ANALYTICAL METHODS FOR MULTICOMPONENT DRUG SYSTEM

Pharmaceutical products formulated with more than two drugs, typically referred to as combination products or multicomponent drug system or polypills. With the increase in various drugs as well as their formulation in the market, the availability of multicomponent drug system proves to be quite comfortable when it comes to the treatment of multiple symptoms at a same time, hence preventing the intake of several formulations. These formulations prove to be a "one for many" and are intended to meet previously unmet patients' need by combining the therapeutic effects of two or more drugs in one product. The combination products present daunting challenges to the quality control analyst, for the development and validation of analytical methods.

The analytical methods used for pharmaceutical products containing more than one active ingredient are:- various UV spectrophotometric methods such as: simultaneous equation method, absorbance ratio method, geometric correction method, orthogonal polynomial method, difference spectrophotometry, derivative zero crossing spectrophotometry, derivative ratio spectra spectrophotometry and dual wavelength method are used for the simultaneous estimation of drug. Spectroscopic methods like FTIR, NIR and Raman spectroscopy are also used for analysis of multicomponent system.

Chromatographic techniques like HPLC, HPTLC, size exclusion chromatography, ion exchange chromatography are widely used in the analysis of multicomponent drug systems. The various techniques of chromatography are used with different detectors (UV detector, fluorescence, photodiode array, mass, etc.) according to the requirement or properties of the drug components. Among all the chromatographic techniques HPLC is the most preferred.

HPLC Method (1-5)

High-performance liquid chromatography (HPLC), also called high-pressure liquid chromatography, is a separation technique. The separation is achieved by various principles like partition, adsorption or ion-exchange processes based on a solid stationary phase and a liquid mobile phase.

Compounds that are to be analyzed are dissolved in a suitable solvent, and most

separations take place at ambient temperature. Thus, most of the drugs or compounds, whether nonvolatile or thermally unstable, can be analysed by HPLC without decomposition or without derivatisation (e.g. volatile derivatives made for gas chromatography).

A liquid chromatography consists a mobile phase, a pump to force the mobile phase through the system at high pressure, an injector to introduce the sample into the mobile phase, a chromatographic column, a detector and a data collection device such as a computer, integrator, or recorder. Short, small-bore columns containing densely packed particles of stationary phase provide for the rapid exchange of compounds between the mobile and stationary phases.

The performance of HPLC method is assessed by the system suitability (SST) parameters. If the results are adversely affected by the changes in column performance (e.g. unacceptable precision of results due to overlapping peaks), the system suitability results from these experiments will help to determine the limits for system suitability criteria. The various SST parameters alongwith their recommendations are as follows:

1. Resolution (R_s): Resolution describes the separation power of the complete chromatographic system relative to the particular components of the mixture. It is defined as the ratio of the distance between two peak maxima. For baseline separation, the ideal value of R_s is 1.5. It is calculated by using the formula,

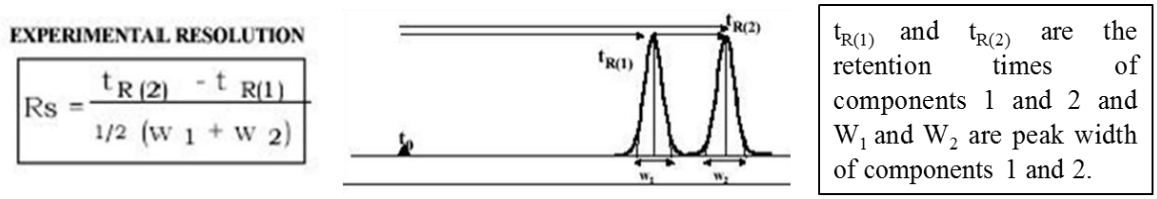


Figure I.1 Resolution between two peaks.

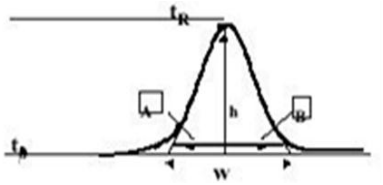
2. Capacity Factor (k'): Capacity factor is a measure of how well the sample molecule is retained by a column during an isocratic separation. It is defined as the ratio of the number of molecules of solute in the stationary phase to the number of molecules of the same in the mobile phase. The ideal value of k' ranges from 2-10. Lower values may give inadequate resolution. Higher values are usually associated with excessively brood peaks and unacceptably long run times. Capacity Factor (k')

changes are typically due to variations in mobile phase composition, changes in column surface chemistry (due to aging) and changes in operating temperature.

Capacity factor can be determined by using the formula,

RETENTION FACTOR or CAPACITY RATIO

$$k' = \frac{t_R - t_0}{t_0} \qquad k' = \phi \frac{C_s}{C_m}$$



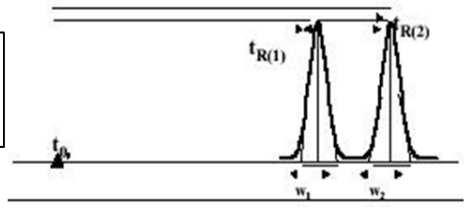
t_R = retention volume at the apex of the peak (solute) and t_0 = void volume of the system.

Figure I.2 Capacity Factor

3. Selectivity (α): The selectivity (or separation factor), α, is a measure of relative retention of two components in a mixture. Selectivity represents the separation power of particular adsorbent to the mixture of these particular components. This parameter is independent of the column efficiency; it only depends on the nature of the components, eluent type and composition and adsorbent surface chemistry. Selectivity (α) values are sensitive to changes in mobile phase composition (pH ionic strength), purity and temperature. In general, if the selectivity of two components is equal to 1, then there is no way to separate them by improving the column efficiency. The ideal value of α is 2. It can be calculated by using formula,

SELECTIVITY FACTOR

$$\alpha = V_2\text{-}V_0 \,/\, V_1\text{-}V_0 = k'_2 / k'_1$$



V_0 = the void volume of the column and V_1 , V_2 = the retention volumes of the first and second peak respectively.

Figure I.3 Selectivity

4. Column Efficiency/ Band broadening: Efficiency, N, of a column is measured by the number of theoretical plates per meter. It is a measure of band spreading of a peak. Similar the band spread, higher is the number of theoretical plates, indicating good column and system performance. Columns with N ranging from 5,000 to 100,000 plates/meter are ideal for a good system. A decline in measured efficiency may be due to age and history of the column, extra column band broadening (such as due to malfunctioning injector or improper tubing ID), inappropriate detector settings (for example, time constant) and change in flow rate and solvent viscosity. Efficiency is calculated by using the formula,

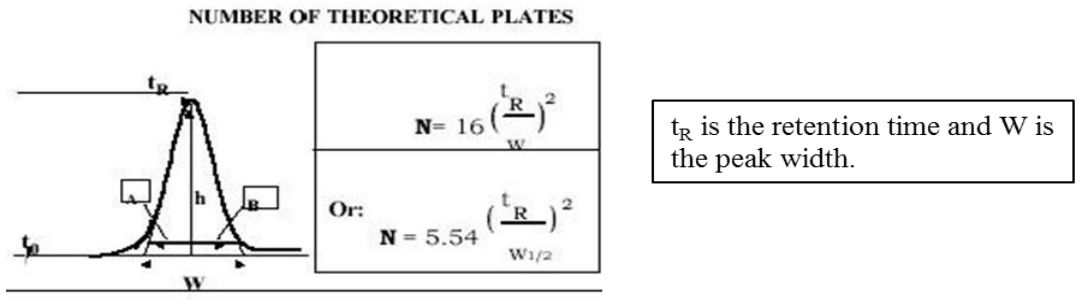


Figure I.4 Number of Theoretical Plates

5. Peak asymmetry factor (T_f): Peak asymmetry factor, T_f, can be used as a criterion of column performance. The peak half width, 'B', of a peak at 10% of the peak height, divided by the corresponding front half width, 'A', gives the asymmetry factor. For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

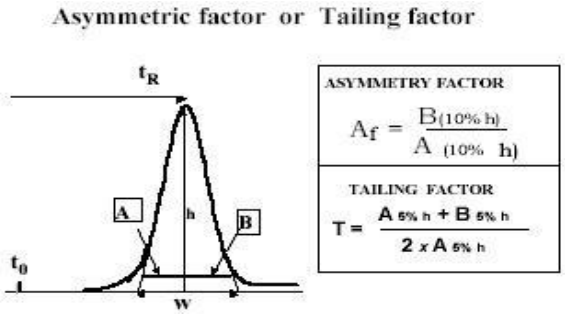


Figure I.5 Asymmetric Factor

Analytical methods in combination with multivariate analysis/ chemometrics (6-13)

The word "kemometri" was first given by a Swedish scientist Svante Wold in 1971 from which the "chemometrics" has derived (6). Now, chemometrics has emerged to have a major role within the analytical chemistry (7).

Chemometrics is the field of extracting information from multivariate chemical data using tools of statistics and mathematics. Chemometrics is typically used for one or more of three primary purposes:

- ✓ To explore patterns in association with the data;
- ✓ To track properties of materials on a continuous basis; and
- ✓ To prepare and use multivariate classification models.

The multivariate calibrations have been particularly subjected to the spectrophotometric determinations. They are used in combination with different analytical techniques like UV spectroscopy, HPLC, polarography, voltametry, IR spectroscopy, etc. for the quantitative determination of drugs in multicomponent pharmaceutical preparations. The application of multivariate calibration to the absorbance signals produced by drugs during their simultaneous determination in pharmaceutical formulations is an effective means for their quality control. The chemometric methods sometimes do offer an interesting alternative to chromatographic techniques.

Various multivariate or chemometric methods such as classical least square (CLS), inverse least square (ILS), principal component regression (PCR) and partial least square (PLS), based on computer assisted instrumentation have been applied for the analysis of multicomponent analysis of mixtures (14-22). All the chemometric methods are found suitable for the resolution of the overlapping spectral bands in quantitative determination.

All these methods comprise of two separate stages. In the first stage, termed "calibration" an empirical model is built, representing the relationship between the data generated from a set of reference samples and the respective concentrations of their component(s) of interest. This is followed by a second stage called "prediction", in which the calibration model is used to determine the concentration of the components in the unknowns from their spectral data.

The regression based chemometric algorithms include partial least squares (PLS) and principal component regression (PCR), which are designed to avoid problems associated with noise and correlations in the data. As the regression algorithms used are based on factor analysis, the entire group of known measurements is considered simultaneously, and information about correlations among the variables is automatically built into the calibration model. Chemometric regression lends itself handily to the on-line monitoring and process control industry, where time saving analysis is required alongwith inexpensive systems that are used to predict and make decisions about the product quality. Multivariate calibration methods allow simultaneous determination, non-resolution analysis and analysis even in the presence of interfering agents (such as excipients).

Principal Component Regression (PCR) Method

PCR is a method by which the dimensionality or complexity of the data is reduced. With reference to application for spectrophotometric analysis, absorbance matrix concerns to a large pool of data containing *n* variables (wavelengths, one column for each) and *m* samples (calibration standards, one row for each). Each sample can be presented as a point in *n* dimensional space according to its absorbance values at different wavelengths. Thus, one sample is described by *n* variables.

PCR reduces number of variables to only a few components, referred to as principal components (PCs). They are computed in such a way that the first PC is the one that carries most information (or in statistical terms: most explained variance). The second PC will then carry the maximum share of the residual information (i.e. not taken into account by the previous PC), and so on. All the PCs are orthogonal to each other and they are ranked in a manner so that first PC will explain maximum variance in the data. After reducing the dimensionality of data to a few PCs, next step is regression to relate these PCs with concentration. There is an individual regression for each drug present in the sample. Thus, PCR deals with only one response variable (concentration) at a time.

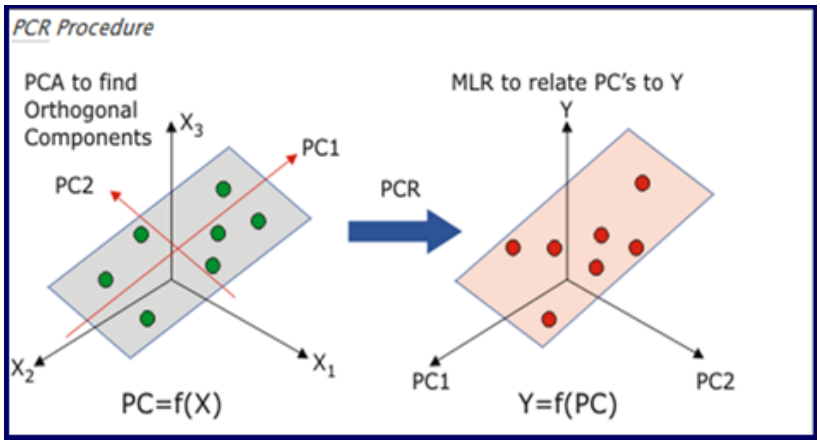


Figure I.6 Pictorial representation of PCR method

The data compression in PCA can be done using inverse regression by which model coefficients are calculated and only the desired constituents or constituents of interest can be calculated.

Advantages

- Can be used for very complex mixtures since only knowledge of constituents of interest is required.
- Can sometimes be used to predict samples with constituents (contaminants) not present in the original calibration mixtures.

Disadvantages

- Optimization requires knowledge of PCA (Principle Component Analysis)
- There is no confirmation that PCA vectors directly correspond to constituents of interest.
- For accurate calibration, generally, a large number of samples are required.
- Collection of calibration samples can be difficult; collinear constituent concentrations must be avoided.

Partial Least Square (PLS) Method

PLS models both the X- and Y-matrices simultaneously to find the latent variables in X that will best predict the latent variables in Y. This method is called projection to latent structures. These PLS latent variables are similar to principal components, and are referred to as PCs or factors.

In PCR, we find PCs for only X matrices (variables) and then these PCs are related directly to Y (sample concentration). Here in PLS, latent variables (LVs) are computed for X and Y both. And then LV of X is related with LV of Y. PLS can handle multiple responses in Y at a time in contrast to PCR.

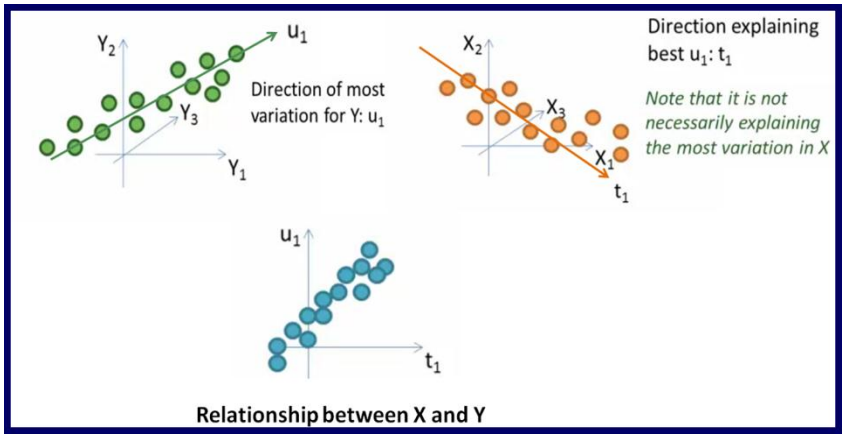


Figure I.7 Pictorial representation of PLS method

Advantages:

- It is a combination of the full spectral coverage of CLS alongwith the partial composition regression of ILS.
- The models built by PLS are more robust if the range of variability as expected in unknown samples is accurately defined by the calibration set.
- As PLS requires only knowledge of constituents of interest, it can be used with complex systems also.

Disadvantages

- For accurate calibration, a large number of samples are required.
- While collection of calibration samples, collinear constituent concentrations should be avoided.

The difference between PCR and PLS lies in the algorithm. PLS uses the information lying in both, X and Y matrices, in order to fit the model. It switches between X and Y iteratively to find the relevant LVs. So PLS often needs fewer LVs to reach the optimal solution because the focus is on the prediction of the Y-variables (not on achieving the best projection of X as in PCR).

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DEVELOPMENT OF RP-HPLC AND CHEMOMETRIC ASSISTED SPECTROPHOTOMETRIC METHODS FOR THE SIMULTANEOUS DETERMINATION OF FIVE ACTIVE INGREDIENTS IN COUGH AND COLD TABLETS AND THEIR APPLICATION TO DISSOLUTION STUDY

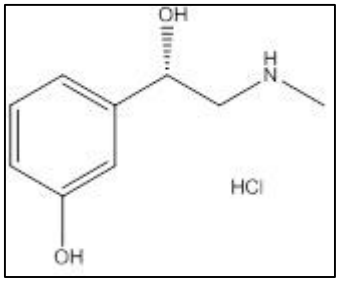
1.1. SELECTION OF FORMULATION

Most of the formulations used for cough and cold, consist of combinations of more than two or three drugs (multicomponent system). The analysis of such multicomponent formulations becomes difficult by conventional UV spectrophotometric techniques mainly because of spectral overlap. However since last few years, the chemometric techniques, such as inverse least squares (ILS), classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS), have widely been applied to the spectrophotometric resolution of such multicomponent formulations without their preliminary separation. PLS and PCR are especially suited for drug mixtures with highly overlapped spectra. Although the HPLC method provides a suitable method for the analysis, but it requires many trials, is expensive and proves to be more time consuming.

The mixture of phenylephrine hydrochloride (PEP), paracetamol (PCM), guaifenesin (GNF), chlorpheniramine maleate (CPM) and bromhexine hydrochloride (BRM) is one of the most widely used combination for cough and cold therapy. It is mainly used in diseases accompanied by cough, pain and fever, common cold and other viral infections and also used as an analgesic, antipyretic, decongestant, antihistaminic and antitussive.

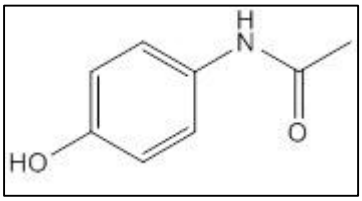
1.2. DRUG PROFILE

(1) Phenylephrine hydrochloride (PEP) (1, 6)



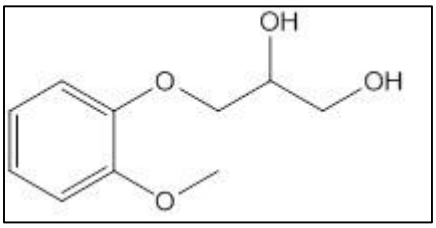
a. **Category :** Decongestant
b. **Molecular formula :** $C_9H_{13}NO_2$. HCl
c. **Molecular Weight :** 204 gm/mole
d. **Nomenclature:** (*R)*-3-[-1-hydroxy-2-(methylamino)ethyl]phenol
e. **Physicochemical Properties:**
    i. Description: White crystalline powder
    i. Solubility: Freely soluble in MeOH, ACN, water
    ii. log P:-0.69
    iii. pK_a: 8.97
    iv. Melting Point: 143-145 ˚C
f. **Official Status:** Official in IP, USP and BP.

(2) Paracetamol (PCM) (2,6)



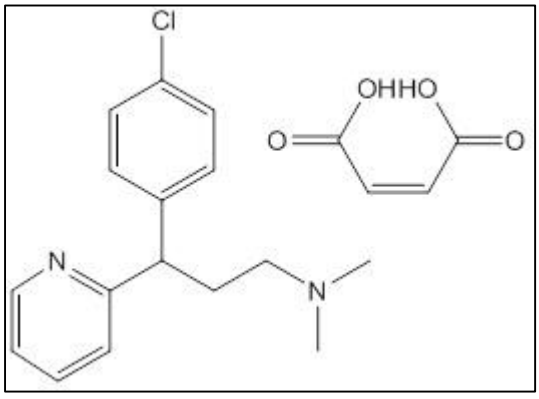
a. **Category :** Analgesic
b. **Molecular formula :** $C_8H_9NO_2$
c. **Molecular Weight :** 151 gm/mole
d. **Nomenclature:** N-(4-hydroxyphenyl)acetamide
e. **Physicochemical Properties:**
    i. Description: White crystalline powder
    ii. Solubility: Freely soluble in MeOH, ACN; sparingly soluble in water
    ii. log P: 0.51
    iii. pKa: 9.46, -4.4
    iv. Melting Point: 168-172 ˚C
f. **Official Status:** Official in IP, USP and BP.

(3) Guaifensin (GNF) (3, 6)



a. **Category** **:** Expectorant
b. **Molecular formula :** $C_{10}H_{14}O_4$
c. **Molecular Weight :** 198 gm/mole
d. **Nomenclature:** 3-(2-methoxyphenoxy)-1,2-propanediol
e. **Physicochemical Properties:**
    i. Description: White crystalline powder
    ii. Solubility: Soluble in MeOH, ACN
    iii. log P:0.76
    iv. pKa: 13.62, -3
    v. Melting Point: 78-82 ˚C
f. **Official Status:** Official in IP, USP and BP.

(4) Chlorpheniramine maleate (CPM) (4, 6)



a. **Category** **:** Antihistaminic
b. **Molecular formula :** $C_{16}H_{19}ClN_2.C_4H_4O_4$
c. **Molecular Weight :** 390 gm/mole
d. **Nomenclature:**3-(4-chlorophenyl)-*N*,*N*-dimethyl-3-pyridin-2-yl-propan-1-amine maleate
e. **Physicochemical Properties:**
    i. Description: White crystalline powder
    ii. Solubility: Soluble in MeOH, ACN

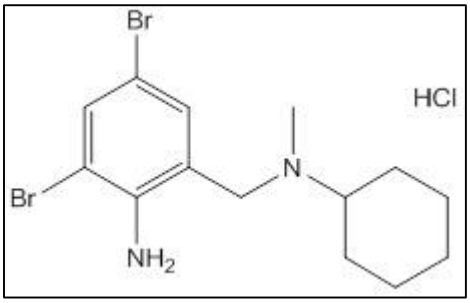
iii. log P:3.74

iv. pKa: 9.47

v. Melting Point: 130-132 ˚C

f. Official Status: Official in IP, USP and BP.

(5) Bromhexine hydrochloride (BRM) (5, 6)



a. Category : Mucolytic

b. Molecular formula : $C_{14}H_{20}Br_2N_2.HCl$

c. Molecular Weight : 412 gm/mole

d. Nomenclature: 2,4-dibromo-6-{[cyclohexyl(methyl)amino]methyl}aniline hydrochloride

e. Physicochemical Properties:

i. Description: White crystals or crystalline powder

ii. Solubility: Freely soluble in MeOH, ACN

iii. log P:4.08

iv. pKa: 19.89, 9.32

v. Melting Point: 242-244 ˚C

f. Official Status: Official in IP, USP and BP.

1.3. MARKETED FORMULATION

A commercial formulation of Intas pharmaceutical (Kuff Q tablet) was used for the study. Each tablet contains paracetamol (450 mg), phenylephrine hydrochloride (10 mg), guaifenesin (100 mg), chlorpheniramine maleate (2 mg) and bromhexine hydrochloride (8 mg).

1.4. LITERATURE REVIEW

Several analytical methods are available for the determination of PEP, PCM, GNF, CPM and BRM in various combinations as cough cold formulations, amongst which some are: high performance liquid chromatography (HPLC) methods (7-12), atomic emission spectrometry (13), mixed ion pair liquid chromatography (14), liquid chromatography–tandem mass spectrometry (15), non-aqueous capillary electrophoresis (16) and gas chromatography (17). Various chemometric methods (18, 19) and HPLC methods of some cough and cold formulations (20-26) have been reported. Chemometric assisted spectrophotometric methods and RP-HPLC method for the five drug combination under study has not been reported yet.

1.5. METHOD 1A. DEVELOPMENT OF RP-HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF PEP, PCM GNF, CPM AND BRM AND ITS APPLICATION TO DISSOLUTION STUDY

1.5.1. EXPERIMENTAL

1.5.1.1. Instrumentation

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AD and Shimadzu PDA-M20A Diode Array Detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µL. Data acquisition and integration was performed using LC Solution software (Shimadzu Corporation, Kyoto, Japan). Separation and quantitation were made on Phenomenex RPC18 column (5µm × 250mm × 4.6mm i.d.).The dissolution was performed on Veego VDA6DR dissolution apparatus.

1.5.1.2. Materials and reagents

BRM, PCM, PEP, GNF and CPM were provided by Ethicare Pharmaceuticals and Alembic Pharmaceuticals (Vadodara, India) as gift samples. HPLC grade MeOH and ACN (Spectrochem), potassium dihydrogen phosphate (AR grade, LobaChem), phosphoric acid (AR grade, LobaChem) and triethylamine (HPLC grade, Spectrochem) were used for HPLC analysis. Dissolution was performed by using AR grade hydrochloric acid (Merck).

1.5.1.3. Experimental conditions

Phosphate buffer (0.01 M) was prepared by dissolving 1.36 g of anhydrous potassium orthophosphate (KH_2PO_4) in 1 L of previously filtered double distilled water, 0.1% of triethylamine was added to it and pH was adjusted to 3.0 using phosphoric acid.The elution was carried out with mobile phase composed of the mixture of MeOH, ACN and 0.01M phosphate buffer (pH 3) in the ratio of 27.5:22.5:50. All determinations were performed at ambient temperature.The flow rate was 1 mL/min. The injection volume was 20 µL and detection wavelength was 218 nm.

1.5.1.4.Preparation of standard solutions

PEP stock solution (1mg/mL): Accurately weighed 25 mg PEP was transferred in 25 ml volumetric flask, dissolved and then diluted with MeOH up to the mark.
PEP working solution (0.1/mg mL): prepared by transferring 2.5mL from PEP stock solution to 25 ml volumetric flask and diluted to the mark with mobile phase.

PCM stock solution (5 mg/mL): Accurately weighed 125 mg PCM was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.
PCM working solution (2.5/mg/mL): prepared by transferring 12.5 mL from PCM stock solution to 25 mL volumetric flask and diluted to the mark with mobile phase.

GNF stock solution (2 mg/mL): Accurately weighed 50 mg GNF was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.
GNF working solution (1 mg/mL): prepared by transferring 12.5 mL from GNF stock solution to 25 mL volumetric flask and diluted to the mark with mobile phase.

CPM stock solution (1 mg/mL): Accurately weighed 25 mg CPM was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.
CPM working solution (0.1 mg/mL): prepared by transferring 2.5 mL from CPM stock solution to 25 mL volumetric flask and diluted to the mark with mobile phase.

BRM stock solution (1 mg/mL): Accurately weighed 25 mg BRM was transferred in 25 mL volumetric flask, dissolved and then diluted with MeOH up to the mark.
BRM working solution (0.1 mg/mL): prepared by transferring 2.5 mL from BRM stock solution to 25 ml volumetric flask and diluted to the mark with mobile phase.

From the working solutions, appropriate aliquots were taken to prepare solution mixtures of PEP, PCM, GNF, CPM and BRM in the range of 5-20 µg/mL PEP, 225-900 µg/mL PCM, 50-200 µg/mL GNF, 1-4 µg/mL CPM and 4-16 µg/mL BRM in the mobile phase, which were analysed by HPLC method under above mentioned chromatographic conditions.

1.5.1.5. HPLC method validation

The developed RP-HPLC method was applied to the simultaneous determination PEP, PCM, GNF, CPM, and BRM and was validated for linearity, accuracy, precision, limit of detection, limit of quantitation, specificity and robustness by the ICH guideline (27).

The linearity of the HPLC detector response for determination of PEP, PCM, GNF, CPM and BRM was evaluated by analysing a series of different concentrations of each compound. The calibration range was established with respect to the practical range necessary, according to the marketed formulation, to give accurate, precise and linear results.

The precision was performed at three concentration levels for each compound in triplicate. The three levels were: 5, 10, 15 for PEP; 225, 450, 675 for PCM; 50, 100, 150 for GNF; 1, 2, 3 for CPM and 4, 8, 12 for BRM.The peak areas of all five drugs were calculated for each trial. The experiment was repeated three times in a day for intra-day precision and on three different days for inter-day precision.

The accuracy was performed by standard addition method. Known amounts of standard drugs were added to a known concentration of the commercial tablet formulation at three levels of standard addition (80%, 100%, 120%). The total concentrations (0, 80, 100, 120%) for accuracy study were resp.: 5, 9, 10, 11 for PEP; 225, 405, 450, 495 for PCM; 50, 90, 100, 110 for GNF; 1, 1.8, 2, 2.2 for CPM and 4, 7.2, 8, 8.8 for BRM. The resulting mixtures were analysed and the percentage recovery of the results was reported.

For determining the limit of detection (LOD) and limit of quantitation (LOQ), the approach based on the standard deviation (S.D.) of the y-intercept and the slope was used and the values were obtained.

Robustness study was performed by making variation in pH of the phosphate buffer by ±0.2 (2.8, 3, 3.2), change in flow rate by ±0.1 (0.9 mL/min, 1.0 mL/min, 1.1 mL/min) and change in the composition of buffer solution by ±1% (49%, 50% and 51%).

The stability of the working stock solutions was studied at room temperature and in refrigerator (8-25ºC).

The specificity of the method was assessed by analysing the formulation, whether any interference of the excipients was there or not.

For system suitability study, six replicates of the solution 10 ppm PEP, 450 ppm PCM, 100 ppm GNF, 2 ppm CPM and 8 ppm BRM was used to evaluate the system suitability and their %RSD was found. The peak purity curves were also assessed.

1.5.1.6. Analysis of tablet formulation

Twenty commercial tablets were accurately weighed and powdered using mortar and pestle. An amount of powder equivalent to 45 mg of PCM, 0.8 mg of BRM, 10 mg GNF, 1 mg PEP and 0.2 mg of CPM was taken in 25 mL of volumetric flask, sufficient mobile phase was added and sonicated for 10 minutes. The volume was then made up to the mark with the mobile phase. This solution was filtered through Whatmann filter paper (No. 42) so as to remove undissolved tablet excipients. From this solution 2.5 mL aliquot was taken and further diluted to 10 mL with mobile phase. This solution was analysed by HPLC.

1.5.1.7. Dissolution study

The dissolution was carried out by USP-II paddle method. The dissolution media comprised of 0.01 N HCl prepared in single distilled water. The conditions for dissolution were 50 rpm at 37ºC temperature for the duration of 90 minutes. The selected dissolution media, 0.01N HCl, is the official media reported for the dissolution of PCM and CPM (28). The dissolution study was performed for the duration of 90 minutes and the sampling was done at different time intervals of 5, 10, 15, 30, 45, 60 and 90 min. 5 mL of aliquot was withdrawn from the dissolution vessel at specific time point. The sample (aliquot) was filtered through 0.2µ membrane filter and injected into the HPLC.

1.5.2. RESULTS AND DISCUSSION

1.5.2.1. Method development

The five drugs PEP, PCM, GNF, CPM and BRM were soluble in MeOH. But the peak shapes of all the five drugs appeared symmetric and sharper when dissolved in the mobile phase hence mobile phase was selected as a solvent for the simultaneous estimation of all the drugs.

For selection of the analytical wavelength, the UV spectra for all five components were extracted from the HPLC chromatogram (an option in PDA post analysis) and accordingly the final wavelength was selected as 218 nm. The overlay of the UV spectra of all five drugs including the zoom (insat) has been shown in Figure 1.1.

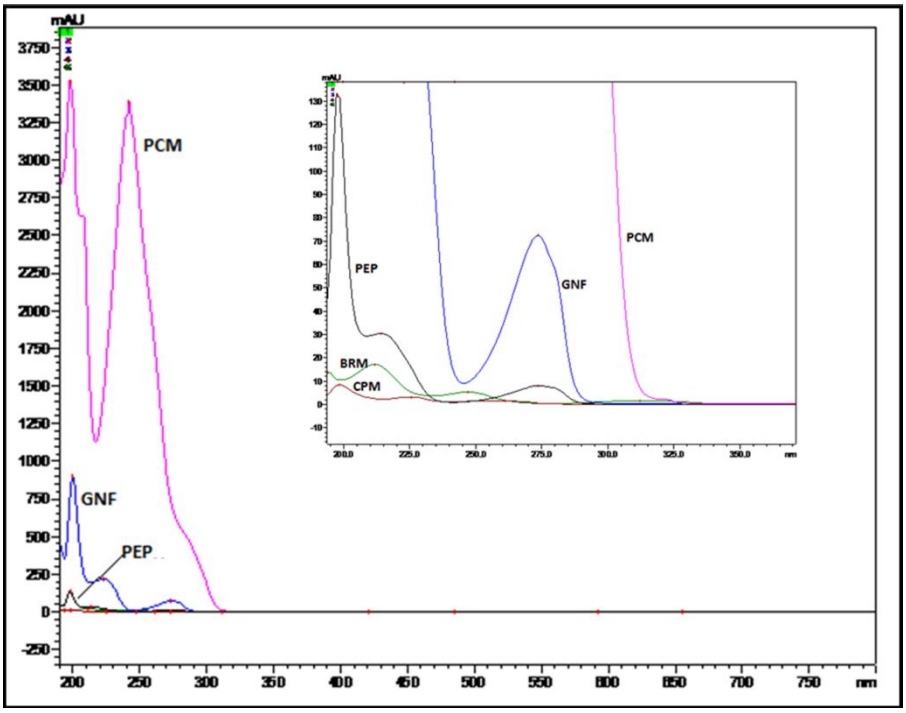


Figure 1.1 Overlay Zero order UV spectra extracted from the chromatogram of solution mixture of 10 ppm PEP, 450 ppm PCM, 100 ppm GNF, 2 ppm CPM and 8 ppm BRM

1.5.2.2. Selection and Optimization of chromatographic condition:

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, pH of buffer, concentration of mobile phase, organic solvent, flow rate and temperature, were studied as shown in Table 1.1. The resulting chromatograms were recorded and the system suitability parameters such as

capacity factor, asymmetric factor, resolution and theoretical plates were calculated. The conditions that gave the best resolution, symmetry and theoretical plate were selected for estimation.

Table 1.1 Optimization of mobile phase

Mobile Phase		PEP	PCM	GNF	CPM	BRM
MeOH: 0.01M phosphate buffer (pH=3.0) (50:50)	Rt	2.5	3.3	5.7	8.3	20
	Peak Shape	Broad Peak	Fronting	Fronting	Fronting	Sharp Peak
ACN: 0.01M phosphate buffer (pH-3.0) (50:50)	Rt	2.0	2.7	3.1	3	4.6
	Peak Shape	Broad Peak	Sharp peak	Fronting	Broad Peak	Sharp peak
[A]MeOH:ACN (50:50) [B] 0.01M phosphate buffer (pH=3) [A:B= 50:50]	Rt	2.3	3.1	4.2	5.1	10.6
	Peak Shape	Broad Peak	Fronting	Fronting	Fronting	Sharp peak
[A]MeOH:ACN (50:50) [B] 0.01M phosphate buffer (pH=4.0) [A:B= 50:50]	Rt	2.5	3.3	5.6	8.5	-
	Peak Shape	Splitted Peak	Fronting	Fronting	Fronting	Not eluted
[A]MeOH:ACN (50:50) [B] 0.01M phosphate buffer (pH=5.0 OPA) [A:B= 50:50]	Rt	2.6	3.3	5.3	9.2	-
	Peak Shape	Not good	Not good	Fronting	Fronting	Not eluted
[A] MeOH: ACN (50:150) [B] 0.01M phosphate buffer (pH=5.0 OPA) [A:B= 50:50]	Rt	2.6	3.0	5.1	8.3	-
	Peak Shape	Fronting	Not good	Sharp peak	Sharp peak	Not eluted
[A]MeOH:ACN (80:20) [B] 0.01M phosphate buffer (pH=5.0) [A:B= 50:50]	Rt	2.6	3.3	5.3	9.2	-
	Peak Shape	Fronting	Sharp peak	Sharp peak	Sharp peak	Not eluted

[A]MeOH:ACN (55:45) [B] 0.01M phosphate buffer (pH=5.0) [A:B= 50:50]	Rt	2.6	3.1	4.4	6.6	-
	Peak Shape	Splitted Peak	Fronting	Fronting	Fronting	Not eluted
Gradient developed on basis of above trial , assymmetric peak for BRM observed						
Trials at pH= 6, 6.6, 7	Assymetric peak shape, long elution time and high tailing factor					
[A]MeOH:ACN (55:45) [B] 0.01M phosphate buffer (pH=3.0) [A:B= 50:50]	Rt	2.6	3.3	4.6	6.0	12.8
	Peak Shape	Sym	Sym	Sym	Sym	Sym

[A] and [B] are pump A for aqueous and pump B for organic solvent resp. for binary pump HPLC system; Sym=symmetric

To optimize the HPLC parameters, several mobile phase compositions and different flow rate were tried. Various buffers e.g. potassium phosphate buffer, formate buffer at different pH and different organic solvents (ACN and MeOH) were tried. The studies suggested that a mobile phase at acidic pH value and the composition of ACN and MeOH (50:50) mixed with the buffer (organic: buffer in the ratio of 50:50) favoured the peak shape of all the five drugs with symmetric peak shape, reasonable retention and resolution.

After trying various mobile phase as described in Table 1.1, finally, the mobile phase potassium phosphate buffer (pH adjusted to 3 with o-phosphoric acid): ACN: MeOH: 50: 22.5: 27.5 (%v/v) was selected. The mixed standard solution containing 10 µg/mL of each of PEP, PCM, GNF, CPM and BRM was analysed using mobile phase of varied ratios of ACN, MeOH and buffer. The chromatograms were recorded. The peaks were separated with good resolution and had intense peak shape with the retention time (t_R) of 2.6 min, 3.3 min, 4.6 min, 6.0 min and 12.8 min for PEP, PCM, GNF, CPM and BRM respectively. The temperature was kept ambient and flow rate was 1 mL/min. The optimised chromatographic conditions are shown in Table 1.2. The chromatogram of standard solution mixture is shown in Figure 1.2.

Table 1.2 Optimised Parameters

Method parameters	Optimised value	
Column	Phenomenex LUNA C18 (250mm × 4.6mm i.d × 5µm particle size)	
Mobile phase	MeOH: ACN: 10 mM potassium dihydrogen phosphate buffer pH=3.0 (27.5: 22.5:50)	
Flow rate (mL/min)	1.0 mL /min	
Temperature	AMBIENT	
Detection wavelength	218 nm	
Retention Time (min)	PEP	2.6
	PCM	3.3
	GNF	4.6
	CPM	6.0
	BRM	12.8
Needle wash	Mobile phase	

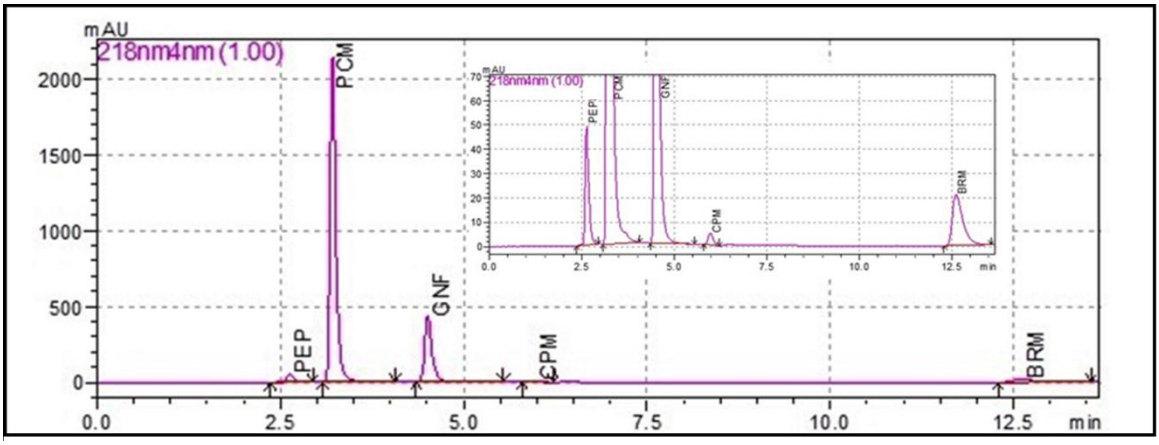


Figure 1.2 Chromatogram of standard solution containing 10 ppm PEP, 450 ppm PCM, 100 ppm GNF, 2 ppm CPM and 8 ppm BRM

1.5.2.3. Method validation

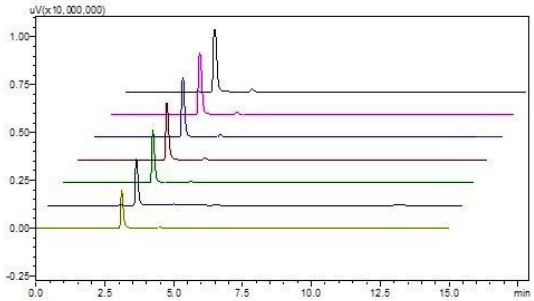
The developed HPLC method was applied to the simultaneous determination PEP, PCM, GNF, CPM, and BRM. The mobile phase composition and pH were studied and optimized. A satisfactory separation was obtained with an isocratic elution. Quantitation was achieved based on peak area with UV detection at 218 nm and runtime of 20 min. The average retention time±standard deviation (for six replicates) for PEP, PCM, GNF, CPM and BRM was found to be 2.63±0.01, 3.23±0.005, 4.59±0.01, 6.07±0.048 and 12.6±0.13 respectively.

1.5.2.3.1. Linearity

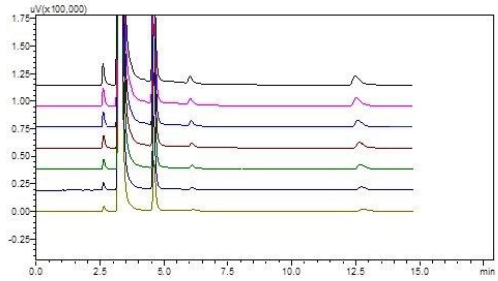
The linearity was analysed in the concentration range of 5-20 µg/mL for PEP, 225-900 µg/mL for PCM, 50-200 µg/mL for GNF, 1-4 µg/mL for CPM and 4-16 µg/mL for BRM**.** Characteristic parameters for regression equations of the HPLC method are given in Table 1.3. Figure 1.3 shows the overlay chromatograms for all the peaks and Figure 1.4 shows the calibration curve plots for all the five components.

Table 1.3 Statistical results of calibration

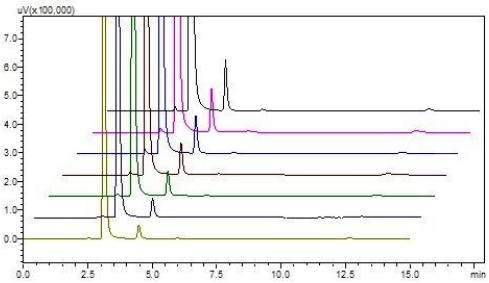
Parameters	**PEP**	**PCM**	**GNF**	**CPM**	**BRM**
Calibration range(µg/mL)	5-20	225-900	50-200	1-4	4-16
Regression equation	y=5722.51 x+2353.41	y=25122.09x +9002.63	y=6312.33x+ 1611.86	y=15356.89x +1421.61	y=11121.77 x+113.76
Regression coefficient (r^2)	0.9994	0.9982	0.9991	0.9992	0.9993



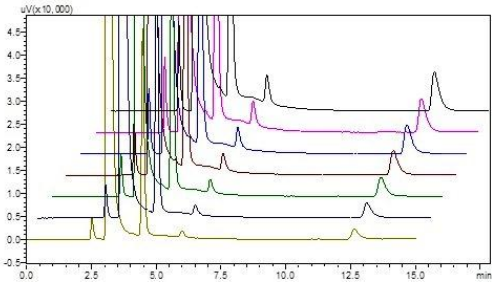
Overlay peaks for PEP (1st peak)



Overlay peaks for PCM (2nd peak)



Overlay peaks for GNF (3rd peak)



Overlay peaks for CPM (4th peak) and BRM (5th peak)

Figure 1.3 Overlay plot of chromatograms

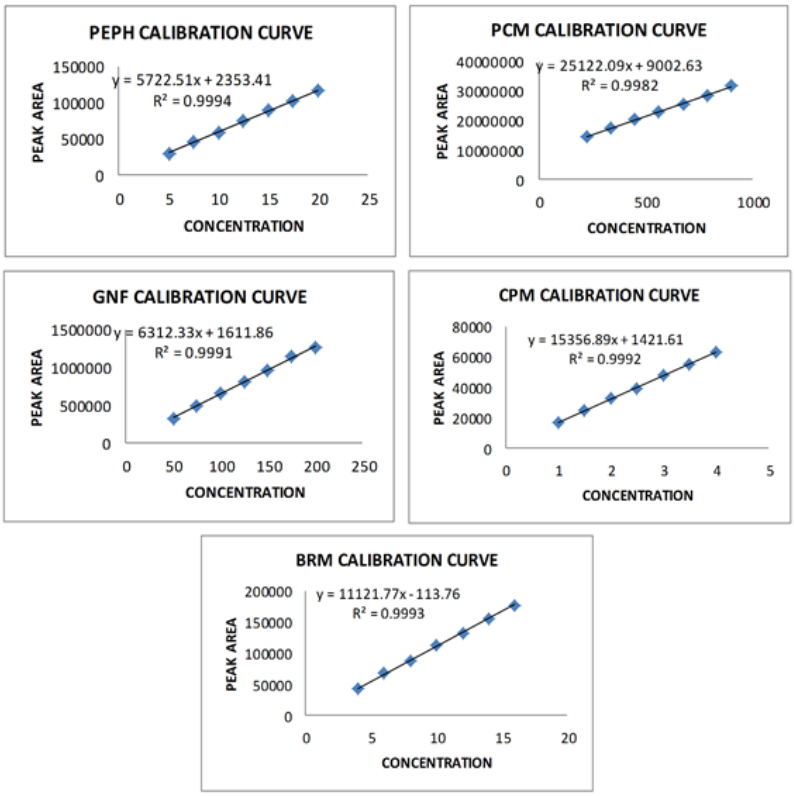


Figure 1.4 Calibration curve plots [Peak area vs Concentration (µg/mL)]

1.5.2.3.2. Precision

The average % RSD (relative standard deviation) of intra-day and inter-day measurements for determination of PEP, PCM, GNF, CPM and BRM are given in Table 1.4 and 1.5 respectively.

Table 1.4 Intra-day precision studies

DRUG CONC.	PEAK AREA			%RSD	MEAN %RSD
			PEP		
5	28921	28911	29519	1.19	
10	58182	59245	58654	0.91	
15	87891	88703	87692	0.60	
					0.90
			PCM		
225	14279132	14575898	14323420	1.11	
450	20933025	20365003	20692873	1.37	
675	26031395	25508496	25927303	1.07	
					1.19
			GNF		
50	334093	322000	330183	1.87	
100	663948	653146	649887	1.12	
150	975473	963721	972653	0.63	
					1.21
			CPM		
1	16153	16526	16281	1.16	
2	32237	32493	32964	1.13	
3	47535	48175	47129	1.11	
					1.13
			BRM		
4	43326	42607	42591	0.98	
8	86990	88585	87393	0.95	
12	130885	132864	131826	0.75	
					0.89

Table 1.5 Inter-day precision studies

DRUG CONC.	PEAK AREA			%RSD	MEAN %RSD
			PEP		
5	28911	29993	29837	1.98	
10	59245	60323	58234	1.76	
15	88703	91273	90263	1.44	
					1.73
			PCM		
225	14575898	14349588	14834756	1.66	
450	20365003	21095867	20932476	1.84	
675	25508496	26138479	25938472	1.24	
					1.58
			GNF		
50	322000	332347	333948	1.97	
100	653146	676948	671329	1.86	
150	963721	984655	993847	1.57	
					1.80
			CPM		
1	16526	16834	17042	1.55	
2	32493	33349	33634	1.79	
3	48175	48234	50234	2.40	
					1.91
			BRM		
4	42607	43464	44334	1.99	
8	88585	89432	91234	1.51	
12	132864	135496	137242	1.63	
					1.71

1.5.2.3.3. Accuracy

The excellent recoveries of standard addition method (Table 1.6) for HPLC suggested good accuracy of the proposed method.

Table 1.6 Recovery from Tablet formulation

Percentage± standard addition				
PEP				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	5	9	10	11
Amount Found (µg/mL)	4.99	8.78	9.85	10.96
Recovery(%) ± S.D.	99.97± 0.46	97.57± 1.16	98.46 ±0.94	99.65 ±1.10
PCM				
% Addition	0	80	100	120
Theoretical Content (µg /mL)	225	405	450	495
Amount Found (µg/mL)	224.54	403.45	449.15	496.87
Recovery(%) ± S.D.	99.79± 0.89	99.6± 0.82	100.99±1.24	100.37±1.01
GNF				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	50	90	100	110
Amount Found (µg/mL)	50.05	89.25	101.32	109.67
Recovery(%) ± S.D.	100.11± 0.94	99.16 ±1.25	101.32±1.15	99.69 ±0.67
CPM				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	1	1.8	2	2.2
Amount Found (µg/mL)	0.99	1.80	2.03	2.19
Recovery(%) ± S.D.	99.62± 0.66	99.91± 0.68	101.39±1.15	99.66± 0.3
BRM				
% Addition	0	80	100	120
Theoretical Content (µg/mL)	4	7.2	8	8.8
Amount Found (µg/mL)	3.97	7.14	7.90	8.71
Recovery(%) ± S.D.	99.25± 0.54	99.18 ±1.07	98.74± 0.94	99.02± 0.6

*Mean value of n=3 replicates for each level of concentrations; S.D. is the standard deviation

1.5.2.3.4. Detection and Quantitation limits (LOD and LOQ)

The LOD and LOQ values are given in Table 1.7 which shows sensitivity of the method.

Table 1.7 LOD and LOQ results

Parameters	PEP	PCM	GNF	CPM	BRM
Detection limit (µg/mL)	0.02	1.34	0.087	0.002	0.05
Quantitation limit (µg/mL)	0.074	4.062	0.265	0.008	0.16

1.5.2.3.5. Robustness

The changes made in various parameters, did not affected the chromatographic resolution in the HPLC method as shown in Table 1.8.

Table 1.8 Robustness results

PARAMETERS		PEP	PCM	GNF	CPM	BRM
pH	2.8	2.58	3.25	4.64	6.02	12.61
	3	2.63	3.23	4.59	6.07	12.60
	3.2	2.57	3.28	4.68	5.99	12.62
	Mean	2.59	3.25	4.63	6.03	12.61
	SD	0.031	0.025	0.044	0.039	0.008
FR	0.9	2.73	3.28	4.64	6.24	12.99
	1	2.63	3.23	4.59	6.07	12.60
	1.1	2.56	3.17	4.43	5.91	12.50
	Mean	2.64	3.23	4.56	6.07	12.69
	SD	0.081	0.057	0.108	0.168	0.257
BUFFER CONC.	49	2.60	3.22	4.61	5.95	12.67
	50	2.63	3.23	4.59	6.07	12.60
	51	2.63	3.27	4.63	6.05	12.74
	Mean	2.62	3.24	4.61	6.02	12.67
	SD	0.020	0.023	0.021	0.064	0.068

1.5.2.3.6. Solution stability

The drug solutions prepared in the mobile phase exhibited no changes in HPLC or UV data for 24 h when kept at room temperature, and for 48 h when stored in refrigerator (8-25ºC). No additional peak was found and no significant decrease in the peak area was found which indicated the stability of the solutions.

1.5.2.3.7. Specificity

The method was found to be specific as the results were unaffected by the presence of the tablet excipients as shown in Figure 1.5.

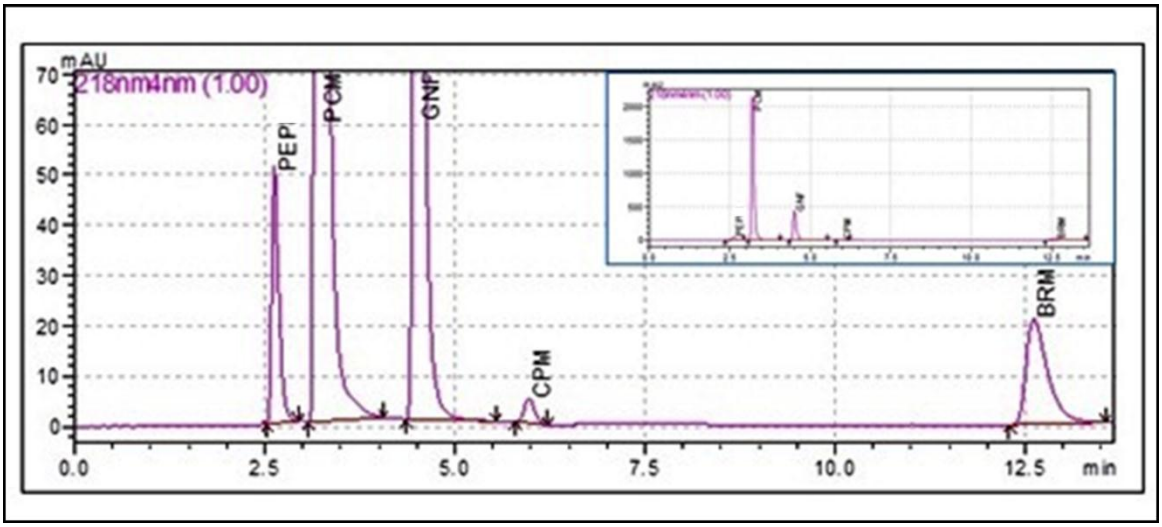


Figure 1.5 HPLC chromatogram of sample solution containing 10 ppm PEP, 450 ppm PCM, 100 ppm GNF, 2 ppm CPM and 8 ppm BRM

1.5.2.3.8. System suitability parameters

Following parameters were calculated for system suitability of HPLC method. Six replicate solutions were injected and their %RSD was found to be less than 2 as shown in Table 1.9. The peak purity results of each peak have been shown in Table 1.10 and the peak purity curves have been shown in Figure 1.6.

Table 1.9 Results for system suitability parameters

Parameters	PEP	PCM	GNF	CPM	BRM
Retention Time	2.63±0.01	3.23±0.005	4.59±0.01	6.07±0.048	12.6±0.13
Theoretical Plates	3600.81±1.23	3772.68±0.95	7847.76±1.71	7248.06±1.38	9194.40±1.54
Tailing factor	1.79±0.04	1.67±0.039	1.51±0.003	1.58±0.031	1.83±0.095
Resolution	--	3.11±0.11	6.51±0.28	5.99±0.17	16.16±0.26

Mean (for n=6 determinations) ±standard deviation

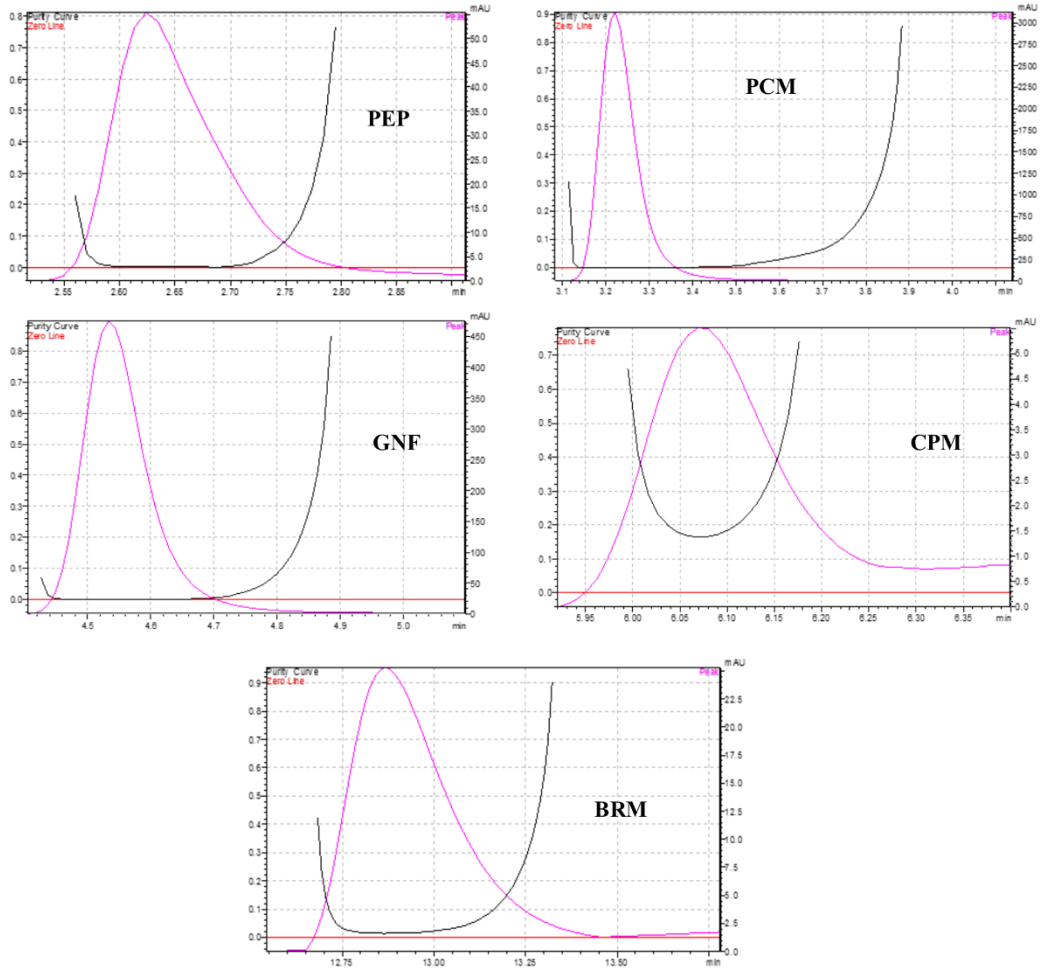


Figure 1.6 Peak purity curves

Table 1.10 Peak purity results

Drug	Peak Purity Index	Peak Threshold
PEP	0.9995	0.9839
PCM	0.9999	0.9999
GNF	1.0000	0.9999
CPM	0.9998	0.5029
BRM	0.9999	0.9658

1.5.2.4. Analysis of tablet formulation

The HPLC method was applied to the simultaneous determination of PEP, PCM, GNF, CPM, and BRM in commercial tablet. The determinations were made in six replicates. Satisfactory results were obtained for each compound in good agreement with the label claims (Table 1.11).

Table 1.11 Determination of PEP, PCM, GNF, CPM and BRM in commercial tablet using the proposed methods

Commercial tablet	Label claim (mg/tablet)	HPLC*
PEP	10	99.48 ± 0.54
PCM	450	98.35± 1.32
GNF	100	101.32± 1.23
CPM	2	98.98±0.76
BRM	8	98.66± 0.68

*Percentage assay ± SD.

1.5.2.5. Dissolution study by HPLC method

The dissolution study was performed for tablet formulation and the samples collected at specific time intervals were analysed by the HPLC method. The results are shown in Table 1.12 which indicated that all the five drugs showed percentage release above 90% in 60 minutes. The dissolution profile is shown in Figure 1.7.

Table 1.12 Dissolution data for HPLC method

HPLC (PERCENTAGE RELEASE)					
TIME (min)	**PCM**	**PEP**	**GNF**	**CPM**	**BRM**
5	23.80 ± 3.77	22.94 ± 2.98	17.90 ±1.71	14.43 ± 1.77	16.89 ±1.54
10	35.02 ± 2.35	50.21 ± 3.29	29.50 ± 1.80	27.01 ± 1.52	25.48 ± 1.15
15	55.98 ± 2.57	85.76 ± 2.23	31.47 ± 1.24	53.42 ± 1.44	38.60 ± 1.15
30	83.19 ± 2.23	87.53 ± 1.64	69.35 ± 1.90	86.71 ± 1.39	57.17 ± 1.40
45	92.98 ± 2.01	90.55 ± 1.68	87.31 ± 1.58	88.50 ± 1.32	73.04 ± 1.66
60	93.99 ± 1.87	93.63 ± 1.38	91.56 ± 1.37	91.31 ± 1.24	92.76 ± 1.16
90	94.64 ± 1.48	95.88 ± 1.24	94.52 ± 1.67	94.88 ± 1.16	94.34 ± 1.06

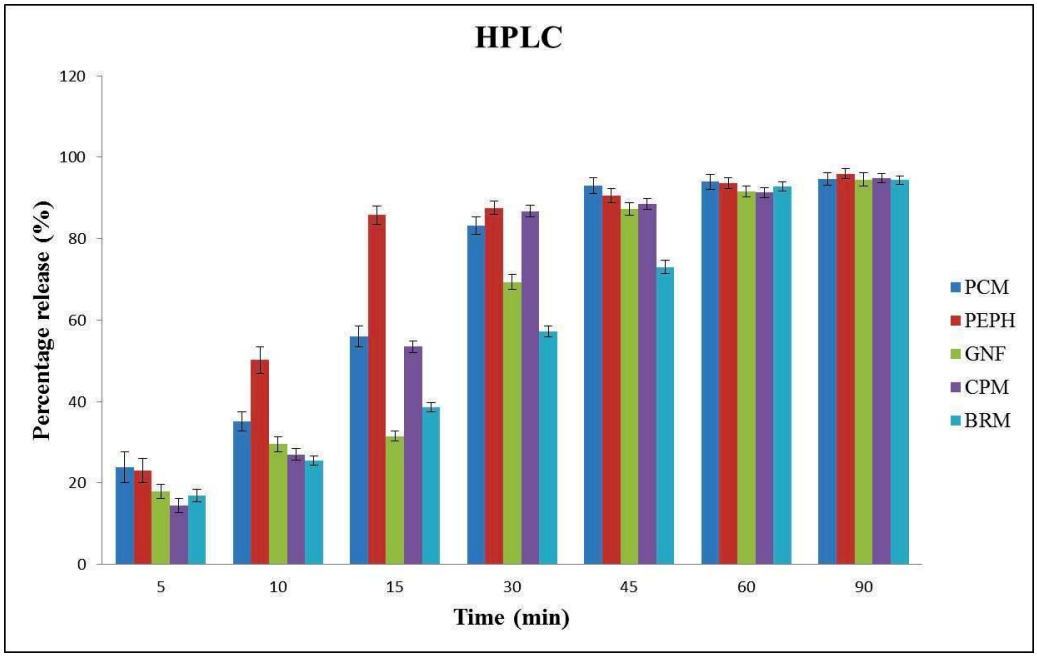


Figure 1.7 Dissolution plots for PEP, PCM, GNF, CPM and BRM analysed by HPLC method

1.6. METHOD 1B. DEVELOPMENT OF CHEMOMETRIC ASSISTED SPECTRO-PHOTOMETRIC METHOD FOR THE SIMULTANEOUS ESTIMATION OF PEP, PCM GNF, CPM AND BRM AND ITS APPLICATION TO DISSOLUTION STUDY

1.6.1. EXPERIMENTAL

1.6.1.1. Instrumentation

Shimadzu UV-1700 double beam spectrophotometer connected to a computer loaded with the Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were measured in 1cm quartz cells over the range of 200-400 nm. PLS and PCR analysis were carried out by using trial version of Unscrambler® software version 2013. The dissolution was performed on Veego VDA6DR dissolution apparatus.

1.6.1.2. Experimental conditions

The UV absorption spectra of appropriate solutions (in MeOH) were recorded in the wavelength range 200-400 nm. The range of 210-320 nm with the intervals of 2 nm ($\Delta\lambda$ = 2 nm) was selected for PLS and PCR model.

1.6.1.3. Preparation of standard solutions

Stock solutions of 1000 ppm each of PEP, PCM, GNF, CPM and BRM were prepared in MeOH. From that the working stock solutions of 100 ppm each of PEP, PCM, GNF, CPM and BRM were prepared in MeOH.

1.6.1.4. Preparation of mixture solutions for chemometrics

From the working stock solution, appropriate aliquots were taken and the final mixture solutions were prepared in the range of 3-15 µg/mL PEP, 4-15 µg/mL PCM, 3-15 µg/mL GNF, 5-15 µg/mL CPM and 4-15 µg/mL BRM for chemometric study. A training set of 26 synthetic mixtures (Table 1.13) and validation set of 14 mixtures (Table 1.14) with different concentrations of each compound were prepared. The UV absorption spectra were recorded over the range 200-400 nm.

Table 1.13 Training set

MIXTURE	PEP	GNF	CPM	PCM	BRM
1	15	1	5	7	12
2	15	5	8	11	2
3	15	12	1	3	9
4	1	15	5	7	12
5	11	15	1	3	9
6	1	5	15	7	12
7	7	12	15	1	6
8	11	1	15	3	9
9	1	5	8	15	12
10	3	8	12	15	2
11	11	1	5	15	9
12	7	12	1	3	15
13	3	5	5	3	6
14	7	8	8	7	9
15	1	5	5	3	6
16	1	8	8	7	9
17	3	1	5	3	6
18	7	1	8	7	9
19	7	8	1	7	9
20	3	5	5	1	6
21	3	5	5	3	1
22	7	8	8	7	1
23	11	12	8	7	9
24	7	8	12	11	9
25	7	8	8	11	12
26	11	8	8	7	12

Table 1.14 Validation set

MIXTURE	PEP	GNF	CPM	PCM	BRM
1	3	5	3	10	6
2	3	5	3	15	6
3	3	5	3	6	12
4	3	5	3	6	15
5	7	8	7	15	9
6	4	4	4	9	4
7	7	8	7	6	6
8	7	8	3	6	9
9	7	3	15	8	9
10	15	3	5	4	4
11	3	15	11	6	4
12	11	4	4	8	6
13	3	11	4	6	6
14	3	12	4	8	6

1.6.1.5. Analysis of tablet formulation

Twenty commercial tablets were accurately weighed and powdered. An amount of powder equivalent to 45 mg of PCM, 0.8 mg of BRM, 10 mg GNF, 1 mg PEP and 0.2 mg of CPM was taken in 25 mL of volumetric flask. To this powder standard addition of 20 mg PEP, 20 mg CPM and 20 mg BRM was done owing to their too much low quantity in the formulation (as they were below LOD of the UV range and hence difficult to determine). This powder mixture was dissolved in sufficient MeOH, sonicated for 10 min and diluted up to 25 mL with MeOH. The solution was filtered through Whatmann filter paper (No. 42) so as to remove undissolved tablet excipients. From this filtered solution 0.1 mL aliquot was taken and was further diluted to 10 mL with MeOH. This solution was analysed by UV-chemometric models for PLS and PCR.

1.6.1.6. Dissolution study

The dissolution was carried out by USP-II paddle method. The dissolution media comprised of 0.01 N HCl prepared in single distilled water. The conditions for dissolution were 50 rpm at 37ºC temperature for the duration of 90 min. The sampling was done at different time intervals of 5, 10, 15, 30, 45, 60 and 90 min. 0.1 mL aliquot was taken in 10 mL volumetric flask followed by the standard addition of 1 mL (10µg/mL) of PEP, CPM and BRM each. The solution was made up to the mark with MeOH, filtered through 0.2 µ membrane filter and analysed by UV spectrophotometer.

1.6.2. RESULT AND DISCUSSION

The simultaneous determination of PEP, PCM, GNF, CPM, and BRM in tablet by conventional spectrophotometric methods is hindered by strong spectral overlap throughout the wavelength range and also the wide difference in the ratio of APIs in the formulation. Simple chemometric techniques such as CLS and ILS were not suitable for the analysis of this combination as model could not be developed due to complex overlapped spectra and comparatively larger data size. Generally ILS and CLS are suitable for combination upto three components but beyond that as the data complexity increases the methods don't prove to be promising. PLS and/or PCR calibration methods are necessary for such determination due to the presence of this spectral interference.

1.6.2.1. PCR method (29)

PCR is the method which works on the principal of reducing the dimensionality of the original data. Absorbance matrix and concentration matrix as shown above were generated and data was fed to software. The absorbance matrix (X) used for calibration contains total 51 variables i.e. wavelengths at which absorbance values are measured. PCR will compute a few PCs and will perform regression of these PCs with concentration (Y). The algorithm used for PCR was NIPALS i.e. nonlinear iterative partial least squares. Validation was set as full cross validation. The data of absorbance values at 51 wavelengths were used as X space (predictors) and the data containing concentrations of all five components in 26 calibration standards were used as Y space (responses).

1.6.2.2. PLS method (29)

PLS computes factors for X and Y both and then correlates them. It models both the X- and Y-matrices simultaneously to find the latent variables in X that will best predict the latent variables in Y. Full cross validation method is used for determining the optimum number of factors. The algorithm used for PLS was NIPALS i.e. nonlinear iterative partial least squares.

The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used. The spectral resolution was assayed with absorbance spectra for PLS and PCR methods, measured at 2 nm intervals over the range 210–320 nm. Wavelengths less than 210 nm were rejected as they were not found to be of much significant contribution for determining the concentration of the five components. Wavelengths more than 320 nm were not used because all the five drugs do not absorb in this region; so any absorbance values obtained below 210 nm and above 320 nm were not used as these wavelengths would have introduced a significant amount of noise in the calibration matrix, thereby decreasing the precision.

The predicted concentrations of the components in each sample were compared with the actual concentrations in the training samples and the root mean square error of prediction (RMSEP) was calculated for each method. The RMSEP value was used as a diagnostic test for examining the errors in the predicted concentrations.

Due to the wide difference in the ratio of APIs in the marketed formulation (i.e. PEP: PCM: GNF: CPM: BRM = 5: 225: 50: 1: 4), a standard addition of some components (i.e. PEP, CPM and BRM) was required, to bring them at the proper the quantitation limit of UV range.

For PCR and PLS methods, 26 calibration spectra were used for the selection of the optimum number of factors by using the cross validation technique. This allowed modelling of data with the optimum amount of information and avoidance of overfitting or underfitting. The cross-validation procedure consists of systematically removing one of a group of training samples in turn and using only the remaining ones for the construction of latent factors and applied regression.

1.6.2.3. Determining Optimum Number of Principal Factors for PLS andPCR and Model Validation

The number of factors to be taken into account was determined by full cross validation method and following parameters were considered:

Total residual variance is computed as the sum of squares of the residuals for all the variables, divided by the number of degrees of freedom.

Total explained variance is computed as:

Explained variance=100*(initial variance - residual variance)/ (initial variance).

It is the percentage of the original variance in the data which is taken into account by the model. The model should explain most of the variance. Models with small (close to 0) total residual variance or large (close to 100%) total explained variance explain most of the variation in Y. For this, number of PCs should be optimized. Normally, 2-3 PCs will explain nearly (not exactly) 100% of variance in data.

The software validates the model by full cross validation method, where one sample from the calibration set is left out each time and model is calibrated using remaining samples. Then the prediction is made for left out sample and its residual is calculated. The same process is repeated until each sample is left out once. There were total 26 segments for validation, because there were 26 calibration standards or samples.

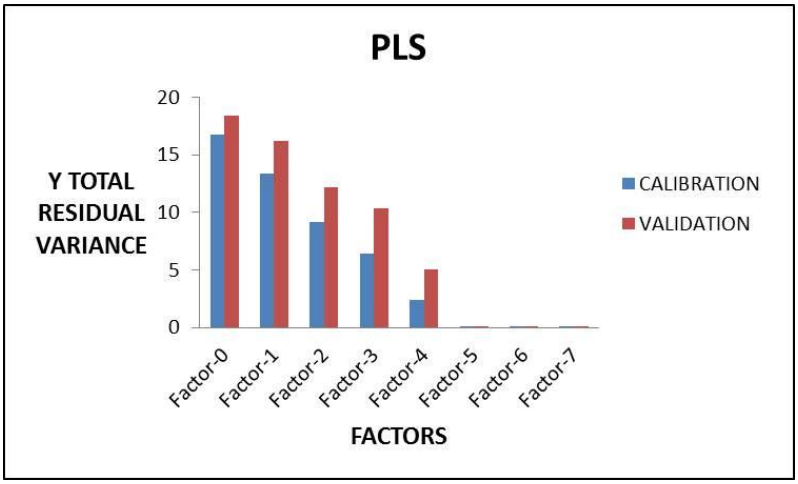
Finally, from the plots of residual variance (Figure 1.8) and explained variance (Figure 1.9) (for calibration and validation set) versus number of PCs (for PCR) and number of latent factors (for PLS) respectively, we can find the optimum number of PCs and latent factors. Once the model is calibrated with optimum number of PCs, the model can predict the unknown concentration from its absorbance data. Maximum number of PCs (and factors) was fixed to 7 and the parameters are discussed in Table 1.15 for PLS and Table 1.16 for PCR.

Table 1.15 Table for Y total residual values and Explained variance for PLS model

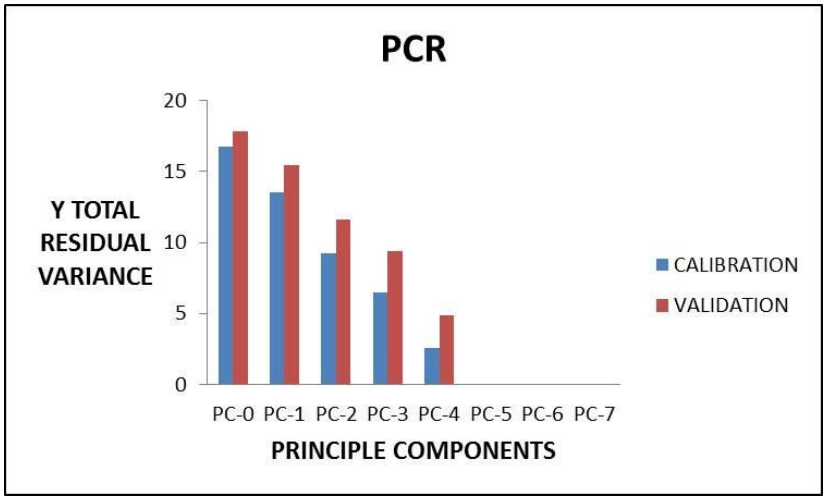
	Y total residual variance		Explained variance	
	Calibration	**Validation**	**Calibration**	**Validation**
Factor-0	16.79	18.38	0	0
Factor-1	13.42	16.18	77.24	74.03
Factor-2	9.20	12.20	98.41	98.1
Factor-3	6.39	10.35	99.46	99.11
Factor-4	2.38	5.09	99.81	99.59
Factor-5	1.56E-02	2.53E-02	99.99	99.99
Factor-6	7.84E-03	1.50E-02	99.99	99.99
Factor-7	5.57E-03	1.52E-02	99.99	99.99

Table 1.16 Table for Y total residual values and Explained variance for PCR model

	Y total residual variance		Explained variance	
	Calibration	**Validation**	**Calibration**	**Validation**
Factor-0	16.79	17.86	0	0
Factor-1	13.50	15.47	19.58	13.37
Factor-2	9.20	11.63	45.19	34.90
Factor-3	6.48	9.40	61.43	47.35
Factor-4	2.59	4.84	84.59	72.88
Factor-5	1.56E-02	2.53E-02	99.91	99.86
Factor-6	7.98E-03	1.46E-02	99.95	99.92
Factor-7	7.40E-03	1.49E-02	99.96	99.92

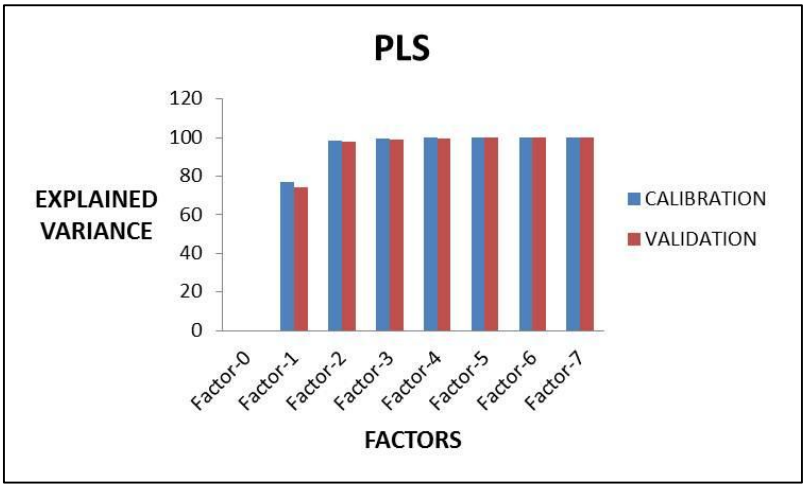


(A)

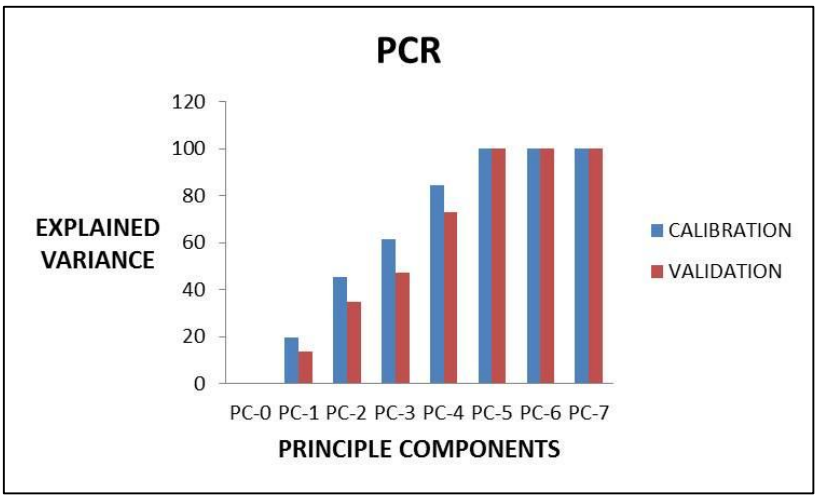


(B)

Figure 1.8 Y total residual variance plots of PLS model (A) and PCR model (B) for PEP, PCM, GNF, CPM and BRM



(A)



(B)

Figure 1.9 Total explained Y-variance plots of PLS model (A) and PCR model (B) for PEP, PCM, GNF, CPM and BRM

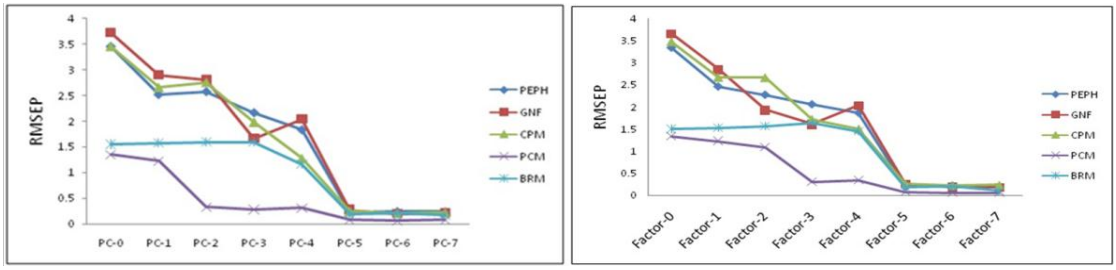
1.6.2.4. RMSEP value

At the selected principal components (for PCR) and factors (for PLS), the concentration of each sample was then predicted and compared with known concentration (actual value). The PRESS (prediction residual error sum of squares) value was calculated as the difference between the real and the calculated concentrations, squared and summed, over all references for each component (equation 1). The RMSEP value (root mean squares error of prediction) was calculated by using equation 2. The selected model was that with the fewest number of factors such that its RMSEP values were not significantly greater than that for the

model. A plot of RMSEP values against number of components is shown in Figure 1.10. The RMSEP values are indicated in Table 1.17.

$$\text{PRESS} = \sum_{i=1}^{n} \left(C_i^{Added} - C_i^{Found} \right)^2 \quad (1)$$

$$\text{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n} \left(C_i^{Added} - C_i^{Found} \right)^2}{n}} \quad (2)$$



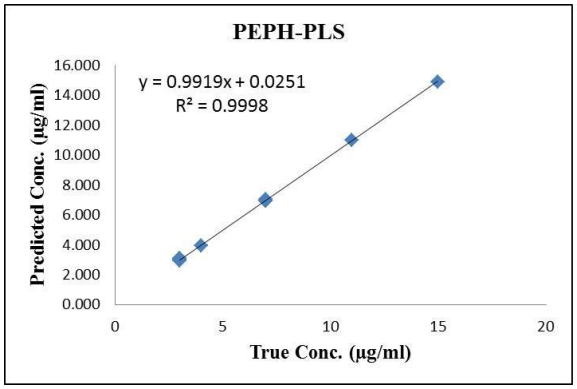
(A) (B)

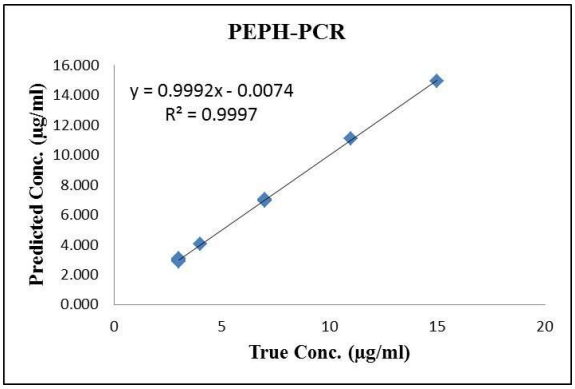
Figure 1.10 RMSEP plot of a calibration set prediction using cross-validation of PCR model (A) PLS model (B) and for PEP, PCM, GNF, CPM and BRM

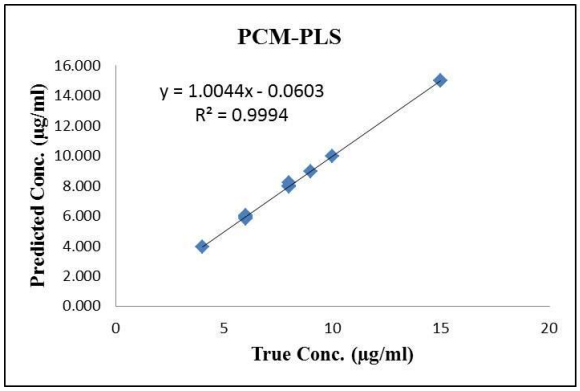
1.6.2.5. Validation of chemometric methods

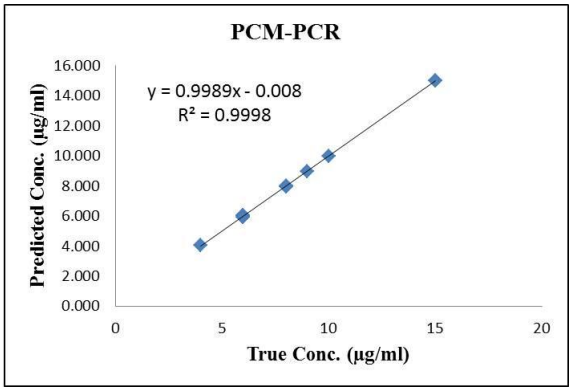
1.6.2.5.1. Model accuracy

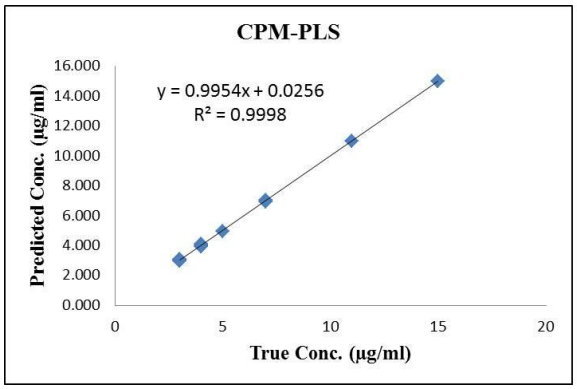
The predictive ability i.e. the validation of PCR and PLS models was thus assessed by the PRESS value, RMSEP value and residual values of actual concentration and predicted concentration (positive difference of actual and predicted values) (27). The plots of predicted versus true concentrations and the residual values versus true concentration have been shown for both methods in Figure 1.11 and 1.12 resp. The results are shown in Table 1.17. A satisfactory value of regression coefficient as well as a very small range of residual values showed good predictive ability of the chemometric models.

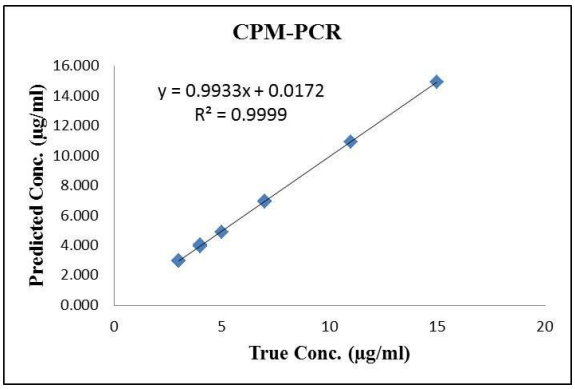

PEPH-PLS
y = 0.9919x + 0.0251
R² = 0.9998
Predicted Conc. (µg/ml)
True Conc. (µg/ml)

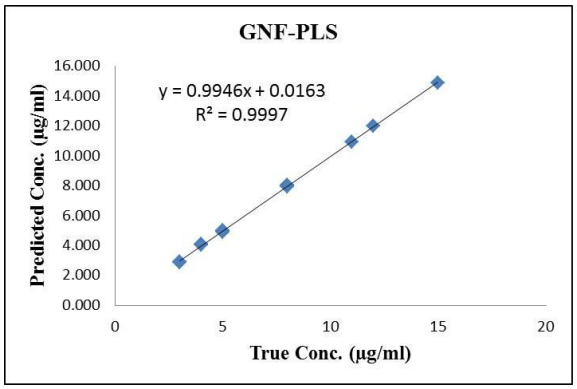

PEPH-PCR
y = 0.9992x - 0.0074
R² = 0.9997
Predicted Conc. (µg/ml)
True Conc. (µg/ml)

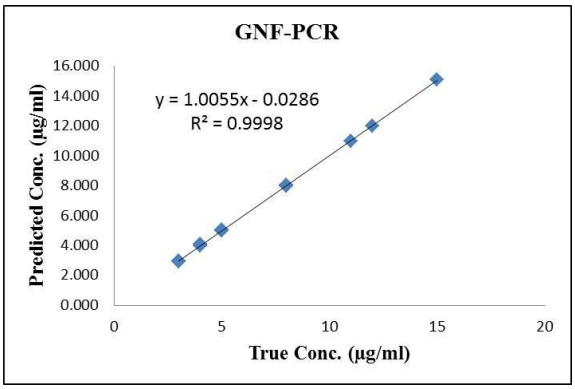

PCM-PLS
y = 1.0044x - 0.0603
R² = 0.9994
Predicted Conc. (µg/ml)
True Conc. (µg/ml)

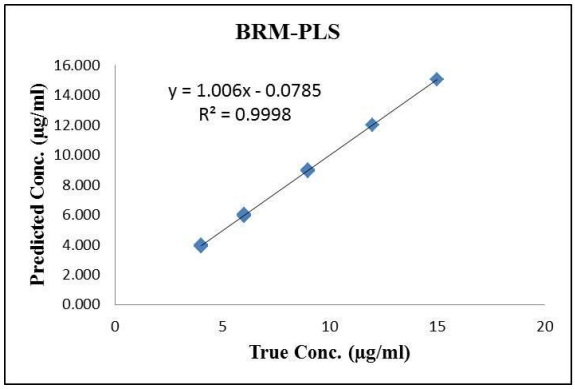

PCM-PCR
y = 0.9989x - 0.008
R² = 0.9998
Predicted Conc. (µg/ml)
True Conc. (µg/ml)


CPM-PLS
y = 0.9954x + 0.0256
R² = 0.9998
Predicted Conc. (µg/ml)
True Conc. (µg/ml)


CPM-PCR
y = 0.9933x + 0.0172
R² = 0.9999
Predicted Conc. (µg/ml)
True Conc. (µg/ml)


GNF-PLS
y = 0.9946x + 0.0163
R² = 0.9997
Predicted Conc. (µg/ml)
True Conc. (µg/ml)


GNF-PCR
y = 1.0055x - 0.0286
R² = 0.9998
Predicted Conc. (µg/ml)
True Conc. (µg/ml)



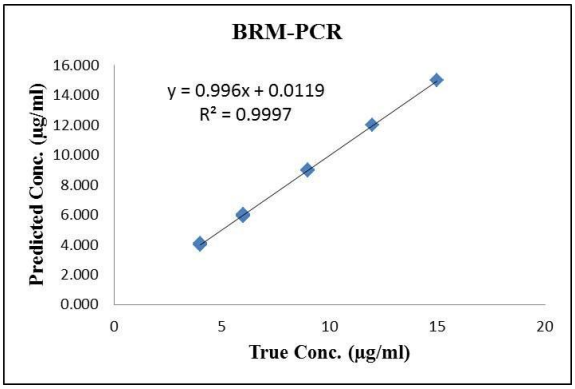
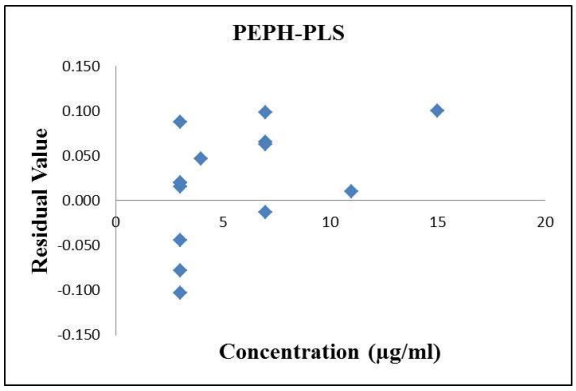
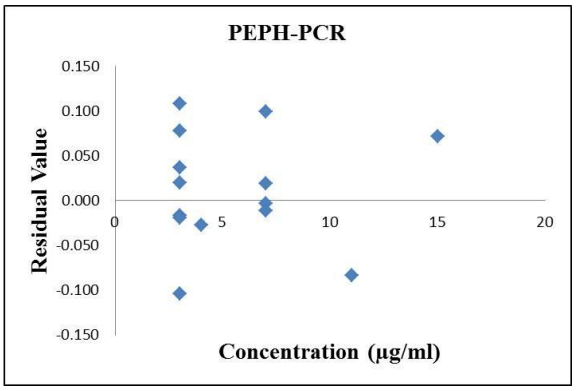
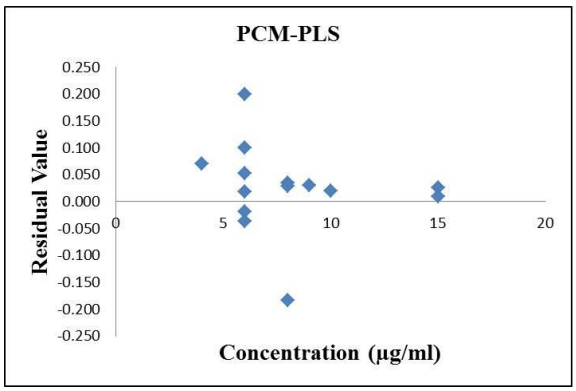
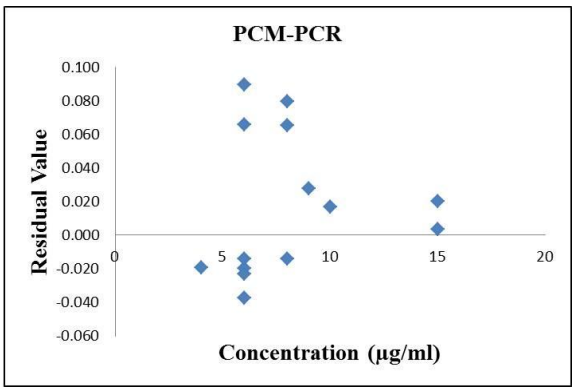


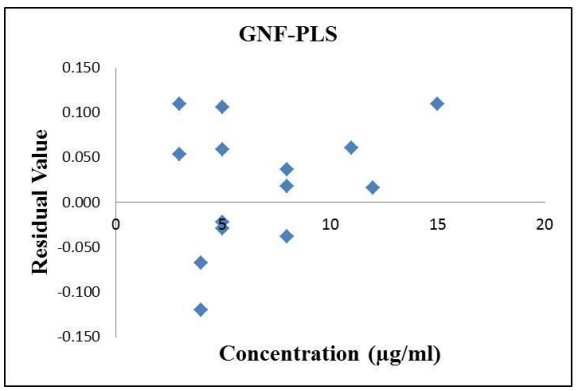
Figure 1.11 Calibration plots of predicted versus actual (true) concentrations

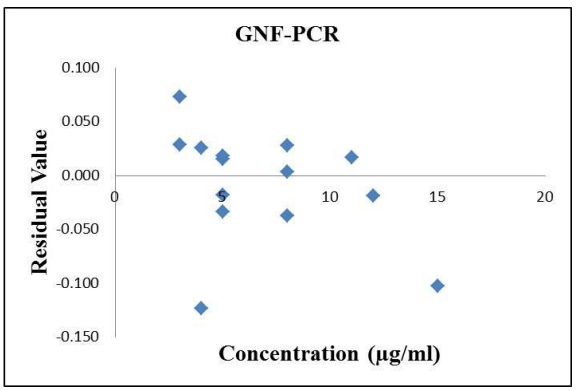












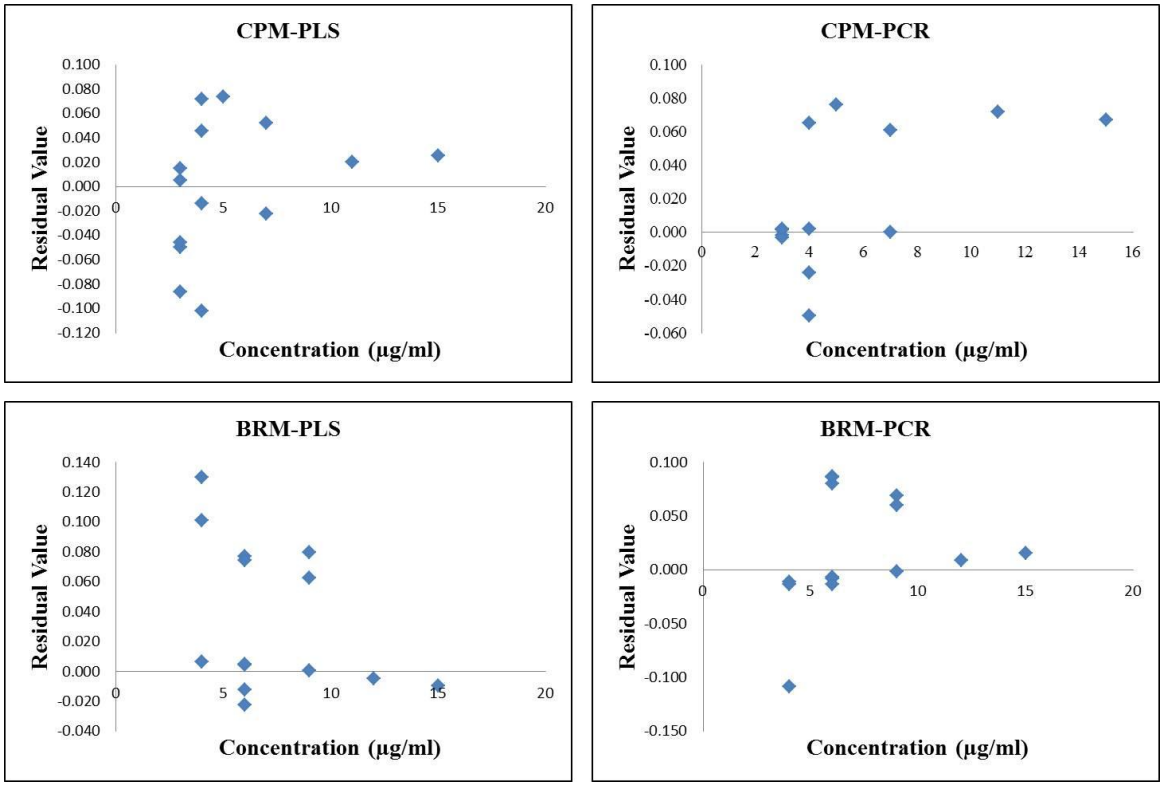


Figure 1.12 Plots of true concentration values versus the residual values

1.6.2.5.2. Accuracy

The accuracy of the chemometric methods was performed by standard addition method at three levels (80%, 100%, and 120%). The resulting mixtures were analysed and results obtained were compared with the true values as shown in shown in Table 1.17. The influence of the commonly used tablet excipients was investigated before the determination of the studied compounds in tablet. No interference could be observed with the proposed methods.

1.6.2.5.3. Precision

The precision of PLS and PCR method was performed at three concentration levels for each compound. The solutions were prepared and analysed three times in a day for intra-day precision and on three different days for inter-day precision. The average % RSD of intra-day and inter-day measurements for determination of PEP, PCM, GNF, CPM and BRM are given in Table 1.17.

1.6.2.5.4. Solution stability

The stability of the solutions prepared in MeOH (for chemometric study), exhibited no spectrophotometric changes for seven days in refrigerator (8-25 ºC).

Table 1.17: Characteristic parameters of the proposed PLS and PCR method for simultaneous determination of PEP, PCM, GNF, CPM and BRM

Parameters		PEP	PCM	GNF	CPM	BRM
RMSEP	PLS	0.034	0.022	0.047	0.037	0.0047
	PCR	0.019	0.019	0.017	0.0015	0.0081
$R^{2\#}$	PLS	0.9997	0.9994	0.9996	0.9997	0.9998
	PCR	0.9997	0.9998	0.9998	0.9999	0.9996
Regression Equation#	PLS	y = 0.991x + 0.025	y = 1.004x - 0.060	y = 0.994x + 0.016	y = 0.995x + 0.025	y = 1.006x - 0.078
	PCR	y = 0.999x - 0.007	y = 0.998x - 0.008	y = 1.005x - 0.028	y = 0.993x + 0.017	y = 0.996x + 0.011
Accuracy*		Mean % Recovery ±SD				
80%	PLS	99.65±0.779	100.37±0.573	100.11±0.61	99.77±0.22	100.28±0.40
	PCR	99.64±0.56	99.88±0.577	99.44±0.49	100.46±0.81	100.08±0.42
100%	PLS	100.37±0.83	99.78±0.64	99.65±0.27	100.42±0.56	99.83±1.14
	PCR	99.62±0.78	100.17±0.34	100.38±0.86	100.83±0.72	100.44±0.80
120%	PLS	99.94±0.18	100.09±0.32	99.96±0.41	100.17±0.49	100.37±0.55
	PCR	99.97±0.48	99.85±.67	99.98±0.41	99.81±0.51	100.20±0.45
Precision$		Mean % Recovery ±SD				
Intraday	PLS	99.67±0.59	99.33±1.16	98.88±0.77	99.59±0.56	99.36± 0.61
	PCR	99.61±0.41	99.02±0.77	98.96±0.74	99.61±0.61	99.89 ±0.98
Interday	PLS	99.67±0.85	98.93±1.45	99.66±1.11	101.18 ±0.72	99.59±1.39
	PCR	99.31±0.79	99.26±1.53	100.65± 0.89	101.71 ±0.76	99.31±1.14

#R^2 and regression equation for the predicted concentration versus true concentration plot

$ Mean-value of three determinations of three concentrations

*Mean recovery of the predicted concentrations at 80%, 100% and 120% level of standard addition

1.6.2.6. Analysis of tablet formulation

The proposed PLS, PCR methods were applied to the simultaneous determination of PEP, PCM, GNF, CPM, and BRM in commercial tablet. The determinations were made in six replicates. Satisfactory results were obtained for each compound in good agreement with the label claims (Table 1.18).

Table 1.18 Determination of PEP, PCM, GNF, CPM and BRM in commercial tablet using the proposed methods

Commercial tablet	Label claim (mg/tablet)	PLS*	PCR*
PEP	10	99.07 ± 0.99	98.7 ± 1.22
PCM	450	100.63 ± 0.98	99.34 ± 0.71
GNF	100	99.51 ± 0.89	100.17 ± 0.65
CPM	2	98.8 ± 1.19	99.4 ± 0.71
BRM	8	98.9 ± 1.20	98.48 ± 1.31

*Percentage assay ± SD.

1.6.2.7. Dissolution study by chemometric methods

The dissolution study was performed for tablet formulation and the samples collected at specific time intervals were analysed by the two methods i.e. PCR and PLS. The results showed that all the five drugs showed percentage release above 85% in 60 minutes. The results are shown in Table 1.19 and 1.20 for PLS and PCR methods resp. The dissolution profiles for all the three methods are shown in Figure 1.13 and 1.14.

Table 1.19 Dissolution data for PLS method

PLS (PERCENTAGE RELEASE)					
TIME (min)	**PCM**	**PEP**	**GNF**	**CPM**	**BRM**
5	22.09 ± 4.57	19.18 ± 4.18	17.33 ± 1.75	13.35 ± 1.72	16.56 ± 1.65
10	31.78 ± 2.81	42.60 ± 4.02	24.60 ±1.62	24.35 ± 1.60	20.21 ± 1.13
15	53.45 ± 3.00	82.18 ± 3.40	29.57 ± 1.25	51.75 ± 1.78	31.97 ± 1.23
30	79.58 ± 2.60	85.18 ± 3.10	64.45 ± 2.05	84.75 ± 1.60	53.83 ± 1.48
45	90.12 ± 2.99	88.88 ± 2.90	83.60 ± 2.20	86.48 ± 1.57	62.58 ± 1.61
60	91.07 ± 2.30	91.05 ± 2.18	88.71 ± 2.05	89.31 ± 1.32	89.86 ± 1.63
90	92.18 ± 1.55	92.51 ± 1.66	91.85 ± 1.72	90.77 ± 1.05	92.28 ± 1.19

Table 1.20 Dissolution data for PCR method

PCR (PERCENTAGE RELEASE)					
TIME (min)	**PCM**	**PEP**	**GNF**	**CPM**	**BRM**
5	21.89 ± 4.28	19.57 ± 4.35	16.39 ± 2.53	13.07 ± 1.53	16.33 ± 1.61
10	31.98 ± 3.15	42.87 ± 4.32	24.19 ± 1.85	23.86 ± 1.85	20.08 ± 1.40
15	53.46 ± 2.84	81.74 ± 3.98	29.06 ± 1.29	50.85 ± 1.72	32.13 ± 1.05
30	78.93 ± 2.93	84.01 ± 3.34	64.18 ± 2.22	84.72 ± 1.91	53.52 ± 1.58
45	89.71 ± 2.64	88.20 ± 3.16	83.30 ± 2.68	86.39 ± 2.04	63.32 ± 2.10
60	90.85 ± 2.26	91.01 ± 2.08	88.28 ± 2.34	88.71 ± 1.99	90.39 ± 1.43
90	91.97 ± 1.77	92.33 ± 1.77	92.01 ± 2.18	91.22 ± 1.37	92.61 ± 1.25

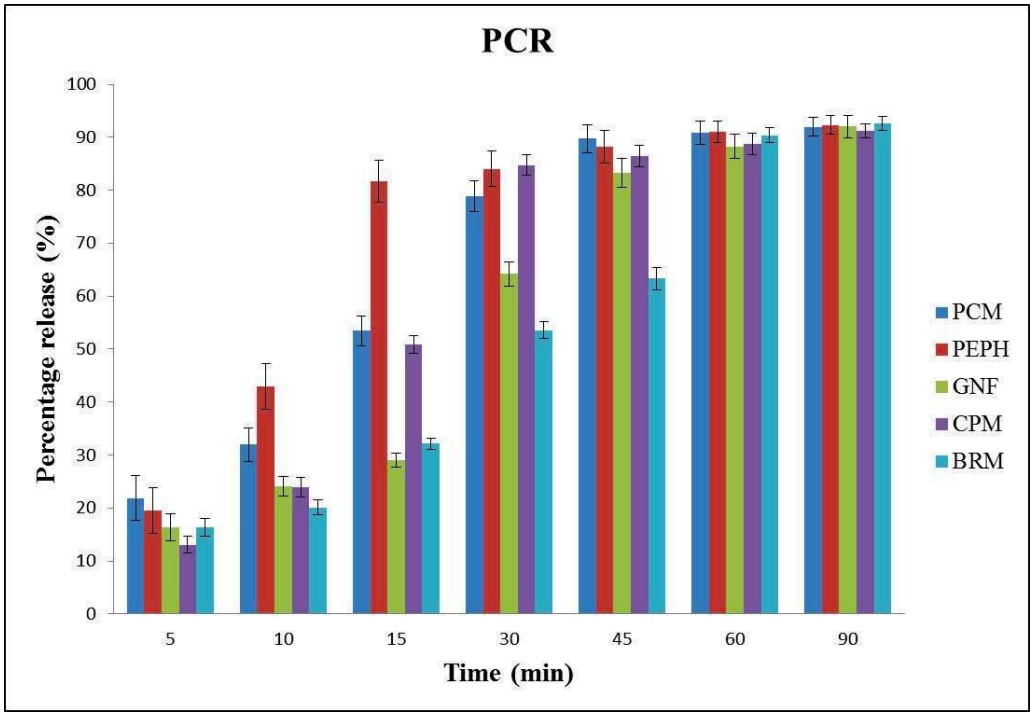


Figure 1.13 Dissolution plots for PEP, PCM, GNF, CPM and BRM analysed by PCR method

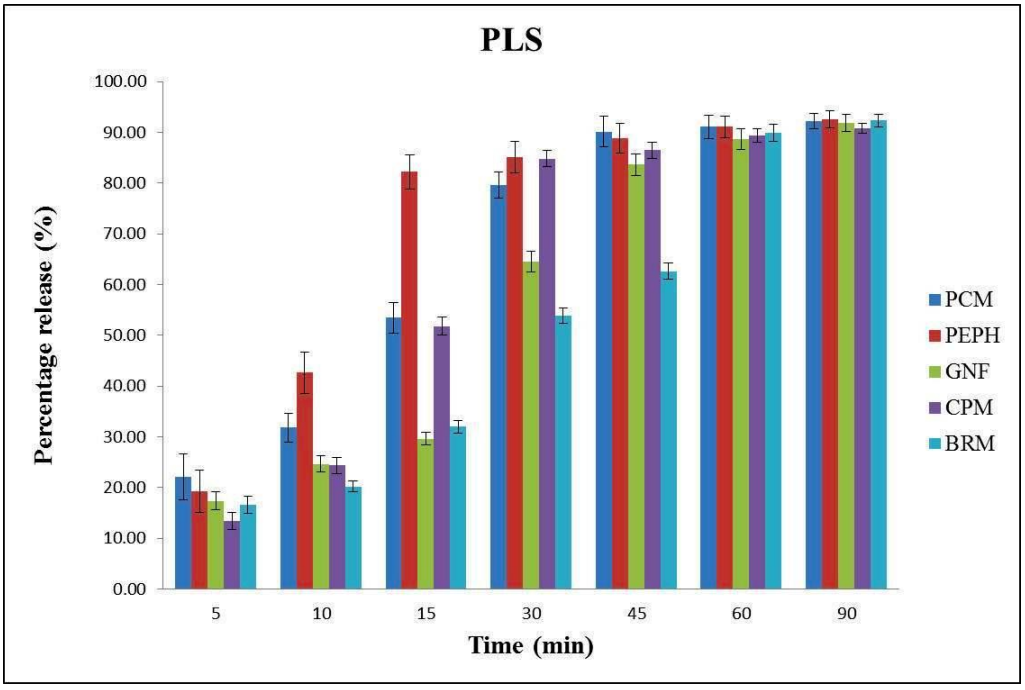


Figure 1.14 Dissolution plots for PEP, PCM, GNF, CPM and BRM analysed by PLS method

1.7. STATISTICAL COMPARISON OF THE THREE METHODS

The results of the assay obtained from the PLS and PCR methods were compared with those of the RP-HPLC method. Statistical comparison between the results of the two chemometric methods with respect to HPLC method was performed using ANOVA, and Student's *t*-test value and *F*-ratio were determined at 95% confidence level. There was no significant difference between the results as stated in Table 1.21.

Table 1.21 Statistical evaluation for the three analytical methods

	Methods	PEP	PCM	GNF	CPM	BRM
**F-value	PLS	0.18	3.76	0.25	3.24	0.89
	PCR	0.13	1.53	0.77	3.25	0.60
**t-value	PLS	0.43	1.94	0.50	1.80	0.95
	PCR	0.35	0.12	0.87	1.27	0.78

**Theoretical values for F and t are 4.96 and 2.23 respectively.

1.8. CONCLUSION

The chemometric assisted spectrophotometric methods (PLS and PCR) and RP-HPLC method have been proposed and successfully applied for the simultaneous determination of PEP, PCM, GNF, CPM, and BRM in their commercial tablet formulation. The assay and dissolution results obtained by chemometric methods were found to be in a good coincidence with that of HPLC method. The dissolution results showed that the release of the five drugs by all three methods was above 85% in 60 min. The HPLC method is more specific than the chemometric assisted spectrophotometric methods, but it needs expensive equipment and materials such as columns and HPLC grade solvents. Chemometric methods are less expensive and do not require sophisticated instrumentation and any prior separation step. This can be considered as an advantage of the chemometric techniques over HPLC. The methods thus developed i.e., PLS, PCR and HPLC, were found to be suitable and can be successfully used for the determination of the PEP, PCM, GNF, CPM and BRM in pharmaceutical tablet formulation as well as for dissolution study.

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