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Design, Optimization, and Validation of Thin-Layer Chromatography–Densitometry and Chemometry-Assisted Spectrophotometry: A Comparative Study Applied on Quaternary Mixture

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Summary

This work presents a comparative study on the development and validation of two analytical techniques applied for the simultaneous determination of hydrocortisone acetate (HCA), fusidic acid (FSA), methyl paraben (MPB), and propyl paraben (PPB) formulated as a topical cream. The first technique was thin-layer chromatography (TLC)–densitometric method, which was developed by separating the four components on silica gel 60 F_{254} using methylene chloride–methanol–benzene in the ratio of 10:2:5, *v/v*, as the developing system, followed by densitometric measurement of the bands at 240 nm. The second technique was the chemometric method using two models: principle component regression model (PCR) and partial least squares (PLS). The suggested techniques were validated in compliance with the International Conference on Harmonization (ICH) guidelines and were successfully applied for the determination of the quaternary mixtures as prepared synthetically in laboratory and in the commercial topical pharmaceutical formulation.

1 Introduction

Hydrocortisone acetate (HCA) is a corticosteroid with both glucocorticoid and, to a lesser extent, mineralocorticoid activity. It is used for topical application in the treatment of various skin disorders. A survey of the literature revealed the reported methods for the determination of HCA such as ultraviolet (UV) spectrophotometry [1, 2], high-performance liquid chromatography (HPLC) [3–5], thin-layer chromatography (TLC) [6, 7], micellar electrokinetic capillary chromatography [8, 9], and capillary electrophoresis [10]. Fusidic acid (FSA) is an antimicrobial substance produced by the growth of certain strains of *Fusidium coccineum*. It is used topically in the treatment of eye and skin infections. Different methods were reported for FSA such as UV spectrophotometry [11, 12], HPLC [13, 14], and TLC [15]. Several methods were reported for the determination of the preservatives (MPB and PPB) in different pharmaceutical formulations [3, 16–18]. All those components are official [19] and they are formulated together as a quaternary mixture in a topical cream. Two HPLC methods were reported for the simultaneous determination of HCA and FSA [13, 20]; spectrophotometric methods were reported for the simultaneous determination of HCA and FSA in the presence of total parabens [21], but there was no method reported for the simultaneous determination of each of the four components. The structural formulae of the components of interest are shown in **Figure 1.**

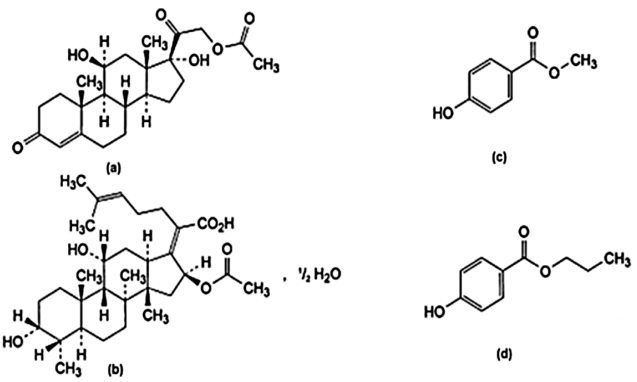

Figure 1

The structural formulae of (a) hydrocortisone acetate (HCA), (b) fusidic acid (FSA), (c) methyl paraben (MPB), and (d) propylparaben (PPB).

The aim of the work was to design, optimize, and validate two analytical techniques based on UV spectrophotometry for the determination of this quaternary mixture. The developed methods were TLC–densitometric method and chemometric-assist-

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ed spectrophotometric method using principle component regression (PCR) and partial least squares (PLS). A comparative study was conducted to evaluate the efficiency of each technique.

2 Materials and Methods

2.1 Apparatus and Software

Shimadzu UV 1800 double beam UV–visible spectrophotometer (Japan) with matched 1-cm quartz cells at 200–800 nm range was used for all absorbance measurements. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software. For the PLS model, Matlab® Version 7.9 with PLS toolbox 2.0 was used.

TLC–densitometric system: CAMAG TLC Scanner 3 S/N 130319 operated with winCATS software, Linomat 5 autosampler (Muttenz, Switzerland), CAMAG microsyringe (100 µL), and TLC aluminum sheet (20 × 20 cm) precoated with silica gel 60 F_{254} (Merck KgaA, Darmstadt, Germany) were used.

2.2 Chemicals and Reagents

2.2.1 Pure Samples

Hydrocortisone acetate (HCA) and fusidic acid (FSA) were kindly supplied by Sigma Pharmaceutical Industries Limited, Al-Monofeya, Egypt, with purity of 99.91 ± 1.072 and 99.58 ± 1.331, tested by the official methods [19], respectively. The preservatives, methyl paraben (MPB) and propyl paraben (PPB), were kindly supplied by Arab Drug Co., Cairo, Egypt, with purity of 97.94 ± 1.721 and 98.10 ± 1.881, tested by the official methods [19].

2.2.2 Market Sample

Fusi-zon® cream, labeled to contain 2 g of FSA and 1 g of HCA per 100 g in the presence of MPB and PPB, was manufactured by Pharaonia Pharmaceuticals, New Borg El-Arab city, Alexandria, Egypt.

2.2.3 Solvents

Spectroscopic analytical grade methanol and methylene chloride were supplied by S.D. Fine-Chem Limited (Mumbai, India), and analytical grade benzene by Adwic – El Nasr Pharmaceutical Chemicals Co. (Egypt).

2.3 Standard Solutions

2.3.1 Stock Solutions

Solutions were prepared in methanol of concentrations: 2 mg mL^{-1} HCA, 5 mg mL^{-1} FSA, and 4 mg mL^{-1} of MPB and PPB.

2.3.2 Working Solutions

Working solutions were freshly prepared by dilution from the stock solutions with methanol to obtain different concentrations: for TLC–densitometric method, 1 mg mL^{-1} HCA, 2.5 mg mL^{-1} FSA, and 2 mg mL^{-1} of MPB and PPB; for chemometric method, 80 µg mL^{-1} of HCA and FSA, and 80 µg mL^{-1} of MPB and PPB.

2.4 Procedure

2.4.1 For TLC–Densitometric Method

2.4.1.1 Chromatographic Conditions

TLC aluminum sheets, 20 × 10 cm, precoated with 0.25 mm silica gel 60 F_{254} were used. The samples were applied as bands (bandwidth: 6 mm, bands were spaced 1 cm apart from each other and 1.5 cm from the bottom edge of the plate). The developing system was methylene chloride–methanol–benzene in the ratio of 10:2:5 (*v*/*v*). Linear ascending development was done in a chromatographic tank previously saturated with the developing system for 1 h, at room temperature to a distance of approximately 8 cm from the lower edge. The developed plates were air-dried and scanned at 240 nm. Detection was performed using CAMAG TLC Scanner 3 operated in the absorbance mode, with deuterium lamp as the source of radiation; the slit dimension was kept at 3 mm × 0.45 mm, and 20 mm s^{-1} scanning speed was employed.

2.4.1.2 System Suitability

Ten microliters of working solutions were injected and applied to the chromatographic conditions. The system suitability parameters were calculated according to United States Pharmacopeia (USP) guidelines [22].

2.4.1.3 Linearity

Different aliquot volumes were separately transferred from each working solution into 10-mL volumetric flasks and diluted to volume with methanol to form working solutions with concentrations of: 100–800 µg mL^{-1} of HCA, 250–1500 µg mL^{-1} of FSA, and 200–1200 µg mL^{-1} of MPB and PPB. Ten microliters of each solution were applied to a TLC plate using a 100-µL syringe. The chromatographic conditions were applied, and the chromatograms were recorded. The calibration curves were plotted between the recorded peak area $\times 10^{-3}$ and the corresponding concentrations, from which the linear and polynomial regression equations were calculated. The calibration curves were made from the average of three experiments.

2.4.1.4 Application to Pharmaceutical Preparation

A five-gram portion of cream was transferred to a 50-mL volumetric flask, taking care to avoid sticking cream to the walls of the volumetric flask. A 35-mL portion of methanol was added to the flask, and the cream was allowed to melt by warming at 60°C in a water bath with constant shaking. The solution was allowed to cool to room temperature. The volume was made up to the mark with methanol and mixed. The solution was centrifuged at 3500 rpm for 5 min, and a clear supernatant solution was obtained. Further dilution was done to obtain a final concentration 500 µg mL^{-1} of HCA and 1 mg mL^{-1} of FSA. The following procedure was as detailed under Section 2.4.1.1. The concentrations of the four components were calculated using the corresponding regression equation. When carrying out the standard addition technique, different known concentrations of the pure standard of each drug were added to the pharmaceutical dosage form before proceeding in the previously mentioned procedure.

2.4.2 For Chemometric Method

2.4.2.1 Construction of Calibration Set

Multilevel partial factorial design [23] was used for the construction of the calibration and validation sets. A five-level, five-factor calibration design was used. Thirteen mixtures were used for

building the calibration model. The laboratory-prepared mixtures of HCA, FSA, MPB, and PPB were prepared within their corresponding concentration ranges. The absorption spectra of the prepared mixtures were recorded in the range of 200–400 nm and transferred to Matlab® for subsequent data manipulation.

2.4.2.2 Application to Validation Set

Into a series of 10-mL volumetric flask, accurate aliquots of each component were transferred from their working solutions to prepare twelve mixtures containing different ratios of the cited drugs. The spectra of the prepared solutions from 200 to 400 nm were recorded and transferred to Matlab®. The concentration of each component was calculated using the constructed model.

2.4.2.3 Application to Pharmaceutical Preparation

A 1-g portion of cream was transferred to a 50-mL volumetric flask, taking care to avoid sticking cream to the walls of the volumetric flask. A 35-mL portion of methanol was added to the flask, and the cream was allowed to melt by warming at 60°C in a water bath with constant shaking. The solution was allowed to cool to room temperature. The volume was made up to the mark with methanol and mixed. The solution was centrifuged at 3500 rpm for 5 min, and a clear supernatant solution was obtained. Further dilution was done to obtain a final concentration 8 µg mL^{-1} of HCA and 16 µg mL^{-1} of FSA. The concentration of each component was calculated using the constructed PCR and PLS models. When carrying out the standard addition technique, different known concentrations of the pure standard of each drug were added to the pharmaceutical dosage form before proceeding in the previously mentioned procedure.

3 Results and Discussion

The aim of this work was to design, optimize, and validate simple, accurate, selective, and precise analytical techniques which were TLC–densitometry and chemometric-assisted spectrophotometry (PCR and PLS), for the simultaneous estimation of the quaternary mixture of HCA, FSA, MPB, and PPB in their pure form and topical pharmaceutical formulation.

3.1 TLC–Densitometry

This method offers a simple way for quantification directly on TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing to a standard curve from reference materials chromatographed simultaneously under the same condition [5, 24–27].

3.1.1 Optimization of the Method

To optimize the method conditions, it was necessary to test the effect of different variables. In order to resolve this quaternary mixture, several ratios of different developing systems were checked. Changing the ratio of benzene affected the resolution of MPB and PPB. Increasing the ratio of methylene chloride affected the symmetry of HCA bands. Increasing methanol ratio caused an increase in R_F of both HCA and FSA bands but also showed tailing of FSA bands. Finally, it was found that the best separation was obtained by applying the developing system using methylene chloride–methanol–benzene in the ratio of 10:2:5 (*v*/*v*). The four components were separated at R_F = 0.13 ± 0.01, 0.32 ± 0.01, 0.49 ± 0.02, and 0.59 ± 0.02 for HCA, FSA, MPB, and PPB, respectively, using detection at 240 nm where the separated peaks were sharp and symmetrical with minimum noise, as shown in **Figure 2.**

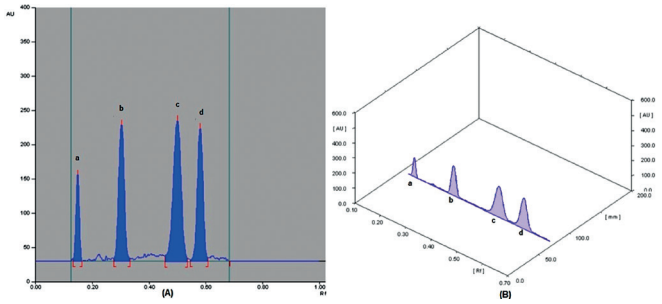

Figure 2

(A) 2D-TLC chromatogram; (B) 3D-TLC chromatogram of (a) 10 µg band^{-1} of FSA, (b) 5 µg band^{-1} of HCA, (c) 2 µg band^{-1} of MPB, and (d) 2 µg band^{-1} PPB using methylene chloride–methanol–benzene (in the ratio of 10:2:5 *v*/*v*) as the developing system.

3.1.2 Method Validation

Method validation was performed according to the International Conference on Harmonization (ICH) guidelines [28] as follows:

Table 1

Linear and polynomial regression equations obtained by the TLC–densitometric method.

	HCA		FSA		MPB		PPB	
	Polynomial regression	Linear regression	Polynomial regression	Linear regression	Polynomial regression	Linear regression	Polynomial regression	Linear regression
Concentration range (µg band^{-1})	1–8	3–7	2.5–15	5–15	2–12	4–12	2–12	4 – 12
No. of calibration curve points	7	5	6	5	6	5	7	5
x^2-Coefficient	−0.1976	–	−0.0103	–	−0.1047	–	−0.0495	–
x-Coefficient	3.4529	1.2296	0.3324	0.1246	3.8594	2.1785	1.3256	0.526
y-Intercept	−1.9471	4.0447	1.003	1.9257	1.4971	7.4113	−0.3614	2.481
r^2	0.9996	0.9953	0.9994	0.9554	0.9993	0.9869	0.9995	0.9633

Table 2

Assay parameters and validation sheet obtained by applying the proposed TLC–densitometric method using polynomial regression equation for standard solutions.

Parameters	HCA	FSA	MPB	PPB
Mean[a)]	100.06	100.32	99.98	99.59
RSD %	1.749	1.505	1.420	1.778
Accuracy[a),b)]	99.95 ± 0.99	99.14 ± 0.83	99.47 ± 1.22	99.54 ± 1.54
Repeatability[a),c)]	1.094	0.892	0.657	1.016
Intermediate precision[a),c)]	0.957	0.768	0.912	0.887
Robustness[a),d)]	99.95 ± 0.754	99.75 ± 0.599	100.29 ± 0.433	99.77 ± 0.521

[a)]Average of three experiments
[b)]Mean ± standard deviation of 5 concentrations of each drug
[c)]Relative standard deviation (RSD %) of triplet measures of 3 concentrations for each drug (n = 9)
[d)]Robustness were checked by changing methanol portion (1.8, 2.2, 2.4 by volume)

Table 3

Application of standard addition technique to the analysis of Fusi-zon® cream by applying the proposed methods.

	TLC–densitometry			PLS		
	Found in µg band^{-1}[a)]	Found[c)] Recovery% ±SD	Pure added[c)] Recovery% ±SD	Found in µg mL^{-1}[b)]	Found[c)] Recovery% ±SD	Pure added[c)] Recovery% ±SD
HCA	4.980	99.60 ± 0.72	99.72 ± 0.86	8.047	100.58 ± 0.69	100.79 ± 1.06
FSA	10.023	100.23 ± 0.59	100.11 ± 0.84	16.040	100.25 ± 0.76	101.06 ± 1.06
MPB	2.625		100.94 ± 0.67	4.223		100.16 ± 0.75
PPB	1.313		100.55 ± 0.59	2.134		100.05 ± 0.66

[a)]HCA claimed to be 5 µg band^{-1}, FSA: 10 µg band^{-1}
[b)]HCA claimed to be 8 µg mL^{-1}, FSA: 16 µg mL^{-1}
[c)]Average of six experiments

3.1.2.1 Range and Linearity

The concentration ranges were selected on the basis of the anticipated drugs concentration during the assay of the pharmaceutical formulation. The linearity of the proposed methods was evaluated by processing the different calibration curves on three different days. The results were analyzed using peak area ($\times 10^{-3}$) of the developed bands. Both the linear and polynomial functions were evaluated where the polynomial regression was superior to that of the linear ones as shown in **Table 1.** The goal of polynomial regression is to model a non-linear relationship between the independent and dependent variables (technically, between the independent variable and the conditional mean of the dependent variable). This is similar to the goal of nonparametric regression, which aims to capture non-linear regression relationships. An advantage of traditional polynomial regression is that the inferential framework of multiple regressions can be used [29]. This can be explained as the usual Beer's law, which is the basis of spectrophotometry, is not valid for densitometry where the absorption and scattering of radiations occur during direct zone measurement on a layer, so densitometric calibration plots are linear towards the lower concentrations but it is lower downwards the x-axis at higher concentrations [30]. Thus, for a wide concentration range, better accuracy and precision can be obtained using polynomial regression.

3.1.2.2 Accuracy

To study the accuracy of the proposed method, procedure under study of linearity was repeated three times for the determination of three blind concentrations of each component within the linearity range. The accuracy expressed as mean percentage recoveries and relative standard deviation (RSD) is shown in **Table 2.** The interference of excipients in the pharmaceutical

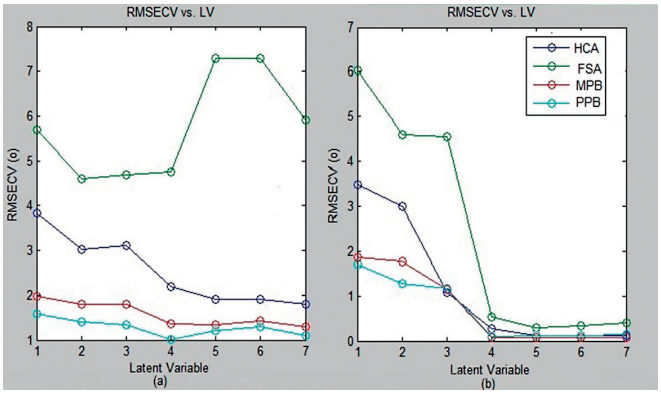

Figure 3

RMSECV of the calibration set of HCA, FSA, MPB, and PPB as a function of latent variables of (a) PCR and (b) PLS models.

Table 4

System suitability parameters of the proposed TLC–densitometric method for the simultaneous determination of the cited components.

Parameters	HCA	FSA	MPB	PPB	Reference USP value [22]
R_F (retardation factor)	0.32 ± 0.01	0.13 ± 0.01	0.49 ± 0.02	0.59 ± 0.02	
N (efficiency)	4302	3775	6195	23391	$N > 2000$
k' (capacity factor)	2.13	6.69	1.04	0.69	1–10 acceptable
α (separation factor)	3.15		2.04	1.50	>1
T (tailing factor)	1.04	0.88	1.02	0.92	$T \leq 2T = 1$ for symmetric peak
R_s (experimental resolution)	3.51		2.67	1.45	$R_s > 1.5$

formulations was studied by applying standard addition to the pharmaceutical formulation. The good accuracy proved that the excipients in the pharmaceutical formulation did not interfere in the analysis of these compounds in the pharmaceutical formulation as shown in **Table 3.**

3.1.2.3 Precision

Precision was studied with respect to both repeatability and intermediate precision through the analysis of three different concentrations of each component, within the linearity range, by three replicate analyses on a single day and on three consecutive days, respectively. The results expressed as mean percentage recoveries and RSD are illustrated in Table 2.

3.1.2.4 Selectivity

Selectivity was ascertained by how accurately and specifically the components of interest were determined in the presence of each other as shown in **Figure 3.** Furthermore, good results obtained by applying the method to Fusi-zon® cream proved that the additives in the capsules did not interfere with any of the four separated components, as shown in Table 3.

3.1.2.5 Robustness

The analysis of samples was done under a variety of experimental conditions, such as small changes in proportions of different components by up to ±0.5% mainly of the organic parts (methanol) of the developing systems. The effect on R_F values and peak parameters was studied. The methods proved to be robust, and percentage recoveries and RSD were calculated, as shown in Table 2.

3.1.2.6 System Suitability

System suitability was checked by calculating different parameters as shown in **Table 4.** The obtained values were in the acceptable ranges when compared to the reference values [22].

3.2 Chemometric Method

Among the different regression methods existing for multivariate calibration, the factor analysis based on principal component regression model (PCR) and partial least squares (PLS) regression have received considerable attention in the chemometrics literature [31]. PCR predates PLS. In cases where only partial knowledge of components is present, PCR and PLS can work well. PCR assumes that error is only in the instrumental response and concentration matrix is error-free, while PLS assumes that error is equally distributed between concentration matrix and instrumental response (spectral) matrix. Thus, PLS produces more robust model as it removes noise from both absorbance and concentration data [32].

Table 5

The concentrations of HCA, FSA, MPB, and PPB in the calibration and validation sets using PCR and PLS models.

Experiment No.[a)]	Concentration (μg mL^{-1})			
	HCA	FSA	MPB	PPB
1	12	12	4	4
2	20	8	6	4
3	20	12	3	3
4	8	8	5	6
5	16	20	5	4
6	12	20	6	2
7	20	20	2	5
8	20	4	5	2
9	16	4	4	5
10	4	12	5	5
11	16	16	3	2
12	16	8	2	3
13	4	8	4	2
14	12	4	2	6
15	4	4	6	3
16	4	20	3	6
17	8	20	4	3
18	12	8	3	5
19	8	16	6	5
20	20	16	4	6
21	16	12	6	6
22	4	16	2	4
23	12	16	5	3
24	8	4	3	4
25	8	12	2	2

[a)]Calibration set (1–13); validation set (14–25)

3.2.1 Model Construction

The calibration set was constructed using the absorption spectra set of 13 mixtures, as listed in **Table 5.** The initial models were found to give bad results, so the regions below 205 and above 300 nm were rejected. Cross-validation methods leaving out one sample at a time was employed. The root mean squares error of cross-validation (RMSECV) was calculated which is used as a diagnostic test for examining the errors in the predicted concentrations. It indicated both precision and accuracy of predictions. The selected model was that with the smallest number of factors such that RMSECV for that model was not significantly greater than RMSECV from the model with additional factor. Four factors were found to be optimum for the mixture, as shown in Figure 3 for PCR and PLS.

3.2.2 Model Validation

To assess the prediction ability of the suggested models, an external validation set of 12 mixtures was used as listed in Table 5. The predicted concentrations were compared with the true concentrations of each component in each sample. The root mean squared errors of prediction (RMSEP) and the regression equations for the predicted *versus* actual concentration are listed in **Table 6** as diagnostic tools for model validation. The results indicated the higher predictive ability of the PLS model than that of PCR model to analyze the laboratory-prepared mixtures (validation set) within the accepted range, as shown in Table 6, where PCR was unable to interpret this complex model, as it might require a larger number of samples for accurate calibration. The proposed model was also applied for the determination of Fusi-zon® cream, and the validity of the proposed procedures was further assessed by applying the standard addition technique showing no excipients interference. The results obtained are shown in Table 3.

4 Statistical Analysis

Table 7 showed statistical comparison of the results obtained by the proposed methods and official methods [2]. The calculated *t* and *F* values were less than the theoretical ones indicating that there was no significant difference between the proposed and the official methods with respect to accuracy and precision.

Table 6

Summary of results obtained by applying the diagnostic tools for model validation of the PCR and PLS models.

Validation parameters	HCA		FSA		MPB		PPB	
	PCR	PLS	PCR	PLS	PCR	PLS	PCR	PLS
Predicted vs. known conc. plot: • Slope	0.9927	0.9945	0.9897	0.9949	0.9945	1.0039	1.0005	0.9883
• Intercept	0.1101	0.0426	0.1374	0.0426	0.0375	−0.0080	−0.0031	0.0645
• Correlation coefficient *r*	0.9992	0.9994	0.9990	0.9998	0.9994	0.9996	0.9990	0.9997
RMSEP	0.1199	0.0974	0.2661	0.1052	0.0429	0.0323	0.0459	0.0349
Recovery % ± SD	100.75 ± 1.52	100.14 ± 1.26	100.12 ± 2.38	99.99 ± 0.89	100.66 ± 1.73	100.09 ± 1.11	99.96 ± 1.37	100.54 ± 0.89

Table 7

Statistical comparison between the results obtained by the proposed methods and the official BP methods for the determination of HCA, FSA, MPB, and PPB in pure powder form.

Items	HCA			FSA			MPB			PPB		
	TLC	PLS	Official method[a)]	TLC	PLS	Official method[a)]	TLC	PLS	Official method[a)]	TLC	PLS	Official method[a)]
Mean	100.06	99.82	99.91	100.33	100.08	99.58	99.98	100.16	99.85	99.59	100.67	99.78
SD	1.75	1.60	1.07	1.51	1.34	1.33	1.42	1.56	1.14	1.79	1.13	1.11
Variance	3.0480	2.5720	1.1503	2.2720	1.7972	1.7721	2.0089	2.4364	1.2953	3.1995	1.2842	1.2320
n	7	7	5	6	7	5	6	7	5	7	6	5
Student's *t*-test	0.165 (2.228)	0.107 (2.228)		0.861 (2.262)	0.633 (2.228)		0.172 (2.262)	0.387 (2.228)		1.346 (2.228)	0.205 (2.262)	
F value	2.651 (6.163)	2.237 (6.163)		1.282 (6.256)	1.014 (6.163)		1.551 (6.256)	1.708 (6.163)		1.042 (6.163)	2.597 (6.256)	

[a)]BP method for HCA is zero order absorption method, for FSA it is acid-base titration method, while for MPB and PPB it is potentiometric titration method

Figures between parentheses represent the corresponding tabulated values

5 Conclusion

This work presented a comparative study on two analytical techniques based on UV spectrophotometry which were TLC–densitometric method and chemometric-assisted spectrophotometric method (PLS). Both techniques were successfully applied for the simultaneous estimation of the quaternary mixture of HCA, FSA, MPB, and PPB in their pure form and topical pharmaceutical formulation. The TLC–densitometric method has the advantage over HPLC methods as it minimizes the usage of reagents which supports the eco-friendly behavior of green chemistry, it minimizes the time required for analysis, and it utilizes the merit of applying several sample bands on TLC plate, which may be more advantageous for regulatory quality control laboratories. In addition, the method is inexpensive and does not require certain types of stationary phases, but still, the method fulfills the same validation parameters and efficiency when compared to reported HPLC method. Meanwhile, the chemometric method has the advantage of being simpler as it does not require special reagents or chemicals, and it is considered to be time- and cost-saving, but it requires a special software (Matlab). It was found that PLS preceded PCR in the analysis of such complex mixtures. As a final conclusion, the results obtained by the two proposed methods were reliable, accurate, and precise. Hence, both methods can be employed for routine quality control analysis as alternative methods to different HPLC techniques in quality control laboratories lacking the required facilities for those expensive techniques.

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