Use of a model procedure for transfer of Minilab qualitative screening TLC methods for lumefantrine and artemether in a combined tablet formulation to individual and simultaneous quantitative HPTLC-densitometry methods

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ABSTRACT
Individual and simultaneous quantitative high performance thin layer chromatography (HPTLC)-densitometry methods for lumefantrine and artemether in a combined tablet formulation were developed by transfer of qualitative screening methods contained in the Global Pharma Health Fund E. V. Minilab manual using a model procedure published earlier including sample and standard solution preparation, calibration curve establishment, assay of pharmaceutical products versus the label values, and validation of precision, accuracy, and sample peak purity and identification. The new methods involve use of inexpensive, relatively nontoxic and readily available “green” mobile phase solvents specified for Minilab methods, EMD Millipore Corp. Premium Purity HPTLC silica gel 60 F_{254} glass plates, automated standard and sample solution application with a CAMAG Linomat 4, and automated densitometry for detection, identification, and quantification with a CAMAG Scanner 3. Direct transfer of individual Minilab methods for the two individual drugs as well as modifications made in developing the simultaneous method are reported. To our knowledge, no previous quantitative HPTLC simultaneous method using the Minilab mobile phases and our reagent free thermochemical activation detection method for artemether has been reported for a combined dosage form with lumefantrine.

KEYWORDS: artemether, lumefantrine, thin-layer chromatography, densitometry, pharmaceutical product analysis, fake drugs, transfer of qualitative TLC methods to HPTLC-densitometry

INTRODUCTION
Development of a model procedure for transfer of thin layer chromatography (TLC) screening methods used to identify pharmaceutical products with quality defects, to high performance TLC (HPTLC)-densitometry methods that can be used to support regulatory sanctions was reported in three earlier papers [1-3]. The model method includes assay of the drug active ingredients relative to label value, evaluation of precision of replicate analyses and accuracy using the standard addition method, and peak purity and identification tests. The TLC screening methods are contained in the Global Pharma Health Fund E. V. (GPHF) Minilab manual [4] for use in developing countries to help detect mislabeled, substandard, and fake pharmaceutical products. In this paper, we report the use of the model procedure to transfer Minilab individual TLC methods for the anti-malaria drugs artemether (CAS No. 71963-77-4) and lumefantrine (CAS No. 82186-77-4) in a combination tablet dosage form to individual and simultaneous HPTLC-densitometry methods.

EXPERIMENTAL
Standard and sample solutions were prepared for the two drugs as described in the next section.
Detailed general sample preparation methods for the preparation of these solutions as described earlier [1-3] were used unless otherwise noted.

**Standard and sample preparation**

For the artemether individual method, 10.0 mg of artemether standard (Sigma-Aldrich, Co., St. Louis, MO, USA; No. A9361) was dissolved in 25.0 mL of acetone to prepare the 100% Standard Solution at a concentration of 0.400 µg/µL. A combination tablet with a label declaration of 20 mg of artemether and 120 mg of lumefantrine (Ipca Laboratories Ltd., Mumbai, India) was ground using a mortar and pestle and dissolved in 50.0 mL of acetone using magnetic stirring and sonication for 10 minutes each to prepare the 100% Sample Solution with a theoretical concentration of 0.400 µg/µL relative to the label value. This solution was syringe filtered to remove undissolved inert ingredients prior to HPTLC analysis.

For the lumefantrine individual method, 16.0 mg of lumefantrine standard (Sigma-Aldrich No. L5420) was dissolved in 100 mL of acetone to prepare the 100% Standard Solution with a concentration of 0.160 µg/µL. A combination tablet was ground and dissolved in 100 mL of acetone, a portion of the solution was syringe filtered, and a 1.00 mL aliquot of filtrate was diluted with 6.50 mL of acetone to make the 100% Sample Solution with a theoretical concentration of 0.160 µg/µL.

For the simultaneous method, 13.3 mg of artemether standard was dissolved in 100 mL of acetone to make the 0.133 µg/µL 100% Standard Solution, and 8.00 mg of lumefantrine standard was dissolved in 10.0 mL of acetone to make a separate 0.800 µg/µL 100% Standard Solution. A combination tablet was ground and dissolved in 50.0 mL of acetone, a portion was syringe filtered, and a 2.00 mL aliquot of filtrate was diluted with 4.00 mL of acetone to make the 100% Sample Solution with theoretical concentrations of 0.800 µg/µL lumefantrine and 0.133 µg/µL artemether.

**HPTLC**

HPTLC-densitometry for the tablet formulation analysis was carried out as described in detail earlier [1-3] using EMD Millipore Corp., Billerica, MA, USA (a division of Merck KGaA, Darmstadt, Germany) silica gel 60 F254 Premium Purity HPTLC glass plates (20 x 10 cm, Part No. 1.05648.0001) without prewashing. The 100% Standard Solutions were applied in 7.00, 9.00, 11.0 and 13.0 µL aliquots (representing 70-130% content versus the drug label value) for the two individual methods, and these volumes of the two 100% Standard Solutions were over-spotted on lane origins for the combination method; 10.0 µL aliquots of the 100% Sample Solutions were applied for assay of each of three different tablets in triplicate (n = 3). Standard and sample solutions were applied on the plate in 6 mm length bands using a CAMAG (Wilmington, DE, USA) Linomat 4 spray-on bandwise applicator equipped with a 100-µL syringe with an application rate of 4 s/µL. Plates were developed in a vapor saturated CAMAG HPTLC twin trough chamber with ethyl acetate-glacial acetic acid-toluene (4:2:18) mobile phase (runtime 15 minutes). After drying the plates, lumefantrine bands that quenched fluorescence of the phosphor in the F-plates were scanned in the absorbance-reflectance mode using a CAMAG TLC Scanner 3 at 254 nm with 4 mm slit length, in both the individual method and the simultaneous method. For the individual artemether method, the plates were sprayed with methanol-96% sulfuric acid (19:1) detection agent, heated at 100 °C for 5 minutes on a CAMAG Plate Heater, and air dried for 10 minutes, and the visible brown bands were scanned at 610 nm. Artemether bands in the simultaneous method were detected by heating the plate at 160 °C for 5 minutes, and after cooling the plate the fluorescence quenching zones were scanned at 254 nm. Sample peak purity and identity for the two individual methods were tested using the respective Scanner 3 winCATS software options. Accuracy of the developed methods was validated by standard addition as described earlier using a 70-130% calibration curve as described by Popovic and Sherma [3]. The curve was constructed by over-spotting triplicate sample aliquots with an extra 50, 100, and 150% of the drug standard (double over-spotting of both standards for the combination method) and leaving three aliquots unspiked to perform the sample assay on the same plate.
The mean recovery (\%, \( n = 3 \)) for each sample at each level was calculated as the difference between the experimental weight and the weight in the unspiked sample divided by the weight of the spike and multiplying the quotient by 100.

**RESULTS**

All assays and validations were performed using polynomial regression of peak areas except for the validation of artemether analysis in the combination method, for which linear regression gave better results in terms of calibration curve r-values, assay values closer to the label value, accuracy of the standard addition validation, and lower RSDs; r-values of all calibration curves in our assay and validation experiments were 0.999. Standard weights applied represented 70-130% of the theoretical weight of the sample solution applied (100% of the label value). All assays and validation analyses were performed in triplicate (\( n = 3 \)).

**Artemether individual method**

Tablet 1 gave a mean weight of 4.20 µg and a mean assay of 105% relative to the label value (4.00 µg theoretical) with relative standard deviation (RSD) of 0.652%, Tablet 2 gave a mean weight of 4.24 µg and a mean assay of 106% with RSD = 0.106%, and Tablet 3 gave a mean weight of 4.21 µg and mean assay of 105% with RSD = 0.540%. Accuracy estimation based on the standard addition recovery results was a mean of 103% with RSD 0.442% at the 50% spike level, 97.6% with RSD 0.174% at the 100% spike level, and 101% with RSD 0.383% at the 150% spike level for a fourth, unspiked tablet that assayed at 99.0% of the label value.

**Lumefantrine individual method**

Tablet 1 gave a mean weight of 1.54 µg and a mean assay of 96.5% relative to the label value (1.60 µg theoretical) with an RSD of 1.79%. Tablet 2 gave a mean weight of 1.61 µg and a mean assay of 101% with RSD = 0.896%, and Tablet 3 gave a mean weight of 1.54 µg and mean assay of 96.5% with RSD = 1.76%. Accuracy estimation based on the standard addition recovery results was a mean of 100% with RSD 2.63% at the 50% spike level, 101% with RSD 0.589% at the 100% spike level and 102% with RSD 1.13% at the 150% spike level for a fourth, unspiked tablet that assayed at 99.0% of the label value.

**Artemether and lumefantrine simultaneous method**

For artemether, Tablet 1 gave a mean weight of 1.24 µg and a mean assay of 93.3% relative to the label value (1.33 µg theoretical) for the three replicate analyses with RSD of 2.88%, Tablet 2 gave a mean weight of 1.30 µg and a mean assay of 97.9% with RSD = 0.596%, and Tablet 3 gave a mean weight of 1.28 µg and mean assay of 96.0% with RSD = 2.15%. For lumefantrine, Tablet 1 gave a mean weight of 7.54 µg and a mean assay of 94.2% relative to the label value (8.00 µg theoretical) for the three replicate analyses with RSD of 0.400%, Tablet 2 gave a mean weight of 7.23 µg and a mean assay of 90.3% with RSD = 1.63%, Tablet 3 gave a mean weight of 7.28 µg and mean assay of 91.0% with RSD = 1.06%. Accuracy estimation based on the standard addition recovery results was a mean of 98.4% with RSD 0.442% at the 50% spike level, 97.6% with RSD 0.174% at the 100% spike level, and 99.2% with RSD 0.427% at the 150% spike level for a fourth, unspiked tablet that gave assay values of 92.3% and 89.9% of label value for artemether and lumefantrine, respectively.

For all assays and validation experiments sample peak identity and purity were confirmed by r-values of 0.999 for these tests, and densitometer peaks were compact and symmetrical for the standards and samples and no additional zones of excipients, impurities or degradation products were detected in sample chromatograms. Rf values were 0.34 for lumefantrine and 0.61 for artemether. A densitogram of a tablet 100% Sample Solution is shown in Figure 1.

**DISCUSSION**

Both of the individual quantitative HPTLC-densitometry methods we developed for the combination tablets are direct transfers from
currently available individual Minilab TLC screening methods in terms of the use of acetone as the standard and sample solution solvent, the weights applied in the 100% Sample Solution and 100% Standard Solution, and the detection methods. There is currently no simultaneous Minilab TLC screening method for combination tablets of artemether and lumefantrine. For our simultaneous HPTLC-densitometry method, some of the conditions of the individual methods we developed were modified. These included changing the sample preparation method so that the concentration of artemether was reduced from 4.00 µg/10 µL in the individual method to 1.33 µg/10 µL in the combination method and the concentration of lumefantrine was increased from 1.60 µg/10 µL to 8.30 µg/10 µL, and heating the plate without a detection reagent for detection of artemether. This novel method based on reagent free thermochemical activation of fluorescence quenching on a silica gel plate was first reported for the HPTLC-densitometry quantification of creatine in nutritional supplements (heating at 160 °C for 5 minutes) [5] and then for the assay of artesunate in combination tablets of artesunate and amodiaquin (heating at 180 °C for 10 minutes) [6].

For artemether detection different temperatures and times of heating were tested, and 160 °C for 5 minutes was optimum in terms of calibration curve r-value, accuracy and precision. All validation data for the three new methods met the requirements of the model transfer procedure, i.e., spike recovery of 100% ± 5% and RSD less than 3%.

In the simultaneous method, aliquots of individual 100% Standard Solutions of the drugs were overspotted to prepare the mixed standards at the plate origins for constructing the calibration curves, and double over-spotting of unspiked 100% Sample Solution with different aliquots of individual 100% Standard Solutions was successfully performed for the standard addition.
validations of accuracy and precision. The ability to over-spot using the Linomat applicator is a time saving advantage in that the mixed standard and spiked sample solutions do not have to be individually prepared.

Two earlier simultaneous TLC-densitometry methods for artemether and lumefantrine in combination formulations [7, 8] used solvents other than those on the approved Minilab list, which includes acetone, ethyl acetate, methanol, ethanol, toluene, ammonium hydroxide, glacial acetic acid, hydrochloric acid and sulfuric acid. In one of these [7], artemether was scanned at 561 nm and so a detection reagent was necessary rather than our simple detection by heating and scanning at 254 nm. The other [8] included scanning at 354 nm, which is not as convenient as scanning easily visible fluorescence quenching zones at 254 nm.

Depending on the applications of the methods described in this paper, they should be fully validated for parameters such as accuracy, precision (repeatability and intermediate precision), specificity, linearity, range and robustness under guidelines such as those described by the International Conference on Harmonization [9], or subjected to an interlaboratory study [10] to prove that they are suitable for their intended purpose.

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REFERENCES