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Transfer of TLC Screening Methods Designed for Use in Developing Countries to Quantitative HPTLC–Densitometry Methods for Diazepam and Amodiaquine Tablets

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Summary. Transfer of two rapid thin-layer chromatography (TLC) screening methods used to detect markedly substandard and fake pharmaceutical products to quantitative high-performance TLC (HPTLC)–densitometry methods is demonstrated using a model procedure that was published earlier. These TLC methods for diazepam and amodiaquine are contained in a Compendium of methods by Kenyon and Layloff and a Minilab method manual from Global Pharma Health Fund E.V., respectively, for use in countries with limited resources. Merck HPTLC Premium Purity silica gel 60 F_{254} glass plates, automated standard and sample solution application with a CAMAG Linomat 4, and automated densitometry with a CAMAG Scanner 3 were used for detection, identification, and quantification. Sample peak identity and purity validation were carried out by spectral comparison checks available in the winCATS software. Accuracy was estimated by the standard addition approach with overspotting standard and sample solutions. HPTLC gives better efficiency, selectivity, and resolution than TLC, and the new methods overcome the deficiencies in technology related to manual application and visual zone comparison that do not allow the Compendium and Minilab TLC procedures to support regulatory compliance actions. These new methods can be fully validated according to the International Conference on Harmonization guidelines or by interlaboratory studies if required by their applications.

Key Words: diazepam, amodiaquine, thin-layer chromatography, densitometry, pharmaceutical product screening, drug analysis, fake drugs, transfer of TLC methods to HPTLC–densitometry

Introduction

A previously published paper [1] described a model method for transfer of qualitative thin layer chromatography (TLC) pharmaceutical product screening methods to quantitative high performance TLC (HPTLC)–densitometry methods that can be used to support regulatory compliance actions. These screening methods have been published in a U.S. Food and Drug Administation Compendium [2] and in manuals published by the Global Pharma Health Fund E.V. (GPHF) [3] primarily for use in developing countries to help detect mislabeled, markedly substandard, and fake products; these methods are limited to the use of the inexpensive, relatively

nontoxic, and readily available solvents specified in the Compendium or Minilab: acetone, ethyl acetate, methanol, ethanol, toluene, ammonium hydroxide, glacial acetic acid, hydrochloric acid, and sulfuric acid in sample and standard solutions, mobile phases, and detection reagent solutions. In a later paper [4], sample peak purity and identity tests were added to the model transfer procedure. In this paper, the model procedure is further expanded to include estimation of accuracy by a standard addition method, and it is applied to the transfer of the Compendium method for analysis of diazepam tablets, used as an anticonvulsant and for treatment of anxiety disorders, and of the Minilab method for tablets containing the antimalaria drug amodiaquine. To our knowledge, no previous quantitative HPTLC method has been reported for analysis of a pharmaceutical product containing amodiaquine as the single active ingredient.

Experimental

Analytical procedures given below are described in more detail by Lianza and Sherma [4].

Standard and Sample Preparation

One milliliter of diazepam 1.00 mg mL^{-1} standard solution (Sigma-Aldrich, St. Louis, MO USA; CAS No. 439-14-5; Catalog No. D9900) was mixed with 4.00 mL of absolute ethanol to prepare the "100% Standard Solution" at a concentration of 0.200 μg μL^{-1}. Generic diazepam tablets with a 10-mg active pharmaceutical ingredient (API) label value, but no indication of inactive ingredients, were obtained from a local pharmacy; the tablets were light blue in color and marked with the identification no. 3927. Tablets were dissolved in 10.0 mL of absolute ethanol with magnetic stirring and sonication to prepare a 1.00-mg mL^{-1} stock standard solution. A portion was filtered to remove undissolved excipients using a 5-mL plastic syringe (Becton Dickinson, Franklin Lakes, NJ, USA) equipped with an Acrodisk 13-mm syringe filter with 0.45-μm PVDF membrane μμμPall Life Sciences, Ann Arbor, MI, USA), and 1.00 mL of clear filtrate was mixed with 4.00 mL of absolute ethanol to prepare the 0.200 μg μL^{-1} "100% Sample Solution."

Amodiaquine dichloride dihydrate standard was purchased from Sigma-Aldrich (CAS No. 6398-98-7; catalog no. A2799). The 100% standard solution was prepared by dissolving 100 mg of standard with methanol in a 100-mL volumetric flask and then mixing 1.35 mL of this solution with 8.65 mL of methanol to give a 0.135-μg μL^{-1} solution of amodiaquine hydrochloride, which is equivalent to 0.103 μg μL^{-1} of amodiaquine free

base after multiplying by the factor 0.765 representing the ratio of molecular weights of the free base and hydrochloride. Amobin brando amodiaqione tablets with a label value of 200 mg free base and no indication of inactive ingredients were obtained from a pharmacy in Dar el Salaam, Tanzania, and the 100% sample solution was prepared by dissolving each tablet in 100 mL of methanol in a volumetric flask with magnetic stirring and sonication, syringe filtering, and mixing 1.00 mL of the clear filtrate with 19.0 mL of methanol to give a concentration of 0.100 μg μL^{-1}.

HPTLC

HPTLC was carried out in general according to the standardized procedures recommended by Reich and Schibli [5]. EMD Millipore, Billerica, MA USA, a division of Merck KGaA, Darmstadt, Germany silica gel 60 F_{254} Premium Purity HPTLC glass plates (20 × 10 cm, Part No. 1.05648.001) were used as received without prewashing. The 100% standard solution was applied in 7.00-, 9.00-, 11.0-, and 13.0-μL aliquots (1.40 to 2.60 μg for diazepam and 0.721 to 1.34 μg for amodiaquine) and 10.0 μL of the 100% sample solution for each of three different tablets in triplicate (n = 3; 2.00 μg theoretical for diazepam and 1.00 μg for amodiaquine based on the label values) in the form of bands with a CAMAG (Wilmington, DE USA) Linomat 4 spray-on applicator equipped with a 100-μL syringe using the following settings: band length 6 mm, application rate 4 s μL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from the left edge of the plate 18 mm, and distance from the bottom of the plate 1.5 cm.

After the applied zones dried, the plates were developed to a distance of 7 cm beyond the bottom of the plate in a CAMAG HPTLC twin-trough chamber containing between 10 and 12.5 mL of mobile phase in each trough and a saturation pad (Analtech, Newark, DE USA) in the rear trough; the chamber was pre-equilibrated with the vapors of the mobile phase for 15 min before insertion of the plate into the front trough. The ambient temperature in the laboratory was approximately 20°C, and the relative humidity was approximately 15%. The mobile phase for diazepam determination was ethyl acetate, and the one for amodiaquine was ethyl acetate–methanol–concentrated ammonium hydroxide (24:3:1).

The developed plates were dried in a fume hood under a hair dryer for 5 min, and the areas of the standard and analyte zones were measured immediately by absorption remission reflectance densitometry using a CAMAG Scanner 3 with the deuterium UV light source set at 254 nm, slit dimensions 4.00 × 0.45 mm Micro, and scanning rate 20 mm s^{-1}. The winCATS software automatically created a calibration curve by 2nd-order

polynomial regression correlating the weights of the standard zones to their scan areas and interpolated the weight of bracketed sample zones from the curve based on their areas. The result of the sample assay was calculated using the following equation: % = (experimental weight/theoretical weight predicted by the label declaration) × 100.

Sample peak purity and identity tests were carried out using the spectra comparison options of the scanner winCATS software. Purity parameters were set at a correlation limit of 0.9900 and used a 90% constant peak level for testing, and identity parameters were tested with a correlation limit also at 0.9900. Spectra were compared at three different regions of a sample peak, i.e., the start (s), middle or apex (m), and end (e), and high *r*(sm) and *r*(me) values confirmed sample peak purity. For sample peak identity tests, spectra for a standard and sample zone were overlaid, and a high *r* value confirmed sample peak identification.

Accuracy (recovery) of the diazepam analysis was estimated by standard addition using overspotting with an additional 50%, 100%, and 150% of standard diazepam and analyzed by the proposed method. A wider calibration curve covering 40% to 160% of the label value was used rather than the 70–130% curve specified for tablet assay above. At each level, three determinations were performed. Specifically, 4.00, 7.00, 10.0, 13.0, and 16.0 aliquots of the 100% standard solution were applied on the first five lanes, and 6.00 µL of the 100% Sample Solution of a fourth tablet was applied to lanes 6–17. Lanes 6–8 were not overspotted and were used for triplicate assay of the sample, and lanes 9–11, 12–14, and 15–17 were overspotted with 3.00, 6.00, and 9.00 µL of the 100% standard solution for assay at the 50%, 100%, and 150% levels, respectively. The recovery (%) for each sample was determined by dividing the difference between the mean weight of diazepam in the unspiked sample and the mean weight in the spiked sample divided by the added weight and multiplying the quotient by 100.

Accuracy of the amodiaquine analysis was estimated by standard addition at 50–150% spiking levels by a different approach using the 70–130% standard curve with a fourth tablet. Aliquots of 7.00, 9.00, 11.0, and 13.0 µL of the 100% standard solution were applied in lanes 1–4, aliquots of 10.0 µL of the 100% sample solution in lanes 5–7 for the unspiked sample assay, aliquots of 6.00 µL of the 100% sample solution overspotted with 3.00 µL of the 100% standard solution in lanes 8–10 (50% spike), aliquots of 5.00 µL of the 100% sample solution overspotted with 5.00 µL of the 100% standard solution in lanes 11–13 (100% spike), and aliquots of 4.00 µL of the 100% sample solution overspotted with 6.00 µL of the 100% standard solution in lanes 14–16 (150% spike). The recovery (%) for each sample was

calculated as the difference between the experimental weight and the weight in the unspiked sample based on the assay divided by the weight of the spike and multiplying the quotient by 100.

Results

Diazepam

The polynomial calibration curve used for diazepam assay (70–130%) had an r value of 0.999 and the equation peak area = −1314.092 + (1676.244 × weight) + (−26.625 × weight^2). *Tablet 1* gave a mean weight of 1.93 µg and a mean assay of 96.3% relative to the label value for the three replicate analyses with relative standard deviation of 2.49%, *Tablet 2* gave a mean weight of 1.96 µg and a mean assay of 98.0% with RSD = 1.33%, and

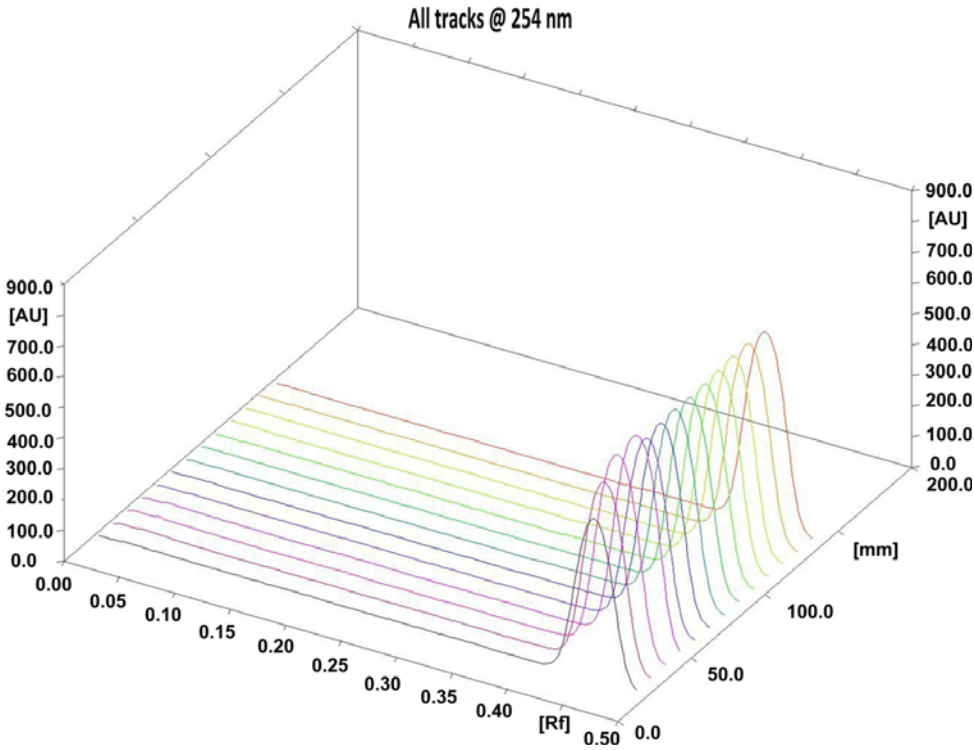

Fig. 1. Three-dimensional overlay of HPTLC densitograms obtained in the diazepam assay with Merck Premium Purity silica gel 60 F_{254} plates and ethyl acetate mobile phase. Peaks 1–4 (front to back) represent standard zones (1.40, 1.80, 2.20, and 2.60 µg, respectively), and peaks 5–13 represent analyte zones *(5–7: Tablet 1, 8–10: Tablet 2, 11–13: Tablet 3)*

Tablet 3 gave a mean weight of 2.00 µg and mean assay of 100% with RSD = 1.20%. Sample peak identity and purity were confirmed by *r* values of 0.999 for these tests. Accuracy estimation based on the standard addition recovery results using the expanded polynomial calibration curve (60–140%; peak area = 3681.2172 + (11.3418 × weight + (−0.0013 × weight^2) ; r = 0.999) was a mean of 97.8% with RSD 0.8% at the 50% spike level, 99.4% with RSD 2.71% at 100%, and 100% with RSD 2.00% at 150% for a fourth, unspiked tablet that assayed at 102% of the label value (n = 3 for all results). Densitometer peaks were compact and symmetrical for the standards and samples (*Fig. 1*), and no additional zones of excipients, impurities, or degradation products were detected in sample chromatograms.

Amodiaquine

The polynomial calibration curve used for amodiaquine assay (70–30%) had an *r* value of 0.999 and the equation peak area = −5755.8220 + (29.2362 × weight) + (−0.0064 × weight^2) (*Fig. 2*). *Tablet 1* gave a mean weight of 1.02 µg and a mean assay of 102% relative to the label value for the three

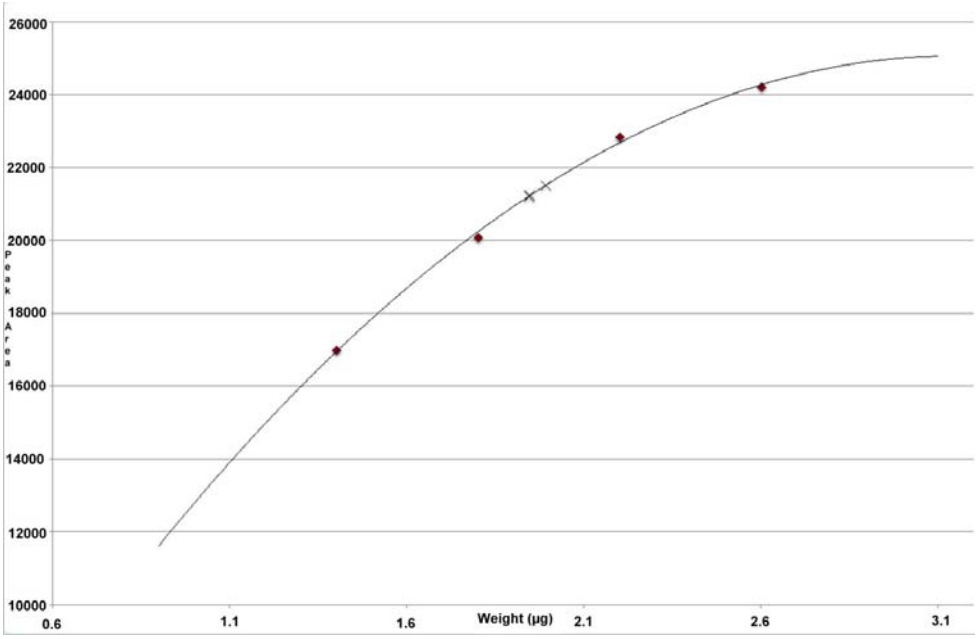

Fig. 2. Polynomial calibration curve used for amodiaquine assay (70–130%) with an *r* value of 0.999. The squares represent the four standard zones and the crosses the three replicate sample zones for *Tablet 2*

replicate analyses with an RSD of 1.37%, *Tablet 2* gave a mean weight of 1.02 μg and mean assay of 102% with RSD = 2.43%, and *Tablet 3* gave a mean weight of 1.03 μg and mean assay of 103% with RSD = 0.25%. Sample peak identity and purity were confirmed by *r* values of 0.999 for these tests. Accuracy estimation based on the standard addition recovery results for a fourth tablet using a polynomial calibration curve over the same 70–130% range as for the assays of the first three tablets was a mean of 101% with 0.94% RSD at the 50% spike level, 101% with RSD 1.68% at 100%, and 98.2% with RSD 0.61% at 150% (n = 3 for all results). No additional zones of excipients, impurities, or degradation were detected in sample chromatograms.

Discussion

The Compendium method for 2 mg diazepam tablets [2] had to be altered in several ways to develop the described HPTLC–densitometry method. The solvent for preparing the standard and sample solutions was changed from 95% ethanol to absolute ethanol to allow faster drying of the applied nonaqueous solutions using the Linomat. Lower concentration sample and standard solutions (0.2 mg mL^{-1} in the developed method versus 1 mg mL^{-1} in the Compendium method) to produce lighter zones and a better calibration curve. The mobile phase was changed from methanol-concentrated ammonium hydroxide (33:0.5) to the single solvent ethyl acetate in order to obtain a lower, more optimum R_F value of 0.45 compared to 0.71 for the Compendium mobile phase. The elutropic series of solvent strengths and solvent selectivity triangle [6] are great aids in devising mobile phases that provide optimum R_F values and resolution of mixtures; ethyl acetate is a weaker solvent than the methanol-aqueous ammonia mixture in the elutropic series. The Compendium mobile phase used to develop the Merck plates used in our previous method transfer studies (Part No. 5642-6) [1, 4] produced a dark line (beta front) immediately above the standard and sample zones that interfered with their scanning and spectral analysis; prewashing with methanol or the mobile phase did not solve this problem, and the disturbing front was still seen when zones were measured with the scanner. Changing to Merck Premium Purity plates and ethyl acetate mobile phase eliminated the interfering dark line above the scanned zones. Assay values for the three analyzed diazepam tablets ranged from 96% to 100% relative to the label value with a mean RSD of 1.67% (n = 3).

Volume II of the GPHF-Minilab Manual on Thin Layer Chromatographic Tests [3] contains a screening method for 153 or 200 mg amodiaquine free base tablets, but direct transfer of its parameters to HPTLC–

densitometry was not possible. The weights of sample and standard applied for the respective methods were approximately the same, but the Minilab method specified the mobile phase methanol:ethyl acetate:concetrated ammonium hydroxide (20:5:0.5). This mobile phase gave an R_F value above the optimum 0.2–0.8 range and a dark line directly above the zones that interfered with scanning and spectral analysis. Our modified mobile phase with a higher proportion of the weaker solvent ethyl acetate according to the elutropic series and a lower proportion of the stronger solvent methanol gave a lower R_F value of 0.72 with the Merck Premium Purity plates, and the dark line, although not completely eliminated, was lighter and did not interfere significantly with scanning of the standard and sample zones or their spectral analysis. Assay results for the three amodiaquine tablets were 102–103% with a mean RSD of 1.35% (n = 3).

For tablets of both drugs, polynomial regression provided better r values for the calibration curves and recoveries closer to 100% in the standard addition experiments compared to linear regression. Results for the standard addition method were within 95–105% with RSD values <3% as specified by Ferenczi-Fodor et al. for accuracy testing of HPTLC assays of APIs in finished products [7]. Standard addition estimation of accuracy by assaying an unspiked sample and overspotting additional aliquots of sample with extra standard at different levels on the same plate has been reported in the literature [8]. Spiking levels of 50–150% have been used in many cases for pharmaceutical analysis validation [e.g., 9, 10]; we applied two overspitting procedures to cover these levels, one with an expanded calibration curve range of 40–160% and the other with our usual 70–130% range. The Linomat with its bandwise spray-on technique offers great advantages of multilevel calibration by application of different volumes of the same standard solution, overspotting of different standards on the same origin in the assay of mixed drug formulations [11], and overspotting of samples and standards for accuracy estimation as described in this paper rather than having to prepare sample solutions spiked with different amounts of added standards.

The HPTLC–densitometry methods described in this paper are not compromised by the limitations in technology related to the ability of operators to manually apply the sample and standard initial zones or visually compare zone sizes/intensities as in the Compendium and Minilab methods. They are therefore capable of providing much more information and better results compared to the qualitative TLC screening methods in support of regulatory compliance actions. Users of these new methods should fully validate them according to the International Conference on Harmonization (ICH) guidelines for performance characteristics such as accuracy, precision (repeatability and intermediate precision), specificity,

linearity, range, and robustness [7, 12] or subject them to an interlaboratory study [13] to assure they are fit for their intended purpose.

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