Transfer of TLC Screening Methods to Quantitative HPTLC–Densitometry Methods for Pharmaceutical Products Containing Amlodipine Besylate, Cefpodoxime Proxetil, Cetirizine 2HCl, Diclofenac Sodium, Efavirenz, Mefenamic Acid, and Atovaquone + Proguanil HCl

B. Zeng, Y. Gu and J. Sherma*

Department of Chemistry, Lafayette College, Easton, PA, USA

Received: 25 July 2018; accepted: 25 July 2018

Transfer of seven thin-layer chromatography (TLC) Global Pharma Health Fund E.V. Minilab protocols for screening counterfeit pharmaceutical products in the field to quantitative high-performance TLC (HPTLC)–densitometry methods was performed using a model process published previously. The developed and validated methods for tablets containing amlodipine besylate, cefpodoxime proxetil, cetirizine 2HCl, diclofenac sodium, efavirenz, mefenamic acid, and atovaquone + proguanil HCl involved the use of only relatively inexpensive and nontoxic solvents, Merck KGaA Premium Purity HPTLC silica gel 60 F₂₅₄ plates, semi-automated sample and standard solution application with a CAMAG Linomat 4, and automated densitometry with a CAMAG Scanner 3 for detection, identification, and quantification. In addition, previously transferred HPTLC–densitometry methods for azithromycin and for cephalexin were used to analyze a new product of each drug to demonstrate the applicability of the methods.

Keywords: Amlodipine besylate, cefpodoxime proxetil, cetirizine HCl, diclofenac sodium, efavirenz, mefenamic acid, atovaquone + proguanil HCl, azithromycin, cephalexin, thin-layer chromatography, TLC, densitometry, counterfeit drug analysis

Introduction

A model process was previously developed [1–3] for transferring visual, semiquantitative thin-layer chromatography (TLC) screening methods for pharmaceutical products with quality defects in the Global Pharma Health Fund E.V. (GPHF) Minilab manual [4] to quantitative high-performance TLC (HPTLC)–densitometry methods. An earlier study in the literature [5] described the application of this model process to antimicrobial and antituberculosis products containing cefixime, cefuroxime axetil, cephalexin-H₂O, ciprofloxacin HCl, levofloxacin, and metronidazole. In this article, the application of the transfer process is extended by seven products containing eight important drugs of different types: amlodipine besylate (calcium channel blocker, CAS No. 111470-99-6), cefpodoxime proxetil (antibiotic, CAS No. 87239-81-4), cetirizine 2HCl (antihistamine, CAS No. 83881-52-1), diclofenac sodium (nonsteroidal anti-inflammatory, CAS No. 15307-79-6), efavirenz (HIV antiviral, CAS No. 154598-52-4), mefenamic acid (nonsteroidal anti-inflammatory, CAS No. 61-68-7), and atovaquone (antifungal and anti-parasite, CAS No. 95233-18-4) + proguanil HCl (antimalarial, CAS No. 637-32-1). In addition, two previously transferred HPTLC–densitometry methods for the antibiotics azithromycin dihydrate [6] and cephalexin [5] were successfully applied to analyze a new product of each drug to demonstrate their applicability.

Experimental

Standard and Sample Preparation. Standard and sample solution preparation was carried out as described in the model

process [1–3] unless otherwise specified. Standards, tablets ground using mortar and pestle, and capsule contents were dissolved in their respective solvents with the assistance of 10 min magnetic stirring, followed by 10 min sonication. Sample stock solutions were syringe filtered to remove undissolved excipients before further dilution or direct application. Dilutions were made using appropriate volumetric flasks, volumetric pipets, and measuring pipettes if necessary. Solutions were stored in Parafilm-sealed vials in a refrigerator. Sources of samples and procedures for standard and sample solution preparation are shown in Table 1.

HPTLC. Premium Purity silica gel 60 F₂₅₄ plates (20 cm × 10 cm; Merck KGaA, Darmstadt, Germany; Catalog No. 1.05648.0001) were used as received without prewashing. Calibration curves were created by spotting 7.00, 9.00, 11.0, and 13.0 µL of the 100% sample solution, representing 70–130% of the label value of the active pharmaceutical ingredient. Assays were carried out by applying 10.0 µL of each sample solution in triplicate. A CAMAG (Wilmington, NC, USA) Linomat 4 was used for semi-automated bandwise standard and sample solution zone application. An application rate of 4 s/µL was used for all solutions. The band length was 6 mm, the table speed was 10 mm/s, the distance between bands was 4 mm, the distance from the left edge of the plate was 17 mm, and the distance from the bottom of the plate was 1 cm. Mobile phases used to develop plates in a CAMAG twin trough chamber and their respective *R_F* values are listed in Table 2. Automated HPTLC–densitometry in the absorption–reflectance mode was performed using a CAMAG Scanner 3 controlled by winCATS software, with 4.00 mm × 0.45 mm Micro slit dimensions and a 20 mm/s scan rate. All drugs for which the methods are detailed in this paper quenched fluorescence of the phosphor indicator in the silica gel. All were scanned under 254 nm ultraviolet (UV) light from a

* Author for correspondence: sherma@lafayette.edu

This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium for non-commercial purposes, provided the original author and source are credited, a link to the CC License is provided, and changes - if any - are indicated.

Table 1. Preparation of 100% standard and 100% sample solutions

Pharmaceutical product	100% standard solution	100% sample solution ^a
Amlodipine besylate (13.9 mg ^b ; Cipla Ltd., Mumbai, India)	1.39 µg 10.0 µL ⁻¹ : dissolve 139 mg standard (Sigma-Aldrich, St. Louis, MO, USA, No. PHR1185) in 100 mL methanol, then dilute 1.00 mL with 9.00 mL methanol	1.39 µg 10.0 µL ⁻¹ : dissolve a tablet in 100 mL methanol
Cefpodoxime proxetil (261 mg ^c ; Pharma International Co., Amman, Jordan)	1.00 µg 10.0 µL ⁻¹ : dissolve 100 mg standard (USP, Rockville, MD, USA, Cat. No. 1098027) in 100 mL methanol, then dilute 1.00 mL with 9.00 mL methanol	1.04 µg 10.0 µL ⁻¹ : dissolve a tablet in 100 mL methanol, then dilute 1.00 mL with 24.0 mL methanol
Cetirizine 2HCl (10 mg; Square Pharmaceuticals Ltd., Dhaka, Bangladesh)	10.0 µg 10.0 µL ⁻¹ : dissolve 100 mg standard (Sigma-Aldrich, No. PHR1656) in 100 mL methanol	10.0 µg 10.0 µL ⁻¹ : dissolve a tablet in 10.0 mL methanol
Diclofenac sodium (50 mg; Zenufa Laboratories (T) Ltd., Dar es Salaam, Tanzania)	1.00 µg 10.0 µL ⁻¹ : dissolve 100 mg standard (Sigma-Aldrich, No. PHR1144) in 100 mL methanol, then dilute 1.00 mL with 9.00 mL methanol	1.00 µg 10.0 µL ⁻¹ : dissolve a tablet in 100 mL methanol, then dilute 1.00 mL with 9.00 mL methanol
Diclofenac sodium (100 mg; Sichuan Huaxin Pharmaceutical Co., Ltd., Leshan, China)		1.00 µg 10.0 µL ⁻¹ : dissolve a tablet in 50.0 mL methanol, then dilute 1.00 mL with 9.00 mL methanol
Efavirenz (600 mg; Hetero Drugs Ltd., Hyderabad, India)	1.00 µg 10.0 µL ⁻¹ : dissolve 25.0 mg standard (Sigma-Aldrich, No. SML0536) in 100 mL methanol, then dilute 2.00 mL with 3.00 mL methanol	1.00 µg 10.0 µL ⁻¹ : dissolve a tablet with 100 mL methanol, then dilute 1.00 mL with 9.00 mL methanol, and then dilute 1.00 mL with 5.00 mL methanol
Mefenamic acid (500 mg; Blue Cross Laboratories Ltd., Mumbai, India)	0.500 µg 10.0 µL ⁻¹ : dissolve 125 mg standard (Sigma-Aldrich, No. 92574) in 100 mL methanol, then dilute 1.00 mL with 24.0 mL methanol	0.500 µg 10.0 µL ⁻¹ : dissolve a tablet in 100 mL methanol, then dilute 1.00 mL with 99.0 mL methanol
Atovaquone + proguanil HCl (250 mg + 100 mg; Glaxo Wellcome S.A., Aranda de Duero, Spain)	1.00 µg 10.0 µL ⁻¹ (atovaquone): dissolve 100 mg standard (Sigma-Aldrich, No. PHR1591) in 100 mL methanol–acetone (3:1, v/v), then dilute 1.00 mL with 9.00 mL methanol–acetone (3:1, v/v) 0.400 µg 10.0 µL ⁻¹ (proguanil HCl): dissolve 100 mg standard (Sigma-Aldrich, No. LR117301) in 100 mL methanol–acetone (3:1, v/v), then dilute 1.00 mL with 24.0 mL methanol–acetone (3:1, v/v)	1.00 µg 10.0 µL ⁻¹ (atovaquone) + 0.400 µg 10.0 µL ⁻¹ (proguanil HCl): dissolve a tablet in 100 mL methanol–acetone (3:1, v/v), then dilute 1.00 mL with 24.0 mL methanol–acetone (3:1, v/v)

^aConcentrations indicated for 100% sample solutions are theoretical concentrations.

^bThe tablet contained an equivalence of 10.0 mg amlodipine free base, so the amount of amlodipine besylate is 10.0 mg ÷ 408.879 g/mol (molecular weight of the free base) × 567.05 g/mol (molecular weight of the besylate) = 13.9 mg.

^cThe tablet contained an equivalence of 200 mg cefpodoxime free base, so the amount of cefpodoxime proxetil is 200 mg ÷ 427.45 g/mol (molecular weight of the free base) × 557.593 g/mol (molecular weight of the proxetil) = 261 mg.

Table 2. Mobile phases used in the development of plates for the analyses of pharmaceutical products containing amlodipine besylate, cefpodoxime proxetil, diclofenac sodium, efavirenz, mefenamic acid, and atovaquone + proguanil HCl

Pharmaceutical product	Mobile phase ^a	R _F
Amlodipine besylate	Methanol–toluene–glacial acetic acid–water (26:6:4:4)	0.74
Cefpodoxime proxetil	Toluene–methanol–ethyl acetate (20:4:16)	0.42
Cetirizine 2HCl	Ethyl acetate–methanol–ammonia (28:6:4)	0.29
Diclofenac sodium	Ethyl acetate–methanol–acetone (30:9:1)	0.31
Efavirenz	Toluene–methanol–ethyl acetate (28:8:4)	0.55
Mefenamic acid	Toluene–ethyl acetate–glacial acetic acid (30:10:2)	0.56
Atovaquone	Acetone–methanol–glacial acetic acid (30:10:1.1)	0.70
Proguanil HCl	Acetone–methanol–glacial acetic acid (30:10:1.1)	0.24

^aAll solutions are shown in volume proportions.

deuterium lamp except amlodipine besylate, which was scanned as fluorescent zones under 366 nm UV light from a mercury lamp. The winCATS software created two calibration curves for each analysis using linear and second order polynomial regressions of the scan areas versus applied weights of standards. Sample weights were interpolated from calibration curves based on the bracketed scan areas of the samples. Spectral comparison was used to test peak purity and identity. Validation of the developed methods was performed using standard addition by spiking at 50, 100, and 150% levels as described by Popovic and Sherma [3].

Results

Assay results of the seven pharmaceutical products are displayed in Table 3, all of which were between the 85 and 115% specification limits of the label value as specified by the model process guidelines except for cefpodoxime proxetil. However, good validation data for this drug confirmed the accuracy of these assays. As required by the model process, *r*-values of

calibration curves for assays and validations and those of peak purity and peak identity were at least 0.99. Results of validation by standard addition are listed in Table 4, in which recoveries were between 95% and 105%. Relative standard deviations (RSDs) for assays and validations were no greater than 3%, which complies with the model process. The preferred regression mode for assays and validation of each pharmaceutical product was selected based on the best results in terms of higher calibration curve *r*-value, assay and validation recoveries closer to 100%, and lower RSDs.

Previously published transferred Minilab methods for azithromycin dihydrate and cephalixin were used to analyze a new product of each drug. The exact procedures published earlier were followed, except that instead of using primary standards of the two drugs from Sigma-Aldrich, previously analyzed tablets of azithromycin dihydrate (Shelys Pharmaceuticals Ltd., Dar es Salaam, Tanzania) and cephalixin (North China Pharmaceutical Hebei Huamin Pharma Co., Ltd., Shijiazhuang, China) were used, respectively, as secondary standards for the analysis of Azibial-500 azithromycin dihydrate tablets (Bafna

Table 3. Assay results for pharmaceutical products containing amlodipine besylate, cefpodoxime proxetil, diclofenac sodium, efavirenz, mefenamic acid, and atovaquone + proguanil HCl

Pharmaceutical product	Regression mode	Tablet 1		Tablet 2		Tablet 3	
		Assay (%)	RSD (%)	Assay (%)	RSD (%)	Assay (%)	RSD (%)
Amlodipine besylate	Polynomial	103	0.206	102	1.27	104	1.60
Cefpodoxime proxetil	Linear	121	1.08	125	1.74	131	1.39
Cetirizine 2HCl	Linear	114	3.00	111	1.13	107	0.962
Diclofenac sodium (50 mg)	Linear	96.1	2.08	94.8	1.01	94.0	1.11
Diclofenac sodium (100 mg)	Linear	103	0.443	104	1.30	105	1.24
Efavirenz	Linear	106	1.62	107	1.69	107	2.29
Mefenamic acid	Polynomial	90.5	0.536	107	1.03	94.7	2.13
Atovaquone	Polynomial	98.9	2.79	102	2.06	115	0.654
Proguanil HCl	Polynomial	108	1.52	107	0.131	111	0.957

Table 4. Validation results for pharmaceutical products containing amlodipine besylate, cefpodoxime proxetil, diclofenac sodium, efavirenz, mefenamic acid, and atovaquone + proguanil HCl

Pharmaceutical product	Regression mode	50% spike		100% spike		150% spike	
		Rec ^a (%)	RSD (%)	Rec (%)	RSD (%)	Rec (%)	RSD (%)
Amlodipine besylate	Polynomial	102	0.893	100	0.902	99.1	1.50
Cefpodoxime proxetil	Linear	96.1	1.18	100	0.890	100	1.17
Cetirizine 2HCl	Linear	105	0.850	104	0.889	100	2.42
Diclofenac sodium (50 mg)	Linear	99.6	3.36	102	1.06	101	1.09
Diclofenac sodium (100 mg)	Linear	100	1.09	99.3	1.57	95.0	1.94
Efavirenz	Linear	104	2.76	103	2.76	104	1.21
Mefenamic acid	Polynomial	99.8	1.93	98.1	0.811	102	1.26
Atovaquone	Polynomial	101	0.750	102	1.03	99.5	1.50
Proguanil HCl	Polynomial	101	1.34	99.9	0.979	104	0.678

^aRec = Recovery

Pharmaceuticals Ltd., Chennai, India) and Aurocef-250 cephalixin capsules (Aurobindo Pharma Ltd., Hyderabad, India). Assay values for triplicate analyses of three different tablets averaged 110, 102, and 96.1% for azithromycin dihydrate and 97.8, 104, and 106% for cephalixin, all of which were within the 85–115% guideline for acceptable assay results in the model process.

Discussion

The direct transfer of a TLC screening method to an HPTLC–densitometry method according to the model process involves the use of the same solvents in sample and standard solution preparation, the application of the same weights in 10.0 μ L of 100% standard and sample solutions as in 2.00 μ L in the Minilab method, and the use of the same mobile phase and detection mode. The Minilab method for amlodipine besylate (Minilab manual Volume II, Supplement 2017, Method 6.91, pp. 4–7) could be directly transferred based on these parameters.

For the transfer of the cefpodoxime proxetil Minilab method (Volume II, Supplement 2017, Method 6.92, pp. 8–11), both the applied weight and the mobile phase were changed. The applied weight was changed to 0.800 μ g instead of 2.00 μ g in the Minilab manual to achieve better scan results. Five mobile phases were tested in total, namely, toluene–methanol (20:20, from the Minilab method), ethyl acetate–methanol (30:10) [7], toluene–ethyl acetate–methanol (20:16:4) [8], methanol–water (20:20) [9], and ethanol–water (20:20) [9]. Among these, toluene–ethyl acetate–methanol produced the best peak shape and highest *r*-value and was chosen for use. All the assays of this drug were over 120% of the label value, but the accuracy of the results was verified by the validation data.

The cetirizine 2HCl Minilab method (Volume II, Supplement 2018, Method 6.97, pp. 4–7) specifies the use of an iodine vapor staining step for zone detection. Detection was simplified in the densitometry method by eliminating the need for this reagent by increasing the applied weight to 10.0 μ g from 2.50 μ g to obtain sufficiently dark fluorescence quenching

zones for scanning at 254 nm. The mobile phase was changed to the one reported by Makhija and Vavia [10], because the original mobile phase for the Minilab method, methanol–ethyl acetate–toluene (14:4:2), produced zones with long, upward tails rather than the more compact zones with the new mobile phase, ethyl acetate–methanol–ammonia (28:6:4). In the Minilab screening method, iodine vapor detection could also be eliminated by applying higher weights of drug sample and standard and thereby giving increased intensity of the spots under 254 nm UV light.

For the transfer of the diclofenac Minilab method (Volume II, Supplement 2018, Method 6.99, pp. 12–15), the spotted weight was lowered to 1.00 μ g because the weight of 2.50 μ g in the Minilab protocol was too high for successful densitometry. This method was applied successfully to both 50 and 100 mg tablets.

For the transfer of the efavirenz Minilab method (Volume II, Supplement 2017, Method 6.95, pp. 20–23), the only modification was the applied weight, which was changed to 1.00 μ g as opposed to 2.00 μ g in order to obtain less dark bands and higher *r*-value for the densitometry calibration curve.

Transfer of the mefenamic acid Minilab method (Volume II, Supplement 2018, Method 6.100, pp. 16–19) required modifications of the applied weight and the standard and sample solution solvent. The applied weight of 2.50 μ g in the Minilab method was reduced to 0.500 μ g to achieve an acceptable *r*-value for the densitometry calibration curve. The Minilab method uses methanol–ammonia (23:2) as the solvent for the stock solutions and methanol for dilution to prepare the working solutions. It was found that the use of methanol to prepare both the stock and working solutions gave excellent densitometry results.

The atovaquone + proguanil HCl simultaneous Minilab method (Volume II, Supplement 2010, Method 6.43, pp. 8–11) could not be directly transferred because the applied weights in the Minilab protocol were too high for successful densitometry. The applied weight was lowered to 1.00 μ g + 0.400 μ g to obtain satisfactory calibration curves. The Minilab method mobile phase was modified by increasing the amount of acetic acid by 0.1 mL to 1.1 mL. This mobile phase decreased the *R_F* value of atovaquone and moved it further away from the

Figure 1. Densitogram of 10.0 µL of atovaquone + proguanil HCl 100% sample solution, showing peaks for atovaquone (R_F : 0.70) and proguanil HCl (R_F : 0.24)

mobile phase front. It also gave good drug separation and zone scans (Figure 1).

Conclusion

The previously developed model process was applied to the transfer of seven Minilab TLC screening methods for pharmaceutical products containing eight different drugs to HPTLC–densitometry methods. 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 relevant guidelines such as those described by the International Conference on Harmonization [11] or subjected to an interlaboratory study [12] to prove that they are suitable for their intended purpose by users.

Acknowledgments. The authors thank Thomas Layloff, Senior Quality Assurance Advisor, Supply Chain Management System (SCMS), Arlington, VA, USA, for his assistance in designing the model process and its application to the analysis of pharmaceutical products, and for arranging delivery of drug samples supplied by the Dar es Salaam, Tanzania, Office of

SCMS. We also thank Dr. Gerd Battermann, Head of Instrumental Analytics Franchise, Merck KGaA, Darmstadt, Germany, for providing the Premium Purity HPTLC plates used in our experiments. Bingsong Zeng and Yiru Gu were supported by the Lafayette College EXCEL Scholar Program.

References

1. O'Sullivan, C.; Sherma, J. *Acta Chromatogr.* **2012**, *24*, 241–252.
2. Lianza, K.; Sherma, J. *J. Liq. Chromatogr. Relat. Technol.* **2013**, *36*, 2446–2462.
3. Popovic, N.; Sherma, J. *Acta Chromatogr.* **2014**, *26*, 615–623.
4. Global Pharma Health Fund e.V., <http://www.gphf.org> (accessed July 1, 2018).
5. Zhang, D.; Armour, E.; Sherma, J. *Acta Chromatogr.* **2017**, *29*, 484–486.
6. Armour, E.; Sherma, J. *J. Liq. Chromatogr. Relat. Technol.* **2017**, *40*, 282–286.
7. Zhang, D.; Strock, J.; Sherma, J. *J. Liq. Chromatogr. Relat. Technol.* **2016**, *39*, 277–280.
8. Nguyen, K.; Zhang, D.; Sherma, J. *Trends Chromatogr.* **2017**, *11*, 12–17.
9. Sharma, S.; Singh, S.; Baghel, S. *J. Environ. Res. Dev.* **2006**, *1*, 46–48.
10. Makhija, S. N.; Vavia, P. R. *J. Pharm. Biomed. Anal.* **2001**, *25*, 663–667.
11. Ferenczi-Fodor, K.; Vegh, Z.; Nagy-Tuak, A.; Renger, B.; Zeller, M. *J. AOAC Int.* **2001**, *84*, 1265–1276.
12. Kaale, E.; Risha, P.; Reich, E.; Layloff, T. P. *J. AOAC Int.* **2010**, *93*, 1836–1843.