD values less than 2.00 and 97% of the RSD values at 2.60% or less. To simulate a real-life situation, no experiments were repeated to improve values. The overall average RSD was 1.90%, and the overall average interlaboratory RSD was 2.74% with a range of 2.09–3.21%.

The differences observed in $R_f$ values at the participating laboratories can be explained by the effects of relative humidity on the plate moisture levels. However, these differences did not affect the quality of the assessments or correlations.

## Conclusions

TLC and the related HPTLC are among the most facile, robust, and easily learned chromatographic procedures. The capillary action development eliminates the need for pumps, valves, etc., that are required to perform HPLC, and which are difficult to maintain in nonindustrialized economies. In addition, the chromatographic medium is single use, which eliminates medium maintenance issues. The densitometer and spotting device used in these studies are also very robust and easily learned and maintained; the densitometer has maintenance requirements similar to other spectrometers, and none of its parts is exposed to analytes or solvents.

These experiments have shown that, where applicable, the HPTLC technology with the spotting device and densitometer provides analytical results comparable to HPLC. The linearity demonstrated that the technique can be successfully applied to determine dosage uniformity and to perform dissolution pharmaceutical assessments in addition to assays. The technique also can be used for fixed-dose-combination drug products. The successful collaboration between the LPA and CAMAG is further evidence of the technologies' robustness and also demonstrates that it can be successfully used to control commerce and in forensic applications.

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