Original article

Spectrophotometric determination of binary mixtures of prednisolone with some antibiotics

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Abstract:

Multivariate (classical least squares and principal components regression) techniques and derivative spectrophotometric techniques (first derivative and derivative ratio) were developed for the assay of two binary mixtures of prednisolone with tetracycline (mix I) and chloramphenicol (mix II) in pharmaceutical combination containing these compounds. The simultaneous determination of these compounds were firstly accomplished by first derivative ($dA/d\lambda$) spectrophotometric technique applying zero-crossing technique and first derivative of the ratio spectrum. The ratio spectrum was obtained by dividing the absorption spectrum of the mixture by that of one of the components, the concentration of the other component was determined from its respective calibration graph treated similarly, the influence of $\Delta\lambda$ for obtaining the first derivative of the ratio spectra and the effect of the divisor concentration on the calibration graphs were studied. The second method depends on application of classical least squares (CLS) and principle component regression (PCR) models. These calibration models were evaluated by internal validation (prediction of compounds in its own designed training set of calibration), by cross-validation (obtaining statistical parameters that show the efficiency for a calibration fit model) and by external validation over synthetic and pharmaceutical mixtures. The four described procedures were successfully applied to the determination of these compounds in laboratory prepared mixtures and in pharmaceutical preparations with high percentage of recovery, accuracy and precision. The procedures do not require any separation step.

Key words: Chloramphenicol; Classical least squares; Derivative ratio spectrum; First derivative spectrophotometry; Prednisolone; Principle component regression; Tetracycline

Introduction

Prednisolone is a synthetic glucocorticoid, it has five times the potency of cortisone acetate but in equivalent doses causes less sodium and fluid retention although more gastric symptoms [1, 2]. Chromatographic [3, 4] and spectrophotometric methods [5] were introduced for determination of prednisolone. Tetracycline is classical, typical, generic and comparatively cheap tetracyclines member. It is the drug of choice in infections with *Mycoplasma pneumoniae,* chlamydiae, rickettsiae and some spirochetes. They are used in combination regimens to treat gastric and duodenal ulcer disease caused by *Helicobacter pylori* [1, 2]. The recent methods for determination of tetracycline include volumetric methods [6], electrochemical methods [7], spectrophotom-etric methods [8] and chromatographic methods [9].

Chloramphenicol is bacteriostatic by virtue of inhibition of protein biosynthesis in both bacterial and to a lesser extent in the host ribosomes. It is a broad-spectrum antibiotic active against both aerobic and anaerobic gram-positive and gram-negative organisms. It is also active against rickettsiae but not chlamydiae [1, 2]. The recent methods for determination of chloramphenicol include chromatographic [10-12] and spectrophotometric methods [13]. The combinations of prednisolone with tetracycline or chloramphenicol exerts highly local antibacterial activity against Gram-positive and many Gram-negative microorganisms, specific antiinflammatory and antiallergic action [1, 2].

Derivative spectrophotometry is an analytical technique of great utility for resolving some mixtures of compounds with overlapping spectra [14-17]. Zero-crossing is measured by finding the value of the derivative of the sum curve of the analyte and interference at an abscissa value (wavelength) corresponding to a zero-crossing of the derivative of the interfering band. Derivative ratio spectrum is able to resolve the strong overlapping of spectra. In this method, the absorption spectrum of the mixture is recorded and divided, amplitude-by-amplitude, by the absorption spectrum of a standard solution of one of the components, and then the first derivative of the ratio spectrum is obtained. The concentration of another component is then determined from a calibration graph [14-16].

Multivariate calibration methods applied to spectral data are being increasingly used for pharmaceutical analysis. Classical least squares (CLS) and principal components regression (PCR) analysis are the most simplest multivariate methods that can be performed with easily accessible statistical software [18-21]. CLS technique assumes that responses (absorbance) at each frequency (wavelengths) are proportional to component concentration units. Model errors are assumed to derive from the measurement of spectral absorbance. So CLS requires that all interfering chemical components are known and included in the calibration data set. CLS has the advantage of improved precision when using many frequencies, due to signal averaging.

Calibration is realized by recording the spectra at *n*-wavelengths of *m* standard mixtures, of known composition of *c* components. The spectra (absorbance or emission) are arranged into the columns of matrix *Y* (dimensions $n \times m$), with the composition of each mixture forming the columns of concentration matrix *X* ($c \times m$)

$$Y = K \,.\, X \qquad (1)$$

With a prior knowledge of *X* and by recording data for *Y*, then the matrix of sensitivities, *K*, can be calculated, but after the rearrengment of equation 1 to the following equation by multiplying the equation components by X^t value as: $Y \,.\, X^t = K \,.\, X \,.\, X^t$

then; $$K = (X \,.\, X^t)^{-1} \,.\, Y \,.\, X^t \qquad (2)$$

To avoid being under-determined, there must be measurements at more wavelengths than there are components (i.e. $n \geq c$). If n > c then the component concentrations in an unknown mixture are obtained from its spectrum by,

$$X_{\text{unknown}} = (K^t \,.\, K)^{-1} \,.\, K^t \, Y_{unknown}$$

This CLS method is intuitively appealing since it is based on some generally assumed relationship, e.g. Beer's law, and it can be used for moderately complex composition of the calibration mixtures, i.e. the concentration of each absorbing species. PCR is a two-step procedure, in the first step, one estimating the number of principal components by one or more of the following criteria, the percentage of explained variance, given value-one criterion, the Scree-test and Cross validation. They can be considered as new variables that summarize in an optimal way the variation present in the spectra, in the second step, CLS is applied to the newly obtained latent variables. When co-linearity between original variables occurs, principal component plots often allow better interpretation of the variations observed in the data set than plots of original variables selected by CLS. As modeling method, it is less performant than CLS when performing prediction within the calibration domain and when the model is indeed linear. It is more reliable if extrapolation may be required. It is a linear method, but it is able to perform quite well for moderately nonlinear data. As CLS, it is a global method [22-25].

The aim of this paper was to demonstrate the capability of first derivative, derivative ratio spectrophotometry, classical least squares (CLS) and principle component regression (PCR) for the simultaneous analysis of the studied drugs in mixtures without the need or preliminary separation steps.

Experimental

Apparatus

Spectrophotometric measurements were carried out on a computerized Spectronic Gensys 2PC, UV/visible Spectrophotometer (Milton Roy, USA), using 1.00 cm quartz cells. The obtained spectral data were saved in PC apparatus program and the subsequent statistical manipulation was performed by transferring the spectral data to Microsoft excel XP program and processing them with the standard curve fit package and matrix calculations, Curve Expert version 1.37 Copyright©1995-2001 by Daniel Hyams and GraphPad Instat version 3.05-32 bit for win 95/NT created Sep. 27, 2000 Copyright© 1992-2000 by GraphPad software (SMAC, New Jersey, USA). Other apparatus were sonicator (Bransonic 220, Bender-Hobein, Jasco, Germany), heater (Salvis, Kreuzwertheim, Germany) and analytical balance (Mettler Toledo, Tokyo, Japan).

Materials

Pharmaceutical compounds

All materials and reagents used were of analytical grade. Prednisolone and tetracycline hydrochloride were supplied by Aldrich Co., USA and ADCO Co. Egypt. Chloramphenicol was supplied by Aldrich Co., USA and CID CO., Egypt. All drugs were used as working standards without further purification and analyzed to one of the official methods or reported methods to determine their purity and compliance with the requirements.

Formulations

Tetracort® ointment with batch number 460043 ADCO; Egypt, labeled to contain 3 g of tetracycline and 0.5 g of prednisolone per 100 g was purchased from the local market and subjected to analysis by the proposed methods. Cortiphen® eye drops and ointment labeled to contain 2 mg of chloramphenicol and 5 mg of prednisolone per 1 mL and 1% of chloramphenicol and 0.5% of prednisolone per 5 g with batch No. 322074 and 322034, respectively (Misr Co., Egypt) as commercial pharmaceutical preparations were purchased from the local market and subjected to analysis by the proposed methods.

Solvents and solutions

Solvents

Absolute ethanol from Riedel-De-Haen AG, Germany was used through all procedures.

Preparation of stock and working standard solutions

Stock solutions of authentic were prepared by dissolving an accurately weighed amount (50 mg) of

the studied drugs in 50 mL ethanol. Suitable aliquots of the stock solutions were completed quantitatively with the solvent to obtain the suitable working standard solutions according to the linear calibration range for each drug.

Preparation of sample solutions from pharmaceutical dosage forms

For ointments: Five ointment tubes were evacuated in clean beaker and mixed well; an accurately weighed 5 g of ointment was extracted on heating and stirring with the suitable solvent and filtrated to 50 mL volumetric flask. The first portion of filtrate was discarded. Different aliquots of prepared solution were diluted with ethanol to produce different dilutions of ratio similar to that in the dosage form.

For eye drops: 10 ml of cortiphen® eye drops equivalent to 50 mg of prednisolone and 20 mg of chloramphenicol was diluted with ethanol to 50 mL in 50 mL volumetric flask, 5 mL was diluted to 50 mL with ethanol in 50 mL volumetric flask where each 1 mL containing 100 µg prednisolone and 40 µg chloramphenicol. Different aliquots of prepared solution were diluted with ethanol to produce different dilutions of ratio similar to that in the dosage form.

General procedures

Procedures for determination of linearity range of standard solutions

In order to obtain the calibration curve for applying quantitative analysis six solutions of each of the pure components of each mixture were prepared with concentrations in the calibration range. These ranges were previously verified to obey Beer's law for each of the studied drugs.

Procedures for preparation of laboratory prepared mixture solutions

Laboratory prepared mixtures were prepared by mixing known amounts of working solution of one of the mixture components with known amounts of working solution of the other component in different proportions (ratios) in order to verify the precision of the method for analysis of such mixtures and match the commercial formulations with those having comparable concentrations

Procedures for preparation of dosage form solutions

Different dilutions of dosage form working solutions were assayed for its drug content as a procedure for prediction step.

Procedures for standard addition technique

Portion of dosage form working solution was quantitatively transferred to six volumetric flasks, then serial portions of authentic working solutions of both drugs were added to each flask and the solution was completed with the used solvent and measured at the specified wavelength.

Optimization

Data processing

Data were processed on an Intel Pentium III 750 MHz PC-compatible computer. For CLS calculations, the spectral data were transferred to Microsoft excel XP program and processing them with the standard curve fit package and matrix calculations. The MVSP version 3.13g (1985-2003), and VISTA 6 version 6.4.3436-EWU (May 10, 2001) software (Zatourn Co., Hamburg, Germany) were used for the principal component regression applications.

Degree of spectral overlapping

The absorption spectra for the studied drugs showed a considerable degree of spectral overlapping. The degree of spectral overlapping can be conveniently given by $(D_i)^{0.5}$ [25], where D_i is the magnitude of the dependency which can be calculated for a two component mixture from the equation:

$$D_i = \frac{\Sigma (k_1 \, k_2^t)^2}{\Sigma k_1 \, k_1^t \, \Sigma . \, k_2 \, k_2^t}$$

; where k_1 and k_2 are the $I \times n$ matrices of regression coefficients for studied drugs and k^t is the transposed k matrix.

Results and Discussion

Derivative spectrophotometric analysis

Zero-crossing technique

Figures 1 and 2 show that prednisolone absorption spectrum overlapped with the absorption spectrum of tetracycline and chloramphenicol, respectively. In the corresponding first derivative (^{1}D) curves (Figure 3), tetracycline hydrochloride showed a well absorption first derivative (^{1}D) value at λ = 245.5 and 350.5 nm while prednisolone had no contribution. On the other hand, prednisolone exhibited an absorption first derivative (^{1}D) value at λ = 236.5 and 266.5 nm where tetracycline hydrochloride absorbance was nil. In Figure 4, the chloramphenicol shows a well absorption first derivative (^{1}D) value at λ = 242.5 and 293.5 nm while prednisolone have no contribution. Prednisolone exhibited an absorption maximum first derivative (^{1}D) value at λ = 234.5 and 272.5 nm where chloramphenicol absorbance was nil. The analytical parameters for the assay of binary mixtures (mix I and mix II) of the studied drugs are presented in Table 1

Derivative ratio technique

The influence of $\Delta\lambda$ for obtaining the first derivative of the ratio spectra as well as the effect of divisor concentration on the calibration graphs for the proposed mixture was studied in order to select the best factors affecting the determination. Results indicated that $\Delta\lambda$ = 3 nm was considered the most suitable one, while the divisor concentration has no significant effect on the assay results for the studied mixtures.

For determination of prednisolone, the absorption spectra of prednisolone were divided by that of standard solutions of tetracycline hydrochloride (12.4 µg/mL) and chloramphenicol (18 µg/mL) as shown in Figures 5 and 6, respectively. The first derivative of the developed ratio spectra were calculated with $\Delta\lambda$ = 3 nm. Figures 7 and 8 show that prednisolone can be determined by measuring the amplitude at many wavelengths where tetracycline hydrochloride and chloramphenicol have no contribution, but it was found that the amplitude at 260.5 and 266.5 nm (for prednisolone in mix I) and 227.5 and 245.5 nm (for prednisolone in mix II) give the most accurate and sensitive results.

In the determination of tetracycline hydrochloride and chloramphenicol by the derivative ratio technique, the absorption spectra of standard solutions of tetracycline hydrochloride or chloramphenicol were divided (amplitude by amplitude at appropriate wavelengths) by absorption spectrum of a standard solution of 10 and 20 µg/ml prednisolone for mix I and mix II, respectively, to obtain the corresponding ratio spectra (Figures 9 and 10). The first derivative of the obtained ratio spectra were calculated with $\Delta\lambda$ = 3 nm, (Figures 11 and 12). From these figures, it is noticed that, both tetracycline hydrochloride and chloramphenicol can be determined in presence of prednisolone by measuring the amplitude at 227.5 and 287.5 nm (mix I) and at 218.5 and 287.5 nm (mix II), where there is no contribution from prednisolone.

Under the specified conditions and the specified wavelengths for each drug, regression equations for the drugs were derived using the least-squares regression analysis, Table 1 summarizes the obtained results for all the used techniques, the results include the intercepts (a), slope (b), correlation coefficient (r), determination coefficient (r^2), limit of detection (LOD) and limit of quantification (LOQ).

Validation of the derivative ratio technique:

(1) Linearity

The linearity of the proposed method was evaluated for each drug by analyzing a series of different concentrations of each studied drug within the range stated in Table 1 and in the absence and presence of a certain concentration of the other component in the mixture. The assay was performed according to the experimental conditions previously established. The first derivative ratios for each drug were measured, at the specified wavelengths (Table 1) and plotted against concentration. A straight line was obtained in each case. The statistical analysis of these graphs using least square method was made for the slope, intercept and correlation coefficients. The results obtained show that

the linearity of calibration graphs and the compliance with Beer's law were validated, as illustrated by the excellent values of correlation coefficients of the regression equation and the small values of intercepts.

Furthermore, the slope of the calibration graph for each drug was independent on the concentratin of the other component in the mixture (Table 1).

Table 1 Analytical parameters for determination of prednisolone, tetracycline hydrochloride and chloramphenicol with the proposed derivative spectrophotometric techniques.

Standard solution of	Technique	Conc. (μg/mL)	λ(nm)	Linear regression equation parameters					
				a	b	r	r^2	LOD (μg/mL)	LOQ (μg/mL)
Tetracycline	^{1}D (zero crossing)	5 - 30	245.5	-0.0055	0.00075	0.9998	0.9996	0.25	0.84
hydrochloride		5 - 30	350.5	0.00048	0.00057	0.9999	0.9998	0.32	1.06
(mix I)	^{1}D (derivative ratio)	5 - 30	227.5	-0.0199	-0.0071	0.9999	0.9998	1.44	4.80
		5 - 30	287.5	1.04548	0.9304	0.99999	0.99998	1.11	3.71
Prednisolone	^{1}D (zero-crossing)	5 - 30	236.5	-0.0005	0.00078	0.9999	0.9998	0.32	1.04
(mix I)		5 - 30	266.5	-0.0004	-0.0013	0.9999	0.9998	0.60	2.00
	^{1}D (derivative ratio)	5 - 30	260.5	-0.0025	-0.0148	0.9998	0.9996	1.02	3.39
		5 - 30	266.5	0.00133	-0.0133	0.9998	0.9996	1.12	3.74
Chloramphenicol	^{1}D (zero-crossing)	10 - 35	242.5	0.00329	0.00073	0.9999	0.9998	0.60	2.00
(mix II)		10 - 35	293.5	0.0002	0.00051	0.9999	0.9998	0.62	2.08
	^{1}D (derivative ratio)	10 - 35	218.5	0.00354	0.00559	0.9997	0.9994	2.20	7.35
		10 - 35	287.5	0.0446	0.03808	0.9999	0.9998	0.48	1.60
Prednisolone	^{1}D (zero-crossing)	10 - 35	234.5	0.01134	0.00255	0.9999	0.9998	0.40	1.33
(mix II)		10 - 35	272.5	0.00059	0.00144	0.9999	0.9998	0.63	2.08
	^{1}D (derivative ratio)	10 - 35	227.5	-0.0309	0.02317	0.9997	0.9994	1.64	5.46
		10 - 35	245.5	0.01237	-0.0180	0.9998	0.9996	1.25	4.18

a: intercept; b: slope; r: correlation coefficient; r^2: coefficient of determination; LOD: limit of detection = $3\sigma / S$; LOQ: limit of quantitation = $10\sigma / S$ (where σ is the standard deviation of the intercept and S is the sensitivity).

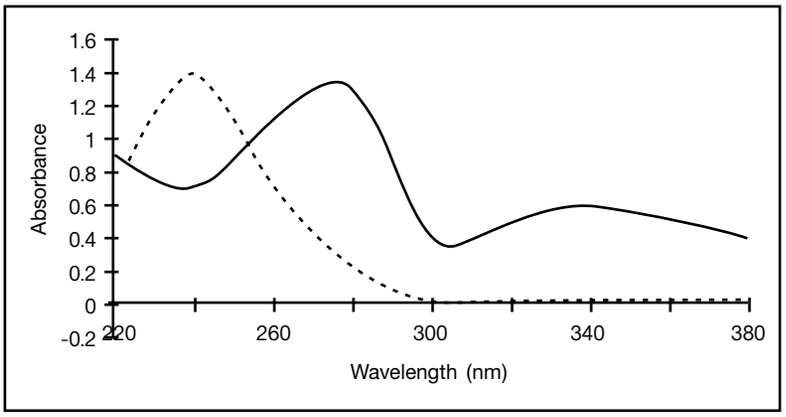

Figure 1 Degree of overlapping as indicated by absorption spectra of tetracycline hydrochloride (—) (30 μg/mL) and prednisolone (---) (30 μg/mL).

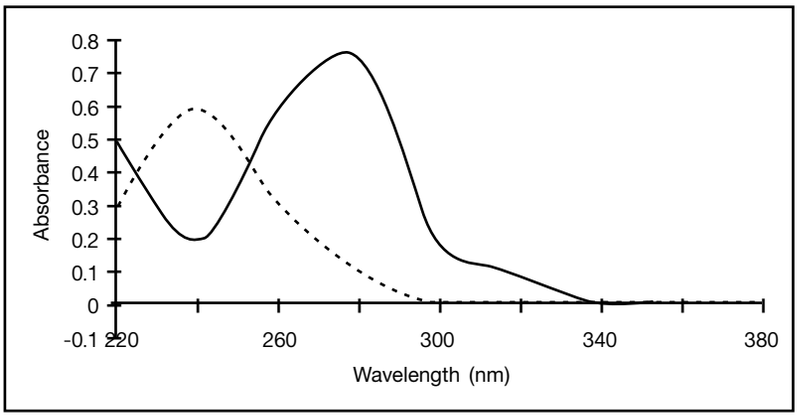

Figure 2 Degree of overlapping as indicated by absorption spectra of chloramphenicol (—) (30 μg/mL) and prednisolone (---) (15 μg/mL).

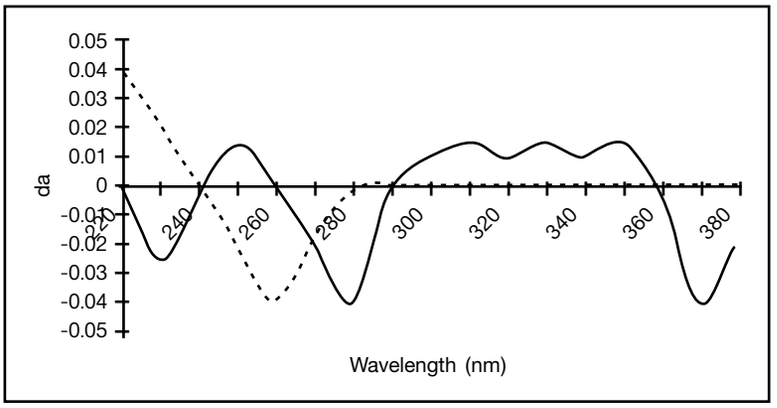

Figure 3 First derivative determination of tetracycline hydrochloride (—) at 245.5 and 350.5 nm and prednisolone (---) at 236.5 and 266.5 nm using zero-crossing spectrophotometric techniques.

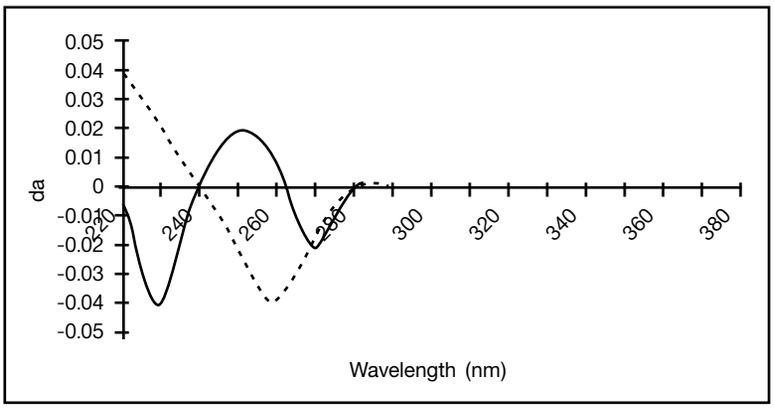

Figure 4 First derivative determination of chloramphenicol (–) at 242.5 and 293.5 nm and prednisolone (---) at 234.5 and 272.5 nm using zero-crossing spectrophotometric techniques.

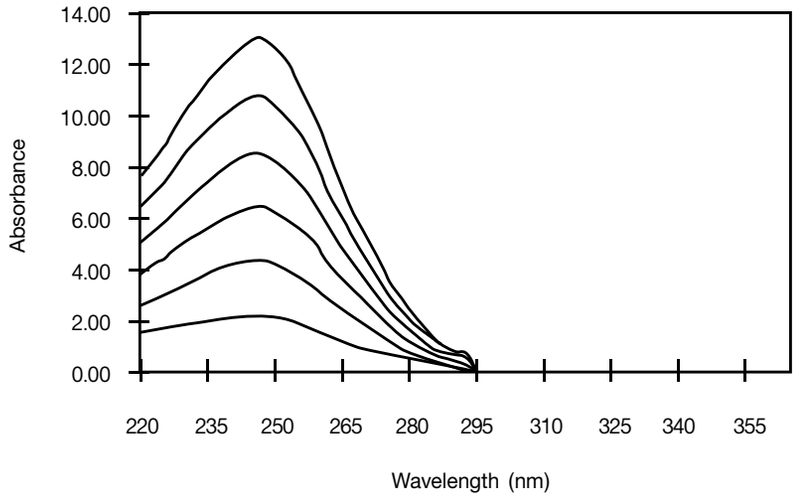

Figure 5 Ratio spectra of prednisolone (5 - 30 µg/mL). Divisor is 12.40 µg/ml tetracycline hydrochloride.

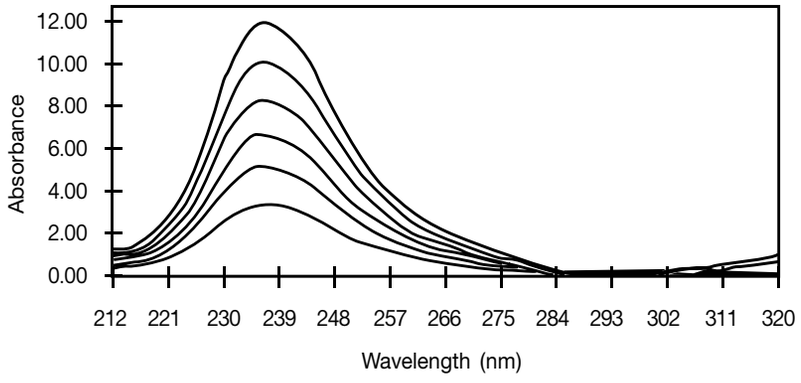

Figure 6 Ratio spectra of prednisolone (10 - 35 µg/mL). Divisor is 18 µg/ml chloramphenicol.

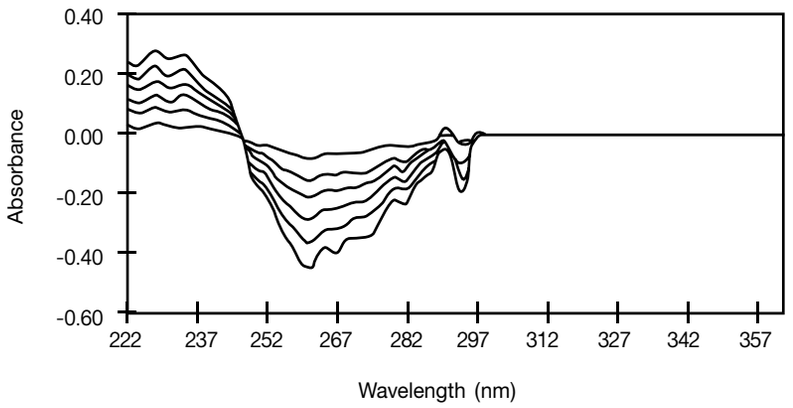

Figure 7 First derivative ratio spectra of prednisolone (5 - 30 µg/mL). Divisor is 12.40 µg/mL tetracycline hydrochloride.

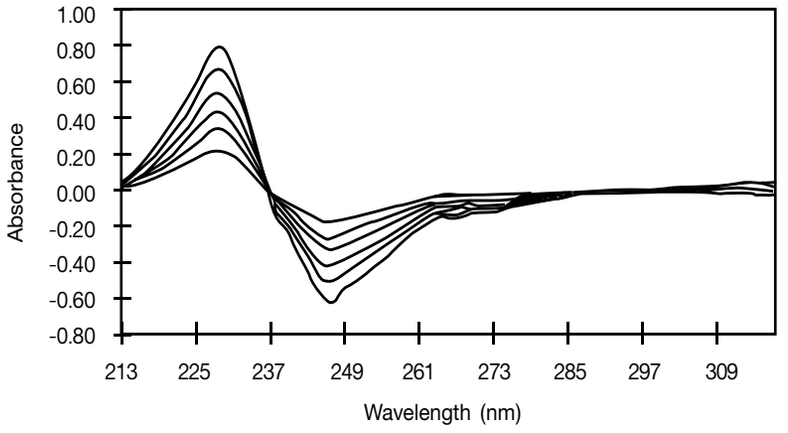

Figure 8 First derivative ratio spectra of prednisolone (10 - 35 µg/mL) Divisor is 18 µg/mL chloramphenicol.

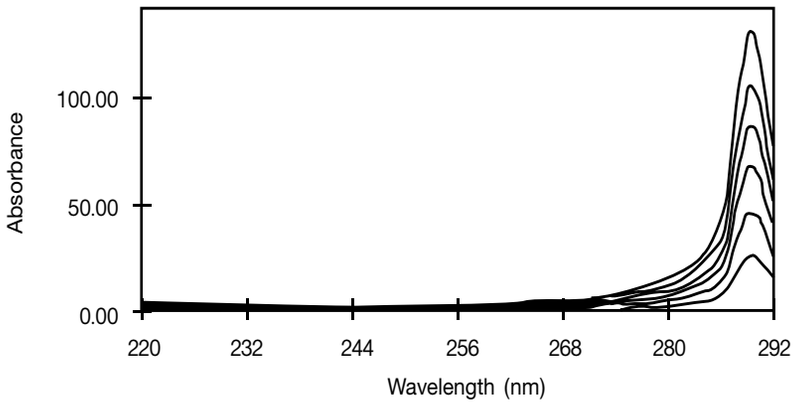

Figure 9 Ratio spectra of tetracycline hydrochloride (5 - 30 µg/mL) Divisor is 10 µg/mL prednisolone.

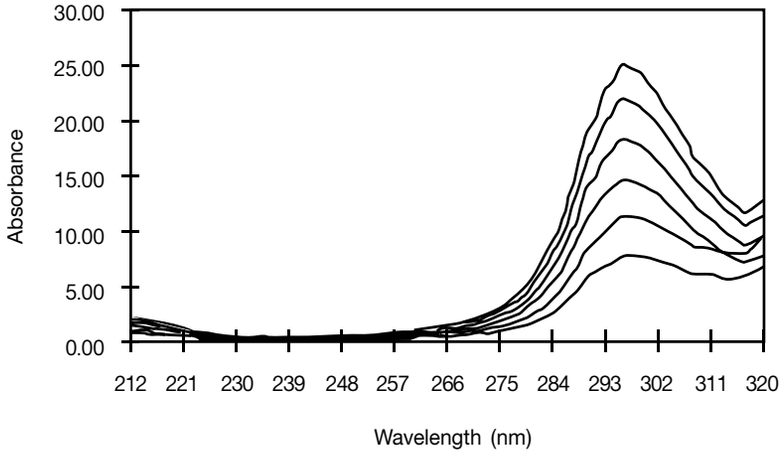

Figure 10 Ratio spectra of chloramphenicol (10 - 35 μg/mL) Divisor is 20 μg/mL prednisolone.

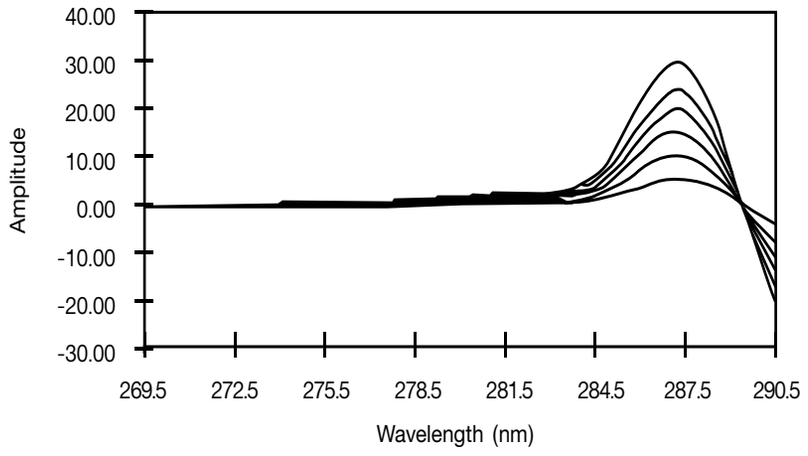

Figure 11 First derivative ratio spectra of tetracycline hydrochloride (5 - 30 μg/ mL) Divisor is 10 μg/mL prednisolone.

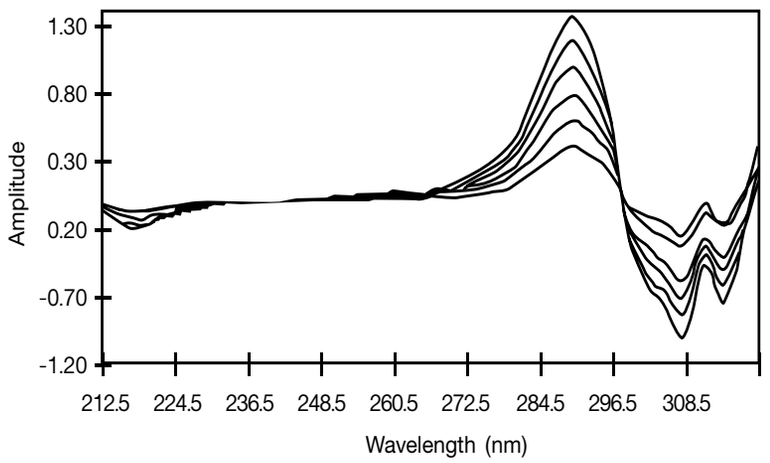

Figure 12 First derivative ratio spectra of chloramphenicol (10 - 35 μg/ mL) Divisor is 20 μg/mL prednisolone.

(2) Accuracy

This study was performed by the addition of known amounts of each drug to placebo containing either excipients only or certain concentration of the other component in the mixture. Each analyte was tested at different levels below and above the label claim of each one. The resulting mixtures were assayed and the accuracy was then calculated from the test results as the percentage of analyte recovered by the assay. The excellent recoveries obtained (Tables 2-5) suggest that good accuracy of the proposed method and there is no interference from excipients and other component which are present in dosage forms.

Table 2 Statistical analysis of the results obtained for assay prednisolone in mix I by the proposed derivative spectrophotometric methods.

	Statistical parameter	Reported methods*	^{1}D (zero-crossing)		^{1}D derivative ratio	
			236.5 nm	266.5 nm	260.5 nm	266.5 nm
Pure	X	99.60	100.25	100.10	99.56	99.68
	± S	0.72	0.82	0.69	0.89	0.88
	n	6	6	6	6	6
	S^2	0.51	0.67	0.48	0.79	0.77
	t	-	1.459	1.228	0.086	0.172
	F	-	1.297	1.089	1.528	1.494
Laboratory	X	99.0	99.82	99.91	99.38	99.36
prepared	± S	0.45	0.76	0.79	1.00	1.32
mixtures	n	5	5	5	5	5
	S^2	0.20	0.58	0.62	1.00	1.74
	t	-	2.076	2.238	0.723	0.734
	F	-	2.852	3.082	5.812	4.938
Tetracort®	X	99.0	98.50	99.04	100.00	99.00
Ointment	± S	0.45	0.20	1.10	1.00	1.00
30/5	n	5	5	5	5	5
	S^2	0.20	0.04	1.21	1.00	1.00
	t	-	1.816	0.000	2.039	0.00
	F	-	5.062	5.975	4.938	4.938
Standard	X	-	99.95	99.99	98.22	99.01
addition	± S	-	1.10	0.28	0.32	0.45
technique	S^2	-	1.21	0.08	0.11	0.09

Theoretical values at 95% confidence limit are; t = 2.306 and F = 6.39 (n_1 = 5, n_2 = 5); t = 2.228 and F = 5.05 (n_1 = 6 , n_2 = 6); X = mean; n = number of observations; S = standard deviation; S^2 = variance

* Reported methods for authentic [27], reported method for laboratory prepared mixtures and pharmaceutical preparations [28].

(3) Precision

To test the repeatability of the proposed method, separate determinations at different concentration levels were carried out for each drug either alone or in the presence of certain concentration of the other component. The results obtained (Tables 2-5) showed that, the relative standard deviation was less than 2%, which indicated high degree of precision of the proposed method.

(4) Selectivity

Method selectivity was achieved by preparing different mixtures of the tested drugs within the linearity range. The mixtures contain varying amounts of one component and constant amount of the other. The laboratory prepared mixtures were analyzed according to the previous procedure. The first derivatives of the ratio values for each component were measured at the specified wavelengths (Table 1). Statistical analysis of the data shows that the slope of the calibration graph for each drug was independent on the concentration of the other component of the mixture. This means that the first derivative of the ratio value amplitudes of the mixture was only a function of the concentration of the drug at the specified wavelength. Consequently, the result obtained (Tables 2-5) well indicated the high selectivity of the proposed method and its potential for the simultaneous determination of these mixtures.

Multivariate calibration analysis

The absorption spectra of the studied drugs are shown in Figures 1 and 2. As can be seen, a considerable degree of spectral overlapping occurs in the region from 220 to 364 nm and from 212 to 320 nm, for the components of mix I and mix II, respectively. The degree of spectral overlapping was given by $(Di)^{0.5}$. In case of the presently studied compounds, the spectra lead to D_i = 0.50 implying a 70.70% and 0.464 implying a 68.12% of spectral overlap for mix I and mix II, respectively. Table 6 shows the actual and predicted amounts ± errors (%) of the studied drugs. The results confirm the high degree of agreement and indicate that both methods are suitable for analysis in the given domain for each drug.

Several laboratory prepared mixtures were subjected to the CLS and PCR analysis in order to confirm the suitability of the calibration model for determination of the studied drugs in the pharmaceutical sample solutions. Table 7 summarizes the results obtained for the suggested laboratory prepared binary mixtures. As could be seen, the concentrations predicted by the model are very close to the real concentrations, the results in all cases were satisfactory.

On the other hand, the results for commercial dosage forms and laboratory prepared mixtures with comparable concentrations were found closely matched. This indicated that, the present or added excipients and additives did not interfere with the determinations.

Moreover, the results for dosage form were compared with those obtained by applying reported methods. As shown in Table 8, the results are in good agreement with those of the reported procedure as indicated by the calculated t and F values.

Conclusion

Derivative, derivative ratio, CLS and PCR methods can be used for the simultaneous determination of tetracycline hydrochloride, chloramphenicol and prednisolone as binary mixtures either in their pure powder forms or in their pharmaceutical preparations. The methods are precise, accurate and simple. Also, no separation step is required. They are rapid and do not require any expensive or sophisticated apparatus if compared with the chromatographic methods. So, the methods were completely validated and suitable for quality control laboratories, where economy and time are essential.

Table 3 Statistical analysis of the results obtained for assay tetracycline hydrochloride by the proposed derivative spectrophotometric techniques.

	Statistical parameters	Reported* methods	^{1}D (zero-rossing)		^{1}D derivative ratio	
			245.5 nm	350.5 nm	227.5 nm	287.5 nm
Pure	X	100.8	100.21	99.91	99.98	100.12
	± S	0.68	1.11	1.1	1.21	0.32
	n	6	6	6	6	6
	S^2	0.46	1.23	1.21	1.46	0.10
	t	-	1.110	1.686	1.447	2.138
	F	-	2.665	2.617	3.166	3.202
Laboratory	X	100.50	100.17	99.53	99.75	99.92
prepared	± S	0.40	1.00	0.96	0.91	0.65
mixtures	n	5	5	5	5	5
	S^2	0.16	1.00	0.92	0.83	0.42
	t	-	0.685	2.086	1.687	1.699
	F	-	6.250	5.760	5.176	2.641
Tetracort®	X	100.50	101.0	100.57	100.11	101.0
Ointment	± S	0.40	0.42	0.65	0.20	0.37
30/5	n	5	5	5	5	5
	S^2	0.16	0.18	0.42	0.04	0.14
	t	-	1.976	0.472	2.000	1.976
	F	-	1.000	3.062	4.000	1.000
Standard	X	-	100.05	100.05	99.00	98.99
addition	± S	-	0.82	1.39	0.91	0.86
technique	S^2	-	0.67	1.93	0.58	0.60

Theoretical values at 95% confidence limit are; t = 2.306 and F = 6.39 (n_1 = 5, n_2 = 5); t = 2.228 and F = 5.05 (n_1 = 6, n_2 = 6); X = mean; n = number of observations; S = standard deviation; S^2 = variance

*Reported methods for authentic [27], reported method for laboratory prepared mixtures and pharmaceutical preparations [28].

Table 4 Statistical analysis of the results obtained for assay prednisolone in mix II by the proposed derivative spectrophotometric methods.

	Statistical parameter	Reported methods*	^{1}D (zero-crossing)		^{1}D derivative ratio	
			234.5 nm	272.5 nm	272.5 nm	245.5 nm
Pure	X	99.60	99.91	99.93	99.91	99.98
	± S	0.72	0.47	0.89	0.75	0.98
	N	6	6	6	6	6
	S^2	0.51	0.22	0.79	0.57	0.96
	t	-	0.88	0.70	0.74	0.77
	F	-	2.35	1.55	1.11	1.88
Laboratory	X	99.00	100.03	99.81	99.80	99.87
prepared	± S	0.45	0.97	0.79	0.77	0.72
mixtures	N	5	5	5	5	5
	S^2	0.20	0.94	0.63	0.59	0.52
	t	-	2.16	1.98	2.01	2.30
	F	-	4.64	3.11	2.91	2.56
Cortiphen®	X	99.54	100.20	100.20	99.53	99.93
Drops	± S	0.49	0.20	0.79	0.57	0.39
6/15	n	3	3	3	3	3
	S^2	0.24	0.40	0.62	0.33	0.15
	t	-	2.160	1.230	0.023	1.079
	F	-	6.002	2.599	1.353	1.579
Cortiphen®	X	99.54	99.80	100.20	100.60	100.00
Ointment	± S	0.49	0.31	0.80	0.81	0.86
1/0.5	n	3	3	3	3	3
	S^2	0.24	0.10	0.64	0.66	0.74
	t	-	0.777	1.219	1.939	0.805
	F	-	2.498	2.666	2.733	3.080
Standard	X	-	99.84	99.60	98.14	98.07
addition	± S	-	0.35	0.30	0.42	0.26
technique	S^2	-	0.12	0.09	0.18	0.31
	X	-	99.50	99.67	97.90	98.00
	± S	-	0.41	0.53	0.51	0.45
	S^2	-	0.17	0.28	0.15	0.68

Theoretical values at 95% confidence limit are; t = 2.306 and F = 6.39 (n_1 = 5, n_2 = 5); t = 2.228 and F = 5.05 (n_1 = 6, n_2 = 6); t = 2.776 and F = 19.0 (n_1 = 3, n_2 = 3); X = mean; n = number of observations; S = standard deviation; S^2 = variance

*Reported methods for authentic [27] , reported method for laboratory prepared mixtures and pharmaceutical preparations [28].

Table 5 Statistical analysis of the results obtained for assay chloramphenicol by the proposed derivative spectrophotometric techniques.

	Statistical parameter	Reported methods*	^{1}D (zero-crossing)		^{1}D derivative ratio	
			242.5 nm	293.5 nm	218.5 nm	287.5 nm
Pure	X	99.90	100.11	100.56	100.04	100.21
	± S	0.93	0.63	0.96	0.96	0.58
	n	6	6	6	6	6
	S^2	0.87	0.40	0.92	0.92	0.34
	t	-	0.458	1.210	0.257	0.693
	F	-	2.179	1.066	1.066	2.571
Laboratory	X	99.68	99.81	100.11	100.74	99.10
prepared	± S	0.84	0.91	0.84	1.19	1.15
mixtures	n	5	5	5	5	5
	S^2	0.71	0.83	0.71	1.42	1.32
	t	-	0.235	0.809	1.627	0.911
	F	-	1.174	1.000	2.007	1.874
Cortiphen®	X	99.50	100.80	100.33	100.50	98.50
Drops	± S	0.87	0.47	1.26	1.36	0.59
6/15	n	3	3	3	3	3
	S^2	0.75	0.22	1.59	1.84	0.35
	t	-	2.277	0.939	1.073	1.648
	F	-	3.426	2.098	2.444	2.174
Cortiphen®	X	99.50	99.00	99.00	99.70	99.00
Ointment	± S	0.87	0.32	0.37	1.10	0.77
1/ 0.5	n	3	3	3	3	3
	S^2	0.75	0.10	0.14	1.20	0.59
	t	-	0.934	1.916	0.247	0.745
	F	-	7.392	5.529	1.599	1.277
Standard	X	-	100.20	99.70	99.58	99.54
addition	± S	-	0.56	0.71	0.66	0.65
technique	S^2		0.31	0.50	0.51	0.55
	X	-	98.80	100.10	99.01	98.78
	± S	-	0.40	0.36	0.34	0.40
	S^2		0.16	0.13	0.10	0.12

Theoretical values at 95 % confidence limit are; t = 2.306 and F = 6.39 (n_1 = 5, n_2 = 5); t = 2.228 and F = 5.05 (n_1 = 6 , n_2 = 6); t = 2.776 and F = 19.0 (n_1 = 3, n_2 = 3) X = mean; n = number of observations; S = standard deviation; S^2 = variance

*Reported methods for authentic [26] , reported method for laboratory prepared mixtures and [29] reported method for pharmaceutical preparations [28].

Table 6 Actual and predicted amounts of the studied drugs given by applying CLS and PCR analysis within its linear domain.

Real (μg/ml)	CLS			PCR		
	Predicted (μg/mL)	Recovery (%)	RRMSE* (%)	Predicted (μg/mL)	Recovery (%)	RRMSE* (%)
Prednisolone (mix I)						
5	5.03	100.60	0.60	4.95	99.00	1.00
10	10.03	100.30	0.30	9.92	99.20	0.80
15	14.9	99.33	0.67	14.9	99.33	0.67
20	19.8	99.00	1.00	19.8	99.00	1.00
25	25.1	100.40	0.40	24.8	99.20	0.80
30	30.1	100.33	0.33	29.5	98.33	1.67
Tetracycline (mix I)						
5	4.93	98.60	1.40	4.97	99.40	0.60
10	9.91	99.10	0.90	9.92	99.20	0.80
15	14.8	98.67	1.33	14.9	99.33	0.67
20	19.9	99.50	0.50	19.8	99.00	1.00
25	25.1	100.40	0.40	24.8	99.20	0.80
30	30.1	100.33	0.33	29.4	98.00	2.00
Prednisolone (mix II)						
10	9.94	99.40	0.60	9.9	99.00	1.00
15	15.2	101.33	1.33	14.5	96.67	3.33
20	19.8	99.00	1.00	19.7	98.50	1.50
25	24.7	98.80	1.20	24.4	97.60	2.40
30	30.0	100.00	0.00	29.4	98.00	2.00
35	35.3	100.86	0.86	34.6	98.86	1.14
Chloramphenicol (mix II)						
10	9.84	98.40	1.60	9.91	99.10	0.90
15	14.8	98.67	1.33	14.7	98.00	2.00
20	20.1	100.50	0.50	19.7	98.50	1.50
25	25.1	100.40	0.40	24.3	97.20	2.80
30	30.2	100.67	0.67	29.1	97.00	3.00
35	34.9	99.71	0.29	34.5	98.57	1.43

*RRMSE is the Relative Root Mean Squared Error.

Table 7 Results obtained by applying CLS and PCR analysis to the laboratory prepared mixtures.

Mix	Component	Real (μg/ml)	CLS			PCR		
			Found (μg/ml)	Found (%)	RRMSE (%)	Found (μg/ml)	Found (%)	RRMSE (%)
1	Tetracycline HCl	10	10.02	100.20	0.20	9.95	99.50	0.50
	Prednisolone	25	24.6	98.40	1.60	24.8	99.20	0.80
2	Tetracycline HCl	15	14.9	99.33	0.67	15.1	100.67	0.67
	Prednisolone	30	29.5	98.33	1.67	30.1	100.33	0.33
3	Tetracycline HCl	15	15.1	100.67	0.67	14.9	99.33	0.67
	Prednisolone	20	19.9	99.50	0.50	19.9	99.50	0.50
4	Tetracycline HCl	25	25.1	100.40	0.40	24.9	99.60	0.40
	Prednisolone	10	9.83	98.30	1.70	9.2	92.00	8.00
5	Tetracycline HCl	25	25.1	100.40	0.40	25.3	101.20	1.20
	Prednisolone	25	24.4	97.60	2.40	25.1	100.40	0.40
6	Tetracycline HCl	30	29.8	99.33	0.67	28.9	96.33	3.67
	Prednisolone	15	14.7	98.00	2.00	15.2	101.33	1.33
7	Tetracycline HCl	30	30.3	101.00	1.00	30.2	100.67	0.67
	Prednisolone	5	5.0	100.00	0.00	4.9	98.00	2.00
8	Tetracycline HCl	5	4.97	99.40	0.60	4.9	98.00	2.00
	Prednisolone	30	29.6	98.67	1.33	29.4	98.00	2.00
1	Chloramphenicol	12	12.2	101.67	1.67	11.9	99.17	0.83
	Prednisolone	30	29.8	99.33	0.67	29.7	99.00	1.00
2	Chloramphenicol	13	13.2	101.54	1.54	12.9	99.23	0.77
	Prednisolone	32.5	32.3	99.39	0.61	31.6	97.23	2.77
3	Chloramphenicol	25	25.1	100.40	0.40	24.7	98.80	1.20
	Prednisolone	15	14.9	99.33	0.67	15.1	100.67	0.67
4	Chloramphenicol	20	20.3	101.5	1.50	20.1	100.50	0.50
	Prednisolone	10	9.9	99.00	1.00	10.0	100.00	0.00
5	Chloramphenicol	30	29.5	98.33	1.67	29.8	99.33	0.67
	Prednisolone	15	14.9	99.33	0.67	14.9	99.33	0.67
6	Chloramphenicol	20	20.4	102.0	2.00	19.5	97.50	2.50
	Prednisolone	20	19.6	98.00	2.00	19.8	99.00	1.00
7	Chloramphenicol	30	29.4	98.00	2.00	29.2	97.33	2.67
	Prednisolone	10	10.1	101.0	1.00	9.9	99.00	1.00
8	Chloramphenicol	10	10.2	102.0	2.00	9.9	99.00	1.00
	Prednisolone	10	9.8	98.20	1.80	9.9	99.00	1.00

Table 8 Results obtained by applying CLS and PCR analysis to commercial dosage form.

Dosage Form	Component	CLS (% ± S)	RRMSE (%)	PCR (% ± S)	RRMSE (%)	Reported (% ± S)
Tetracort® Ointment	Tetracycline HCl	101.0 ± 0.45 t = 1.857 F = 1.266	1.00	99.5 ± 0.70 t = 2.774 F = 3.062	0.50	100.5 ± 0.4 [28]
	Prednisolone	98.6 ± 0.25 t = 1.737 F = 3.240	1.40	99.4 ± 0.31 t = 1.637 F = 2.107	0.60	99.04 ± 0.4 [28]
Cortiphen® Drops	Chlorampenicol	100.5 ± 0.50 t = 1.726 F = 3.028	0.50	98.83 ± 0.56 t = 1.122 F = 2.414	1.17	99.5 ± 0.87 [29]
	Prednisolone	99.33 ± 0.20 t = 0.683 F = 6.002	0.67	95.33 ± 0.40 t = 1.152 F = 1.501	4.67	99.04 ± 0.4 [29]
Cortiphen® Ointment	Chloramphenicol	99.00 ± 1.04 t = 0.639 F = 1.429	1.00	99.7 ± 0.83 t = 3.601 F = 1.099	3.00	99.5 ± 0.87 [29]
	Prednisolone	98.00 ± 0.92 t = 2.559 F = 3.525	2.00	98.00 ± 0.90 t = 2.603 F = 3.374	2.00	99.04 ± 0.4 [29]

Theoretical values at 95% confidence limit are t = 2.776 and F = 19.0 (n_1 = 3, n_2 = 3).

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