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# Liquid Chromatographic and Spectrophotometric Determination of Diflucortolone Valerate and Chlorquinaldol in Creams

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## ABSTRACT

A new reversed phase high pressure liquid chromatography and a spectrophotometric method are proposed for the simultaneous determination of diflucortolone valerate (DIF) and chlorquinaldol (CHL) in creams. An isocratic system consisted of an ACE® C18 analytical column and a mobile phase composed of methanol/phosphate buffer (pH 5.5, 0.1 M) (95:5, v/v) at a flow rate 1.0 mL/min was used for the optimal chromatographic separation using UV detection at 220 nm. Ephedrine hydrochloride was used as internal standard. Principal component regression (PCR) was used as the chemometric technique in spectrophotometry. In this technique, the concentration data matrix was prepared by using the synthetic mixtures containing these drugs in methanol/water (3:1). The absorbance data matrix corresponding to the concentration data matrix was obtained by measuring the absorbances at 60 wavelengths in the range of 230-348 nm with appropriate interval $\Delta\lambda$ of 2 nm for DIF and CHL in the zero-order spectra for the binary combinations. The linear ranges were found 2.40-300 μg/mL for DIF, 0.72-240 μg/mL for CHL for LC method, and 0.88-3.0 μg/mL for DIF and 1.0-11.2 μg/mL for CHL for PCR method. The accuracy, precision and the linear ranges of the methods have been evaluated and they have been validated by analyzing synthetic mixtures containing the title drugs. These two methods were successfully applied to two pharmaceutical cream preparations and the results were compared with each other.

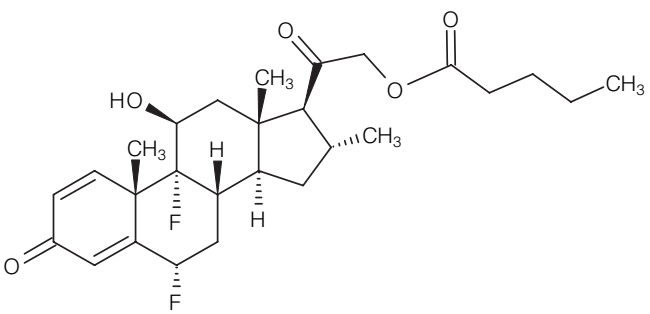
Key words: diflucortolone valerate, chlorquinaldol, principal component regression, pharmaceutical preparation, liquid chromatography

## INTRODUCTION

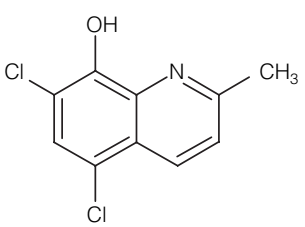
Chlorquinaldol (CHL) (5-7-Dichloro-2-methylquinolin-8-ol) is used as antimicotic and antifungal in the treatment of infection of skin and vagina that is administrated topically. Diflucortolone valerate (DIF) (6α9αDifloro-11β21-dihydroxy-16αmethylpregna-1,4-diene-3,20-dione 21 valerate) is a corticosteroid that is used in the inflammation of skin. Binary combinations of these drugs are frequently prescribed as antimicrobial drugs. The structures of these drugs are shown in Figure 1.

There are few examples of published methods for the determination of CHL and DIF alone or in their mixture with other substances. A gas-liquid chromatographic method was used for the determination of CHL and chlortalidone in biological materials[1]. A polarographic technique was applied for the determination of CHL alone in a pharmaceutical preparation[2]. A spectrofluorimetric method was used to determination CHL in the presence of chloroxine in pharmaceutical preparations[3]. High pressure liquid chromatography (HPLC) methods are described for the analysis of DIF alone in pharmaceutical dosage forms[4-7]. However, no information concerning the simultaneous determination of CHL and DIF in their binary mixture or in pharmaceutical preparations could be seen in the literatures.

Chromatographic techniques are most widely used for resolving mixtures. However chemometric techniques (multivariate calibration techniques), based on the computer aided instrumentation and algoritms, are employed for the analysis of multicomponent samples.




Diflucortolone valerate




Chlorquinaldol

**Figure 1.** The structures of chlorquinaldol and diflucortolone valerate

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A certain number of calibration methods are available as an affordable commercial software is used with existing instruments. The most popular among them include CLS (classical least squares), ILS (inverse least squares), PCR (principle component regression) and PLS (partial least squares). The joining spectrophotometric data and multivariate calibration techniques for the resolution of mixtures of analytes with overlapped spectra becomes a useful tool for developing new analytical methods. All of the chemometric spectral analysis techniques are useful for the resolution of spectral bands overlapping in quantitative determination. Main advantage of these techniques is the simultaneous analysis of the mixture components without chemical pre-treatment or graphical procedure of spectra such as derivative and ratio spectra derivative. They also require shorter time, less costs and simple instrumentation.

The aim of this study was to develop a LC method and a chemometric technique in spectrophotometry that allow the simultaneous determination of DIF and CHL in their mixture and in cream formulations.

## MATERIALS AND METHODS

I. *Apparatus*

Shimadzu 1601 PC double beam spectrophotometer with a fixed slit width (2 nm) connected to a computer loaded with Shimadzu UVPC was used for the spectrophotometric measurements.

In spectrophotometric methods, zero-order spectra of the solution of DIF and CHL in methanol in the range of 200-300 nm were used.

An Agilent Technologies HP 1100 chromatographic system equipped with a model series of G13 79A degasser, G1311A quaternary pump, 61313A injector and G1315B DAD detector was used. ACE® C18 column 250 × 4.6 mm, particle size 5 µm was used. The chromatograms were recorded and the peaks were quantitated using the automatic integrator. The mobile phase was methanol - phosphate buffer (pH 5.5, 0.1 M) (95:5, v/v). The flow rate was set at 1 mL/min with 20 mL as injection volume and the detection wavelength was 220 nm. Ephedrine hydrochloride was used as internal standard (IS).

II. *Computer Software and Hardware*

In chemometric procedure, Multivariate Analysis Add-in for Excel v1.3 [8] software was used and run on a Pentium III, 128 MB RAM, 1500 MHz computer.

III. *Materials*

Chlorquinaldol and diflucortolone valerate were obtained from Intendis, Turkey and they were used without further purification. Methanol Chromasolv® of HPLC gradient grade was provided by Sigma Aldrich. Water for preparation of solution was produced in-house by the PurelabUHQ water purification system (Elba). The mobile phase and the solution for injection were degassed in an ultrasonic bath and were filtered through the 0.45 µm nylon membrane before use.

IV. *Standard Solutions*

Solutions of DIF (10 mg/50 mL) and CHL (10 mg/50 mL) were prepared in methanol/water (3:1) for PCR method and solutions of DIF (50 mg/100 mL) and CHL (50 mg/100 mL) were prepared in methanol for LC method. Solution of 100 mg/100 mL ephedrine hydrochloride was prepared in same solvent as IS as stock solution for LC method.

V. *Sample Preparation*

(1) For LC: 1 g of Nerisona® C or Impetex® cream was weighed in 50-mL volumetric flask and diluted to volume with methanol. After 30 min of mechanically shaking and 15 min of standing in the dark, the solution was filtered through a 0.45-µm Millipore filter into another 50-mL volumetric flask. Then the volume was completed to the mark with methanol (I). I (12.5 mL) and IS stock solution (2.5 mL) were put into a 25-mL volumetric flask and the volume was completed to 25 mL with methanol (II). Solution II can be injected into the chromatographic system.

(2) For PCR: 1 g of Nerisona® C or Impetex® cream was weighed in 50-mL volumetric flask and diluted to volume with methanol/water (3:1). After 30 min of mechanically shaking and 15 min of standing in the dark, the solution was filtered through a 0.45-µm Millipore filter into another 50-mL volumetric flask. Then the volume was completed to the mark with methanol/water (3:1) (I). Then 1.25 mL of I was added into a 25-mL volumetric flask and the volume was completed to 25 mL with methanol/water (3:1) (II). The absorbances of the solution II were measured at the selected wavelengths.

VI. *Commercial Pharmaceutical Preparations*

NERISONA C cream (1 mg diflucortolone valerate and 10 mg chlorquinaldol/1 g cream) from Intendis, Turkey (batch no: 59098) and IMPETEX cream (1 mg diflucortolone valerate and 10 mg chlorquinaldol/1 g cream) from Roche, Turkey (batch no: IT0004) were assayed.

## RESULTS AND DISCUSSION

I. *LC Method*

First, the pH and concentration of mobile phase were optimized for the separation on ACE® C18 column.

0.1 M Phosphate buffer (pH 5.5, 0.1 M) was selected for suitable separation. Beside this, the solvent content in mobile phase was studied on the same column. The optimal mobil phase for good separations was found to be methanol/phosphate buffer (pH 5.5, 0.1 M) (95:5, v/v). Furthermore, the flow rate, column temperature and injection volumes were also optimized to be 1 mL/min, 25°C and 20 µL, respectively. As for the IS, pseudoephedrine hydrochloride, timolol maleat, dorzolamid, ephedrine hydrochloride and phenylephrine hydrochloride were tested. Ephedrine hydrochloride could be successfully separated from other compounds so that it was used as IS in this study. Also, the detection wavelength, 220 nm, was chosen according to absorption spectra of all three substances.

Thereupon, ACE® C18 column (250 × 4.6 mm, 5 µm), mobile phase composed of methanol/phosphate buffer (pH 5.5, 0.1 M) (95:5, v/v) at 1 mL/min flow rate, 25°C column temperature and 20 µL injection volume were finally employed for the optimal separations. System suitability test were done and the results were shown in Table 1. For separation factor, the IS was taken as the reference peak.

Under these chromatographic conditions, ephedrine hydrochloride, DIF and CHL peaks were well resolved and their retention times were found 2.53, 3.44 and 4.46 min, respectively. A typical chromatogram of the drugs and internal standard was illustrated in Figure 2.

Each solution was injected three times and the areas of peaks, as measured at 220 nm, were integrated. The ratios of the peaks areas of investigated substances to that of IS were calculated for each injection. Regression equation was established by plotting the ratio of peak areas to the concentration of each substance. Linearity was found in the range of 2.40-300 µg/mL for DIF and 0.72-240 µg/mL for CHL. The calculation method of LOQ (limit of quantitation) is based on the standard deviation (SD) of the response and the slope (m) of the calibration curve according to the formula: LOQ = 10 (SD/m). LOD (limit of detection) can be calculated based on the SD of the response and the slope (m) of the calibration curve according to the formula: LOD = 3.3 (SD/m). LOQ values were calculated as 2.40 µg/mL for DIF and 0.037 µg/mL for CHL. LOD values were calculated as 0.79 and 0.24 µg/mL for DIF and CHL respectively (Table 2). Mean recoveries, relative standard deviations and confidence interval for this method found in synthetic mixtures were shown in Table 2.

**Table 1.** System suitability tests results for DIF and CHL in LC method

| Parameter | DIF | CHL |
|---|---|---|
| Retention factor | 5.86 | 7.85 |
| Separation factor | 1.45 | 1.33 |
| Resolution | 4.51 | 4.97 |
| Asymmetry | 0.906 | 0.899 |

II. *PCR Method*

(I) *Method*

The original data obtained in absorbances (A) and concentrations (C) of analytes were reprocessed by standardizing as $A_0$ and $C_0$, respectively. Using the ordinary linear regression with coefficients a and b:

$$C = a + b \times A$$

$b = P \times q$, where $P$ is the matrix of eigenvectors and q is the $C$ – loadings given by $q = D \times T^T \times A_0$. Here $T^T$ is the transpose of the score matrix $T$. $D$ is a diagonal matrix with the inverse of the selected eigenvalues as components. Knowing $b$ one can easily find $a$ by the formula $a = C_{mean} - A^T_{mean} \times b$, where $A^T_{mean}$ represents the transpose of the matrix with the entries of the mean absorbance values and $C_{mean}$ is the mean concentration of the calibration set. Multivariate Analysis Add-in for Excel v1.3 software [8] was used for the calculation.

(II) *Procedure*

The zero-order absorption spectra for DIF and CHL and their binary mixture in methanol/water (3:1) were

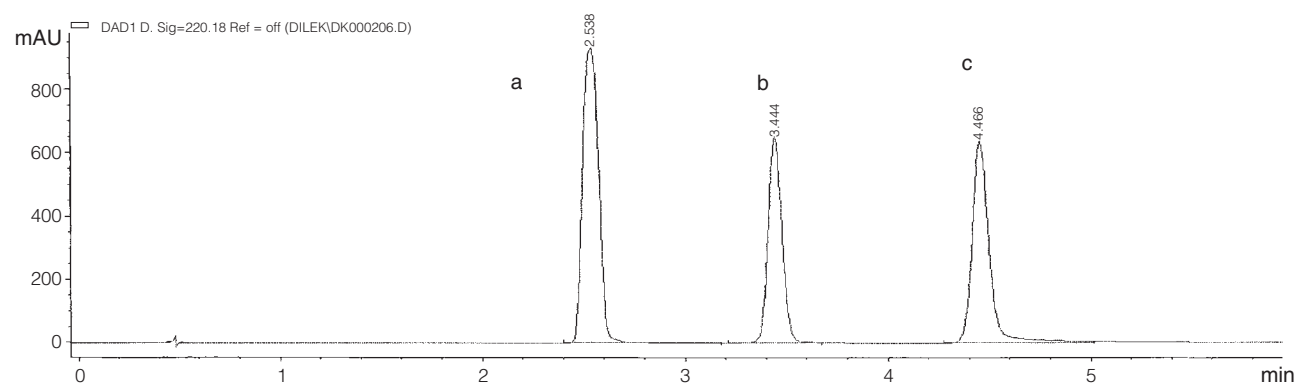


**Figure 2.** Typical chromatogram of a) ephedrine HCl (internal standard) (250 mg/mL), b) diflucorotolone valerate (80 mg/mL) and, c) chlorquinaldol (80 mg/mL) in HPLC method for pharmaceutical preparations.

**Table 2.** Linearity parameters and recovery results of DIF and CHL in LC method

| | DIF | CHL |
|---|---|---|
| Linearity range (μg/mL) | 2.40-300 | 0.72-240 |
| Slope of the calibration curve ± standard error | 0.1504 ± 0.0002 | 0.8210 ± 0.0026 |
| Intercept of the calibration curve ± standard error | 0.2224 ± 0.0087 | 0.014 ± 0.0030 |
| r | 0.9998 | 0.9999 |
| LOD (μg/mL) | 0.79 | 0.24 |
| LOQ (μg/mL) | 2.40 | 0.72 |
| Mean recovery % | 100.5 | 100.6 |
| *RSD % | 1.58 | 0.99 |
| Confidence interval for p = 0.05 | 1.18 | 0.80 |

*RSD: relative standard deviation.

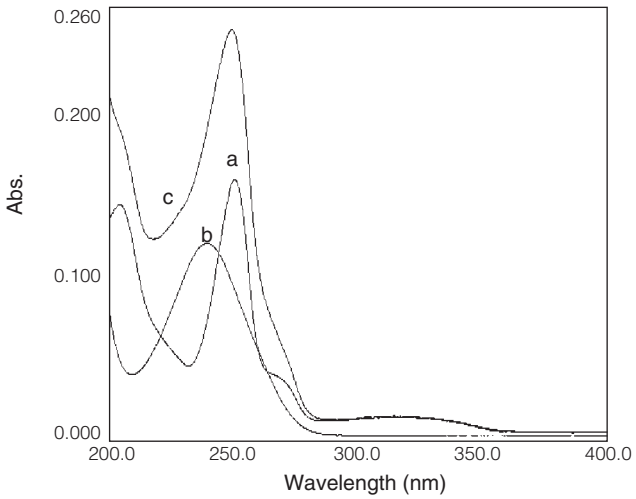


**Figure 3.** Zero-order absorption spectra of a) 3.2 mg/mL solution of DIF, b) 0.96 mg/mL solution of CHL, c) solution containing 3.2 mg/mL DIF and 0.96 mg/mL CHL mixture in methanol/distilled water (3:1).

**Table 3.** Training set used in PCR method for DIF and CHL.

| Mixture | CHL (μg/mL) | DIF (μg/mL) |
|---|---|---|
| 1 | 1 | 0,88 |
| 2 | 1 | 2,6 |
| 3 | 1 | 3 |
| 4 | 4 | 0,88 |
| 5 | 4 | 1,2 |
| 6 | 4 | 2,6 |
| 7 | 4 | 3 |
| 8 | 6 | 0,88 |
| 9 | 6 | 1,2 |
| 10 | 6 | 2,6 |
| 11 | 6 | 3 |
| 12 | 11,2 | 0,88 |
| 13 | 11,2 | 2,6 |
| 14 | 11,2 | 3 |

**Table 4.** Linearity parameters, recovery and statistical results of DIF and CHL in PCR technique

| Parameter | DIF | CHL |
|---|---|---|
| Linearity range (μg/mL) | 0.88-3.00 | 1.0-11.2 |
| Regression coefficient | 0.9986 | 0.9999 |
| LOD (μg/mL) | 0.32 | 0.30 |
| LOQ (μg/mL) | 0.86 | 1.00 |
| Mean recovery % | 99.9 | 100.1 |
| RSD % | 2.7 | 0.6 |
| Confidence interval for p = 0.05 | 1.80 | 0.40 |

shown in Figure 3. The spectra of both components were overlapped in the range of 200-350 nm. So, it is impossible to determine DIF or CHL in their mixture (Figure 3C) by measuring the absorbances at their $\lambda_{max}$ or any other wavelengths without interference of each other. PCR technique was employed for the simultaneous determination of DIF and CHL in their binary combination using the zero-order absorption spectra of their mixture without any prior separation technique. Other chemometric techniques such as CLS, ILS and PLS methods have been proven unsuccessful in the simultaneous analysis of DIF and CHL in their mixture.

In PCR technique, for the determination of DIF and CHL in their binary mixture, optimal conditions were investigated and absorbance data matrix were obtained by measuring the absorbance between 230-348 nm with a interval $\Delta\lambda$ of 2 nm at 60 wavelengths for DIF, CHL and their binary mixtures. The calibration was obtained by using the absorbance data matrix mentioned above and the concentration data matrix prepared as the concentrations in the mixtures for the prediction of the unknown concentrations of DIF and CHL in their binary mixtures. Good results were obtained by using standardized data in calculation procedures. Two factorial four level design was used for Design of Experiments (DOE) in the preparation of training sets (Table 3).

The linearity parameters, mean recoveries, relative standard deviations and confidence interval for this method shown in Table 4. Linearity was found in the range of 0.88-3.00 μg/mL for DIF and 1.0-11.2 μg/mL for CHL. LOQ values were selected as 0.88 μg/mL for DIF and 1.0 μg/mL for CHL. LOD values were calculated as 0.32 and 0.30 μg/mL for DIF and CHL respectively (Table 4). For calculations we used algoritms as described in the literatures[9,10].

To select the number of factors, in order to model

the system without overfitting the concentration data in PCR algorithm, a cross-validation method, leaving out one sample at a time was employed using training sets. In this technique; three factors for DIF and CHL in their binary mixture were found optimal for the determinations. We obtained the prediction error sum of squares (PRESS) minimum with these factors (Table 5).

The predictive ability of a model can be defined in various ways. The most general expression is the standard error of prediction (SEP). Another statistical value is the SEC (standard error of calibration). SEP and SEC were calculated and illustrated in Table 5.

These results for the application of PCR method to the same binary mixture are shown in Table 5 and were found satisfactory.

III. *Precision*

The precision was determined by means of one-way ANOVA including 10 replicates carried out on three successive days by PCR method and LC method for synthetic mixtures of DIF and CHL. Snedecor *F* values below the tabulated levels were obtained in all cases ($F = 4.21$, $n_1 = 2$, $n_2 = 27$; Table 6), so there were no significant differences among the results obtained in the determination of each drug in the presence of other on 3 different days (Table 6).

IV. *Applications*

Comparison of the spectra of DIF and CHL in standard and drug formulation solutions indicated that the $\lambda_{max}$ in the zero-order spectra did not change and after the addition of known amount of active ingredients to the commercial formulations, the amount of these drugs did not change. In LC method, no interfering peak was observed in the chromatogram of the commercial cream formulations in our conditions (Figure 4A). Also, as seen in the chromatogram of the placebo in the same conditions (Figure 4B), the excipients placed in the commercial formulation did not interfere the quantitation of DIF and CHL. All the results obtained by the meth-

**Table 5.** Summary of statistics in PCR method for DIF and CHL in the mixture

| | |
|---|---|
| SEP | |
| DIF | 0.030 |
| CHL | 0.027 |
| SEC | |
| DIF | 0.032 |
| CHL | 0.029 |
| PRESS | |
| DIF | 0.005 |
| CHL | 0.006 |

**Table 6.** Analysis of variance (ANOVA) for the proposed methods

| | PCR | | LC | |
|---|---|---|---|---|
| | CHL | DIF | CHL | DIF |
| Between-days variance | 0.062 | 0.074 | 0.067 | 0.096 |
| Within-days variance | 0.197 | 0.203 | 0.200 | 0.370 |
| F ratio | 3.18 | 2.74 | 3.03 | 3.85 |
| Mean value | 41.7 | 63.6 | 40.6 | 62.8 |
| Between-days RSD (%) | 0.072 | 0.134 | 0.080 | 0.152 |
| Within-days RSD (%) | 0.017 | 0.078 | 0.030 | 0.072 |

Between-day and within-day degrees of freedom 2 and 27 respectively. The critical F ratio value for 2 and 27 degrees of freedom and a confidence level of 95% is 4.21.

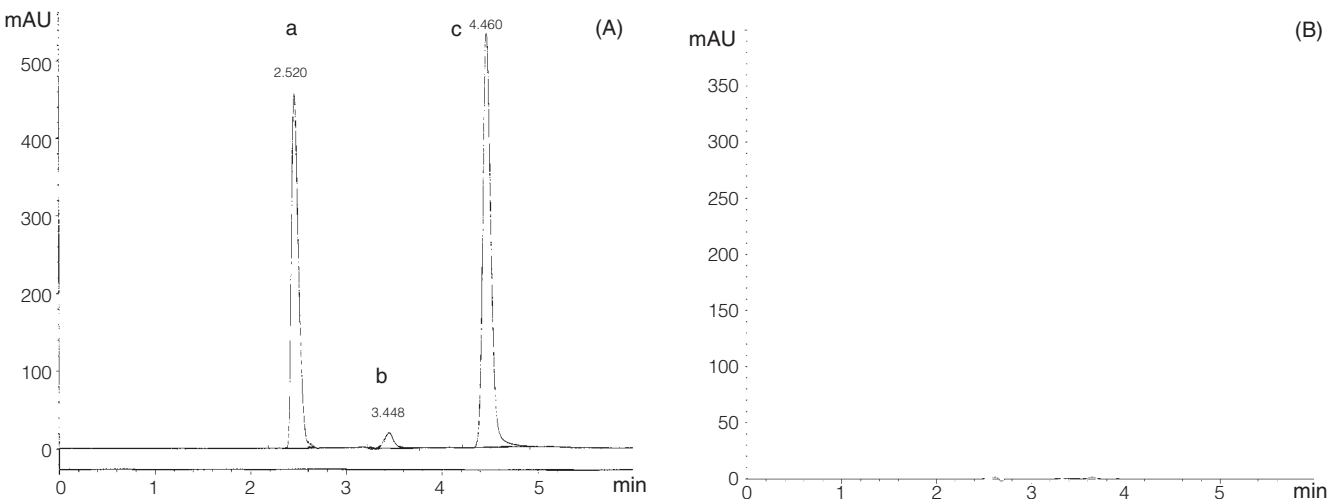


**Figure 4.** Chromatogram of (A) cream preparation; a) ephedrine (internal standard), b) DIF and c) CHL, (B) placebo in LC method developed.

ods described above were compared statistically with each other and no significant difference was observed for the amount of drugs found theoretical values for *t* in p = 0.05 level for commercial formulations (Table 7).

**Table 7.** Assay results of commercial preparations (NERISONA C cream) and (IMPETEX cream) (Label claims = 10 mg CHL and 1 mg DIF/1 g cream)

| Methods | DIF | | | CHL | | |
|---|---|---|---|---|---|---|
| | mean* ± SD** | | t value | mean* ± SD** | | t value |
| NERISONA C cream | | | | | | |
| PCR | 0.96 | 0.11 | 1.28 | 10.08 | 0.06 | 0.06 |
| LC | 1.01 | 0.02 | | 10.02 | 0.03 | |
| IMPETEX cream | | | | | | |
| PCR | 1.12 | 0.03 | 0.52 | 10.32 | 0.52 | 0.12 |
| LC | 1.09 | 0.06 | | 10.28 | 0.56 | |

*Average of ten assay.
**SD: standard deviation.
***Theoretical value for t at P: 0.05 level = 2.26.

## CONCLUSIONS

In this study, a new LC method and PCR method in spectrophotometry were developed for the simultaneous analysis of DIF + CHL combination. These methods could be applied with great success for the simultaneous determination of DIF and CHL in their binary mixtures and in two pharmaceutical cream preparations. Generally, it is very difficult to study the cream preparations and often it is necessary to do an extraction, which is a time-consuming and precision-decreasing procedure in the assay, prior to the application of analytical methods. Our methods described in this work don't need any prior separation procedure. Satisfactory results were obtained by these methods, but, PCR method needs softwares for mathematical calculations. Using only zero-order spectra in the procedures without any other graphical mode such as derivative and ratio spectra derivative, is one of the advantages for the chemometric methods. Without any time-consuming preparation procedures and methanol/water mixture as solvent, spectrophotometric method proposed in this article is easier and cheaper when compared with the LC method. Both methods proposed in this article were compared with each other due to the absence of official or published method for the simultaneous analysis of DIF and CHL in their binary mixture. These methods were found suitable for the simple and precise routine analysis of the pharmaceutical preparations selected. Good agreement was achieved in the assay results of pharmaceutical preparations widely used in Turkey, such as cream, for two methods proposed in the text.

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