Journal of Applied Pharmaceutical Science Vol. 10(09), pp 134-139, September, 2020
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2020.10916
ISSN 2231-3354

Box–Behnken design-based HPLC optimization for quantitative analysis of chloramphenicol and hydrocortisone acetate in cream

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ARTICLE INFO

Received on: 31/05/2020
Accepted on: 30/07/2020
Available online: 05/09/2020

Key words:
Chloramphenicol, hydrocortisone acetate, Box–Behnken design, HPLC method, cream formulation.

ABSTRACT

Inserting chloramphenicol (CL) and hydrocortisone acetate (HCA) in cream preparation is intended to have activity against skin infection and dermatitis and such a product is available in the Indonesian market. Due to its capability as a separation technique, chromatography is widely used for the analysis of mixture in pharmaceutical products. The objective of this study was to develop high-performance liquid chromatography (HPLC) combined with an experimental design for an effective analysis of CL and HCA in a cream formulation. In this study, the experimental Box–Behnken design (BBD) was used. BBD is one of the useful experimental designs for the optimization of chromatographic separation and analysis and for getting a better understanding of the interaction of studied factors on HPLC separation quality. Separation and HPLC analysis of CL and HCA were performed using a Shimadzu LC-20AD chromatograph, a Waters X-Bridge C-18 column (250 × 4.6 mm ID, 5 µm), and a UV-Vis detector at 261 nm. HPLC method was validated according to the International Conference on Harmonization by determining several analytical performances intended for the method's purpose. Based on BBD, the optimal condition of HPLC was obtained using a mobile phase of acetonitrile 47%– 53%, with a flow rate of 0.9 ml/minutes and a column temperature of 38°C. The validation of HPLC resulted in the selectivity of a method with a resolution value of ≥1.5, linearity with a correlation coefficient of >0.999, intraday and inter-day precisions with relative standard deviation values of ≤1.9%, and recovery values in the range of 98%−102%. The validated method is successfully used for the analysis of CL and HCA in cream formulations. BBD could be an effective design to get the optimum reversed HPLC condition for the separation of CL and HCA in a cream formulation.

INTRODUCTION

Chloramphenicol (CL), chemically D-(-)-threo-1-p-nitrol-phenyl-2-dichloroacetamido 1,3-propanediol, is an antibiotic having a broad-spectrum antibacterial activity to treat either Gram-negative or Gram-positive bacterial infections and is widely used for the treatment of bacterial conjunctivitis. CL is one of the first synthetic antibiotics available in clinical practice (Al-Rimawi and Kharoaf, 2011). Besides, the corticosteroid of hydrocortisone acetate (HCA), chemically 11,17-Dihydroxy-3, 20-dioxopregn-4-en-21-yl acetate, is used to reduce the inflammation due to dermatitis. The symptom onset of inflammation induced by radiation, chemicals, infection, and allergen could be suppressed and prevented by HCA (Kristiningrum and Rakhmawati, 2012). The chemical structures of both drugs are shown in Figure 1. The combination of CL and HCA in some pharmaceutical products, especially the cream, is typically used for the treatment of dermatitis and skin infections. To perform quality control of both drugs in pharmaceutical products, an analytical method capable of analyzing CL and HCA must be developed.

Several analytical methods have been developed, validated, and applied for the analysis of CL and HCA in some pharmaceutical preparations either individually or in combination with other drugs, including ultraviolet spectrophotometry (Al-Sabha and Rasheed, 2010), thin-layer chromatography combined

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with densitometric measurement for the quantitative analysis of HCA using absorbance mode at 250 nm (Pyka *et al*., 2011), analysis of HCA and its related compounds (Dołowy *et al*., 2014) as well as simultaneous analysis of CL and HCA (Kristiningrum and Rakhmawati, 2012), high-performance liquid chromatography (HPLC) for quantitative analysis of HCA in antibiotics preparation (Bracey *et al*., 1966), analysis of CL and other drugs with a UV detector at 254 nm (Iqbal *et al*., 2006), and analysis of CL and HCA with UV detection at 259 nm (Katakam, 2014). Among these methods, HPLC is the most reported one due to its capability to provide the best separation of analytes and to get versatility in selecting the HPLC condition. However, the optimization of HPLC used was a traditional approach. In this study, the experimental Box–Behnken design (BBD) was used.

Currently, the application of chemometrics, especially the experimental designs in chromatographic-related techniques, is important to reduce the experiment numbers. BBD is one of the useful experimental designs for the optimization of chromatographic separation and analysis (Ferreira *et al*., 2007). BBD has been used for the separation of levofloxacin and ciprofloxacin using six factors, namely, the percentage of mobile phase composition, the concentration of buffer, the pH of the mobile phase, and the percentage of ion-pairing (Czyrski and Sznura, 2019). Our group also used BBD to optimize HPLC conditions for the separation of curcuminoid (curcumin, dimethoxy curcumin, and *bis*-desmethoxycurcumin), using variables of column temperature, a solvent ratio of acetonitrile-acetic acid, flow rate, and a percentage of acetic acid (Prabaningdyah *et al*., 2017), as well as the separation of Acid Orange-7 and Sudan II in cosmetic products (Purba *et al*., 2019). However, using the literature review, the application of BBD on HPLC optimization of CL and HCA is not yet reported. In this study, we worked on the optimization of HPLC separation of CL and HCA using three variables, namely, mobile phase composition, flow rate, and column temperature.

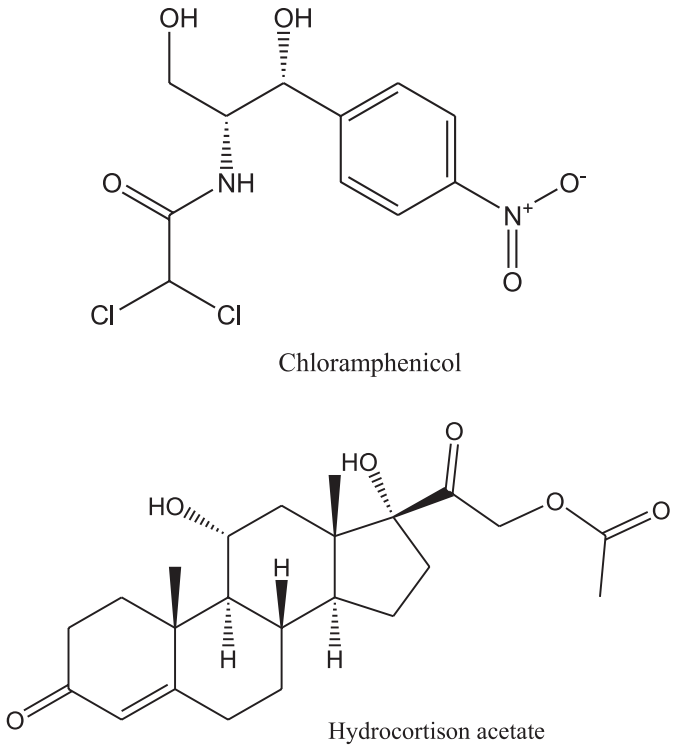

Figure 1. The chemical structures of CL and HCA.

MATERIALS AND METHODS

The reference standards of CL and HCA were obtained from *Badan Pengawas Obat dan Makanan* (The National Agency of Drug and Food Control), the Republic of Indonesia. Methanol and acetonitrile (ACN) for HPLC grade (*Lichrosolv)* were obtained from E. Merck (Darmstadt, Germany). Aqua pro injection was purchased from Ikapharmindo (Indonesia). The other solvents and reagents were of preanalytical grade and bought from E. Merck (Darmstadt, Germany). The commercial samples (Kemiderm® from PT. Berlico, Yogyakarta, Indonesia) were obtained from several pharmacies around Yogyakarta, Indonesia.

Experimental design

Approximately 4 mg of CL and 5 mg HCA were accurately weighed using a microanalytical balance with a sensitivity of 0.001 mg (Metler Toledo MX5) and each amount was added with methanol in a 10 ml volumetric flask. A 0.5 ml of each solution was pipetted and diluted with ACN-water (1:1) in a 20 ml volumetric flask. Each solution was subjected to scanning using an ultraviolet spectrophotometer at a wavelength range of 200−400 nm. The isobathic point of two UV spectra was used as a wavelength for the detection. BBD experimental design was performed using *Design Expert 10.1*. The variables used for BBD optimization were composed of ACN at a concentration range of 45%−55%, a flow rate of 0.7−1.3 ml/minutes, and a column temperature of 30°C−40°C. The HPLC responses were retention time, peak area, resolution (Rs), tailing factor, and theoretical plate. The design of BBD for HPLC separation using three variables was compiled in Table 1.

Table 1. BBD experimental design in HPLC optimization for the separation and analysis of CL and HCA.

Std	*Run*	Ratio of ACN (%)	Flow rate (ml/minute)	Column temperature (°C)
17	1	50	1.0	35
12	2	50	1.3	40
6	3	55	1.0	30
7	4	45	1.0	40
1	5	45	0.7	35
13	6	50	1.0	35
11	7	50	0.7	40
14	8	50	1.0	35
9	9	50	0.7	30
16	10	50	1.0	35
5	11	45	1.0	30
8	12	55	1.0	40
15	13	50	1.0	35
2	14	55	0.7	35
4	15	55	1.3	35
10	16	50	1.3	30
3	17	45	1.3	35

HPLC analysis

Analysis of CL and HCA was performed by Shimadzu LC-20AD autosampler (SIL-20A) using a UV detector (SPD-20) and a photo-diode array detector (SPD-20MA) equipped with Labsolutions software. The separation was carried out on a Waters X-Bridge C-18 column (250 × 4.6 mm ID, 5 µm) using a mobile phase composed of ACN-water. The mobile phase was delivered isocratically at a flow rate of 1.0 ml/minutes. The use of ACN-water as a mobile phase is due to its viscosity which is lower than methanol:water so that the pressure of the pump is low. Besides, ACN exhibited higher eluent strength than methanol in the C-18 column.

System suitability test and validation of HPLC method

The system suitability test (SST) of HPLC was carried out according to Siregar *et al*. (2017) by injecting six replicate standard solutions using conditions obtained during optimization with BBD. The parameters of SST evaluated were the precision expressed by relative standard deviation (RSD) of retention time, peak area, tailing factor, efficiency, and height equivalent to the theoretical plate (HETP). RSD values should be <2%. Validation of the HPLC method was done according to the guidelines of the International Conference on Harmonization (ICH) by evaluating several performance characteristics which included selectivity, linearity, sensitivity, precision, accuracy, and robustness. The acceptance criteria of validation protocols were based on several standards such as ICH and Association of Official Analytical Chemists.

Analysis of CL and HCA in pharmaceutical preparation

Cream samples containing CL and HCA equivalent to 4 mg CL and 5 mg HCA were accurately weighed using an analytical balance, added to 5 ml methanol, sonicated 10 minutes, and then transferred to a 10-ml volumetric flask. A 4.0 ml solution was taken and diluted with ACN-water (1:1) and transferred to a 20.0 ml calibrated volumetric flask. The solution was filtered using microfilter Polytetrafluoroethylene (PTFE) 0.45 µm and then injected into HPLC systems using a Waters X-Bridge C-18 column (250 × 4.6 mm ID, 5 µm) with a mobile phase of ACN-water (47%:53%) delivered isocratically at a flow rate of 0.9 ml/minutes.

RESULTS AND DISCUSSION

In this study, the experimental design of BBD was used to seek the optimum condition of HPLC intended for the analysis of CL and HCA in cream samples. The optimization process was performed using three independent variables, namely, ACN composition (%) (X_1), flow rate (X_2), and column temperature (X_3). The responses investigated were retention time of CL (Y_1), retention time of HCA (Y_2), peak area CL (Y_3), peak area of HCA (Y_4), Rs of matrix sample-1 (M1)-CL (Y_5), Rs of matrix sample-2 (M2)-CL (Y_6), Rs of M2-HCA (Y_7), tailing factor CL (Y_8), tailing factor HCA (Y_9), theoretical plate CL (10), and theoretical plate HCA (11). Based on the running order as in Table 1, the responses obtained were compiled in Table 2.

All responses were analyzed statistically using the analysis of Variance and resulted in a polynomial equation having significant effects of all variables toward responses. For selecting the optimum condition, some criteria were used, namely the minimum use of an organic solvent (ACN), the retention time for

Table 2. The running and responses obtained during BBD approach of HPLC method for the analysis of CL and HCA.

		Dependent variables (X)			Responses (Y)									
Std	*Run*	Conc. ACN (%) X_1	Flow rate (ml/minute) X_2	Column temp. (°C) X_3	Rt CHL (Minute) Y_1	Rt (Minute)Y_2	Area CHL Y_3	Area HID Y_4	R M1-CHL Y_5	R CHL-M2 Y_6	R M2-HID Y_7	TF CHL Y_8	TF HID Y_9	Theoritical plate CHL Y_{10}	Theoritical plate HID Y_{11}
17	1	50	1.0	35	3.484	5.120	975,198	1168,284	1.508	2.171	7.695	1.284	1.188	8,960.22	11,497.55
12	2	50	1.3	40	2.661	3.889	749,324	899,834	1.400	1.932	7.398	1.320	1.196	8,127.01	10,338.11
6	3	55	1.0	30	3.275	4.467	1,259,699	1,105,319	0.000	2.057	5.429	0.944	1.214	6,234.89	10,187.69
7	4	45	1.0	40	3.776	6.167	974,893	1,222,167	2.094	2.045	11.267	1.267	1.148	9,573.48	13,278.45
1	5	45	0.7	35	5.462	8.957	1,393,103	1,753,677	2.851	2.298	11.765	1.254	1.133	10,193.72	15,226.27
13	6	50	1.0	35	3.485	5.121	974,093	1,172,185	1.598	2.181	7.704	1.281	1.195	8,981.14	11,403.54
11	7	50	0.7	40	4.875	7.094	1,380,854	1,670,385	1.344	2.130	8.035	1.267	1.179	10,106.00	13,288.58
14	8	50	1.0	35	3.486	5.123	972,781	1,160,883	1.476	2.173	7.675	1.277	1.190	8,988.04	11,335.50
9	9	50	0.7	30	5.022	7.398	1,385,541	1,655,280	1.406	2.481	7.965	1.275	1.168	10,104.91	13,002.87
16	10	50	1.0	35	3.486	5.120	973,841	1,172,382	1.530	2.176	7.687	1.280	1.195	8,954.81	11,355.65
5	11	45	1.0	30	3.926	6.480	981,018	1,206,342	1.969	2.356	10.980	1.251	1.146	9,050.66	12,631.05
8	12	55	1.0	40	3.194	4.297	1,255,902	1,116,542	0.000	1.861	5.507	0.875	1.226	7,104.65	10,642.05
15	13	50	1.0	35	3.482	5.119	971,683	1,183,029	1.478	2.183	7.656	1.294	1.192	9,006.38	11,234.05
2	14	55	0.7	35	4.593	6.210	1,798,992	1,595,174	0.000	2.047	5.764	0.895	1.206	7,175.51	11,987.85
4	15	55	1.3	35	2.497	3.383	975,883	8,649,35	0.000	1.833	5.199	0.923	1.234	5,852.85	9,519.84
10	16	50	1.3	30	2.746	4.064	751,172	904,448	1.329	2.194	7.273	1.319	1.186	7,795.40	10,105.50
3	17	45	1.3	35	2.986	4.926	753,840	949,323	1.944	2.064	10.636	1.288	1.156	8,466.95	11,473.03

the first analyte should be minimum 2.5 minutes and maximum 10 minutes, the tailing factor should not exceed 1.5, the number of theoretical plates should take the highest values, the Rs should be minimum 2.0, and the peak area should be the highest (Vanbel and Schoenmakers, 2009) (Wu *et al*., 2018). The optimum conditions suggested by BBD were the composition of ACN of 47%, a flow rate of 0.9 ml/minutes, and a column temperature of 38°C. The HPLC chromatogram obtained using the suggested condition was shown in Figure 2. CL and HCA, matrix component-1 (M1), and matrix component-2 (M2) were well separated from others. The Rs values for CL-M1, CL-M2, and HCA-M2 were 2.080, 2.140, and 9.762, respectively. The tailing factors obtained were in the range of 1.158−1.283, and the number of theoretical plates (HETP) was of > 9,000. Based on the independent *t*-test, the HPLC separation parameters during optimization resulted by BBD agreed with those obtained in actual HPLC running.

The optimum HPLC condition was then validated by determining several performance characteristics as suggested in the ICH. During SST, RSD values of retention times, the number of theoretical plates, and peak area were of <2.00%; as shown in Table 3, indicating that the HPLC condition is suitable for further analysis (Ravisankar *et al*., 2015). HPLC was selective for the separation of CL and HCA as indicated by Rs values of >2.00. Over a concentration range of 20−140 µg/ml, CL was linear with a correlation coefficient of $r \geq 0.997$ with an equation for the relationship between concentration (x-axis) and peak area (y-axis) of $y = 14{,}495.5\ x - 3{,}501.9$. In addition, HCA over-concentration of 25−175 µg/ml was linear with an equation of $y = 12{,}822.1\ x + 7{,}538.7$ with a correlation coefficient of $r \geq 0.999$ (Araujo, 2009). The RSD values of CL and HCA obtained during precision analysis were <2.00%, lower than the required value in Horwitz function (Table 4). The recovery values obtained through standard addition method for CL and HCA were acceptable, namely, in the range of 99.33%−100.93% and 99.29%−100.82%, respectively (Table 5). The robustness of HPLC method was evaluated by adjustment of HPLC conditions (± 50% from optimum flow rate, ± 30% of mobile phase composition, and ± 10°C of column temperature). Based on the Student *t*-test between adjusted condition and optimum HPLC

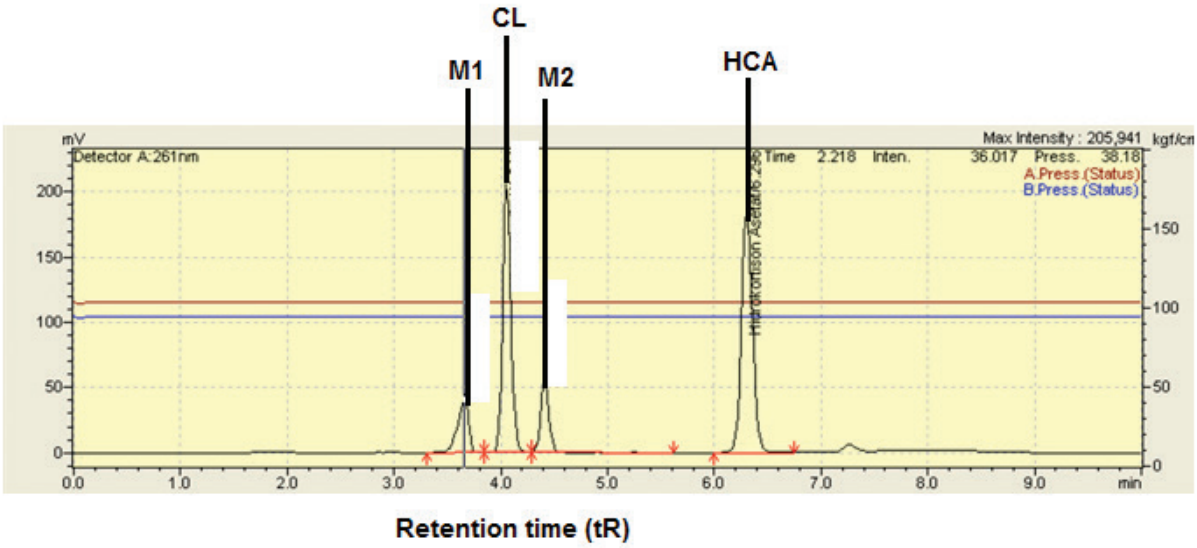

Figure 2. Separation of CL, matrix component-1 (M1), matrix component-2 (M2), and HCA using HPLC condition as suggested by BBD. Separation was carried out on a Waters X-Bridge C-18 column (250 x 4.6 mm ID, 5 µm) using a mobile phase composed of ACN-water (47%: 53%). The mobile phase was delivered isocratically at a flow rate of 0.9 ml/minutes. Detector: UV at 261 nm.

Table 3. The SST of optimum HPLC condition for the separation of CL and HCA.

No.	Retention time (minutes)		Peak area		Tailing factor		Rs			Theoritical plate	
	CL	HCA	CL	HCA	CL	HCA	M1-CL	CL-M2	M2-HCA	CL	HCA
1	4.049	6.299	1,098,873	1,325,970	1.283	1.163	2.664	2.138	9.731	9,321.129	12,852.061
2	4.050	6.300	1,099,107	1,344,506	1.282	1.164	2.644	2.140	9.739	9,350.126	12,854.441
3	4.050	6.300	1,099,737	1,333,280	1.280	1.163	2.607	2.142	9.750	9,342.676	12,856.958
4	4.050	6.299	1,099,475	1,318,750	1.282	1.162	2.619	2.136	9.734	9,340.575	12,908.649
5	4.049	6.299	1,098,104	1,340,425	1.282	1.163	2.597	2.141	9.749	9,357.433	12,890.998
6	4.050	6.300	1,092,236	1,343,675	1.280	1.165	2.606	2.141	9.733	9,402.749	12,849.407
Mean	4.050	6.300	1,097,922	1,334,434	1.282	1.163	2.623	2.140	9.739	9,352.448	12,868.752
SD	0.001	0.001	2,842	10,409	0.001	0.001	0.026	0.002	0.008	27.487	24.833
RSD (%)	0.013	0.009	0.259	0.780	0.096	0.089	0.988	0.105	0.085	0.294	0.193

SD = standard deviation; RSD = relative standard deviation; M1 = matrix component 1; M2 = matrix component 2.

Table 4. The precision studies of HPLC for the analysis of CL and HCA.

Time	No.	Sample weight (mg)	CL			HCA		
			Peak area	Conc. (mg/g)	Conc. (%)	Peak area	Conc. (mg/g)	Conc. (%)
First day	1	203.9	1,103,470	18.71	93.53	1,336,354	25.09	100.34
	2	200.6	1,093,484	18.84	94.21	1,322,219	25.23	100.92
	3	203.5	1,112,555	18.90	94.49	1,346,654	25.33	101.32
	4	200.9	1,085,551	18.68	93.39	1,326,068	25.26	101.06
	5	202.4	1,092,006	18.65	93.25	1,335,545	25.26	101.03
	6	206.3	1,121,028	18.78	93.92	1,364,948	25.32	101.30
Second day	1	208.2	1,125,405	18.78	93.89	1,358,220	25.23	100.93
	2	205.7	1,108,848	18.73	93.63	1,355,425	25.49	101.95
	3	204.1	1,115,892	18.99	94.96	1,345,932	25.51	102.03
	4	207.9	1,134,279	18.95	94.76	1,382,151	25.71	102.86
	5	204.7	1,121,059	19.02	95.12	1,353,379	25.57	102.29
	6	206.3	1,133,405	19.08	95.42	1,354,483	25.39	101.58
Third day	1	208.0	1,139,375	18.86	94.28	1,365,709	25.13	100.52
	2	201.7	1,106,911	18.89	94.46	1,338,186	25.39	101.58
	3	202.2	1,099,634	18.72	93.61	1,334,827	25.27	101.07
	4	200.6	1,093,151	18.76	93.80	1,331,584	25.41	101.63
	5	204.4	1,117,898	18.83	94.14	1,362,851	25.52	102.08
	6	207.5	1,135,579	18.84	94.20	1,383,727	25.52	102.10
Average					94.17		Average	101.48
SD					0.615		SD	0.655
% RSD					0.653		% RSD	0.645

Conc. = concentration; SD = standard deviation; RSD = relative standard deviation.

Table 5. The accuracy studies of HPLC for the analysis of CL and HCA, as expressed by recovery percentages.

Level	Sample weight (mg)	Conc. of CL in sample (mg)	Referance standard of CL added (mg)	Total theoritical conc. of CL (mg)	Peak area	Conc. of CL (mg)	*Recovery* (%)	Range of *recovery* (%)
80%	111.8	2.0855	1.2014	3.2869	953,063	3.2944	100.23	99.40–100.23
	117.0	2.1825	1.2014	3.3839	975 096	3.3706	99.61	
	108.0	2.0146	1.2014	3.2160	924 840	3.1968	99.40	
100%	109.9	2.0501	2.0023	4.0524	1,172,178	4.0518	99.99	99.99–100.24
	115.0	2.1452	2.0023	4.1475	1,202,208	4.1556	100.19	
	114.1	2.1284	2.0023	4.1307	1,197,895	4.1407	100.24	
120%	112.8	2.1042	2.8033	4.9074	1,432,882	4.9530	100.93	99.33–100.93
	105.6	1.9699	2.8033	4.7731	1,376,255	4.7572	99.67	
	111.0	2.0706	2.8033	4.8739	1,400,612	4.8414	99.33	

Conc. = concentration.

condition, there is no significant difference at $p > 0.05$; therefore, HPLC method was robust enough (Budiarti *et al*., 2015).

The validated HPLC method was then used for the analysis of commercial cream products containing CL and HCA. The average levels of CL were of 18.65 mg/g (equivalent to 93.27% from declared level) and 25.22 mg/g (equivalent to 100.88% from declared level). The developed method is accurate and reliable for the simultaneous determination of CL and HCA in cream samples.

CONCLUSION

The experimental design of BBD has been successful to achieve an effective and efficient separation of CL and HCA in cream preparation. There is no difference between HPLC conditions suggested by BBD and actual HPLC running ($p > 0.05$ via the independent *t*-test). HPLC method exhibited acceptable performance characteristics as required by ICH. The validated method is also successfully used for the analysis of CL and HCA in cream formulations.

ACKNOWLEDGEMENTS

The authors thank the Ministry of Research and technology scheme World Class Research 2020 with contract number of 869/UN1/DITLIT/DIT-LIT/PT/2020.

CONFLICT OF INTEREST

The authors have no conflict of interest during this study.

FUNDING

None.

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How to cite this article:
Khotimah K, Martono S, Rohman A. Box–Behnken design-based HPLC optimization for quantitative analysis of chloramphenicol and hydrocortisone acetate in cream. J Appl Pharm Sci, 2020; 10(09):134–139.