Vol. 17 (2014) 70-78

Stability Indicating Liquid Chromatographic Method for Determination of Lamivudine and Tenofovir Disoproxil Fumarate in Fixed Dose Combination Formulations

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This study describes the development and validation of a stability indicating high performance liquid chromatographic method for the analysis of lamivudine and tenofovir disoproxil fumarate and their degradants. The method uses a Reprosil®-pur basic C18 column (250 mm × 4.6 mm, 5 µm) maintained at 30°C, methanol and a mixture of buffers (2.3 g/L ammonium dihydrogen phosphate and 1.32 g/L of *di*ammonium hydrogen phosphate, pH 3.9) for gradient elution at a flow rate of 1.0 mL/min, and UV detection at 270 nm. Good separation of lamivudine and tenofovir disoproxil fumarate and their potential impurities was achieved. The stability indicating ability of the developed method was validated by subjecting both active ingredients to hydrolytic and oxidative stress conditions and separating the degradation products from their respective intact drugs. The calibration curve was linear over the 80-120 µg/mL concentration range for both active ingredients with r^2> 0.99. A recovery rate of 99.8 % for lamivudine and 99.3 % for tenofovir disoproxil fumarate confirmed the accuracy of the method for the simultaneous determination of both drugs in the fixed-dose combination.

Key words: Stability indicating liquid chromatography, lamivudine, tenofovir, validation

INTRODUCTION

Products formulated with more than one active pharmaceutical ingredient (API), typically referred to as fixed-dose combinations (FDCs), are intended to fulfil unmet patients' needs by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods [1,2]. Lamivudine/tenofovir disoproxil fumarate formulation is one such FDC used for the management of Human Immunodeficiency Virus. The chemical structures of lamivudine and tenofovir disoproxil fumarate are given in Figure 1.

Lamivudine is also referred generically as (-)-2′,3′-dideoxy-3′-thiacytidine while its chemical name is (-)-4-amino-1-[(2*R*,5*S*)-2-(hydroxyl-methyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one [3]. Lamivudine is a synthetic nucleoside that is phosphorylated intracellularly to the active lamivudine 5'-triphosphate metabolite. Lamivudine triphosphate competes with the natural substrate deoxycytidine triphosphate for incorporation into viral DNA by reverse transcriptase and once incorporated, causes premature termination of viral DNA synthesis.

Tenofovir disoproxil fumarate is a fumaric acid salt of the bis-isopropoxycarbonyl-oxymethyl ester derivative of tenofovir. The chemical name of tenofovir disoproxil fumarate is 9-[(*R*)-2[[bis[[(isopropoxy-carbonyl)oxy]-methoxy]phosphinyl]methoxy]-propyl]adenine fumarate (1:1) [4]. Tenofovir disoproxil is an oral prodrug of tenofovir developed to increase oral bioavailability of tenofovir [5].

High performance liquid chromatography (HPLC) has increasingly been shown to be a selective and sensitive method for stability studies of pharmaceutical products, both in the bulk as well as in single and FDC dosage forms due to increased separation efficiency of the stationary phase compared to other chromatographic techniques [6, 7]. So far, no liquid chromatographic (LC) stability indicating method has been reported for purity

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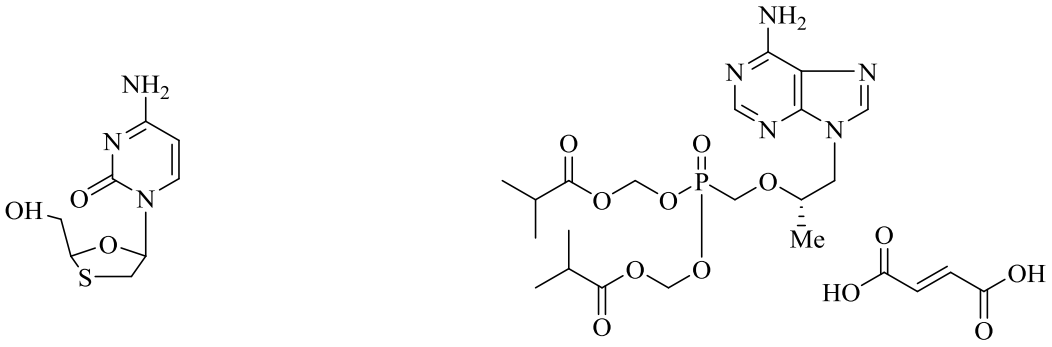
control and assay of tenofovir disoproxil fumarate and lamivudine in the literature. Some LC methods with different detection systems have been described for the quantitative determination of tenofovir and lamivudine in human plasma. These include LC with mass spectrometric (MS) detection whereby spray ionization was operated in multiple-reaction monitoring mode for the analysis [8]. However, no gradient LC methods for purity control have been published in any pharmacopoeia for analysis of lamivudine and tenofovir disoproxil fumarate (LT) formulations.



Lamivudine **Tenofovir disoproxil fumarate**

Figure 1. Chemical structures of lamivudine and tenofovir disoproxil fumarate.

To establish the stability study, a forced degradation study was used to test the selectivity of lamivudine and tenofovir disoproxil fumarate product and its degradants. The study was conducted by subjecting the product of lamivudine and tenofovir disoproxil fumarate in various stress conditions such as 0.1 N hydrochloric acid, 0.1 N sodium hydroxide and 3% hydrogen peroxide [9,10]. Therefore the aim of this study was to develop and validate lamivudine and tenofovir disoproxil fumarate stability indicating assay in line with specificity, linearity, precision, accuracy and robustness according to ICH and USP guidelines [11-14].

EXPERIMENTAL

Reagents and chemicals

HPLC-grade methanol, sodium hydroxide pellets and hydrochloric acid were purchased from Scharlau Chemie (Scharlau, Spain), whereas hydrogen peroxide, ammonium dihydrogen phosphate and *di*ammonium hydrogen phosphate were products of May & Baker (Dagenham, England). Trifluoroacetic acid was from Carlo Erba (Strada, Rivoltana, Spain). Filtered demineralised water was used. Tenofovir disoproxil fumarate and lamivudine commercial samples (drug substances and tablets) were obtained from Hetero Labs Limited (Hyderabad, India).

Preparation of assay solutions

All samples and reference standard solutions were prepared in methanol immediately prior to use. Standard solutions of tenofovir disoproxil fumarate and lamivudine were prepared by dissolving 50 mg of tenofovir disoproxil fumarate and 50 mg of lamivudine in 50 ml methanol and diluted to a final concentration of 0.1 mg/mL.

A total of 20 tablets of LT were weighed and finely powdered using mortar and pestle. A 50 mg powder sample was weighed into a 50 ml volumetric flask followed by the addition of the diluent. The resulting solution was shaken, ultrasonicated for 15 min, made to volume using the diluent and then filtered. For chromatography, the filtrate was diluted in the ratio of 1:10 v/v with the diluents.

Instrumentation and chromatography

The HPLC analyses were performed using Lab Solution LC-2010 CHT equipment I (Shimadzu, Japan) on a Reprosil®-pur basic C18 column 250 mm × 4.6 mm, 5 µm maintained at 30°C, mobile phase flow-rate of 1.0 mL/min and UV detection at 270 nm. The HPLC data acquisition was supported by Lab Solution 5.35 SP3 software (Shimadzu, Japan). For intermediate precision studies, analyses were performed by a second analyst using the

same LC equipment and under the same experimental conditions. The pH measurements were performed using a Hanna HI 211 pH meter (Rhodes Island, U.S.A.).

Mobile phase

Since gradient elution was applied (Table 1), two mobile phases were used: methanol as mobile phase A and a mixture of buffers (2.3 g/L ammonium dihydrogen phosphate and 1.32 g/L of *di*ammonium hydrogen phosphate, pH 3.9) as mobile phase B. The buffer was prepared by dissolving 2.3 g of ammonium dihydrogen phosphate and 1.32 g of *di*ammonium hydrogen phosphate in 800 mL of purified water, adjusting the pH to 3.9 by adding 50% trifluoroacetic acid and diluting to 1000 mL with purified water.

Table 1: Elution programme for the analysis of lamivudine and tenofovir disoproxil fumarate

Time (min)	Mobile phase A	Mobile phase B	Elution
0-15	97	3	Isocratic
15-70	97	3	Isocratic
70-80	50	50	Linear gradient
80-82	40	60	Return to initial conditions
82-90	97	3	Isocratic re-equilibration

Forced degradation studies

The drug substance was subjected to forced degradation under acidic, basic and neutral conditions. The acidic (0.1 M HCl) and basic (0.1 M NaOH) hydrolyses were carried out by refluxing in a water bath for 60 min. Before injection into the chromatograph, the solutions were neutralized using either NaOH or HCl. Oxidative stress studies were carried out at room temperature for up to 1 h in 3% H_2O_2. For all degradation studies in solution, 1 mg/mL drug concentration was used.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT

An LC method described by Reddiah *et al.* was used as a starting point [15]. Since this method was able to separate lamivudine in lamivudine-zidovudine-abacavir combination, the separation conditions of the method were improved to make it suitable for separation of both tenofovir disoproxil fumarate and lamivudine. The method uses an Intensil® ODS-3V (250 mm × 4.6 mm, 5µm) column at 50°C and sample compartment at 5°C. Two mobile phases were used: methanol as mobile phase A and a mixture of buffers (2.3 g/L ammonium dihydrogen phosphate and 1.32 g/L of *di*ammonium hydrogen phosphate, pH 3.9) mobile phase B. The gradient used was: 0 min 97/3; 15 min 97/3; 70 min 60/40; 80 min 40/60; 82 min 97/3; and 90 min 97/3.

Several trials were conducted using the adopted method. It was observed that at 70 min (60/40), there was poor separation of tenofovir disoproxil fumarate and hence the gradient system was adjusted to enhance the separation (Figure 2). Chromatographic conditions were optimized by changing the gradient composition at 70 min to 50/50 and reducing the column temperature to 30°C due to the instability of tenofovir. Different experiments were performed to optimize the elution gradient and adequate separation of the two drugs. The optimized mobile phase was composed of methanol as mobile phase A and mixture of buffers (2.3 g/L ammonium dihydrogen phosphate and 1.32 g/L of *di*ammonium hydrogen phosphate, pH 3.9) as mobile phase B.

METHOD VALIDATION

Method robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small changes. When the method is applied place in a different laboratory by different analysts and equipment. In this study, the influence of three chromatographic parameters on the separation was investigated. The

parameters examined were the flow rate of the mobile phase, the column temperature and the pH of the mobile phase. Each of these parameters was investigated at three levels, low (-1), central (0) and high (+1) (Table 2). Their effects on the separation, between lamivudine and tenofovir were evaluated by means of a central composite face centered design using Modde 4.0 statistical graphic software (Umetrics, Umea, Sweden). A central composite face centred design which prescribes 17 experiments was applied.

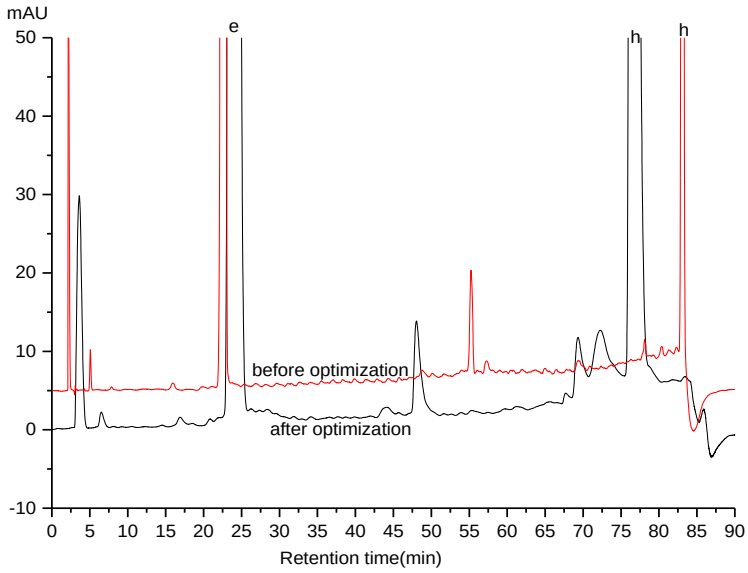


Figure 2. Lamivudine (e) and tenofovir disoproxil fumarate (h) before and after method optimization.

The central composite design permitted the response surface to be modelled by fitting a second-order polynomial model (Figure 3). A positive effect means that an increase of the factor value increases the response while a negative gives the opposite response. The interactive effect of temperature and pH on the theoretical plates of lamivudine showed maximum response at intermediate pH and lowest extreme of temperature. For TDF, temperature had negligible effect on k' while a decrease in pH caused a significant decrease with least effect observed at intermediate pH. However, the tailing factor for TDF decreased with increased temperature and was highest at intermediate pH (Figure 3).

Specificity and selectivity

Excipients mixture that were used for preparation of LT tablets without the APIs was used as a placebo in order to check possible interference with the analyte peaks during analysis. No interference was observed between analytes, placebo and solvent chromatograms (Figure 4).

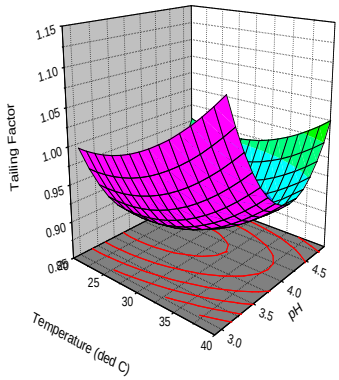
Table 2: Chromatographic parameters investigated for robustness

Parameter	Low level (-1)	Central level (0)	High level (1)
pH	2.9	3.9	4.9
Temperature (°C)	20	30	40
Elution time (min)	10	15	20

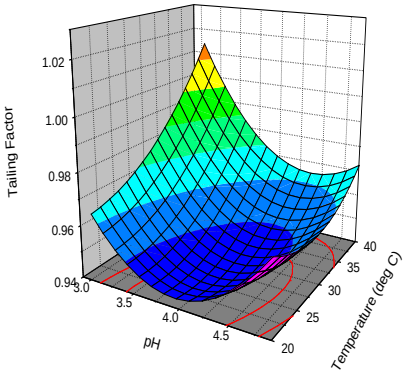
Linearity

Evaluation of linearity of lamivudine and tenofovir disoproxil fumarate (Table 3) assay were demonstrated by preparing five standard concentrations in the range 80-120% using serial dilutions from stock solution. The solutions were applied on LC for each

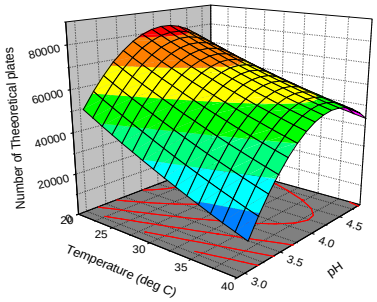
concentration starting with lowest concentration to avoid carryover effect. The procedure was repeated three times and peak areas were calculated and analysed from the developed peaks. The correlation coefficient of 0.995 for lamivudine and 0.998 for tenofovir disoproxil fumarate were obtained as shown in Table 3. The results indicate that the method is linear in the range investigated.



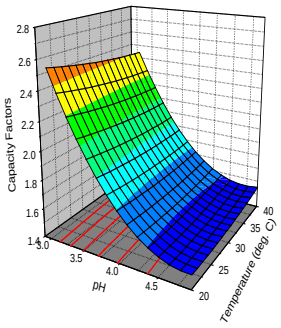
Lamivudine



Tenofovir disoproxil fumarate



Lamivudine



Tenofovir disoproxil fumarate

Figure 3. Response surface plots of the number of theoretical plates, tailing and capacity factors between lamivudine and tenofovir disoproxil fumarate examined. Other parameters were kept constant at their central value.

Table 3: Linear regression equation for lamivudine and tenofovir disoproxil fumarate

Parameter	Lamivudine	Tenofovir disoproxil fumarate
Concentration range (µg/ml)	80-120	80-120
Slope	2e+7	6e+7
y-Intercept	-40584	-76984
r^2	0.998	0.995

n = 5.

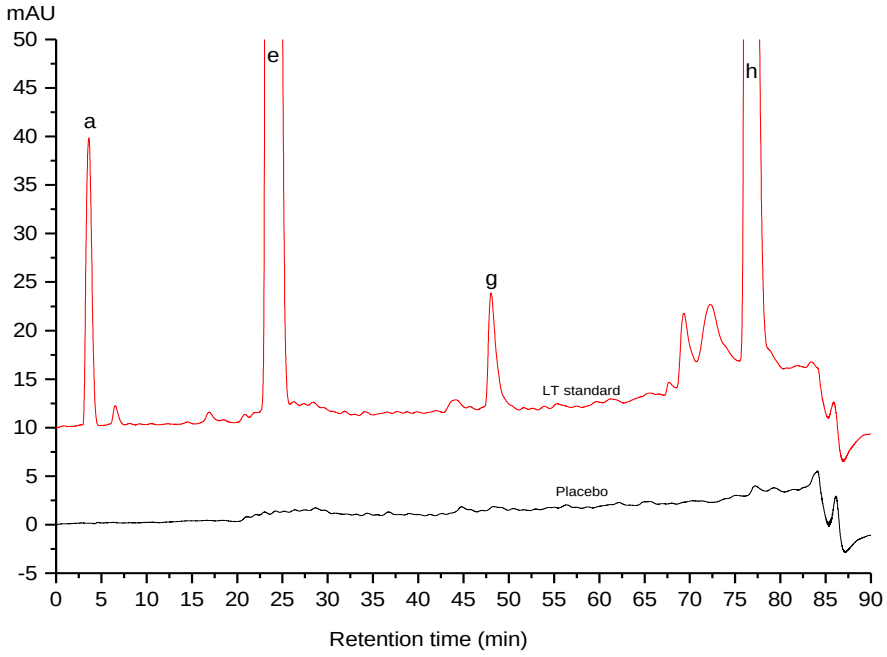


Figure 4. Overlaying chromatograms obtained for placebo and lamivudine/tenofovir disoproxil fumarate standard.

Precision

Repeatability and intermediate precision (Table 4) were performed by independently preparing six replicate sample solutions equivalent to 0.1 mg/mL lamivudine, and 0.1 mg/ml tenofovir disoproxil fumarate. Intermediate precision was done using two analysts on different days. The mean, standard deviation and calculated percentage relative deviations (% RSD) of peak responses were evaluated (Table 4). The % RSD from peak areas were found to be 0.19 for lamivudine and 0.61 for tenofovir disoproxil fumarate as calculated by analyst I whereas 0.38 % RSD for lamivudine and 0.56 % RSD for tenofovir disoproxil fumarate were obtained by analyst II. The RSD values for tenofovir disoproxil fumarate and lamivudine were less than 2 %, indicating that the method is precise.

Table 4: Repeatability and intermediate precision of lamivudine and tenofovir disoproxil fumarate in tablets using two analysts at nominal concentration

Precision	Analyst/day	Parameter	Lamivudine	TDF
Repeatability	Analyst 1 on Day 1	% Concentration level	100.00	100.00
		Mean	6047943.75	2067569
		SD	11904.65	12616.67
		% RSD	0.196	0.610
	Analyst 2 on Day 2	% Concentration level	100.00	100.00
		Mean	6043162.5	2044654.17
		SD	222789.23	11559.72
		% RSD	0.377	0.56
Intermediate precision	Two different days	% Concentration level	100.00	100.00
		Mean	6045552	2039260
		SD	17513.86	17376.35
		% RSD	0.289	0.852

SD = standard deviation; RSD = relative standard deviation; TDF = tenofovir disoproxil fumarate; n = 6 (Repeatability); n = 12 (intermediate precision).

Accuracy

Accuracy was evaluated using recovery method by spiking APIs in the placebo (Table 5). Three solutions of the controls were prepared at each level of the concentration namely 80%, 100% and 120%, by independently weighing each analyte in triplicate. Accuracy was determined by evaluating the mean recovery of the analyte at these three levels from a spiked placebo solution. The controls were weighed in triplicate for each concentration and injected in triplicate. Assay of sample tablet was derived as percentage assay at 100%, which was determined using the calibration curve. The accuracy was within limits at concentration of 95% and 105% according to USP2 specifications.

Table 5: Accuracy determinations of lamivudine and tenofovir disoproxil fumarate from a placebo mixture spiked with lamivudine/tenofovir reference standard

Concentration level	% Recovery (% RSD)		
	Lamivudine	Tenofovir disoproxil fumarate	n
80%	100.41 (0.37)	100.73 (0.74)	3
100%	100.46 (0.81)	100.04 (0.95)	3
120%	99.5 (0.55)	99.7 (0.31)	3

n = 9; RSD = relative standard deviation.

Degradation behaviour

Both lamivudine and tenofovir disoproxil fumarate were found to be relatively stable under oxidative stress conditions with recoveries of 98% for tenofovir disoproxil fumarate and 96% for lamivudine. Tenofovir disoproxil fumarate was found to be unstable under acidic and basic conditions. The drug was completely degraded in both conditions while lamivudine remained stable with a recovery of about 98%. New unknown peaks a-g were seen in the chromatograms of alkaline, acidic and oxidative degraded samples of the drug (Figure 5) as compared to the standard LT.

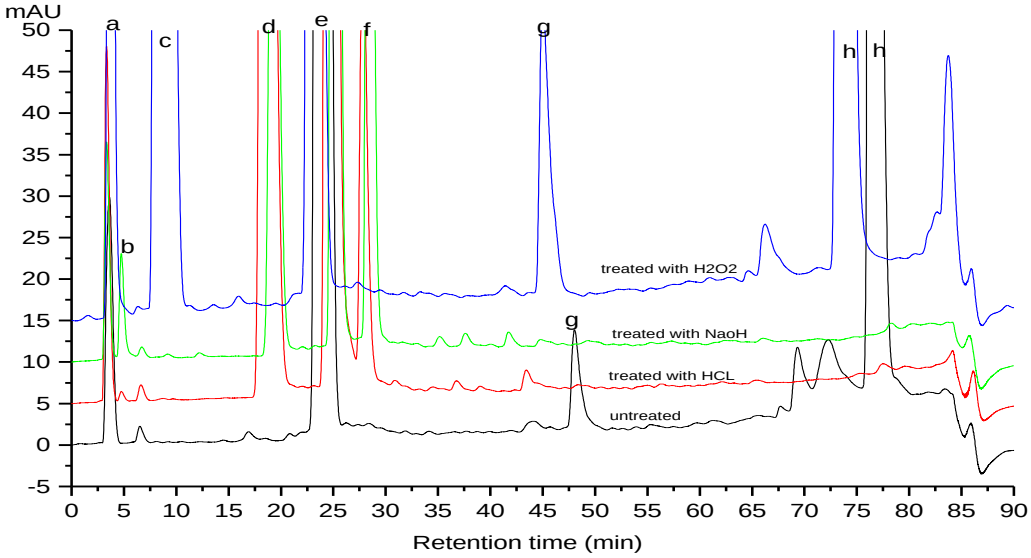


Figure 5. Comparisons of chromatograms obtained before treated and after treated for lamivudine, tenofovir disoproxil fumarate and LT to different stress conditions. Acid hydrolysis for 2 h in a boiling water bath, base hydrolysis for 2 h in boiling water bath and oxidative stress in 3% H_2O_2 for 2 h at room temperature, where e and h are lamivudine and tenofovir disoproxil fumarate; a, b, c, d, f and g are unknown degradants.

The developed method was applied in the assay of commercial tablets of the two drugs. The results obtained met the USP acceptance criteria of between 95% and 105%. The developed method was found to be simple, rapid, sensitive, accurate, precise and specific (Tables 3, 4 and 5), for the determination of LT as well as stability testing of pharmaceutical dosage forms.

CONCLUSION

The developed gradient HPLC method for the simultaneous determination of lamivudine and tenofovir disoproxil fumarate in a pharmaceutical dosage form is specific, precise, accurate, linear and robust. An excellent correlation exists between peak area and concentration for the two drugs. The developed method is stability indicating and can be conveniently used by quality control outfits to determine the contents of LT simultaneously in routine and stability analyses.

ACKNOWLEDGEMENT

We wish to thank Action Medeor Tanzania country office for financial support by providing the reference standards and reagents. We also acknowledge the technical contribution from the staff of Pharm R&D Lab, Muhimbili University of Health and Allied Sciences (MUHAS), Mr. Prosper Tibalinda, Ms. Ruth Ng'wananogu, Ms. Bertha Francis, and Mr. Maro Mhando of Tanzania Pharmaceutical Industries for allowing us to use their HPLC.

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