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Original Article

Comparative study of two different chromatographic approaches for quantitation of hydrocortisone acetate and pramoxine hydrochloride in presence of their impurities

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ABSTRACT

In the present study, we compare the performance of two reversed-phase liquid chromatographic approaches using different eluents either conventional hydro-organic eluent or micellar one for simultaneous estimation of hydrocortisone acetate and pramoxine hydrochloride in presence of their degradants and process-related impurities; hydrocortisone and 4-butoxyphenol, respectively. For conventional reversed-phase liquid chromatography (RPLC), separation of the studied compounds was completed on an Inertsil ODS 3-C18 column (150 mm × 4.6 mm, 5 μm particle size) with a mobile phase consists of 50 mM phosphate buffer (pH 5.0): acetonitrile (50: 50, v/v). For micellar liquid chromatography (MLC), an Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 μm particle size) was chosen for the separation with a green mobile phase consists of 0.15 M sodium dodecyl sulfate, 0.3% triethylamine and 10% *n*-butanol in 20 mM orthophosphoric acid (pH 5.0). Both methods were extended to analyze hydrocortisone acetate and pramoxine hydrochloride in their co-formulated cream. RPLC was superior to MLC with regard to sensitivity for the estimation of impurities. While, MLC represents an eco-friendly, less hazardous and biodegradable approach. Furthermore, the direct injection of the cream to the system without the need to laborious samples pretreatment, excessive amount of analysis time and/or use of large amount of toxic organic solvents is one of the outstanding advantages of MLC.

Abbreviations: MLC, micellar liquid chromatography; RPLC, reversed-phase liquid chromatography; HCA, hydrocortisone acetate; PMX, pramoxine hydrochloride; HC, hydrocortisone; BPH, 4-butoxyphenol.

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1. Introduction

Hydrocortisone acetate (HCA), 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate (Fig. 1a), is a corticosteroid with glucocorticoids and to a lesser extent mineralocorticoids activity [1]. It is applied topically for the treatment of different skin disorders. Pramoxine hydrochloride (PMX) (Fig. 1b), also known as pramocaine HCl, is chemically defined as 4-[3-(4-butoxyphenoxy) propyl] morpholine hydrochloride [1]. It is a local anesthetic used for surface anesthesia. It can relief the pain and itching associated with some of skin conditions, hemorrhoids, minor wound care and anorectal disorders. Mainly, it is used alone or with other drugs like corticosteroids, usually in a concentration of 1%, in a wide range of formulations. Hydrocortisone (HC), 11 β,17,21-trihydroxypregn-4-ene-3,20-dione (Fig. 1c), and 4-butoxyphenol (BPH) (Fig. 1d), are reported as the potential impurities and degradants of HCA and PMX in both British pharmacopeia (BP) [2] and United States pharmacopeia (USP) [3], respectively. HCA is the topic of a monograph in both BP [2] and USP [3], while PMX is an official drug in USP [3]. Both HCA and PMX are combined in a topical dosage form at a pharmaceutical ratio 1:1 for the treatment of hemorrhoids. Reviewing the literature, few analytical methods were reported for assaying PMX in various matrices. Most of them are HPLC [4–8] and TLC [8] methods. For HCA, many methods were published for its determination in different matrices including spectrophotometry [9–12], HPLC [8,13–17] and TLC [8,18–20]. Only one report is available for the simultaneous determination of PMX and HCA in coformulated cream using HPLC and TLC [8].

Quantitation of drugs in presence of their potential impurities has become very important. This is because the existence of impurities in the drug substance may affect its safety. To the extent of our knowledge, there is no published method for the simultaneous determination of PMX and HCA in the presence of their related-impurities and degradation products. This inspired us to study the stability of both HCA and PMX and develop analytical methods that can detect and quantitate HC and BPH in HCA and PMX drugs, respectively.

Nowadays, there is a great concern about the use of micellar liquid chromatography (MLC) as an efficient alternative to conventional HPLC with hydro-organic mobile phases. In micellar system, multiple interactions occurred between the solutes and both the mobile and stationary phases, e.g. hydrophobic, electrostatic and steric interactions [21–23]. MLC has a lot of advantages over conventional RPLC. The most important advantage of MLC over RPLC is reduced amount of organic solvent consumed and decreased generated waste without affecting the chromatographic performance. Additionally, easy sample treatment and direct on-column injection of physiological fluids are another advantages [24]. Also, its ability to simultaneously separate hydrophilic and hydrophobic compounds in the same run with no need to gradient elution is a great merit [25]. In our laboratory, MLC was confirmed to be a highly effective approach in the quality control of several drugs in different dosage forms either in combinations [26] or in the presence of their potential impurities [27].

In our study, we aimed to establish a stability-indicating method by either micellar or conventional liquid chromatographic approach to simultaneously determine HCA and PMX in the presence of their potential related-impurities HC and BPH, respectively. The RPLC method allowed the detection of trace amounts of BPH and HC impurities in PMX/HCA combination with easy procedure. While MLC method can separate the four compounds using small amount of organic modifier and thus generates little amount of toxic waste. This make the approach less hazardous and environmentally friendly. Additionally, it allowed the separation of the drugs in their combined cream with no need to laborious samples pretreatment, excessive amount of analysis time and/or use of large amount of toxic organic solvents.

2. Experimental

2.1. Instrumentation

Chromatographic separation was performed on an Agilent 1220 LC system (G4294B configuration, Agilent technologies,

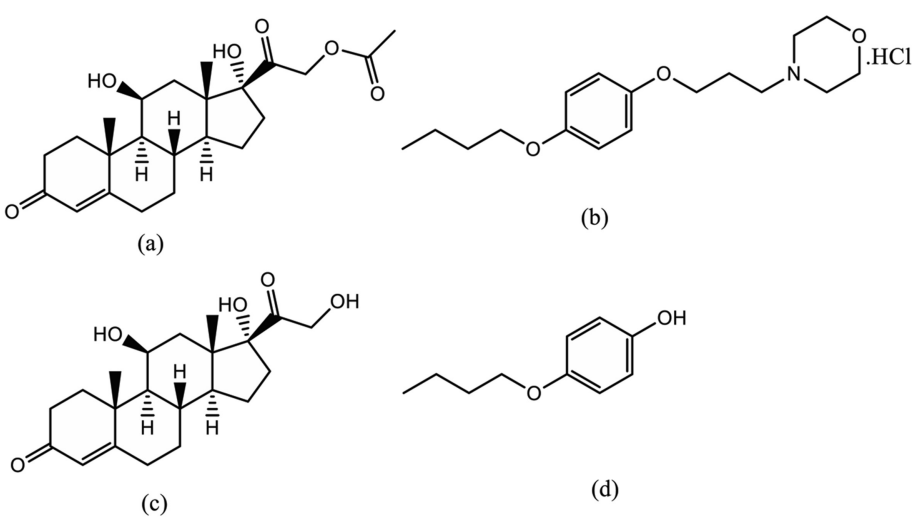

Fig. 1 – Structural formulas of (a) HCA, (b) PMX, (c) HC and (d) BPH.

USA), equipped with a dual solvent deliver system, an auto sampler and diode array detector. A Docu pH-meter (Sartorius, USA) was used for pH adjustment. An ultrasonic bath (S 100 H, Elmasonic, Germany) was used.

2.2. Chemicals and reagents

Analytical Reagent Grade chemicals and HPLC grade solvents were used. HCA was purchased from Tokyo Chemical Industry Co, Ltd. (Tokyo, Japan). PMX and BPH were purchased from Sigma–Aldrich Co. (Darmstadt, Germany). HC, orthophosphoric acid (85%), sodium dodecyl sulfate (SDS, 90%), sodium hydroxide, triethylamine (TEA), sodium dihydrogen phosphate, disodium hydrogen phosphate, methanol, 2-propanol, acetonitrile and *n*-butanol (HPLC grade) were purchased from Wako Pure Chemical industries, Ltd. (Osaka, Japan). Pramosone® cream (batch no.# 14131B), labeled to contain 1% HCA and 1% PMX, was manufactured by Ferndale Laboratories, Inc., Ferndale, MI 48220 U.S.A.

2.3. Chromatographic conditions

2.3.1. MLC

A mobile phase containing 0.15 M SDS, 10% *n*-butanol and 0.3% TEA, prepared in 20 mM orthophosphoric acid adjusted at pH 5.0, running through an Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 μm particle size) (Agilent Technologies, USA) was used.

2.3.2. RPLC

An Inertsil ODS 3-C18 column (150 mm 4.6 mm, 5 mm particle size) (Agilent Technologies, USA) was used as the stationary phase. A mobile phase consists of acetonitrile: 50 mM phosphate buffer (pH 5.0) (50: 50, v/v) was used.

In each method, the mobile phase was filtered, degassed and pumped at a flow rate of 1.0 mL/min and detection was monitored at 230 nm.

2.4. Standard solutions

Stock solutions (1000 μg/mL) of HCA and PMX and (200 μg/mL) of HC and BPH were prepared in methanol. Further dilution of the stock solutions was made with the mobile phase to prepare working solutions of the studied compounds. HCA, HC and BPH solutions were stable for at least seven days, while PMX was stable for at least two days when kept at 4 °C in refrigerator.

2.5. Procedures

2.5.1. Construction of calibration graphs

2.5.1.1. *MLC method*. Aliquots of HCA, PMX, HC and BPH stock solutions, over the concentration ranges of 10.0–100.0, 5.0–100.0, 1.0–30.0 and 3.0–25.0 μg/mL for HCA, PMX, HC and BPH, respectively, were transferred into four series of 10 mL volumetric flasks and completed with the mobile phase. Twenty μL injections were made. Calibration plots of the peak area *versus* the final concentrations of each drug in μg/mL were constructed and the regression equations were also derived.

2.5.1.2. *RPLC method*. Aliquots of HCA, PMX, HC and BPH stock solutions were transferred into four series of 10 mL volumetric flasks and completed with the mobile phase so that the final concentrations were in the ranges of 10.0–200.0, 15.0–100.0, 0.5–25.0 and 1.0–20.0 μg/mL for HCA, PMX, HC and BPH, respectively. Twenty μL injections were made. Calibration plots of the peak area *versus* the final concentrations of the drugs in μg/mL were constructed and the corresponding regression equations were also derived.

2.5.2. Analysis of HCA/PMX laboratory-prepared mixtures

Aliquots of HCA and PMX stock solutions were transferred into a set of volumetric flasks maintaining the pharmaceutical ratio of 1:1, then diluted with the mobile phase. Procedure in Section 2.5.1 were then followed. Percentages found were calculated from the regression equations.

2.5.3. Analysis of HCA/HC/PMX/BPH synthetic mixtures

Aliquots of HCA, HC, PMX and BPH stock solutions in different ratios were transferred into a set of volumetric flasks and completed with the mobile phase. Procedure in Section 2.5.1 were then followed. Percentages found were calculated from the regression equations.

2.5.4. Analysis of HCA and PMX in coformulated cream

2.5.4.1. *MLC method*. An accurately weighed amount of cream (5.0 g), equivalent to 50.0 mg HCA and 50.0 mg PMX, was transferred to a 100-mL volumetric flask, dissolved in micellar mobile phase and then sonicated for 15 min. Further dilution of the stock solution was made with the mobile phase to prepare working solutions. The resulted solutions were filtered before injection. Procedure described in construction of calibration graphs section of MLC were then followed. Percentages found were calculated from the corresponding regression equations.

2.5.4.2. *RPLC method*. An accurately weighed amount of cream (5.0 g), equivalent to 50.0 mg HCA and 50.0 mg PMX, was transferred to a 100-mL volumetric flask followed by 30.0 mL methanol. The solution was sonicated for 30 min followed by filtration. The extraction steps were repeated three times and the filtrates were collected and completed with methanol. Further dilutions of the stock solution were made with mobile phase. The resulted solutions were filtered before injection. Procedure in Section 2.5.1.2 were then followed. Percentages found were calculated from the corresponding regression equations.

3. Results and discussion

3.1. Optimization of chromatographic performance

The developed methods allow good separation and quantitation of the studied compounds. Under the above mentioned experimental conditions, clear baseline separation with a good resolution was attained in a reasonable run time (less than 10 min). Various experimental parameters affecting the separation and chromatographic performance of the concerned compounds in both methods have been investigated and optimized. One-by-one sequential strategy is performed

for optimizing each experimental variable. These variables including column, detection wavelength, mobile phase composition and flow rate. Variables are optimized by changing each in a sequential order while maintaining all others constant to achieve the highest plate counts, the highest sensitivity and good resolution within short chromatographic run time.

3.1.1. *MLC method*

Throughout the study two columns were tried, including Inertsil ODS 3-C18 column (150 mm × 4.6 mm, 5 μm particle size) and Eclipse XDB-C8 column (150 mm × 4.6 mm, 5 μm particle size). The second column was the most suitable since it could separate all the studied compounds in a short chromatographic run time. While PMX was highly retained upon using C18 column. This attributed to the hydrophobicity of PMX. It will interact with the C18 column and highly retained for longer time. Thus, a shorter chain length column (C 8 column) is more appropriate.

To attain the highest sensitivity, different detection wavelengths in the range of (190–400 nm) were tried. The most appropriate wavelength regarding the sensitivity for all the studied compounds was found to be 230 nm (Fig. 2a).

In order to design an eco-friendly green analytical protocol, micellar mobile phase was investigated where it consists of an aqueous solution of a surfactant with a small proportion of organic modifier, thus it is environmentally friendly [25]. So, modifications in the mobile phase composition were done to study the chance of enhancing the chromatographic performance (Table 1).

The influence of change in pH of the mobile phase on the chromatographic performance was tried. The working pH range that maintains the lifetime of the column is restricted to acid and neutral pH. So, different values over the working range of column 3.0–6.0 were studied. Slight change in the retention time of all compounds was observed by changing the pH. The other chromatographic parameters (efficiency and resolution) were increased by increasing the pH until pH

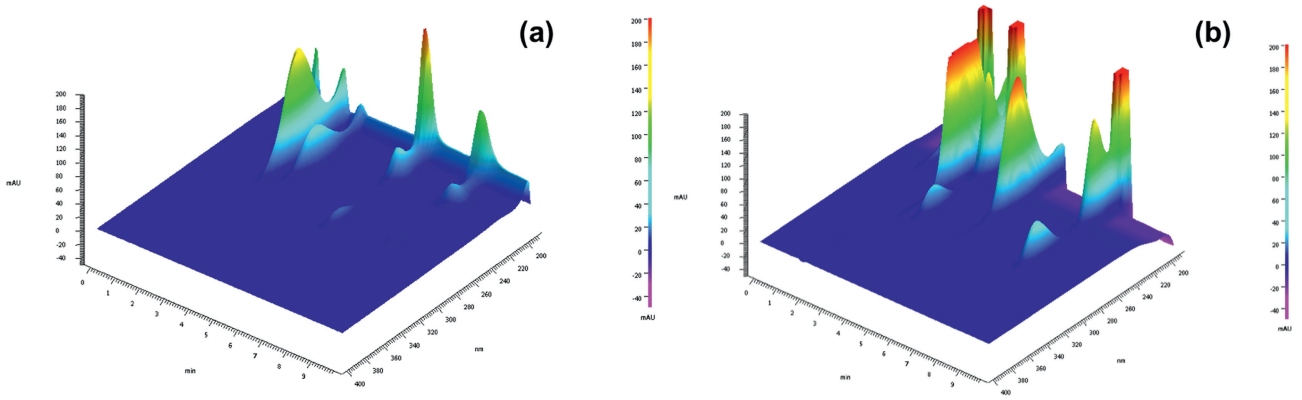

Fig. 2 – Three-dimensional (3D) plot of a chromatographic run of the studied drugs using DAD detector for (a) MLC and (b) RPLC.

Table 1 – Optimization of the chromatographic conditions for the separation of a mixture of HC, HCA, PMX and BPH by the proposed MLC method.

Parameter		Number of theoretical plates[a]				Resolution[a]			Separation factor[a]		
		HC	HCA	BPH	PMX	HC/HCA	HCA/BPH	BPH/PMX	HC/HCA	HCA/BPH	BPH/PMX
pH	**3.0**	804	1366	2107	2379	3.60	10.80	3.49	1.61	2.41	1.52
	4.0	1104	1383	2418	2467	3.89	11.21	7.10	1.63	2.37	1.51
	5.0	1359	2261	2721	2547	4.50	12.55	7.15	1.59	2.37	1.50
	6.0	1035	1355	2223	2295	4.00	10.94	6.50	1.62	2.39	1.48
Conc. of SDS (mM)	**0.075**	914	1629	2090	2745	3.60	11.25	7.84	1.44	2.34	1.56
	0.10	966	1499	2269	2798	4.00	12.65	7.10	1.59	2.33	1.60
	0.13	1043	1762	2647	2632	4.35	2.94	7.50	1.58	2.45	1.48
	0.15	1359	2261	2721	2547	4.50	12.55	7.15	1.59	2.37	1.50
	0.175	1059	1569	2169	2171	3.88	10.91	6.51	1.59	2.37	1.50
n-butanol conc. (%)	**4**	1246	2019	2551	16363	4.29	18.14	29.73	1.43	3.18	2.49
	7	1086	1433	3207	5076	4.19	14.54	10.14	1.56	2.60	1.54
	8	1198	1283	3463	4705	5.00	15.09	8.30	1.77	2.74	1.43
	10	1359	2261	2721	2547	4.50	12.55	7.15	1.59	2.37	1.50

[a] Calculations were done according to the USP guidelines [3].

5.0 and then decreased at pH 6.0. The plate counts and resolution were comparatively better while using pH 5.0. So, it was selected as the optimum pH throughout the work.

The anionic sodium dodecyl sulfate (SDS) is the most commonly used surfactant in MLC and has revealed as one of the most effective silanol suppressors, giving rise to almost symmetrical peaks [24]. Thereby, the effect of SDS concentration was studied using five different concentrations of 0.075, 0.10, 0.13, 0.15 and 0.175 M of SDS, all modified by using 10% *n*-butanol and adjusted at pH 5.0. The relationship between the concentration of SDS and the retention factor k′ value of the four studied compounds is indicated in Fig. 3a. http://pubs.rsc.org/en/content/articlehtml/2009/gc/b815182b-imgfig4. It was clear that the retention factor k′ of the compounds especially that of PMX was decreased by increasing the concentration of the SDS solution. Additionally, the number of theoretical plates increased by increasing the SDS concentration until 0.15 M SDS and then decreased. The use of 0.15 M and 0.17 M SDS give the same retention time, while 0.15 M resulted in better sensitivity. So, 0.15 M SDS was selected as the optimum concentration (Table 1).

Addition of a small volume of short chain alcohol to the micellar mobile phase produces an enhancement in efficiency of the method [25]. This attributed to reducing the amount of adsorbed surfactant on the stationary phase as well as improving the poor wetting of the stationary phase when only aqueous micellar mobile phases are employed. Hence, the mass transfer of the analyte can be greatly improved and the retention time is reduced significantly [25]. So that, different organic modifiers viz. acetonitrile, methanol, 2-propanol and *n*-butanol were examined to study the effect of the nature of the organic solvents. Among these solvents, only *n*-butanol could elute all the studied compounds while PMX was highly retained by using the other solvents. That is because the addition of these solvents increases the polarity of the mobile phase relative to *n*-butanol. Since the studied analytes are hydrophobic compounds; this lead to an increase in the retention time especially for PMX which is associated with larger peak width and lower plate counts. So, *n*-butanol was chosen as the optimum organic modifier through the work. Consequently, different concentrations of *n*-butanol (4–10%) were tried. The relationship between the concentration of *n*-butanol and the retention factor k′ value of the studied compounds is shown in Fig. 3b. The retention factor of all the studied compounds, especially that of PMX, decreased upon increasing the concentration of *n*-butanol. The use of 4% *n*-butanol resulted in overlapping the peaks of HCA and HC while increasing the retention time of PMX to 25 min. By increasing the concentration of *n*-butanol, good separation of the studied compounds occurs. The use of 7% results in good separation of the compounds and elution of PMX at 11.5 min. While, the use of 10% of *n*-butanol elute the compounds in a short run time. So, 10% of *n*-butanol was chosen as the optimum concentration (Table 1).

Finally, the flow rate of the mobile phase was studied (0.5, 0.8 and 1.0 mL/min). The retention times of the studied compounds were delayed upon decreasing the flow rate with unsymmetrical peaks. A flow rate of 1.0 mL/min was the optimum for good separation in an adequate time with good peak symmetry.

3.1.2. *RPLC method*

Inertsil ODS 3-C18 column (150 mm × 4.6 mm, 5 μm particle size) and Phenomenex-C18 column (250 mm × 4.6 mm, 5 μm particle size) were tried. The first column was chosen as it resulted in good separation of the four drugs in a short run time.

To attain the highest sensitivity, different detection wavelengths in the range of (190–400 nm) were tried. The most appropriate wavelength with a regard to sensitivity for all the studied compounds was found to be 230 nm (Fig. 2b).

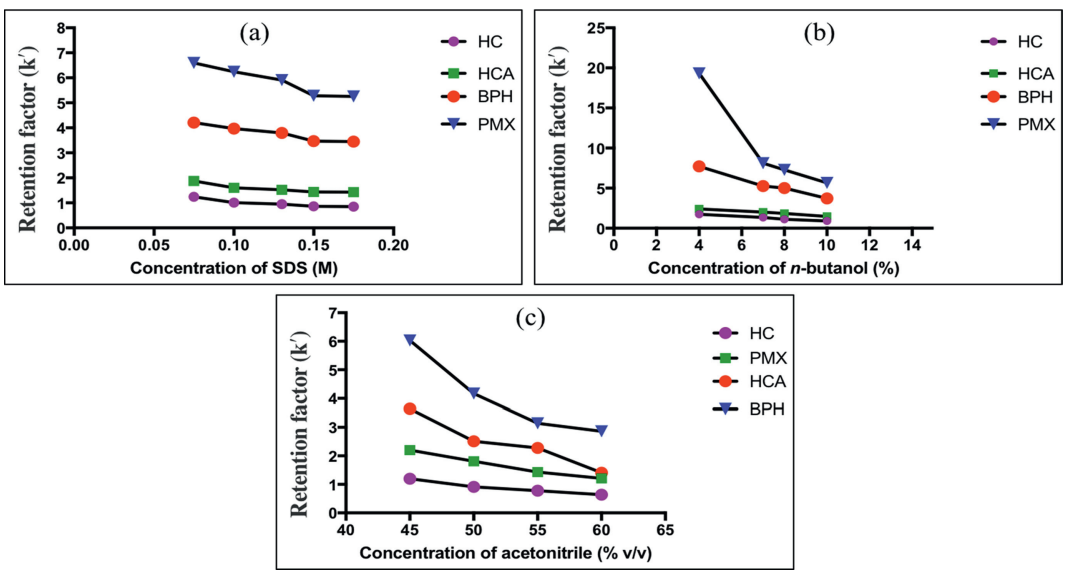

Fig. 3 – (a) The influence of the concentration of *n*-butanol in the micellar mobile phase on the retention factor k′ of the studied compounds. (b) The influence of the concentration of SDS in the micellar mobile phase on the k′ of the studied compounds. (c) The influence of the concentration of acetonitrile (%) in the RPLC mobile phase on the k′ of the studied compounds.

The effect of the nature of the organic solvent was studied using different organic solvents (methanol and acetonitrile). Acetonitrile was the suitable solvent resulting in high resolution of the compounds, while methanol resulted in overlapping of PMX and HCA (Table 2) and broad peaks of PMX and BPH. Consequently, different ratios of acetonitrile (45–60%, v/v) were tried. Acetonitrile concentration was found to have a considerable effect on the retention of the four compounds as indicated in Fig. 3c. Fifty %, v/v acetonitrile was the optimum one, as it can separate the concerned drugs within a short time. Decreasing acetonitrile concentration to 45%, v/v increased the retention times especially of BPH to 10 min, while increasing it to 60%, v/v resulted in co-elution of PMX and HCA (Table 2).

Furthermore, the effect of phosphate buffer pH was tested in the range of 3.0–6.0. pH 5.0 was chosen since it gave narrow symmetrical peaks with the best resolution and the highest plate counts (Table 2). Additionally, different phosphate buffer concentrations (20–50 mM) were tried. Fifty mM phosphate buffer was the optimum resulted in the highest resolution and number of theoretical plates (Table 2). Finally, the mobile phase was pumped at different flow rates (0.5, 0.8 and 1.0 mL/min). Flow rate of 1.0 mL/min was the optimum for better separation in a short run time.

Good separation of the four concerned compounds was attained in a reasonable chromatographic run time.

For MLC, retention times are HC ($t_R = 2.35$), HCA ($t_R = 3.03$), BPH ($t_R = 5.54$), and PMX ($t_R = 7.72$) as indicated in Fig. 4a.

Table 2 – Optimization of the chromatographic conditions for the separation of a mixture of HC, PMX, HCA and BPH by the proposed RPLC method.

Parameter		Number of theoretical plates[a]				Resolution[a]			Separation factor[a]		
		HC	PMX	HCA	BPH	HC/PMX	PMX/HCA	HCA/BPH	HC/PMX	PMX/HCA	HCA/BPH
Acetonitrile conc. (%)	**45**	2336	4986	5833	8582	9.46	11.37	14.89	1.84	1.64	1.66
	50	2742	2150	5960	7260	7.94	5.60	13.03	2.00	1.38	1.66
	55	732	2216	2930	1165	5.94	6.48	4.00	2.22	1.60	1.37
	60	2035	1374	2796	2931	5.31	1.26	7.03	1.94	1.12	1.62
pH of phosphate buffer	**3.0**	1601	1930	3325	4595	1.67	12.00	10.22	1.22	2.50	1.66
	4.0	2758	1030	5912	6155	6.60	3.60	12.57	2.07	1.30	1.66
	5.0	2742	2150	5960	7260	7.94	5.60	13.03	2.00	1.38	1.66
	6.0	2804	2768	5912	7180	7.59	6.87	13.14	1.85	1.46	1.66
Conc. of phosphate buffer (mM)	**10**	2804	1302	6155	5934	9.05	1.78	11.89	2.46	1.12	1.61
	20	2585	1133	6057	6213	7.48	2.98	12.27	2.22	2.69	1.66
	30	2626	829	5936	6099	5.00	5.11	12.48	1.81	1.49	1.65
	40	2713	753	5834	5934	5.85	3.27	12.39	2.06	1.30	1.66
	50	2742	2150	5960	7260	7.94	5.60	13.03	2.00	1.38	1.66

[a] Calculations were done according to the USP guidelines [3].

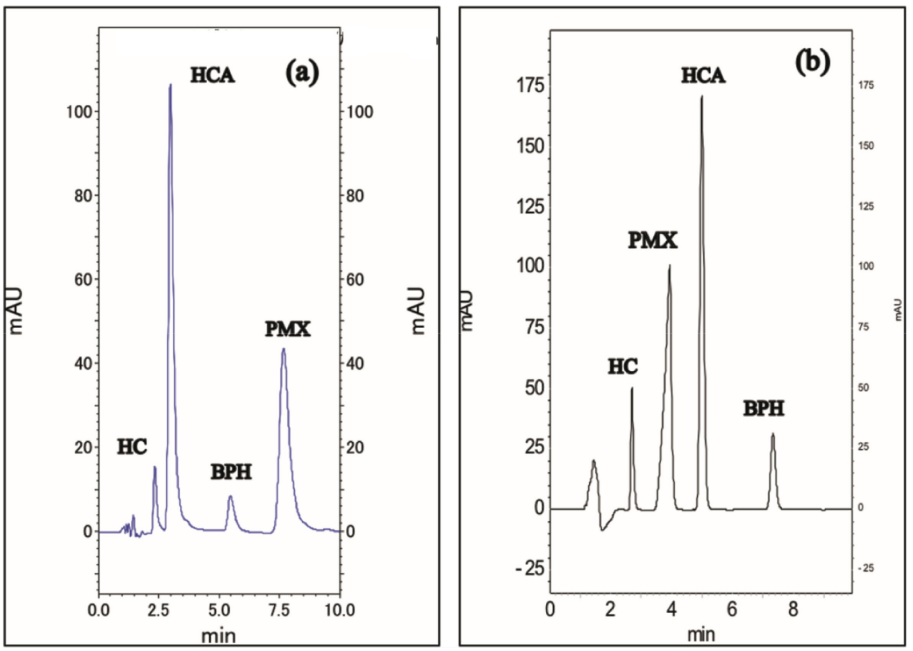

Fig. 4 – Typical chromatograms for synthetic mixture of HC (1.0 μg/mL), HCA (50.0 μg/mL), PMX (50.0 μg/mL) and BPH (3.0 μg/mL) by (a) MLC and (b) RPLC.

For RPLC, retention times were HC (t_R = 2.67), PMX (t_R = 3.94), HCA (t_R = 4.92), and BPH (t_R = 7.24) indicated in Fig. 4b.

3.2. Method validation

Validation of the developed methods was performed by testing linearity and range, detection limit (DL), quantitation limit (QL), accuracy, precision, robustness and specificity according to the International Conference on Harmonization (ICH) guidelines [28].

Under the aforementioned experimental conditions, a linearity was checked by analyzing different concentrations of the studied drugs. In case of MLC, excellent linearity was proven over the ranges of 10.0–100.0, 5.0–100.0, 1.0–30.0 and 3.0–25.0 μg/mL for HCA, PMX, HC and BPH, respectively. While RPLC was found to be rectilinear over the ranges of 10.0–200.0, 15.0–100.0, 0.5–25.0 and 1.0–20.0 μg/mL for HCA, PMX, HC and BPH, respectively (Table 3). The high values of correlation coefficients ($r > 0.9998$) and small values of relative standard deviation (RSD) indicate the good linearity of both methods over the working concentration ranges (Table 3). DL and QL were calculated as per the ICH Q2(R1) recommendations using the following equations: DL = 3.3 S_a/b and QL = 10 S_a/b, where S_a = standard deviation of the intercept and b = slope of the calibration curve (Table 3).

Precision of both methods was assured with regard to intra-day and inter-day precision through analysis of three different concentrations of the drugs in triplicate for three successive times within the same day or on three successive

Table 3 – Analytical performance data for the determination of HC, HCA, PMX and BPH by both MLC and RPLC methods.

	Parameters	HCA	PMX	HC	BPH
For MLC	Linearity range (μg/mL)	10.0–100.0	5.0–100.0	1.0–30.0	3.0–25.0
	Slope (b)	6.5×10^4	7.9×10^4	7.4×10^4	7.7×10^4
	Correlation coefficient (r)	0.9998	0.9999	0.9999	0.9998
	SD of intercept (Sa)	3.8×10^4	2.7×10^4	7.3×10^3	1.04×10^4
	SD	1.43	1.28	1.10	1.35
	% RSD	1.43	1.27	1.10	1.34
	DL (μg/mL)	1.94	1.16	0.32	0.45
	QL (μg/mL)	5.87	3.50	0.98	1.36
For RPLC	Linearity range (μg/mL)	10.0–200.0	15.0–100.0	0.5–25.0	1.0–20.0
	Slope (b)	6.9×10^4	6.6×10^4	8.6×10^4	1.2×10^5
	Correlation coefficient (r)	0.9999	0.9998	0.9999	0.9999
	SD of intercept (Sa)	4.2×10^4	3.4×10^4	1.7×10^3	1.1×10^4
	SD	1.07	1.38	0.67	0.76
	% RSD	1.07	1.38	0.67	0.75
	DL (μg/mL)	2.02	1.67	0.07	0.32
	QL (μg/mL)	6.12	5.06	0.20	0.98

Table 4 – Assay results for the determination of HCA and PMX in Laboratory-prepared mixtures by the proposed MLC and RPLC methods.

	Proposed method						Comparison method [8]	
	Conc. Taken (μg/mL)		Conc. Found (μg/mL)		% Found[a]		% Found	
	HCA	PMX	HCA	PMX	HCA	PMX	HCA	PMX
For MLC	20.0	20.0	19.90	19.87	99.51	99.33	99.07	100.53
	50.0	50.0	50.07	50.68	100.15	101.36	98.61	99.03
	100.0	100.0	99.04	98.60	99.04	98.60	100.45	101.36
Mean					99.57	99.76	99.38	100.31
SD					0.56	1.43	0.96	1.18
t-test					0.297	0.507		
F-test					2.953	1.467		
	Conc. Taken (μg/mL)		Conc. Found (μg/mL)		Percent Found		Percent Found	
	HCA	PMX	HCA	PMX	HCA	PMX	HCA	PMX
For RPLC	20.0	20.0	19.81	20.18	99.04	100.92	99.07	100.53
	50.0	50.0	50.68	49.50	101.35	98.99	98.61	99.03
	100.0	100.0	99.17	99.11	99.17	99.11	100.45	101.36
Mean					99.85	99.67	99.38	100.31
SD					1.29	1.08	0.96	1.18
t-test					0.512[b]	0.685[b]		
F-test					1.837[b]	1.193[b]		

[a] Each result is the mean of three individual determinations.
[b] The *t*- and *F*- values at $P = 0.05$ are 2.776 and 19.0, respectively [29].

days, respectively. The results indicate that the relative standard deviation (%RSD) was less than 2% which confirms the high precision of both methods (Table 4).

Accuracy of the developed methods was tested by analysis of pure samples of the studied compounds over the working concentration ranges. The methods were also applied for the determination of the concerned drugs simultaneously in laboratory prepared mixtures (Fig. 5) and their co-formulated cream (Fig. 6). Percentages found of HCA and PMX were calculated from the corresponding regression equations. The results obtained were compared with those obtained using the comparison method [8]. The comparison method depends on the separation of HCA/PMX using C18 column and a mobile phase consisting of distilled water: acetonitrile: TEA (530: 470: 0.1, v/v) at pH 3.0. Statistical analysis of the results using the Student's *t*-test and the variance ratio *F*-test [29] revealed no significant difference between the performance of the proposed and the comparison methods (Tables 5 and 6).

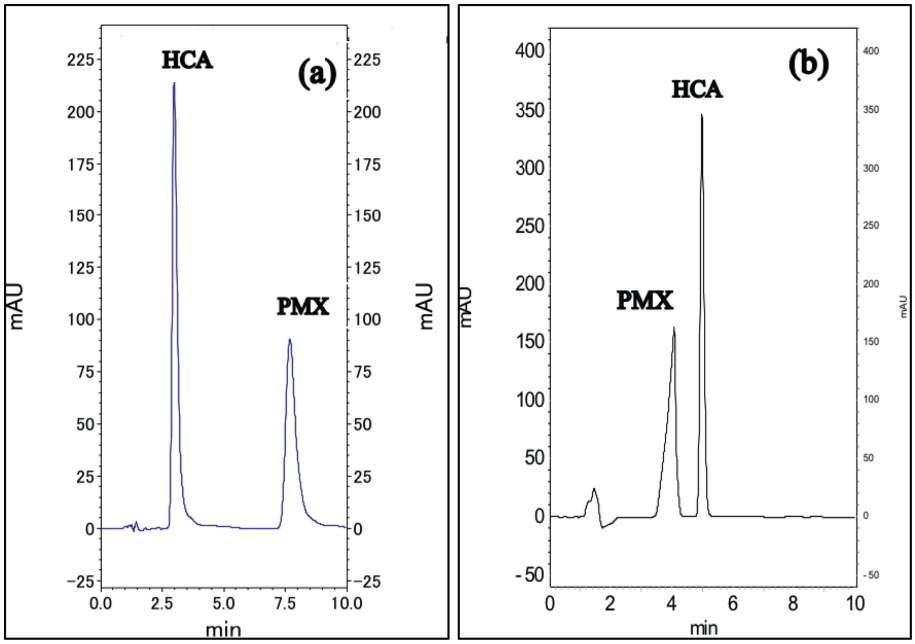

Fig. 5 – Typical chromatograms for HCA (100.0 μg/mL) and PMX (100.0 μg/mL) in laboratory prepared mixture by (a) MLC and (b) RPLC.

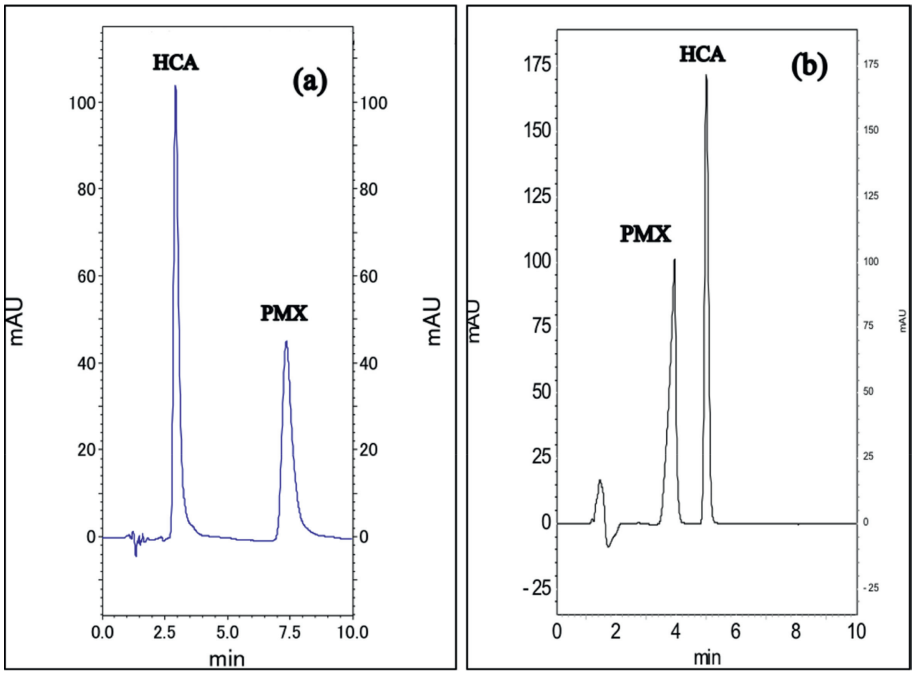

Fig. 6 – Typical chromatograms for HCA (50.0 μg/mL) and PMX (50.0 μg/mL) in Pramosone® cream by (a) MLC and (b) RPLC.

Table 5 – Assay results for the determination of HCA and PMX in Pramosone® cream by the proposed MLC and RPLC methods.

Pramosone® cream	Proposed method						Comparison method [8]	
	Conc. Taken (μg/mL)		Conc. Found (μg/mL)		% Found[a]		% Found	
	HCA	PMX	HCA	PMX	HCA	PMX	HCA	PMX
For MLC	20.0	20.0	20.35	19.95	101.74	99.77	99.06	100.05
	50.0	50.0	50.21	50.82	100.41	101.64	99.71	101.24
	100.0	100.0	98.64	101.00	98.64	100.99	100.49	99.75
Mean					100.26	100.80	99.75	100.35
SD					1.56	0.95	0.72	0.79
t-test					0.642[b]	0.636[b]		
F-test					4.718[b]	1.451[b]		
	Conc. Taken (μg/mL)		Conc. Found (μg/mL)		% Found[a]		% Found	
	HCA	PMX	HCA	PMX	HCA	PMX	HCA	PMX
For RPLC	20.0	20.0	20.41	19.87	102.07	99.33	99.06	100.05
	50.0	50.0	50.23	50.94	100.46	101.87	99.71	101.24
	100.0	100.0	99.63	99.02	99.63	99.02	100.49	99.75
Mean					100.72	100.07	99.75	100.35
SD					1.24	1.56	0.72	0.79
t-test					1.169[b]	0.270[b]		
F-test					3.002[b]	3.937[b]		

[a] Each result is the mean of three individual determinations.
[b] The *t*- and *F*- values at $P = 0.05$ are 2.776 and 19.0, respectively [29].

Table 6 – Precision data for the determination of HCA and PMX by both MLC and RPLC methods.

Compound		Conc. (μg/mL)	Inter-day precision			Intra-day precision		
			Mean[a] ± SD	% RSD	% Error	Mean[a] ± SD	% RSD	% Error
For MLC	**HCA**	**20.0**	100.05 ± 1.58	1.58	0.91	100.22 ± 0.24	0.23	0.14
		50.0	99.23 ± 0.59	0.59	0.34	100.15 ± 1.18	1.18	0.68
		100.0	100.17 ± 1.49	1.49	0.86	100.27 ± 0.55	0.55	0.32
	PMX	**20.0**	100.11 ± 0.85	0.85	0.49	100.07 ± 0.72	0.72	0.42
		50.0	99.02 ± 0.45	0.46	0.26	100.18 ± 0.60	0.59	0.35
		100.0	100.23 ± 1.32	1.32	0.76	99.47 ± 1.15	1.16	0.67
For RPLC	**HCA**	**20.0**	100.26 ± 0.95	0.94	0.54	99.47 ± 1.04	1.05	0.60
		50.0	99.95 ± 0.50	0.50	0.29	101.04 ± 1.11	1.10	0.64
		100.0	100.29 ± 0.95	0.94	0.54	99.76 ± 1.00	1.00	0.58
	PMX	**20.0**	100.51 ± 1.13	1.13	0.65	99.99 ± 0.60	0.60	0.35
		50.0	99.63 ± 0.63	0.63	0.36	99.86 ± 0.13	0.13	0.08
		100.0	100.14 ± 0.28	0.28	0.16	100.18 ± 0.87	0.87	0.50

[a] Each result is the mean of three individual determinations.

The robustness of the developed methods was checked by confirming that small deliberate changes in experimental parameters did not have a significant effect on the chromatographic behavior of the cited drugs. For MLC, these parameters include pH of the mobile phase (5.0 ± 0.1), *n*-butanol concentration (10 ± 0.5%, v/v) and SDS concentration (0.12 M ± 0.01). For RPLC, they include acetonitrile concentration (50 ± 1%, v/v), pH of phosphate buffer (5.0 ± 0.1), and concentration of phosphate buffer (50 ± 5 mM).

The specificity of the proposed methods was investigated through the determination of HCA and PMX in coformulated cream with no interference from common excipients (Fig. 6). In addition, the specificity was confirmed by analyzing different synthetic mixtures of HCA/HC/PMX/BPH (Fig. 4).

3.3. *Applications*

3.3.1. *Analysis of HCA/PMX/HC/BPH synthetic mixtures*

Both MLC and RPLC were applied for simultaneous analysis of HCA and PMX in synthetic mixtures with HC and BPH (Fig. 4) in different ratios. The average percent recoveries of both HCA and PMX were calculated based on the average of three replicate determinations. The obtained results revealed the ability of the developed methods to determine the studied drugs in presence of different concentrations of their impurities (HC and BPH). MLC can analyze HCA in presence of HC ranged from (1–8%) and determine PMX in presence of BPH ranged from (3–20%). While, RPLC can analyze HCA in presence of HC ranged from (1–16%) and determine PMX in

Table 7 – Application of the proposed method for the determination of synthetic mixtures of HCA/HC and PMX/BPH by MLC.

Parameter	Conc. taken (μg/mL)		Conc. found of HCA (μg/mL)	% Found[a] of HCA
	HCA	HC		
HCA/HC synthetic mixtures	100.0	1.0	98.43	98.43
		4.0	98.61	98.61
		6.0	100.07	100.07
		8.0	99.14	99.14
X' ± S.D		99.06 ± 0.73		
% RSD		0.74		
% Error		0.37		
	Conc. taken (μg/mL)		Conc. found of PMX (μg/mL)	% Found[a] of PMX
	PMX	BPH		
PMX/BPH synthetic mixtures	100.0	3.0	98.73	98.73
		5.0	98.55	98.55
		10.0	99.82	99.82
		20.0	99.51	99.51
X' ± S.D		99.15 ± 0.61		
% RSD		0.61		
% Error		0.31		

[a] Each result is the mean of three individual determinations.

Table 8 – Application of the proposed method for the determination of synthetic mixtures of HCA/HC and PMX/BPH by RPLC.

Parameter	Conc. taken (μg/mL)		Conc. found of HCA (μg/mL)	% Found[a] of HCA
	HCA	HC		
HCA/HC synthetic mixtures	50.0	0.5	50.13	100.26
		1.0	49.83	99.66
		5.0	49.66	99.31
		8.0	49.20	98.40
X' ± S.D		99.40 ± 0.78		
% RSD		0.78		
% Error		0.39		
	Conc. taken (μg/mL)		Conc. found of PMX (μg/mL)	% Found[a] of PMX
	PMX	BPH		
PMX/BPH synthetic mixtures	50.0	1.0	49.92	99.83
		5.0	49.16	98.32
		8.0	50.03	100.05
		10.0	49.54	99.09
X' ± S.D		99.32 ± 0.78		
% RSD		0.79		
% Error		0.40		

[a] Each result is the mean of three individual determinations.

presence of BPH ranged from (2–20%). The results are presented in (Tables 7 and 8).

3.3.2. *Application of the developed methods to the analysis of HCA/PMX in Pramosone® cream*

The developed methods were successfully extended to the assay of both HCA and PMX in their co-formulated cream. The results of both methods were also compared with those obtained using the comparison method [8]. No significant difference between the performance of the methods regarding the accuracy and precision upon statistical analysis of the results using the Student's *t*-test and variance ratio *F*-test [29] (Table 6).

4. Conclusion

In this study, a comparison of the performance of MLC and RPLC methods for the simultaneous determination of HCA and PMX in presence of their potential impurities, HC and BPH, was performed. MLC was found to be superior in being eco-friendly, less hazardous, and biodegradable. This is because it consumes less amount of toxic organic solvent and thus generates little amount of toxic waste. Furthermore, the ability of direct injection of the cream dosage form to the system without the need to tedious multi-step extraction and pretreatment is one of its outstanding advantages. On the other hand, RPLC was superior in terms of sensitivity to

degradation products and efficiency. The methods were successfully used for the analysis of HCA and PMX in coformulated cream with a satisfactory recovery of both drugs and small standard deviation.

Conflicts of interest

The authors declare that they have no conflict of interest.

REFERENCES

[1] Sweetman Sean C. Martindale: the complete drug reference. 36th ed. UK: The Pharmaceutical Press; 2009. https://doi.org/10.1017/CBO9781107415324.004.

[2] The British pharmacopoeia. London: Her Magesty's Stationary Office; 2012.

[3] The United States pharmacopoeia 30, the national formulary 25. Rockville, MD: US Pharmacopeial Convention; 2007.

[4] Tavlarakis P, Greyling J, Snow NH. Simultaneous determination of pramoxine HCl and benzalkonium chloride in wound care solutions by HPLC. Anal Methods 2010;2:722. https://doi.org/10.1039/b9ay00284g.

[5] Wang L, Li H. Determination of content of compound pramoxine hydrochloride cream by HPLC 2007;37:52–4. Xibei Daxue Xuebao, Ziran Kexueban.

[6] Chang ZL, Boller JP, Pacenti DM, Wong CF. Rapid high-performance liquid chromatographic determination of pramoxine hydrochloride in topical cream and suppositories. J Chromatogr 1984;291:428–33.

[7] Weinberger R, Mann B, Posluszny J. High-pressure liquid chromatographic analysis of pramoxine hydrochloride in high lipoid aerosol foam dosage form. J Pharm Sci 1980;69:475–7.

[8] Merey HA, Mohammed MA, Morsy FA, Salem MY. Simultaneous determination of pramocaine hcl and hydrocortisone acetate in pharmaceutical dosage form. J Liq Chromatogr Relat Technol 2013;36:2774–84.

[9] Lotfy HM, Saleh SS, Hassan NY, Salem H. Novel two wavelength spectrophotometric methods for simultaneous determination of binary mixtures with severely overlapping spectra. Spectrochim Acta Part A Mol Biomol Spectrosc 2015;136:1786–96. https://doi.org/10.1016/j.saa.2014.10.084.

[10] Myneni H, Kumar GS. Simultaneous uv-spectrophotometric estimation of hydrocortisone acetate and sulphacetamide sodium in combined dosage form. Int J Pharm Sci Res 2013;4:1901–4.

[11] Medenica M, Ivanovic D, Markovic S, Malenovic A, Jancic B. Second-derivative spectrophotometric analysis of lidocaine and hydrocortisone acetate in suppositories. Pharm Ind 2004;66:330–3.

[12] Blanco M, Coello J, Iturriaga H, Maspoch S, Villegas N. Kinetic spectrophotometric determination of hydrocortisone acetate in a pharmaceutical preparation by use of partial least-squares regression. Analyst 1999;124:911–5.

[13] Sudhakar P, Sharma P, Shrivastava B. Validation of stability indicating hplc method for assay of fusidic acid, hydrocortisone acetate and potassium sorbate in topical pharmaceutical. World J Pharm Pharmaceut Sci 2016;5:1923–38.

[14] Ascaso M, Perez-Lozano P, Garcia M, Garcia-Montoya E, Minarro M, Tico JR, et al. Development and validation of a stability indicating RP-HPLC method for hydrocortisone acetate active ingredient, propyl parahydroxybenzoate and methyl parahydroxybenzoate preservatives, butylhydroxyanisole antioxidant, and their degradation products in a rectal gel formulation. J AOAC Int 2015;98:27–34.

[15] Jancic-Stojanović B, Malenović A, Marković S, Ivanović D, Medenica M. Optimization and validation of an RP-HPLC method for analysis of hydrocortisone acetate and lidocaine in suppositories. J AOAC Int 2010;93:102–7.

[16] Chauhan V, Conway B. Optimisation of a selective liquid chromatography procedure for hydrocortisone acetate, hydrocortisone alcohol and preservatives in a pharmaceutical emulsion. Chromatographia 2005;61:555–9. https://doi.org/10.1365/s10337-005-0555-2.

[17] Hájková R, Solich P, Dvořák J, Šicha J. Simultaneous determination of methylparaben, propylparaben, hydrocortisone acetate and its degradation products in a topical cream by RP-HPLC. J Pharm Biomed Anal 2003;32:921–7. https://doi.org/10.1016/S0731-7085(03)00193-6.

[18] Dolowy M, Maryszczak J, Pyka A. Comparison of the detection and quantitative limits of hydrocortisone acetate in different chromatographic conditions in tlc. J Liq Chromatogr Relat Technol 2014;37:2929–41.

[19] Widiretani D, Luailia I, Indrayanto G. Simultaneous densitometric determination of hydrocortisone acetate and 2-phenoxyethanol in creams. J Planar Chromatogr Mod TLC 2013;26:37–42. https://doi.org/10.1556/JPC.26.2013.1.6.

[20] Kristiningrum N, Rakhmawati M. Simultaneous determination of chloramphenicol and hydrocortisone acetate in cream using TLC desitrometry method. Int Curr Pharmaceut J 2012;2:7–10.

[21] Armstrong DW, Nome F. Partitioning behavior of solutes eluted with micellar mobile phases in liquid chromatography. Anal Chem 1981;53:1662–6. https://doi.org/10.1021/ac00234a026.

[22] Love LJ, Fett JJ. Optimization of selectivity in micellar chromatographic procedures for the determination of drugs in urine by direct injection. J Pharm Biomed Anal 1991;9:323–33.

[23] Westerlund D. Direct injection of plasma into column liquid chromatographic systems. Chromatographia 1987;24:155–64. https://doi.org/10.1007/BF02688478.

[24] Ruiz-Ángel MJ, García-Álvarez-Coque MC, Berthod A. New insights and recent developments in micellar liquid chromatography. Sep Purif Rev 2009;38:45–96. https://doi.org/10.1080/15422110802178876.

[25] Boichenko AP, Loginova LP, Kulikov AU. Micellar liquid chromatography (Review). Part 1. fundamentals, retention models and optimization of separation 2007;2:92–116.

[26] Ibrahim F, El-Deen AK, El Abass SA, Shimizu K. An ecofriendly green liquid chromatographic method for simultaneous determination of nicotinamide and clindamycin phosphate in pharmaceutical gel for acne treatment. J Food Drug Anal 2016:1–7. https://doi.org/10.1016/j.jfda.2016.09.009.

[27] Ibrahim F, Sharaf El-Din MK, El-Deen AK, Shimizu K. Micellar HPLC method for simultaneous determination of ethamsylate and mefenamic acid in presence of their main impurities and degradation products. J Chromatogr Sci 2016:1–7. https://doi.org/10.1093/chromsci/bmw143.

[28] ICH Harmonised Tripartite Guideline. Validation of analytical procedures: text and methodology, Q2(R1). Geneva. 2005. n.d., http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf. [Accessed 23 September 2015].

[29] Miller JN, Miller JC. Statistics and chemometrics for analytical chemistry. 5th ed. Harlow, UK: Prentice Hall; 2005.