REPORT

Simultaneous determination of naphazoline hydrochloride, chlorpheniramine maleate and methylene blue in their ternary mixture

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Abstract: Validated spectrophotometric and chemometric methods were developed for determination of Naphazoline Hydrochloride (NAP), Chlorpheniramine maleate (CLO) and Methylene blue (MB) in their ternary mixture. Method A was a spectrophotometric method, where NAP and MB were determined using second derivative (D^2) spectrophoto metric method using the peak amplitudes at 299 nm and 337 nm for NAP and MB respectively , while CLO was determined using second derivative ratio (DD^2) spectrophotometric method using the peak amplitude at 276.6 nm. Method B used the chemometric techniques; principal component regression (PCR) and partial least squares (PLS) for determination of NAP, CLO and MB using the information contained in the absorption spectra of their ternary mixture solutions. The proposed methods have been successfully applied for the analysis of NAP, CLO and MB in their pharmaceutical formulation and the obtained results were statistically compared with the reported methods.

Keywords: Naphazoline hydrochloride; chlorpheniramine maleate; methylene blue; derivative spectrophotometry; chemometrics.

INTRODUCTION

Naphazoline hydrochloride (NAP), fig. 1a [2-(1-naphthylmethyl)-2-imidazoline monohydrochloride] is a sympathomimetic drug, which belongs to the imidazole group. It is used as vasoconstrictor of relatively long-lasting action that acts on the α receptors of the vascular smooth muscle (Goodman-Hillman *et al*., 1996).

Chlorpheniramine maleate (CLO), fig.1b; [3-(*p*-chlorophenyl)-3-(2-pyridyl)-N,N-dimethylpropylamine] is a powerful H_1-receptor antagonist (anti-histamine), widely used for symptomatic relief of common cold, allergic rhinitis and conjunctivitis, with weak sedative properties (Paton and Webster, 1983).

Methylene blue (MB), fig. 1c; is a classical basic dye used for its antioxidant and antiseptic effects (Mills *et al*., 2011).Surveying the literature reveals several reported methods for determination of NAP, CLO and MB either alone or in the presence of other drugs including spectrophotometric (Szabolcs, 1980; Strelets *et al*., 1986; Kelani, 1998; Goicoechea *et al*., 2002; Qin and Mo, 1995; Nie, 1992; Leung and Law, 1989; Muralikrishna *et al*., 1976; David *et al*., 1997; Onur and http://www.sciencedirect.com/science/article/pii/037851739290359A - COR1 Acar**,** 1992; Korany, 1990), fluorimetric (Bai and Tongbao, 1988; Díaz *et al*, 2004; Chen, 1985), liquid chromatographic (Chabenat and Boucly, 1992; Ruo-wan *et al*, 2010; Xu *et al*, 2009; Turnipseed *et al*, 1997), Gas chromatographic (Massaccesi, 1987), TLC-Densitometric (Kelani, 1998; Lu *et al*, 1988; Gaitonde and Rivankar, 1987), Polarographic (Liang and Zhang, 1992), nuclear magnetic resonance spectrometric (Hanna and Lau-Cam, 1993), Complexometric (Chen and Wang, 1987; Abdel Fattah *et al*, 1987; Qi *et al*, 1986), and Potentiometric (Selig, 1981) methods.

The ternary mixture of NAP, CLO and MB is used for their decongestant, antihistaminic and antiseptic effects, respectively. Reviewing literature in hand reveals that few methods were reported for determination of NAP and CLO in their binary mixture (Kelani, 1998; Ruo-wan *et al*, 2010). The admixture of MB with NAP and CLO in the form of ternary mixture makes their simultaneous determination a difficult task even when using the reported methods. In addition, no analytical methods are reported for determination of NAP, CLO and MB in their ternary mixture.

Spectrophotometric methods are considered the methods of choice used on a large scale in pharmaceutical industry especially in quality control laboratories. The reported spectrophotometric methods involve use of techniques that can only be applied for binary mixtures of NAP and CLO and not ternary ones. No reported methods have

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been found for determination of NAP and CLO in presence of MB.

Therefore, the aim of this work is to develop validated spectrophotometric and chemometric methods for the simultaneous determination of NAP, CLO and MB in their ternary mixture. This is the first report for determination of NAP and CLO in presence of MB. The proposed methods can be used for routine quality control analysis of the studied drugs in bulk powders and in pharmaceutical formulations.

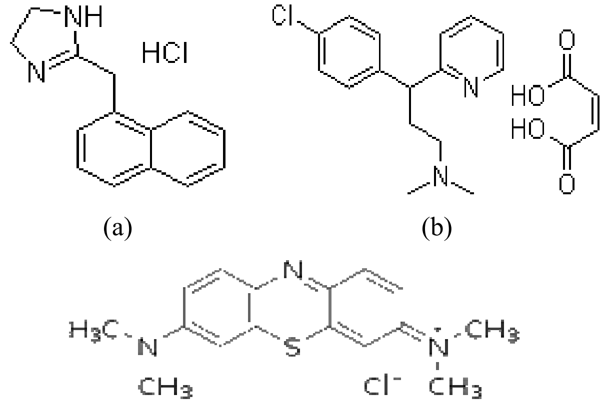
(a) (b) (c)

Fig. 1: Chemical structure of (a) Naphazoline Hydrochloride, (b) Chlorpheniramine Maleate and (c) Methylene Blue.

MATERIALS AND METHODS

Instruments

A double beam UV–visible spectrophotometer (Shimadzu, Japan) model UV-1601 PC with quartz cell of 1 cm path length, connected to IBM compatible computer. The software was UVPC personal spectroscopy software version 3.7. The spectral bandwidth was 2nm and wavelength-scanning speed 2800 nm/min.

Materials

Ppure samples

NAP, CLO were kindly provided by Egyptian International Pharmaceutical Industries Co. (E.I.P.I.CO) (Egypt). Their purity was found to be 98.82% and 98.58%, respectively according to the company certificate of analysis. MB was kindly supplied from Kahira Pharmaceutical & Chemical Industries Co. (Egypt). Its purity was found to be 99.75% according to the company certificate of analysis.

Pharmaceutical formulation

Prisoline Blue® eye drops (Batch No. 06891), labeled to contain 50 mg Naphazoline HCl, 50 mg Chlorphenirmine maleate, 3 mg Methylene blue and 20 mg of methylparaben per 100 ml, manufactured by Kahira Pharmaceutical & Chemical Industries Co. (Egypt).

Chemicals and solvents

Hydrochloric acid and Methanol of HPLC grade (E. Merck, Germany). All chemicals and solvents used were of analytical grade and were used without further purification.

Standard solutions

Stock standard solutions

Stock standard solutions of NAP, CLO and MB ($1 mg.mL^{-1}$) were prepared by weighing accurately 100 mg of pure powder of each into three separate 100-mL volumetric flasks and dissolved in methanol. The volume was completed to the mark with methanol.

Working standard solutions

Working standard solution of NAP, CLO and MB (100 $\mu g.mL^{-1}$) were prepared by accurately transferring 10 mL of stock standard solution of NAP, CLO and MB ($1 mg.mL^{-1}$) into three separate 100-mL volumetric flasks. The volume was completed to the mark with methanol.

Procedures

Derivative Spectrophotometric methods

Construction of calibration curve

The D^2 spectra corresponding to concentration range of 5-80 $\mu g.mL^{-1}$ for NAP and 1- 15 $\mu g.mL^{-1}$ for MB were recorded (Δ λ= 8 and scaling factor = 100). The peak amplitudes at 299 nm for NAP and 337 nm for MB were measured. The DD^2 spectra corresponding to concentration range of 5-80 $\mu g.mL^{-1}$ for CLO were recorded using the spectra of 30 $\mu g\ mL^{-1}$ of NAP as a divisor (Δ λ = 8 and scaling factor = 100). The peak amplitudes at 276.6 nm were measured. The calibration curves relating the peak amplitudes to the corresponding drug concentrations were constructed then the regression equations were computed.

Analysis of laboratory prepared mixtures of NAP, CLO and MB

Mixtures containing variable ratios of NAP, CLO and MB were prepared and analyzed using the proposed methods stated above.

Chemometric-assisted spectrophotometric methods

Construction of both training and validation sets

Different mixtures of varying ratios of NAP, CLO and MB were prepared as represented in table 1. The absorbance of these mixtures was recorded in the wavelength range between 200 and 400 nm at 1 nm intervals against a blank of 0.1N HCl. The composition of the samples was designed randomly using five level five factor experimental design (Brereton, 1997; Kramer, 1998). PCR and PLS calibration models were constructed using the data obtained. Constructing the models For both PCR and PLS models, the PLS-Toolbox 2.0 software using training set absorbance and concentration matrices was used for the calculations.

Application to Prisoline Blue® eye drops

Accurate volume of 20 mL of Prisoline Blue® eye drops were transferred into a 100-mL volumetric flask and diluted to the mark with methanol to obtain working standard solution containing 100 µg.mL^{-1} of NAP, 100 µg.mL^{-1} of CLO and 60 µg.mL^{-1} of MB. Then the procedure detailed under linearity and construction of calibration curve for each method was applied. In case of derivative spectrophotometric methods, the peak amplitudes of the D^2 spectra at 299 nm and 337 nm were recorded for NAP and CLO, respectively while the peak amplitude of the at DD^2 at 276.6 nm was recorded for MB. The concentrations of NAP, CLO and MB were calculated from their corresponding regression equations. For the chemometric method, the absorbance values of the obtained spectra in the specified wavelength range were introduced to the models and the concentrations of NAP, CLO and MB were computed automatically.

Table 1: The concentration of mixtures of Naphazoline HCl, Chlorpheniramine maleate and Methylene blue used in the training and validation sets.

Mixture No.	Naphazoline HCl (µg.mL^{-1})	Chlorpheniramine maleate (µg.mL^{-1})	Methylene blue (µg.mL^{-1})
1	15	20	1.5
2	20	10	1
3	5	15	1.5
4	10	20	0.5
5	15	10	0.5
6	5	10	2.5
7	5	30	1
8	25	15	2.5
9	10	30	1.5
10	25	20	1
11	15	15	1
12	10	15	2
13	10	25	2.5
14	20	30	2
15	25	25	1.5
16	20	20	2.5
17	15	30	2.5
18	25	30	0.5
19	25	10	2
20	5	25	0.5
21	20	10	1.5
22	5	20	2
23	15	25	2
24	20	25	1
25	20	15	0.5
26[a]	15	20	1.5
27[a]	10	25	2
28[a]	20	25	1.5
29[a]	20	20	2
30[a]	15	25	1
31[a]	20	15	1
32[a]	10	15	1.5
33[a]	15	15	2

[a] mixtures of validation set

RESULTS

Simultaneous determination of two or more compounds in the same sample using analytical methods that do not require previous chemical separation is always of interest. Spectrophotometric methods and chemometric assisted spectrophotometric methods offer the advantage of determineation of mixtures of compounds without previous treatment steps or separation techniques. The aim of this work was to develop and apply validated spectrophtotmetric and chemometric analytical methods for the determination of NAP, CLO and MB in their ternary mixture that can be further used for their determination either in bulk powder or in their pharmaceutical formulation.

Derivative Spectrophotometric methods

Second derivative (D^2) Spectrophotometric method

The application of derivative spectrophotometry offers greater selectivity if compared with the normal spectrophotometry, as it decreases spectral overlap and allows better resolution (Espinosa Mansilla *et al*., 1993).

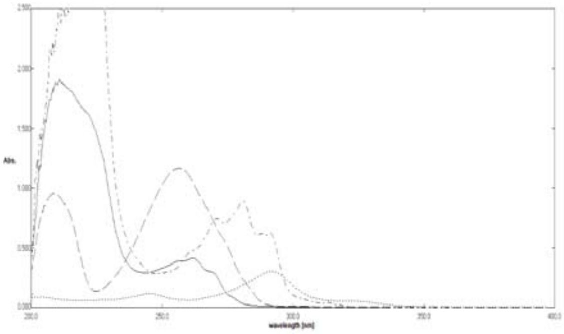

Fig. 2: Zero- order absorption spectra of 30 µg.mL^{-1} of Naphazoline HCl (-.-.-.), 30 µg.mL^{-1} of Chlorpheniramine maleate (—), 2 µg.mL^{-1} Methylene Blue (….) and 10 µg.mL^{-1} Methylparaben (-----) using methanol as a blank.

It is obvious in fig. 2 that there is severe overlapping in the zero-order absorption spectra of NAP, CLO and MB which makes the attempt for direct determination of each drug with the required specificity and accuracy not possible. Different solvents were tested, e.g. methanol, 0.1 N HCl and 0.1 N NaOH. Methanol was chosen as it offered the best spectral resolution by applying D^2 for determination of NAP at 299 nm and MB at 337 nm. Studying different parameters that may affect D^2 spectra was done e.g. different smoothing factor (Δλ) intervals and scaling factor values. The optimum conditions were using 8 nm as Δλ interval and scaling factor 100, respectively for both NAP and MB, as shown in fig. 3.

Linearity was evident for D^2 spectra of NAP at 299 nm in the concentration range 5-80 µg.mL^{-1} and for D^2 spectra of MB at 337 nm in the concentration range 1-15 µg.mL^{-1} from which the following regression equations were computed:

$Y_1 = 0.0325C_1 + 0.0172$, $r_1 = 0.9999$, for NAP at 299 nm
$Y_2 = 0.0106C_2 - 0.0003$, $r_2 = 0.9999$, for MB at 337 where Y_1 and Y_2 are the peak amplitudes at 299 nm and 337 nm for NAP and MB, respectively while C_1 and C_2 are the concentrations in μg mL^{-1} for NAP and MB, respectively and r_1 and r_2 are the corresponding correlation coefficients. Results described in Table 2 show that this method is selective and applicable for the determination of NAP in presence of CLO and MB, and MB in presence of NAP and CLO.

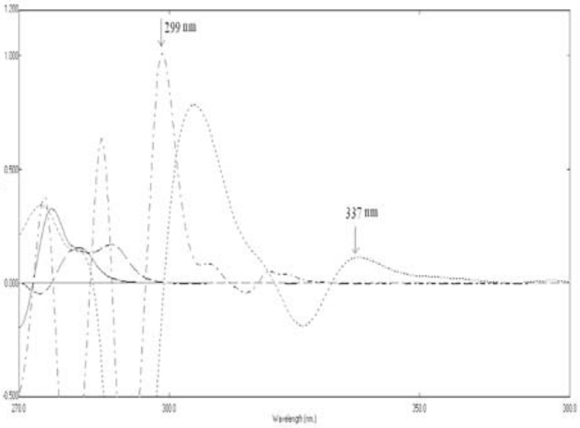

Fig. 3: Second derivative spectra of 30 μg.mL^{-1} Naphazoline HCl (-.-.-.), 30 μg.mL^{-1} Chlorpheniramine maleate(—), 2 μg.mL^{-1} Methylene blue (……) and 10 μg.mL^{-1} Methylparaben (----) using methanol as a solvent.

Second derivative of ratio (DD$^{2)}$ pectrophotometric method

Derivative ratio spectrophotometry represents another method for resolving binary or ternary mixtures without previous separation, which was first introduced by Salinas *et al*. (Salinas *et al*., 1993). First derivative of ratio (DD^1), second derivative of ratio (DD^2), third derivative of ratio (DD^3) and fourth derivative of ratio (DD^4) spectra were obtained, DD^1 method gave a zero crossing for NAP and MB at 276.6 nm for determination of CLO in the presence of NAP and MB, good recoveries were obtained from laboratory prepared mixtures, however the DD^1 method failed to determine CLO in Prisoline blue® eye drops due to interference from methyl paraben. On applying DD^2 method, good recoveries for laboratory prepared mixtures as well as dosage form without interference from methyl paraben were obtained. Therefore, DD^2 method was chosen for determination of CLO in the mixture. In this method, the recorded absorption spectra of NAP, CLO and MB were divided by the spectrum of 30 μg.mL^{-1} of NAP (as a divisor) to get the corresponding ratio spectra. DD^2 spectra of these ratio spectra were obtained, in which it was obvious that CLO could be determined at 276.6 nm (corresponding to negligible absorbance of MB and NAP and zero-crossing of methyl paraben) as shown in fig.4.

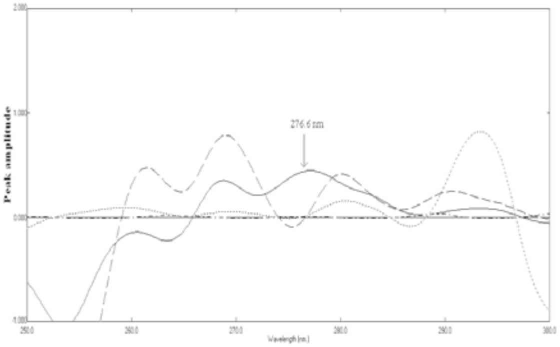

Fig. 4: Second derivative ratio of 30 μg.mL^{-1} Naphazoline HCL (-.-.-.), 30 μg.mL^{-1} Chlorpheniramine maleate (—) 2 μg.mL^{-1} Methylene blue (…….), 10μg.mL^{-1}Methyl-paraben (---) using 30 μg.mL^{-1} Naphazoline HCl as a divisor.

The choice of the proper divisor and its concentration can affect the applicability of the method in terms of signal-to-noise ratio, sensitivity and selectivity. Therefore, the selection of the divisor and its concentration is of great importance. Different concentrations of NAP (10, 15 and 20, 30 μg.mL^{-1}) and MB (2, 4, 6, 8 μg.mL^{-1}) were tried as divisors. The best results were obtained by using the spectrum of 30 μg.mL^{-1} of NAP as a divisor for determination of CLO. Linearity was evident for CLO at 276.6 nm in the concentration range 5–80 μg.mL^{-1} from which the following regression equation was computed:

$Y = 0.0039C + 0.0144$, $r = 0.9999$ where Y is the peak amplitude at 276.6 nm, C is the concentration of CLO in μg.mL^{-1} and r is the correlation coefficient. Results shown in table 2 indicate neither NAP nor MB interferes with the determination of CLO in their laboratory prepared mixtures upon applying the suggested DD^2 method.

Multivariate Calibration Method

Two chemometric approaches, namely; PCR and PLS were applied for the determination of NAP, CLO and MB in their ternary mixture. These multivariate calibrations have the advantage of simultaneous inclusion of many spectral wavelengths instead of single wavelength which greatly improves their precision and predictive ability (Ni and Gong, 1997). The first step involved constructing the calibration matrix for the studied ternary mixture. The calibration set was obtained by using the absorption spectra of a set of 25 mixtures of NAP, CLO and MB containing varying ratios of each compound as shown in table 1. Best results were obtained using 0.1 N HCl as a solvent as it enhances the absorption of CLO in the selected region where there is no interference from methyl paraben as shown in fig. 5. The spectral region above 345 nm and below 289 nm was rejected. To ensure the suitability of the calibration models, the 'leave one out' cross-validation method was used and the rmsecv values of different developed models were compared. Nine factors for PLS and 10 factor for PCR were found suitable.

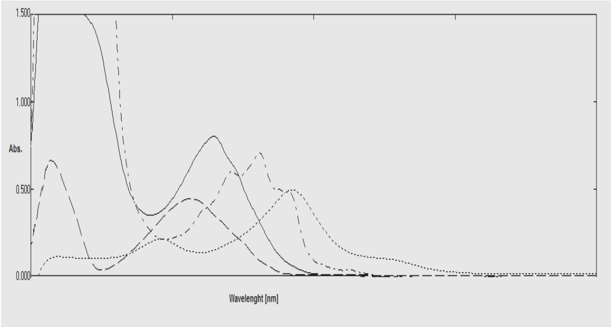

Fig. 5: Zero- order absorption spectra of 30 µg. mL^{-1} of naphazoline HCl (-.-.-.), 30 µg. mL^{-1} of chlorpheniramine maleate (—), 2 µg. mL^{-1} methylene blue (…….) and 10 µg. mL^{-1} methylparaben using 0.1 N HCl as a blank.

The predictive ability of the suggested models were validated by using these models to predict the concentration of NAP, CLO and MB in laboratory-prepared mixtures. Table 1 shows different concentrations of NAP, CLO and MB used in the validation set. Satisfactory results for PLS method were obtained, PCR had a lower prediction ability than PLS as shown in table 3.

For further evaluation of the predictive abilities of the developed models, other diagnostic tools e.g. predicted versus actual concentration plot (model and sample diagnostic); and root mean square error of prediction (RMSEP) (model diagnostic) were calculated and the results are represented in table 4.

DISCUSSION

The proposed methods are proven valid and applicable for the determination of NAP, CLO and MB in their pharmaceutical formulation (Prisoline Blue® eye drops). Furthermore, the accuracy of the proposed methods was further assessed using the standard addition technique that showed accurate results revealing no interference from eye drops excipients as shown in table 5. Method validation was performed according to USP guidelines (The United States Pharmacopeia, 2007). Table 6 shows results of accuracy, repeatability and intermediate precision of the derivative spectrophotometric method.

Statistical comparison of the results obtained by the proposed methods and the reported methods (Kelani, 1998; Korany, 1990) is shown in table 7. There is no significant difference between the proposed methods and the reported methods with respect to accuracy and

Table 2: Determination of Naphazoline HCl, Chlorpheniramine maleate and Methylene blue in laboratory prepared mixtures by the proposed second derivative (D^2) and second derivative ratio (DD^2) methods.

Concentration (µg.mL^{-1})				Derivative method recovery % a		Derivative ratio method recovery % a
Ratio NAP:CLO:MB	NAP	CLO	MB	NAP	MB	CLO
16.6:16.6:1	25	25	1.5	97.71	98.24	99.64
16.6:16.6:1	16.6	16.6	1	98.19	103.23	98.03
5:4:1	25	20	5	98.32	100.56	97.66
6:5:1	12	10	2	99.82	102.45	102.62
5:5:1	10	10	2	98.20	99.86	100.32
5:6:1	10	12	2	97.15	102.03	102.58
Mean ± SD				98.23 ± 0.892	101.06 ± 1.856	100.14 ± 2.144

[a] average of three determinations

Table 3: Recoveries % of Naphazoline HCl ,Chlorpheniramine maleate and Methylene blue in the validation set by PLS method

Mixture No.	Concentration (µg.mL^{-1})			PLS recovery %		
	NAP	CLO	MB	NAP	CLO	MB
1	15	20	1.5	98.67	102.25	102.67
2	10	25	2	99.09	99.80	101.50
3	20	25	1.5	101.12	99.04	97.00
4	20	20	2	100.00	99.04	98.38
5	15	25	1	100.60	101.28	103.25
6	20	15	1	98.59	97.36	100.92
7	10	15	1.5	99.80	98.47	102.36
8	1.5	1.5	2	100.07	101.24	102.08
Mean ±S.D.				99.74± 0.903	99.81 ± 1.654	101.02± 2.203

Table 4: Results of assay validation parameters of the proposed multivariate method for the determination of Naphazoline HCl, Chlorpheniramine maleate and Methylene blue

Validation parameters	NAP	CLO	MB
Mean ±S.D	99.74 ± 0.903	99.81 ± 1.654	101.02± 2.203
RMSEP	0.159	0.329	0.039
Predicted versus actual concentration plot			
a- slope	1.0249	0.9865	0.9913
b- intercept	-0.3909	0.1925	0.0349
c- correlation coefficient (r)	0.9997	0.9995	0.9996

Table 5: Quantitative determination of Naphazoline HCl, Chlorpheniramine maleate and Methylene blue in Prisoline Blue eye drops by the proposed methods and application of standard addition technique.

The proposed method	Prisoline blue eye drops Batch No. (06891)			Standard addition technique					
	Recovery ^{a} % ± S.D.			NAP		CLO		MB	
	NAP	CLO	MB	Pure added	Recovery^{b} %	Pure added	Recovery ^{b}%	Pure added	Recovery^{b} %
Derivative and derivative ratio Spectrophotometric methods Mean ± S.D.	99.37 ± 0.503	103.00 ± 0.014	108.03 ± 0.722	10	99.02	10	102.34	0.5	102.77
				15	101.69	15	100.09	1	102.77
				20	97.79	20	99.80	2	102.67
					99.50 ± 1.991		99.12 ± 2.214		100.20 ± 2.085
Multivariate calibration method Mean ± S.D. PLS	102.57 ± 1.410	99.65 ± 1.311	105.15 ± 2.706	5	103.20	5	102.36	0.5	103.22
				5.5	101.65	5.5	97.54	0.6	103.25
				6	101.42	6	99.83	0.7	102.68
					102.09 ± 1.096		99.91± 2.411		103.05 ± 0.321

^{a}Average of 6 determinations, ^{b}Average of 3 determinations

Table 6. Assay parameters and method validation obtained by applying the proposed derivative spectrophotometric methods, for the determination of Naphazoline HCl, Chlorpheniramine maleate and Methylene blue

Parameters	D^{2} spectrophoto-metric method		DD^{2} spectrophoto-metric method
	MB	NAP	CLO
Range	1-15 µg.mL^{-1}	5-80 µg.mL^{-1}	5-80 µg.mL^{-1}
Linearity			
Slope	0.0106	0.0325	0.0039
Intercept	- 0.0003	0.0172	0.0144
Correlation coefficient	0.9999	0.9999	0.9999
Accuracy Mean ± S.D.	99.84 ± 1.481	100.37 ± 1.235	100.78 ± 0.687
Specificity	101.06 ± 1.856	98.23 ± 1.892	100.14 ± 2.144
Precision (R.S.D. %)			
Repeatability^{a}	0.260	0.451	0.521
Intermediate Precision^{b}	0.692	0.498	0.666

^{a}The intra-day precision (n = 3), average of three different concentrations repeated three times within day.
^{b}The inter-day precision (n = 3), average of three different concentrations repeated three times in three successive days

precision as the calculated t and F values are less than the theoretical ones.

CONCLUSION

In this work, different selective analytical techniques were developed for the determination of NAP, CLO and MB in their ternary mixture either in bulk powder or in pharmaceutical formulation. The spectrophotometric methods can be regarded as an alternative to chromatographic techniques especially in the routine quality control analysis of pharmaceutical formulations, allowing qualitative and quantitative measurements to be simultaneous and rapid at relatively low costs.

Table 7: Statistical comparison of the results obtained by the proposed methods and the reported methods

Item	MB			NAP			CLO		
	D^2 method	PLS method	Reported method [14]	D^2 method	PLS method	Reported method [6]	DD^2 method	PLS method	Reported Method [6]
Mean	99.84	101.02	99.91	100.37	99.74	100.11	100.78	99.81	99.88
S.D.	1.481	2.203	1.439	1.235	0.903	0.739	0.687	1.654	1.459
R.S.D %	1.484	2.181	1.440	1.230	0.905	0.738	0.682	1.657	1.461
n	8	8	6	9	8	6	10	8	6
Variance	2.194	4.853	2.071	1.525	0.815	0.545	0.472	2.736	2.128
Studdent's t-test	0.076 (2.262)[a]	0.989 (2.201)[a]	—	1.802 (2.145)[a]	1.288 (2.131)[a]	—	1.483 (2.160)[a]	0.081 (2.179)[a]	—
F-value	1.237 (6.256)[b]	2.345 (6.094)[b]	—	1.942 (4.147)[b]	1.334 (3.500)[b]	—	2.576 (3.687)[b]	1.070 (4.876)[b]	—

[a]Figures between parentheses represent the corresponding tabulated values of t at $P = 0.05$.
[b]Figures between parentheses represent the corresponding tabulated values of t at $P = 0.05$.

Chemometric methods allow simple, selective, accurate, and economical simultaneous determination of NAP, CLO and MB in their ternary mixture as the simultaneous inclusion of many spectral wavelengths, instead of a single wavelength, greatly improve the precision and predictive ability of these multivariate calibrations. The proposed methods offer the distinct possibility of assaying NAP, CLO and MB in their pharmaceutical formulations without interference due to co-formulated excipients.

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