Original Article

Comparison of Mean Centering of Ratio Spectra Based Spectrophotometric Approach and HPLC Method for Quantitative Determination of Pirenoxine in the Presence of Methylparaben and Propylparaben

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ABSTRACT

Objective: The mean centering of ratio spectra method (MCR) was developed for determination of pirenoxine in the presence of methylparaben and propylparaben. **Background:** The UV spectrum of pirenoxine was suffered from spectra overlapping of methylparaben and propylparaben, the preservatives used in the eye drop formulation. Since, MCR method was introduced to overcome this limitation. **Methods:** The developed MCR method was performed using 39 synthetic mixtures of pirenoxine, methylparaben and propylparaben. The amplitudes at 320 nm of the second ratio spectra were used to construct a calibration model for pirenoxine. Performance characteristics of the method such as linearity, accuracy and precision, were calculated. A high-performance liquid chromatography (HPLC) method was also developed and validated. Then, two methods were used to determine a set of commercial eye drop samples for comparison. **Results:** The developed and validated MCR method was simple, rapid, accurate, and precise and could be applied to determine pirenoxine in eye drop samples. Measurement of pirenoxine in eye drop samples by MCR and HPLC methods were not significantly different (*P*-value = 0.21).

Key words: Pirenoxine, Mean centering of ratio spectra, HPLC.

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INTRODUCTION

Pirenoxine (PRN) is an active ingredient in eye drop preparations that is used for treatment of cataract (Figure 1).[1] Cataract is a clouding of the lens in the eye leading to a decrease in vision. Three types of crystalline, α, β and γ are the major lens proteins. Denature and degradation of these proteins from eye inflammation, UV radiations, drug-induced side effects, age, gender and genetic factors cause clouding of the lens and cataract development. Among many risk factors associated with cataract formation, selenite and calcium have been demonstrated to cause lens crystalline aggregation.[2,3,4] Mechanism of action of pirenoxine for cataract treatment is protection of crystalline from UV-C, selenite-, and calcium-induced lens protein turbidity.

Pirenoxine is commercially available in eye drop preparations that contain not only the

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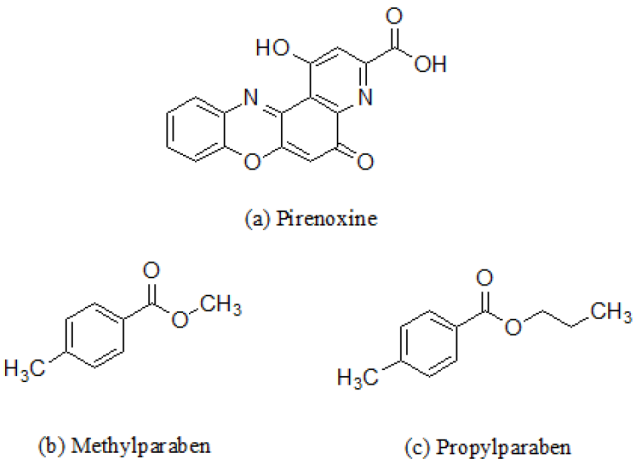

Figure 1: Chemical structures of pirenoxine (a), methylparaben (b) and propylparaben (c).

active ingredient, but also some preservatives. Methylparaben (MP) and propylparaben (PP) (Figure 1) are commonly used in eye drop preparation as preservatives.[5,6,7,8] Parabens have a broad spectrum of antimicrobial activity of bacteria, yeasts and molds. The single use of either methylparaben or propylparaben in eye drop preparation is not recommended due to eye irritation side effect.[5,6,7,9] Combination of parabens in pharmaceutical preparation gives synergistic effects as preservatives to achieve a maximal efficacy.[10]

Pirenoxine determination have been done by potentiometric titration,[11] and noninvasive dual-λ iris imaging technique.[12] In general, high-performance liquid chromatography (HPLC) is commonly used for determining active and excipient compounds in pharmaceutical preparations due to its high accuracy and sensitivity, time consuming can be a weakness. Spectrophotometric method is simple, rapid and allows high sample throughput, but the direct determination of multi-component samples can be problematic due to overlapping spectra. Several advanced spectrophotomeric approaches such as derivative spectrometry, partial least squares regression (PLSR), principle component regression (PCR), multiple linear regression (MLR), and mean centering of ratio spectra (MCR) have been applied to overcome this limitation. For these spectrophotometric based methods, MCR is an interesting one especially it can be performed on widely available Microsoft Excel®.

In this research, a new spectrophotometric method, mean centering of ratio spectra (MCR) was attempted to assay pirenoxine in the presence of methylparaben and propylparaben. A high-performance liquid chromatography (HPLC) based on C8 stationary phase was also developed and validated in comparison with MCR method.

MATERIAL AND METHODS

Instrumentation

HPLC analysis was performed on a HPLC system (Thermo separation, Waltham, USA) equipped with a P4000 pump, a AS3000 auto injector, a UV2000 UV detector, and a Colibrick® communication bus module. Data acquisition, analysis, and report were demonstrated by Clarity® (Data Apex, Prague, Czech Republic) chromatography software. The analytical column was Symmetry® C8, 4.6 × 150 mm, 5 µm (Waters, Wexford, Ireland). Separations were carried out using an isocratic system with a flow rate of 1.0 ml/min and the analytical column was set at room temperature. UV spectrophotometry was carried out by a double-beam UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a pair of 1 cm quartz cells. The spectral bandwidth was 1 nm over the wavelength range of 200-400 nm with a medium scanning speed.

Chemicals and Reagents

Standard pirenoxine sodium was purchased from Hangzhou Dayang Chem., China. Standard, methylparaben and propylparaben were kindly donated from the Government Pharmaceutical Organization (GPO), Bangkok, Thailand. Samples of ophthalmic solution were obtained from Senju Pharmaceutical, Osaka, Japan. Analytical grade of tetrabutylammonium hydroxide (TBAH) and orthophosphoric acid were purchased from Carlo Erba, Milan, Italy. Meth

Preparation of standard solutions

For HPLC method, stock standard solutions of pirenoxine sodium, methylparaben and propylparaben were separately prepared by dissolving accurately weighed amount of each standard in 25% acetonitrile. Standard mixture solutions were prepared by mixing each stock standard solution and diluted with 25% acetonitrile to achieve the desired concentrations. For MCR method, stock standard solution of pirenoxine sodium, methylparaben and propylparaben were prepared by dissolving accurately weighed amounts of each compound in 50 % methanol. Mixture standard solutions of each compound were prepared by diluting the stock standard solution with 50 % methanol to obtain an appropriate concentration.

MCR modeling

An absorbance value of the eye drops sample at a certain wavelength are contributed from PRN, PP and MP (Eq. (1)) since spectra of the three compounds are overlap in the UV region. Mean centering of ratio spectra for the three components composes of four steps. First, Eq. (1) is divided by the absorptivity coefficient of MP

resulting in Eq. (2). Then, Eq. (2) is mean centered to obtain Eq. (3), by this way, term of MP is deleted because mean centering of a constant is zero.[13,14] Next, Eq. (3) is divided by the mean center of the ratio of absorptivity coefficient of PP and MP to obtain Eq. (4). Finally, Eq. (4) is mean centered again to delete PP term. The new relation between concentration of pirenoxine and mean centered of the second ratio spectra is illustrated in Eq. (5). The calibration curve for pirenoxine can be constructed between the mean centering of second ratio spectra (*MC (D)*, *y*-axis) and concentration of PRN (*cPRN*, *x*-axis).

$$A_m = a_{PRN} b c_{PRN} + a_{PP} b c_{PP} + a_{MP} b c_{MP} \quad \text{Eq-1}$$

$$B = \frac{A_m}{a_{MP}} = \frac{a_{PRN} c_{PRN}}{a_{MP}} + \frac{a_{PP} c_{PP}}{a_{MP}} + c_{MP} \quad \text{Eq-2}$$

$$MC(B) = MC\left[\frac{a_{PRN}\ c_{PRN}}{a_{MP}}\right] + MC\left[\frac{a_{PP}\ c_{PP}}{a_{MP}}\right] \quad \text{Eq-3}$$

$$D = \frac{MC(B)}{MC(a_{PP} / a_{MP})} = \frac{MC(a_{PRN} c_{PRN} / a_{MP})}{MC(a_{PP} / a_{MP})} + c_{PP} \quad \text{Eq-4}$$

$$MC(D) = MC\,\frac{MC(a_{PRN} c_{PRN} / a_{MP})}{MC(a_{PP} / a_{MP})} \quad \text{Eq-4}$$

Where "A_m" is the absorbance at a certain wavelength, "a" is absorptivity coefficient, "b" is path length and equal 1 for a 1 cm quartz cells and "c" is concentration. A number of 39 synthetic mixtures [Supporting document 1] was contributed in MCR model construction. The absorption data of this set were recorded and subjected to Microsoft Excel® for MCR model construction.

Validation of MCR model

The final MCR model was validated by determination of 12 samples in the test set, which were not contributed in model building. Compositions of test set samples were shown in Table 1. The model was tested for linearity, recovery and precision according to USP 39 and ICH Q2 (R1) guidelines.[15,18] Linearity was checked by plotting the amplitude of spectra at 320 nm of the second ratio spectra of pirenoxine (*y*-axis) versus the concentrations 1.0-10.1 µg/ml (*x*-axis). Linearity was expressed by the square of the correlation coefficient (r^2) of the plot. Recovery of the method was performed by determination of five synthetic mixtures of pirenoxine combined with methylparaben and propylparaben. The percent recovery between amount found and the amount added was used to display recovery of the method. For precision, the same experiment as recovery was repeated for another two consecutive days. The precision of the method was expressed in term of percent relative standard deviation (% RSD).

Development of HPLC method

An ion-pair reversed-phase HPLC method was developed for simultaneous determination of pirenoxine sodium, methylparaben and propylaraben in eye drop preparations. Standard mixture containing of each compound was used for method development. Symmetry® C8 (4.6 × 150 mm, 5µm) column was used as stationary phase. Mobile phase conditions such as types and concentrations of ion-pairing agents, concentrations of organic modifier, and pH of mobile phase were studied to obtain an optimum condition. System suitability parameters such as tailing factor, the number of theoretical plates, and resolution of interested compounds were evaluated to justify an optimum chromatographic condition.[15]

Table 1: Recovery data of test set samples by the developed MCR method.

Sample	Added Conc. (µg/ml)	Found Conc. (µg/ml)	% Recovery
T01	3.23	3.17	98.0
T02	4.34	4.38	101.0
T03	4.85	4.71	97.2
T04	5.56	5.65	101.7
T05	5.76	5.63	97.8
T06	6.46	6.60	102.1
T07	7.58	7.56	99.8
T08	7.88	7.93	100.7
T09	8.28	8.12	98.0
T10	9.39	9.48	101.0
T11	9.49	9.73	102.5
T12	9.49	9.53	100.3

Validation of HPLC method

The developed HPLC method was validated to demonstrate that the performance characteristics of analytical method meet the requirements for the intended purpose.[16,17] Validation of the developed HPLC method was performed by evaluating the analytical performance characteristics such as linearity, range, accuracy, intra-day and inter-day precision and specificity according to USP 39 and ICH Q2(R1) guidelines.[15,18] All solutions used in validation assay were injected into the HPLC system with the optimized chromatographic condition in three replicates.

Assay of eye drop samples

Eleven commercial samples were assayed for pirenoxine contents by using the developed and validated MCR and HPLC methods. For HPLC, eye drop sample solutions were prepared by transferring 2.0 ml of the sample into a 10.0 ml volumetric flask and diluted with 25% acetonitrile to the volume. Standard mixture and sample solutions were filtered using 0.22 µm nylon syringe filter before injecting into the HPLC instrument. For MCR method, 29 mixtures in the calibration set were utilized. The mean centered values of the second ratio spectra at 320 nm for PRN in these mixtures were measured and plotted against the corresponding concentrations to construct the calibration graph. The regression equation was used to determine PRN in eye drop samples. Sample solutions were carried out by transferring 0.5 ml of eye drop solutions into 10 ml volumetric flask and adjusted with 50 % methanol to volume.

RESULTS AND DISCUSSION

Development of MCR models

The developed MCR method depends on the mean centering of ratio spectra and derivative steps can be avoided. Therefore, signal-to-noise ratios are enhanced and the technique has been applied for resolving binary and ternary mixtures in complex samples with unknown matrices.[13,14] The mathematical explanation of the method was illustrated by Afkhami and Bahram.[13,14] A number of 39 synthetic mixtures [Supporting document 1] were used to construct the MCR model. Only the absorption data of mixtures 1-29 were used in the model. Solutions number 30-34 and 35-39 contained only MP and PP, respectively and were used for testing effects of divisor. In order to optimize the developed MCR method, the effects of divisor concentrations on the selectivity of the method were tested. Different concentrations each of PRN (1, 5, and 10 µg/ml) were challenged. It was found that the divisor had a great effect on the selectivity of determination of PRN where reproducible and good results were obtained upon using 18 µg/ml of MP and 7 µg/ml of PP as divisors. By following the calculation steps of Eq.1-Eq.5 as described in material and methods section, mean centering of the first and second ratio spectra plots of pirenoxine could be performed as showed in Figure 2 (a) and (b), respectively. As seen in Figure 2(b), the plot of mean centering of the second ratio spectra presented the maximum amplitude at 320 nm. A calibration curve of the mean centered values at 320 nm against PRN concentrations of PRN was constructed and obtained regression equation ($y = 0.426x - 0.084$, $r^2 = 0.9977$).

Method validation of MCR method

The developed MCR method was validated according to USP 39 and ICH Q2 (R1) guidelines [15,18] to ensure its suitability. Validation studies were performed by determination the test set samples, which were not contributed in the calibration step and performance parameters

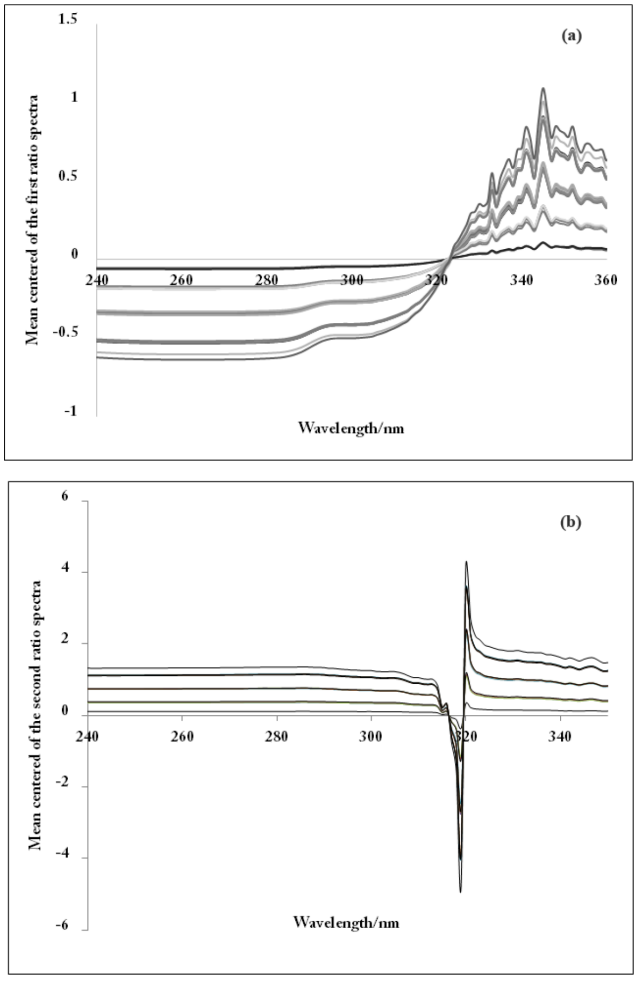

Figure 2: The first ratio spectra of pirenoxine (a), and the second ratio spectra of pirenoxine (b).

Table 2: Summary of validation results of the developed MCR and HPLC methods.

Parameters	MCR method	HPLC method
Linearity		
Range	1.0-10.1 μg/ml	1.0-20.0 μg/ml
Slope	0.422	26.77
Intercept	-0.077	21.85
r^2	0.9976	0.9963
Accuracy (% Recovery, n = 3)	97.3-106.8%	99.8-102.0%
Precision		
Intra-day precision (% RSD, n = 3)	0.02 – 0.51 %	0.27 – 1.87 %
Inter-day precision (% RSD, n = 9)	1.57 – 8.34 %	0.74 - 1.56 %

for validation such as specificity, accuracy, precision, linearity and range were investigated.

Results of the test set samples, expressed as the percentage recovery between amount drug found by the method and amount drug added were shown in Table 1. Good recoveries with low %RSD were obtained for every mixture in the set. These results implied that the developed MCR method had specificity to PRN and was able to assay the new samples containing different ratios of PRN and parabens.

Accuracy and precision were performed by using 5 mixtures of pirenoxine in the presence of methylparaben and propylparaben (Table 2). Percent recovery or the percent of ratios between amounts found and true concentrations was used to express accuracy of the method and relative standard deviation (%RSD) was used to confirm the method precision. Accuracy and precision of the method were confirmed by good percentage recoveries with low % RSD of pirenoxine in different synthetic mixtures (Table 2). Moreover, the specificity of the method was confirmed since it could determine pirenoxine in mixtures in the presence of methylparaben and propylparaben.

Linearity and range of the method was obtained by using linear least square regression. The plot of pirenoxine concentrations (*x*-axis) and their corresponding amplitudes at 320 nm of the second ratio spectra (*y*-axis) was constructed. The method showed acceptable linearity between pirenoxine concentrations of 1.0-10.1 μg/ml ($r^2 > 0.99$) (Table 2).

Development of HPLC method

Standard mixture of pirenoxine sodium 10 μg/ml, methylparaben 10 μg/ml and propylparaben 10 μg/ml were used to optimize HPLC conditions. Method optimization was attempted by varying mobile phase pH between 2 to 7, concentration of organic solvent (acetonitrile, 35%-50%), type and concentration of ion-pairing agent (octane sulfonic acid sodium salt and tetrabutylammonium hydroxide (TBAH), 1%-10%). Finally, the suitable condition was obtained using 5% TBAH in DI water, pH 6.0 and acetonitrile (50:50, v/v) as mobile phase and Symmetry® C8 (4.6 × 150 mm, 5μm) column as stationary phase. Under the optimized condition, a baseline resolution of the three compounds was achieved in less than six min. The retention time of pirenoxine sodium, methylparaben and propylparaben were 2.1, 2.8, and 4.6 min, respectively. A typical chromatogram of pirenoxine in the presence of methylparaben and propylparaben at optimized chromatographic conditions is shown in Figure 3.

Validation of HPLC method

Validation of the developed HPLC method was performed by evaluating analytical parameters such as linearity and range, accuracy, intra-day and inter-day precision and specificity according to USP 39 and ICH Q2 (R1) guidelines.[15,18]

Linearity for HPLC method was constructed using seven concentrations range from 1.0 to 20.0 μg/ml for pirenoxine sodium. The linearity was demonstrated by

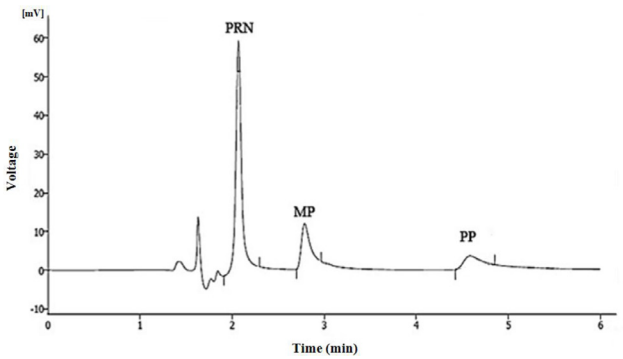

Figure 3: A typical chromatogram of a standard mixture of PRN, MP and PP under the optimized condition. Column, Symmetry® C8; mobile phase, 5% TBAH in DI water, pH 6.0 and acetonitrile (50:50, v/v). PRN 10 μg/ml, MP 10 μg/ml, PP 10 μg/ml.

Table 3: Assay data of pirenoxine in eye drop samples obtained from HPLC and MCR methods.

Sample number	Actual value (µg/ml)	HPLC (µg/ml)	MCR (µg/ml)
1	56.67	54.56	56.50
2	56.67	56.68	56.54
3	56.67	56.18	56.64
4	56.67	56.46	56.59
5	56.67	56.83	58.80
6	56.67	54.51	58.65
7	56.67	54.87	55.66
8	56.67	57.09	55.82
9	56.67	54.98	56.20
10	56.67	54.10	54.44
11	56.67	56.38	54.38
Average	56.67	55.69	56.38
SD		1.09	1.41
% RSD (n = 11)		1.96	2.49

least-squares linear regression. The linearity of calibration graphs between concentrations and peak areas was repeated three times and results showed acceptable with r^2 of 0.9963.

Accuracy was performed by a standard addition method using pirenoxine eye drop preparations containing methylparaben and propylparaben. Three concentration levels of spiked standards mixture at 80%, 100% and 120% concentration levels were evaluated. Three replicate determinations were performed for each concentration level. The accuracy of the method was expressed as percent recovery between the amount of the standard added and the standard found from each determination. Results showed that the mean recovery of pirenoxine sodium was between 99.8 and 102.0 %. The accuracy results were acceptable and complied with accuracy criteria for assay of pharmaceutical products.[16]

Intra-day and inter-day precision of developed HPLC method for pirenoxine sodium were performed and evaluated. The studies were performed at three different concentration levels and the results were expressed as percentage of the relative standard deviation (% RSD). Results demonstrated a good reproducibility with % RSD between 0.27 and 1.87% for intra-day precision and between 0.74 and 1.56% for inter-day precision. These results were less than 2.00% and met a satisfactory precision for pharmaceutical analysis.[17]

Specificity of the HPLC method was evaluated from chromatograms of unspiked and spiked eye drop samples and standard mixture of pirenoxine sodium, methylparaben and propylparaben. Specificity was performed to prove that the method has an ability to determine the interested compound unequivocally in the presence of other expected components such as preservatives and excipients in pharmaceutical preparations.[17] It was found that there was no interference peak presented at the same retention time of the desired compounds. Summary of validation results of the developed HPLC method was in Table 2.

Assay of eye drop samples

Eleven commercial eye drop samples in the market were enrolled in the study. The sample was twenty times diluted with deionized water before the measurement of pirenoxine content. As displayed in Table 3, the drug contents obtained from HPLC and MCR methods were closed to actual value. In addition, the concentrations from HPLC and MCR methods were not significant different (*P*-value = 0.21) at 95% confidence limit.

CONCLUSION

In summary, the mean centering of ratio spectra (MCR) and HPLC methods for quantitation of pirenoxine in eye drop preparation were successfully developed and validated. Under the optimum HPLC condition, pirenoxine sodium, methylparaben and propylparaben were well separated within six min. The developed MCR method was simple, accurate and precise. The other advantages of this method could be listed, including only spectra data were required, data analysis could be performed by Microsoft Excel® that was widely used, high sample throughput and less time consuming. Applications of the developed MCR method was also illustrated by quantitative determination of pirenoxine in commercial eye drop samples. The determination results of pirenoxine obtained from HPLC and MCR methods were statistically compared.

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CONFLICT OF INTERSET

The authors declare no conflict of interest.

ABBREVIATIONS USED

MCR: Mean centering of ratio spectra; **HPLC:** High-performance liquid chromatography; **UV:** Ultraviolet; **UV-C:** Ultraviolet-C; **PRN:** Pirenoxine; **MP:** Methylparaben; **PP:** Propylparaben; **PLSR:** Partial least squares regression; **PCR:** Principle component regression; **MLR:** Multiple linear regression; **C8:** Octlysilane stationary phase; **GPO:** The Government Pharmaceutical Organization, Thailand; **TBAH:** Tetrabutylammonium hydroxide; **USP:** The United State Pharmacopeia; **ICH:** International Conference on Harmonization; **µg:** Microgram; **µm:** Micrometer; **pH:** Potential hydrogen; **mm:** Millimeter; **nm:** Nanometer; **ml:** Milliliter; r^2**:** The square of correlation coefficient; **% RSD:** Percentage of the relative standard deviation; **DI:** Deionized.

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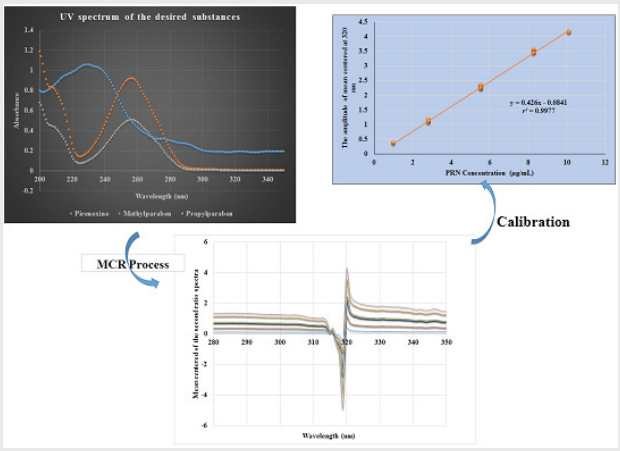
Supporting document: Composition of calibration set and test set solutions.							
Sample	**PRN (µg/ml)**	**MP (µg/ml)**	**PP (µg/ml)**	**Sample**	**PRN (µg/ml)**	**MP (µg/ml)**	**PP (µg/ml)**
C1	1.01	13.61	5.56	T01	3.23	5.24	5.25
C2	10.10	13.61	5.56	T02	4.34	8.47	4.44
C3	5.56	5.04	5.56	T03	4.85	7.66	7.68
C4	5.56	22.18	5.56	T04	5.56	18.35	7.58
C5	5.56	13.61	3.03	T05	5.76	18.65	7.27
C6	5.56	13.61	8.08	T06	6.46	15.52	3.84
C7	2.83	8.57	4.04	T07	7.58	17.34	5.86
C8	2.83	8.57	7.07	T08	7.88	15.93	4.65
C9	2.83	18.65	4.04	T09	8.28	14.92	5.25
C10	2.83	18.65	7.07	T10	9.39	12.50	7.27
C11	8.28	8.57	4.04	T11	9.49	5.75	6.46
C12	8.28	8.57	7.07	T12	9.49	11.59	7.88
C13	8.28	18.65	4.04				
C14	8.28	18.65	7.07				
C15	5.56	13.61	5.56				
C16	5.56	13.61	5.56				
C17	5.56	13.61	5.56				
C18	5.56	13.61	5.56				
C19	5.56	13.61	5.56				
C20	5.56	13.61	5.56				
C21	5.56	13.61	5.56				
C22	5.56	13.61	5.56				
C23	5.56	13.61	5.56				
C24	5.56	13.61	5.56				
C25	1.01	0.00	0.00				
C26	2.83	0.00	0.00				
C27	5.56	0.00	0.00				
C28	8.28	0.00	0.00				
C29	10.10	0.00	0.00				
C30	0.00	5.04	0.00				
C31	0.00	8.57	0.00				
C32	0.00	13.61	0.00				
C33	0.00	18.65	0.00				
C34	0.00	22.18	0.00				
C35	0.00	0.00	3.03				
C36	0.00	0.00	4.04				
C37	0.00	0.00	5.56				
C38	0.00	0.00	7.07				
C39	0.00	0.00	8.08				

PICTORIAL ABSTRACT



SUMMARY

The mean centering of ratio spectra method (MCR) was developed for determination of pirenoxine in the presence of methylparaben and propylparaben. The assay could be performed without prior separation of pirenoxine from excipients or interferences. The amplitudes at 320 nm of the second ratio spectra were used to construct a calibration model for pirenoxine. Performance characteristics of the method such as linearity, accuracy and precision, were calculated. Results showed that the proposed method was simple, rapid, accurate, and precise and could be applied to determine pirenoxine in eye drop samples. The same samples were also determined by a developed and validated high-performance liquid chromatography (HPLC) method for comparison. Measurement of pirenoxine in eye drop samples by MCR and HPLC methods were not significantly different (*P*-value = 0.21).

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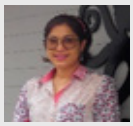

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