APPLICATION OF CHEMOMETRIC METHODS FOR THE DETERMINATION OF CIPROFLOXACIN HYDROCHLORIDE AND PHENAZOPYRIDINE HYDROCHLORIDE IN THEIR PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
For simultaneous analysis of Ciprofloxacin Hydrochloride (CIP), phenazopyridine Hydrochloride (PHE), in its drug-dose type, chemometric-assisted UV spectrophotometric methods have been established and validated. The chemometric methods used were partial least square (PLS) regression, principal component regression (PCR) and classical least square (CLS) models. Two sets of standard mixtures, calibration sets and validation sets have been prepared. All three models have been optimized to quantify each drug in the mixture using the information included in the UV absorption spectra of the appropriate solution in the range of 205-515 nm with the range of 5 nm. Optimized models have been successfully applied to the simultaneous estimation of these drugs in synthetic mixture and pharmaceutical dosage form. The methods were validated in linearity, precision, sensitivity, specificity and robustness in the range of 5-25μg / ml for CIP and 4-20μg / ml for PHE for spectrophotometric-assisted UV chemometrics as per the ICH guideline. The PLS, PCR, CLS methods developed were used to determine CIP and PHE in tablets.

Keywords: Ciprofloxacin Hydrochloride (CIP), Phenazopyridine Hydrochloride (PHE), Chemometrics, UV Spectrophotometric.

INTRODUCTION
Ciprofloxacin Hydrochloride (CIP), chemically 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid monohydrochloride monohydrate, is a fluoroquinolone class and the structure is shown in Fig.-1. CIP is official in Indian Pharmacopoeia (Indian Pharmacopoeia 2010), British Pharmacopoeia (British Pharmacopoeia 2004), and United State Pharmacopoeia (United State Pharmacopeia 2012). It is a broad-spectrum anti-infective agent that is used in the treatment of different pathological conditions like bacterial conjunctivitis, corneal ulcer, bacterial infection of the respiratory tract, urinary tract infection. The literature survey revealed that there are several analytical methods reported for CIP either individually such as spectrophotometric method1, RP-HPLC2 or in combination with other drugs by spectrophotometric method3-4 and RP-HPLC5. Phenazopyridine Hydrochloride (PHE) is chemically 3-(phenylazo)-2, 6-pyridine diamine monohydrochloride and the structure is shown in Figure 1(b). It is broadly used as urinary tract mucosal anesthesia or analgesia for relief in pain, burning and other discomfort resulting from irritation of the lower urinary tract mucosa caused by infection, trauma conditions. PHE is official in United States Pharmacopoeia. The literature survey revealed that there are several analytical methods have been employed for the quantification of PHE either individually such as HPLC in human plasma6 and force acid and heat degradation by HPLC7 or in combination with other drugs by chemometrics8, Spectrophotometric and HPLC9. The combination of CIP and PHE is used for the treatment of urinary tract infection. The combination of CIP and PHE has been commercially available in the tablet dosage form.

CIP and PHE analysis in combination can not be carried out without separation by direct UV spectrophotometry, because their UV spectrum is overlapping. For the study of drug substances by
multicomponent using the spectrophotometric technique, chemometrics approach, multivariate calibration method, is applied. The Partial Least Square (PLS)\textsuperscript{10}, Principal Component Regression (PCR) and Classical Least Square (CLS) techniques are full-spectrum methods\textsuperscript{11}, more effective than those based on single or dual wavelength measurements, such as direct spectrophotometry, simultaneous equation or absorption ratio method, since the simultaneous inclusion of multiple spectral intensities can greatly enhance performance.\textsuperscript{12-14}

![Chemical Structure](image)

Fig.-1: Chemical Structure of (a) Ciprofloxacin Hydrochloride and (b) Phenazopyridine Hydrochloride

This study aims to implement a spectrophotometric chemometric approach for the CIP and PHE analysis in tablets. The tablet sample was tested using the spectrophotometric approach optimized by chemometrics. Also, this work is the first application of multivariate calibration methods, principle component regression method (PCR) and partial least square regression (PLS-1), Classical least square (CLS) for quantifying CIP and PHE combination on the tablet.

**EXPERIMENTAL**

**Material and Methods**

A pharmaceutically pure CIP sample was acquired as a free sample from Zydus Cadila Healthcare Ltd., Ahmedabad and PHE from Sris Pharmaceuticals, Hyderabad. Methanol was purchased from Loba chemicals, Mumbai, India. Glass distillation assembly of Durga scientific, Vadodara was used to prepare triple distilled water. Marketed formulation UTIStat® containing 250 mg of CIP and 200 mg of PHE procured from local market.

**Instrumentation and Software**

Shimadzu UV-1800, UV-Visible double beam spectrophotometer with matching 1 cm quartz cuvette (Shimadzu Corporation, Kyoto, Japan) was used to record UV spectra of solutions. The width of the spectral band is 0.5 nm. Unscrambler ® and MICROSOFT EXCEL were used for PCR and PLS model development and data analysis, and Matlab ® and MICROSOFT EXEL were used for CLS model development and data analysis.

**General Procedure**

**Preparation of Standard Solutions**

The CIP powder (100 mg) has been precisely measured into a 100mL flask. It was dissolved and diluted to 100mL with methanol to obtain a CIP stock solution with a final concentration of 1mg / mL, while PHE powder (100 mg) was carefully weighed and transferred to a 100mL volumetric flask. It was dissolved and diluted to 100mL with methanol to obtain a PHE stock solution with a final concentration of 100μg / mL.

**Preparation of Working Standard Solutions**

The standard CIP (10mL) solution has been transferred to the 100mL volumetric flask and diluted with 100 mL methanol for the preparation of the working standard solution of CIP with the final 100μg / mL concentration.

The standard PHE solution (10mL) has been transferred with methanol into a 100mL volumetric bottle and diluted with methanol to achieve the standard working PHE solution with a final concentration of 100μg / mL.
Calibration of PLS, PCR, CLS Methods

One Component Calibration

Calibration of one variable was performed to find the dynamic linear concentration range of each drug. The range of 5-25 μg / ml for CIP and 4-20 μg / ml for PHE has been studied for Linear Dynamic Ranges. Absorption values in a 1 cm quartz cell against methanol as blank were recorded as λmax (281 nm CIP and 414 nm PHE). For each compound, the linear dynamic range was calculated by the least-square linear concentration regression and the resulting absorption.

Binary Standard Solutions

Two sets of standard solutions were planned calibration set and validation set. Fourteen calibration specifications and eight testing level mixtures were prepared by combining appropriate quantities of CIP and PHE working standard solutions and dilution to methanol quantities. This demonstrated the combination of CIP and PHE in Table-1. The spectra of absorption of prepared solutions were measured at an interval of 5 nm from 205-505 nm. Calibration collection absorbance data were subjected to Unscambler ® for PLS, PCR and Matlab ® for CLS models. The concentration of CIP and PHE in the validation set was predicted with the use of proposed PLS, PCR and CLS models to test these PLS, PCR and CLS models.

Table-1: Composition of the Calibration Set and Validation Set

| Std. No. | CIP (μg/ml) | PHE (μg/ml) |
|----------|-------------|-------------|
| 1C       | 5           | 12          |
| 2C       | 5           | 16          |
| 3C       | 5           | 24          |
| 4C       | 10          | 4           |
| 5C       | 10          | 8           |
| 6C       | 10          | 24          |
| 7C       | 15          | 4           |
| 8C       | 20          | 8           |
| 9C       | 20          | 12          |
| 10C      | 20          | 16          |
| 11C      | 20          | 20          |
| 12C      | 20          | 24          |
| 13C      | 25          | 20          |
| 14C      | 25          | 24          |
| 1V       | 10          | 20          |
| 2V       | 15          | 8           |
| 3V       | 15          | 16          |
| 4V       | 15          | 20          |
| 5V       | 15          | 24          |
| 6V       | 20          | 4           |
| 7V       | 20          | 8           |
| 8V       | 25          | 16          |

*c = Solution of calibration set, v = Solution of validation set

Analysis of marketed formulation

Twenty tablets were measured and finely powdered. Tablet powder equal to 100 mg Ciprofloxacin hydrochloride was correctly weighed and transferred to 100mL volumetric flask and added 50mL of methanol. Sonicated for 20 min, the mixture was diluted with methanol (Solution A) to the mark and filtered through Whatman filter paper No. 41, 10 ml aliquot was separated from this Solution A into 100
ml flask and diluted with methanol (Solution B). From this Solution B, 0.5 ml aliquot was inserted into 10 ml volumetric flask and diluted as the final test solution using methanol for chemometrics (Solution C had 5μg / ml CIP and 4μg / ml PHE). Concentration CIP and PHE were identified using established calibration model PCR, PLS and CLS for chemometrics.

**Detection Method**

**Chemometrics Methods**

CIP and PHE chemical structures are shown in Fig.-1 whereas Fig.-2 Shows UV spectra and combination of medications. As this figure indicates, they overlap. Such drugs' spectral similarity prohibits mixture resolution through direct spectrophotometric measurements.

![Fig.-2: Overlain Spectra of CIP, PHE and their Mixture One Component Calibration](image)

To find each component's linear dynamic range, calibration graphs were obtained. Absorption spectra were reported over 200-600 nm against a blank solvent. For each compound, the linear range was calculated by plotting the absorbance at its $\lambda_{\text{max}}$ (CIP, 281 nm and PHE, 414 nm) versus sample concentration. Calibration curves were linear between 5.0-25 μg / ml CIP and 4.0-20.0μg / ml PHE. Characteristic parameters for individual calibration regression equations are shown in Table-2.

| Comp. | Regression Equation | $r^2$ | SD of slope | SD of intercept |
|-------|---------------------|-------|-------------|----------------|
| CIP   | $Y = 0.039x - 0.002$ | 0.996 | 0.00669     | 0.0253         |
| PHE   | $Y = 0.082x + 0.019$ | 0.999 | 0.00584     | 0.0201         |

**Multivariate Methods**

The first step in multivariate methods involved constructing the calibration matrix. The wavelength range used was 205 to 515 nm. Sixty-three spectral points with 5 nm intervals were selected within this range. The composition of calibration mixtures was random. Designed to obtain full information from the mixtures' spectra. The efficiency of the study of multiple components depends on the range of wavelength and spectrum mode. The UV spectrum of CIP, PHE and mixture are shown in the Fig.-2. The calibration set and validation set were prepared randomly with the CIP and PHE methanol mixtures (Table-1). The absorbance was observed inside the region between 200-600 nm at the wavelength point of 63 in the region between 205-515 nm with an interval of 5 nm. The model PCR and PLS were developed by the program Unscrambler® and the model CLS was developed by the program Matlab®. The development of the model was carried out using calibration standards. The LOO-CV was used in the development of the model to validate PCR, PLS and CLS models, and to obtain latent equilibrium variables (number of factors) of the model. A cross-validation approach was used with high calibration spectra to pick the optimally latent variables (number of factors) in the PLS, PCR and CLS algorithms. Each sample has been compared to actual component
concentrations for each validation sample and each test the root average cross-validation square error (RMSECV) is determined. The RMSECV was used to check the error in expected concentrations as a diagnostic tool. This model is important if PLS, PCR and CLS calibrations are to be quantified correctly. Table-3 displays the parameters of optimal models. The resulting models were also validated by predicting the analyte concentration in a separate validation method not used in the design of the model. Tables-4 and 5 demonstrate the outcomes of the forecast and the rate of recoveries. Assessing the predictive ability of the model was carried out with the plotting of the actual known concentrations against predicted concentrations and the plot of the actual known concentrations against the expected concentrations is stated in Fig.-3. As observed, the predicted(calculated) was consistent with the actual drug concentration. Recoveries of means and the relative standard deviation from our proposed methods have been estimated and shown for CIP and PHE in Tables-4 and 5. In the validation package, optimized PLS, PCR and CLS models suggesting the strong predictive capacity of the models were provided with satisfactory correlation coefficient (r2) values for-compound. Another diagnostic test was performed by measuring the residual concentrations against the concentrations expected. The residuals are distributed uniformly about zero, suggesting appropriate models. Table-6 shows the statistical parameters of the validation package.

Analytical Discussion

Statistical Analysis

The ability to calibrate can be described in many ways. For this article, the figures were determined for the standard variation of the chemometric calibrations in the case of the mixtures examined. The following expression shows the normal calibration error (SEC) and prediction:

\[
\text{SEP}, \ \text{SEC}(\text{SEP}) = \sqrt{\frac{\sum_{i=1}^{n} (C_i^{\text{added}} - C_i^{\text{Found}})^2}{n-1}} \quad (1)
\]

Here, \(C_i^{\text{added}}\) represents the added concentration, \(C_i^{\text{Found}}\) denotes the determined concentration and \(n\) is the total number of samples. The numerical values of SEC were indicated in Table 3. The SEP of the same mixtures is displayed in Table-3.

The prediction residual error sum-of-squares (PRESS) of the calibration step was calculated as:

\[
\text{PRESS} = \sum_{i=1}^{N} (C_i^{\text{added}} - C_i^{\text{Found}})^2 \quad (2)
\]

The root mean squares error of cross-validation (RMSECV) was calculated for each method as follows:

\[
\text{RMSECV} = \sqrt{\frac{\text{PRESS}}{n}} \quad (3)
\]

Where, \(n\) = number of predicted samples

RESULTS AND DISCUSSION

Recovery Study

For calibration purposes, the zero-order absorbance measured on selected wavelength ranges is used and used in PLS, PCR, CLS calibrations. The quantity of each drug was calculated in the synthetic mixture prediction (validation). Table-7 and Table-8 display the findings of the recovery analysis.

Analysis of Market Formulation

UV-assisted chemometrics was used for the study of URISTAT ® marketed formulation, reporting 250 mg for CIP and 200 mg for PHE per tablet. The drug check findings are consistent with the label statement (Table-9).
### Table-3: Statistical Parameters of Optimum PLS, PCR and CLS Models for the Calibration Set

| Parameter          | CIP          | PHE          |
|--------------------|--------------|--------------|
| Range (µg/ml)      | PLS 5-25     | PCR 4-20     |
| Wavelength Region  | 205 – 515    | 205 – 515    |
| λΔ (nm)            | 5            | 5            |
| Factor             | 7            | 7            |
| SD                 | 1.46         | 1.44         |
| Correlation Coefficient (r²) | 0.997 | 0.997 |
| Intercept          | -0.366       | -0.368       |
| Slope              | 1.023        | 1.023        |
| RMSECV             | 0.0609       | 0.0659       |
| RMSEP              | 0.2923       | 0.2893       |

### Table-4: Results of the Prediction Set of CIP by PLS, PCR and CLS Methods

| CIP (µg/ml) | Predicted Conc. | % Recovery | Residual |
|-------------|-----------------|------------|----------|
|             | PLS  | PCR  | CLS  | PLS  | PCR  | CLS  | PLS  | PCR  | CLS  |
| 10          | 10.01 | 10.01 | 9.97  | 100.10 | 100.10 | 99.78  | -0.010 | -0.010 | 0.022 |
| 15          | 15.01 | 15.02 | 15.14 | 100.13 | 100.18 | 100.98  | -0.020 | -0.026 | -0.146 |
| 15          | 14.82 | 14.83 | 15.00 | 98.87  | 98.90  | 100.01  | 0.173  | 0.164  | -0.001 |
| 15          | 14.73 | 14.74 | 15.38 | 98.20  | 98.29  | 102.55  | 0.269  | 0.256  | -0.382 |
| 15          | 15.26 | 15.25 | 15.13 | 101.73 | 101.72 | 100.93  | -0.266 | -0.258 | -0.139 |
| 20          | 20.29 | 20.28 | 20.04 | 101.45 | 101.45 | 100.25  | -0.297 | -0.289 | -0.049 |
| 20          | 19.67 | 19.67 | 19.60 | 98.35  | 98.38  | 98.04   | 0.326  | 0.324  | 0.391 |
| 25          | 25.40 | 25.42 | 24.79 | 101.60 | 101.68 | 99.19   | -0.409 | -0.420 | 0.201 |

### Table-5: Results of the Prediction Set of PHE by PCR, PLS and CLS Methods

| PHE (µg/ml) | Predicted Conc. | % Recovery | Residual |
|------------|-----------------|------------|----------|
|            | PLS  | PCR  | CLS  | PLS  | PCR  | CLS  | PLS  | PCR  | CLS  |
| 20         | 19.92 | 19.93 | 19.93 | 99.65 | 99.66 | 99.67 | 0.072 | 0.067 | 0.065 |
| 8          | 7.81  | 7.83  | 7.86  | 97.75 | 97.89 | 98.30 | 0.181 | 0.168 | 0.135 |
| 16         | 15.67 | 15.69 | 15.58 | 98.00 | 98.12 | 97.40 | 0.324 | 0.505 | 0.416 |
| 20         | 19.91 | 19.96 | 20.14 | 99.55 | 99.80 | 100.75 | 0.660 | 0.040 | -0.149 |
| 24         | 23.93 | 23.90 | 23.94 | 99.71 | 99.62 | 99.77 | 0.538 | 0.092 | 0.055 |
| 4          | 4.06  | 4.03  | 3.99  | 101.75 | 100.90 | 99.82 | -0.067 | -0.036 | 0.007 |
| 8          | 7.88  | 7.91  | 7.93  | 98.63 | 98.95 | 99.17 | 0.114 | 0.083 | 0.066 |
| 16         | 16.08 | 16.14 | 16.05 | 100.50 | 100.90 | 100.32 | -0.080 | -0.144 | -0.050 |

### Table-6: Statistical Parameters of Optimum PLS, PCR and CLS Models for the Validation Set

| Parameters | CIP          | PHE          |
|------------|--------------|--------------|
| RMSECV     | 0.0609       | 0.0659       | 0.0889 | 0.0993 | 0.2553 |
| RMSEP      | 0.2923       | 0.2893       | 0.2191 | 0.2093 | 0.2025 | 0.1689 |
| Slope      | 1.023        | 1.023        | 0.974  | 0.998  | 0.997  | 0.997  |
| Intercept  | -0.366       | -0.368       | 0.450  | -0.059 | -0.066 | -0.066 |
| R2         | 0.997        | 0.997        | 0.998  | 0.999  | 0.999  | 0.999  |
Table 7: Result of CIP for Accuracy by PLS, PCR and CLS Models (n = 3)

| Level (µg/ml) | Sample Conc. (µg/ml) | Standard Added (µg/ml) | Mean Amount Recovered (µg/ml) | %Mean |
|--------------|----------------------|------------------------|------------------------------|-------|
|              | PLS                  | PCR                    | CLS                          | PLS   | PCR | CLS |
| 50           | 7.51                 | 7.52                   | 7.38                         | 100.23 | 100.28 | 98.41 |
| 100          | 10.03                | 10.05                  | 9.87                         | 100.37 | 100.52 | 98.72 |
| 150          | 12.24                | 12.25                  | 12.58                        | 98.03  | 98.02  | 100.65 |

Fig.-3: Plot of Predicted Vs Known Concentration for (A) CIP and (B) PHE for PLS method, (C) CIP and (D) PHE for PCR method and (E) CIP and (F) PHE for CLS Method

Table 8: Result of PHE for Accuracy by PLS, PCR and CLS Models (n = 3)

| Level (µg/ml) | Sample Conc. (µg/ml) | Standard Added (µg/ml) | Mean Amount Recovered (µg/ml) | %Mean |
|--------------|----------------------|------------------------|------------------------------|-------|
|              | PLS                  | PCR                    | CLS                          | PLS   | PCR | CLS |
| 50           | 5.93                 | 5.94                   | 5.92                         | 98.94 | 99.01 | 98.74 |
| 100          | 7.84                 | 7.85                   | 7.84                         | 98.02 | 98.03 | 99.13 |
| 150          | 9.98                 | 10.01                  | 10.02                        | 99.98 | 100.10 | 100.20 |
Table 9: Results of the Assay in Commercial Samples

| Sample No. | % Amount Found |
|-----------|----------------|
|           | PLS            | PCR            | CLS            |
|           | CIP            | PHE            | CIP            | PHE            | CIP            | PHE            |
| 1         | 98.63          | 97.97          | 99.26          | 98             | 99.88          | 98.02          |
| 2         | 98.26          | 98             | 98.88          | 98             | 99.56          | 98.03          |
| 3         | 101.41         | 99.38          | 101.99         | 99.50          | 100.18         | 98.11          |
| 4         | 99.84          | 99.08          | 99.40          | 99.22          | 99.92          | 98.03          |
| 5         | 99.60          | 99.03          | 99.07          | 99.28          | 99.57          | 98.02          |
| 6         | 99.57          | 99.23          | 99.70          | 99.42          | 100.20         | 98.12          |
| Mean      | 99.22          | 98.78          | 99.71          | 99.89          | 99.98          | 98.06          |
| SD        | 1.16           | 0.64           | 1.15           | 0.72           | 0.28           | 0.04           |

Fig. 4: Plot of Expected Vs Residual Concentration for (A) CIP and (B) PHE for PLS method, (C) CIP and (D) PHE for PCR method and (E) CIP and (F) PHE for CLS Method.
CONCLUSION

The Partial least-square regression (PLS), Principal component regression (PCR) and Classical Least square (CLS) models have been successively built in the standard mixing set (validation set) for the determination of CIP and PHE. Three multivariate calibration models achieved equal accuracy. The proposed Chemometric assisted spectrophotometric method in the combination of the pharmaceutical formulation is applicable, rapid and accurate for the simultaneous determination of CIP and PHE. The findings of the testing of commercial formulation obtained using PLS, PCR and CLS models were not substantially different. This means that the proposed PLS, PCR and CLS models should be used in combined pharmaceutical formulation to evaluate the quality of CIP and PHE.

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