DEVELOPMENT AND VALIDATION OF CHEMOMETRICS - ASSISTED SPECTROPHOTOMETRY AND LIQUID CHROMATOGRAPHY FOR THE SIMULTANEOUS DETERMINATION OF SOME NOVEL DRUGS IN PHARMACEUTICALS

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Development and validation of chemometrics - assisted spectrophotometry and liquid chromatography for the simultaneous determination of some novel drugs in pharmaceuticals"** is a record of research work done by **D. NAGAVALLI** at C.L. Baid Metha College of Pharmacy, Chennai - 600 096 under my supervision during the years 2005-2010 and this thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title. I also certify that the thesis represent work done by the candidate and that this has not formed in part or fully the basis for the award of any other previous research degree.

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CONTENT

LIST OF ABBREVIATIONS USED

% RSD	-	Percentage Relative Standard Deviation
%	-	Percentage
µg	-	Microgram
µl	-	Microlitre
λ_{max}	-	absorption maximum
a	-	Number of PCs
A_f	-	Asymmetry factor
AR	-	Analytical reagent
BP	-	British Pharmacopoeia
CAS NO	-	Chemical Abstracts Series Number
CDS	-	Chromatographic data system
CLS	-	Classical least squares
CV	-	Cross validation
E	**-**	Residual matrix
eik	-	Residual of observation k for variable i
Eur.P	-	European Pharmacopoeia
g	-	Grams
GC/MS	-	Gas Chromatography/ Mass Spectroscopy
HPLC	-	High Performance Liquid Chromatography
HPTLC	-	High performance thin layer chromatography
Int.P	-	International Pharmacopoeia
IP	-	Indian Pharmacopoeia

L	-	litre
LC/MS	-	Liquid Chromatography/ Mass Spectroscopy
LOD	-	Limit of Detection
LOQ	-	Limit of Quantification
m	-	Number of observations
mg	-	milligram
mL	-	milliliter
MLR	-	Multiple linear regression
n	-	Number of variables
N	-	Number of theoretical plates
NIPALS	-	Non-linear iterative partial least squares
nm	-	nanometer
^{o}C	-	Degree Centigrade
OSC	-	Orthogonal signal correction
p	-	Loading vector
PARAFAC	-	Parallel factor analysis
PC	-	Principal component
PCA	-	Principal component analysis
PCR	-	Principal component regression
PLS	-	Partial least squares regression
PRESS	-	Prediction error sum of squares
r	-	Regression coefficient
RMSEP	-	Root mean square error of prediction
RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
RS	-	Chromatographic resolution

Rs	-	Resolution
RSD	-	Relative standard deviation
RSEP	-	Relative standard error of prediction
s	-	Standard deviation
s0	-	Pooled residual standard deviation
SD	-	Standard Deviation
SS	-	Sum of squares
t	-	Score vector
TEA	-	Tri Ethyl Amine
T_f	-	Tailing factor
USP	-	United States Pharmacopoeia
UV-Vis	-	Ultraviolet-visible
X	-	Matrix of x-variables
Y	-	Matrix of dependent y-variables
β	-	Regression coefficient in experimental design
λ	-	Eigenvalue

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1. INTRODUCTION

1.1 Analytical chemistry

The science of analytical chemistry can be described in simplified terms as the process of obtaining knowledge of a sample by chemical analysis of some kind. The sample under investigation may consist of any solid, liquid or gaseous compound and the result of the analysis is data of some kind that is related to the initial question raised about the sample. From the data obtained in the analysis some knowledge about the sample can be extracted. This knowledge may be either qualitative or quantitative (or both). Examples of qualitative information are types of atoms, molecules, functional groups etc., while the quantitative information provides numerical information like the content of different compounds in the sample. The qualitative information thus answers the question "What?" and the quantitative information is more related to the question "How much?" In the beginning of the 19th century performing chemical analysis was a tedious and labour-intensive task. Analytical instruments were primitive and most analysis was carried out using wet chemistry. Furthermore, the results of an analysis were often a change in the colour of a reagent, the boiling point, the solubility, the odour, the optical activity and so on. However, enormous developments have taken place in analytical chemical techniques, microelectronics and computers during the last five to six decades and it has increased and revolutionized the scope for performing analytical chemical analysis. Nowadays an analytical chemical analysis generally includes some sort of analytical instrument that performs the actual analysis, while the data processing and instrument control are taken care by software that runs on a computer.

The shape of the data of analytical chemical analysis has moreover changed. From a single sample, it is now possible after a very short period of analysis to obtain enormous amounts of data. By means of techniques like Ultraviolet-Visible (UV-Vis) spectroscopy, Fluorescence spectroscopy, Infrared (IR) spectroscopy, Near infrared (NIR) spectroscopy, Raman spectroscopy, Mass spectrometry (MS) and Nuclear magnetic resonance spectroscopy (NMR), large amount of data on a sample can be collected in a short period of time.

1.2 Chemometrics

The science of chemometrics can briefly be described as the interaction of certain mathematical and statistical methods to solve chemical problems. It has developed as a consequence of the change in the data obtained within chemistry with the emergence of new analytical techniques as well as microprocessors. Chemometrics has been developed by chemists using special calculation methods to solve chemical problems and has the unique ability that many of the methods in use have been developed from within the field itself.

The breakthrough represented by microcomputers has also revolutionised the field of chemometrics; it may even be the case that developments in microprocessors have indeed made the full emergence of chemometrics possible. Since, majority of multivariate methods depend heavily upon the extensive ability of computers to perform large numbers of calculations, this was probably a prerequisite for the emergence of chemometrics. From occupying a very unobtrusive and mostly theoretical position, the scientific field of chemometrics has grown tremendously, starting around the seventies to become a very important and influential part of chemistry, including analytical chemistry. The applications using chemometric techniques in analytical chemistry are now numerous and applications have been

revealed in spectroscopy (1-5), chromatography (6-12), Near IR (13-15), Spectrofluorimetry (16-17) and other disciplines of analytical chemistry (18-20). A major strength of chemometric techniques lies in their ability to find and extract information from given large amount of data. As mentioned above, with the development of analytical instruments the type of data has changed from being uni- and low -variate (≤2 variables) to truly become multivariate. The field of chemometrics has thus found its natural connection with analytical chemistry.

A firm connection has also been established between chemometrics and spectroscopy. Figure 1 show in a simplified manner why this is a powerful combination.

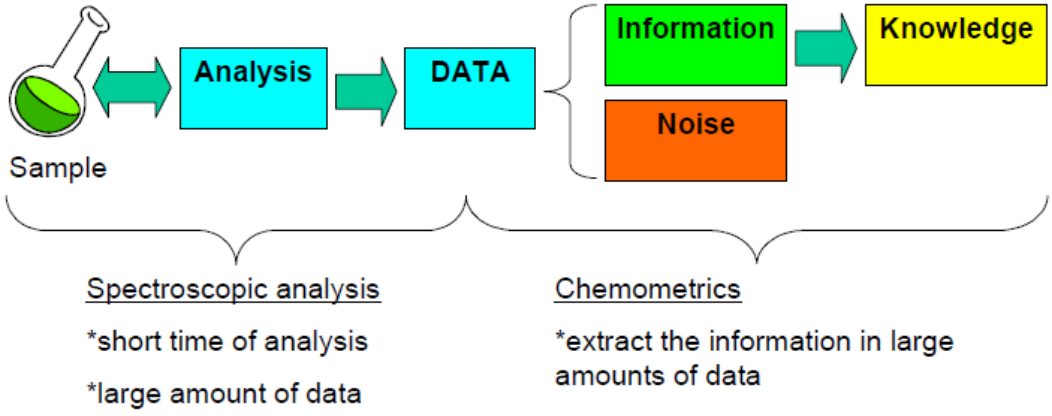


Figure 1. Illustration of why spectroscopy and chemometrics work well in conjunction.

Spectroscopic techniques are generally fast, with the analysis taking from a few seconds to a few minutes. As previously mentioned, spectroscopy also produces large amount of data for each sample analyzed. Roughly speaking, this data can be said to consist of two parts: information and noise. The information part of the data is what eventually leads to knowledge about the sample, while the noise is a non-information part. A matter of concern is always to minimize and, if possible, to get rid of disturbing noise in the data since it impairs the information gained. This is where

chemometrics comes in, since multivariate methods are constructed to extract the information from large sets of data. Usage of multivariate data with many variables instead of univariate data, offers many advantages in qualitative and quantitative spectroscopic analysis. The methods generally become more robust, precise and less sensitive to spectral artifacts.

One could therefore say that multivariate methods are the optimal choice for the evaluation of spectroscopic data and that the conjunction of spectroscopic analysis techniques with multivariate data analysis offers further possibilities in analytical chemistry.

Pharmaceutical analysis can be regarded as the application of analytical chemistry to pharmaceutical formulations, products and substances. These samples may consist of liquids, gels, powders, tablets, aerosols etc., of various types and contents. In the case of a pharmaceutical process, the samples may also consist of various solid or liquid mixtures of different kinds, thereby making the types of samples in pharmaceutical analysis numerous. The analytical answers wanted in pharmaceutical analysis are generally of a quantitative as well as qualitative type. Examples of quantitative analysis are the determination of the content of the active compound or the content of a major impurity in a pharmaceutical formulation. Examples of qualitative analysis are the identification of an active compound, i.e. to ensure that the compound is actually the one that is wanted. An example of an analysis that is both quantitative and qualitative is of the purity of a pharmaceutical formulation, i.e. whether there are any impurities, degradation products or synthesis intermediates etc., in the sample and, if so, how many and in what concentration.

In the pharmaceutical industry generally there is a strong tradition of using separation techniques in many analysis of both quantitative and qualitative type, such

as the determination of content, identity and purity. Very often these analyses are carried out by means of high performance liquid chromatography (HPLC) (21-24) with UV-Vis detection. This detection is, moreover, for the most part univariate, i.e. detection at a single wavelength, and consequently there is generally an ambition to obtain completely separated or baseline - separated peaks in order to be able to determine the compounds in the sample. Furthermore, HPLC is often applied to the analysis of samples that do not really need any separation since they contain one only active substance. One of the aims of this work is to show how spectroscopy and chemometrics in conjunction can be used as an alternative to HPLC for quantitative and qualitative analysis. These spectrometric methods consisting of spectroscopic analysis, a high level of automation and chemometric data evaluation can lead to rapid methods having a high analytical capacity, and the term high capacity analysis (HCA) is suggested in the work for these methods. HCA methods might very well be alternatives to HPLC and, in some cases, even outperform the chromatographic methods.

As mentioned above chemometrics has emerged from chemistry and has introduced new methods capable of dealing with the large amounts of chemical data by means of multivariate data analysis. It also includes methods of performing experiments in a more rational and structured way by means of experimental design. The name chemometrics can be divided into chemo (from chemistry) and metric (meaning measurement). Chemometrics thus deals with chemical data and how to obtain information from it.

One of the corner-stones of chemometrics is that data and information is not the same thing. As previously mentioned, data consists mainly of noise and information and the tools of chemometrics can be used to extract the information in

the data. In chemometrics the extraction of information is performed by the use of a mathematical model. This model, however, can never describe a chemical system in detail, since it is always an approximation valid only in a limited interval. Moreover, models must always be validated before they are applied (discussed below). Another important aspect of chemometrics is that experiments should be performed in a structured manner, varying all the experimental variables at the same time, i.e. by using experimental design. All data should also be analysed simultaneously multivariate and not univariate since this approach generally gives more and deeper information. A final cornerstone of chemometrics is that its results are generally best illustrated graphically. People generally have difficulty looking at large data sets and drawing conclusions from the figures they contain. This can probably be explained by the fact that in the history of mankind we have always (until very recently) studied the world and reality surrounding us in the form of pictures. Human beings are thus probably more accustomed to discovering patterns in images than in large data sets since each figure in the data set is actually a symbol for a value that needs to be translated in our brain before we can understand it. The number of textbooks in the field is numerous (25-28).

To sum up, some of the cornerstones of chemometrics are:

- Data = information + noise
- The model concept, i.e. models are always approximations and always have to be validated
- All variables should be changed and analysed simultaneously (experimental design and multivariate data analysis)
- Results are most easily investigated graphically

1.2.1 Validation

Validation is a crucial step of the model concept in chemometrics. Since it is always possible to make a mathematical model of any system at all, regardless of whether it only contains random variation, it is essential to validate the model always. The validation is needed in order to be able to decide whether or not the conclusions drawn from it are reliable, i.e. to make sure that the results can be extrapolated to new data.

Validation can be divided into two main types: **internal validation** and **external validation**. Internal validation means utilising the data that has been used to construct the model to validate it, while external validation means that new data that not has been previously used in the model is used for the validation. It goes without saying that the best form of validation is external validation, since using completely new data represents the most objective way to validate a model. However, it is generally recommended that one should first use an internal validation of the model and then apply new data sets and perform a thorough external validation of the model before it is further used. In internal validation a number of calculations and statistics can be applied and the discussion that follows applies to the chemometric methods used in this thesis.

Pre-treatment

Pre-treatment of the data is carried out before the multivariate data analysis takes place. The reasons for performing data pre-treatment are generally to reduce the effect of noise, improve the predictive ability of the model and simplify the model by making the data more normally distributed.

Scaling

Mean centring of the data is more or less standard in chemometric analyses, and especially for the rank reduction methods as mean centring often allows the analyst to use one less component in the model. Mean centring is done by subtracting the mean of each column from all the elements in the respective columns in the data matrix. In auto scaling the data are mean centred and additionally all the elements in each column are divided by the standard deviation of the column. This gives all the variables unit variance which is useful when the variables have different units. Auto scaling is rarely used for spectroscopic data, and it is therefore not included in the experimental designs presented herein.

Smoothing is a low-pass filter used for removing high-frequency noise from samples. Often used on spectra, this operation is done separately on each row of the data matrix and acts on adjacent variables. Smoothing assumes that variables which are near to each other in the data matrix (*i.e.*, adjacent columns) are related to each other and contain similar information which can be averaged together to reduce noise without significant loss of the signal of interest.

Derivation

Derivation is used to remove baseline effects from the data. The first derivative can be used, where as the second derivative is occasionally found useful.

The most commonly employed validation criterion is to divide the dataset into two subsets, one calibration set and one validation set. The calibration model is calculated using the calibration set. Then, the root mean square errors of calibration and validation, RMSEC, and RMSEP, are calculated by using the calibration model under investigation to predict the samples in the calibration set and validation set,

respectively. Next, the deviations between the measured and calculated values are determined and squared:

$$\mathrm{RMSEC} = \sqrt{\frac{(y - y_{pred})^i (y - y_{pred})}{m - 1}}$$

$\boldsymbol{y}$ contains the m measured values for the samples in the calibration set and $\boldsymbol{y}_{\mathrm{pred}}$ contains the m values predicted by the calibration model. The RMSEP values are calculated analogously using the values for the validation set. Obviously, both values should be minimised.

In order to obtain an estimate of the predictive ability of a regression model, a often used method is Cross Validation (CV) (29). The basic principle of CV is as follows, in the used data set, one sample is kept out of the calibration and a regression model is calculated from the remaining observations. The y value of the object taken away is then predicted with the calibration model constructed. The prediction error of this observation can then be calculated as the difference between the predicted and true value. This procedure is then repeated leaving out every sample in the calibration set once, the summed prediction error then indicating the predictive ability. This 'leave one out' cross validation is, however, generally valid only when the number of samples is small. For larger calibration sets, the procedure should be carried out by the data matrix which is divided into a number of segments containing one or more samples. One by one the segments are left out and a model is calibrated on the remaining samples and used to predict the samples in the left out segment. The prediction error is estimated as the root mean square error of cross validation (RMSECV) which is calculated by comparing the predicted values with the reference values as given below:

$$RMSECV = \sqrt{\sum_{i=y}^{N} \frac{(y_{ipred} - y_{iref})^2}{N}}$$

where y_{pred} is the predicted value, y_{ref} is the reference value and N is the number of samples. Test-set validation is used when the data set is large enough to be divided into two subsets: a calibration set which is used for calibration of the model, and a validation set which is used to estimate the prediction error. Since different samples are used for calibration and validation, test-set validation is a stronger test of the model than cross-validation and the estimate of the prediction error, root mean square error of prediction (RMSEP) will usually be more conservative and closer to the true value than RMSECV. A test set may be a dependent test set, when it consists of samples which are selected randomly or orderly from the original data set, or it may be independent, when a new series of samples is analyzed and used as a test set.

$$RMSEP = \sqrt{\sum_{i=1}^{N} \frac{(y_{pred} - y_{obs})^2}{P}}$$

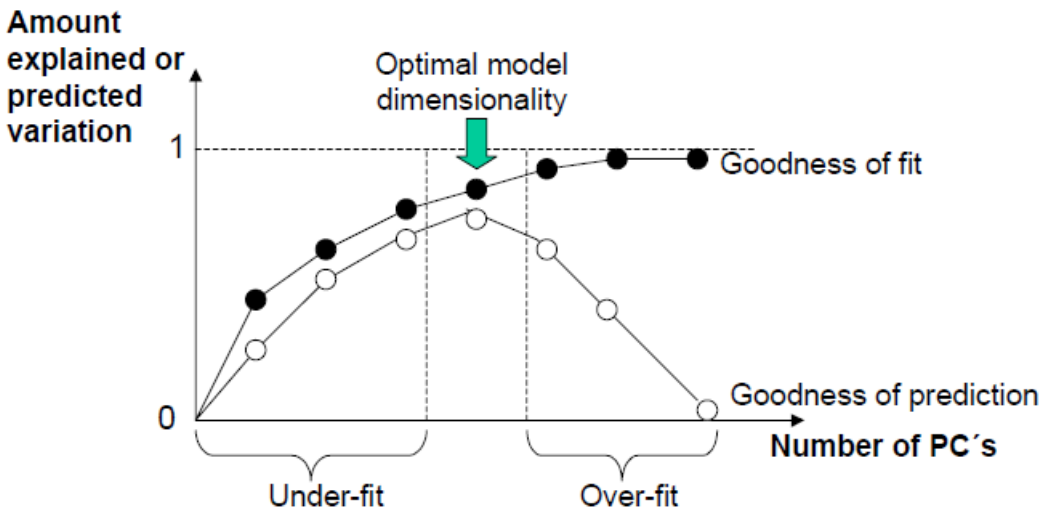


Figure 2. Illustration of how the model dimensionality affects the fit and predictive ability.

1.2.2 Experimental design

In order to obtain knowledge about any chemical system under investigation, chemists perform experiments and analyses and draw conclusions from the results of these experiments. The reasons for using experimental design is that it is a structured, planned and well-organised approach that minimises the number of experiments needed and maximises the information obtained. It also enables one to find interactions between factors and is a good way to find the optimum of the system under investigation. Some important concepts in the design of experiments are *experiment*, *factor*, *response*, *experimental domain* and *experimental design*. A factor is a controllable x-variable that is varied in a predefined interval. Factors can be both quantitative and qualitative in nature. Quantitative factors can be measured on a continuous scale, examples being pH, concentration, pressure, flow, temperature etc. Qualitative factors have discrete values, e.g. type of mobile phase, type of solvent, type of catalyst etc. Responses are the dependent variables (Y) that we measure the result of the experiments on. In the design of experiments the factors are varied independently at predefined intervals (with a minimum and a maximum). These factors intervals span the *experimental domain* under investigation. The low and high levels of each factor setting can be denoted by "+" and "–", and the centre by "0". The choice of which experiments to perform within this experimental domain is given by the *experimental design*. An example of an experimental design (fractional factorial design) with three factors and one response variable is Five-level calibration designs, using cyclic generator $-2 \rightarrow -1 \rightarrow 2 \rightarrow 1 \rightarrow -2$ and repeater of 0.

1.2.3 Classical least squares (CLS)

This method assumes Beer's law model with the absorbance at each frequency being proportional to the component concentrations. In matrix notation, Beer's law model for *m* calibration standards containing *l* chemical components with the spectra of *n* digitized absorbances is given by:

$$A = C \times K + E_A \quad (1)$$

where A is the $m \times n$ matrix of calibration spectra, C is the $m \times l$ matrix of component concentration, K is the $l \times n$ matrix of absorptivity-path length products, and E_A is the $m \times n$ matrix of spectral errors. K, then represents the matrix of pure component spectra at unit path length. The classical least squares solution to equation (1) during calibration is,

$$\hat{K} = (C^T C)^{-1} C^T \times A \quad (2)$$

where $\hat{K}$ indicates least-squares estimation of K.

Analysis based on the spectrum a, of unknown components concentration (samples),

$$C_0 = (\hat{K}\hat{K}^T)^{-1} \hat{K} \times a \quad (3)$$

Where C_o is vector of predicted concentration and $\hat{K}^T$ is transpose of the matrix $\hat{K}$

1.2.4 Multiple linear regressions (MLR)

If absorbance measurements for several solution containing mixtures of the analytes are made in numbers exceeding the number of mixture components, then the system composed of the absorbance and concentration matrices will be over dimensioned and with take the following matrix form:

$$\mathbf{A = KC} \quad (4)$$

Where **A** is the data absorbance calibration matrix, **K** is the matrix form which the proportionality constants are calculated from spectra for standard solutions of the pure analytes and or their mixtures, and **C** is the concentration matrix. The **C** prediction concentration matrix can be calculated from the following equation:

$$\mathbf{C} = (\mathbf{K}^{\mathbf{T}}\mathbf{K})^{-1}\mathbf{K}^{\mathbf{T}}\mathbf{A} \quad (5)$$

Where **K'** is the transpose of **K** and **A** is the absorbance matrix of unknown samples. Matrix **K** can be obtained in various ways. We calculated **K** values by **MLR** of data for mixtures of analytes of known composition (29).

1.2.5 Principle component regression (PCR)

In the spectral work, the following steps can explain the fundamental concept of PCR.

The original data obtained in absorbance's (A) and concentrations (C) of analytes were reprocessed by mean-centring as A_0 and C_0, respectively. Using the ordinary linear regression

$$C = a + b \times A \quad (6)$$

The coefficients a and b: b = P× q, where P is the matrix of eigenvectors and q is the C- loadings given by $q = D \times T^T \times A_0$. Here T^T is the transpose of the score matrix T. D is a diagonal matrix having the components on the inverse of the selected eigenvalues. Knowing 'b', one can easily find a by using the formula $a = C_{mean} - A^T_{mean} \times b$, where A^T_{mean} represents the transpose of the matrix having the entries of the mean absorbance values and C_{mean} is the mean concentration of the calibration set.

1.2.6 Partial least squares (PLS)

In the UV-Vis spectra, the absorbance data (A) and concentration data (C) are mean centered to give data matrix A0 and vector C0. The orthogonalized PLS algorithm has the following steps. The loading weight vector W has the following expression:

$$W = \frac{A_0^T C_0}{C_0^T C_0} \tag{7}$$

The scores and loadings are given by:

$$t_1 = A_0 W \tag{8}$$

$$P_1 = \frac{A_0^T t_1}{t_0^T t_1} \tag{9}$$

$$q_1 = \frac{C_0^T t_1}{t_1^T t_1} \tag{10}$$

The matrix and vector of the residuals in A_0 and C_0 are:

$$A_1 = A_0 - t_1 P_1^T \tag{11}$$

$$C_1 = C_0 - t_1 q_1^T \tag{12}$$

From the general linear equation, the regression coefficients were calculated by:

$$b = W (P^T W)^{-1} q, \tag{13}$$

$$a = C_{mean} - A^T{}_{meanb}. \tag{14}$$

The builded calibration equation is used for the estimation of the compounds in the samples (30).

The calibration samples should be in the calibration set span of the calibration experimental domain as far as possible and experimental design can be used to find suitable samples to include in the calibration set. Also the best calibration is generally obtained when the calibration model contains all the sources of variation that can

occur in the actual measurement. The order in which the samples are analysed is one source of variation and randomisation of the order of analysis minimises the error caused by day-to-day variations. The evaluation of the predictive ability of a quantitative multivariate calibration model can be made, by means of the root mean squares error of cross validation (RMSECV) root mean square error of prediction (RMSEP).

2. REVIEW OF LITERATURE

The literature review describes the various reported methods for the estimation of these drugs individually or in combination with other drugs. Although there are several analytical methods reported for the estimation of these drugs individually or in combination with other drugs, the review will deal only with the simultaneous estimation of drugs when present in the multiple dosage forms.

2.1 Losartan potassium

Polinko Michelle et al (31) have quantified 'Simultaneous determination of losartan and EXP3174 in human plasma and urine utilizing liquid chromatography/ tandem mass spectrometry'.

Sivakumar T et al (32) have studied 'Development of a HPLC method for the simultaneous determination of losartan potassium and atenolol in tablets'. The separation was achieved on Supelcosil ODS analytical column (25×0.46 cm, i.d., 5 µm) using acetonitrile and 25 mM potassium dihydrogen phosphate (45:55 v/v, pH 3.00±0.05) as mobile phase at a flow rate of 1.2 mL/min. Detection was carried out using a UV detector at 227 nm.

Lastra, Olga C et al (33) have reported 'Development and validation of an UV derivative spectrophotometric determination of Losartan potassium in tablets'. Based on the spectrophotometric characteristics of Losartan potassium, a signal at 234 nm of the first derivative spectrum (ID234) was found adequate for quantification.

Randal A et al (34) have studied 'Photosensitized degradation of losartan potassium in an extemporaneous suspension formulation'. Formation of the two major degradates required exposure to light (UV or visible) and the presence of oxygen. Experiments using Rose Bengal (a singlet oxygen photosensitizer) and 1,4-

diazabicyclooctane (DABCO; a singlet oxygen quencher) established that the major photodegradates are formed via the intermediacy of singlet oxygen.

Lee H et al (35) have estimated 'Simultaneous determination of losartan and active metabolite EXP3174 in rat plasma by HPLC with column switching'. The plasma sample was injected onto a precolumn of Lichroprep RP-8 after dilution with 5% acetonitrile in 50 mM phosphoric acid. Polar plasma components were eluted using this diluent. After valve switching, the concentrated drugs were eluted in the back-flush mode and separated by an Inertsil ODS-2 column with acetonitrile-acetate buffer.

Shankar MB et al (36) have developed 'Simultaneous Spectrophotometric Determination of Losartan Potassium and Hydrochlorothiazide in Tablets'. Two simple, accurate and precise methods for simultaneous estimation of losartan potassium and hydrochlorothiazide in combined dosage form have been described. First method employs formation and solving of simultaneous equations using 206.6nm and 270.6nm as two analytical wavelengths. Second method is dual wavelength method, which uses the difference of absorbance values at 206.6 and 261.4 nm for the estimation of losartan and absorbance values at 270.6 nm for the estimation of hydrochlorothiazide.

Maja lusina et al (37) has studied 'Stability study of losartan/hydrochlorothiazide tablets'. The results of stress test suggested that losartan/hydrochlorothiazide tablets are sensitive to moisture. It was demonstrated that the developed analytical methods are stability indicating.

Shailesh A. Shah et al (38) have studied 'Simultaneous Determination of Losartan and Hydrochlorothiazide in Combined Dosage Forms by First-Derivative Spectroscopy and High-Performance Thin-Layer Chromatography'. This paper

describes the development of 2 methods that use different techniques, first-derivative spectroscopy and high-performance thin-layer chromatography (HPTLC), to determine LST and HCTZ in the presence of each other. LST and HCTZ in combined preparations were quantitated by using the first-derivative responses at 271.6 nm for LST and 335.0 nm for HCTZ in spectra of their solutions in water. The linearity ranges are 30 –70 μg/mL for LST and 7.5 –17.5 μg/ mL for HCTZ with correlation coefficients of 0.9998 and 0.9997, respectively. In the HPTLC method, a mobile phase of chloroform–methanol–acetone–formic acid (7.5 + 1.5 + 0.5 + 0.03, v/v) and a prewashed Silica Gel G60 F_{254} TLC plate as the stationary phase were used to resolve LST and HCTZ in a mixture.

Ruben M. Maggio et al (39) have developed 'A multivariate approach for the simultaneous determination of losartan potassium and hydrochlorothiazide in a combined pharmaceutical tablet formulation'. The procedure, based on the multivariate analysis of spectral data in the 220–274 nm region by the partial least squares algorithm.

Lusina M et al (40) have estimated 'Stability study of losartan/hydrochlorothiazide tablets'. The purpose of stability testing is to investigate how the quality of a drug product changes with time under the influence of environmental factors, to establish a shelf life for the product and to recommend storage conditions. Stability study of losartan/hydrochlorothiazide tablets is presented in this paper.

Baing MM et al (41) have reported 'Simultaneous RP-LC Determination of Losartan Potassium, Ramipril, and Hydrochlorothiazide in Pharmaceutical Preparations'. The three drugs were separated on a 150 mm × 4.6 mm i.d., 5 μm particle, Cosmosil C_{18} column. The mobile phase was 0.025 m sodium perchlorate–

acetonitrile, 62:38 (*v*/*v*), containing 0.1% heptanesulphonic acid, pH adjusted to 2.85 with orthophosphoric acid, at a flow rate of 1.0 mL min^{-1}. UV detection was performed at 215 nm.

Mehdi Ansari et al (42) have quantified 'Derivative Spectrophotometric Method for Determination of Losartan in Pharmaceutical Formulations'. The first derivative spectrum recorded between 220 and 320 nm, and a zero-crossing technique for first derivative measurement at 232.5 nm was selected. It is found that the selectivity and sensitivity of method to be in desirable range.

Obando MA et al (43) quantified 'Simultaneous determination of hydrochlorothiazide and losartan potassium in tablets by high-performance low-pressure chromatography using a multi-syringe burette coupled to a monolithic column'. The system comprised a multisyringe module, three low-pressure solenoid valves, a monolithic C (18) column (25 mm x 4.6 mm i.d.), and a diode-array detector. The mobile phase was 10 mmol L (-1) potassium dihydrogen phosphate (pH 3.1)-acetonitrile-methanol (65:33:2 v/v/v) at a flow rate 0.8 mL min (-1). UV detection was carried out at 226 nm. The multi-syringe chromatographic (MSC) method with UV spectrophotometric detection was optimized and validated.

Gandhimathi M et al (44) developed 'A simple and accurate method for the Simultaneous estimation of losartan potassium (LP) and hydrochlorothiazide (HZ) has been developed'. The method employs simultaneous equations to estimate these drugs. in methanol, losartan potassium and hydrochlorothiazide showed maximum absorbance at 236 and 270nm respectively. Losartan potassium and hydrochlorothiazide obeyed Beer Lambert's law in the concentration range from 2-20mug/ mL and 1-50mug/ mL, respectively. The results of analysis have been validated statistically and by recovery studies.

Ensafi AA et al (45) have quantified 'Determination of losartan and triamterene in pharmaceutical compounds and urine using cathodic adsorptive stripping voltammetry'. A square-wave voltammetric procedure for the electroanalytical determination of losartan and triamterene in Britton-Robinson buffer (pH 3.0, 0.1 mol L (-1)) as a supporting electrolyte containing 30 ng mL (-1) of copper ions was developed. Opposite to the case of triamterene, losartan can not be reduced at a mercury electrode alone, but a new peak appears at -0.25 V in the presence of copper due to the formation of a complex between copper (II) and losartan.

Suhagia BN et al (46) have developed 'Development of a RP-HPLC method for evaluating losartan Potassium and hydrochlorthiazide tablets'. A simple, specific, accurate and precise reverse phase high performance liquid chromatographic method was developed for the simultaneous determination of losartan potassium and hydrochlorthiazide in tablet dosage forms. A Lichrospher 100 C-18, 5-microM column having 20x4.6 mm i.d. in isocratic mode, with mobile phase containing 20 mM KH under 2 PO under 4 buffers (pH 3): acetonitrile: tetrahydrofuran in 60:30:10 were used. The flow rate was 1.0 mL/min and effluent was monitored at 215 nm. The retention time of losartan potassium and hydrochlorthiazide were 7.94 min and 3.26 min, respectively.

Valiyare GR et al (47) have reported 'HPLC Determination of Amlodipine, Losartan and Ramipril in Pharmaceutical Formulations'.

Mahadik KR et al (48) have studied 'Simultaneous HPTLC Estimation of amlodipine besylate and Losartan Potassium in Tablet Dosage Form'.

Kulkarni AP et al (49) have quantified 'HPLC Method for Determination of Losartan Potsssium and Amlodipine Besylate in tablets'.

Lande NR et al (50) have developed ‘Simultaneous Spectrophotomertic Estimation of Losratan potassium and hydrochlorthiazide in tablet drugs’.

Kanumula GV and Bhanu Raman (51) estimated ‘Simultaneous determination of hydrochlorthiazide and losartan potassium in pharmaceutical dosage by reverse phase high performance liquid chromatography’.

Baing MM et al (52) have quantified ‘Simultaneous RP-LC Determination of Losartan Potassium, Ramipril, and Hydrochlorothiazide in Pharmaceutical Preparations’. The three drugs were separated on a 150 mm × 4.6 mm i.d., 5 μm particle, Cosmosil C18 column. The mobile phase was 0.025 m sodium perchlorate–acetonitrile, 62:38 (v/v), containing 0.1% heptanesulphonic acid, pH adjusted to 2.85 with orthophosphoric acid, at a flow rate of 1.0 mL min−1. UV detection was performed at 215 nm.

Gandhimathi M et al (53) have reported ‘RP-HPLC Determination of Losartan Potassium and Ramipril in Tablet’.

Topale PR et al (54) have studied ‘Simultaneous Uv- Spectrophotometric Estimation of Losartan Potassium and Amlodipine in tablet’.

Sankar DG et al (55) have carried out ‘Extractive Spectrophotometic Determination of Losartan Potassium using Acidic and basic dyes’.

Thomas AB et al (56) have reported ‘Simultaneous Estimation of Losartan Potassium and Atenolol in Tablet Dosage Form’.

Sathyanarayana D et al (57) have quantified ‘Back-propagation neural network Model for simultaneous spectrophotometric estimation of losartan potassium and hydrochlorothiazide in tablet dosage’. The calibration sets were designed such that the concentrations were orthogonal and span the possible mixture space fairly evenly. The back-propagation neural network model was optimized with respect to

the spectral input, training parameters and topology including transfer functions for each layer so as to yield accurate and precise estimations on model validation.

Suhagia BN et al (58) have quantified 'Development of a RP-HPLC method for evaluating losartan Potassium and hydrochlorthiazide tablets'. A Lichrospher 100 C-18, 5-microM column having 20x4.6 mm i.d. in isocratic mode, with mobile phase containing 20 mM KH under 2 PO under 4 buffers (pH 3): acetonitrile: tetrahydrofuran in 60:30:10 were used. The flow rate was 1.0 mL/min and effluent was monitored at 215 nm.

Durga Rao D et al (59) have developed 'Simultaneous Determination of Losartan Potassium, Atenolol and Hydrochlorothiazide in Pharmaceutical Preparations by Stability-Indicating UPLC'. A stability-indicating UPLC method was developed for the simultaneous quantitative determination of losartan potassium, atenolol, and hydrochlorothiazide in pharmaceutical dosage forms in the presence of degradation products. The separation was achieved on a simple isocratic method (water: acetonitrile: triethyl amine: ortho phosphoric acid (60:40:0.1:0.1, v/v) at 0.7 mL min−1, a detection wavelength of 225 nm).

Balesteros et al (60) reported 'Determination of losartan associated with chlorthalidone or hydrochlorothiazide in capsules by capillary zone electrophoresis'. An alternative methodology by capillary zone electrophoresis (CZE) is proposed for the determination of losartan potassium (LOS) associated with chlorthalidone (CTD) or hydrochlorothiazide (HCT) in capsules, using 50 mmol L^{-1} of sodium carbonate buffer with detection at 226 nm.

Argekar AP et al (61) have developed 'A Gradient Reversed Phase High Performance Liquid Chromatography Method for Simultaneous Determination of Hydrochlorothiazide (HCT) and Losartan Potassium (LOS) from Tablets'. The

stationary phase was Microbondapak C18 column (10 μ, 300 mm ×3.9 mm ID). A gradient elution with an aqueous methanolic mobile phase (pH=3) was employed for the separation. Detection was carried out at 270 nm using UV detector.

Yoonho Choi et al (62) have reported 'Determination of Losartan in Human Plasma by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS): Application to Bioequivalence Study'. Losartan and I.S. were successfully separated on a CN column with a mobile phase of acetonitrile - 0.2% formic acid solution (68:32, v/v). Detection was performed on a single quadrupole mass spectrometer by a selected ion monitoring (SIM) mode via electrospray ionization (ESI) source.

Maggio RM et al (63) have reported 'A multivariate approach for the simultaneous determination of losartan potassium and hydrochlorothiazide in a combined pharmaceutical tablet formulation'. The procedure, based on the multivariate analysis of spectral data in the 220-274 nm region by the partial least squares algorithm, is linear in the concentration range 1.06-5.70 mg L(-1) for hydrochlorothiazide and 4.0-22.2 mg L(-1) for losartan.

Prabhakar et al (64) have studied 'A rapid colorimetric method for the determination of Losartan potassium in bulk and in synthetic mixture for solid dosage form'. Two new rapid reproducible and economical spectrophotometric methods are described for the determination of Losartan potassium in bulk and in synthetic mixture for solid dosage forms. Both methods are based on the formation of an orange-red and orange ion-pair complex due to the action of Calmagite (CT) and Orange-II (O-II) on Losartan potassium in acidic medium (pH 1.2).

Quaglia MG et al (65) have developed 'Determination of losartan and hydrochlorothiazide in tablets by CE and CEC'. The CE separation was carried out in

an uncoated capillary filled with a 100 mM sodium borate pH 9 solution containing trimethyl-beta-cyclodextrins. CEC was performed using a capillary packed with a RP-18 stationary phase. The mobile phase was a mixture of 50 mM ammonium acetate pH 7, water, acetonitrile (1/1.5/7.5).

2. 2 Hydrochlorothiazide

Liu, et al (66) have developed 'Determination of hydrochlorothiazide in human plasma by liquid chromatography/tandem mass spectrometry'. The analyte and irbesartan, used as the internal standard, were precipitated and extracted from plasma using methanol. Analysis was performed on a Phenomenex Kromasil C(8) column with water and methanol (27:73, v/v) as the mobile phase. Linearity was assessed from 0.78 to 200 ng/ mL in plasma.

Li, et al (67) have carried out 'A liquid chromatography/tandem mass spectrometry method for the simultaneous quantification of valsartan and hydrochlorothiazide in human plasma'. After a simple protein precipitation using acetonitrile, the analytes were separated on a Zorbax SB-Aq C18 column using acetonitrile-10mM ammonium acetate (60:40, v/v, pH 4.5) as mobile phase at a flow rate of 1.2 mL/min.

Ramakrishna, et al (68) have reported 'Sensitive liquid chromatography-tandem mass spectrometry method for quantification of hydrochlorothiazide in human plasma'. The analyte and internal standard, tamsulosin (II) were extracted by liquid-liquid extraction with diethyl ether-dichloromethane (70:30, v/v) using a Glas-Col Multi-Pulse Vortexer. The chromatographic separation was performed on a reversed-phase column (Waters symmetry C18) with a mobile phase of 10 mm ammonium acetate-methanol (15:85, v/v).

Van der Meer et al (69) have reported ‘Simultaneous determination of amiloride and hydrochlorothiazide in plasma by reversed-phase high-performance liquid chromatography’.

Medvedovici et al (70) have reported ‘Liquid extraction and HPLC-DAD assay of hydrochlorothiazide from plasma for a bioequivalence study at the lowest therapeutic dose’. The final selected method, considered being rapid and simple, was applied to determine the pharmacokinetics of hydrochlorotiazide (HCT) after administration of Capozide (Bristol-Myers Squibb) tablets containing 50 mg Captopril and 25 mg HCT, to 4 healthy volunteers. The results obtained were in accordance with the pharmacokinetic parameters of HCT carried out in the literature.

Vonaparti et al (71) have studied ‘Development and validation of a liquid chromatographic/electrospray ionization mass spectrometric method for the determination of benazepril, benazeprilat and hydrochlorothiazide in human plasma’. The MS system was operated in selected ion monitoring (SIM) modes. HPLC was performed isocratically on a reversed-phase porous graphitized carbon (PGC) analytical column (2.1 x 125.0 mm i.d., particle size 5 microm). The mobile phase consisted of 55% acetonitrile in water containing 0.3% v/v formic acid and pumped at a flow rate of 0.15 mL min(-1). Chlorthalidone was used as the internal standard (IS) for quantitation.

Erk Nevin (72) has quantified ‘Simultaneous determination of irbesartan and hydrochlorothiazide in human plasma by liquid chromatography’. Good chromatographic separation was achieved using a Supelcocil C(18) (5 micrometer 15 cmx4.6 mm) column and a mobile phase consisting of 10 mM potassium dihydrogen phosphate:methanol:acetonitrile (5:80:15 v/v/v) (pH:2.5) while at a flow-rate of 1.0 mL min(-1).

Parekh Sagar A et al (73) have reported ‘Simultaneous determination of hydrochlorothiazide, quinapril and quinaprilat in human plasma by liquid chromatography-tandem mass spectrometry;. An isocratic mobile phase consisting of 0.5% (v/v) formic acid: acetonitrile (25:75, v/v) was used to separate all these drugs. The precursor and product ions of these drugs were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring mode (MRM) without polarity switch.

De Vries et al (74) have developed ‘Simple determination of hydrochlorothiazide in human plasma and urine by high performance liquid chromatography’. Samples were purified by solvent extraction and analysed by reversed phase HPLC with ultraviolet detection, using hydroflumethiazide as the internal standard; plasma was eluted using gradient elution and urine was analysed isocratically.

Soldin et al (75) has reported ‘High performance liquid chromatographic analysis of hydrochlorothiazide in serum and urine’. We report a high performance liquid chromatographic method for the analysis of hydrochlorothiazide in 500microliter of serum of 25 microliter of urine.

Azumaya C T (76) has reported ‘Sensitive liquid chromatographic method for the determination of hydrochlorothiazide in human plasma’.

Zendelovska Dragica et al (77) have estimated ‘Development of solid-phase extraction method and its application for determination of hydrochlorothiazide in human plasma using HPLC’. This method involves solid-phase extraction on RP-select B cartridges followed by isocratic reversed-phase chromatography on a Hibar Lichrospher 100 RP-8 column with UV detection at 230 nm

Huang Taomin et al (78) have quantified 'Simultaneous determination of captopril and hydrochlorothiazide in human plasma by reverse-phase HPLC from linear gradient elution'. Effective chromatographic separation was achieved using a C(18) column (DIAMONSIL 150 mmx4 mm i.d., 5 microm) based on an acetonitrile-trifluoroacetic acid-water gradient elution at a flow rate of 1.2 mL/min.

Vignaduzzo et al (79) have studied 'PLS and first derivative of ratio spectra methods for determination of hydrochlorothiazide and propranolol hydrochloride in tablets'. The methods are based on the first derivative of ratio spectra (DRS) and on partial least square (PLS) analysis of the ultraviolet absorption spectra of the samples in the 250-350-nm region.

Dinç E and Ozdemir (80) have carried out 'A linear regression analysis and its application to multivariate chromatographic calibration for the quantitative analysis of two-component mixtures'. The mathematical algorithm of multivariate chromatographic calibration technique is based on the use of the linear regression equations constructed using relationship between concentration and peak area at the five-wavelength set.

Belal F et al (81) have reported 'A stability-indicating LC method for the simultaneous determination of ramipril and hydrochlorothiazide in dosage forms'. Acetonitrile: sodium perchlorate solution (0.1 M) adjusted to pH 2.5+/-0.2 with phosphoric acid (46:54 v/v), was used as the mobile phase, at a flow rate of 1.5 mL/min. A supelcosil LC-8 column (5 microm), 15 cm x 4.6 mm i.d. was utilized as stationary phase. Detection was affected spectrophotometrically at 210 nm.

Dinç E and Ustündağ O (82) have developed 'Spectophotometric quantitative resolution of hydrochlorothiazide and spironolactone in tablets by chemometric analysis methods'. Spectrophotometric simultaneous determination of

hydrochlorothiazide and spironolactone in tablets was performed by classical least-squares (CLS), inverse least-squares (ILS), principal component regression (PCR) and partial least-squares (PLS).

Tomsu D et al (83) have reported 'Automated simultaneous triple dissolution profiles of two drugs, sulphamethoxazole-trimethoprim and hydrochlorothiazide-captopril in solid oral dosage forms by a multicommutation flow-assembly and derivative spectrophotometry'.

Salem H (84) has repoted 'High-performance thin-layer chromatography for the determination of certain anti hypertensive mixtures'. The methods consist of dissolving the drugs in methanol and spotting these solutions on a thin layer Merck HPTLC plates (0.25 mm thickness) pre-coated with 60 GF254 silica gel on aluminum sheet as the stationary phase, using dioxane:acetonitrile: 1-propanol:hexane (30:18:23:1 v/v/v/v), ethylacetate: chloroform: methanol: acetic acid (11:8:7.5:1.5 v/v/v/v), ethylacetate:chloroform: 1-propanol:25% ammonia solution (12:9:1:0.2 v/v/v/v), dioxan:acetonftrile:l-propanol:tetrahydrofuran (20:13:4:15, v/v/v/v) and dioxane:ethylacetate: acetonitrile:1-propanol (10:7:5.5:3 v/v/v/v) as the mobile phases for mixtures I, II, III, IV and V, respectively. Detection was carried out densitometrically using UV detector.

Sultan M et al (85) have reported 'High performance liquid chromatographic method for the simultaneous determination of labetalol and hydrochlorothiazide in tablets and spiked human plasma'. The chromatographic separation was performed using a Microbondapak C18 column (4.6 W. x 250 nm) and paracetamol as internal standard. A mobile phase consisting of 0.05 M phosphate buffer/acetonitrile of pH 4 (7:3) at a flow rate of 0.7 mL/min was used. The detection was affected spectrophotometrically at 302 nm.

Razak OA (86) has carried out ‘Electrochemical study of hydrochlorothiazide and its determination in urine and tablets’. The development and validation of a voltammetric method for the determination of hydrochlorothiazide in human urine with no prior extraction and in commercial tablets, is described.

Ferraro MC et al (87) have studied ‘Chemometric determination of amiloride hydrochloride, atenolol, hydrochlorothiazide and timolol maleate in synthetic mixtures and pharmaceutical formulations’. The development and validation of different chemometric methods such as classical least squares, principal components regression and partial least squares with 1 dependent variable applied on UV spectral data and on their first derivatives.

Erk N (88) has developed ‘Application of first derivative UV-spectrophotometry and ratio derivative spectrophotometry for the simultaneous determination of candesartan cilexetil and hydrochlorothiazide’.The first method depends on zero-crossing and peak to base measurement. The first derivative amplitudes at 270.1 and 255.5 nm were selected for the assay of (CAN) and (HYD), respectively. The second method depends on first derivative of the ratio spectra by division of the absorption spectrum of the binary mixture by a normalized spectrum of one of the components and then calculating the first derivative of the ratio spectrum.

Ertuerk S et al (89) have developed ‘Simultaneous determination of moexipril hydrochloride and hydrochlorothiazide in tablets by derivative spectrophotometric and high-performance liquid chromatographic methods’. The UV spectrophotometric and isocratic reversed-phase liquid chromatography determination of binary mixtures of moexipril hydrochloride and hydrochlorothiazide are evaluated.

Hillaert S and Van-den-Bossche W (90) have quantified ‘Simultaneous determination of hydrochlorothiazide and several angiotensin-II-receptor antagonists

by capillary electrophoresis'. We have investigated the capability of the capillary zone electrophoretic (CZE) and micellar electrokinetic capillary chromatographic (MEKC) methods to simultaneously separate hydrochlorothiazide and six angiotensin-II-receptor antagonists (ARA-IIs): candesartan, eprosartan mesylate, irbesartan, losartan potassium, telmisartan, and valsartan.

Belal F et al (91) have estimated 'Spectrofluorometric determination of labetolol in pharmaceutical preparations and spiked human urine through the formation of coumarin derivative'. The method is based on the reaction between LBT and ethylacetoacetate in the presence of sulphuric acid to give yellow fluorescent product with excitation wavelength of 312 nm and emission wavelength of 432 nm.

Dinc E and Baleanu D (92) have studied 'Spectrophotometric quantitative determination of cilazapril and hydrochlorothiazide in tablets by chemometric methods'. Our chemometric methods were applied to simultaneous determination of cilazapril and hydrochlorothiazide in tablets. Classical least-square (CLS), inverse least-square (ILS), principal component regression (PCR) and partial least-squares (PLS) methods do not need any priori graphical treatment of the overlapping spectra of two drugs in a mixture.

Erk N (93) reported 'Simultaneous determination of fosinopril and hydrochlorothiazide in pharmaceutical formulations by spectrophotometric methods;. Three new spectrophotometric procedures for the simultaneous determination of fosinopril and hydrochlorothiazide are described.

Jonczyk A and Nowakowska Z (94) have studied 'Determination of hydrochlorothiazide and pindolol mixtures by UV spectrophotometry and high performance liquid chromatography (HPLC)'.

El Walily AF et al (95) have reported ‘Simultaneous determination of enalapril maleate and hydrochlorothiazide by first-derivative ultraviolet spectrophotometry and high performance liquid chromatography’.Two methods based on UV derivative spectrophotometry and reversed-phase HPLC are described for the simultaneous determination of enalapril maleate and hydrochlorothiazide in tablets.

2.3 Amlodipine Besylate

Abdel-Wadood et al (96) have carried out ‘Validated spectrofluorometric methods for determination of amlodipine besylate in tablets’. The first method was based on the condensation reaction of AML with ninhydrin and phenylacetaldehyde in buffered medium (pH 7.0) resulting in formation of a green fluorescent product, which exhibits excitation and emission maxima at 375 and 480 nm, respectively. The second method was based on the reaction of AML with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) in a buffered medium (pH 8.6) resulting in formation of a highly fluorescent product, which was measured fluorometrically at 535 nm (lambda(ex), 480 nm).

Malesuik et al (97) have quantified ‘Determination of amlodipine in pharmaceutical dosage forms by liquid chromatography and ultraviolet spectrophotometry’. The isocratic LC analyses were performed on an RP18 column using a mobile phase composed of 0.1% (v/v) ortho-phosphoric acid (pH 3.0) - acetonitrile (60 + 40, v/v) at a flow rate of 1.0 mL/min. The UV spectrophotometric method was performed at 238 nm.

Ragno et al (98) have reported ‘Photodegradation monitoring of amlodipine by derivative spectrophotometry’. A derivative spectrophotometric method for the simultaneous determination of amlodipine and its pyridine photodegradation product has been developed.

Zarghi et al (99) have studied ‘Validated HPLC method for determination of amlodipine in human plasma and its application to pharmacokinetic studies’. The separation was performed on an analytical 125 x 4.6 mm i.d. Nucleosil C8 column. The wavelength was set at 239 nm. The mobile phase was a mixture of 0.01 M sodium dihydrogen phosphate buffer and acetonitrile (63:37, v/v) adjusted to pH 3.5 at a flow rate of 1.5 mL min(-1).

Josefsson et al (100) have reported ‘Sensitive high-performance liquid chromatographic analysis of amlodipine in human plasma with amperometric detection and a single-step solid-phase sample preparation’. By using a single-step solid-phase extraction procedure on Bond Elut C2 columns, the sample preparation step has been considerably simplified and less time-consuming compared to earlier presented works. With a linear and reproducible calibration curve over the range 0.5-20 ng mL-1 plasma, the assay has successfully been used in the analysis of more than 500 plasma samples from a multicenter trial.

Barman et al (101) have studied ‘Simultaneous high-performance liquid chromatographic determination of atenolol and amlodipine in pharmaceutical-dosage form’. The method is based on High Performance Liquid Chromatography (HPLC) on a reversed-phase column, shim-pack CLC, ODS (C18), 4.6 mmx25 cm and 0.5 mm, using a mobile phase of ammonium acetate buffer (the pH was adjusted to 4.5+/-0.05 with glacial acetic acid), acetonitrile and methanol (35::30:35 v/v).

Suchanova et al (102) have developed ‘Liquid chromatography-tandem mass spectrometry in chiral study of amlodipine biotransformation in rat hepatocytes’. In this work a novel reversed phase liquid chromatography (RP-LC) separation method for amlodipine and its metabolites was developed. Based on this separation chiral aspects of amlodipine biotransformation were studied by incubation of amlodipine

and its two individual enantiomers with primary culture of rat hepatocytes. Structure of the metabolites was elucidated using a liquid chromatography (LC) separation with ultraviolet (UV) and mass spectrometry (MS) detection. An LC-tandem MS (MS/MS) method was used to establish fragmentation pattern of amlodipine and its metabolites.

Ma et al (103) have reported 'Determination and pharmacokinetic study of amlodipine in human plasma by ultra performance liquid chromatography-electrospray ionization mass spectrometry'. The analysis was carried out on an ACQUITY UPLC BEH C (18) column (50 mm x 2.1 mm, i.d., 1.7 microm) with gradient elution at a flow-rate of 0.35 mL/min. The mobile phase was water and acetonitrile under gradient conditions (both containing 0.3% formic acid) and nimodipine was used as the internal standard. Detection was performed on a triple-quadrupole tandem mass spectrometer by multiple reactions monitoring (MRM) mode via Turbo ion spray ionization (ESI).

Dongre et al (104) have carried out 'Simultaneous determination of metoprolol succinate and amlodipine besylate in pharmaceutical dosage form by HPLC'. The chromatographic separation was achieved on Hypersil BDS cyano (250 mm x 4.6 mm, 5 microm) column using PDA detector. The mobile phase consisting of buffer (aqueous triethylamine pH 3) and acetonitrile in the ratio of 85:15 (v/v) at a flow rate of 1.0 mL/min was used. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness.

Naidu et al (105) have estimated 'Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product'. The proposed RP-HPLC method utilizes a Zorbax SB C18, 5 microm, 250 mm x 4.6 mm i.d. column, mobile phase consisting of phosphate

buffer and acetonitrile in the proportion of 65:35 (v/v) with apparent pH adjusted to 7.0, and UV detection at 240 nm using a photodiode array detector.

Sarkar et al (106) have reported 'Simultaneous determination of metoprolol succinate and amlodipine besylate in human plasma by liquid chromatography-tandem mass spectrometry method and its application in bioequivalence study'. Both the drugs were extracted by simple liquid-liquid extraction with chloroform. The chromatographic separation was performed on a reversed-phase peerless basic C18 column with a mobile phase of methanol-water containing 0.5% formic acid (8:2, v/v). The protonated analyte was quantitated in positive ionization by multiple reactions monitoring with a mass spectrometer.

Altiokka et al (107) have reported 'Determination of amlodipine in pharmaceutical formulations by differential-pulse voltammetry with a glassy carbon electrode'. The experiments were conducted in a supporting electrolyte consisting of 0.2 M KCl, 0.1 M phosphate buffer, and 10% (v/v) methanol during investigation of initial potential and pH effects. No adsorption effect was observed on using an initial potential of 0 mV and the supporting electrolyte solution at pH 5.5 under both stationary and rotating conditions.

Chaudhari et al (108) have carried out 'Stability indicating RP-HPLC method for simultaneous determination of atorvastatin calcium and amlodipine from their combination drug products'. The proposed RP-HPLC method utilizes a Lichrospher 100 C18, 5 microm, 250 mm x 4.0 mm i.d. column, at ambient temperature, optimum mobile phase consisted of acetonitrile and 50 mM potassium dihydrogen phosphate buffer (60 : 40, v/v), apparent pH adjusted to 3+/-0.1 with 10% phosphoric acid solution, effluent flow rate monitored at 1.0 mL/min, and UV detection at 254 nm. ATV, AML, and their combination drug product were exposed to thermal, photolytic,

hydrolytic, and oxidative stress conditions, and the stressed samples were analyzed by proposed method.

Mohammadi et al (109) have carried out 'A stability-indicating high performance liquid chromatographic (HPLC) assay for the simultaneous determination of atorvastatin calcium and amlodipine in commercial tablets'. Separation was achieved on a Perfectsil Target ODS-3, 5 microm, 250 mm x 4.6 mm i.d. column using a mobile phase consisting of acetonitrile-0.025 M NaH(2)PO(4) buffer (pH 4.5) (55:45, v/v) at a flow rate of 1 mL/min and UV detection at 237 nm. The drugs were subjected to oxidation, hydrolysis, photolysis and heat to apply stress conditions.

Argekar et al (110) have studied 'Simultaneous determination of atenolol and amlodipine in tablets by high-performance thin-layer chromatography'. A new simple, precise, rapid and selective high-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous determination of atenolol (ATL) and amlodipine (AMLO) in tablets, using methylene chloride: methanol: ammonia solution (25% NH3) (8.8:1.3:0.1; v/v) as the mobile phase and Merck HPTLC plates (0.2 mm thickness) precoated with 60F254 silica gel on aluminium sheet as the stationary phase. Detection was carried out densitometrically using a UV detector at 230 nm.

Mohamed et al (111) have reported 'Determination of antihypertensive mixtures by use of a chemometrics-assisted spectrophotometric method'. The mixtures are composed of chlorthalidone with atenolol or chlorthalidone with both amiloride hydrochloride and atenolol. The components of the mixtures result in substantial spectral overlap-between 87.5 and 91.0%. Resolution of the mixtures under investigation has been accomplished mainly by using CLS and PCR methods.

The components in each mixture have been simultaneously determined in three commercial dosage forms with high accuracy and without interference from commonly encountered excipients and additives. Good recoveries were obtained with both synthetic mixtures and commercial tablets.

Tatar et al (112) have quantified 'Determination of amlodipine in human plasma by high-performance liquid chromatography with fluorescence detection'. A sensitive and specific HPLC method has been developed for the assay of amlodipine in human plasma. The assay involves derivatization with 4-chloro-7-nitrobenzofurazan (NBD-Cl), solid-phase extraction on a silica column and isocratic reversed-phase chromatography with fluorescence detection. Nortriptyline hydrochloride was used as an internal standard. The assay was linear over the concentration range of 0.25-18.00 ng/ mL. Both of the within-day and day-to-day reproducibility and accuracy were less than 11.80% and 12.00%, respectively. The plasma profile following a single administration of 10 mg amlodipine to a healthy volunteer was presented.

Bahrami et al (113) have estimated 'Simple and rapid HPLC method for determination of amlodipine in human serum with fluorescence detection and its use in pharmacokinetic studies'. Amlodipine is extracted from serum by ethyl acetate and involves precolumn derivatization with 4-chloro-7-nitrobenzofurazan (NBD-Cl) and reverse-phase chromatography on C_{18} column. The mobile phase was sodium phosphate buffer (pH 2.5) containing 1 mL/l triethylamine and methanol at flow rate of 2.8 mL/min. Propranolol was used as internal standard.

Asai et al (114) have developed 'Misinterpretation of the effect of amlodipine on cytosolic calcium concentration with fura-2 fluorospectrometry'. In ex vivo experiments without cells, due to the autofluorescence of amlodipine excited by

ultraviolet (UV light), amlodipine itself boosted the intensity of F380 much more than it did that of F340, which results in a decrease in F340/F380 ratio of fura-2 fluorescence. Rhod-2/AM, a fluorescent probe of which the excitation wavelength is out of the excitation spectrum of amlodipine, showed that amlodipine did not affect TG-induced intracellular Ca2+ increase in ECs. When the effect of amlodipine on intracellular Ca2+ concentration is assessed, fura-2 fluorospectrometry should not be used due to fluorescent interaction between amlodipine and fura-2, and using rhod-2 is strongly recommended.

Liu et al (115) have reported 'Study of interaction between drug enantiomers and human serum albumin by flow injection-capillary electrophoresis frontal analysis'. The results obtained by the method were compared with those determined by conventional equilibrium dialysis (ED)-CE and fluorescence spectra. Hydroxypropyl-beta-CD (HP-beta-CD) (10 mM) was used as a chiral selector in pH 3.7 phosphate buffer. L-tryptophan (L-try) and ketoprofen (Ket) were used as displacement reagents to investigate the binding sites of AL to HSA. A binding synergism effect between hydrochlorothiazide (QL) and AL was observed and the results suggested that QL can destroy binding equilibrium of R-AL and S-AL toward HSA and they can occupy the same binding site of HSA (site I). The reproducibility was confirmed by RSD (RSD<1.5%) of the plateau height determined by FI-CE frontal analysis (FI-CE-FA). The FI-CE-FA was a good method to study protein-drug interaction.

Altiokka et al (116) have reported 'Determination of amlodipine in pharmaceutical formulations by differential-pulse voltammetry with a glassy carbon electrode'. The experiments were conducted in a supporting electrolyte consisting of 0.2 M KCl, 0.1 M phosphate buffer, and 10% (v/v) methanol during investigation of

initial potential and pH effects. No adsorption effect was observed on using an initial potential of 0 mV and the supporting electrolyte solution at pH 5.5 under both stationary and rotating conditions. The factor affecting the voltammetric current was diffusional in the range of 200-1000 rpm for rotating, and 2-40 mV s-1 for stationary conditions up to a concentration of 0.04 mg mL-1 amlodipine.

Rahman et al (117) have estimated 'Spectrophotometric method for the determination of amlodipine besylate with ninhydrin in drug formulations'. The method is based on the reaction of the primary amino group of the drug with ninhydrin in N, N'-dimethylformamide (DMF) medium producing a coloured complex which absorbs maximally at 595 nm. Beer's law is obeyed in the concentration range of 10-60 microg mL(-1) with RSD of 0.66% and molar absorptivity of 6.52 x 10(3) l mol(-1) cm(-1).

Meyyanathan et al (118) have carried out 'HPTLC method for the simultaneous determination of amlodipine and benazepril in their formulations'. The method uses zolpidem as an internal standard (IS). The stationary phase used is silica gel 60 F254 prewashed with methanol. The mobile phase consists of an ethyl acetate-methanol-ammonia solution (8.5:2.0:1.0, v/v/v). Detection and quantitation are performed densitometrically at lambda = 254 nm. The Rf values of amlodipine, benazepril, and zolpidem (IS) are 0.58, 0.50, and 0.78, respectively.

2.4 Atorvastatin Calcium

Shah DA et al (119) have studied 'RP-HPLC method for the determination of atorvastatin calcium and nicotinic acid in combined tablet dosage form'. A phenomenex Luna C-18, 5 mm column having 250 × 4.6 mm i.d. in isocratic mode, with mobile phase containing 0.02 M potassium dihydrogen phosphate: methanol:

acetonitrile (20:40:40, pH 4) was used. The flow rate was 1.0 mL/ min and effluents were monitored at 240 nm.

Sonawane SS et al (120) have reported 'Simultaneous spectrophotometric estimation of atorvastatin calcium and ezetimibe in tablets'. Simultaneous estimation of atorvastatin calcium and ezetimibe in their tablet dosage form has been developed using, Q-absorbance equation. The method involves, the absorbance measurement at 235.5 nm (iso-absorptive point) and 246.0 nm (λmax ofatorvastatin calcium), in methanol.

Naresh et al (121) have quantified 'Estimation of atorvastatin calcium and Fenofibrate in tablets by derivative spectrophotometry and liquid chromatography'. In the second-derivative responses at 245.64 nm for atorvastatin calcium and 289.56 nm for Fenofibrate in spectra of their solution in methanol. In the LC method, analysis was performed on a Hypersil ODS-C18 column (250 mm x 4.6 mm id, 5 [micro]m particle size) in the isocratic mode using the mobile phase methanol-water (90 + 10, v/v), adjusted to pH 5.5 with orthophosphoric acid, at a flow rate of 1 mL/min. Measurement was made at a wavelength of 246.72 nm.

Sandeep S et al (122) have reported 'Application of UV-Spectrophotometry and RP-HPLC for Simultaneous Determination of Atorvastatin Calcium and Ezetimibe in Pharmaceutical Dosage Form'. The first method was based on UV-spectrophotometric determination of two drugs, using simultaneous equation method. It involves absorbance measurement at 232.5 nm (ëmax of Ezetimibe) and 246.0 nm (ëmax of Atorvastatin calcium) in methanol; linearity was obtained in the range of 5 – 25 µg. ML^{-1} for both the drugs. The second method was based on HPLC separation of the two drugs in reverse phase mode using Luna C18 column.

Zahid Zaheer et al (123) have developed 'Stability-indicating high performance liquid chromatographic determination of atorvastatin calcium in pharmaceutical dosage form'. The chromatographic conditions comprised of a reversed-phase C18 column (250 x 4.6 mm), 5 µ with a mobile phase consisting of a mixture of Methanol: Acetonitrile: Phosphate Buffer solution in the ratio (45:45:10). Flow rate was 1 mL / min. Detection was carried out at 246 nm. The retention time of Atorvastatin calcium was 6.98 min. Atorvastatin calcium was subjected to acid and alkali hydrolysis, oxidation, photochemical degradation and thermal degradation.

Nagaraj et al (124) have estimated 'Simultaneous quantitative resolution of atorvastatin calcium and Fenofibrate in pharmaceutical preparation by using derivative ratio spectrophotometry and chemometric calibrations'. In ratio spectra derivative spectrophotometry, analytical signals were measured at wavelengths corresponding to either maximums or minimums for both drugs in first derivative spectra of ratio spectra obtained by using either spectrum as divisor. For the remaining four methods using chemometric techniques, namely, classical least squares (CLS), inverse least squares (ILS), principal component regression (PCR) and partial least squares (PLS), the calibrations were constructed by using the absorption data matrix corresponding to the concentration data matrix, with measurements in the range of 231- 310 nm ($\Delta\lambda$ = 1 nm) in their zero-order spectra.

Jain N et al (125) have reported 'Simultaneous Determination of Atorvastatin Calcium and Ezetimibe in Pharmaceutical Formulations by Liquid Chromatography'. The separation was achieved by Luna C18 column and methanol : acetate buffer pH 3.7 (82:18 v/v) as mobile phase, at a flow rate of 1.5 mL/min. Detection was carried out at 248 nm.

Borek-Dohalsky V et al (126) have carried out 'Validated HPLC–MS–MS method for simultaneous determination of atorvastatin and 2-hydroxyatorvastatin in human plasma-pharmacokinetic study'. Quantitative determination of the major statin drug atorvastatin calcium (ATV) and its metabolite 2-hydroxyatorvastatin (HATV) detection was performed with an electrospray ionization triple-quadrupole mass spectrometer equipped with an ESI interface operating in positive-ionization mode. Multiple reactions monitoring (MRM) was used for MS–MS detection.

Nakarani, Naresh V et al (127) have quantified 'Estimation of atorvastatin calcium and Fenofibrate in tablets by derivative spectrophotometry and liquid chromatography'. Atorvastatin calcium and Fenofibrate in combined preparations (tablets) were quantitated using the second-derivative responses at 245.64 nm for atorvastatin calcium and 289.56 nm for Fenofibrate in spectra of their solution in methanol. In the LC method, analysis was performed on a Hypersil ODS-C18 column (250 mm x 4.6 mm id, 5 microm particle size) in the isocratic mode using the mobile phase methanol-water (90 + 10, v/v), adjusted to pH 5.5 with orthophosphoric acid, at a flow rate of 1 mL/min.

2.5 Fenofibrate

El-Gindy A et al (128) have studied 'Spectrophotometric and LC determination of Fenofibrate and Vinpocetine and their hydrolysed product'.

Masnatta LD et al (129) have quantified 'Determination of benzafibrate, ciprofibrate and fenofibric acid in human plasma by HPLC'

Komsta Lukasz and Misztal Genowefa (130) have assayed 'Determination of Fenofibrate and gemfibrozil in pharmaceuticals by densitometric and videodensitometric thin-layer chromatography'. New thin-layer chromatography (TLC) methods with densitometric and videoscanning detection were elaborated for

the quantitative determination of Fenofibrate in Fenoratio capsules and gemfibrozil in Gemfibral tablets. Analysis was performed on high-performance TLC diol F254 plates using hexane-tetrahydrofuran (8 + 2, v/v) mobile phase in horizontal DS chambers using the sandwich technique.

2.6 Folic Acid

Klaczkow Gabriela and Anuszewska Elzbieta L (131) have reported 'Determination of impurities in pharmaceutical preparations containing folic acid'. In the presented study, the HPLC method was used for the determination of impurities (p-aminobenzoic acid and p-aminobenzoyl-L-glutamic acid) in single-component pharmaceutical products containing folic acid. The determination was performed using a spectrophotometric detector at lambda = 269 nm wavelength.

Nelson Bryant C et al (132) have reported 'Quantitative determination of folic acid in multivitamin/multielement tablets using liquid chromatography/tandem mass spectrometry'. Two different isotope-dilution liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods for the quantitative determination of folic acid (FA) in multivitamin/multielement tablets are reported. These methods represent distinct improvements in terms of speed and specificity over most existing microbiological and chromatographic methods for the determination of FA in dietary supplements.

Zhang, Bo-Tao et al (133) have reported 'Determination of folic acid by chemiluminescence based on peroxomonosulfate-cobalt (II) system'. Based on the chemiluminescence (CL) phenomena of folic acid in peroxomonosulfate-cobalt (II) system, a rapid and sensitive CL method was developed for determination of folic acid in pharmaceutical preparations and its urinary metabolism processes.

Póo-Prieto Rosalia et al (134) have quantified 'Use of the affinity/HPLC method for quantitative estimation of folic acid in enriched cereal-grain products'. Folic acid and 5-methyltetrahydrofolate were separated and quantified by reversed-phase HPLC with fluorescence and UV detection. A gradient elution with phosphate buffer and acetonitrile was used to separate the different forms of folates.

2.7 Rosiglitazone Maleate

Koltea BL et al (135) have quantified 'Liquid chromatographic method for the determination of rosiglitazone in human plasma'. Isocratic separation of I and II is carried out using a reversed-phase Zorbax SB C18, 15-cm column with mobile phase consisting of methanol and a mixed phosphate buffer (10 mM monobasic sodium phosphate and dibasic sodium phosphate, pH adjusted to 2.6 with ortho-phosphoric acid) in the ratio 30:70 (v/v) and quantified by UV detection at 245 nm.

Patricia Gomes and Martin Steppe (136) have studied 'First-Derivative Spectrophotometry in the Analysis of Rosiglitazone in Coated Tablets'. First-derivative spectrophotometry, applying the peak-zero method, was developed for the determination of rosiglitazone (RSG) in coated tablets. The solutions of standard and sample were prepared in ethanol. Quantitative determination of the drug was performed at 331.4 nm (N = 4; $\Delta\lambda$ = 3.2 nm) and was evaluated for the parameters specificity.

Ceren Yardımcıa et al (137) have estimated Simultaneous determination of rosiglitazone and metformin in plasma by gradient liquid chromatography with UV detection. The analysis was performed on a phenyl column (250 mm × 4.6 mm i.d., 5 μm) using a gradient method starting with mobile phase composed of acetonitrile: 5 mM acetate buffer pH 5.5 (75:25, v/v). The flow rate was 1 mL min^{-1}. UV detection was performed at 245 nm and verapamil was used as internal standard.

2.8 Glibenclamide

Georgita, Cristina et al (138) have repoted 'Simultaneous assay of metformin and glibenclamide in human plasma based on extraction-less sample preparation procedure and LC/(APCI)MS'. Separation of metformin and glibenclamide was achieved within a single chromatographic run on a Zorbax CN column, under isocratic conditions, using acetonitrile and aqueous component (0.01 moles/L ammonium acetate adjusted at pH 3.5 with acetic acid) in volumetric ratio 1/1. Plasma sample preparation is based on protein precipitation by means of organic solvent addition. 1,3,5-Triazine-2,4,6-triamine (IS1) was used as internal standard for metformin, while gliquidone (IS2) played the same role for glibenclamide.

Niopas Ioannis et al (139) have carried out 'A validated high-performance liquid chromatographic method for the determination of glibenclamide in human plasma and its application to pharmacokinetic studies'. The method was linear in the 10-400 ng/ mL concentration range ($r > 0.999$). Recovery for glibenclamide was greater than 91.5% and for internal standard was 93.5%. Within-day and between-day precision, expressed as the relative standard deviation (RSD %), ranged from 1.4 to 5.9% and 5.8 to 6.6%, respectively.

Albu, Florin et al (140) have developed 'Determination of glibenclamide in human plasma by liquid chromatography and atmospheric pressure chemical ionization/MS-MS detection'. The chromatographic separation was achieved on a monolithic octadecyl chemically modified silicagel column and a mobile phase containing 42% aqueous 0.1% HCOOH solution (v/v) and 58% acetonitrile, at a flow rate of 1 mL/min, in isocratic conditions. Preparation of plasma samples was based on protein precipitation with acetonitrile. Gliquidone was used as internal standard.

Bansal Gulshan et al (141) have reported 'Ultraviolet-photodiode array and high-performance liquid chromatographic/mass spectrometric studies on forced degradation behavior of glibenclamide and development of a validated stability-indicating method'. A forced degradation study on glibenclamide was performed under conditions of hydrolysis, oxidation, dry heat, and photolysis and a high-performance column liquid chromatographic-ultraviolet (HPLC-UV) method was developed to study degradation behavior of the drug under the forced conditions. The degradation products formed under different forced conditions were characterized through isolation and subsequent infrared/nuclear magnetic resonance/mass spectral analyses, or through HPLC/mass spectrometric (HPLC/MS) studies.

El Deeb et al (142) have quantified 'Fast HPLC method for the determination of glimepiride, glibenclamide, and related substances using monolithic column and flow program'. The separation was performed on a Chromolith Performance (RP-18e, 100 mm x 4.6 mm) column. As mobile phase, a mixture of phosphate buffer pH 3, 7.4 mM, and ACN (55:45 v/v) was used. Column oven temperature was set to 30 degrees C. The total chromatographic run time was 80 s.

Venkatesh, P et al (143) have reported 'Simultaneous estimation of six anti-diabetic drugs--glibenclamide, gliclazide, glipizide, pioglitazone, repaglinide and rosiglitazone: development of a novel HPLC method for use in the analysis of pharmaceutical formulations and its application to human plasma assay'. The analytes were either injected directly onto the column after suitable dilution (pharmaceutical formulation analysis) or a simple extraction procedure, using acetonitrile, from human plasma spiked with anti-diabetic drugs and internal standard (IS). Ternary gradient elution at a flow rate of 1 mL/min was employed on an Intertisl ODS 3V column

(4.6 x 250 mm, 5 microm) at ambient temperature. The mobile phase consisted of 0.01 m formic acid (pH 3.0), acetonitrile, Milli Q water and methanol.

Rajendran SD et al (144) have reported 'RPHPLC method for the estimation of glibenclamide in human serum'. After precipitation with methanol, the separation of glibenclamide and internal standard was accomplished using reversed phase chromatography. The mobile phase, a combination of acetonitrile and 25 mM phosphate buffer (pH 3.5) at 3:2 ratio was run isocraticaly through a C18 analytical column. The UV detection was done at 253 nm for glibenclamide

Khalil I et al (145) have carried out 'High-Performance Liquid Chromatographic Method for Determination of Glibenclamide in Human Plasma'. The methodology is based on simple one step extraction of glibenclamide and diazepam (internal standard) from human plasma with dichloromethane. The drugs were eluted from a resolve 5 u spherical C_{18} column with a mobile phase consisting of 0.05 M ammonium dihydrogen phosphate: methanol (40:60%, v/v) adjusted to pH 4.0 with phosphoric acid.

Florin Albua et al (146) have studied 'Determination of glibenclamide in human plasma by liquid chromatography and atmospheric pressure chemical ionization/MS-MS detection'.

Susanto F and Reinauer H (147) have reported 'Glibenclamide in serum: comparison of high-performance liquid chromatography using fluorescence detector and liquid chromatography/mass spectrometry with atmospheric-pressure chemical-ionization (APCI LC/MS)'. After acidification of the sample, the analyte and internal standard are extracted with chloroform. For HPLC analysis, the glibenclamide and internal standard are derivatized to a highly fluorescent amine with 7-chloro-4-

nitrobenzo-2-oxa-1,3-diazole (NBD-chloride). The glibenclamide derivative is detected by a fluorescence detector.

Aburuz S et al (148) have developed 'The development and validation of liquid chromatography method for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma'. This article describes the development of SPE and HPLC methods for the simultaneous determination of metformin and glipizide, gliclazide, glibenclamide or glimperide in plasma.

Valdes Santurio JR et al (149) have estimated 'Determination of glibenclamide in human plasma by solid-phase extraction and high-performance liquid chromatography'. A sensitive high-performance liquid chromatographic method for determination of intact glibenclamide in human plasma has been developed.

Rydberg T et al (150) have quantified 'Determination of glibenclamide and its two major metabolites in human serum and urine by column liquid chromatography'. A simple reversed-phase liquid chromatographic method for the measurement of low concentrations of glibenclamide (glyburide) and its two major metabolites, 4-trans- and 3-cis-hydroxyglibenclamide, in human serum and urine has been developed. The compounds were extracted with n-hexane-dichloromethane (1:1). The UV detection wavelength was 203 nm. The minimum detectable serum level of glibenclamide was 1 ng mL (2 nM), and the relative standard deviation was 8.9% (n = 9). When maximum sensitivity was desired the metabolites were chromatographed separately. Metabolites in urine were measured by the same method after five-fold sample dilution. The utility of the method was tested on a healthy volunteer who ingested 3.5 mg of glibenclamide. The parent drug was present in the serum for at least 18 h, and the metabolites in the urine for at least 24 h.

2.9 Metformin Hydrochloride

Zarghi et al (151) have developed 'Rapid determination of metformin in human plasma using ion-pair HPLC'. The separation was performed on an analytical 150 x 4.6 mm i.d. mubondapak C_{18} column. The wavelength was set at 235 nm. The mobile phase was 40% acetonitrile, 0.01 M sodium dodecyl sulphate, 0.01 M sodium dihydrogen phosphate, and distilled water to 100%, adjusted to pH 5.1 at a flow rate of 1.5 mL /min. The calibration curve was linear over the concentration range 0.2-2.5 microg/ mL . The coefficients of variation for inter-day and intra-day assay were within the range of clinical usefulness.

Mistri Hiren N et al (152) have reported 'Liquid chromatography tandem mass spectrometry method for simultaneous determination of antidiabetic drugs metformin and glyburide in human plasma'. After acidic acetonitrile-induced protein precipitation of the plasma samples, metformin, glyburide and IS were chromatographed on reverse phase C_{18} (50 mm x 4.6 mm i.d., 5 microm) analytical column.

Zhang Lu et al (153) have carried out 'Simultaneous determination of metformin and rosiglitazone in human plasma by liquid chromatography/tandem mass spectrometry with electrospray ionization: application to a pharmacokinetic study'. Plasma samples were precipitated by acetonitrile and the analytes were separated on a prepacked Phenomenex Luna 5u CN 100A (150 mm x 2.0 mm I.D.) column using a mobile phase comprised of methanol: 30 mM ammonium acetate pH 5.0 (80:20, v/v) delivered at 0.2 mL /min. Detection was performed on a Finnigan TSQ triple-quadrupole tandem mass spectrometer in positive ion selected reaction monitoring (SRM) mode using electrospray ionization.

2.10 Nebivolol Hydrochloride

Selvan P Senthamil et al (154) have studied 'Simultaneous determination of fixed dose combination of nebivolol and valsartan in human plasma by liquid chromatographic-tandem mass spectrometry and its application to pharmacokinetic study'. Nebivolol and valsartan were extracted from plasma using acetonitrile and separated on a C18 column. The mobile phase consisting of a mixture of acetonitrile and 0.05 mM formic acid (50:50 v/v, pH 3.5) was delivered at a flow rate of 0.25 mL /min. Atmospheric pressure ionization (API) source was operated in both positive and negative ion mode for nebivolol and valsartan, respectively.

Ramakrishna NVS et al (155) have reported 'Rapid quantification of nebivolol in human plasma by liquid chromatography coupled with electrospray ionization tandem mass spectrometry'. The method involved a simple single-step liquid–liquid extraction with diethyl ether/dichloromethane (70/30). The analyte was chromatographed on Waters symmetry® C_{18} reversed-phase chromatographic column by isocratic elution with water: acetonitrile: formic acid (30:70:0.03, v/v) and analyzed by mass spectrometry in the multiple reaction monitoring mode.

Pankaj K. Kachhadia et al (156) have quantified 'Development and Validation of a Stability-Indicating Column High-Performance Liquid Chromatographic Assay Method for Determination of Nebivolol in Tablet Formulation'. Isocratic RP-HPLC separation was achieved on a Phenomenex Luna C8 (2) column (250 mm × 4.6 mm id, 5 µm particle size) using mobile phase composed of acetonitrile–pH 3.5 phosphate buffer (35 + 65, v/v) at a flow rate of 1.0 mL /min, and detection was performed at 280 nm using a photodiode array detector.

Dimal A. Shah et al (157) have carried out 'Determination of Nebivolol Hydrochloride and Hydrochlorothiazide in Tablets by First-Order Derivative

Spectrophotometry and Liquid Chromatography'. Two simple and accurate methods for analysis of nebivolol hydrochloride (NEB) and hydrochlorothiazide (HCTZ) in their combined dosage forms were developed using first-order derivative spectrophotometry and reversed-phase liquid chromatography (LC).

Nikalaje AG et al (158) have estimated 'Derivative spectrophotometric method for estimation of Amlodipine and Nebivolol in combined tablet formulation;.

Kamila MM et al (159) have estimated 'A validated UV spectrophotometric method for estimation of Nebivolol and Hydrochlorothiazide in bulk and pharmaceutical formulation'.

Reddy TS and Devi SP et al (160) have estimated 'Validation of high performance thin layer Chromatographic methods with densitometric detection for quantitative analysis of Nebivolol Hydrochloride in tablet formulations'.

2. 11 Pioglitazone Hydrochloride

Sripalakit Pattana et al (161) have estimated 'High-performance liquid chromatographic method for the determination of pioglitazone in human plasma using ultraviolet detection and its application to a pharmacokinetic study'. Rosiglitazone was used as an internal standard. Chromatographic separation was achieved with a reversed-phase Apollo C18 column and a mobile phase of methanol-acetonitrile-mixed phosphate buffer (pH 2.6; 10 mM) (40:12:48, v/v/v) with a flow rate of 1.2 mL /min.

Y. -J. Xue et al (162) have studied 'Quantitative determination of pioglitazone in human serum by direct-injection high-performance liquid chromatography mass spectrometry and its application to a bioequivalence study'. The on-line extraction was achieved on an Oasis® HLB column (1 mm×50 mm, 30 μm) with a 100% aqueous loading mobile phase containing 5 mM ammonium acetate (pH 4.0) at a flow

rate of 4 mL /min. The extracted analyte was eluted by a mobile phase which contained 5 mM ammonium acetate and acetonitrile. The analytical column was a Luna C18 column (4.6 mm×50 mm, 5 μm). Detection was achieved by positive ion electrospray tandem mass spectrometry.

Kolte BL et al (163) have quantified 'Simultaneous high-performance liquid chromatographic determination of pioglitazone and metformin in pharmaceutical-dosage form'. Separation is achieved with a Zorbax XDB C(18), 15-cm analytical column using buffer-acetonitrile (66:34, v/v) of pH 7.1, adjusted with orthophosphoric acid as the mobile phase. The buffer used in the mobile phase contains 10mM disodium hydrogen phosphate and 5mM sodium dodecyl sulphate in double-distilled water. The instrumental settings are flow rate of 1 mL /min, column temperature at 40 degrees C, and detector wavelength of 226 nm.

Souri E et al (164) have estimated 'Development and validation of a simple and rapid HPLC method for determination of pioglitazone in human plasma and its application to a pharmacokinetic study' After liquid-liquid extraction with diethylether, samples were quantitated on a Nova-Pak C8 column using a mixture of acetonitrile-140mM K2HPO4 (40:60, v/v, pH = 4.45) as mobile phase with UV detection at 269 nm. The flow rate was set at 1.4 mL /min.

Wang M et al (165) have estimated 'Multi-component plasma quantitation of anti-hyperglycemic pharmaceutical compounds using liquid chromatography-tandem mass spectrometry'. Hypoglycemic agents such as metformin, glipizide, glyburide, repaglinide, rosiglitazone, nateglinide, and pioglitazone are widely prescribed to control blood sugar levels. These drugs provide the basis for the development of a quantitative multianalyte bioanalytical method.

Zhong WZ et al (166) have estimated 'Simultaneous quantitation of pioglitazone and its metabolites in human serum by liquid chromatography and solid phase extraction'. Separation of the eight analytes was achieved within 20 min using a reversed-phase Zorbax RX-C8 analytical column (250 mm x 4.6 mm i.d., 5 microns particle size) with a mobile phase of acetonitrile-water (40:60, v/v) containing 3 mL acetic acid per liter mobile phase (apparent pH 5.5). An ultraviolet detector operated at 269 nm.

2.12 Ramipril

Gowda, K Veeran et al (167) have studied 'Liquid chromatography tandem mass spectrometry method for simultaneous determination of metoprolol tartrate and ramipril in human plasma'. Both the drugs were extracted by liquid-liquid extraction with diethyl ether-dichloromethane (70:30, v/v). The chromatographic separation was performed on a reversed-phase C8 column with a mobile phase of 10 mM ammonium formate-methanol (3:97, v/v).

Belal et al (168) have estimated 'Voltammetric determination of benazepril and ramipril in dosage forms and biological fluids through nitrosation'. A simple and highly sensitive voltammetric method was developed for the determination of benazepril (I) and ramipril (II). The compounds were treated with nitrous acid, and the cathodic current produced by the resulting nitroso derivatives was measured. The voltammetric behavior was studied by adopting direct current (DCt), differential pulse (DPP), and alternating current (ACt) polarography.

Zhimeng Zhu et al (169) have quantified 'Liquid chromatography–mass spectrometry method for determination of ramipril and its active metabolite ramiprilat in human plasma'. The method involves a solid-phase extraction from plasma, simple

isocratic chromatography conditions and mass spectrometric detection that enables a detection limit at sub-nanogram levels.

Rahman N et al (170) have estimated 'Kinetic Spectrophotometric Method for the Determination of Ramipril in Pharmaceutical Formulations'. The method was based on the reaction of carboxylic acid group of the drug with a mixture of potassium iodate (KIO_3) and potassium iodide (KI) in aqueous medium at room temperature.

Abdellatef et al (171) have estimated 'A Spectrophotometric and atomic absorption spectrometric determination of ramipril and perindopril through ternary complex formation with eosin and Cu (II)'. This paper describes three sensitive spectrophotometric and spectrofluorimetric methods for determination of ramipril in its pure form and pharmaceutical tablets.

Al-Majed A et al (172) have estimated 'Spectrophotometric determination of ramipril (a novel ace inhibitor) in dosage forms'. The method is based on reacting the drug with potassium permanganate in alkaline medium whereby a bluish green colour peaking at 610 nm is produced.

Veeran Gowdaa K et al (173) has estimated 'Liquid chromatography tandem mass spectrometry method for simultaneous determination of metoprolol tartrate and ramipril in human plasma'. Both the drugs were extracted by liquid–liquid extraction with diethyl ether–dichloromethane (70:30, v/v). The chromatographic separation was performed on a reversed-phase C8 column with a mobile phase of 10 mM ammonium formate–methanol (3:97, v/v).

Hassan et al (174) have estimated 'Determination of Ramipril and Its Precursors by Reverse Phase High Performance Liquid Chromatography'. A simple

isocratic HPLC elution method consisting of acetonitrile: 5% phosphoric acid (30:70) was employed for the separation of ramipril from precursors I and II in 15 minutes.

Joseph L et al (175) have studied 'Simultaneous estimation of atorvastatin calcium and ramipril by RP-HPLC and spectroscopy'. Individual lambda-max for atorvastatin calcium is 247 nm and that of ramipril is 208 nm. They intercept at 215 nm which is fixed as wavelength for reverse phase-high performance liquid chromatographic method.

2.13 Ezetimibe

Sistla R et al (176) have quantified 'Development and validation of a reversed-phase HPLC method for the determination of ezetimibe in pharmaceutical dosage forms'. Ezetimibe belongs to a group of selective and very effective 2-azetidione cholesterol absorption inhibitors that act on the level of cholesterol entry into enterocytes.

Oliveira et al (177) have estimated 'Simultaneous liquid chromatographic determination of ezetimibe and simvastatin in pharmaceutical products'. A reversed-phase liquid chromatographic (LC) method was developed and validated for the simultaneous determination of ezetimibe and simvastatin in pharmaceutical dosage forms. The LC method was carried out on a Synergi fusion C18 column (150 mm x 4.6 mm id) maintained at 45 degrees C.

2.14 Levofloxacin

Altiokka et al (178) have estimated 'The determination of levofloxacin by flow injection analysis using UV detection, potentiometry and conductometry in pharmaceutical preparations'. The solvent system was found to consist of 0.2M acetate buffer at pH 3.0 having 10% MeOH and the active material detected at 388 nm.

Fu et al (179) have estimated 'The determination of levofloxacin hydrochloride in human plasma by RP-HPLC method'. The concentration of levofloxacin in human plasma after a single dose of a domestic capsule or imported tablet and their pharmacokinetics were studied.

Ashour et al (180) have estimated 'A simple extractive colorimetric determination of levofloxacin by acid-dye complexation methods in pharmaceutical preparations'. The developed methods involve formation of coloured chloroform extractable ion-pair complexes of levofloxacin with bromophenol and bromocresolgreen in aqueous medium.

Salem et al (181) have studied 'two colourimetric and atomic absorption spectrometric determination of some fluoroquinolone derivatives'. The developed methods involve the formation of ion-pair complexes between the drugs and ammonium reineckate reagent in acidic medium and the formed precipitates are determined either colourimetrically by dissolving the formed precipitate with acetone at 527 nm or by atomic absorption spectrometric procedure at 358 nm. Other colourimetric method is based on the reaction of the drug with 2,2-diphenyl 1-picrylhydrazyl reagent and the violet colour formed is measured at 520 nm.

Atkosar et al (182) have estimated 'A pulse polarographic determination of levofloxacin in tablets'. The study was conducted to investigate the optimum polarographic conditions for the determination of levofloxacin based on the reduction of the quinolone ring and to apply the method to pharmaceutical preparations.

Lei et al (183) have quantified 'The determination of levofloxacin injection and related substances by HPLC'.

Si et al (184) have estimated 'The comparison of bioassay and HPLC for the determination of levofloxacin in plasma'. Drug levels in plasma determined by bioassay and HPLC were not significantly different.

Zhang et al (185) have estimated the determination of levofloxacin in serum by RP-HPLC.

Lei et al (186) have estimated the determination of concentration of levofloxacin in blood by RP-HPLC

Wong et al (187) have estimated a rapid stereo specific high-performance liquid chromatographic determination of levofloxacin in human plasma and urine

Lakshmi sivasubramanian et al (188) has studied 'The three visible spectrophotometric determination of Levofloxacin in tablet dosage forms'. The first two methods were based on the formation of ion-association complex of the drug with Eriochrome black T and Bromocresol green, the absorbance was measured at 490 nm and 520 nm respectively, and the third method was based on formation of blue coloured complex with Folin Ciocalteau reagent which showed maximum absorbance at 720 nm.

2.15 Ornidazole

Li et al (189) have quantified a HPLC method for the determination of ornidazole in plasma and study on its pharmacokinetics. The method employs C<INF>18</INF>5mum, column using methanol and 0.4%glacial acetic acid (50/50) at a flow rate 0.8mL/min, the detection was carried out at 316n nm.

Padhye et al (190) have estimated a simple colorimetric method for the determination of ornidazole from bulk drug.

Abu et al (191) have estimated a extractional spectrophotometric determination of febendazole and ornidazole in pharmaceutical formulations.

Ozkan et al (192) have estimated the determination of ornidazole in pharmaceutical dosage forms based on reduction at an activated glassy carbon electrode. The electrode was activated by applying a new pretreatment and the dependence of intensities of currents and potentials on pH, concentration, scan rate, nature of the solvent, and surfactants was examined.

Heizmann et al (193) have quantified an HPLC method with UV detection for the determination of ornidazole in plasma, urine, vaginal fluid and CSF.

Rona et al (194) have estimated a simple liquid chromatographic method with diode ray detection for the determination of ornidazole and metronidazole in human serum.

Groppi et al (195) have estimated an HPLC method for the determination of ornidazole in human plasma and red blood cells using high performance liquid chromatography.

Kamble et al (196) have studied a HPLC method for the determination of Ornidazole and Ofloxacin in solid dosage form. The method consists of hypersil ODS C_{18} column with mobile phase consisting of 0.5% triethlyl amine in water, pH adjusted to 3 with dilute Orthophosphoric acid and acetonitrile having the ratio (65:35) and flow rate 0.8ml/min detected at 315 nm

Kasture et al (197) have estimated the simultaneous estimation of Ofloxacin and Ornidazole in tablet dosage form. The maximum absorbance was found to be 287 nm and 320 nm respectively for Ofloxacin and Ornidazole respectively when the drug was dissolved in methanol and further diluted with distilled water.

Patel et al (198) have estimated the simultaneous spectrophotometric estimation of Gatifloxacin and Ornidazole in mixture, the maximum absorption was

found to be 286.2 nm for Gatifloxacin and 319 nm for Ornidazole and the iso-absorptive point was 299.2 nm in distilled water

Kale et al (199) have estimated the spectrophotometric determination of Ornidazole and Norfloxacin in tablets, the maximum absorption was found to be 317 nm for Ornidazole and 272 nm for Norfloxacin in 0.1N NaOH.

3. EXPERIMENTAL WORK

This part of the thesis deals with the experiments part of the simultaneous estimation of the drug combinations by chemometrics assisted UV- Visible spectroscopy and comparison of the same with LC method and development of simple, accurate simultaneous estimation of few drugs in combined dosage form by first order derivative spectroscopic method.

3.1 AIM AND PLAN OF WORK

The object of this project is to develop

1. Spectroscopic techniques in conjugation with chemometric tools
2. Derivative spectroscopic methods
3. RP-HPLC methods

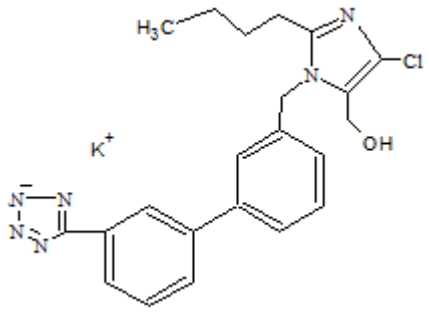
In order to achieve simple, rapid, precise and accurate analysis of combination of drugs in pharmaceutical formulation.

3.2 DRUG PROFILE

This part of the thesis describes the drug profile of the individual drugs selected for the estimation.

3.2.1 LOSARTAN POTASSIUM

Molecular structure



Chemical name

Losartan potassium, a non-peptide molecule, is chemically described as 2-butyl-4-chloro-1-[p-(o-1H-tetrazol-5-ylphenyl) benzyl] imidazole-5-methanol monopotassium salt.

Molecular formula

$C_{22}H_{22}ClKN_6O$

Molecular weight

461.01 g mol^{-1}

Category

Losartan potassium is an angiotensin II receptor (type AT_1) antagonist.

Description

White to off-white free-flowing crystalline powder

Solubility

It is freely soluble in water, soluble in alcohols, and slightly soluble in common organic solvents, such as acetonitrile and methyl ethyl ketone.

Identification

i) Melting Point

Standard Value	Observed Value*
183.5 - 184.5°C	184°C

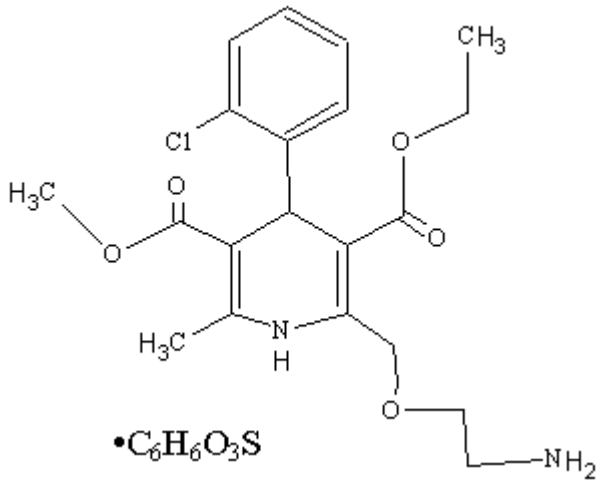
*Average of six observations

Storage

Stored at 25°C (77°F); excursions permitted to 15-30°C (59-86°F). Keep container tightly closed and protected from light.

3.2.2 AMLODIPINE BESYLATE

Molecular structure



Chemical name

Amlodipine besylate is chemically described as 3-Ethyl-5-methyl (±)-2-[(2 aminoethoxy) methyl]-4-(2-chlorophenyl)-1, 4-dihydro-6-methyl-3, 5 pyridine dicarboxylate, monobenzenesulphonate.

Molecular formula

$C_{20}H_{25}ClN_2O_5 \bullet C_6H_6O_3S$

Molecular weight

567.1 g mol^{-1}

Category

Anti-hypertensive and in the treatment of angina

Description

Amlodipine besylate is a white crystalline powder.

Solubility

It is slightly soluble in water and sparingly soluble in ethanol.

Identification

i) Melting Point

Standard Value	Observed Value*
195- 204°C	203°C

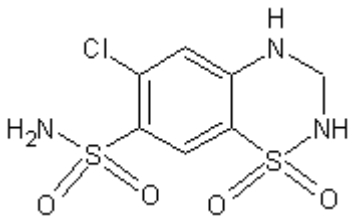
*Average of six observations

Storage

Store at room temperature, keep away from light and moisture.

3.2.3 HYDROCHLOROTHIAZIDE

Molecular structure



Chemical name

6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide.

Molecular formula

$C_7H_8ClN_3O_4S_2$

Molecular weight

297.74 g mol^{-1}

Category

Hydrochlorothiazide is a first line diuretic drug of the thiazide class.

Description

It is a white crystalline powder.

Solubility

It is slightly soluble in water, soluble in methanol, ethanol and acetone but freely soluble in sodium hydroxide solution.

Identification

i) Melting Point

Standard Value	Observed Value*
273 - 275°C	274°C

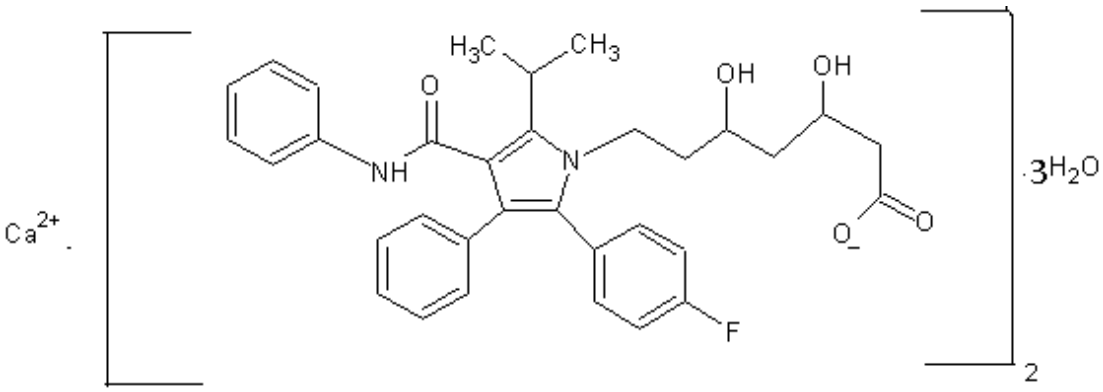
*Average of six observations

Storage

Keep container tightly closed. Protect from light, moisture, freezing, -20°C (-4°F) and store at room temperature, 15 - 30°C (59-86°F).

3.2.4 ATORVASTATIN CALCIUM

Molecular structure



Chemical name

(3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-(propan-2-yl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid, calcium salt (2:1) trihydrate.

Molecular formula

$C_{66}H_{68}CaF_2N_4O_{10}$

Molecular weight

1155.37 g mol^{-1}

Category

HMG-CoA reductase inhibitor

Description

White to off-white crystalline powder

Solubility

Freely soluble in methanol, slightly soluble in ethanol, acetonitrile and phosphate buffer P^H 7.4

Identification

i) Melting Point

Standard Value	Observed Value*
161 – 163°C	162°C

*Average of six observations

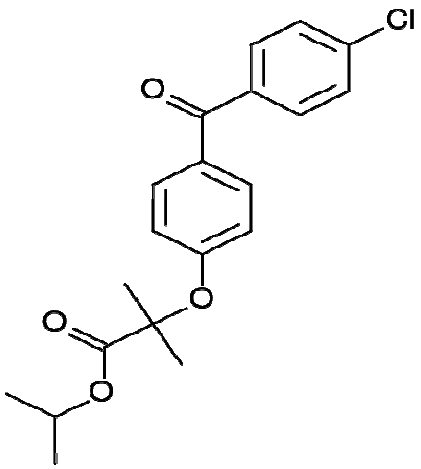
Storage

Store in tightly closed containers in a cool and dry place

3.2.5 FENOFIBRATE

Fenofibrate is second generation pro-drug fibric acid derivative which has greater LDL-CH lowering action than other fibrates.

Molecular structure



Chemical name

Propan-2-yl 2-{4-[(4-chlorophenyl) carbonyl] phenoxy}-2-methylpropanoate

Molecular formula

$C_{20}H_{21}ClO_4$

Molecular weight

360.831 g mol^{-1}

Category

HMG-CoA Reductase Inhibitor

Description

White crystals

Solubility

Practically insoluble in water, slightly soluble in methanol, ethanol and soluble in ether, acetone, benzene, chloroform.

Identification

i) Melting Point

Standard Value	Observed Value*
79 - 82°C	79°C

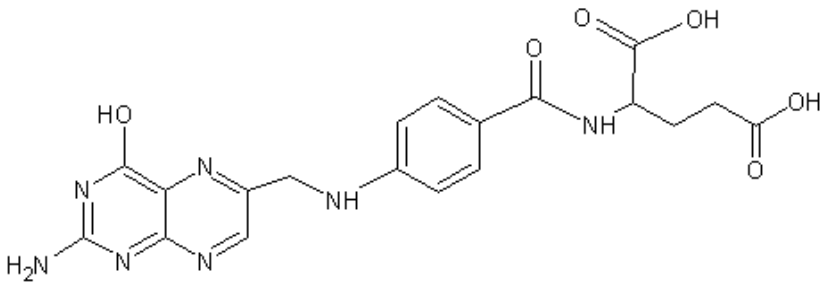
*Average of six observations

Storage

Store in tightly closed containers in a cool and dry place

3.2.6 FOLIC ACID

Molecular structure



Chemical name

(2S) -2 -[(4-{[(2- amino -4-hydroxypteridin-6-yl)methyl] amino}phenyl)formamido] pentanedioic acid.

Molecular formula

$C_{19}H_{19}N_7O_6$

Molecular weight

441.4 g mol^{-1}

Category

Vitamin B_9

Description

Yellow-orange crystalline powde

Solubility

It is slightly soluble in water and in methanol, less soluble in ethanol and butanol, and insoluble in acetone, chloroform, ether and benzene. Soluble in alkali hydroxide and carbonates, Soluble in hydrochloric and sulphuric acid.

Identification

i) Melting Point

Standard Value	Observed Value*
250°C -decompose	250°C

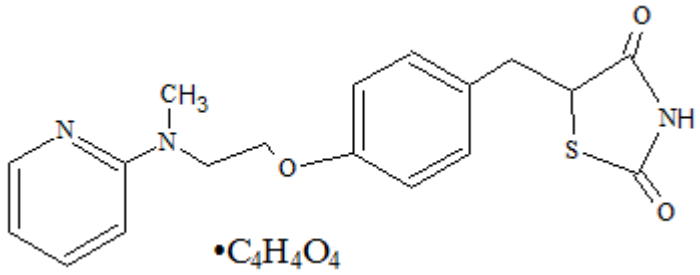
*Average of six observations

Storage

Store in tightly closed container in a cool and dry place

3.2.7 ROSIGLITAZONE MALEATE

Molecular structure



Chemical name

Chemically, rosiglitazone maleate is (±)-5-[[4-[2-(methyl-2-pyridinylamino) ethoxy] phenyl] methyl]-2, 4-thiazolidinedione, (Z)-2-butenedioate (1:1) the molecule has a single chiral center and is present as a racemate. Due to rapid interconversion, the enantiomers are functionally indistinguishable.

Molecular formula

$C_{18}H_{19}N_3O_3S•C_4H_4O_4$

Molecular weight

473.52 g mol^{-1} (357.44 free base).

Category

Oral antidiabetic agent

Description

Rosiglitazone maleate is a white to off-white solid.

Solubility

It is readily soluble in ethanol and a buffered aqueous solution with pH of 2.3; solubility decreases with increasing pH in the physiological range.

Identification

i) Melting Point

Standard Value	Observed Value*
122 - 123°C	123°C

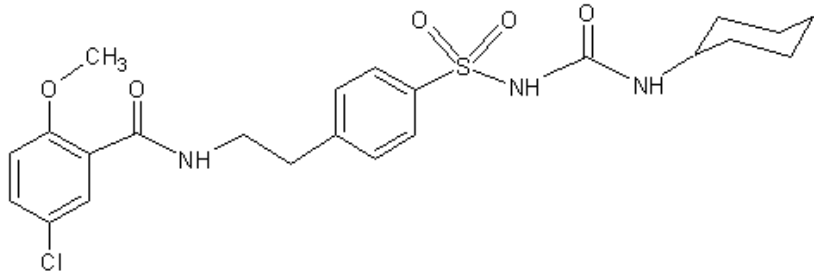
*Average of six observations

Storage

Store at room temperature and kept away from light and moisture.

3.2.8 GLIBENCLAMIDE

Molecular structure



Chemical name

Chemically, Glibenclamide is 5-chloro-N-(4-[N-(cyclohexylcarbamoyl) sulfamoyl] phenethyl)-2-methoxybenzamide.

Molecular formula

$C_{23}H_{28}ClN_3O_5S$

Molecular weight

494.004 g mol^{-1}

Category

Anti-diabetic

Description

White crystalline compound

Solubility

It is sparingly soluble in water, soluble in alcohols, and slightly soluble in common organic solvents.

Identification

i) Melting Point

Standard Value	Observed Value*
172 - 174°C	174°C

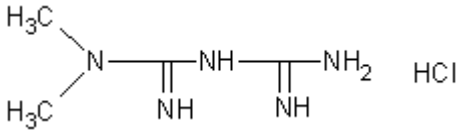
*Average of six observations

Storage

Stored at room temperature and kept away from light and moisture.

3.2.9 METFORMIN HYDROCHLORIDE

Molecular structure



Chemical name

Chemically, Metformin hydrochloride is N, N-dimethylimidodicarbonimidic diamide hydrochloride.

Molecular formula

$C_4H_{11}N_5$•HCl

Molecular weight

165.63 g mol^{-1}

Category

Oral antidiabetic agent

Description

Metformin HCl is a white to off-white crystalline compound.

Solubility

Metformin HCl is freely soluble in water and is practically insoluble in acetone, ether, and chloroform.

Identification

i) Melting Point

Standard Value	Observed Value*
222 - 226°C	226°C

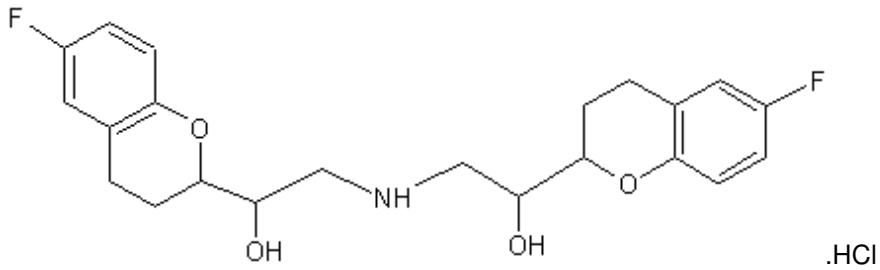
*Average of six observations

Storage

Stored at room temperature.

3.2.10 NEBIVOLOL HYDROCHLORIDE

Molecular structure



Chemical name

Chemically, Nebivolol hydrochloride is 1- (6 - fluoro- 3,4 – dihydro - 2*H* -1-benzopyran – 2 – yl) – 2 - {[2 - (6 – fluoro - 3, 4 – dihydro - 2*H* – 1 - benzopyran-2-yl) - 2- hydroxyethyl] amino} ethan-1-ol HCl.

Molecular formula

$C_{22}H_{25}F_2NO_4.HCl$

Molecular weight

441.90 g mol^{-1}

Category

Nebivolol HCl is antihypertensive.

Description

Nebivolol hydrochloride is a white to almost white powder.

Solubility

Nebivolol HCl is soluble in methanol, dimethylsulfoxide, and N, N-dimethylformamide, sparingly soluble in ethanol, propylene glycol, and polyethylene glycol, and very slightly soluble in hexane, dichloromethane, and methylbenzene

Identification

i) Melting Point

Standard Value	Observed Value*
220-222°C	221°C

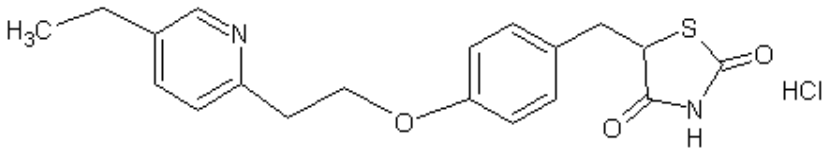
*Average of six observations

Storage

Stored at room temperature between 68-77 degrees F (20-25 degrees C) away from light and moisture.

3.2.11 PIOGLITAZONE HYDROCHLORIDE

Molecular structure



Chemical name

Chemically, Pioglitazone hydrochloride is [(±)-5-[[4-[2-(5-ethyl-2-pyridinyl) ethoxy] phenyl] methyl]-2, 4-] thiazolidinedione monohydrochloride.

Molecular formula

$C_{19}H_{20}N_2O_3S \bullet HCl$

Molecular weight

392.90 g mol^{-1}

Category

Pioglitazone hydrochloride is an oral antidiabetic agent that acts primarily by decreasing insulin resistance.

Description

Pioglitazone hydrochloride is an odorless white crystalline powder.

Solubility

It is soluble in N, N-dimethylformamide, slightly soluble in anhydrous ethanol, very slightly soluble in acetone and acetonitrile, practically insoluble in water, and insoluble in ether.

Identification

i) Melting Point

Standard Value	Observed Value*
183 - 184°C	183°C

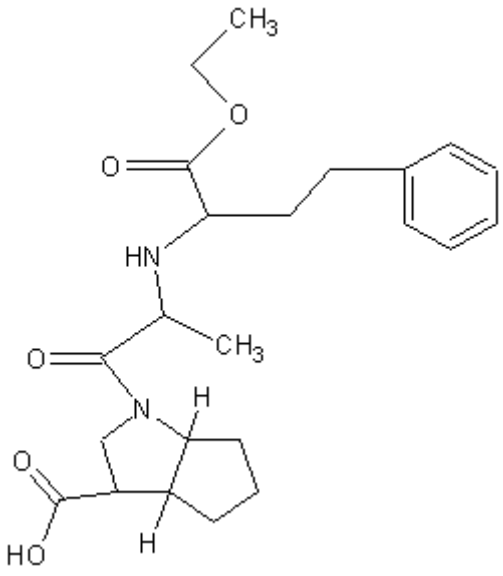
*Average of six observations

Storage

Stored at 25°C (77°F); excursion permitted to 15-30°C (59-86°F) [see USP Controlled Room Temperature]. Keep container tightly closed, and protect from moisture and humidity.

3.2.12 RAMIPRIL

Molecular structure



Chemical name

Ramipril's chemical name is (2S, 3aS, 6aS)-1[(S)-N-[(S)-1-Carboxy-3-phenylpropyl] alanyl] octa hydrocyclopenta [b]pyrrole-2-carboxylic acid, 1-ethyl ester.

Molecular formula

$C_{23}H_{32}N_2O_5$

Molecular weight

416.5 g mol^{-1}

Category

Ramipril is an angiotensin-converting enzyme (ACE) inhibitor, used to treat hypertension and congestive heart failure.

Description

It is a white, crystalline substance.

Solubility

It is soluble in polar organic solvents and buffered aqueous solutions.

Identification

i) Melting Point

Standard Value	Observed Value*
105 - 112°C	108°C

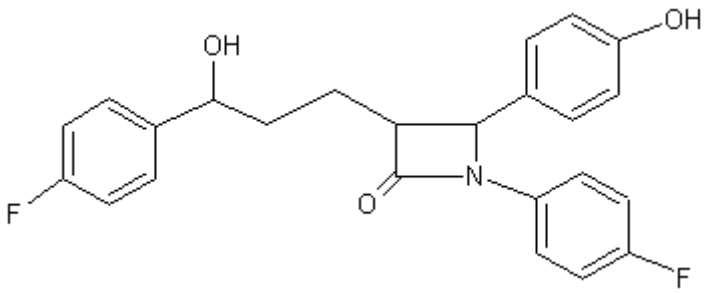
*Average of six observations

Storage

Stored at room temperature between 59-86 degrees F (15-30 degrees C) away from light and moisture.

3.2.13 EZETIMIBE

Molecular structure



Chemical name

(3R, 4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidin-2-one

Molecular formula

$C_{24}H_{21}F_2NO_3$

Molecular weight

409.43 g mol^{-1}

Category

HMG-CoA Reductase Inhibitor

Description

White crystalline solid.

Solubility

Freely soluble in methanol, ethanol, acetone and practically insoluble in water.

Identification

i) Melting Point

Standard Value	Observed Average Value*
164-166°C	164.3°C

*Average of six observations

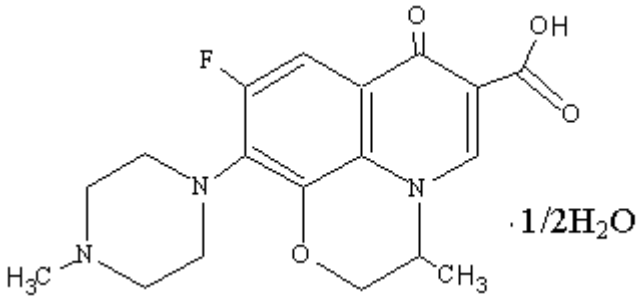
Storage

Stored in tightly closed containers in a cool and dry place

3.2.14 LEVOFLOXACIN HEMIHYDRATE

Levofloxacin is a chiral fluorinated 4-quinolone containing a six-member (pyridobenzoxazine) ring from positions 1 to 8 of the basic ring structure. Levofloxacin is the pure (-)-(S)-enantiomer of the racemic drug substance, ofloxacin.

Molecular structure



Chemical name

(-)-(S)-9-fluoro-2,3-dihydro-3-methyl-10-(4- methyl-1-piperazinyl)-7-oxo-7H-pyrido [1, 2,3-de] -1,4 benzoxazine-6-carboxylic acid hemihydrate

Molecular formula

$C_{18}H_{20}FN_3O_4.1/2H_2O$

Molecular weight

370.38 g mol^{-1}

Category

Anti-bacterial

Description

Light yellow to yellow-white crystal or crystalline powder

Solubility

Freely soluble in water, 0.1M HCl, Dichloromethane, chloroform and sparingly soluble in hexane, toluene etc.

Identification

i) Melting Point

Standard Value	Observed Value*
225-227°C	226°C

*Average of six observations

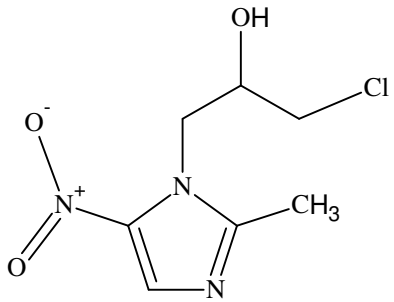
Storage

Stored in tightly closed containers in a cool and dry place

3.2.15 ORNIDAZOLE

Ornidazole is a nitro imidazole derivative which is used in the treatment of susceptible protozoal infections and prophylaxis of anaerobic bacterial infections.

Molecular structure



Chemical Name

1-(3-Chloro-2-hydroxypropyl)-2-methyl-5- Nitroimidazole

Molecular formula

$C_7 H_{10} Cl N_3 O_3$

Molecular weight

219.63 g mol^{-1}

Category

Anti-protozoal

Description

White to off white crystalline powder.

Solubility

Freely soluble in water, 0.1M HCl, isopropanol, chloroform, and insoluble in cyclohexane and dimethyl formamide.

Identification

i) Melting Point

Standard Value	Observed Value*
77 ºC -78 ºC	**77ºC**

*Average of six observations

Storage

Store in tightly closed containers in a cool and dry place (200 - 203).

3. 3 Materials and Methods

3.3.1 Drug Samples used (Raw material)

The following combinations were assayed by chemometrics assisted spectrophotometry, first order derivative spectroscopic and RP-HPLC method

1. Losartan potassium, Amlodipine besylate and Hydrochlorothiazide
2. Atorvastatin calcium, Fenofibrate and Folic acid
3. Rosiglitazone Maleate, Glibenclamide and Metformin HCl
4. Nebivolol HCl, Hydrochlorothiazide
5. Pioglitazone HCl, Metformin HCl
6. Losartan potassium, Ramipril
7. Atorvastatin calcium, Fenofibrate
8. Atorvastatin calcium, Ezetimibe
9. Levofloxacin hemihydrate, Ornidazole

All the drugs mentioned above were gifted by ATOZ India Ltd., India with their analytical report. The drugs were authenticated and used without any further purification.

3.3.2 Chemicals and solvents used

Acetonitrile	HPLC grade
Methanol	HPLC grade
Water	HPLC grade
Sucrose	Analytical reagent grade
Potassium dihydrogen phosphate	Analytical reagent grade
Ammonium acetate	Analytical reagent grade
Hydrochloric acid	Analytical reagent grade
Phosphoric acid	Analytical reagent grade

Sodium hydroxide	Analytical reagent grade

3.3.3 Instrument and software used

1. Shimadzu (Japan) 2550-double beam spectrophotometer was connected to PIVPC.

The bundled software winlab 2.0 (Shimadzu) was used for all the spectrophotometric measurements. The spectral bandwidth of 0.5 nm and the wavelength readability of 0.1 nm were used. The absorption spectra of the reference and test solutions were carried out in 1 cm matched quartz cells over the range of 200-400 nm. The chemometric calculations on the resulting data were carried out in a Pentium IV computer with PLS toolbox (demover 4.1 and 5.0) in MATLAB 7(Math works).

2. HPLC (HITACHI L-7400) instrument was equipped with a model series LC-10 ATVP pump, SPD-10AVP UV–VIS detector; separation and quantization were made on a 250 mm × 4.6 mm (i.d.) Merck RP_{18} column (4.6 μm particle size). Data acquisition was performed on D-7000HSM ADMINI software.

3. Shimadzu AUX- 220 Digital balance
4. Sonicator – Sonica ultrasonic cleaner – model 2200 MH

3.3.4 Pharmaceutical Formulation used

The following drug samples were procured from the local market

1. **TRILOPACE**

Each tablet contains

Losartan potassium USP	50 mg
Amlodipine Besylate BP equivalent to amlodipine	5 mg
Hydrochlorothiazide IP	12.5 mg

2. **LORISK™**

Each tablet contains	
Atorvastatin calcium equivalent to atorvastatin IP	10 mg
Fenofibrate BP (Micronized)	160 mg
Folic acid IP	5 mg

3. **RGM**

Each tablet contains	
Rosiglitazone maleate equivalent to rosiglitazone	2 mg
Glibenclamide IP	5 mg
Metformin Hydrochloride IP	500 mg

4. **NEBICARD-H**

Each tablet contains	
Nebivolol HCl equivalent to Nebivolol	5 mg
Hydrochlorothiazide IP	12.5 mg

5. **GLYCIPHAGE P30**

Each tablet contains	
Pioglitazone HCl equivalent to pioglitazone	30 mg
Metformin Hydrochloride IP	500 mg

6. **LORAM – 2.5**

Each tablet contains	
Losartan potassium	50 mg
Ramipril	2.5 mg

7. **ATORLIP-F**

Each tablet contains

Atorvastatin calcium equivalent to atorvastatin IP	10 mg
Fenofibrate BP (Micronized)	160 mg

8. **ATTOR EZ10**

Each tablet contains

Atorvastatin calcium equivalent to atorvastatin IP	10 mg
Ezetimibe	10 mg

9. **LOXOF-OZ**

Each tablet contains

Levofloxacin hemihydrate equivalent to levofloxacin	250 mg
Ornidazole	500 mg

3.4 Chemometric Methods

All the prepared mixtures of calibration set, prediction set, formulation and recovery set were scanned in the electronic UV absorption spectrophotometer and the spectra were recored each 0.1 nm in the wavelength range of 200–400 nm. The computation of the chemometric calculations on the resulting data were carried out in a Pentium IV computer with PLS toolbox (demover 4.1 and 5.0) in MATLAB 7 (Math works) and the statistical calculations were made in the Xl.sheet.

3.4.1 Preparation of Standard stock and working solutions of Losartan potassium, Amlodipine besylate and Hydrochlorothiazide

Stock solutions of losartan potassium, amlodipine besylate and hydrochlorothiazide containing each 1 mg mL $^{-1}$ were prepared separately in methanol. From this 8 mL of losartan potassium, 1 mL of amlodipine besylate and 1.5 mL of hydrochlorothiazide were diluted to 50 mL with Millipore water to get the concentration of 0.16 mg mL $^{-1}$, 0.02 mg mL $^{-1}$ and 0.06 mg mL $^{-1}$ respectively. A blank solution containing 10.5 mL of methanol in 50 mL Millipore water was also prepared.

Preparation of calibration and prediction mixtures

A calibration set containing 25 standard solutions and prediction sets of 9 standard solutions were prepared in Millipore water from their corresponding working solutions, applying a multilevel multifactor design (204) in which five levels of concentrations of Losartan potassium, Amlodipine besilate and Hydrochlorothiazide were introduced. The levels were in the calibration range of 8 – 40 μg mL^{-1} for losartan potassium, 1 – 5 μg mL^{-1} for amlodipine besylate and 3 – 15 μg mL^{-1} for hydrochlorothiazides which are illustrated in **Table 3 - 5.** The overlaid spectra of the losartan potassium, amlodipine besylate and hydrochlorothiazide are presented in the

Spectrum.1. The calibration curves are shown in the **Graph. 1 - 14.** The results are furnished in the **Table 6 and 7.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. An accurately weighed powder equivalent to 500 mg of losartan potassium, 50 mg of amlodipine and 125 mg of hydrochlorothiazide was dissolved in 50 mL of methanol. The solution was ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 3 mL of the filtrate in to 100 mL standard flask and made up to volume with Millipore water. Aliquots of these solutions were used in such a way that the concentration of each drug obtained within the range of the calibration samples. The dilute solutions were analysed six times and the results are furnished in the **Table 8.**

3.4.2 Preparation of Standard stock and working solutions of Atorvastatin calcium, Fenofibrate and Folic acid

Stock solutions of Atorvastatin calcium 1000 µg mL^{-1}, Fenofibrate 3000 µg mL^{-1} and Folic acid 1000 µg mL^{-1} were prepared in 100 mL volumetric flasks separately by dissolving 100 mg each of atorvastatin calcium and folic acid, 300 mg of fenofibrate in a mixture of methanol and 5% Sucrose in 0.1N Sodium hydroxide (80:20). Working standard solutions were prepared separately by diluting 1ml of atorvastatin calcium, 5mL of fenofibrate and 0.5mL of folic acid stock solutions to 50mL with above solvent to obtain 20 µg mL^{-1}, 300 µg mL^{-1} and 10 µg mL^{-1} of the above drugs respectively. A blank solution containing a mixture of methanol and 5% Sucrose in 0.1N Sodium hydroxide (80:20) was also prepared.

Preparation of calibration and prediction mixtures

Calibration set containing 25 standard solutions and prediction set containing 9 standard solutions were prepared in Millipore water as described in the previous section. The levels were in the calibration range of 1.0 – 5.0 μg mL^{-1} for atorvastatin calcium, 15 – 75 μg mL^{-1} for fenofibrate, and 0.5 – 2.5 μg $mL^{-1}$for folic acid. The results are furnished in the **Table 10 – 14.** The overlaid spectra of the atorvastatin calcium, fenofibrate and folic acid are presented in the **Spectrum.2**. The calibration curves are shown in the **Graph. 15 - 28.**

Market sample Analysis

Twenty tablets were weighed accurately and powdered. An accurately weighed quantity of the powder equivalent to 3.125 mg of atorvastatin, 50 mg of fenofibrate and 1.5625 mg of folic acid was dissolved in a mixture of 50 mL of methanol and 5% Sucrose in 0.1N Sodium hydroxide (80:20). The solution was ultrasonicated for 10 minutes and filtered through whatman filter paper No. 41. Transferred 6.25 mL of the filtrate in to 50ml standard flask and made up to volume with above solvents. Aliquots of these solutions were diluted in such a way that the concentration of each drug obtained was within the linearity range. The diluted solutions were analysed six times and the results are furnished in the **Table 15.**

3.4.3 Preparation of Standard stock and working solutions of Rosiglitazone Maleate, Glibenclamide and Metformin HCl

Stock solutions containing 1000 μg mL^{-1} each of Rosiglitazone, Glibenclamide and Metformin Hydrochloride were prepared in separate 100 mL volumetric flasks by dissolving 100 mg each of the above drugs in methanol. Working standard solutions were prepared separately by diluting the stock solutions to obtain 1 μg mL^{-1} of Rosiglitazone Maleate, 2 μg mL^{-1} of Glibenclamide and 140

μg mL^{-1} of Metformin HCl in methanol. A blank solution was also prepared without samples.

Preparation of calibration and prediction mixtures

A calibration set containing 25 standard solutions and prediction set containing 9 standard solutions were prepared in Millipore water as described in the previous section. The levels were in the calibration range of 0.05 - 0.25 μg mL^{-1} for Rosiglitazone Maleate, 0.5 - 2.5 μg mL^{-1} for Glibenclamide and 7 - 35 μg mL^{-1} for Metformin HCl. The results are furnished in the **Table 17 – 21.** The overlaid spectra of the rosiglitazone maleate, glibenclamide and metformin HCl are presented in the **Spectrum.3**. The calibration curves are shown in the **Graph. 29 - 42.**

Market sample Analysis

Twenty tablets were weighed accurately and powdered. An accurately weighed quantity of the powder equivalent to 0.4 mg of Rosiglitazone, 1 mg of Glibenclamide and 100 mg of Metformin HCl was dissolved in 100 mL of methanol. The solution was ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 12.5 mL of the filtrate into 50mL standard flask and made up to volume with Milli pore water. Aliquots of these solutions were used in such a way that the concentration of each drug was obtained within the range of the calibration. The diluted solutions were analysed six times and the results are furnished in the **Table 22**

3.4.4 Preparation of Standard stock and working solutions of Nebivolol HCl and Hydrochlorothiazide

Stock solutions of nebivolol HCl and hydrochlorothiazide containing 1000 μg mL^{-1} were prepared separately in 100 mL volumetric flasks, by dissolving 100 mg of each compound in methanol. Working standard solutions were prepared separately by

diluting the stock solutions to obtain 10 µg mL^{-1} of nebivolol HCl, 20 µg mL^{-1} of hydrochlorothiazide in methanol. A blank solution was also prepared without samples.

Preparation of calibration and prediction mixtures

A calibration set containing 25 standard solutions and prediction set containing 9 standard solutions were prepared in Millipore water as described in the previous section. The levels were in the calibration range of 1 – 5 µg mL^{-1} for nebivolol HCl, 2 - 10 µg mL^{-1} for hydrochlorothiazide. The results are furnished in the **Table 24 – 28.** The overlaid spectra of the nebivolol HCl and hydrochlorothiazide are presented in the **Spectrum.4**. The calibration curves are shown in the **Graph. 43 - 52.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. An accurately weighed quantity of the powder equivalent to 50 mg of Nebivolol and 125 mg of Hydrochlorothiazide was dissolved in 50 mL of methanol. The solution was ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 5 mL of the filtrate into 50 mL standard flask and made up to volume with milli pore water. Aliquots of these solutions were used in such a way that the concentration of each drug obtained was within the calibration range. The diluted solutions were analysed six times and the results are furnished in the **Table 29.**

3.4.5 Preparation of Standard stock and working solutions of Pioglitazone and Metformin HCl

Stock solutions of pioglitazone HCl and metformin HCl containing 1000 µg mL^{-1} each were prepared in separate 100 mL volumetric flasks, by dissolving 100 mg

of each compound in methanol. Working standard solutions were prepared separately by diluting the stock solutions to obtaine 6 µg mL^{-1} of pioglitazone HCl and 140 µg mL^{-1} of metformin HCl in milli pore water. A blank solution was also prepared without samples.

Preparation of calibration and prediction mixtures

A calibration set containing 25 standard solutions and prediction set containing 9 standard solutions were prepared in Millipore water as described in the previous section. The levels were in the calibration range of 0.3 – 1.5 µg mL^{-1} for pioglitazone HCl, 7 - 35 µg mL^{-1} for metformin HCl. The results are furnished in the **Table 31 – 35.** The overlaid spectra of the pioglitazone HCl and metformin HCl are presented in the **Spectrum.5**. The calibration curves are shown in the **Graph. 53 – 62.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. An accurately weighed quantity of the powder equivalent to 1.5 mg of pioglitazone and 50 mg of metformin HCl was dissolved in 50 mL of methanol. The solution was ultrasonicated for 10 minutes and filtered through whatman filter paper No. 41. Transferred 10 mL of the filtrate into 50 mL standard flask and made up to volume with milli pore water. Aliquots of these solutions were used in such a way that the concentration of each drug obtained was within the linearity range. The diluted solutions were analysed six times and the results are furnished in the **Table 36.**

3.4.6 Preparation of Standard stock and working solutions of Losartan potassium and Ramipril

Stock solutions of losartan potassium and ramipril containing 1000 µg mL^{-1} each were prepared in separate 100 mL volumetric flasks, by dissolving 100 mg of each compound in methanol. Working standard solutions were prepared separately by

diluting the stock solutions to obtaine 160 µg mL $^{-1}$ of Losartan potassium and 12 µg mL $^{-1}$ Ramipril in Millipore water. A blank solution was also prepared without samples.

Preparation of calibration and prediction mixtures

A calibration set containing 25 standard solutions and prediction set containing 9 standard solutions was prepared in Millipore water as described in the previous section. The levels were in the calibration range of 8 – 40 µg mL^{-1} for Losartan potassium and 1.2 - 6.0 µg mL^{-1} for Ramipril. The results are furnished in the **Table 36 – 42.** The overlaid spectra of the losartan potassium and ramipril are presented in the **Spectrum.6**. The calibration curves are shown in the **Graph. 63 – 72.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. An accurately weighed quantity of the powder equivalent to 500 mg of Losartan potassium and 25 mg of Ramipril was dissolved in 50 mL of methanol. The solution was ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 3 mL of the filtrate into 100 mL standard flask and made up to volume with Milli pore water. Aliquots of these solutions were used in such a way that the concentration of each drug obtained was within the linearity range. The diluted solutions were analysed six times and the results are furnished in the **Table 43.**

3.4.7 Recovery

In order to ensure the accuracy of the proposed method, recovery studies were performed by standard addition method at three levels. Known amounts of standard drugs were added to the pre-analyzed samples and they were subjected to the proposed method. Results of recovery studies are shown in the **Table 9** for losartan potassium, amlodipine besylate and hydrochlorothiazide in formulation, **Table 16** for

atorvastatin calcium, fenofibrate and folic acid in formulation, **Table 23** for rosiglitazone maleate, glibenclamide and metformin hydrochloride in formulation, **Table 30** for nebivolol hydrochloride and hydrochlorothiazide in formulation, **Table 37** for pioglitazone hydrochloride and metformin hydrochloride in formulation and **Table 44** for losartan potassium and ramipril in formulation.

3.5 Derivative spectroscopy

Atorvastatin calcium and Fenofibrate, Atorvastatin calcium and Ezetimibe and Levofloxacin hemihydrate and ornidazole combinations of drugs in pharmaceuticals were simultaneously estimated by first order derivative spectroscopic method.

3.5.1 Selection of solvent

The combinations containing atorvastatin calcium and fenofibrate and atorvastatin calcium and ezetimibe were dissolved in methanol. Levofloxacin hemihydrate and ornidazole were dissolved in 0.1M HCl.

3.5.2 Selection of wavelengths in first order derivative spectra for Atorvastatin calcium and Fenofibrate

A suitable standard solution containing 10 µg mL^{-1} each of atorvastatin calcium and fenofibrate were prepared separately and scanned in the entire range from 200 nm - 400 nm and overlaid spectra were recored. The zero order spectrum was derivatised to first order with $\Delta\lambda$ = 1 nm for the entire spectrum and the wavelength 239 nm was selected for the determination of atorvastatin calcium, which is zero crossing for fenofibrate and 246 nm was selected for fenofibrate which is zero crossing for atorvastatin calcium. The overlaid spectra of the atorvastatin calcium and fenofibrate at zero order and first derivative are presented in the **Spectrum.7 and 8.**

For Atorvastatin calcium and Ezetimibe

A suitable standard solution containing 10 µg mL^{-1} of atorvastatin calcium and ezetimibe were prepared separately, scanned in the entire range from 200 nm - 400 nm and overlaid spectra were recored. The zero order spectrum was derivatised to first order with $\Delta\lambda$ = 1 nm for the entire spectrum and the wavelength 224 nm was selected for determination of atorvastatin calcium, which is zero crossing for ezetimibe and 233 nm was selected for ezetimibe which is zero crossing for

atorvastatin calcium. The overlaid spectra of the atorvastatin calcium and ezetimibe at zero order and first derivative are presented in the **Spectrum.9 and 10.**

For Levofloxacin hemihydrate and ornidazole

A suitable standard solution containing 10 µg mL $^{-1}$ each of levofloxacin hemihydrate and ornidazole were prepared separately and scanned in the entire range from 200 nm - 400 nm, overlaid spectra were recored. The zero order spectrum was derivatised to first order with $\Delta\lambda$ = 1nm for the entire spectrum and the wavelength 277.5 nm was selected for the determination of levofloxacin hemihydrate, which is zero crossing for ornidazole and 319 nm was selected for ornidazole which is zero crossing for levofloxacin hemihydrate. The overlaid spectra of the levofloxacin hemihydrate and ornidazole at zero order and first derivative are presented in the **Spectrum.11 and 12.**

3.5.3 Preparation of standard stock solution and working solution

For Atorvastatin calcium and Fenofibrate

Stock solutions of atorvastatin calcium and fenofibrate containing 1000 µg mL^{-1} each were prepared in separate 100 mL volumetric flasks, by dissolving 100 mg of each compound in methanol. Working standard solutions were prepared separately by diluting the stock solutions to obtaine 80 µg mL $^{-1}$ each of atorvastatin calcium and fenofibrate in methanol.

For Atorvastatin calcium and Ezetimibe

Stock solutions of atorvastatin calcium and ezetimibe containing 1000 µg mL^{-1} were prepared separate in 100 mL volumetric flasks, by dissolving 100 mg of each compound in methanol. Working standard solutions were prepared separately by diluting the stock solutions to obtaine 80 µg mL $^{-1}$ of atorvastatin calcium and ezetimibe in methanol.

For Levofloxacin hemihydrate and ornidazole

Stock solutions of levofloxacin hemihydrate and ornidazole containing 1000 µg mL^{-1} were prepared in separate 100 mL volumetric flasks, by dissolving 100 mg of each compound in 0.1M HCl. Working standard solutions were prepared separately by diluting the stock solutions to obtaine 100 µg mL^{-1} of levofloxacin hemihydrate and 200 µg mL^{-1} ornidazole in 0.1M HCl.

3.5.4 Preparation of calibration curves

For Atorvastatin calcium and Fenofibrate

The linearity study was carried out seperately for both the drugs by first order derivative spectrum at the above said wavelengths. The calibration curves were constructed by plotting $\frac{\Delta A}{\Delta \lambda}$ versus concentration of the standard solutions. Calibration curves were linear between 4 – 24 µg mL^{-1} for atorvastatin calcium and 4 - 24 µg mL^{-1} for fenofibrate. The calibration curves are produced in the **Graph. 73 – 74.** The results are furnished in the **Table 45.**

For Atorvastatin calcium and Ezetimibe

The linearity study was carried out separately for both the drugs by first order derivative spectrum at the above said wavelengths. The calibration curves were constructed by plotting $\frac{\Delta A}{\Delta \lambda}$ versus concentration of the standard solutions. Calibration curves were linear between 4 – 24 µg mL^{-1} for atorvastatin calcium and 4 - 24 µg mL^{-1} for ezetimibe. The calibration curves are produced in the **Graph. 75 – 76.** The results are furnished in the **Table 45.**

For Levofloxacin hemihydrate and ornidazole

The linearity study was carried out separately for both the drugs by first order derivative spectrum at the above said wavelengths. The calibration curves were

constructed by plotting $\frac{\Delta A}{\Delta \lambda}$ versus concentration of the standard solutions. Calibration curves were linear between 10–50 μg mL^{-1} for levofloxacin hemihydrate and 20-80 μg mL^{-1} for ornidazole. The calibration curves are produced in the **Graph. 77 – 78.** The results are furnished in the **Table 45.**

3.5.5 Quantification of formulations

For Atorvastatin calcium and Fenofibrate

The average weight of 20 tablets were determined and triturated to fine powder. A quantity of powder equivalent to 10 mg of atorvastatin and 160 mg of fenofibrate was transferred to 100 mL volumetric flask. Since the ratio of atorvastatin and fenofibrate in the tablet formulation is 1:16, the instrumental response produced by atorvastatin is not accurate. Therefore a suitable amount (150 mg) of pure atorvastatin was added to the formulation. Sufficient methanol was added sonicated for 5 minutes and made up to the mark with the same solvent and filtered through Whatman Filter paper no: 41. Transferred 2.5 mL of the filtrate in to 50 mL standard flask and made upto volume with methanol, from this 1.5 mL was diluted to 10 mL to get the final concentration. The $\frac{\Delta A}{\Delta \lambda}$ values were measured at the 239 and 246 nm wavelengths in the first order spectrum and the amount present was calculated from their respective calibration curve. The diluted solutions were analysed six times and the results are furnished in the **Table 46.**

For Atorvastatin calcium and Ezetimibe

The average weight of 20 tablets were determined and triturated to fine powder. A quantity of powder equivalent to 100 mg of ezetimibe and 100 mg of atorvastatin was transferred to 100 mL volumetric flask, Sufficient methanol was added, sonicated for 5 minutes and made up to the mark with the same solvent and

filtered through Whatman Filter paper no: 41. Transferred 4 mL of the filtrate in to 50 mL standard flask and made upto volume with methanol, from this 1.5 mL was diluted to 10 mL to get the final concentration. The $\frac{\Delta A}{\Delta \lambda}$ values were measured at the 224 and 233 nm wavelengths in the first order spectrum and the amount present was calculated from their respective calibration curve. The diluted solutions were analysed six times and the results are furnished in the **Table 46.**

For Levofloxacin hemihydrate and ornidazole

The average weight of 20 tablets were determined and triturated to fine powder. A quantity of powder equivalent to 250 mg of ornidazole and 125 mg of levofloxacin was transferred to 100 mL volumetric flask, sufficient 0.1M HCl was added sonicated for 5 minutes and made up to the mark with the same solvent and filtered through Whatman Filter paper no: 41. Transferred 2 mL of the filtrate in to 50 mL standard flask and made upto volume with 0.1M HCl, from this 2 mL diluted to 10 mL to get the final concentration. The $\frac{\Delta A}{\Delta \lambda}$ values were measured at the 277.5 and 319 nm wavelengths in the first order spectrum and the amount present was calculated from their respective calibration curve. The diluted solutions were analysed six times and the results are furnished in the **Table 46.**

3.5.6 Recovery

In order to ensure the accuracy of the proposed method, recovery studies were performed by standard addition method at three levels. Known amounts of standard drugs were added to pre-analyzed samples and were subjected to the proposed method. Results of recovery studies are shown in the **Table 47.**

3.6 RP-HPLC method development

3.6.1 Optimised chromatographic conditions for losartan potassium, amlodipine besylate and hydrochlorothiazide

Mode of operation	: Isocratic
Stationary phase	: C_{18} ODS column (150 mm X 4.6 mm i.d.)
Mobile phase	: 10mM KH_2PO_4 (adjusted to pH -3): ACN
Ratio	: 60:40
Detection wavelength	: 225 nm
pH	: 3.0
Flow rate	: 1.5 mL min^{-1}
Temperature	: Ambient
Sample load	: 20 µL
Operating pressure	: 94 kgf

The system suitability studies were carried out as specified in USP and the results are furnished in the **Table 49**. The individual Chromatograms of losartan potassium, amlodipine besylate and hydrochlorothiazide are presented in the **Chromatogram.1 - 3.**

Preparation of Calibration curves of losartan potassium, amlodipine besylate and hydrochlorothiazide

The Stock solutions containing 1000 µg mL^{-1} each of losartan potassium, amlodipine besylate and hydrochlorothiazide were prepared in methanol. Working standard solutions were prepared separately by diluting the stock solutions suitably to get 160 µg mL^{-1} of losartan potassium, 20 µg mL^{-1} of amlodipine besylate and 60 µg mL^{-1} of hydrochlorothiazide in HPLC water.

Losartan potassium, amlodipine besylate and hydrochlorothiazide linearity mixture were prepared with in the calibration range of 8 – 40 μg mL^{-1} for losartan potassium, 1 – 5 μg mL^{-1} for amlodipine besylate and 3 – 15 μg mL^{-1} for hydrochlorothiazide in mobile phase from their respective working solution. The aliquots of 20 μL samples were injected and the chromatograms were recorded. The calibration curves were constructed by plotting peak area versus concentration. The optical and regression characteristics of losartan potassium, amlodipine besylate and hydrochlorothiazide are produced in the **Table 50**. The linearity chromatograms of losartan potassium, amlodipine besylate and hydrochlorothiazide are presented in the **Chromatogram.4 - 8.** The calibration curves are produced in the **Graph. 79 – 81.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. The amount of the powder equivalent to 500 mg of Losartan potassium, 50 mg of amlodipine and 125 mg of hydrochlorothiazide was dissolved in 50 mL of methanol, ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 3 mL of the filtrate into 100 mL standard flask and made up to volume with the mobile phase. Aliquots of these solutions were diluted in such a way that the concentration of each drug obtained within the linearity range. The dilute solutions were analysed six times as described in the previous section. The results are furnished in the **Table 51.** The assay chromatogram of losartan potassium, amlodipine besylate and hydrochlorothiazide is presented in the **Chromatogram.9.**

3.6.2 Optimised chromatographic conditions For Atorvastatin calcium, Fenofibrate and folic acid

Mode of operation : Isocratic

Stationary phase : C_{18} BDS column (150 mm X 4.6 mm i.d.)

Mobile phase	: 10mM KH_2PO_4 (adjusted to pH -2.5): ACN
Ratio	: 30:70
Detection wavelength	: 248 nm
pH	: 2.5
Flow rate	: 1.5 mL min^{-1}
Temperature	: Ambient
Sample load	: 20 µL
Operating pressure	: 94 kgf

The system suitability studies were carried out as specified in USP and the results are furnished in the **Table 54**. The individual Chromatogram of atorvastatin calcium, fenofibrate and folic acid are presented in the **Chromatogram.13 - 15.**

Preparation of Calibration samples of Atorvastatin calcium, Fenofibrate and folic acid

The stock solutions containing 1000 µg mL^{-1} of atorvastatin calcium, 4000 µg mL^{-1} of fenofibrate and 1000 µg mL^{-1} of folic acid were prepared separately in mobile phase. Working standard solutions were prepared separately by diluting the stock solutions suitably to get 20 µg mL^{-1} of atorvastatin calcium, 300 µg mL^{-1} of fenofibrate and 10 µg mL^{-1} of folic acid in mobile phase.

Atorvastatin calcium, fenofibrate and folic acid linearity mixtures were prepared with in the linearity range of 1 – 5 µg mL^{-1} for atorvastatin calcium, 15 - 75 µg mL^{-1} for Fenofibrate and 0.5 – 2.5 µg mL^{-1} for folic acid in mobile phase from their respective working solutions. The aliquots of 20 µL samples were injected and the chromatograms were recorded. The calibration curves were constructed by plotting peak area versus concentration. The optical and regression characteristics of atorvastatin calcium, fenofibrate and folic acid are produced in the **Table 55**. The

linearity chromatograms of atorvastatin calcium, fenofibrate and folic acid are presented in the **Chromatogram.16 - 20.** The calibration curves are produced in the **Graph. 82 – 84.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. The amount of the powder equivalent to 50 mg of Fenofibrate, 3.125 mg of atorvastatin and 1.5625 mg of folic acid was dissolved in 50 mL mobile phase, ultrasonicated for 10 minute and filtered through Whatman filter paper No. 41. Transferred 15 mL of the filtrate into 50 mL standard flask and made up to volume with solvent. Aliquots of these solutions were used in such a way that the concentration of each drug is obtained within the linearity range. The dilute solutions were analysed six times as described in the previous section. The results are furnished in the **Table 56.** The assay chromatogram atorvastatin calcium, fenofibrate and folic acid is presented in the **Chromatogram.21.**

3.6.3 Optimised chromatographic conditions for Rosiglitazone maleate, Glibenclamide and Metformin HCl

Mode of operation : Isocratic

Stationary phase : C_{18} ODS column (150 mm X 4.6 mm i.d.)

Mobile phase : 0.02M CH_3COONH_4 : ACN

Ratio : 45:55

Detection wavelength: 230 nm

pH : 4.2

Flow rate : 1.5 mL min^{-1}

Temperature : Ambient

Sample load : 20 µL

Operating pressure : 94 kgf

The system suitability studies were carried out as specified in USP and the results are furnished in **Table 59**. The individual Chromatogram of rosiglitazone Maleate, glibenclamide and metformin HCl are presented in the **Chromatogram.25 - 27.**

Preparation of Calibration samples of Rosiglitazone maleate, Glibenclamide and Metformin HCL

The stock solutions containing 1000 µg mL^{-1} each of Rosiglitazone maleate, Glibenclamide and Metformin Hydrochloride were prepared separately in methanol. Working standard solutions were prepared separately by diluting the stock solutions to get 1 µg mL^{-1} of Rosiglitazone Maleate, 2 µg mL^{-1} of Glibenclamide and 140 µg mL^{-1} of Metformin HCl in methanol.

Rosiglitazone Maleate, Glibenclamide and Metformin HCl linearity mixture was prepared within the linearity range of 0.05 – 0.25 µg mL^{-1} for Rosiglitazone Maleate, 0.1 – 0.5 µg mL^{-1} for Glibenclamide and 7 – 35 µg $mL^{-1}$for Metformin HCl in mobile phase from their respective working solutions. The aliquots of 20 µL samples were injected and the chromatograms were recorded. The calibration curves were constructed by plotting peak area versus concentration. The optical and regression characteristics of rosiglitazone maleate, glibenclamide and metformin hydrochloride are produced in the **Table 60**. The linearity chromatogram of rosiglitazone maleate, glibenclamide and metformin HCl are presented in the **Chromatogram.28 - 32.** The calibration curves are produced in the **Graph. 85 – 87.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. The amount of the powder equivalent to 0.4 mg of rosiglitazone, 1 mg of Glibenclamide and 100 mg of Metformin HCl was dissolved in 100 mL of methanol, ultrasonicated for 10 minutes

and filtered through whatman filter paper No. 41. Transferred 12.5 mL of the filtrate into 50 mL standard flask and made up to volume with mobile phase. Aliquots of these solutions were used in such a way that the concentration of each drug is obtained within the linearity range. The dilute solutions were analysed six times as described in the previous section. The results are furnished in the **Table 61.** The assay chromatogram of rosiglitazone maleate, glibenclamide and metformin HCl is presented in the **Chromatogram.33.**

3.6.4 Optimised chromatographic conditions for Nebivolol HCl and hydrochlorothiazide

Mode of operation : Isocratic

Stationary phase : C_{18} BDS column (150 mm X 4.6 mm i.d.)

Mobile phase : 0.4% Triethylamine: ACN

Ratio : 70:30

Detection wavelength: 240 nm

pH : 7.0

Flow rate : 1 mL min^{-1}

Temperature : Ambient

Sample load : 20 µL

Operating pressure : 94 kgf

The system suitability studies were carried out as specified in USP and the results are furnished **Table 64**. The individual Chromatogram of Nebivolol HCl and Hydrochlorothiazide are presented in the **Chromatogram. 37- 38.**

Preparation of Calibration samples of Nebivolol HCl and Hydrochlorothiazide

The Stock solutions containing 1000 µg mL^{-1} each of Nebivolol HCl and Hydrochlorothiazide were prepared separately in methanol. Working standard

solutions were prepared separately by diluting the stock solutions to get 20 µg mL^{-1} of nebivolol HCl and 20 µg mL^{-1} of hydrochlorothiazide in methanol.

Nebivolol HCl and Hydrochlorothiazide linearity mixtures were prepared within the linearity range of 1- 5 µg mL^{-1} for Nebivolol HCl and 3 - 15 µg mL^{-1}Hydrochlorothiazide in mobile phase from their respective working standard. The aliquots of 20 µL samples were injected and the chromatograms were recored. The calibration curves were constructed by plotting peak area versus concentration. The optical and regression characteristics of nebivolol hydrochloride and hydrochlorothiazide are produced in the **Table 65**. The linearity chromatogram of Nebivolol HCl and Hydrochlorothiazide are presented in the **Chromatogram.39 - 43.** The calibration curves are produced in the **Graph. 88 – 89.**

Market sample analysis

Twenty tablets are weighed accurately and powdered. An amount of the powder equivalent to 50 mg of Nebivolol and 125 mg of Hydrochlorothiazide was dissolved in 100 mL of methanol, ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 3ml of the filtrate into 50 mL standard flask and made up to volume with mobile phase. Aliquots of these solutions were used in such a way that the concentration of each drug was within the linearity range. The dilute solutions were analysed six times as described in the previous section. The results are furnished in the **Table 66.** The assay chromatogram of Nebivolol HCl and Hydrochlorothiazide is presented in the **Chromatogram.44.**

3.6.5 Optimised chromatographic conditions for Pioglitazone HCl and Metformin HCl

Mode of operation : Isocratic

Stationary phase : C_{18} ODS column (150 mm X 4.6 mm i.d.)

Mobile phase : 0.02M CH_3COONH_4 (adjusted to pH -4.2): ACN

Ratio : 45:55

Detection wavelength: 230 nm

pH : 4.2

Flow rate : 1.5 mL min^{-1}

Temperature : Ambient

Sample load : 20 µL

Operating pressure : 94 kgf

The system suitability studies were carried out as specified in USP and the results are furnished in the **Table 69**. The individual Chromatogram of Pioglitazone HCl and Metformin hydrochloride are presented in the **Chromatogram.48 - 49.**

Preparation of calibration samples of Pioglitazone HCl and Metformin HCl

The Stock solutions containing 1000 µg mL^{-1} each of Pioglitazone HCl and Metformin hydrochloride were prepared separately in methanol. Working standard solutions were prepared separately by diluting the stock solutions to obtained 6 µg mL^{-1} of pioglitazone HCl and 140 µg mL^{-1} of metformin HCl in RP-HPLC water.

Pioglitazone HCl and Metformin HCl linearity mixtures were prepared with in the calibration range of 0.3 - 1.5 and 7 – 35 µg mL^{-1} in mobile phase from their respective working standard. The 20 µL samples were injected and recorded the chromatogram. The calibration curves were obtained by plotting peak area versus concentration. The optical and regression characteristics of metformin hydrochloride and pioglitazone hydrochloride are produced in the **Table 70**. The linearity chromatogram of Pioglitazone HCl and Metformin hydrochloride are presented in the **Chromatogram.50 - 54.** The calibration curves are produced in the **Graph. 90 – 91.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. The amount of the powder equivalent to 3 mg of Pioglitazone and 50 mg of Metformin hydrochloride was dissolved in 100 mL of methanol, ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Tranferred 10 mL of the filtrate in to 50 mL standard flask and made up to volume with mobile phase. Aliquots of these solutions were used in such a way that the concentration of each drug is obtained within the range of the calibration. The dilute solutions were analysed six times as described in the previous section. The results are furnished in the **Table 71.** The assay chromatogram of Pioglitazone HCl and Metformin hydrochloride is presented in the **Chromatogram.55.**

3.6.6 Optimised chromatographic conditions for Losartan potassium and Ramipril

Mode of operation : Isocratic

Stationary phase : C_{18} BDS column (150 mm X 4.6 mm i.d.)

Mobile phase : 0.05M KH_2PO_4 (adjusted to pH -2): ACN

Ratio : 62:38

Detection wavelength: 210 nm

pH : 2.0

Flow rate : 1 mL min^{-1}

Temperature : Ambient

Sample load : 20 µL

Operating pressure : 94 kgf

The system suitability studies were carried out as specified in USP and the results are furnished in the **Table 74**. The individual Chromatogram of Losartan

potassium and Ramipril are presented in the **Chromatogram.59 - 60.** The calibration curves are produced in the **Graph. 92 – 93.**

Preparation of Calibration samples of Losartan potassium and Ramipril

Stock solutions 1000 µg mL^{-1} each of Losartan potassium and 1000 µg mL^{-1} of Ramipril were prepared separately in methanol. Working standard solutions were prepared separately by diluting the stock solutions to obtain 160 µg mL^{-1} of losartan potassium and 24 µg mL^{-1} of ramipril in milli pore water.

Losartan potassium and Ramipril linearity mixtures were prepared with in the calibration range of 8 - 40 and 1.2 - 6 µg mL^{-1} in mobile phase from their respective working standard. The 20 µL samples were injected and recorded the chromatogram. The calibration curves were obtained by plotting peak area versus concentration. The optical and regression characteristics of losartan potassium and ramipril are produced in the **Table 75**. The linearity chromatogram of Losartan potassium and Ramipril are presented in the **Chromatogram.61 - 65.**

Market sample analysis

Twenty tablets were weighed accurately and powdered. An amount of the powder equivalent to 500 mg of losartan potassium and 25 mg of ramipril was dissolved in 50 mL of methanol, ultrasonicated for 10 minutes and filtered through Whatman filter paper No. 41. Transferred 2.5 mL of the filtrate and made up to volume with mobile phase. Aliquots of these solutions were used in such a way that the concentration of each drug obtained within the range of the calibration. The dilute solutions were analysed six times as described in the previous section. The results are furnished in the **Table 76.** The assay chromatogram Losartan potassium and Ramipril is presented in the **Chromatogram.66.**

3.6.7 Recovery

In order to ensure the accuracy of the proposed method, Recovery studies were performed by standard addition method at three levels. Known amounts of standard drugs were added to pre-analyzed samples and they were subjected to the proposed method. Results of recovery studies are shown in the **Table 52** for losartan potassium, amlodipine besylate and hydrochlorothiazide, **Table 57** for atorvastatin calcium, fenofibrate and folic acid, **Table 62** for rosiglitazone maleate, glibenclamide and metformin hydrochloride, **Table 67** for nebivolol hydrochloride and hydrochlorothiazide, **Table 72** for pioglitazone hydrochloride and metformin hydrochloride and **Table 77** for losartan potassium and ramipril. The recovery chromatograms of losartan potassium, amlodipine besylate and hydrochlorothiazide are presented in the **Chromatogram.10-12.** The recovery chromatograms of atorvastatin calcium, fenofibrate and folic acid are presented in the **Chromatogram.22-24.**The recovery chromatograms of rosiglitazone maleate, glibenclamide and metformin hydrochloride are presented in the **Chromatogram.34-36.** The recovery chromatograms of nebivolol hydrochloride and hydrochlorothiazide are presented in the **Chromatogram.45-47.** The recovery chromatograms of pioglitazone hydrochloride and metformin hydrochloride are presented in the **Chromatogram.56-58.** The recovery chromatograms of losartan potassium and ramipril are presented in the **Chromatogram.67-69.**

3.7 Validation of the methods

3.7.1 Linearity

The linearity of the proposed methods for determination of above mentioned drugs were evaluated by analysing mixtures containing different concentrations of each compound by chemometry, RP-HPLC and individual drugs for first order derivative spectroscopic method was repeated six times. This approach will provide information on the variation in absorbance for UV and peak area for LC. The linearity of the calibration graphs was validated by the high value of the correlation coefficient.

3.7.2 Precision

Evaluation of the precision, repeatability and intermediate precision were performed at three concentration levels for each compound. The data for each concentration level were evaluated. Statistical comparison of the results was performed using the SD and % RSD. The results of intra day and inter day precision are shown in the **Table 48** for atorvastatin calcium and fenofibrate, atorvastatin calcium and ezetimibe, levofloxacin hemihydrate and ornidazole by first order derivative method **Table 53** for losartan potassium, amlodipine besylate and hydrochlorothiazide, **Table 58** for atorvastatin calcium, fenofibrate and folic acid, **Table 63** for rosiglitazone maleate, glibenclamide and metformin hydrochloride, **Table 68** for nebivolol hydrochloride and hydrochlorothiazide, **Table 73** for pioglitazone hydrochloride and metformin hydrochloride, **Table 78** for losartan potassium and ramipril.

3.7.3 Limit of detection and quantification

According to ICH recommendations the approach based on the S.D. of the response and the slope was used for determining the limit of detection and quantification.

3.7.4 Selectivity

Methods selectivity was achieved by preparing nine physical mixtures of the studied drugs at various concentrations within the linearity range for RP - HPLC. The external validation of the CLS, MLR PCR and PLS models was achieved over set of nine physical mixtures of the six combinations. The concentrations were falling within the calibration range. The physical mixtures were analyzed according to the previous procedures described under the proposed methods.

3.7.5 Accuracy

This study was performed by recovery studies of pharmaceutical formulation, to the preanalysed formulation three levels of known quantity of standard were added and analysed by proposed method. The resulting mixtures were analyzed.

3.7.6 Stability

The studied drug solutions in the solvents and mobile phase exhibited no chromatographic or absorbance changes for 5 h when kept at room temperature, and for 10 h when refrigerated at 15 °C.

4. RESULTS AND DISCUSSION

4.1 Chemometric method

4.1.1 Chemometric method for the Losartan potassium, Amlodipine besylate and Hydrochlorothiazide

A calibration set was randomly prepared as mixtures of Losartan potassium, Amlodipine besylate and Hydrochlorothiazide in their possible compositions in methanol and in Millipore water by applying a multilevel multifactor design in which five levels of the concentration range. The levels were in the calibration range of 8 – 32 μg mL^{-1} for losartan potassium, 1 – 5 μg mL^{-1} for amlodipine besylate and 3 – 5 μg $mL^{-1}$for hydrochlorothiazides. The UV absorbance data were obtained by measuring the absorbance in the region of 200 – 400 nm. The calibration models were constructed with wavelength selection based on best outcome reduced error of the spectral data, selection of wavelength in the range of 230.5 - 350.4nm ($\Delta\lambda$ = 0.1 nm) in their zero order spectra. The obtained data were processed on a Pentium IV computer with PLS-Toolbox software version 4.1 and 5.0 in MATLAB 6.5 (Math works). The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the Classical least squares (CLS), Multiple linear regression (MLR), Principal component regression(PCR) and Partial least squares regression (PLS).

Before constructing the model preprocessing (205) **[**1st Derivative (order: 2, window: 21 pt), Smoothing (order: 0, window: 15 pt), Mean center**]** were carried out to reduce the effect of noise, improve the predictive ability of the model and simplify the model by making the data more normally distributed.

Selection of optimum number of factors for PCR and PLS

The most commonly employed validation criterion is to divide the dataset into two subsets, one calibration set and one validation set. The calibration model was calculated using the calibration set. Then, the root mean square errors of calibration RMSEC and validation RMSEP, were calculated by using the calibration model under investigation to predict the samples in the calibration set and validation set, respectively.

The 25 calibration spectra were used for the selection of the optimum number of factors by using the cross validation with leave out one technique. This allows modelling of the system with the optimum amount of information and avoidance of over fitting or under fitting. The cross-validation procedure consisting of systematically removing one of a group of training samples in turn and using only the remaining ones for the construction of latent variable factors and applied regression. The predicted concentrations were then compared with the actual ones for each of the calibration samples and root mean squares error of prediction (RMSEP) was calculated. The RMSEP was computed in the same manner each time, then, a new factor was added to the PCR and PLS model. The selected model was with the fewest number of factors such that its RMSECV values were not significantly greater than that for the model, which yielded the minimum RMSECV. A plot of RMSECV values against number of components indicates that latent variable factors 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV respectively. At the selected principal components of PCR and PLS, the concentrations of each sample were then predicted and compared with known concentration and the RMSEP was calculated. The RMSEC, RMSECV and RMSEP values are less than 0.3 for Losartan potassium, Amlodipine besylate and Hydrochlorothiazide.

The '*r*' is defined as the correlation between constituent concentrations and shows the absorbance effects relating to the constituent of interest values obtained in the methods close to 1 mean no interference was coming from the other constituents in this set of calibration mixtures.

In order to test the proposed techniques, the validation set of the physical mixtures (from standard) containing the three drugs in variable ratios was carried out. The maximum values of the mean percent errors corresponding to CLS, MLR, and PCR and PLS for the same mixtures were completely acceptable because of their very small numerical (below 0.2) values. The standard deviations corresponding to CLS, MLR, and PCR and PLS for the same mixtures were calculated, which are completely acceptable because of their very small values.

Market sample analysis

Quantification in tablet dosage form was made for Losartan potassium, Amlodipine besylate and Hydrochlorothiazide and its mean of standard errors and the relative standard deviations of our proposed methods were computed. Their percentage of drugs with reference to lable claim was found to be 100 ± 0.03 -101± 0.17% for all the calibration methods.

Recovery studies

To check the accuracy of the proposed methods, recovery studies were carried out by (standard additions method) the addition of standard to the preanalysed formulation. The percentage recovery was found to be in the range between 99 ± 0.6 – 101 ± 0.6%. The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

4.1.2 Chemometric method for the Atorvastatin calcium, Fenofibrate and Folic acid

A calibration set was prepared as mixtures of Atorvastatin calcium, Fenofibrate and Folic acid in their possible compositions in hydrotropic mixture of methanol: Sucrose 5% in 0.1N Sodium hydroxide (80:20) by applying a multilevel multifactor design in which five levels of the concentration range. From the solubility profile of the folic acid, is soluble in sodium hydroxide, atorvastatin calcium and fenofibrate soluble in methanol. In the ratio of methanol and sodium hydroxide calcium produce precipitate, the hydrotropic mixture containing methanol: Sucrose 5% in 0.1N Sodium hydroxide (80:20) were tried, all the three drugs were freely soluble so it was used as common solvent.

The levels were in the calibration range of 1.0 – 5.0 μg mL^{-1} for atorvastatin calcium, 15 – 75 μg mL^{-1} for fenofibrate, and 0.5 – 2.5 μg mL^{-1} for folic acid. The UV absorbance data were obtained by measuring the absorbances in the region of 200 – 400 nm. The calibrations model were constructed with selected wavelength in the range of 224.4 - 249.8 and 303.2 - 360 nm ($\Delta\lambda$ = 0.1 nm) in their zero order spectra. The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the CLS, MLR, PCR and PLS.

Before constructing the model preprocessing [Smoothing (order: 0, window: 15 pt), 2nd Derivative (order: 2, window: 21 pt) Auto scale] was carried out. A fit model constructed by a plot of RMSECV values against number of components indicates that latent variable factors 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV respectively. The RMSEC, RMSECV and RMSEP values are less than 0.6 for Atorvastatin calcium, Fenofibrate and Folic acid. The r

values obtained in the methods close to 1 mean no interference was coming from the other constituents in this set of calibration mixtures.

The validation set of the physical mixtures (from standard) containing the three drugs in variable ratios was carried out. The maximum values of the mean percent errors corresponding to CLS, MLR, and PCR and PLS for the same mixtures were completely acceptable because of their very small numerical (below 0.08) values. The standard deviations corresponding to CLS, MLR, and PCR and PLS for the same mixtures were calculated, which are completely acceptable because of their very small values.

Market sample analysis

Quantification in tablet dosage form was made for Atorvastatin calcium, Fenofibrate and Folic acid and its mean of standard errors and the relative standard deviations of our proposed methods were computed. Their percentage of drugs with reference to lable claim was found to be 98.7 ± 1.3 -103± 1.4% for all the calibration methods.

Recovery studies

To check the accuracy of the proposed methods, recovery studies were carried out by (standard additions method) the addition of standard to the preanalysed formulation. The percentage recovery was found to be in the range between 99 ± 0.12 – 100 ± 1.1%**.** The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

4.1.3 Chemometric method for the Rosiglitazone Maleate, Glibenclamide and Metformin HCl

A calibration set was randomly prepared as mixtures of Rosiglitazone Maleate, Glibenclamide and Metformin HCl in their possible compositions in methanol and in Millipore water; the levels were in the calibration range of 0.05 - 0.25 μg mL^{-1} for roseglitazone maleate, 0.5 - 2.5 μg mL^{-1} for glibenclamide, 7 - 35 μg $mL^{-1}$for metformin HCl. The UV absorbance data were obtained by measuring the absorbance's in the region of 200 – 400 nm. The calibrations model were constructed with selected wavelength in the range of 200-340 nm ($\Delta\lambda$ = 0.1 nm) in their zero order spectra. The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the CLS, MLR, PCR and PLS.

Before constructing the model preprocessing [2nd Derivative (order: 2, window: 15 pt) Mean Center] was carried out. A fit model constructed by a plot of RMSECV values against number of components indicates that latent variable factors 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV respectively. The RMSEC, RMSECV and RMSEP values are less than 0.3 for Rosiglitazone Maleate, Glibenclamide and Metformin HCl.

The validation set of the physical mixtures (from standard) containing the three drugs in variable ratios was carried out. The maximum values of the mean percent errors corresponding to CLS, MLR and PCR and PLS for the same mixtures were completely acceptable because of their very small numerical (below 0.2) values. The standard deviations corresponding to CLS, MLR, and PCR and PLS for the same mixtures were calculated, which are completely acceptable because of their very small

values. The r values obtained in the methods close to 1 mean no interference was coming from the other constituents in this set of calibration mixtures.

Market sample analysis

Quantification in tablet dosage form was made for Rosiglitazone Maleate, Glibenclamide and Metformin HCl and its mean of standard errors and the relative standard deviations of our proposed methods were computed. Their percentage of drugs with reference to lable claim was found to be 98 ± 0.6 – 103 ± 1.9 of all the calibration methods.

Recovery studies

To check the accuracy of the proposed methods, recovery studies were carried out by (standard additions method) the addition of standard to the preanalysed formulation. The percentage recovery was found to be in the range between 99 ± 0.2 – 100 ± 0.1. The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate. Results of recovery studies were found to be 99 ± 0.3 – 100 ± 0.2**.** The good precision of the method was indicated by the standard derivation (< 2).

4.1.4 Chemometric method for the Nebivolol HCl and Hydrochlorothiazide

A calibration set was randomly prepared as mixtures of Nebivolol HCl and Hydrochlorothiazide in their possible compositions in methanol and in Millipore water; the levels were in the calibration range of 1.0 – 5.0 μg mL^{-1} for nebivolol HCl, 2 - 10 μg mL^{-1} for hydrochlorothiazide. The UV absorbance data were obtained by measuring the absorbance's in the region of 200 – 400 nm. The calibrations model were constructed with measurements in the range of 200-400 nm ($\Delta\lambda$ = 0.1 nm) in

their zero order spectra. The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the CLS, MLR, PCR and PLS.

Before constructing the model preprocessing [1st Derivative (order: 2, window: 21 pt), Smoothing (order: 0, window: 15 pt), Mean Center] was carried out. A plot of RMSECV values against number of components indicates that latent variable factors 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV respectively. The RMSEC, RMSECV and RMSEP values are less than 0.3 for Nebivolol HCl and Hydrochlorothiazide. The r values obtained in the methods close to 1 mean no interference was coming from the other constituents in this set of calibration mixtures.

The validation set of the physical mixtures (from standard) containing the three drugs in variable ratios was carried out. The maximum values of the mean percent errors corresponding to CLS, MLR, and PCR and PLS for the same mixtures were completely acceptable because of their very small numerical (below 0.2) values. The standard deviations corresponding to CLS, MLR, and PCR and PLS for the same mixtures were calculated, which are completely acceptable because of their very small values.

Market sample analysis

Quantification in tablet dosage form was made for Nebivolol HCl and Hydrochlorothiazide and its mean of standard errors and the relative standard deviations of our proposed methods were computed. Their percentage of drugs with reference to lable claim was found to be 99 ± 0.4 – 100 ± 1.9 of all the calibration methods.

Recovery studies

To check the accuracy of the proposed methods, recovery studies were carried out by (standard additions method) the addition of standard to the preanalysed formulation. The percentage recovery was found to be in the range between 99 ± 0.2 – 100 ± 0.1. The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

4.1.5 Chemometric method for the Pioglitazone HCl and Metformin HCl

A calibration set was randomly prepared as mixtures Pioglitazone HCl and Metformin HCl in their possible compositions in methanol and in Millipore water; the levels were in the calibration range of 0.3–1.5 μg mL^{-1} for pioglitazone HCl, 7-35 μg mL^{-1} for metformin HCl. The UV absorbance data were obtained by measuring the absorbance's in the region of 200 – 400 nm. The calibrations model were constructed with measurements in the range of 200-350 nm ($\Delta\lambda$ = 0.1 nm) in their zero order spectra. The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the CLS, MLR, PCR and PLS.

Before constructing the model preprocessing [1st Derivative (order: 2, window: 21 pt), Smoothing (order: 0, window: 15 pt), Mean Center] was carried out. A plot of RMSECV values against number of components indicates that latent variable factors 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV respectively. The RMSEC, RMSECV and RMSEP values are less than 0.3 for Pioglitazone HCl and Metformin HCl.

The validation set of the physical mixtures (from standard) containing the three drugs in variable ratios was carried out. The maximum values of the mean

percent errors corresponding to CLS, MLR, and PCR and PLS for the same mixtures were completely acceptable because of their very small numerical (below 0.2) values. The standard deviations corresponding to CLS, MLR, and PCR and PLS for the same mixtures were calculated, which are completely acceptable because of their very small values. The r values obtained in the methods close to 1 mean no interference was coming from the other constituents in this set of calibration mixtures.

Market sample analysis

Quantification in tablet dosage form was made for Pioglitazone HCl and Metformin HCl and its mean of standard errors and the relative standard deviations of our proposed methods were computed. Their percentage of drugs with reference to lable claim was found to be 99 ± 0.2 – 100 ± 0.2 of all the calibration methods.

Recovery studies

To check the accuracy of the proposed methods, recovery studies were carried out by (standard additions method) the addition of standard to the preanalysed formulation. The percentage recovery was found to be in the range between 99 ± 1.8 – 100 ± 0.2%. The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

4.1.6 Chemometric method for the Losartan potassium and Ramipril

A calibration set was randomly prepared as mixtures of Losartan potassium and Ramipril in their possible compositions in methanol and in Millipore water by applying a multilevel multifactor design in which five levels in the concentration range. The levels were in the calibration range 8 - 40 μg mL^{-1} for losartan potassium, 1.2 - 6 μg mL^{-1} for ramipril. The UV absorbance data were obtained by measuring the

absorbance's in the region of 200 - 400 nm. The calibrations model were constructed with measurements in the range of 200 - 350 nm ($\Delta\lambda$ = 0.1 nm) in their zero order spectra. The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the CLS, MLR, PCR and PLS.

Before constructing the model preprocessing [Auto scale was carried out for PLS and PCR, Preprocessing: Smoothing (order: 0, window: 15 pt), 2nd Derivative (order: 2, window: 21 pt) for CLS and MLR] was carried out. A plot of RMSECV values against number of components indicates that latent variable factors 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV respectively. The RMSEC, RMSECV and RMSEP values are less than 0.3 for Losartan potassium, Ramipril.

The validation set of the physical mixtures (from standard) containing the three drugs in variable ratios was carried out. The maximum values of the mean percent errors corresponding to CLS, MLR, and PCR and PLS for the same mixtures are completely acceptable because of their very small numerical (below 0.2) values. The standard deviations corresponding to CLS, MLR, and PCR and PLS for the same mixtures were calculated, which are completely acceptable because of their very small values less than 2. The r values obtained in the methods close to 1 mean no interference was coming from the other constituents in this set of calibration mixtures.

Market sample analysis

Quantification in tablet dosage form was made for Losartan potassium, Ramipril and its mean of standard errors and the relative standard deviations of our proposed methods were computed. Their percentage of drugs with reference to lable claim was found to be 99 ± 0.2 – 100 ± 0.2 of all the calibration methods.

Recovery studies

To check the accuracy of the proposed methods, recovery studies were carried out by (standard additions method) the addition of standard to the preanalysed formulation. The percentage recovery was found to be in the range between 99 ± 0.2 – 100 ± 0.1. The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery revealed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

4.2 Derivative Spectroscopy determination

First order derivative spectroscopic determination of zero crossing points in derivative mode completely eliminates the interference from unwanted component and thus concentration determination of each drug can be easily calculated without prior separation from combined dosage form, from their respective calibration curve.

Atorvastatin calcium and fenofibrate, atorvastatin calcium and ezetimibe were dissolved in methanol. Levofloxacin hemihydrate and ornidazole were dissolved in 0.1M HCl.

From the first order derivative spectrum, the wavelengths 239 nm was selected for the determination of atorvastatin calcium which is zero crossing for fenofibrate and 246 nm for fenofibrate which is zero crossing for atorvastatin calcium, 224 nm for atorvastatin calcium which is zero crossing for ezetimibe and 233 nm for ezetimibe which is zero crossing for atorvastatin calcium and 277.5 nm for levofloxacin hemihydrate which is zero crossing for ornidazole and 319 nm for ornidazole which is zero crossing for levofloxacin hemihydrate.

The linearity study was carried out individual drugs for the three combinations in the first order derivative mode at their wavelengths and the concentration range of 4 – 24 μg mL^{-1} for atorvastatin calcium and 4 - 24 μg mL^{-1} for fenofibrate for the combination of atorvastatin calcium and fenofibrate, 4 –24 μg mL^{-1} for atorvastatin calcium and 4 - 24 μg mL^{-1} for ezetimibe for the combination of atorvastatin calcium and ezetimibe, 10–50 μg mL^{-1} for levofloxacin hemihydrate and 20 - 80 μg mL^{-1} for ornidazole in the combination of levofloxacin hemihydrate and ornidazole.

The calibration curves were obtained by plotting absorbance versus concentration of the standard solution. The correlation coefficient was found to be 0.9999 for atorvastatin calcium, 0.99986 for fenofibrate, 0.9961 for atorvastatin

calcium, 0.9993 for ezetimibe, 0.9992 for levofloxacin hemihydrate and 0.99987 for ornidazole. .

Quantification and recovery studies of tablets were made by measuring the $\frac{\Delta A}{\Delta \lambda}$ at about their wavelengths in the first order derivative mode. The percentage quantification in formulation, recovery and the results of intra day and inter day studies were analysed. The percentage recovery was found to be in the range between 98.0 – 101.5% for all the combinations. The % RSD was found to be less than 2. The low % RSD values for recovery indicated that the method was found to be accurate. The high percentage recovery exposed that no interference produced due to the excipients used in formulation. Therefore, the developed method was found to be accurate.

4.3 RP- HPLC METHOD

4.3.1 Selection of detection wavelength

The sensitivity of HPLC method that uses UV detection depends upon the proper selection of detection wavelength. An ideal is the one that response for the drugs that are to be detected. In the present study individual drug solutions of 10 μg mL^{-1} were prepared in solvent mixture of acetonitrile: water (1:1). These solutions were scanned in the UV region of 200 – 400 nm and the overlaid UV spectra were recorded. From the overlaid spectra 225 nm for Losartan potassium, Amlodipine besylate and Hydrochlorothiazide, 248 nm for atorvastatin calcium, Fenofibrate and folic acid, 230 nm for rosiglitazone maleate, glibenclamide and metformin HCl, 240 nm for nebivolol HCl and hydrochlorothiazide, 230 nm for pioglitazone HCl and metformin HCl and 210 nm for losartan potassium and ramiprile was selected as the detection wavelength.

4.3.2 Selection of chromatographic method

Depending on the nature of the sample (ionic or neutral molecule), molecular weight, pK_a value and solubility, chromatographic method was selected. The drugs were polar in nature and so reverse phase chromatographic technique were selected for the present study.

4.3.4 Initial chromatographic condition

Acetonitrile and Methanol were selected as organic phase in the mobile phase to elute the drug from stationary phase because of its favorable UV transmittance, low viscosity and better solubility for the selected drugs. The pH of the initial mobile phase selected was 3 because of its low pH, C_{18}, ODS and BDS columns were used for the separation of the selected drugs in raw material and in formulations.

4.3.5 Optimization of Losartan potassium, Amlodipine besylate and Hydrochlorothiazide

Initially the chromatogram was developed with water: acetonitrile: methanol (40:30:30) as mobile phase showed splitting peaks, the concentration of organic phase was increased by adjusting the ratio of the acetonitrile and methanol to get splitless, reduce tailing and asymmetric peaks. The peak sharpness increased by the addition of buffer. Under the chromatographic conditions mentioned above, there was tailing, spliting in peaks. Hence, different buffers were tried in the place of water with potassium dihydrogen ortho phosphate which gave sharp peaks. Hence, 10mM KH_2PO_4 (adjusted to pH -3): ACN (60:40) was selected as the mobile phase.

The resolution between the peaks increased by adjusting the pH of the mobile phase, Hence, it was decided to run the chromatogram at specified pH. In the above selected mobile phase various effects of pH were studied by using different acidic pH for buffer like pH 3, 4 and 5. The pH 3 was found to be satisfactory.

With the above selected mobile phase ratio 10mM KH_2PO_4 (adjusted to pH -3): ACN (60:40) different flow rates ranging from 0.8, 1 and 1.5 mL min^{-1} were tried and the chromatograms were recorded. It was observed that the chromatogram obtained with 1.5 mL min^{-1} flow rate, the peaks were symmetrical and with better resolution compared to other flow rates. Hence 1.5 mL min^{-1} was selected.

Optimised chromatographic conditions for Losartan potassium, Amlodipine besylate and Hydrochlorothiazide were Isocratic mode, C_{18} ODS column (150 mm X 4.6 mm i.d.), 10mM KH_2PO_4 (adjusted to pH -3): ACN(60:40) as mobile phase, 225 nm detection wavelength and flow rate 1.5 mL min^{-1}. The system suitability parameters as specified in USP results are found to be acceptable.

The linearity range of losartan potassium was 8 – 40 µg mL^{-1}, 1 – 5 µg mL^{-1} for amlodipine besylate and 3 – 15 µg $mL^{-1}$for hydrochlorothiazide. The calibration curves were obtained by plotting peak area versus concentration. The correlation coefficients were found to be 0.9982 for losartan potassium, 0.9987 for amlodipine besylate and 0.9994 for hydrochlorothiazide.

Market sample analysis

Quantification in tablet dosage form was made for losartan potassium, amlodipine besylate and hydrochlorothiazide. The tabletss were analysed six times as described in the previous section. The percentage of drugs with reference to lable claim was found to be 100.95 ± 0.4 – 101 ± 1.5. The SD and % RSD are found to be within 1.5 indicating good precision and accuracy of the method.

4.3.6 Optimization of Atorvastatin calcium, Fenofibrate and Folic acid

Initially the chromatogram was developed with water: acetonitrile: methanol (20:30:50% v/v) as mobile phase, showed merging peaks. The concentration of organic phase was increased by adjusting the ratio of the acetonitrile and methanol to get well resolved peaks. Under the chromatographic conditions mentioned above, there was tailing of peaks. Hence, acetate buffer of pH - 4.5 was tried in the place of water which showed more than five peaks indicating degradation of the drug. Potassium dihydrogen ortho phosphate was tried, which gave sharp peaks. Hence 10mM of KH_2PO_4 was selected as the buffer. Peak sharpness was achieved with buffer containing 10mM of KH_2PO_4 (adjusted to pH -2.5 with ortho phosphoric acid): ACN (30:70 % v/v), as the mobile phase.

The resolution of the peaks was increased by adjusting the pH of the mobile phase. From the above selected mobile phase the effect of different pH was studied

with different acidic buffers like pH 2.5, 3 and 4. Among tham the buffer pH 2.5 was found to be satisfactory.

The flow rates ranging from 0.8, 1 and 1.5 mL min^{-1} were tried and the chromatograms were recorded. It was observed the peaks were symmetrical and better resolved with 1.5 mL min^{-1} compared to other flow rates. Hence the flow rate of 1.5 mL min^{-1} was selected.

Optimised chromatographic conditions for Atorvastatin calcium, Fenofibrate and Folic acid were Isocratic mode, C_{18} BDS column (150 mm X 4.6 mm i.d.), 10mM KH_2PO_4 (adjusted to pH -2.5): ACN (30:70) mobile phase, 248 nm detection wavelength and flow rate 1.5 mL min^{-1}. The system suitability parameters as specified in USP results are found to be acceptable.

The linearity range of atorvastatin calcium was 1 – 5 μg mL^{-1}, 15 - 75 μg mL^{-1} for fenofibrate and 0.5 – 2.5 μg $mL^{-1}$for folic acid. The calibration curves were obtained by plotting peak area versus concentration. The correlation coefficient was found to be 0.9965 for atorvastatin calcium, 0.9992 for fenofibrate and 0.9997 for folic acid.

Market sample analysis

Quantification in tablet dosage form was made for atorvastatin calcium, fenofibrate and folic acid. The tablets were analysed six times as described in the previous section. The percentage of drugs with reference to lable claim was found to be 99.8 ± 0.2 – 101 ± 0.3. The SD and % RSD were found to be within 0.3 showing the better precision and accuracy of the method.

4.3.7 Optimization of Rosiglitazone maleate, Glibenclamide and Metformin HCl

Initially the chromatogram was developed with water: acetonitrile: methanol (40:30:30), showed splitting, merging, broading and tailing of the peaks, the alteration

of the mobile phase by adjusting the ratio of the mobile phase and the addition of buffer improved the peak sharpness. Different buffers were tried in the place of water like, acetate buffer and phosphate buffer. In the phosphate buffer merging and tailing was produced. Hence, 0.02M CH_3COONH_4 (adjusted to pH - 4.2): ACN (45:55) was selected as the buffer.

The sharpness and resolution of the peaks were improved by adjusting the pH. Hence, it was decided to run the chromatogram at specified pH. In the above selected mobile phase various effect of pH were studied with different acidic buffer like pH 4, 4.2 and 4.6. Among tham the buffer pH 4.2 was found satisfactory.

The flow rates ranging from 0.8, 1 and 1.5 mL min^{-1} were tried and the chromatograms were recorded. It was observed that the chromatogram obtained with 1.5 mL min^{-1} flow rate, the peaks were symmetrical and with better resolution compared to other flow rates. Hence the flow rate 1.5 mL min^{-1} was selected.

Optimized chromatographic conditions for rosiglitazone maleate, glibenclamide and metformin HCl were Isocratic mode, C_{18} ODS column (150 mm X 4.6 mm i.d.), 0.02M CH_3COONH_4: ACN(pH-4.2)(45:55) mobile phase, 230 nm detection wavelength and flow rate 1.5 mL min^{-1}. The system suitability parameters as specified in USP results are found to be acceptable.

The linearity range was 0.05 – 0.25 μg mL^{-1} for rosiglitazone maleate, 0.1 – 0.5 μg mL^{-1} for glibenclamide and 7 – 35 μg $mL^{-1}$for metformin HCl. The calibration curves were obtained by plotting peak area versus concentration. The Correlation coefficient was found to be 0.9959 for rosiglitazone maleate, 0.9985 for glibenclamide and 0.9993 for metformin HCl.

Market sample analysis

The tablets were analysed six times as described in the previous section. The percentage of drugs with reference to lable claim was found to be 100 ± 0.4 – 101 ± 1.5. The SD and %RSD were found to be within 1.5 which shows the good precision and accuracy of the method.

4.3.8 Optimization of Nebivolol HCl and Hydrochlorothiazide

Initially the chromatogram was developed with water: acetonitrile (40:60), nebivolol HCl was not eluted up to 20 minutes. Then a mixture of 0.4% Triethylamine: ACN (50:50) was used which showed tailing for nebivolol HCl. Further, the ratio of the above buffer was changed to 40:60 and 60:40 which showed broadening and tailing of peaks. The mixture of 0.4% Triethylamine: ACN (70:30) gave less tailing and sharp peak. Hence it was selected as the mobile phase. The effects of pH were studied by using different buffers of pH 6 and 7. Among them the buffer of pH 7 was found satisfactory.

Different flow rates ranging from 0.8, 1 and 1.2 mL min^{-1} were tried and the chromatograms were recorded. It was observed that the chromatogram obtained with 1 mL min^{-1} flow rate, the peaks were symmetrical and with better resolution compared to other flow rates. Hence the flow rate of 1 mL min^{-1} was selected.

Optimised chromatographic conditions of nebivolol HCl and hydrochlorothiazide were isocratic mode, C_{18} BDS column (150 mm X 4.6 mm i.d.), 0.4% Triethylamine: ACN pH - 7.0 70:30 mobile phase, 240 nm detection wavelength and flow rate 1 mL min^{-1}. The system suitability parameters as specified in USP results are found to be acceptable.

The linearity range was 1 - 5 µg mL^{-1} for nebivolol HCl and 3 - 15 µg mL^{-1} for hydrochlorothiazide. The calibration curves were obtained by plotting peak area

versus concentration. The correlation coefficient was found to be 0.9961 for nebivolol HCl and 0.9971 for hydrochlorothiazide.

Market sample analysis

The tablets were analysed six times as described in the previous section. The percentage of drugs with reference to lable claim was found to be 100 ± 0.3 – 101 ± 0.4 The SD and %RSD found to be within 0.4 conforming the better precision and accuracy of the method.

4.3.9 Optimization of Pioglitazone HCl and Metformin HCl

Initially the chromatogram was developed with water: acetonitrile: methanol (40:30:30) as mobile phase which showed broad splitting peaks. To increase peak sharpness different buffers were tried in the place of water like triethyl amine, potassium dihydrogen ortho phosphate and ammonium acetate buffer and phosphate buffer which produced merging and negative peaks. The buffer containing 0.02M CH_3COONH_4 (adjusted to pH -4.2): ACN (45:55) gave symmetric and sharp peak, hence it was selected as the mobile phase.

The mobile phase with buffer of different pH like pH 3, 4.2 and 4.5 were used. Among them the buffer pH 4.2 was found satisfactory.

Different flow rates ranging from 0.8, 1 and 1.5 mL /min were tried and the chromatograms were recorded. It was observed that the chromatogram obtained with 1.5 mL min^{-1} flow rate gave symmetrical peaks and better resolution compared to other flow rates. Hence the flow rate 1.5 mL /min was selected.

Optimised chromatographic conditions for pioglitazone HCl and metformin HCl were isocratic mode, C_{18} ODS column (150 mm X 4.6 mm i.d.), mobile phase 0.02M CH_3COONH_4 (adjusted to pH - 4.2): ACN (45:55),detection wavelength 230

nm and flow rate 1.5 mL min^{-1}. The system suitability parameters as specified in USP results are found to be acceptable.

The linearity range of pioglitazone HCl was 0.3 – 1.5 μg mL^{-1} and 7 - 35 μg mL^{-1} for metformin HCl. The calibration curves were obtained by plotting peak area versus concentration. The correlation coefficient was found to be 0.9989 for pioglitazone HCl and 0.9988 metformin HCl

Market sample analysis

The tablets were analysed six times as described in the previous section. The percentage of drugs with reference to lable claim was found to be 100 ± 0.4 – 101 ± 1.04. The SD and %RSD were found to be within 1.04 showing good precision and accuracy of the method.

4.3.10 Optimization of Losartan potassium and Ramipril

Initially the chromatogram was developed with water: acetonitrile: methanol (40:30:30) which showed splitting peaks. Hence, different buffers were tried in the place of water like acetate buffer and phosphate buffer which produced tailless and sharp peak. Hence, 0.05M KH_2PO_4 was selected as the buffer.

The mobile phase with buffer of different pH 2.5, 3 and 4 were tried. The pH 2.5 was found satisfactory.

Different flow rates ranging from 0.8, 1 and 1.5 mL min-1 were tried and the chromatograms were recorded. It was observed that the chromatogram obtained with 1mL min^{-1}flow rate, the peaks were symmetrical and with better resolution compared to other flow rates. Hence the flow rate 1 mL min^{-1} was selected.

Optimised chromatographic conditions for losartan potassium and ramipril were Isocratic mode, C_{18} BDS column (150 mm X 4.6 mm i.d.), mobile phase 0.05M KH_2PO_4 (adjusted to pH -2.5): ACN (62:38), detection wavelength 210 nm with flow

rate 1 mL min^{-1}. The system suitability parameters as specified in USP results are found to be acceptable.

The linearity range of losartan potassium was 8 – 40 μg mL^{-1} and 1.2 - 6 μg mL^{-1} for ramipril. The calibration curves were obtained by plotting peak area versus concentration. The correlation coefficient was found to be 0.9935 for losartan potassium and 0.9937 for ramipril.

Market sample analysis

The tablets were analysed six times as described in the previous section. The percentage of drugs with reference to lable claim was found to be 100 ± 0.1 – 101 ± 0.5. The SD and %rsd were found to be within 1.5 indicating the precision and accuracy of the method.

4.3.11 Recovery

In order to ensure the accuracy of the proposed method, Recovery studies were performed by standard addition method at three levels. To the preanalysed sample solution, a definite concentration was added and then it was recovered. The percentage recovery was found to be in the range between 98.0 – 101.5% for all the combinations. The % RSD was found to be less than 2. The low % RSD values of recovery studies indicated that the method is more accurate. The high percentage recovery revealed that there is no interference produced due to the excipients used in formulation. Therefore, the developed can be adapted for routine analysis of the above combinations.

4.4 Validation of the methods

4.4.1 Linearity

The linearity of the proposed method for determination of above mentioned drugs were evaluated by analysing a series of different concentrations of each compound in mixture for chemometric, RP-HPLC and individual drugs for first order derivative method. Each concentration was analysed six times. This approach will provide information on the variation in absorbance for UV and peak area for LC. The linearity of the calibration graphs was validated by the high value of the correlation coefficient. Optical and regression characteristic parameters for regression equations for RP-HPLC and derivative methods were obtained by least squares treatment and for chemometric methods obtained by CLS, MLR, PCR and PLS.

4.4.2. Precision

Evaluation of the precision, repeatability and intermediate precision were performed at three concentration levels for each compound. The data for each concentration level were evaluated. Statistical comparison of the results was performed using the SD and % RSD.

4.4.3 Limit of detection and quantification

According to ICH recommendations the approach based on the S.D. of the response and the slope was used for determining the detection and quantification limits.

4.4.4 Selectivity

Methods selectivity was achieved by preparing nine physical mixtures of the studied drugs at various concentrations within the linearity range for HPLC. The external validation of the CLS, MLR PCR and PLS models was achieved over set of nine physical mixtures of the six combinations. The concentrations were falling

within the ranges of calibration matrix. The physical mixtures were analyzed according to the previous procedures described under the proposed methods. Satisfactory results were obtained, indicating the high selectivity of the proposed methods for simultaneous determination six combinations.

4.4.5 Accuracy

This study was performed by recovery studies of pharmaceutical formulation (standard addition method). The resulting mixtures were analyzed and the results obtained were compared with the expected results. The excellent recoveries by standard addition method suggested good accuracy of the proposed methods.

4.4.6 Stability

The studied compound solutions in the mobile phase or solvents exhibited no chromatographic or absorbance changes for 5 h when kept at room temperature, and for 10 h when stored at 15 °C.

5. SUMMARY AND CONCLUSION

A simple and precise method involving four chemometric techniques, First order derivative and RP-HPLC was developed for the determination of various combinations of drugs in pharmaceutical formulations.

5.1 Chemometrics-assisted spectrophotometry

Chemometric techniques are wide applied for the resolution of the selected drug combinations. A 25 calibration set of sample mixtures were used to construct the best model. The levels were in the calibration range of 8.0 – 32.0 μg mL^{-1} , 1 – 5 μg mL^{-1} and 3 – 5 μg mL^{-1} for losartan potassium, amlodipine besylate and hydrochlorothiazide, 1.0 – 5.0 μg mL^{-1} , 15 – 75 μg mL^{-1} and 0.5 – 2.5 μg mL^{-1} for atorvastatin calcium, fenofibrate and folic acid, 0.05 - 0.25 μg mL^{-1} , 0.5 - 2.5 μg mL^{-1} and 7 - 35 μg $mL^{-1}$for rosiglitazone maleate, glibenclamide and metformin HCl, 1.0 – 5.0 μg mL^{-1} and 2 - 10 μg mL^{-1} for nebivolol HCl and hydrochlorothizide, 0.3 – 1.5 μg mL^{-1} and 7 - 35 μg mL^{-1} for pioglitazone HCl and metformin HCl, 8 – 40 μg mL^{-1} and 1.2 - 4.8 μg mL^{-1} for losartan potassium and ramipril.

The UV absorbance data were recorded by measuring the absorbances in the region of 200 – 400 nm with 0.1 nm difference for the mixtures. The obtained data were processed on a Pentium IV computer with PLS-Toolbox software version 4.1 and 5.0 in Matlab 6.5 (math works). The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in the CLS, MLR, PCR and PLS.

Before constructing the model preprocessing was carried out to reduce the effect of noise, improve the predictive ability of the model and simplify the model by

making the data more normally distributed and wavelength selection based on best outcome reduced the error of the spectral data.

The predictive ability of a model in chemometric methods was estimated from their RMSEC, RMSECV, r and RMSEP values of the drugs. Hence, good agreement was seen in the assay results of pharmaceutical formulation. We conclude that the chemometric technique in combination with CLS, MLR, PCR and PLS calibration methods is a good approach for obtaining reliable results.

6.2 Derivative spectroscopy determination

Derivative method involving first derivative UV spectroscopic determination of zero crossing points in derivative mode completely eliminates the interference from unwanted component and thus concentration determination of each drug can be easily calculated without prior separation from combined dosage form, from their respective calibration curve. Atorvastatin calcium and fenofibrate, atorvastatin calcium and ezetimibe, Levofloxacin hemihydrate and ornidazole were determined simultaneously without prior separation from tablets dosage form.

6.3 RP- HPLC Method

Selection of mobile phase, column, pH and wavelength

The mobile phase, column, pH and wavelength were selected on the basis of peak purity and acceptable limit of system suitability parameter and resolution of the peak, which were listed below

1. Losartan potassium, amlodipine besylate and hydrochlorothiazide: 10mM KH_2PO_4 (adjusted to pH -3): ACN (60:40), 225 nm (C_{18}, ODS)

2. Atorvastatin calcium, fenofibrate, folic acid: 10mM KH_2PO_4 (adjusted to pH -2.5): ACN (30:70), 248 nm (C_{18}, BDS)

3. Rosiglitazone, glibenclamide, metformin HCl: 0.02M CH_3COONH_4 (adjusted to pH -4.2): ACN (45:55), 230 nm (C_{18}, ODS)

4. Nebivolol HCl, hydrochlorothiazide: 0.4% Triethylamine: ACN (70:30), 240 nm (C_{18}, BDS)

5. Pioglitazone HCl, metformin HCl: 0.02M CH_3COONH_4 (adjusted to pH -4.2): ACN (45:55), 230 nm (C_{18}, ODS)

6. Ramipril, losartan potassium: 0.05M KH_2PO_4 (adjusted to pH -2): ACN (62:38), 210 nm (C_{18}, BDS).

Chromatographic conditions:

Chromatographic separations were performed at ambient temperature with the use of respective mobile phase. The mobile phase was filtered and degassed before use. The flow rate of mobile phase was adjusted to 1.5 and 1 mL min^{-1}. the detection wavelength was set at their nm. The injection volume of the standard and sample solutions was 20 μL^{-1}. The system suitability parameters as specified in USP results are found to be acceptable. The developed method was found to be accurate.

Hence, good agreement was seen in the assay results of pharmaceutical formulation by chemometric, derivative spectroscopic and RP-HPLC. We conclude that the chemometric technique when coupled with CLS, MLR, PCR and PLS calibration methods sounds a good approach for obtaining reliable results, when compared with RP-HPLC method. Chemometric techniques are precise, rapid, selective, sensitive and less expensive. Hence it can be applied for the routine analysis of these combinations of drugs in the tablet formulation without any pre-treatment and without time consumption in quality control laboratories.

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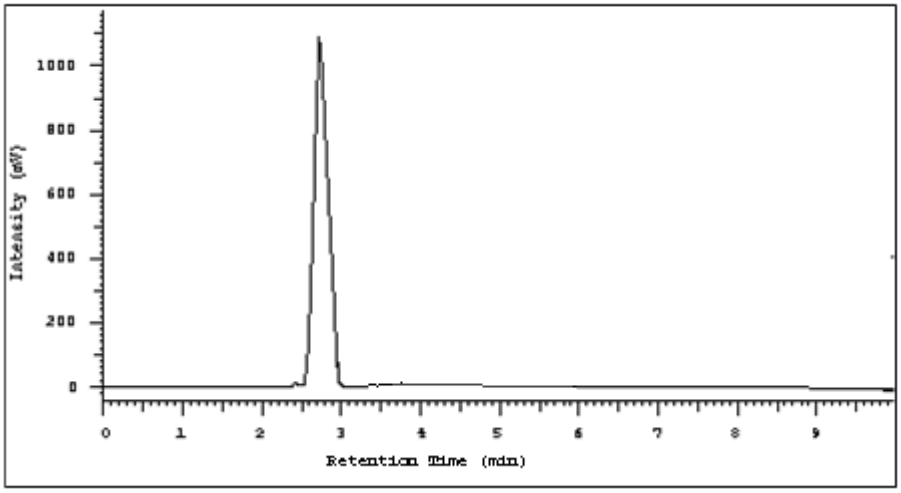
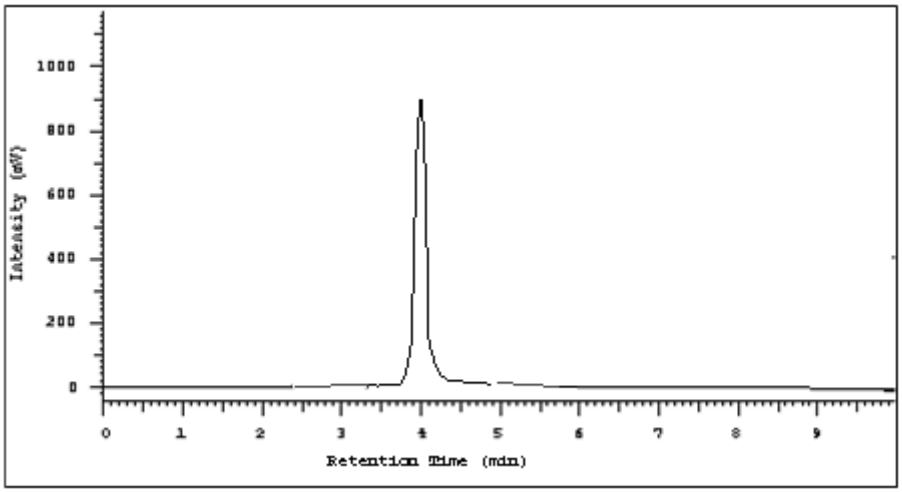
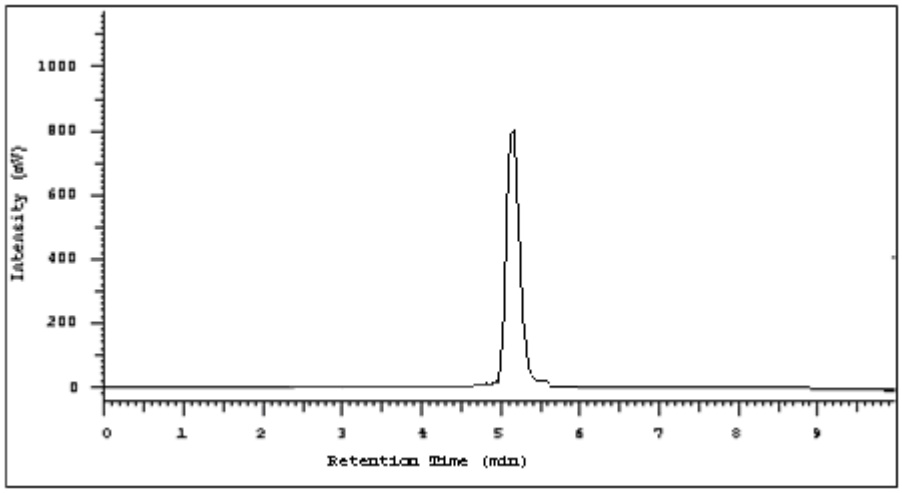
Chromatogram.1 Individual Chromatogram of losartan potassium



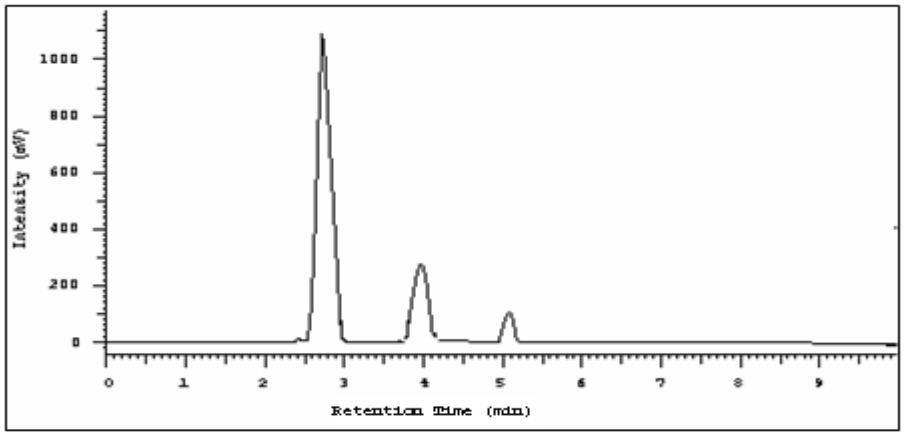
Chromatogram.2 Individual Chromatogram of hydrochlorothiazide



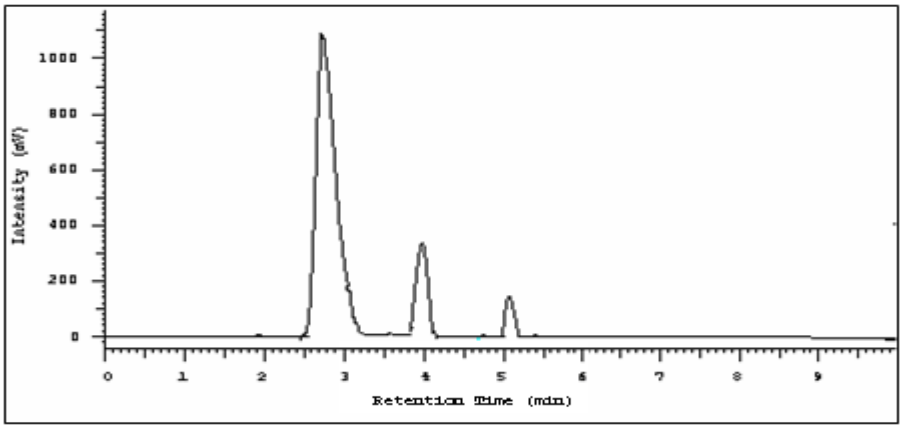
Chromatogram.3 Individual Chromatogram of amlodipine besylate



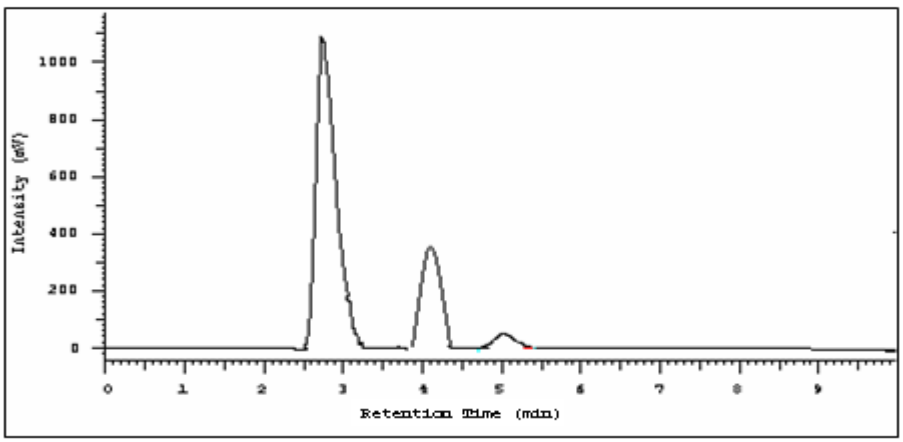
Chromatogram.4 Linearity of losartan potassium, amlodipine besylate and hydrochlorothiazide (8, 1, 3 µg mL^{-1})



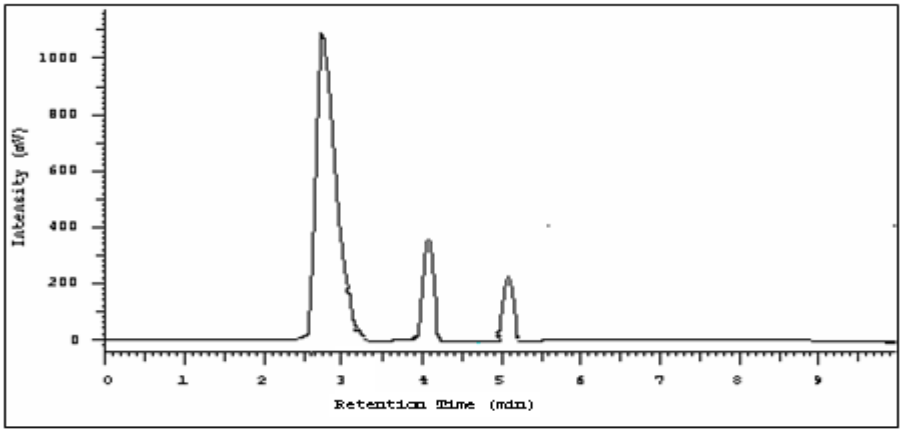
Chromatogram.5 Linearity of losartan potassium, amlodipine besylate and hydrochlorothiazide (16, 2, 6 µg mL^{-1})



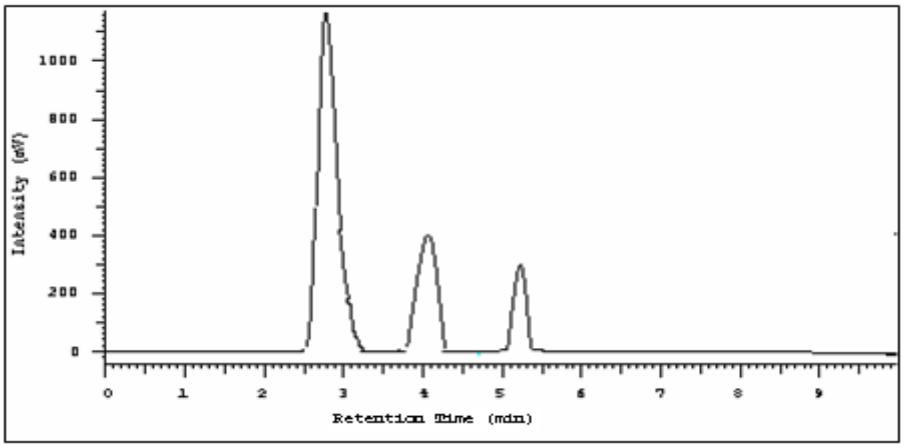
Chromatogram.6 Linearity of losartan potassium, amlodipine besylate and hydrochlorothiazide (24, 3, 9 µg mL^{-1})



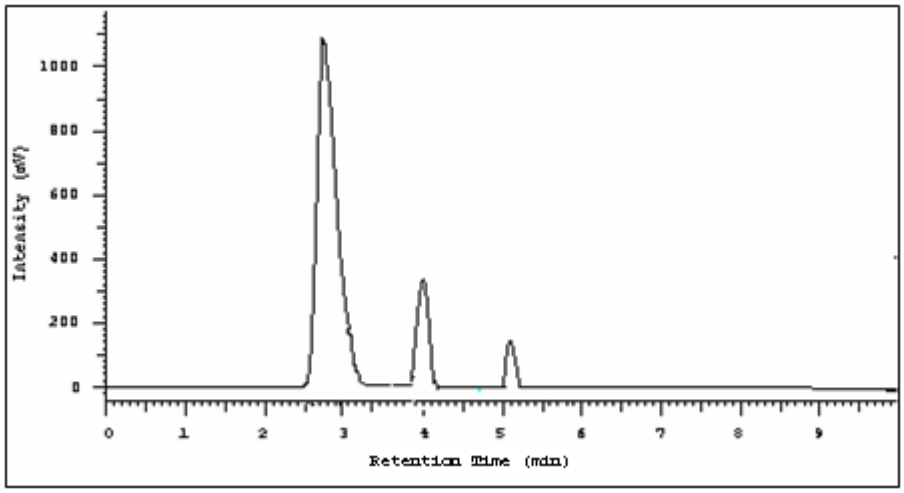
Chromatogram.7 Linearity of losartan potassium, amlodipine besylate and hydrochlorothiazide (32, 4, 12 µg mL^{-1})



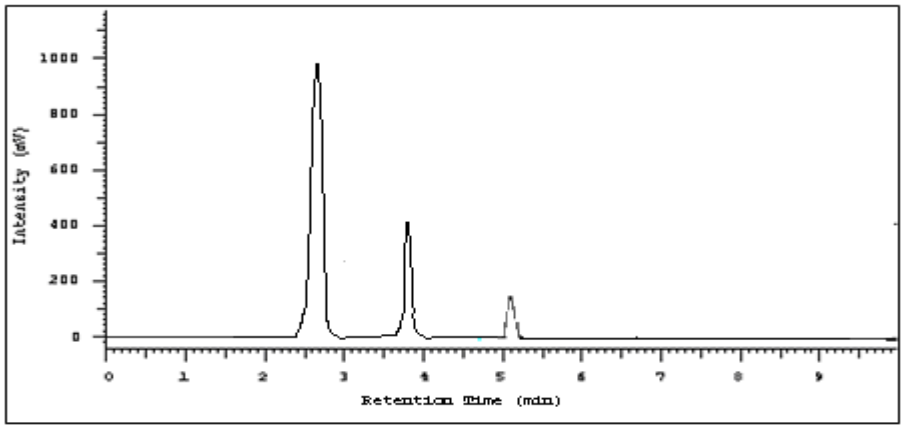
Chromatogram.8 Linearity of losartan potassium, amlodipine besylate and hydrochlorothiazide (40, 5, 15 µg mL^{-1})



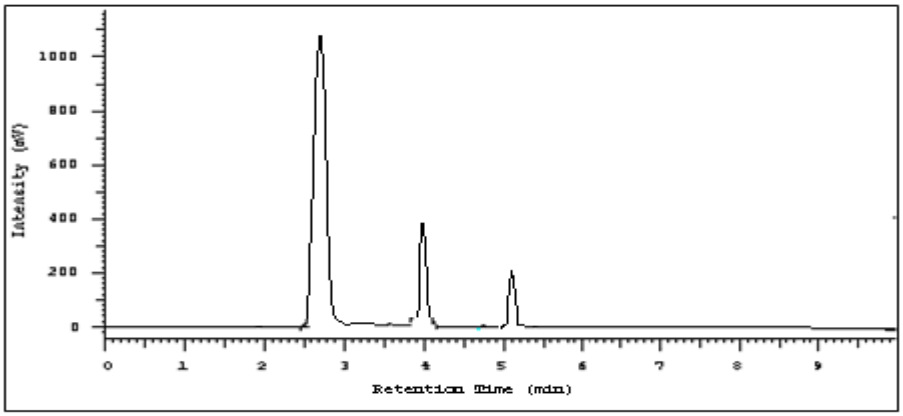
Chromatogram.9 Assay of formulation of losartan potassium, amlodipine besylate and hydrochlorothiazide (30, 3, 7.5 µg mL^{-1})



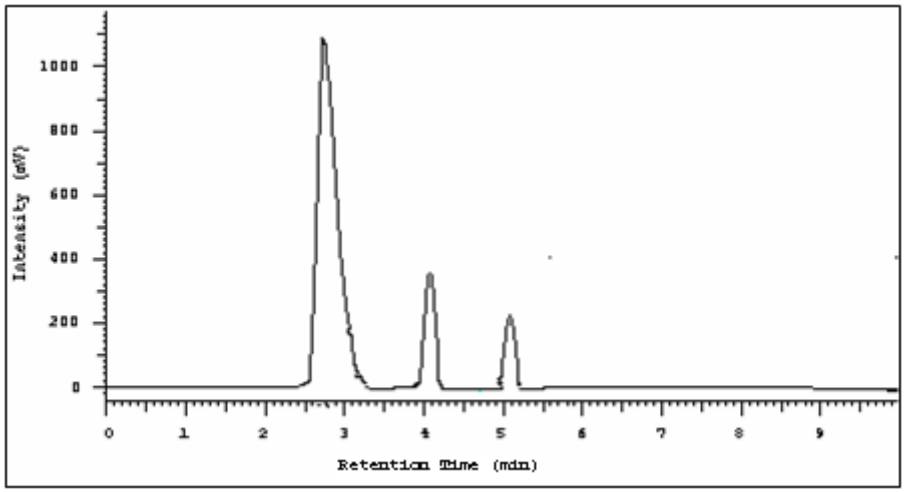
Chromatogram.10 Recovery studies of losartan potassium, amlodipine besylate and hydrochlorothiazide (27, 2.7, 6.75 µg mL^{-1})



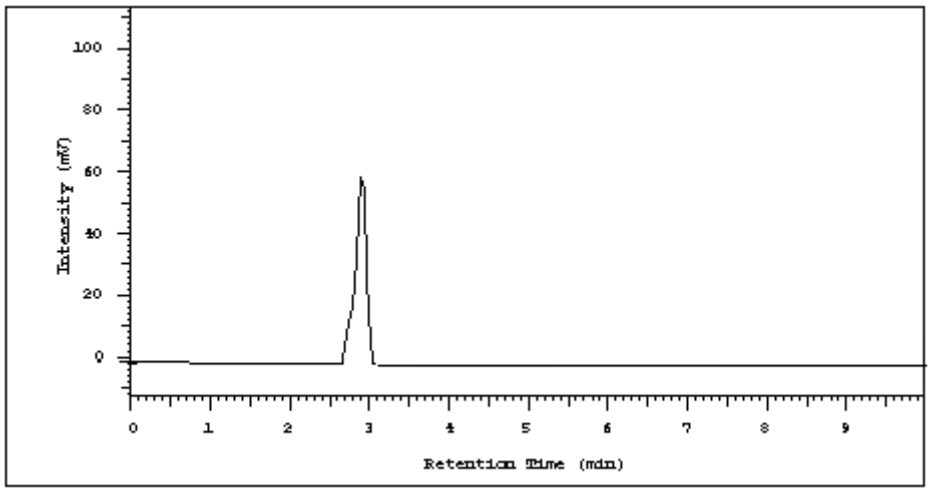
Chromatogram.11 Recovery studies of losartan potassium, amlodipine besylate and hydrochlorothiazide (30, 3, 7.5 µg mL^{-1})



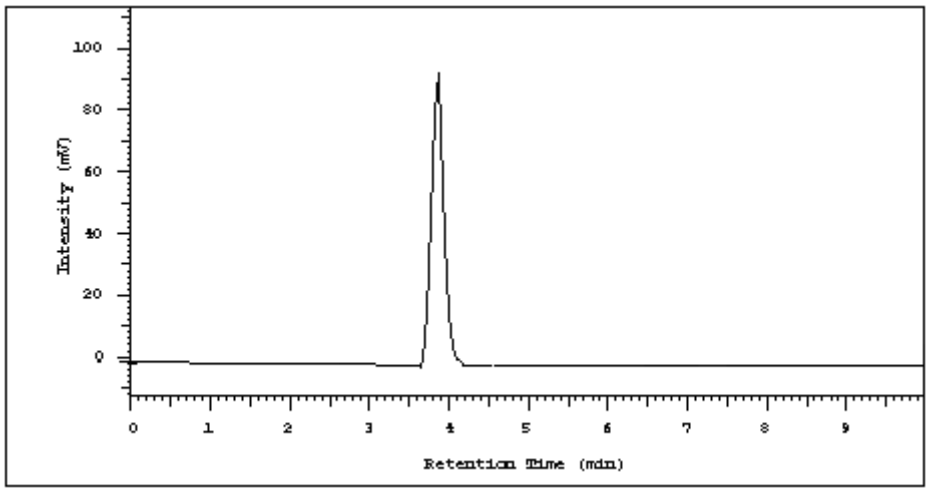
Chromatogram.12 Recovery studies of losartan potassium, amlodipine besylate and hydrochlorothiazide (33, 3.3, 8.25 µg mL^{-1})



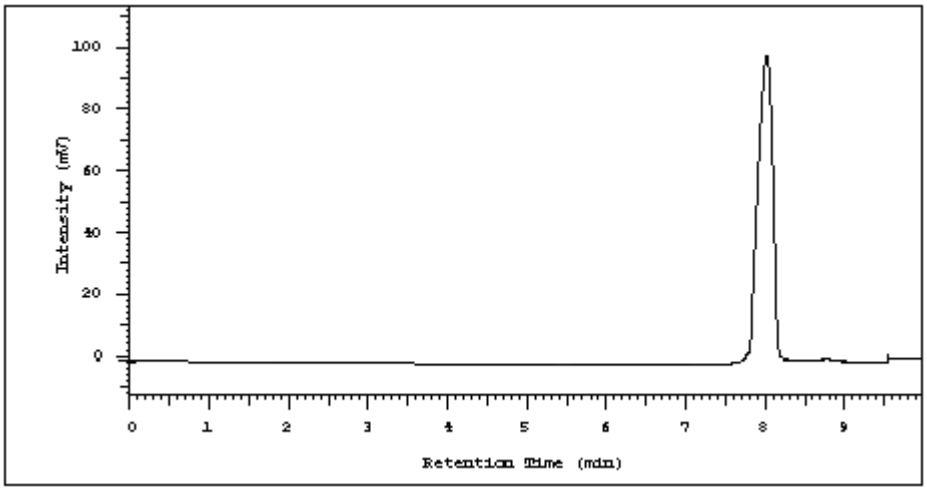
Chromatogram.13 Individual Chromatogram of Folic acid



Chromatogram.14 Individual Chromatogram of Atorvastatin calcium

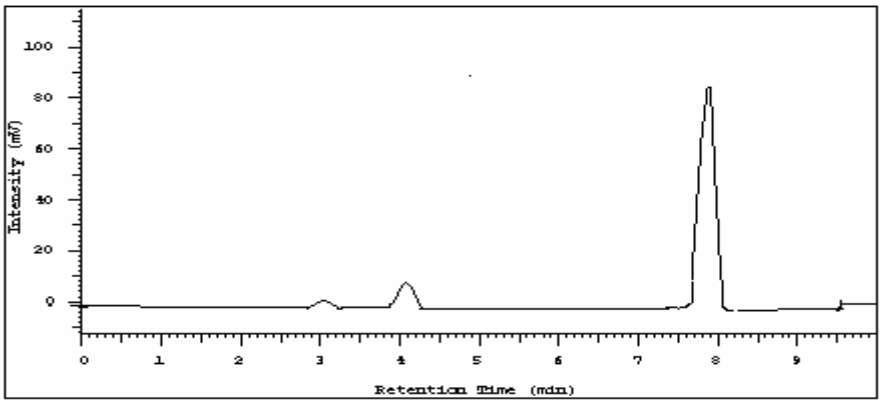


Chromatogram.15 Individual Chromatogram of Fenofibrate



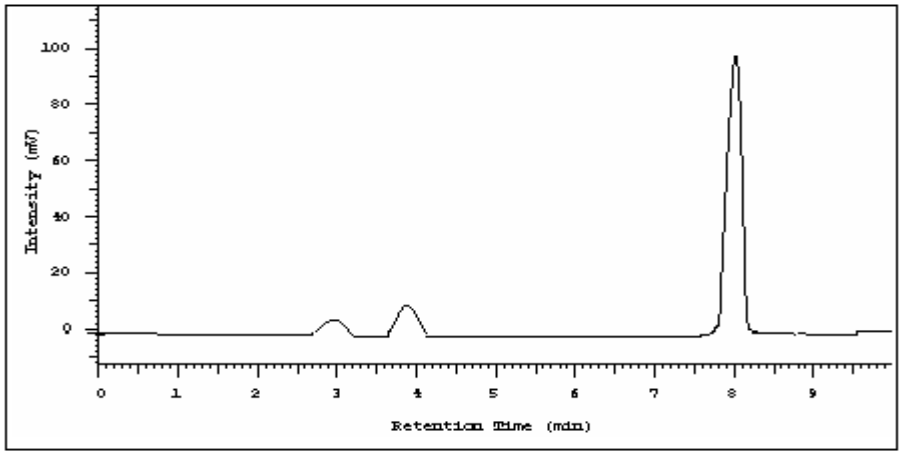
Chromatogram.16 Linearity of Atorvastatin calcium, Fenofibrate and Folic acid

(1, 15, 0.5 µg mL^{-1})



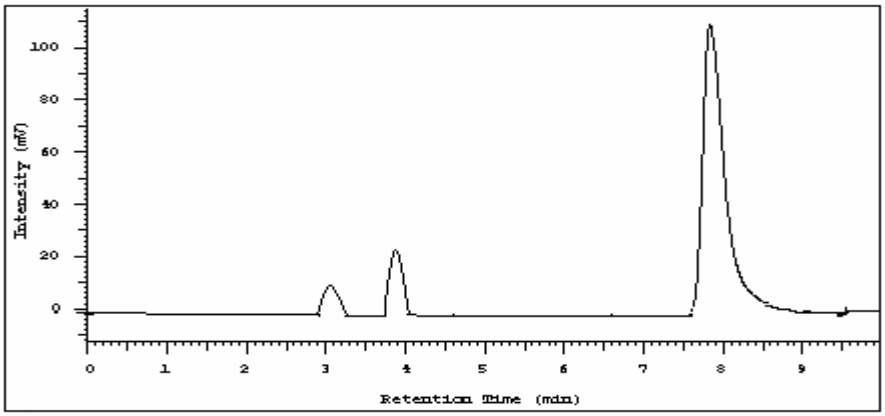
Chromatogram.17 Linearity of Atorvastatin calcium, Fenofibrate and Folic acid

(2, 30, 1 µg mL^{-1})

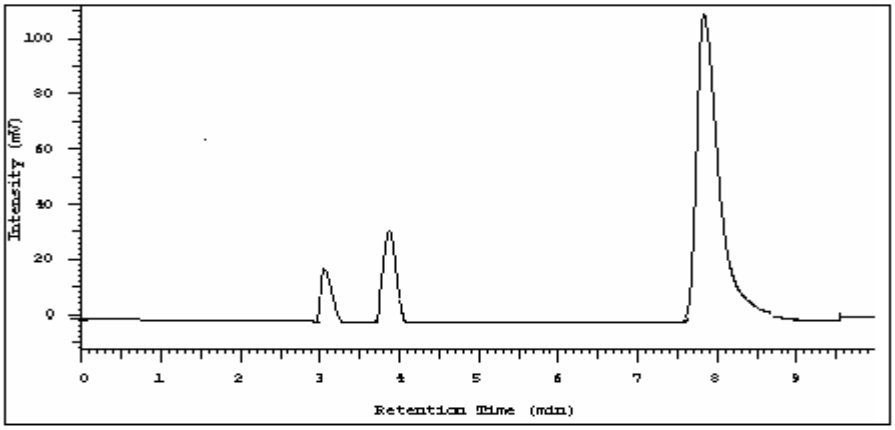


Chromatogram.18 Linearity of Atorvastatin calcium, Fenofibrate and Folic acid

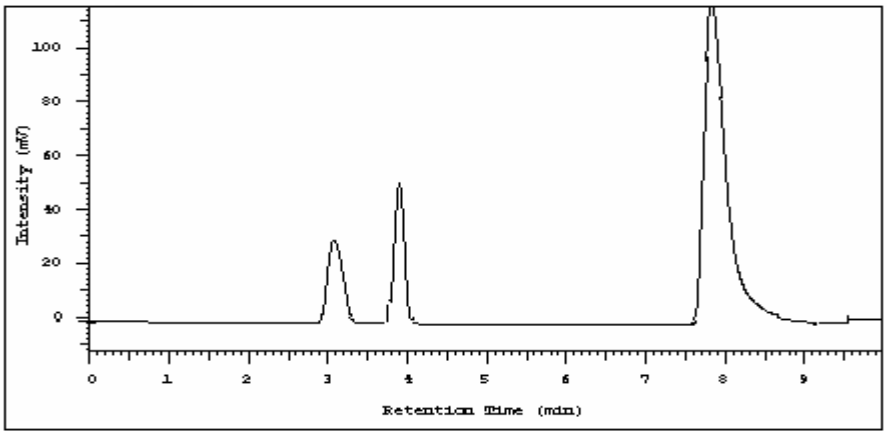
(3, 45, 1.5 µg mL^{-1})



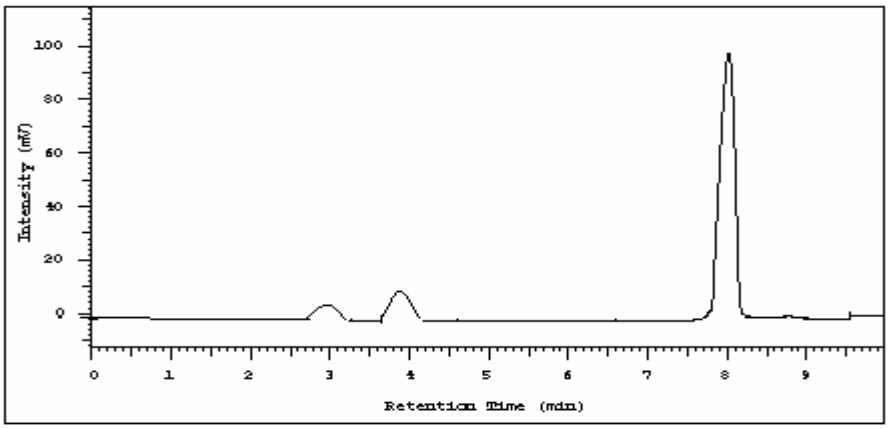
Chromatogram.19 Linearity of Atorvastatin calcium, Fenofibrate and Folic acid (4, 60, 2 µg mL^{-1})



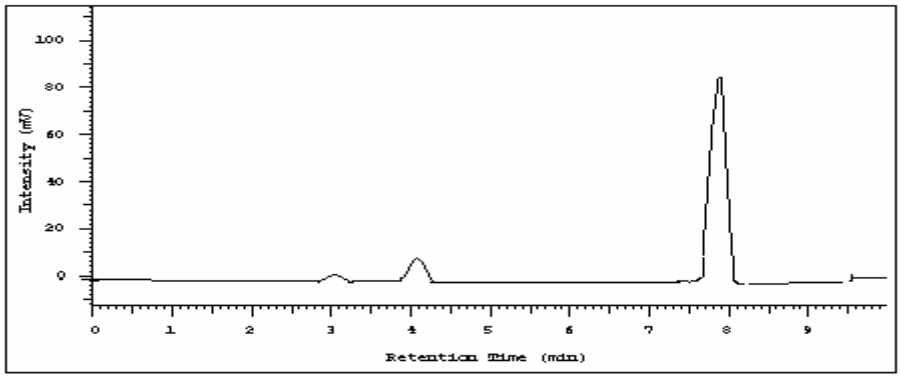
Chromatogram.20 Linearity of Atorvastatin calcium, Fenofibrate and Folic acid (5, 75, 2.5 µg mL^{-1})



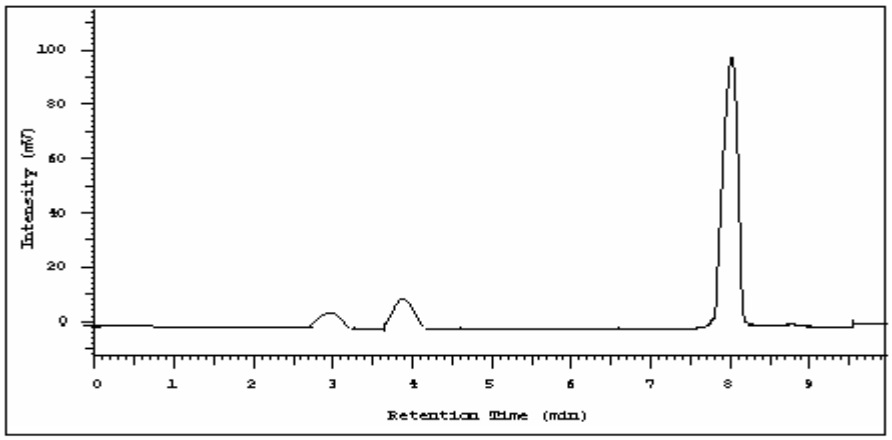
Chromatogram.21 Assay of formulation of Atorvastatin calcium, Fenofibrate and Folic acid (1.875, 30, 0.9375 µg mL^{-1})



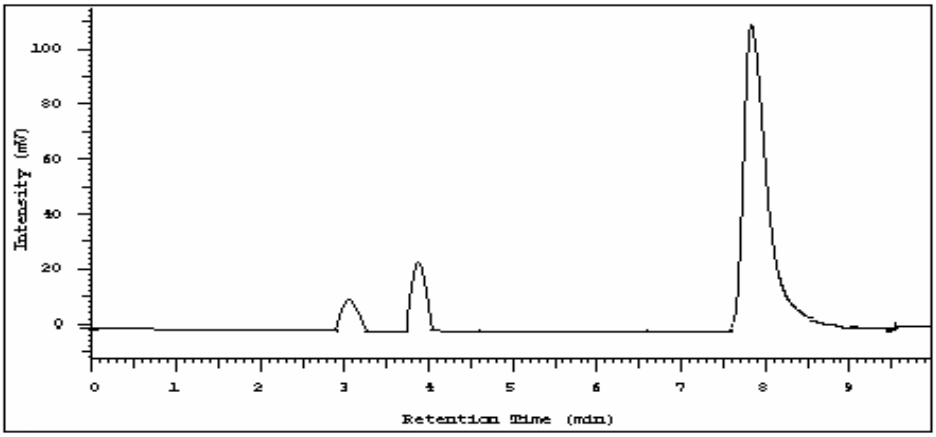
Chromatogram.22 Recovery studies of Atorvastatin calcium, Fenofibrate and Folic acid (2.6, 39, 1.3 µg mL^{-1})



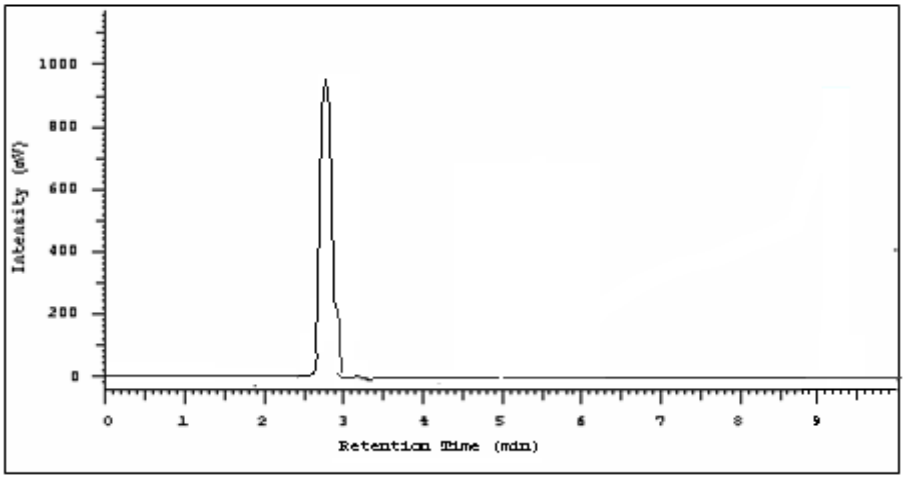
Chromatogram.23 Recovery studies of Atorvastatin calcium, Fenofibrate and Folic acid (3, 45, 1.5 µg mL^{-1})



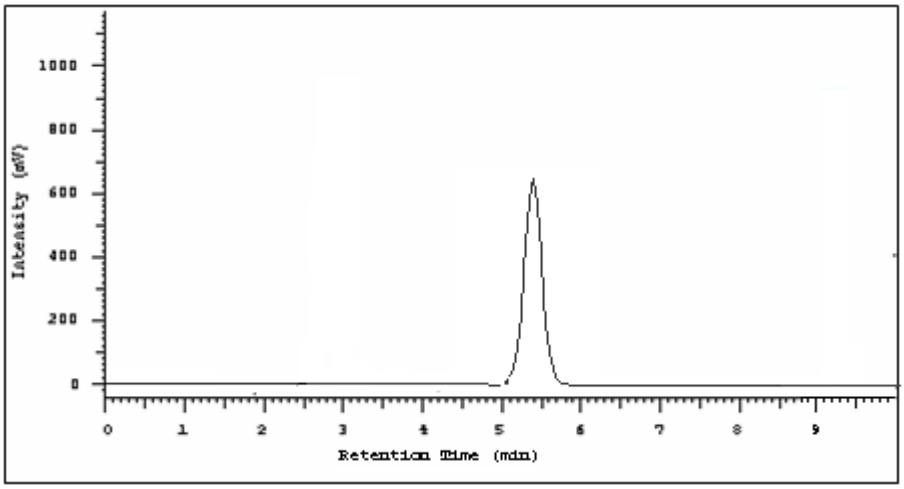
Chromatogram.24 Recovery studies of Atorvastatin calcium, Fenofibrate and Folic acid (3.4, 51, 1.7 µg mL^{-1})



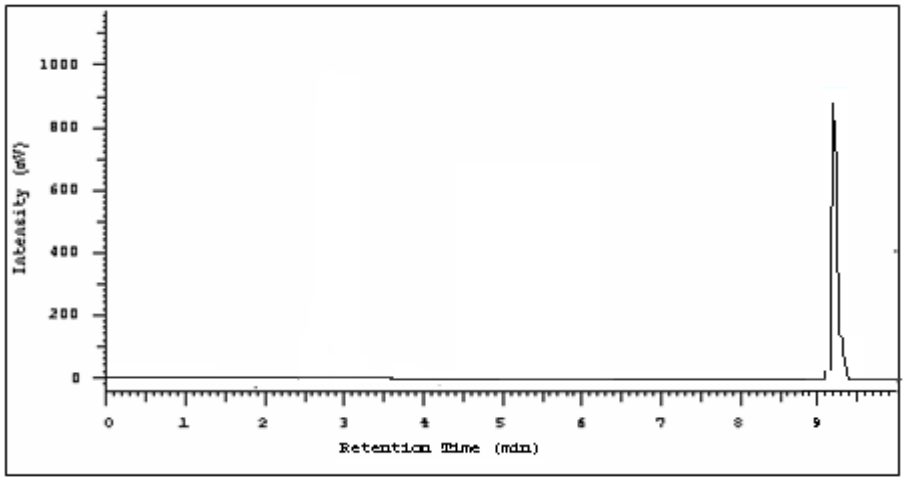
Chromatogram.25 Individual Chromatogram of metformin HCl



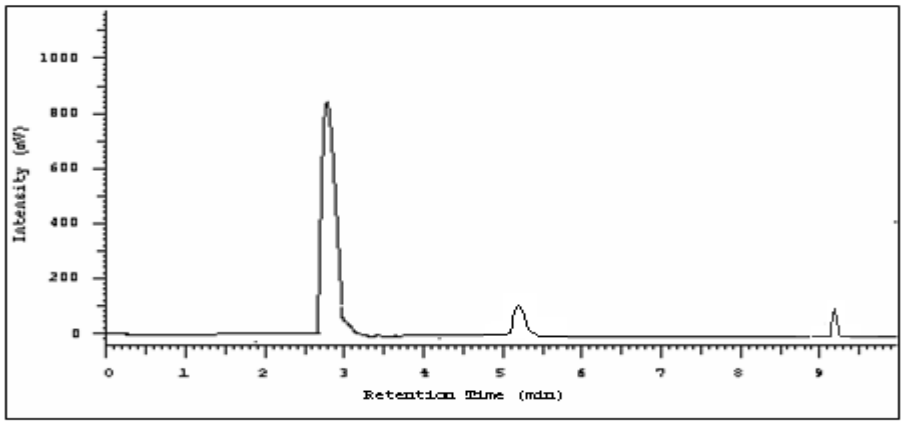
Chromatogram.26 Individual Chromatogram of glibenclamide



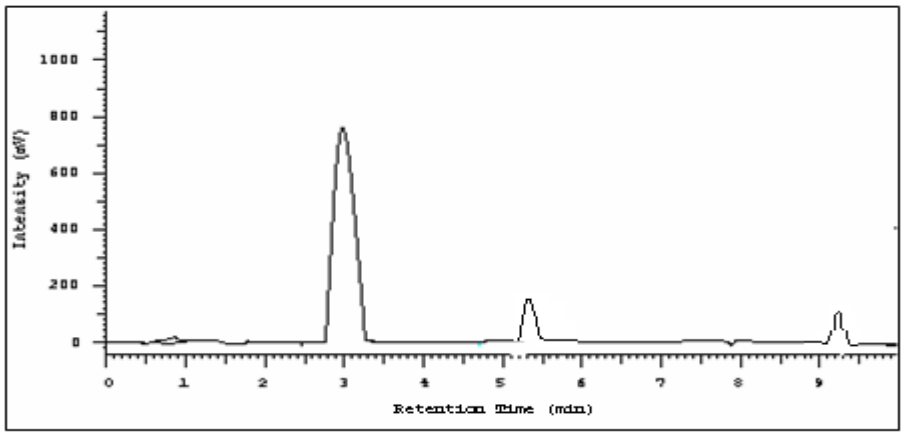
Chromatogram.27 Individual Chromatogram of rosiglitazone maleate



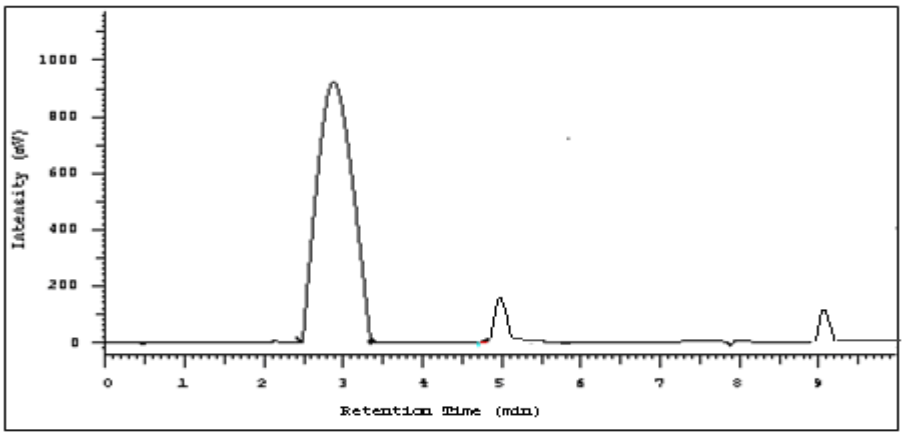
Chromatogram.28 Linearity of rosiglitazonemaleate, glibenclamide and metformin HCl (0.05, 0.1,7 µg mL^{-1})



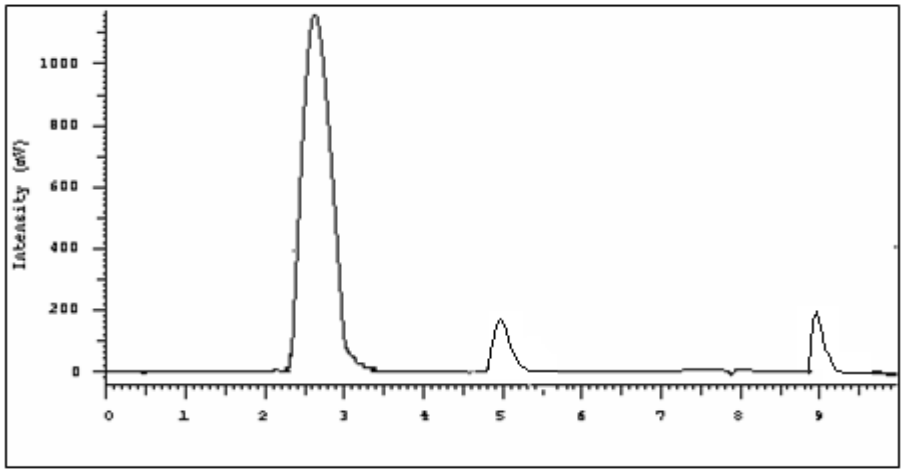
Chromatogram.29 Linearity of rosiglitazonemaleate, glibenclamide and metformin HCl (0.1, 0.2, 14 µg mL^{-1})



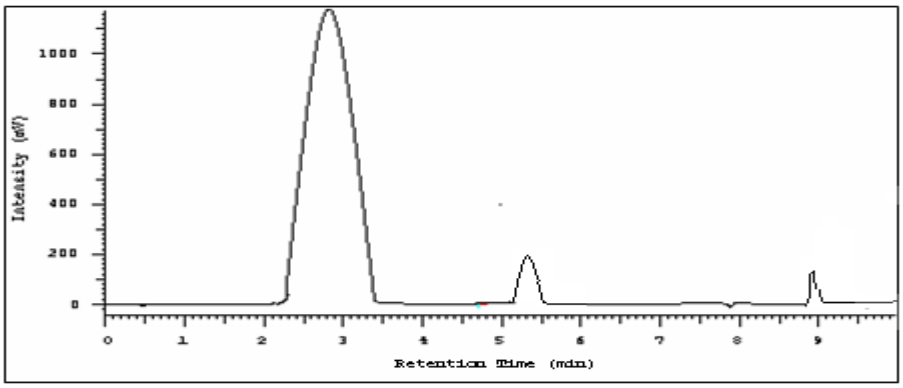
Chromatogram.30 Linearity of rosiglitazonemaleate, glibenclamide and metformin HCl (0.15, 0.3, 21 µg mL^{-1})



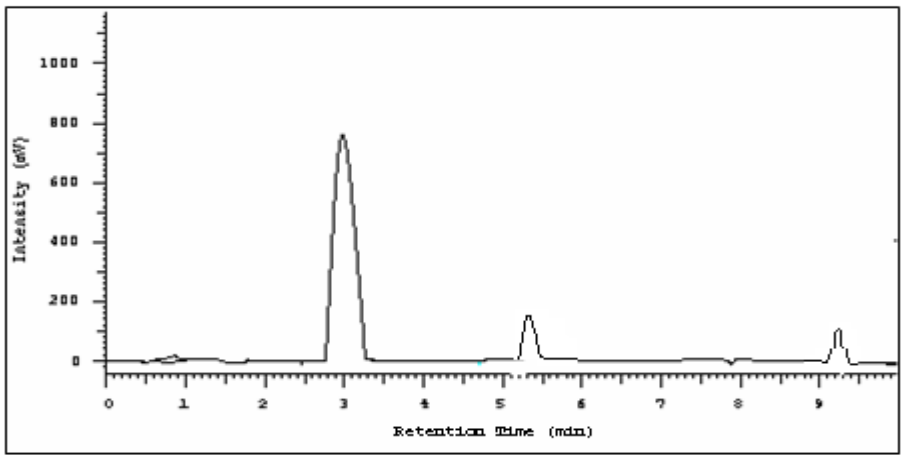
Chromatogram.31 Linearity of rosiglitazonemaleate, glibenclamide and metformin HCl (0.2, 0.4, 28 µg mL^{-1})



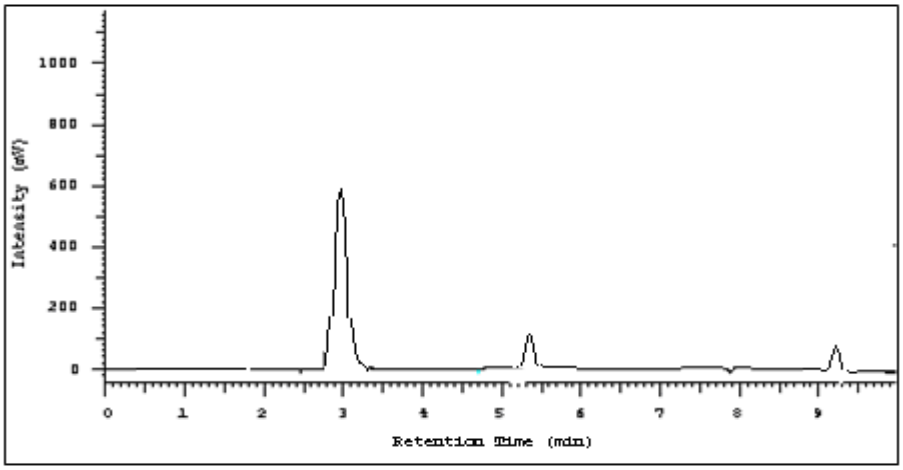
Chromatogram.32 Linearity of rosiglitazonemaleate, glibenclamide and metformin HCl (0.25, 0.5, 35 µg mL^{-1})



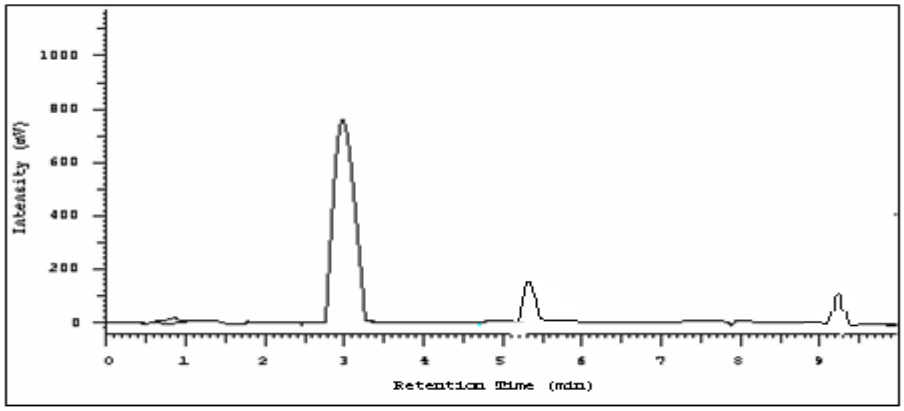
Chromatogram.33 Assay of formulation of rosiglitazone maleate, glibenclamide and metformin HCl (0.1, 0.25, 25 µg mL^{-1})



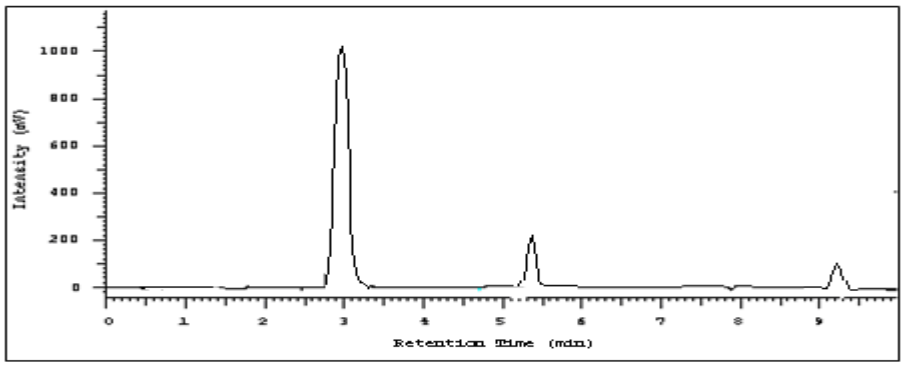
Chromatogram.34 Recovery studies of rosiglitazonemaleate, glibenclamide and metformin HCl (0.09, 0.225, 22.5 µg mL^{-1})



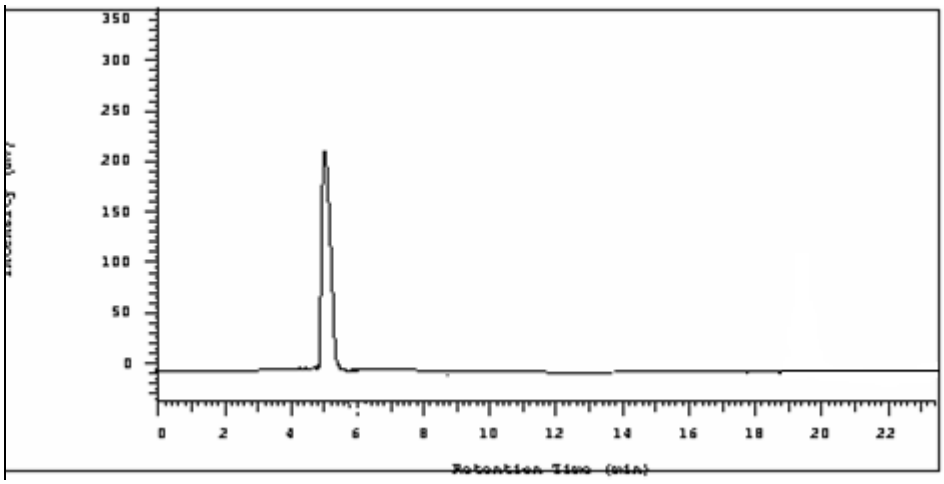
Chromatogram.35 Recovery studies of rosiglitazonemaleate, glibenclamide and metformin HCl (0.1, 0.25, 25 µg mL^{-1})



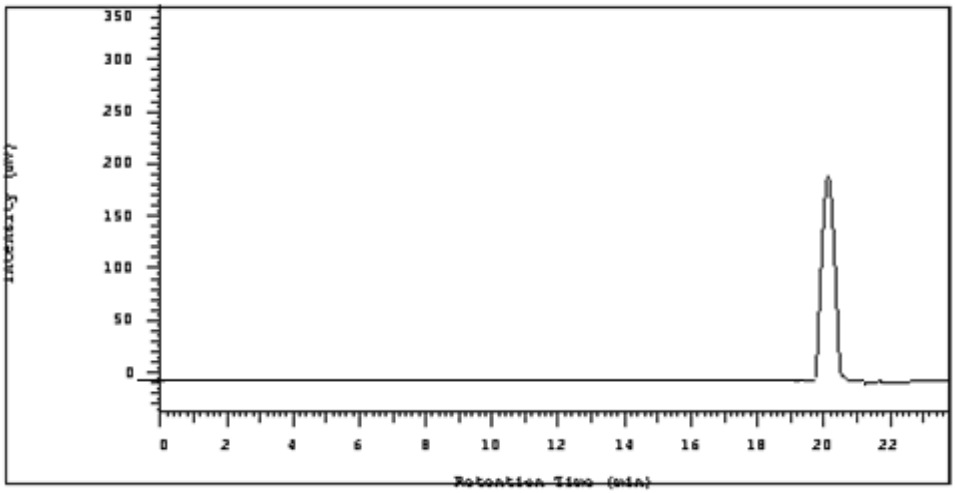
Chromatogram.36 Recovery studies of rosiglitazonemaleate, glibenclamide and metformin HCl (0.11, 0.275, 27.5 µg mL^{-1})



Chromatogram.37 Individual Chromatogram of Hydrochlorothiazide

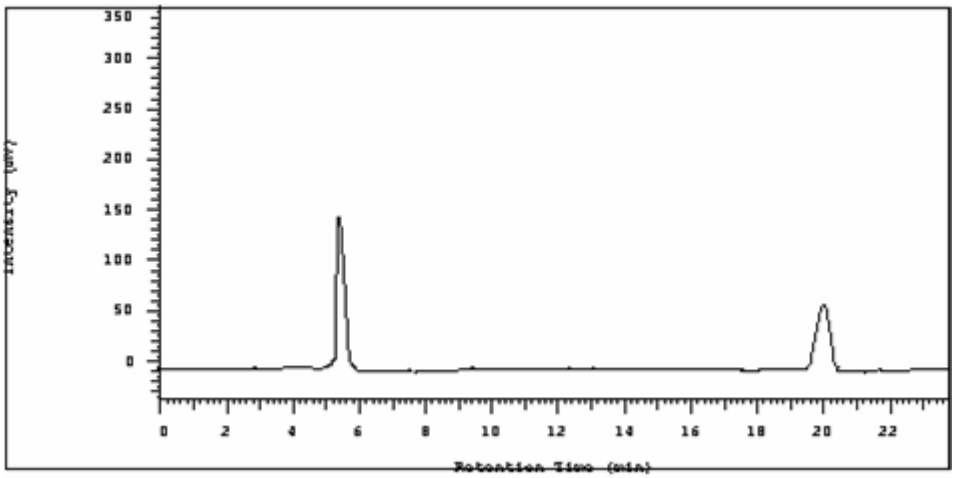


Chromatogram.38 Individual Chromatogram of Nebivolol HCl



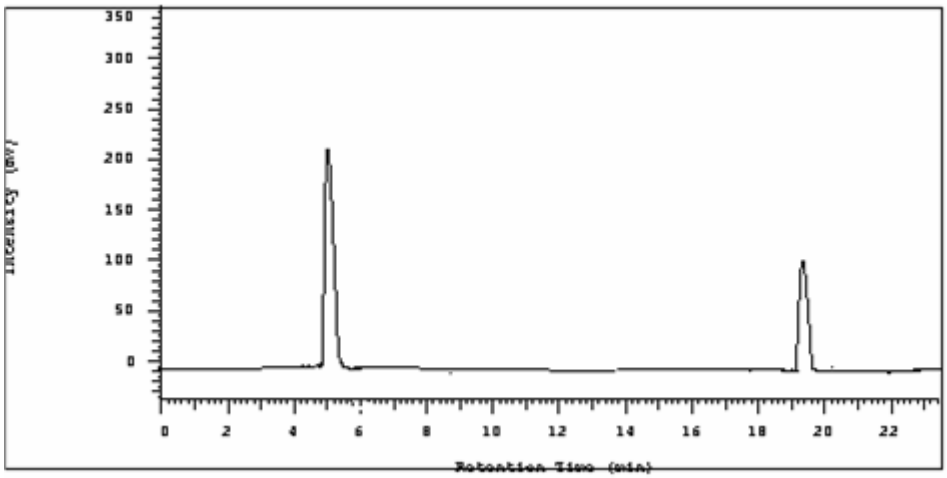
Chromatogram.39 Linearity of Nebivolol HCl and Hydrochlorothiazide

(1, 3 µg mL^{-1})



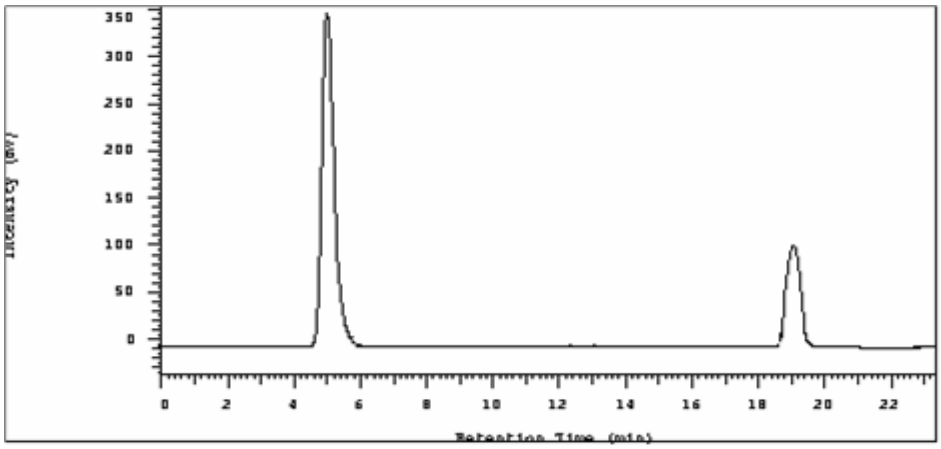
Chromatogram.40 Linearity of Nebivolol HCl and Hydrochlorothiazide

(2, 6 µg mL^{-1})



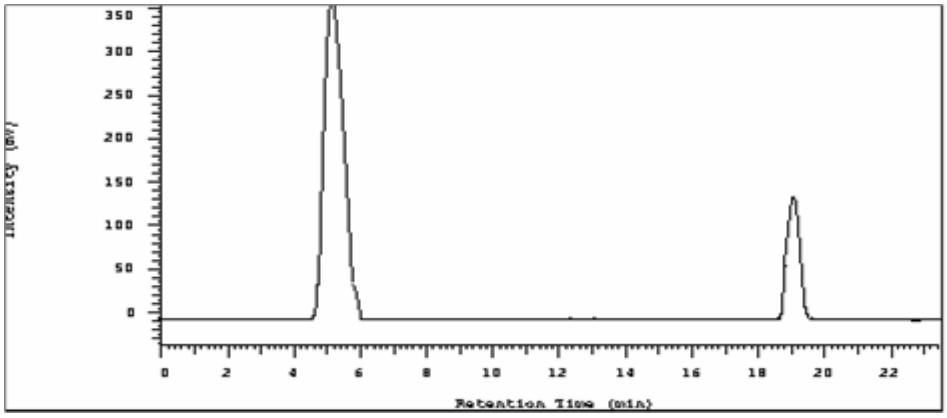
Chromatogram.41 Linearity of Nebivolol HCl and Hydrochlorothiazide

(3, 9 µg mL^{-1})



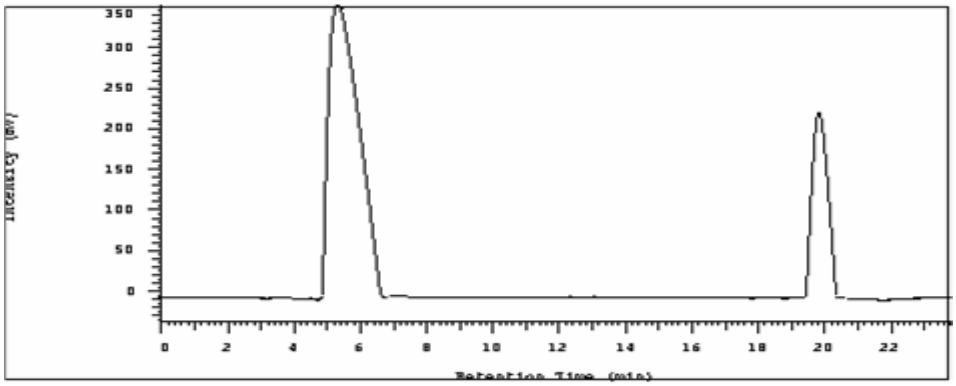
Chromatogram.42Linearity of Nebivolol HCl and Hydrochlorothiazide

(4, 12 µg mL^{-1})



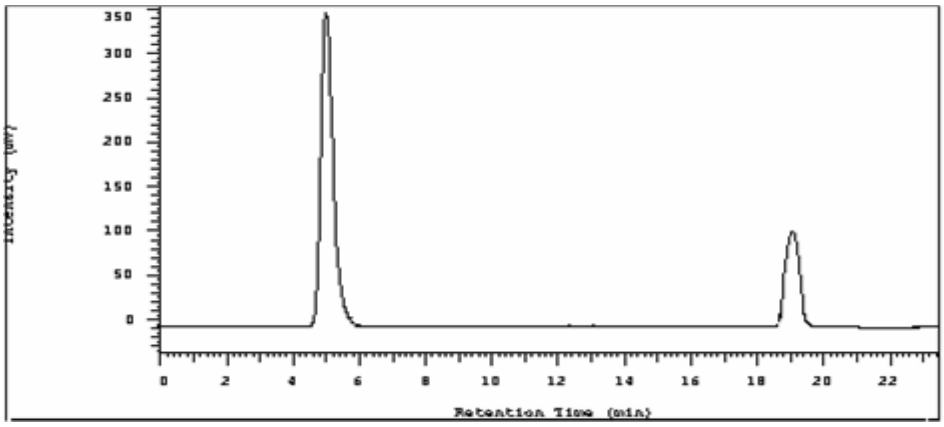
Chromatogram.43 Linearity of Nebivolol HCl and Hydrochlorothiazide

(5, 15 µg mL^{-1})



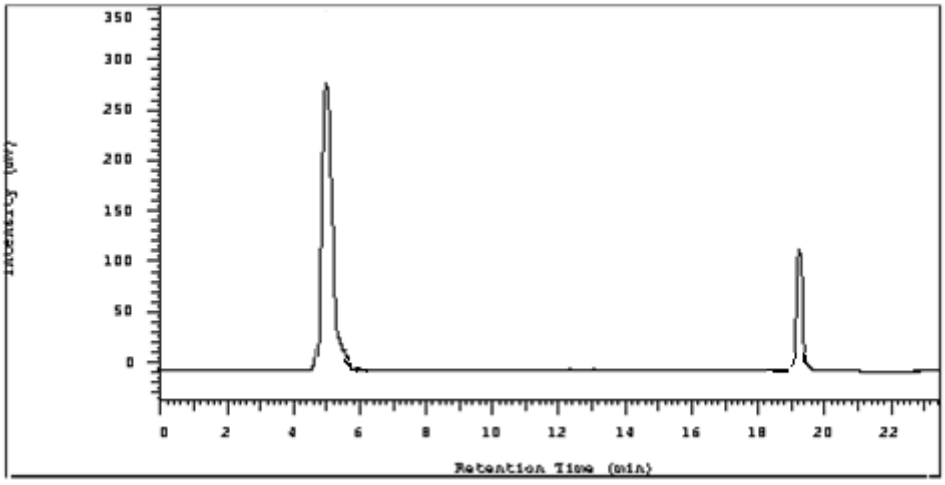
Chromatogram.44 Assay of formulation of Nebivolol HCl and Hydrochlorothiazide

(3, 7.5 µg mL^{-1})



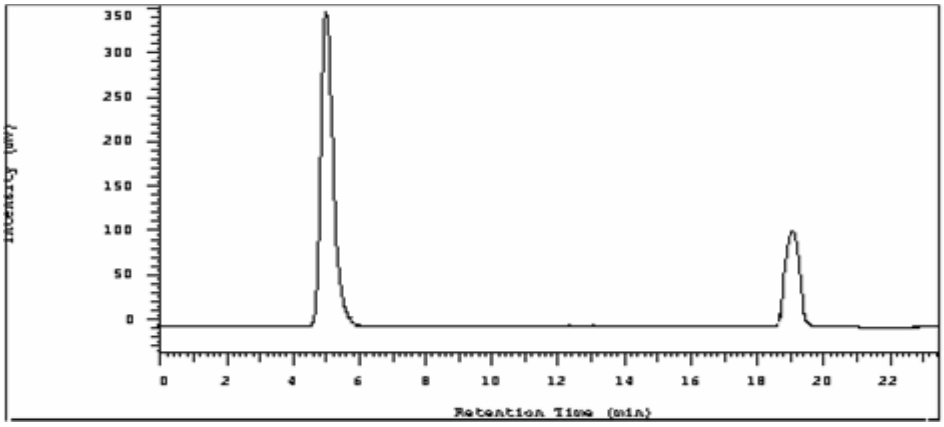
Chromatogram.45 Recovery studies of Nebivolol HCl and Hydrochlorothiazide

(2.7, 6.75 µg mL^{-1})



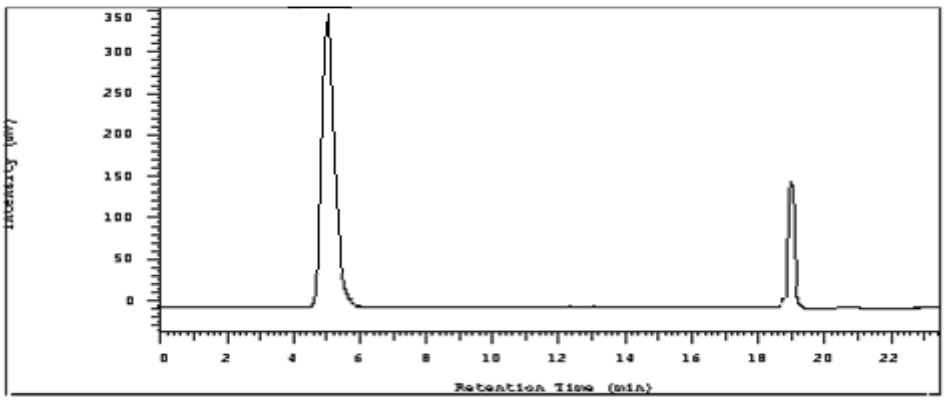
Chromatogram.46 Recovery studies of Nebivolol HCl and Hydrochlorothiazide

(3, 7.5 µg mL^{-1})

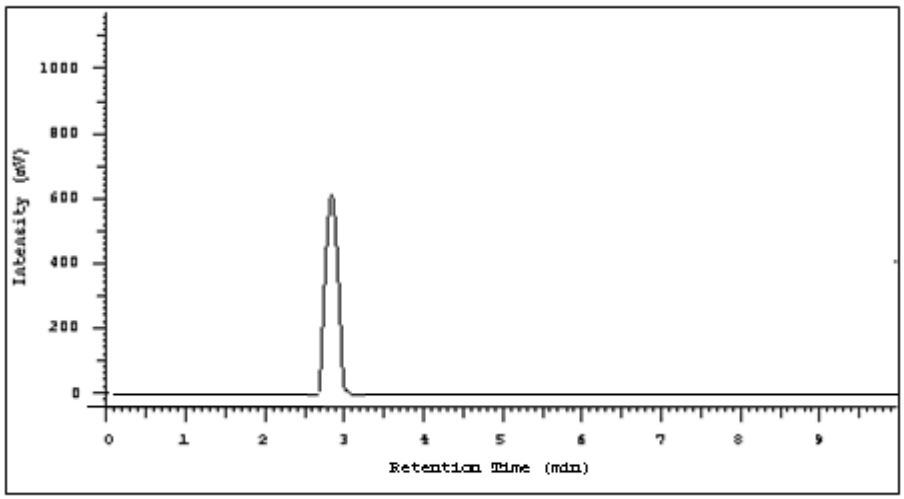


Chromatogram.47 Recovery studies of Nebivolol HCl and Hydrochlorothiazide

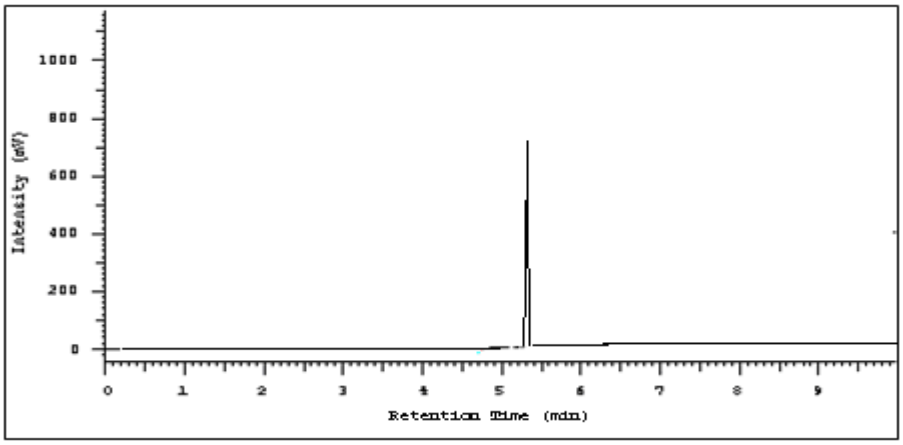
(3.3, 8.25 µg mL^{-1})



Chromatogram.48 Individual Chromatogram of Metformin HCl

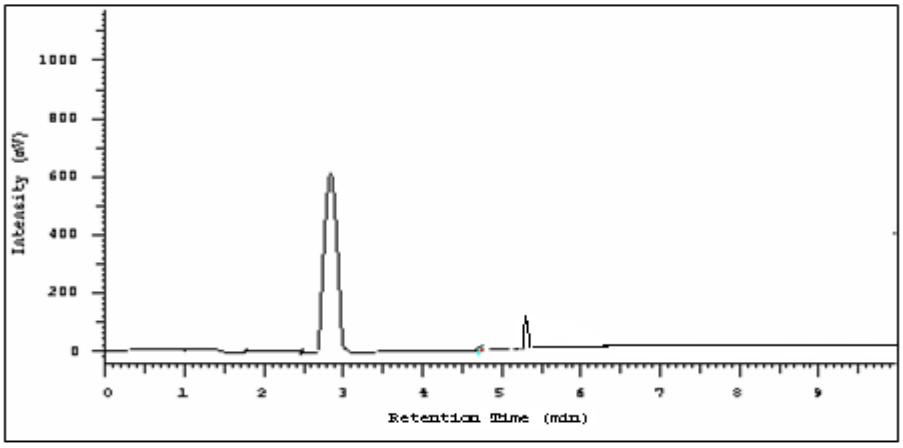


Chromatogram.49 Individual Chromatogram of Pioglitazone HCl



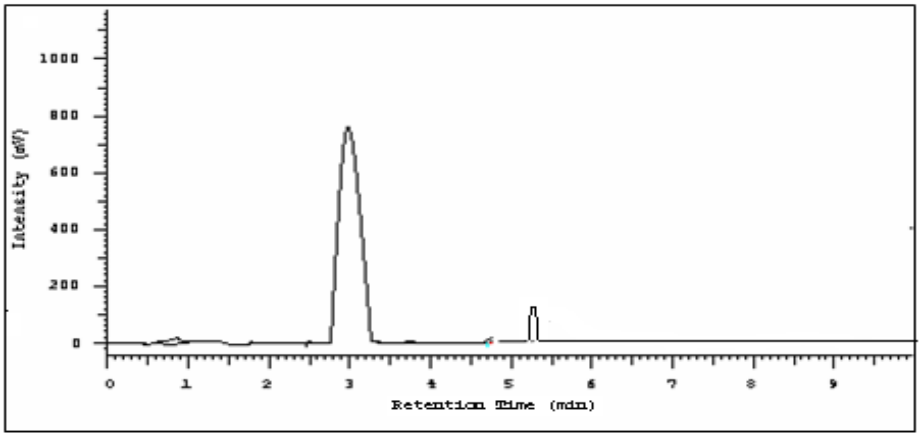
Chromatogram.50 Linearity of Pioglitazone HCl and Metformin HCl

(0.3, 7 µg mL^{-1})



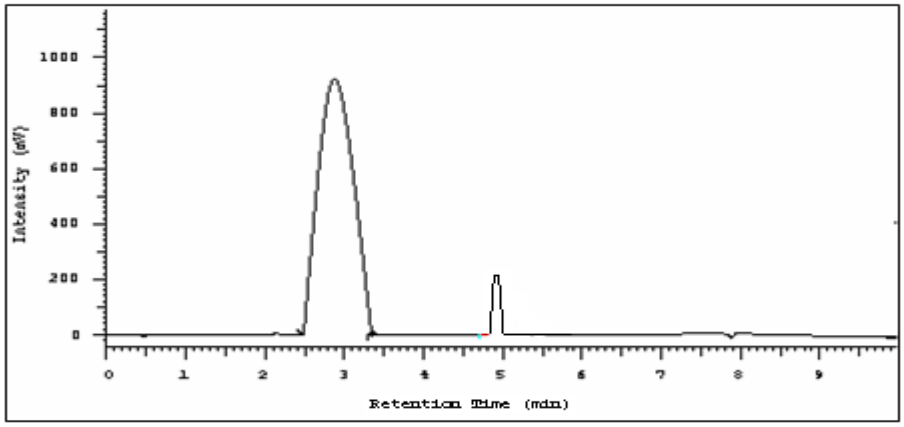
Chromatogram.51 Linearity of Pioglitazone HCl and Metformin HCl

(0.6, 14 µg mL^{-1})



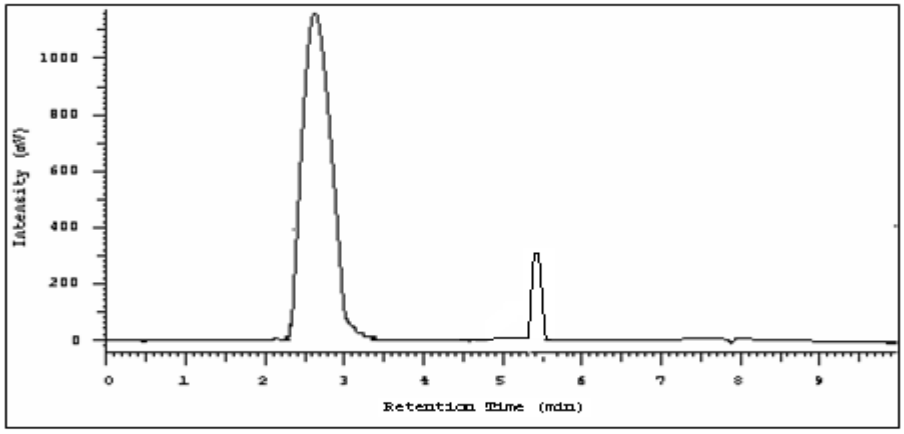
Chromatogram.52 Linearity of Pioglitazone HCl and Metformin HCl

(0.9, 21 µg mL^{-1})



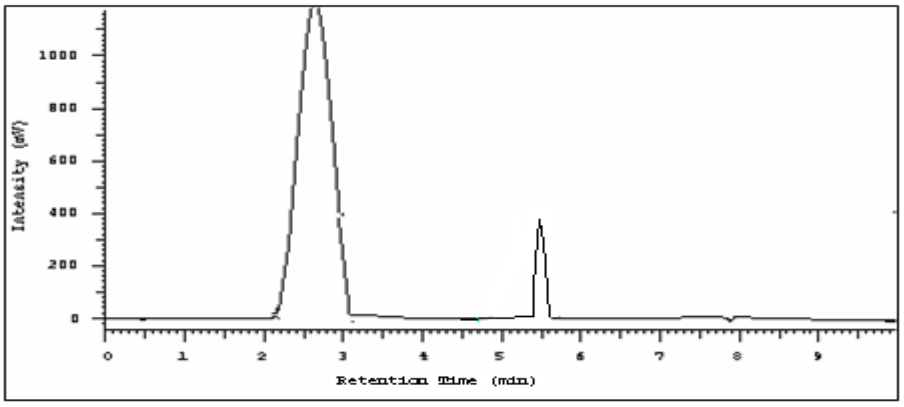
Chromatogram.53 Linearity of Pioglitazone HCl and Metformin HCl

(1.2, 28 µg mL^{-1})



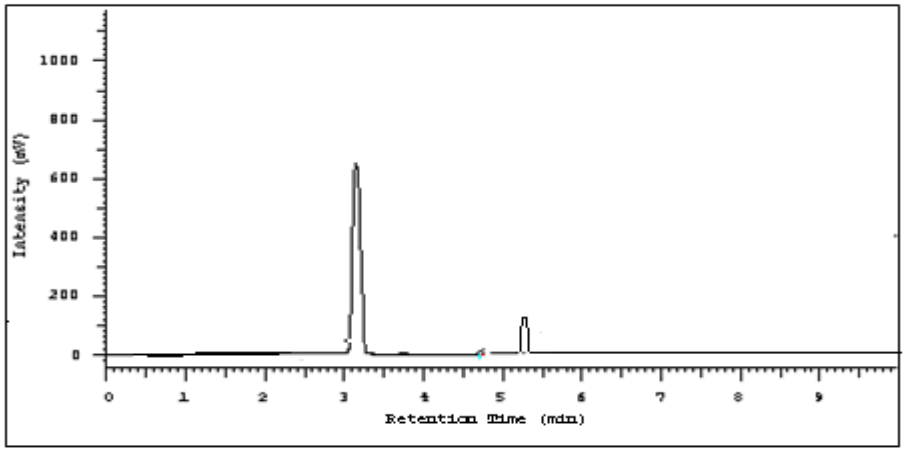
Chromatogram.54 Linearity of Pioglitazone HCl and Metformin HCl

(1.5, 35 µg mL^{-1})



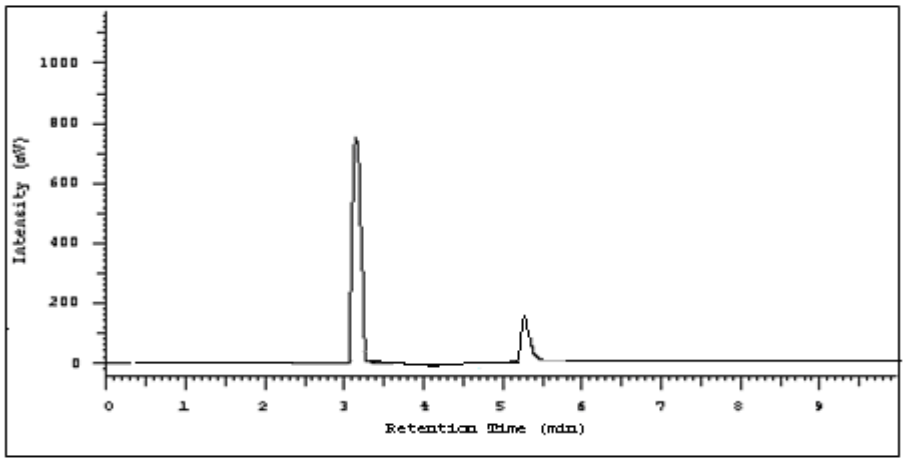
Chromatogram.55 Assay of formulation of Pioglitazone HCl and Metformin HCl

(0.6, 10 µg mL^{-1})



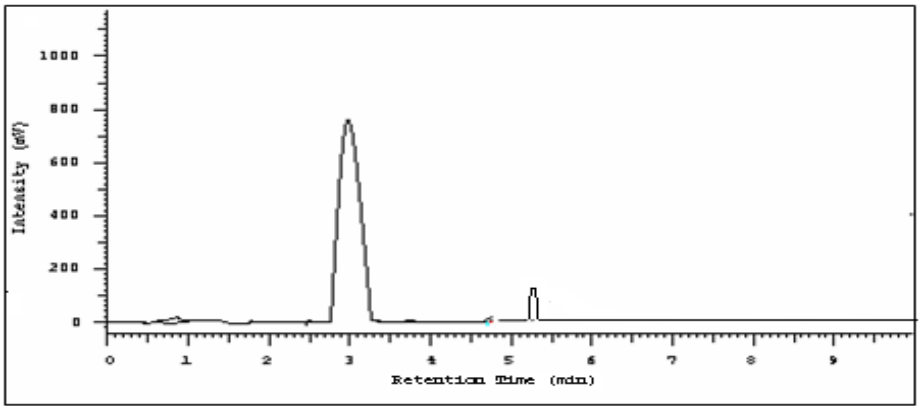
Chromatogram.56 Recovery studies of Pioglitazone HCl and Metformin HCl

(0.78, 13 µg mL^{-1})



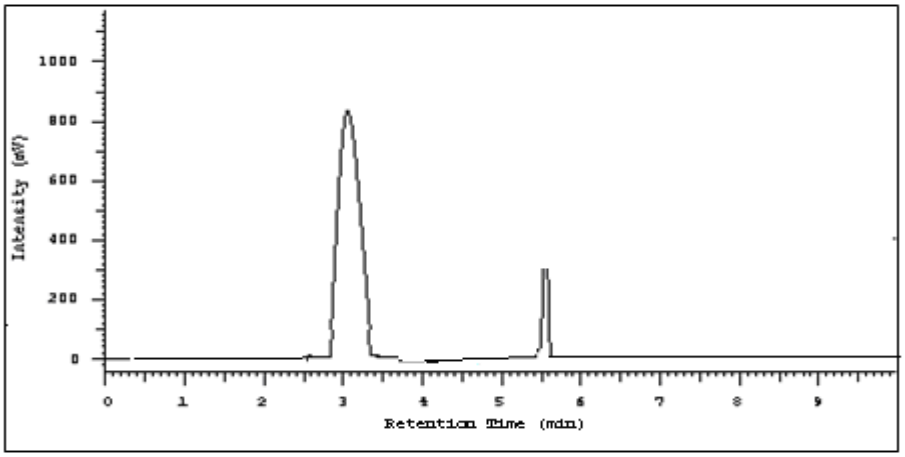
Chromatogram.57 Recovery studies of Pioglitazone HCl and Metformin HCl

(0.9, 15 µg mL^{-1})

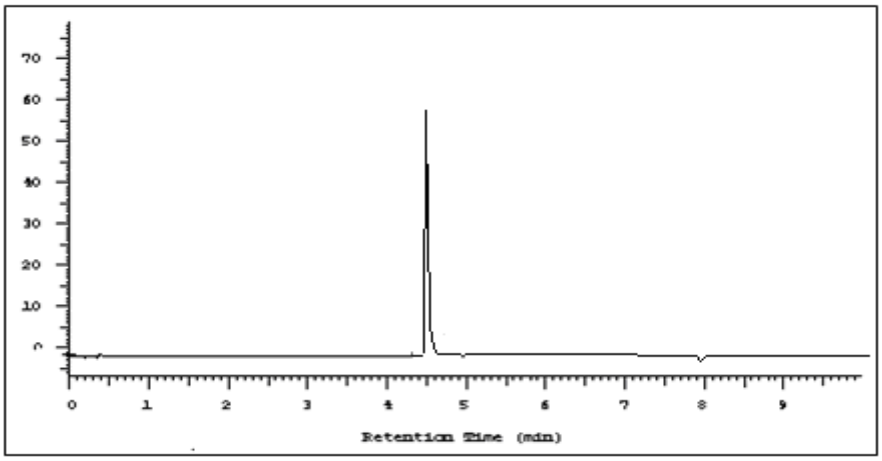


Chromatogram.58 Recovery studies of Pioglitazone HCl and Metformin HCl

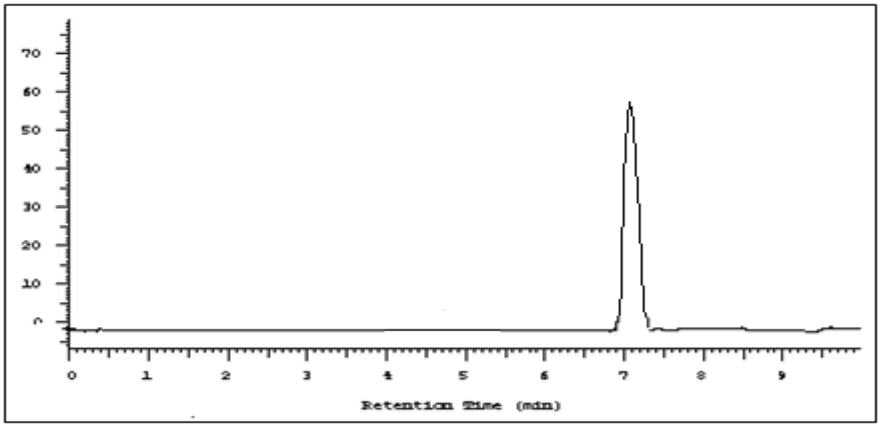
(1.02, 17 µg mL^{-1})



Chromatogram.59 Individual Chromatogram of Ramipril

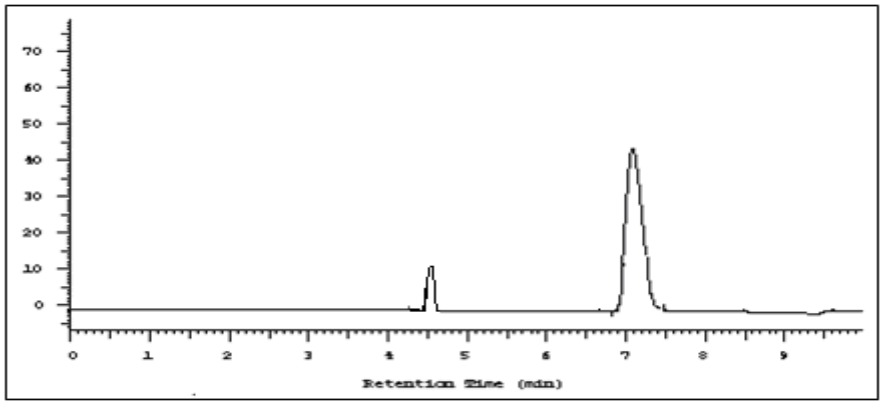


Chromatogram.60 Individual Chromatogram of Losartan potassium



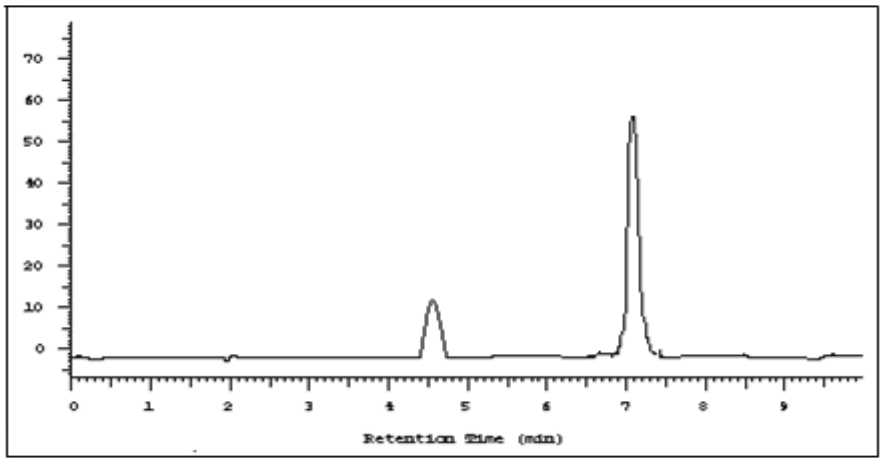
Chromatogram.61 Linearity of Losartan potassium and Ramipril

(8,1.2 µg mL^{-1})



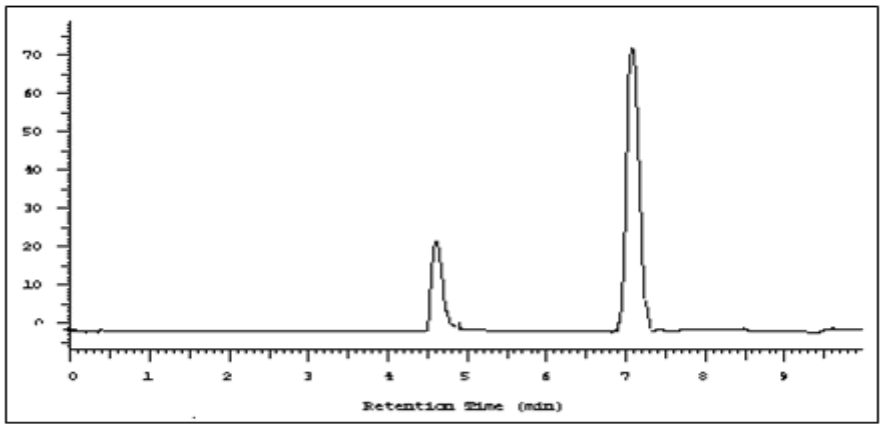
Chromatogram.62 Linearity of Losartan potassium and Ramipril

(16, 2.4 µg mL^{-1})



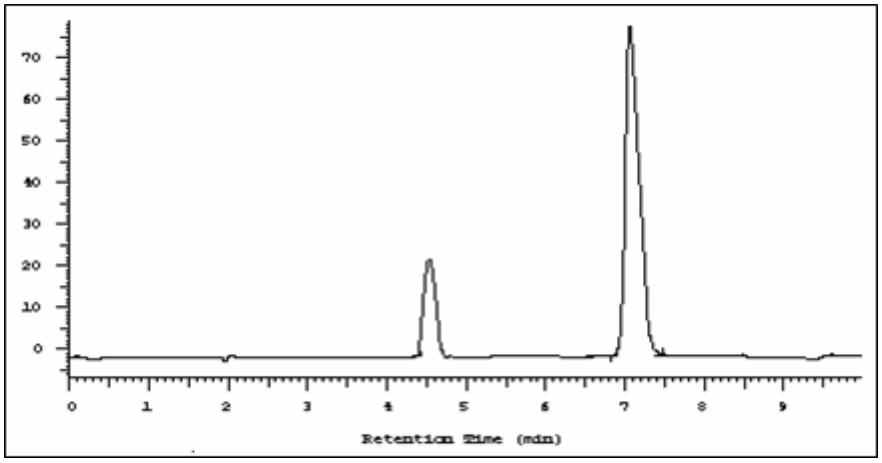
Chromatogram.63 Linearity of Losartan potassium and Ramipril

(24, 3.6 µg mL^{-1})



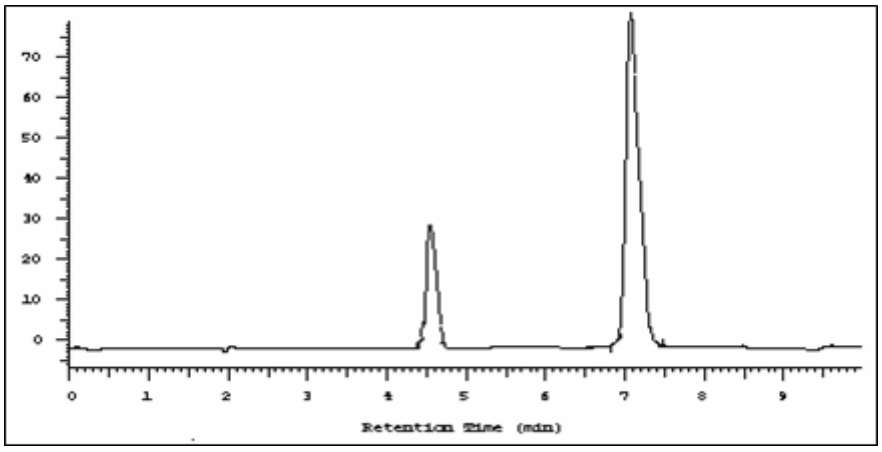
Chromatogram.64 Linearity of Losartan potassium and Ramipril

(32,4.8 µg mL^{-1})



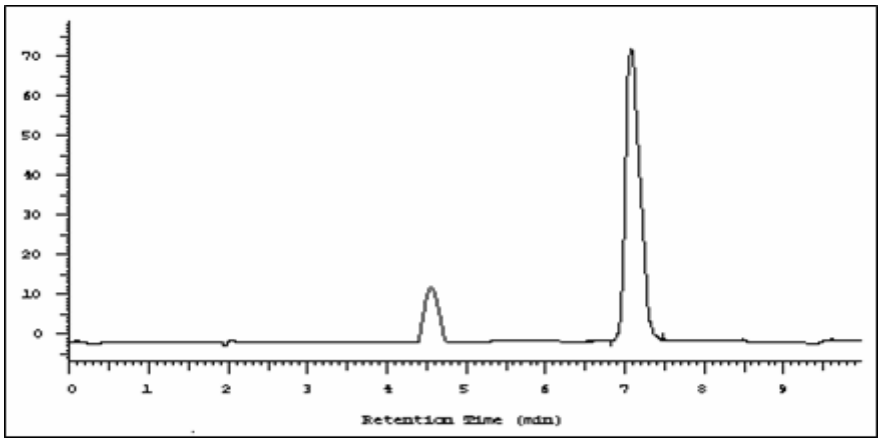
Chromatogram.65 Linearity of Losartan potassium and Ramipril

(40, 6 µg mL^{-1})



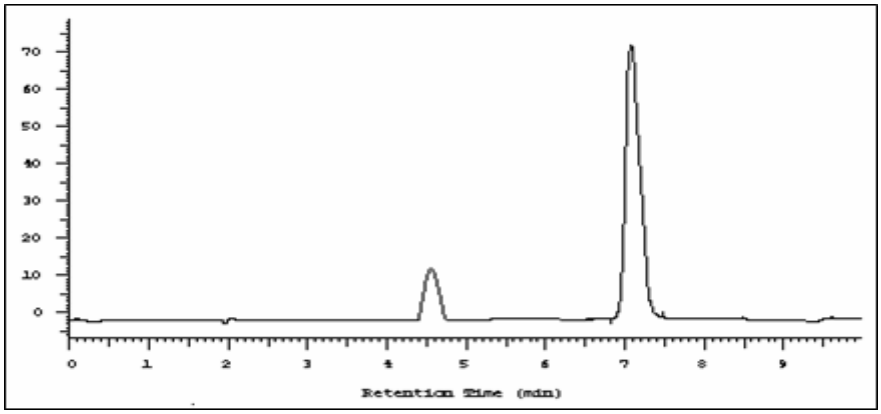
Chromatogram.66 Assay of formulation of Losartan potassium and Ramipril

(30, 1.5 µg mL^{-1})



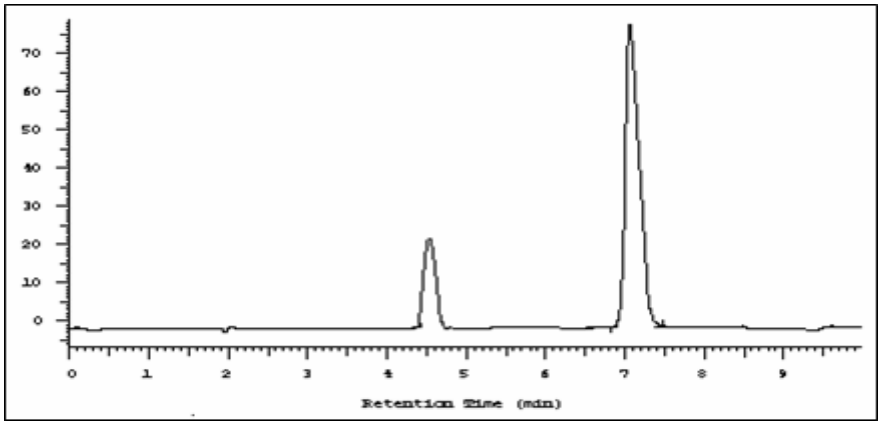
Chromatogram.67 Recovery studies of Losartan potassium and Ramipril

(27, 1.35 µg mL^{-1})



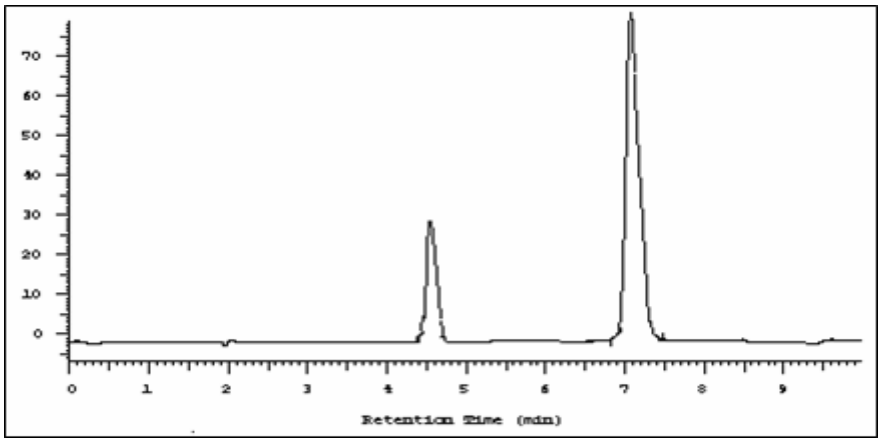
Chromatogram.68 Recovery studies of Losartan potassium and Ramipril

(30, 1.5 µg mL^{-1})

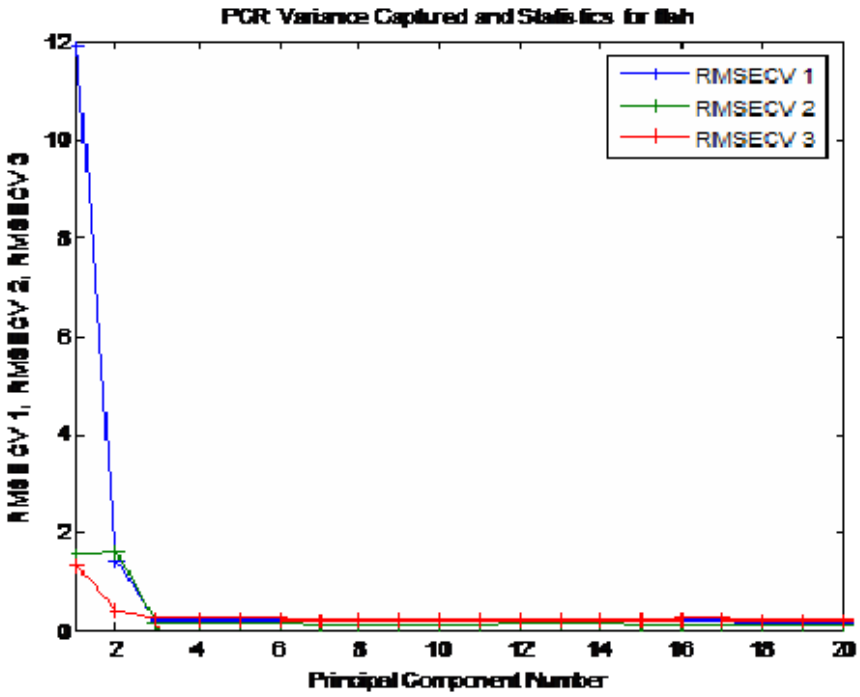


Chromatogram.69 Recovery studies of Losartan potassium and Ramipril

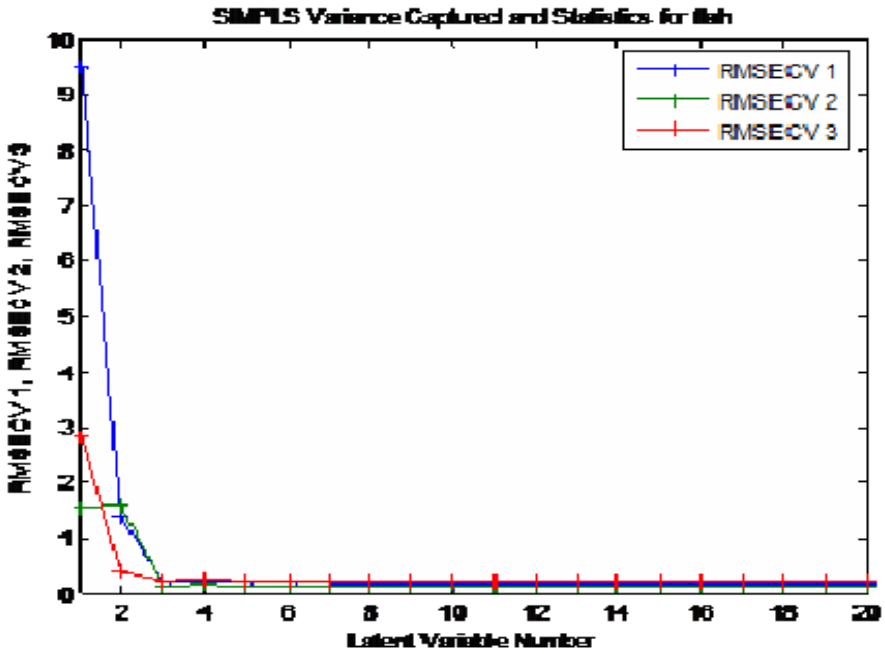
(33, 1.65 µg mL^{-1})



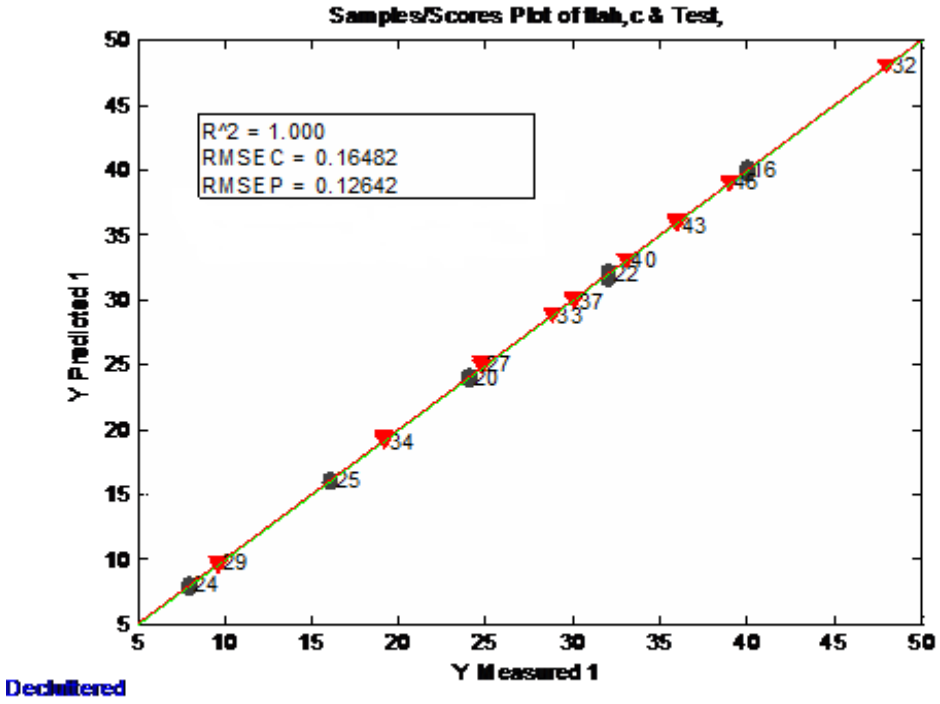
Graph.1 RMSECV values generated from calibration by PCR: losartan potassium, amlodipine besylate and hydrochlorothiazide



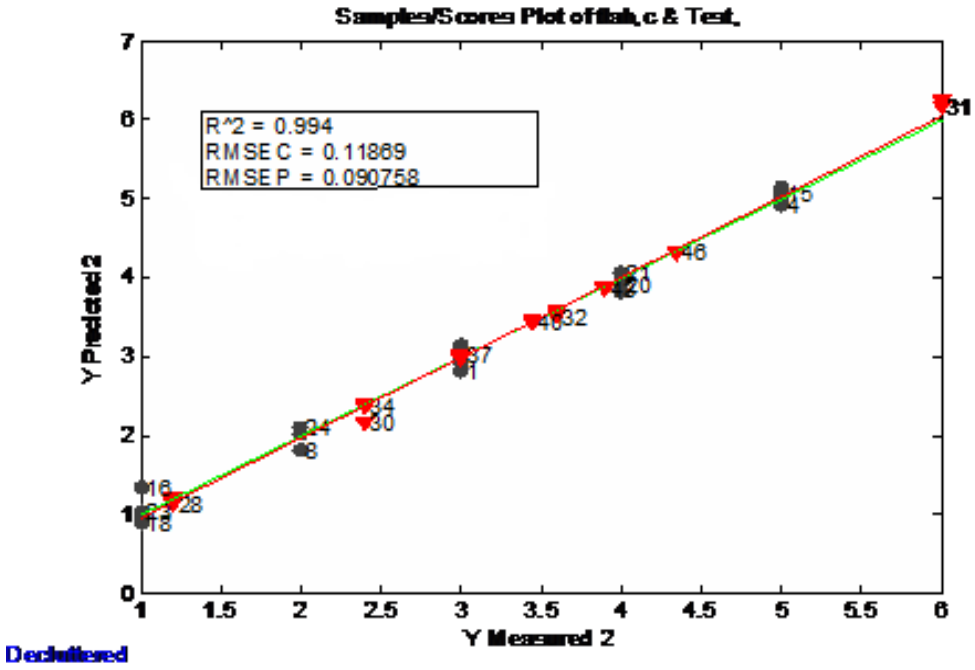
Graph.2 RMSECV values generated from calibration by PLS: losartan potassium, amlodipine besylate and hydrochlorothiazide



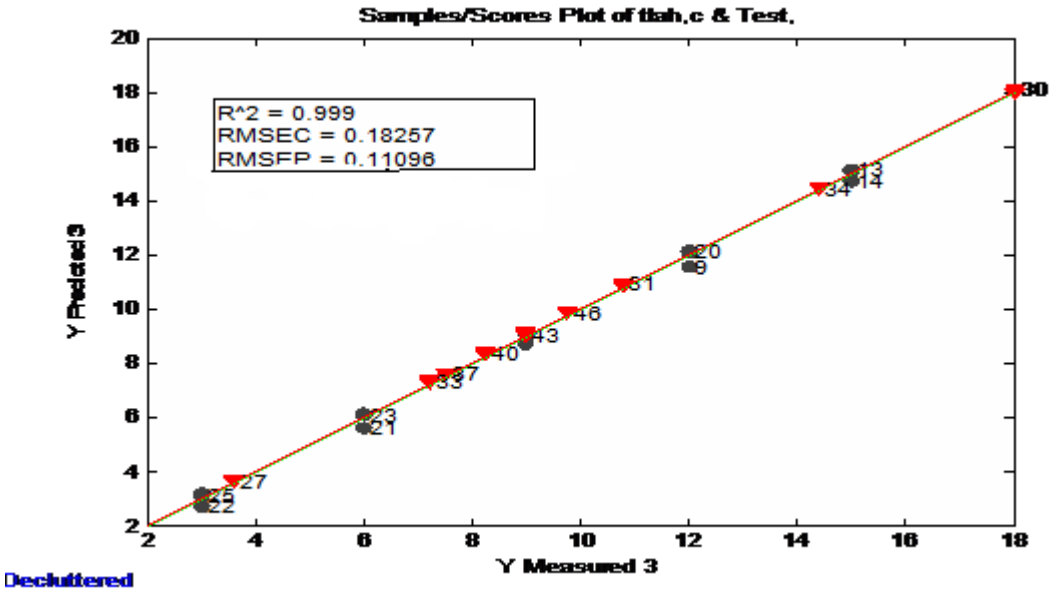
Graph.3 CLS Calibration and prediction curves of losartan potassium



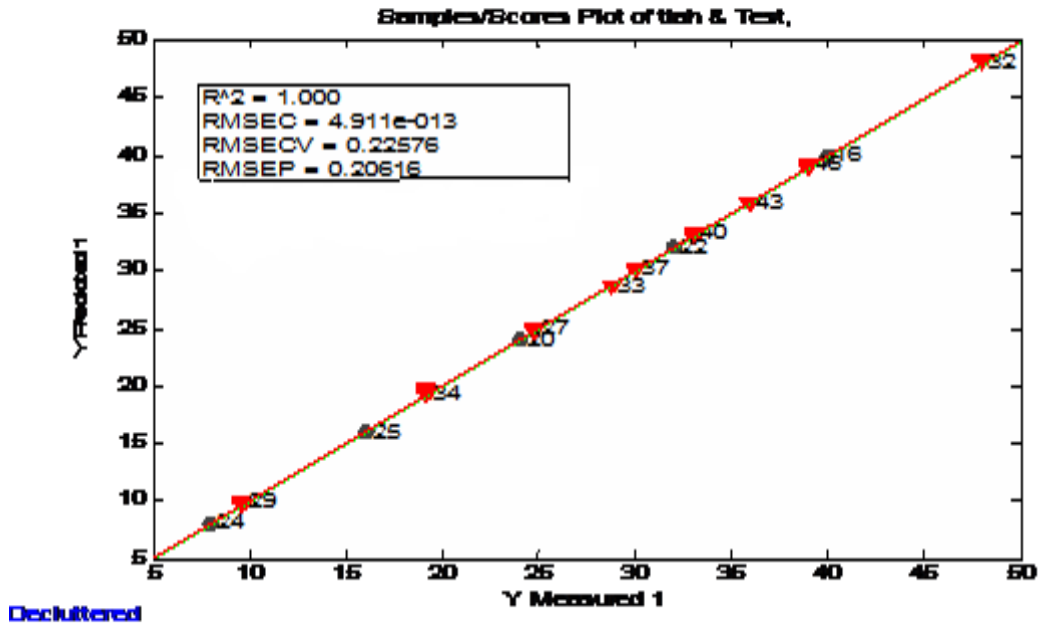
Graph.4 CLS Calibration and prediction curves of amlodipine besylate



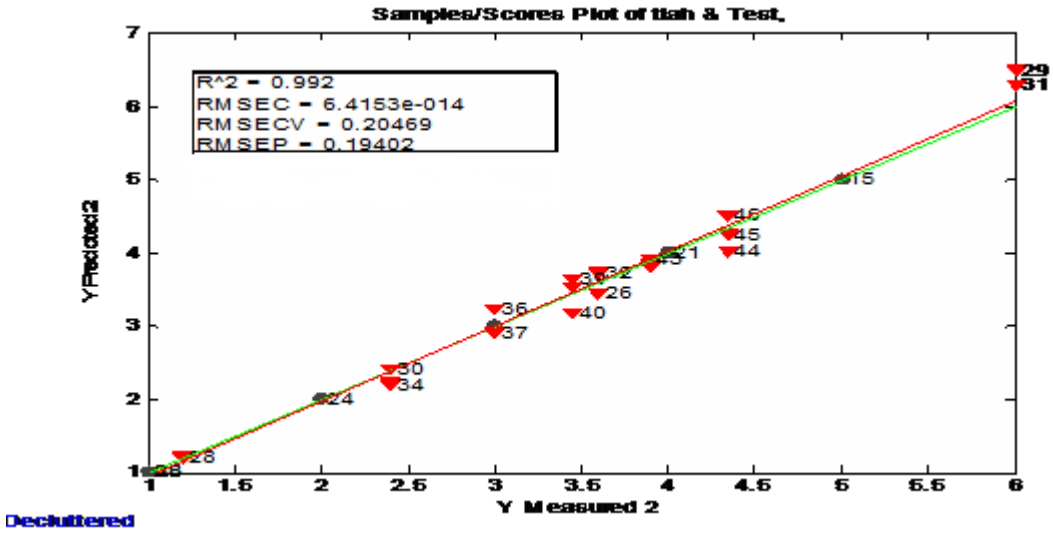
Graph.5 CLS Calibration and prediction curves of hydrochlorothiazide



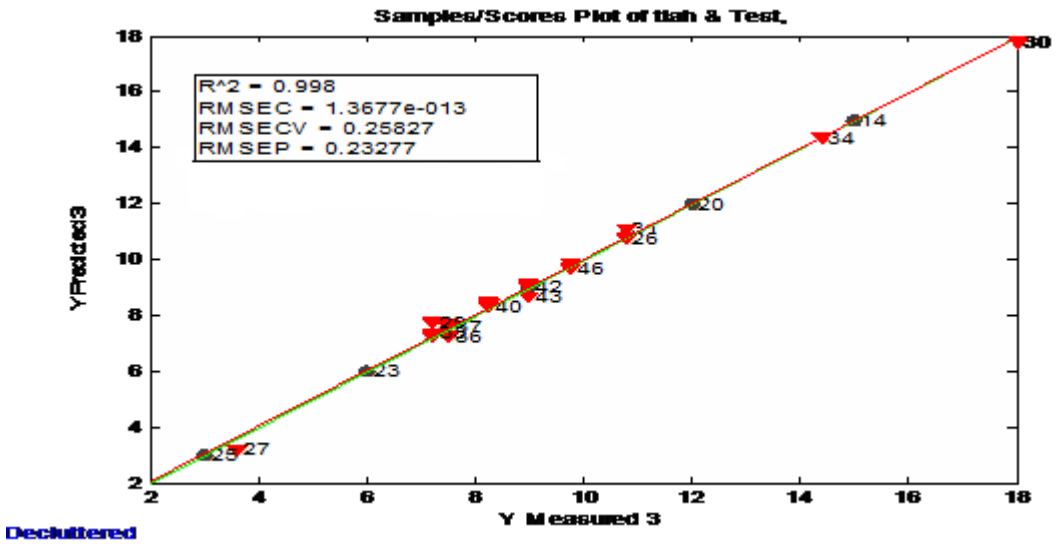
Graph.6 MLR Calibration and prediction curves of losartan potassium



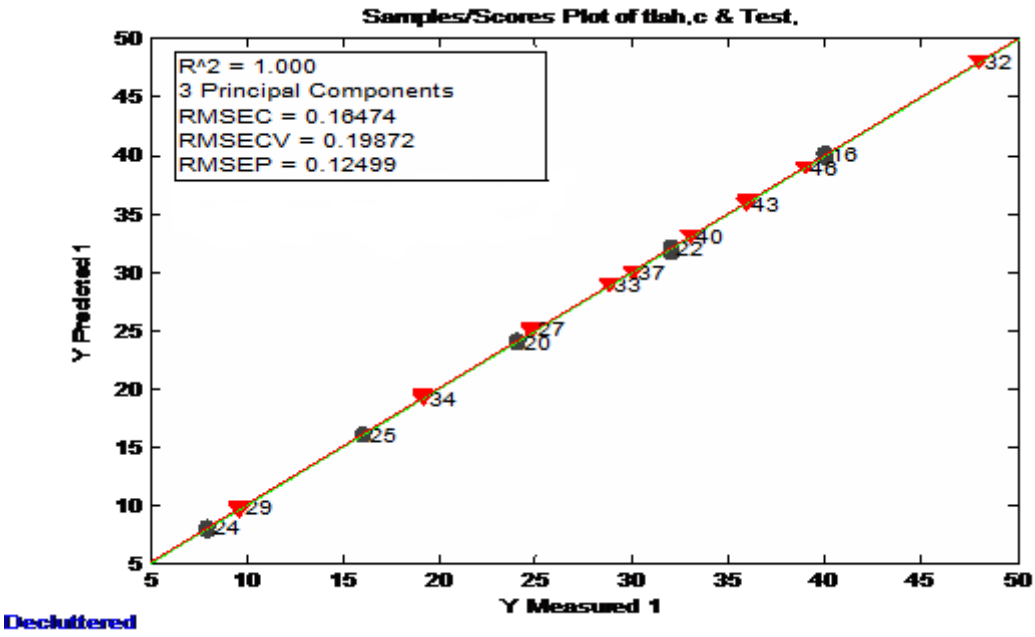
Graph.7 MLR Calibration and prediction curves of amlodipine besylate



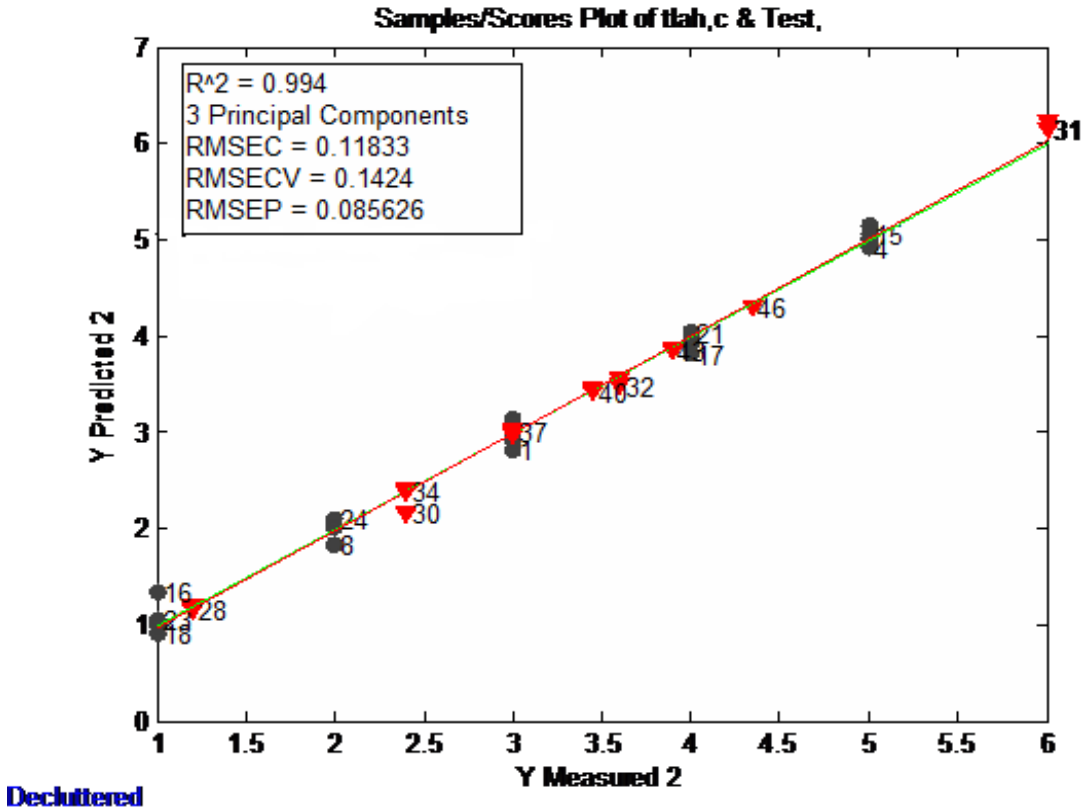
Graph.8 MLR Calibration and prediction curves of hydrochlorothiazide



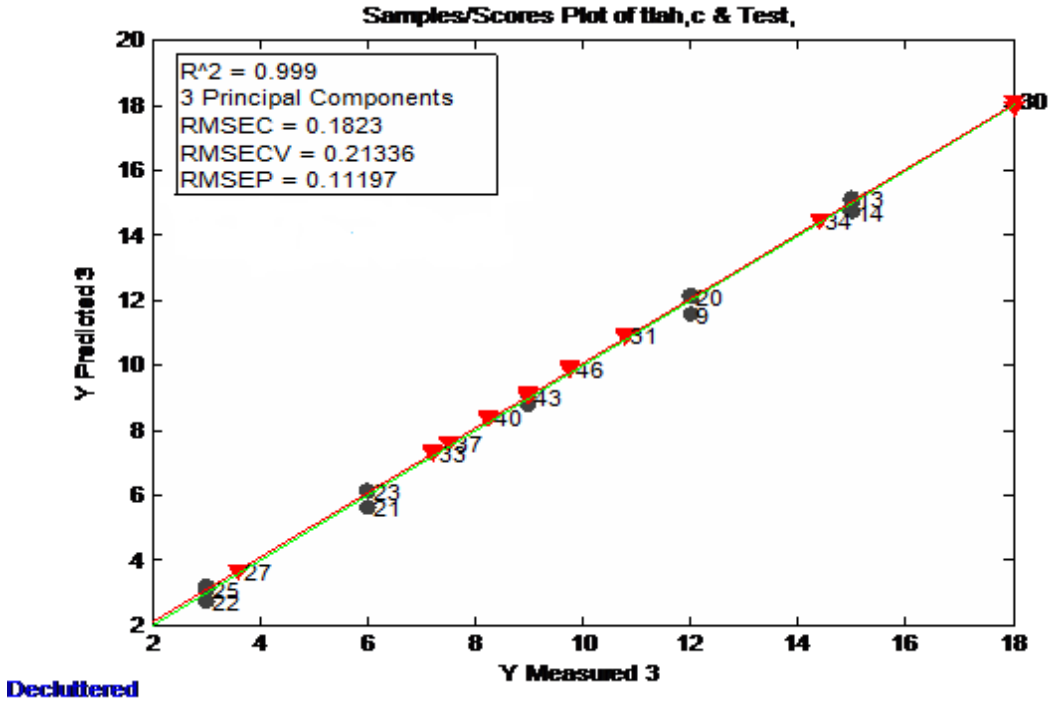
Graph.9 PCR Calibration and prediction curves of losartan potassium



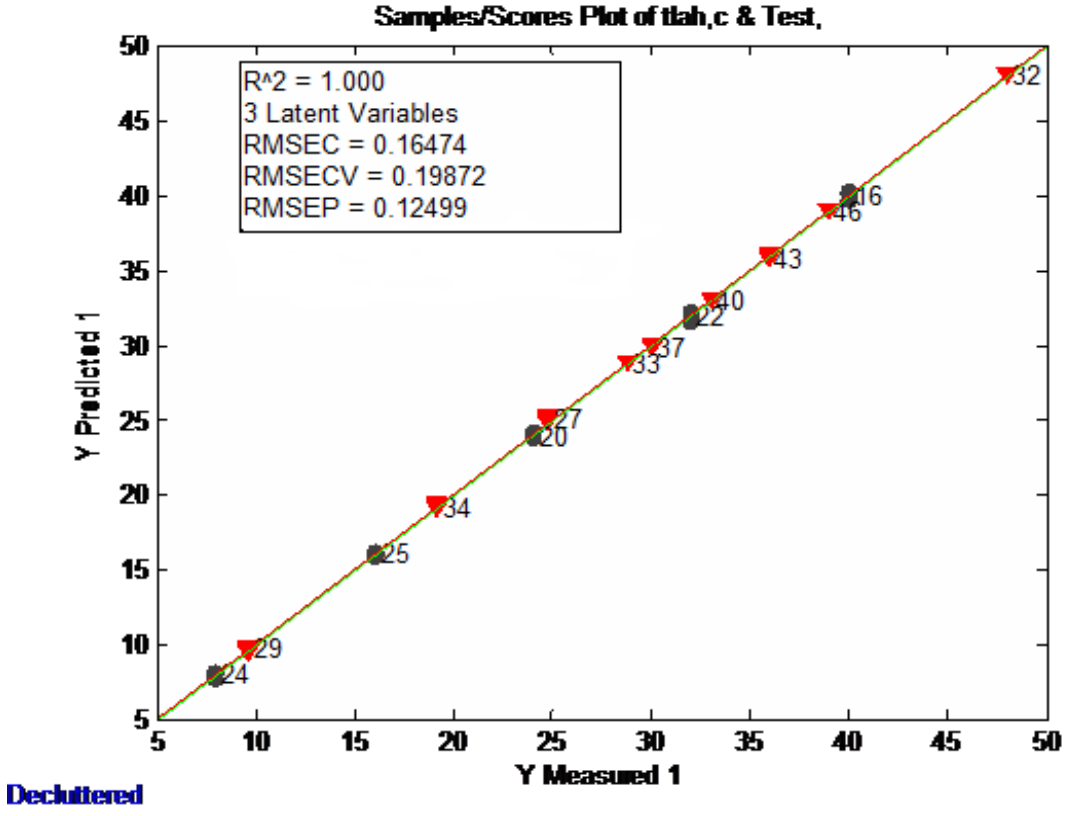
Graph.10 PCR Calibration and prediction curves of amlodipine besylate



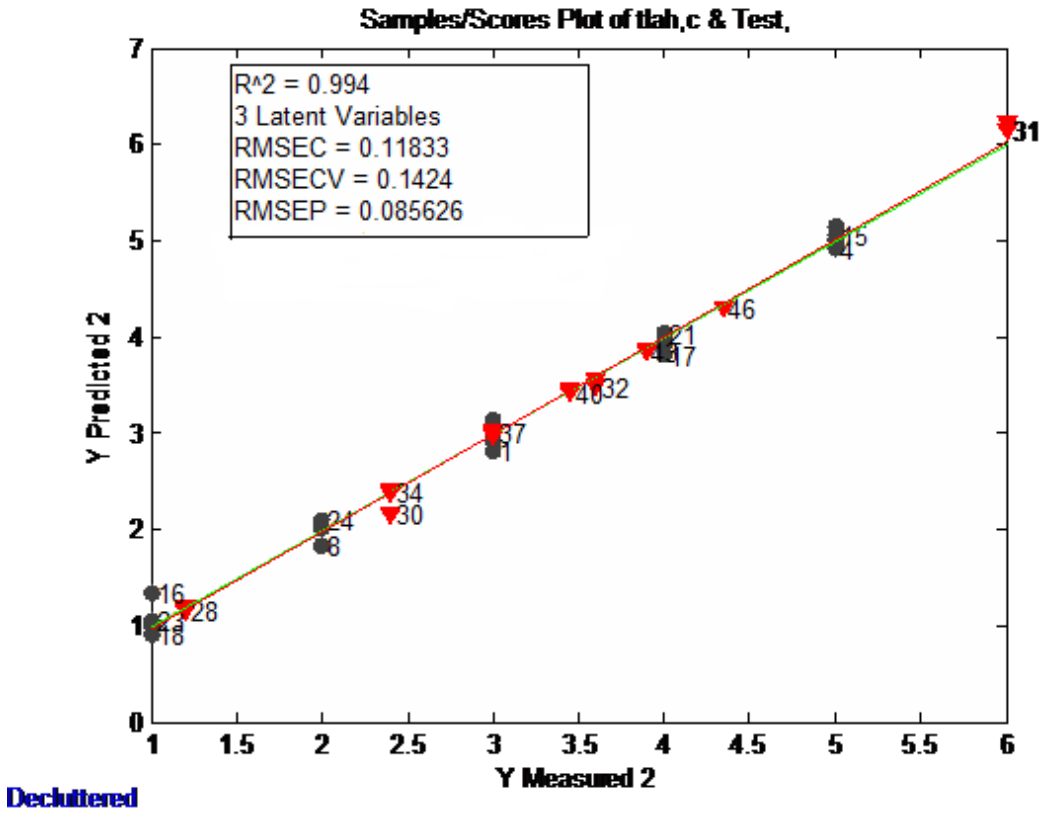
Graph.11 PCR Calibration and prediction curves of hydrochlorothiazide



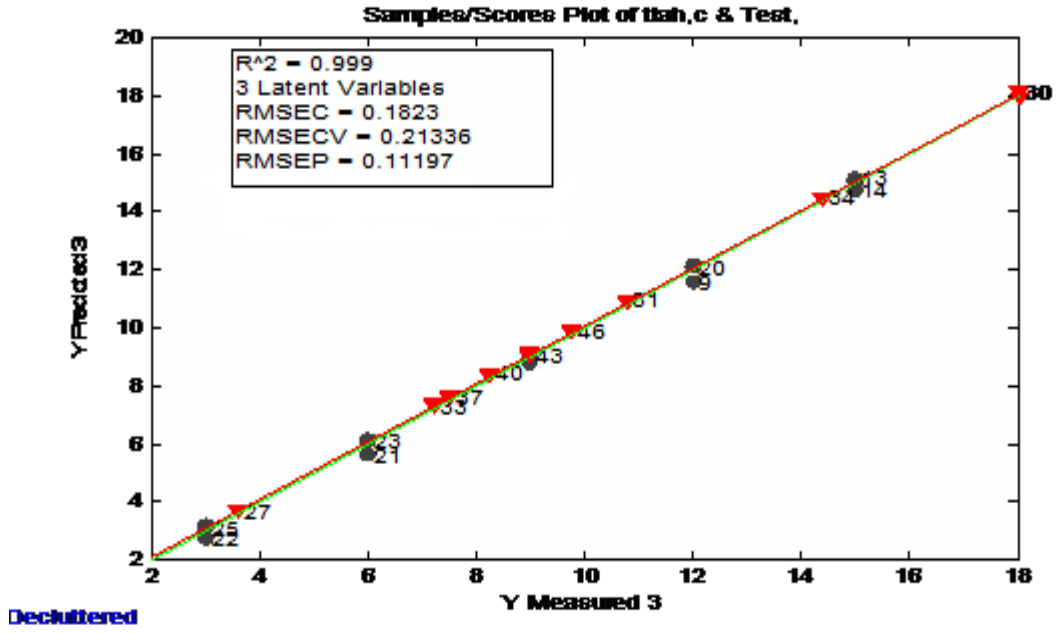
Graph.12 PLS Calibration and prediction curves of losartan potassium



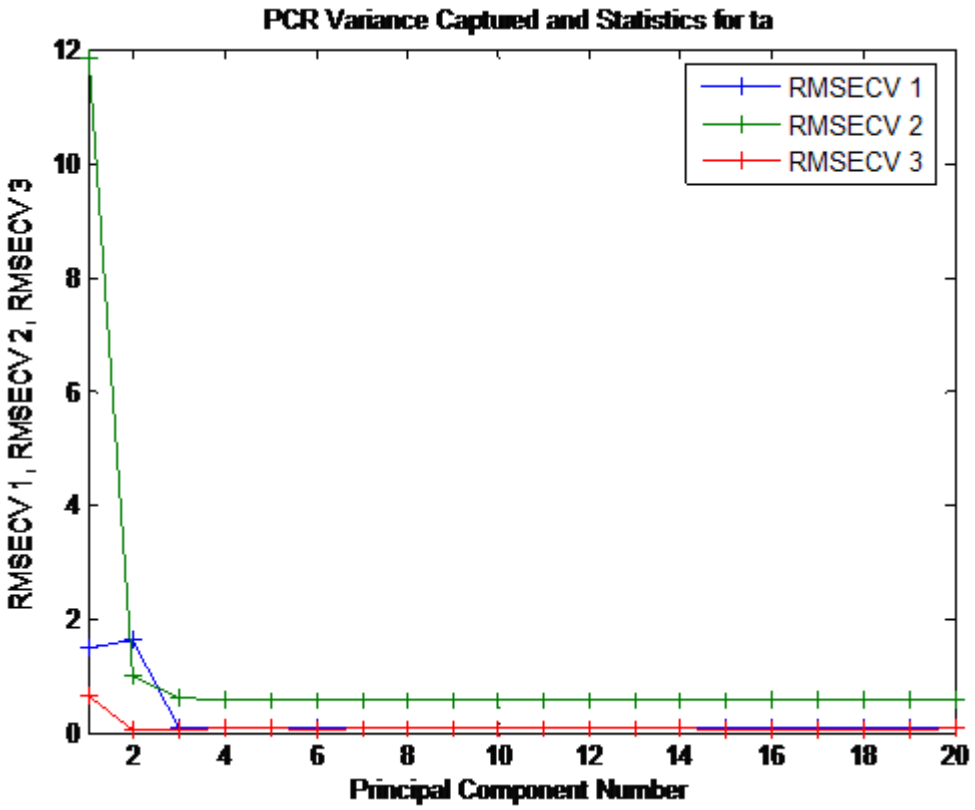
Graph.13 PLS Calibration and prediction curves of amlodipine besylate



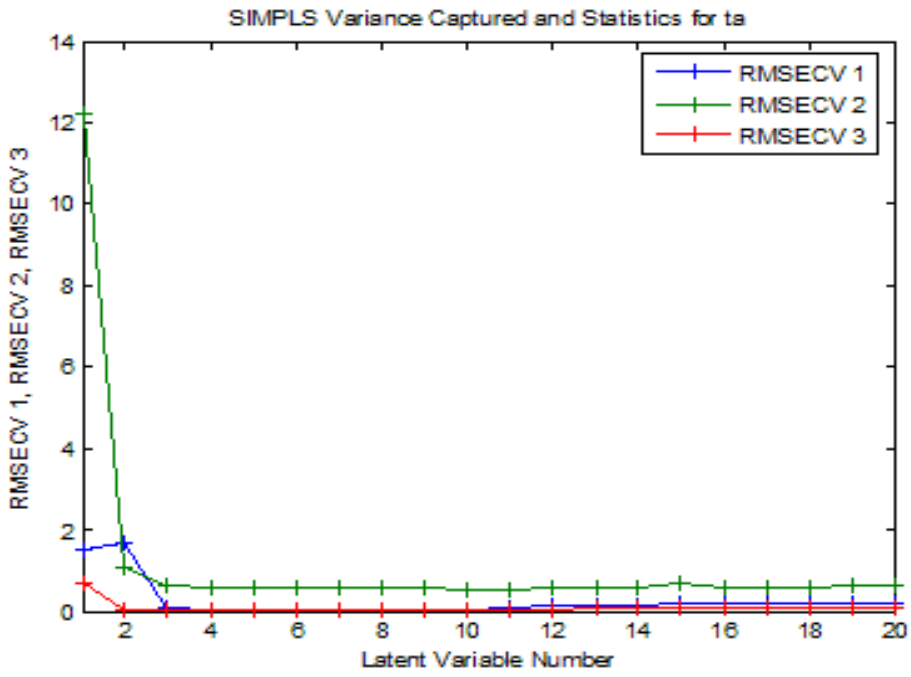
Graph.14 PLS Calibration and prediction curves of hydrochlorothiazide



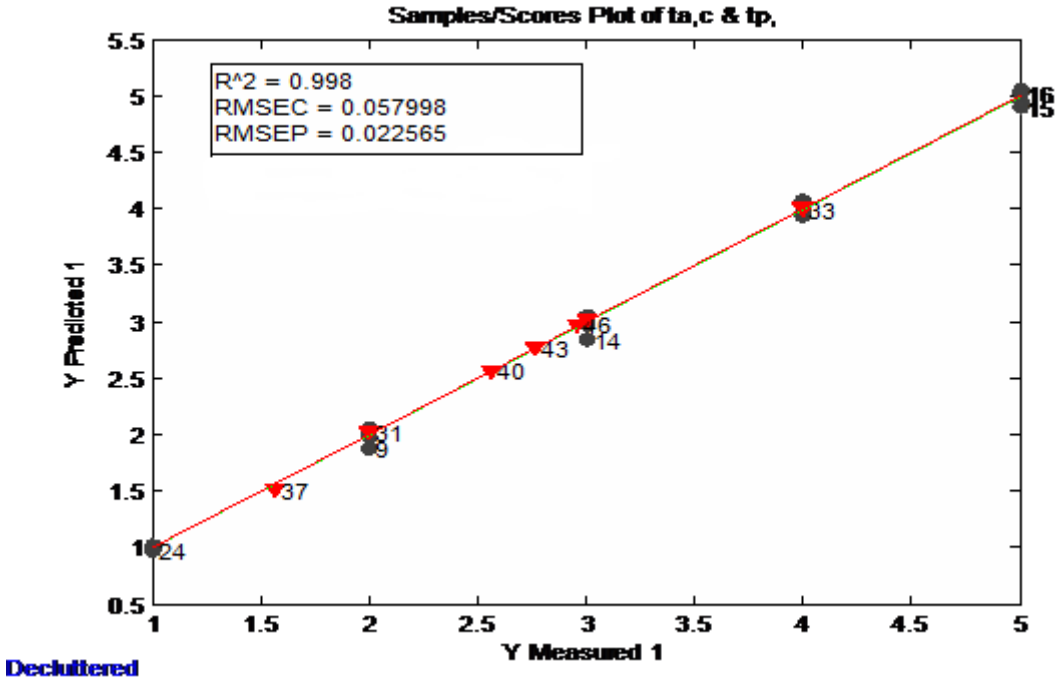
Graph.15 RMSECV values generated from calibration by PCR: atorvastatin calcium, fenofibrate and folic acid



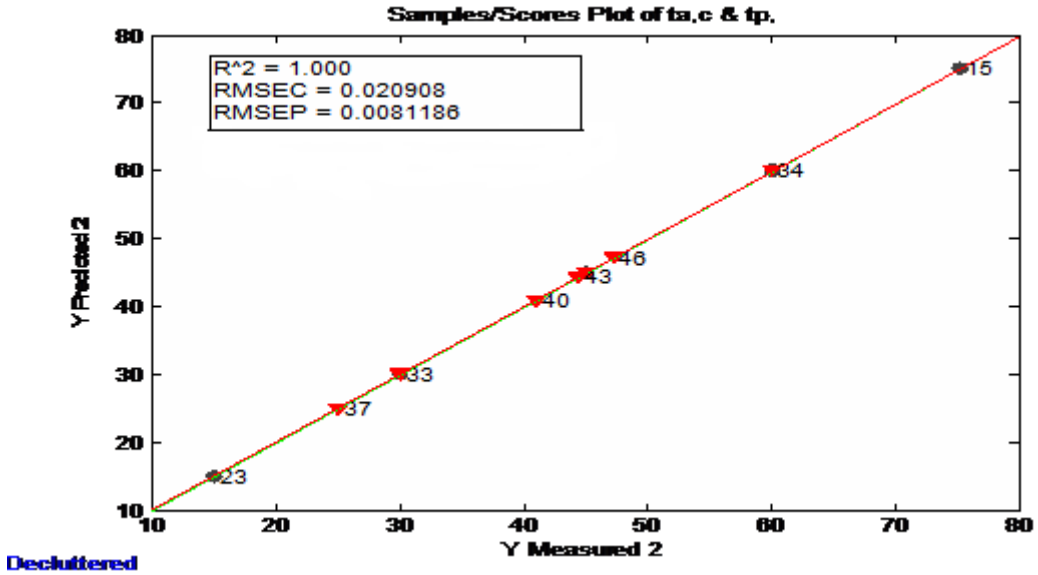
Graph.16 RMSECV values generated from calibration by PLS: atorvastatin calcium, fenofibrate and folic acid



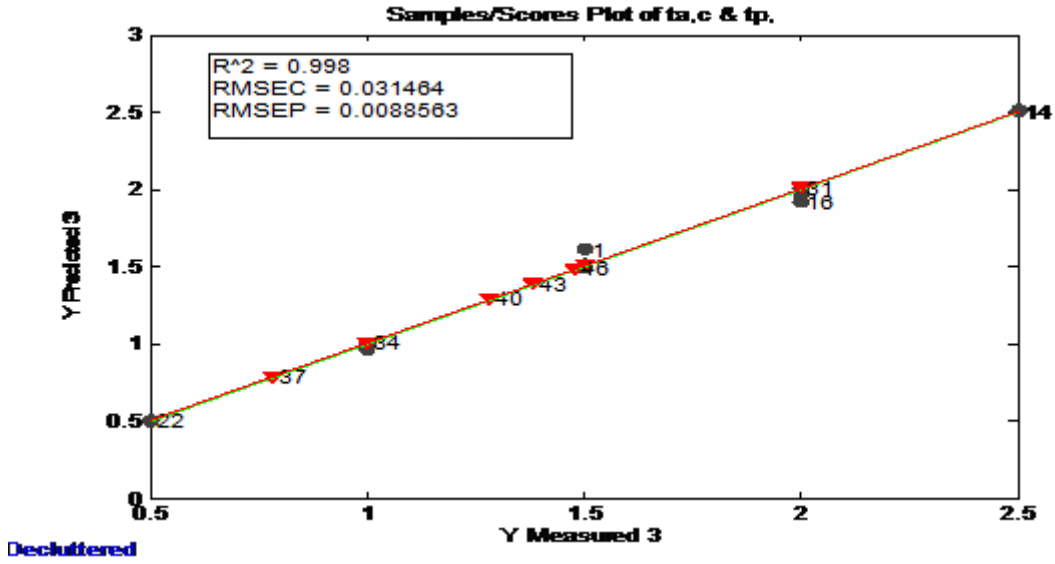
Graph.17 CLS Calibration and prediction curves of atorvastatin calcium



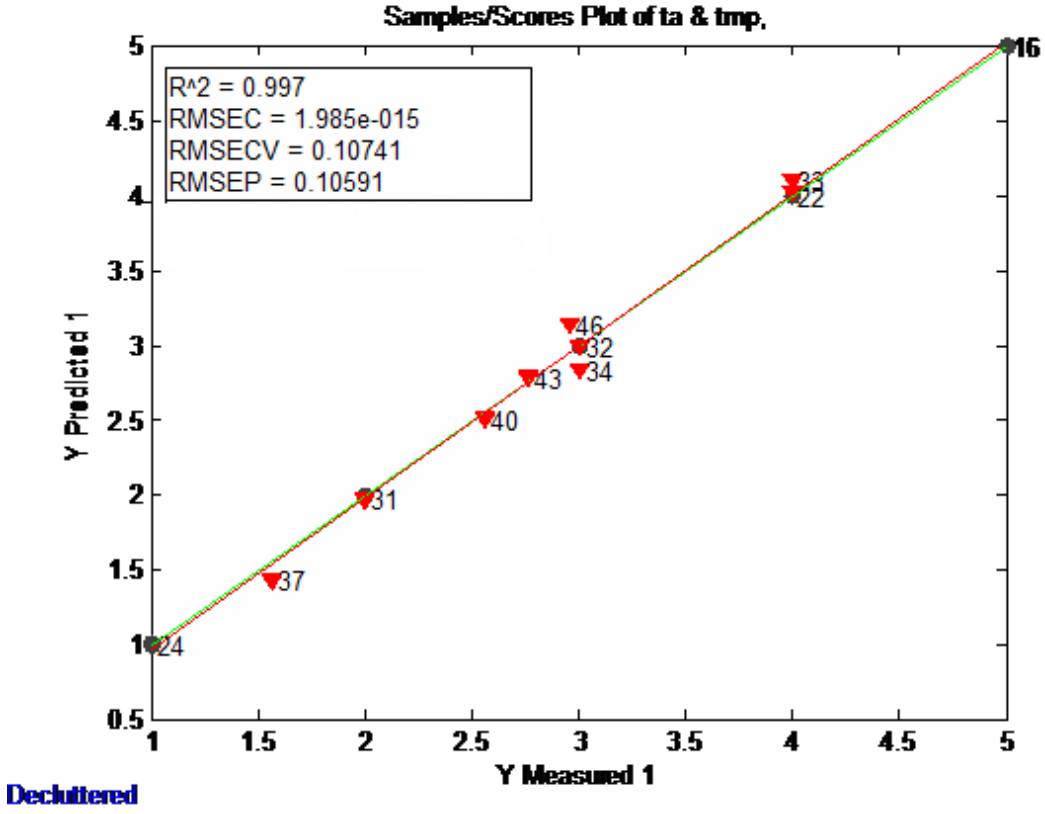
Graph.18 CLS Calibration and prediction curves of fenofibrate



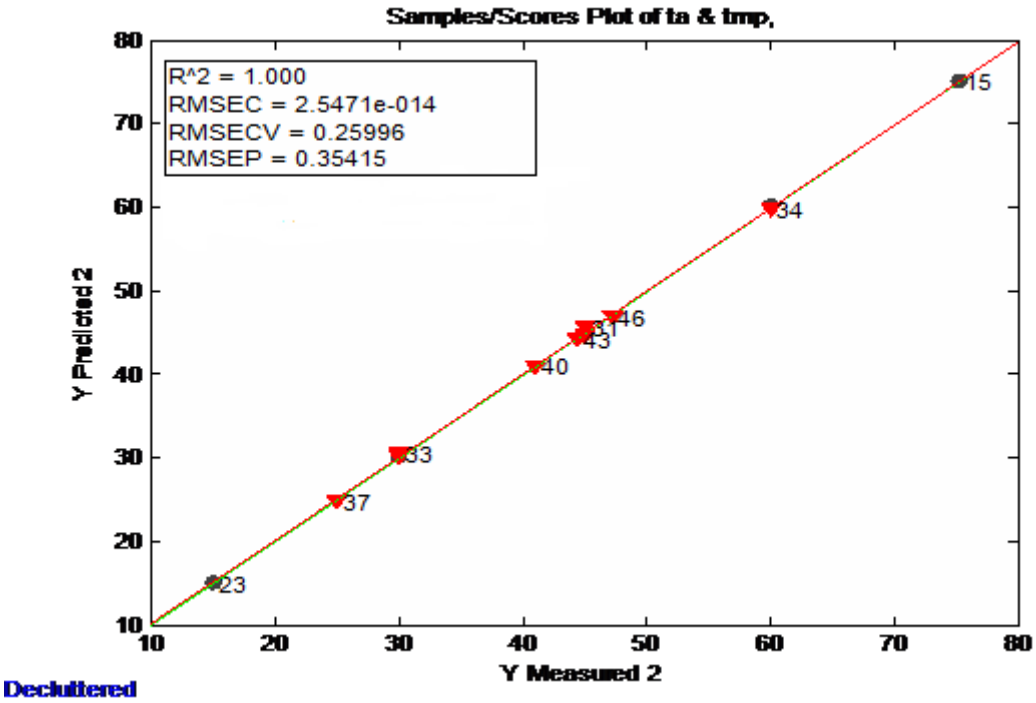
Graph.19 CLS Calibration and prediction curve of folic acid



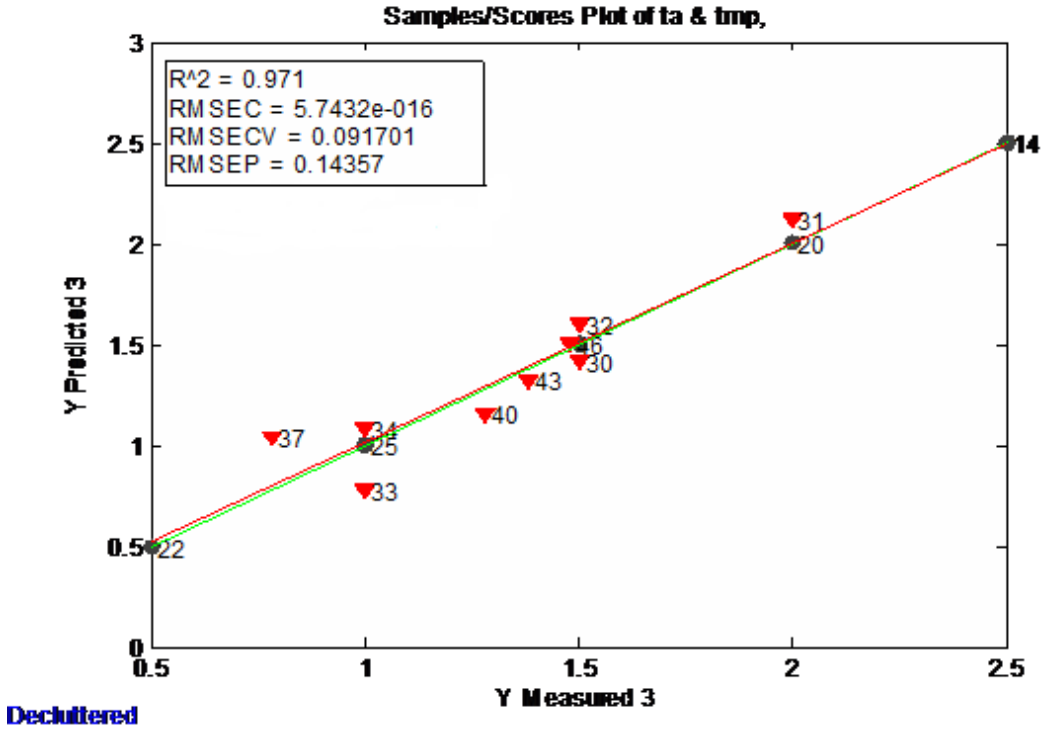
Graph.20 MLR Calibration and prediction curves of atorvastatin calcium



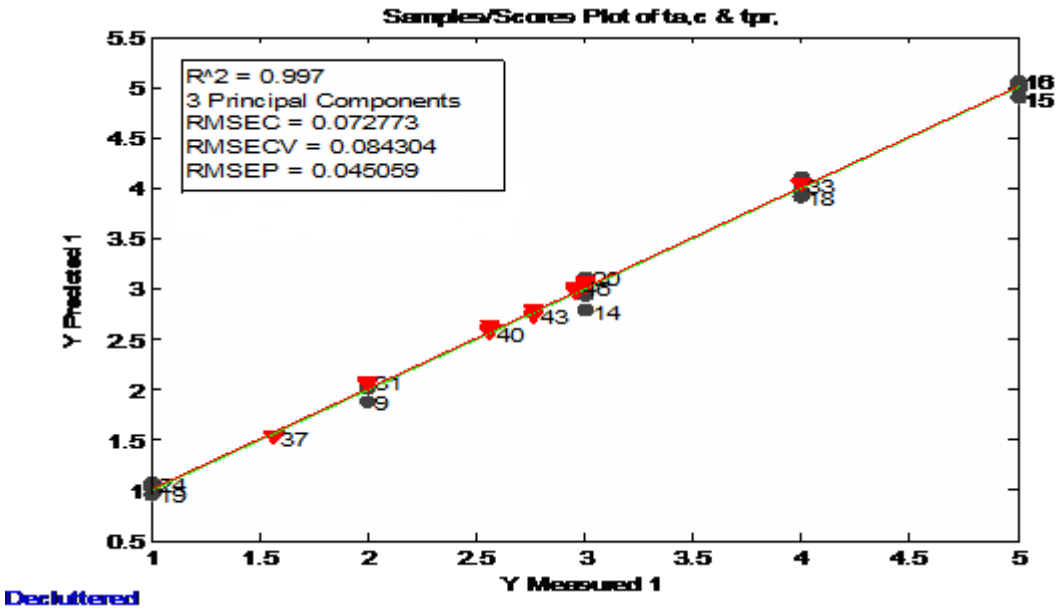
Graph.21 MLR Calibration and prediction curves of fenofibrate



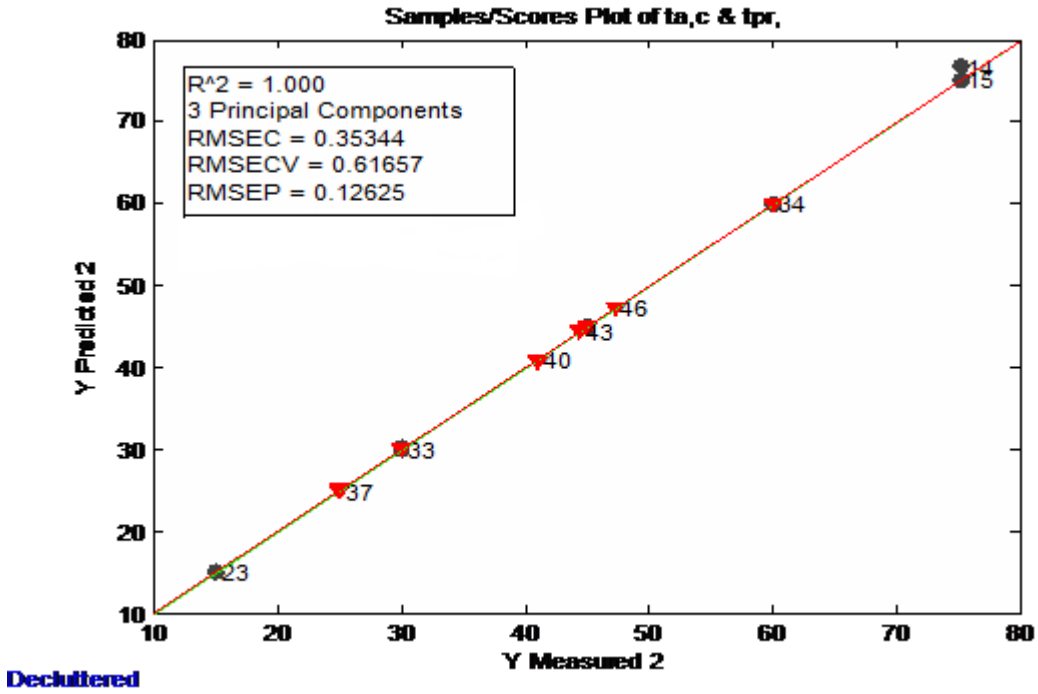
Graph.22 MLR Calibration and prediction curves of folic acid



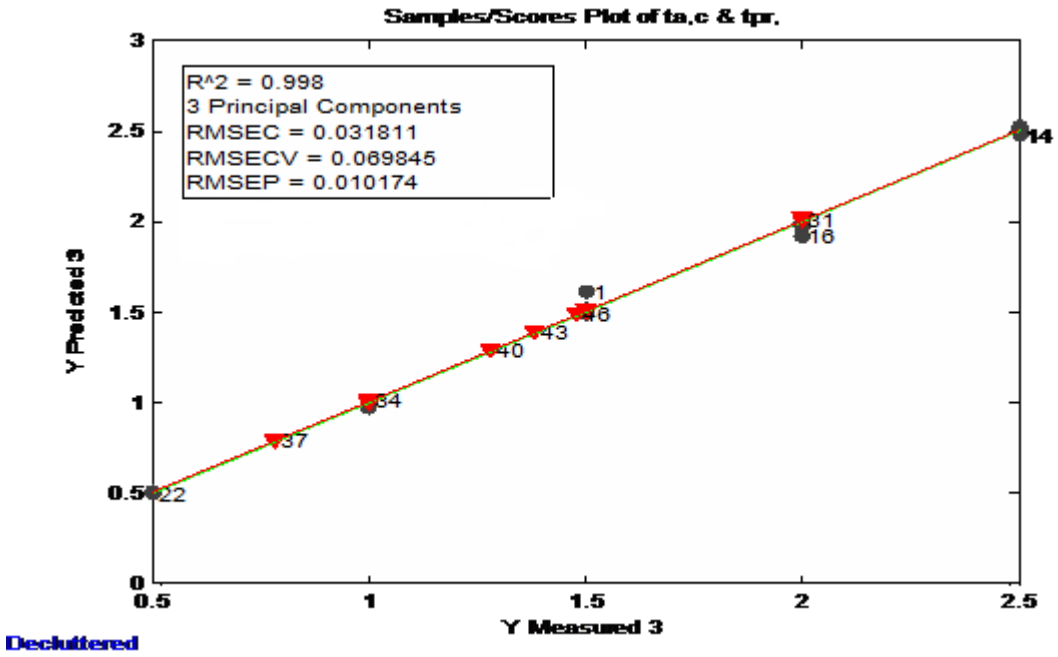
Graph.23 PCR Calibration and prediction curves of atorvastatin calcium



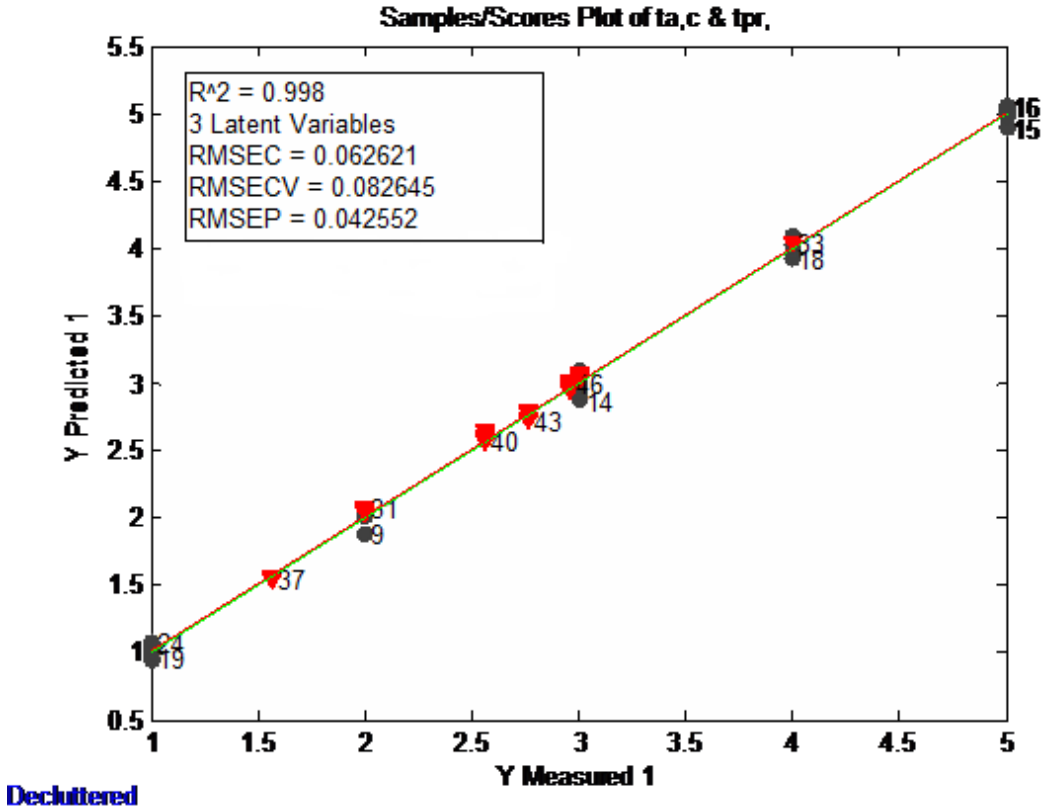
Graph.24 PCR Calibration and prediction curves of fenofibrate



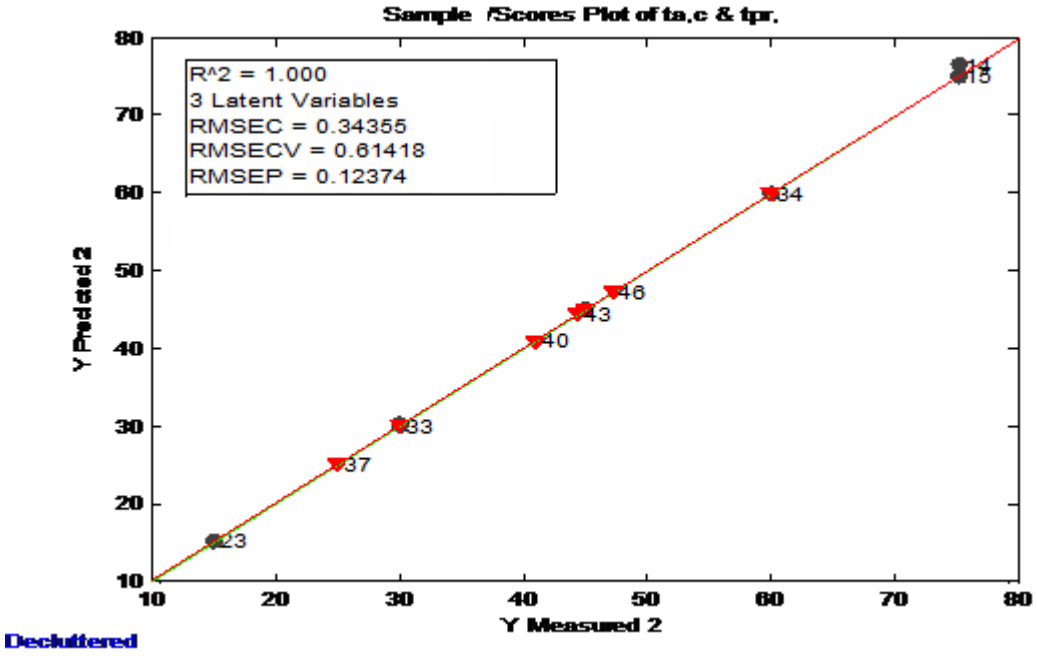
Graph.25 PCR Calibration and prediction curves of folic acid



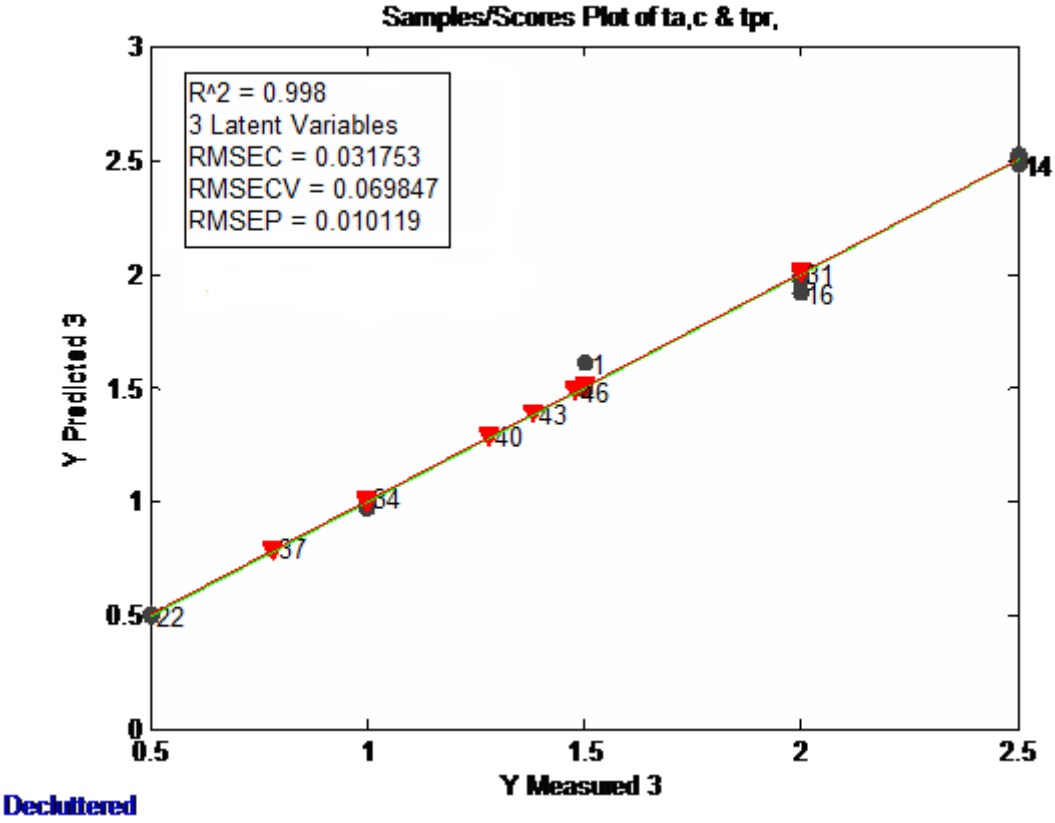
Graph.26 PLS Calibration and prediction curves of atorvastatin calcium



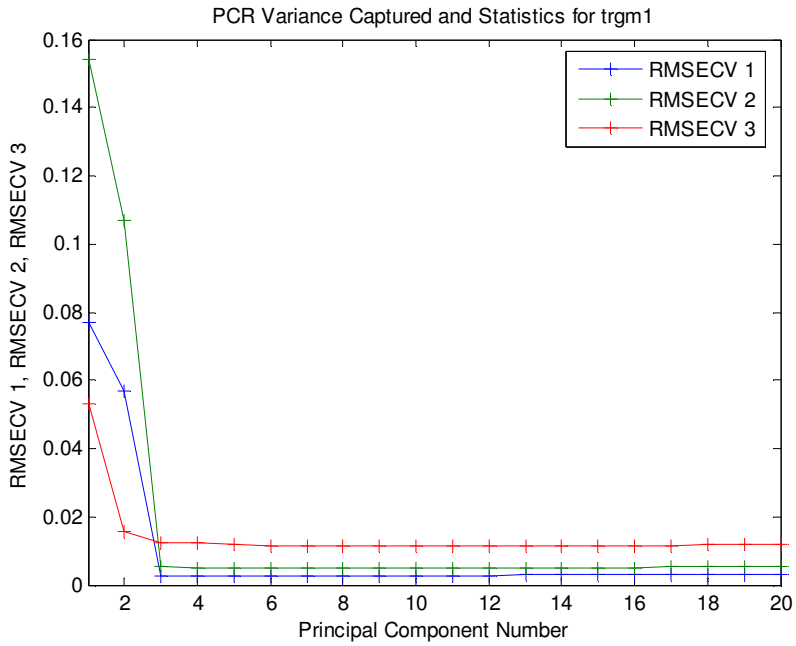
Graph.27 PLS Calibration and prediction curves of fenofibrate



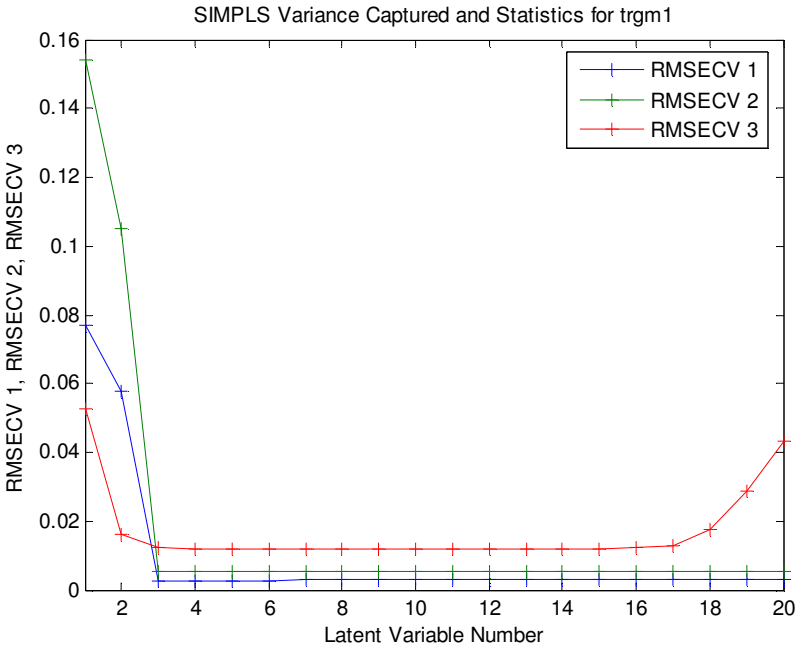
Graph.28 PLS Calibration and prediction curves of folic acid



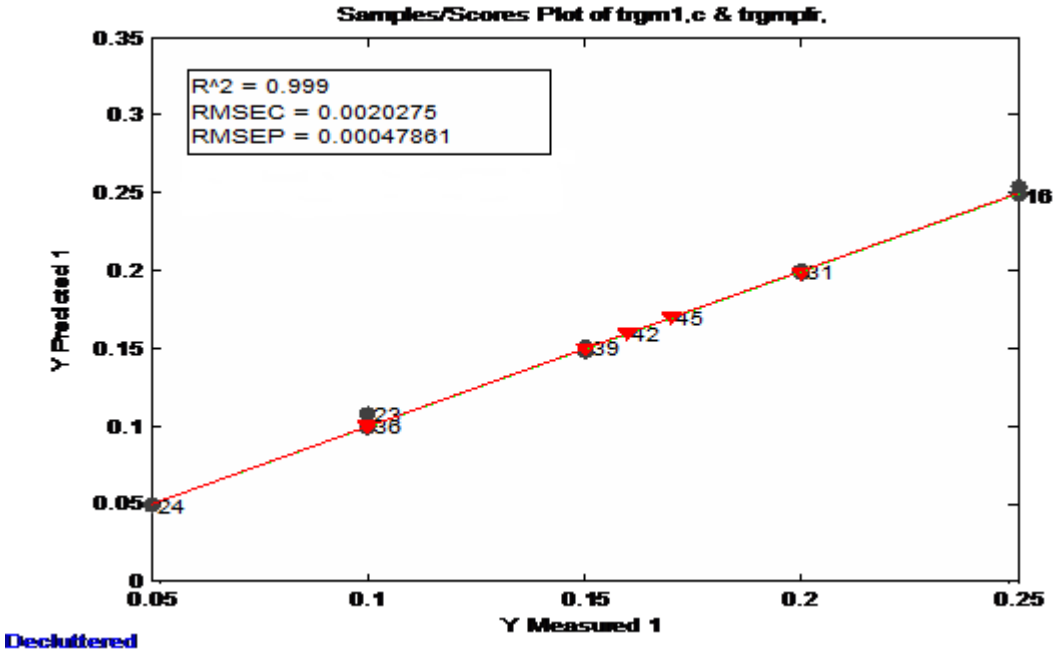
Graph.29 RMSECV values generated from calibration by PCR: rosiglitazone maleate, glibenclamide and metformin hydrochloride



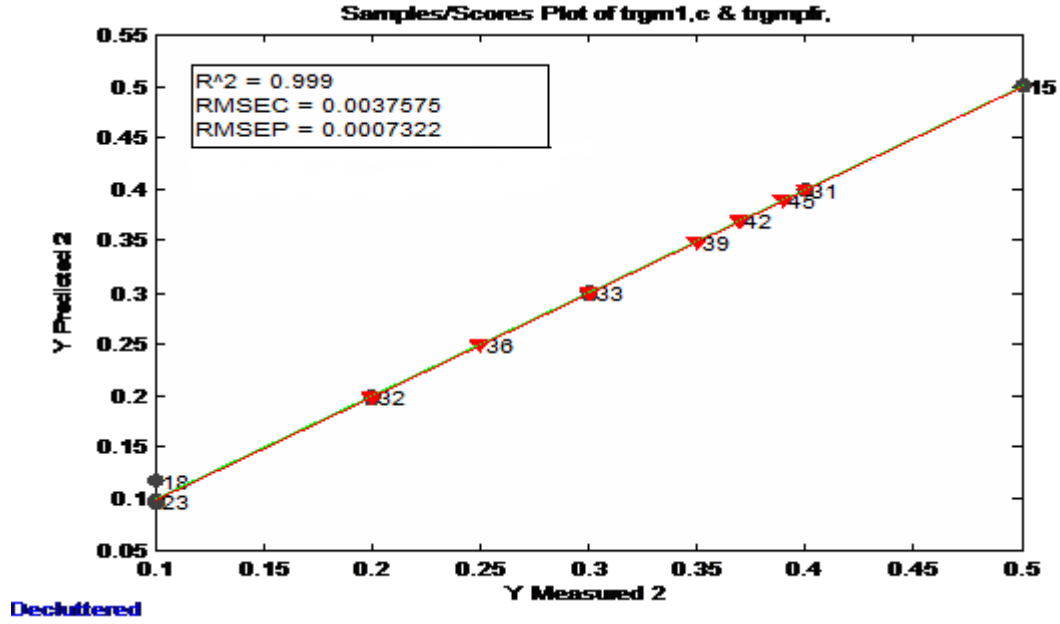
Graph.30 RMSECV values generated from calibration by PLS: rosiglitazone maleate, glibenclamide and metformin hydrochloride



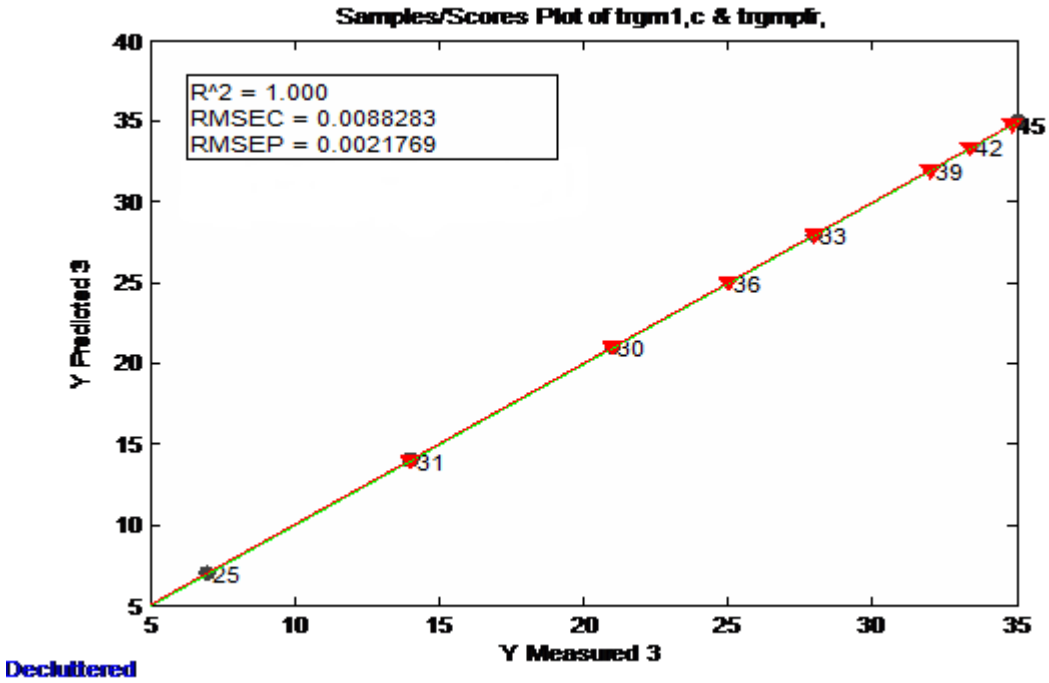
Graph.31 CLS Calibration and prediction curves of rosiglitazone maleate



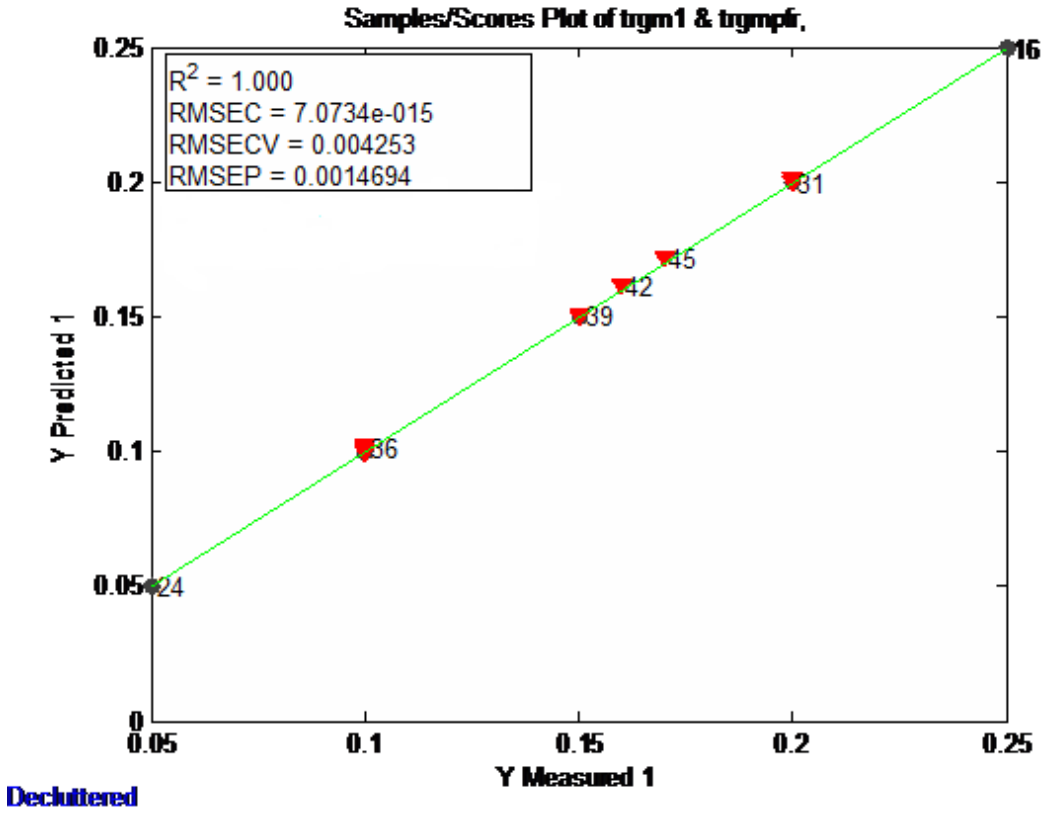
Graph.32 CLS Calibration and prediction curves of glibenclamide



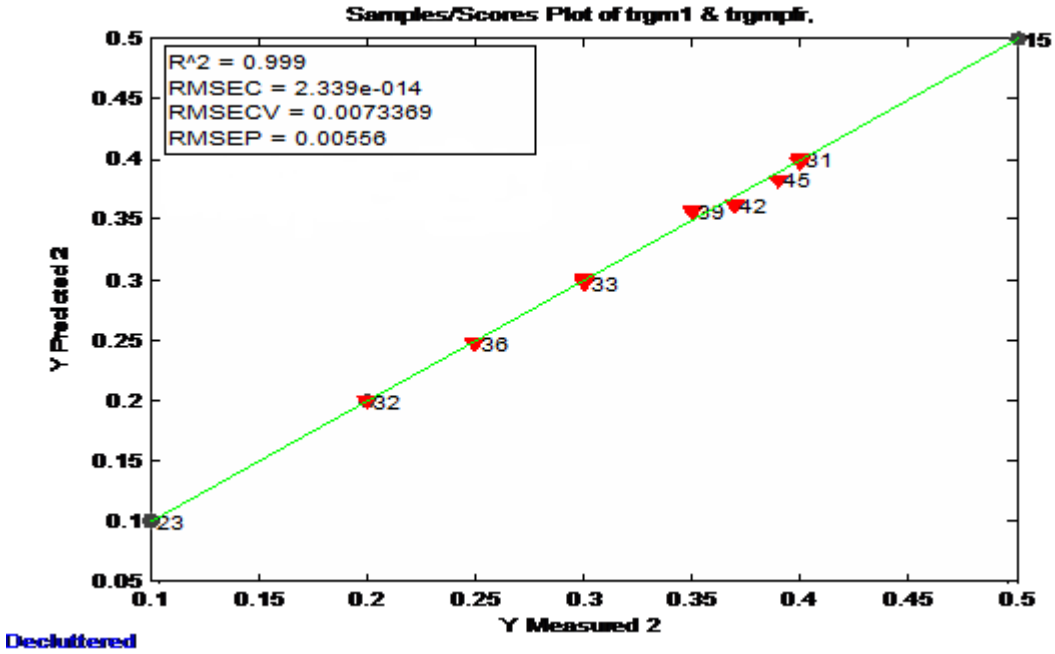
Graph.33 CLS Calibration and prediction curves of metformin hydrochloride



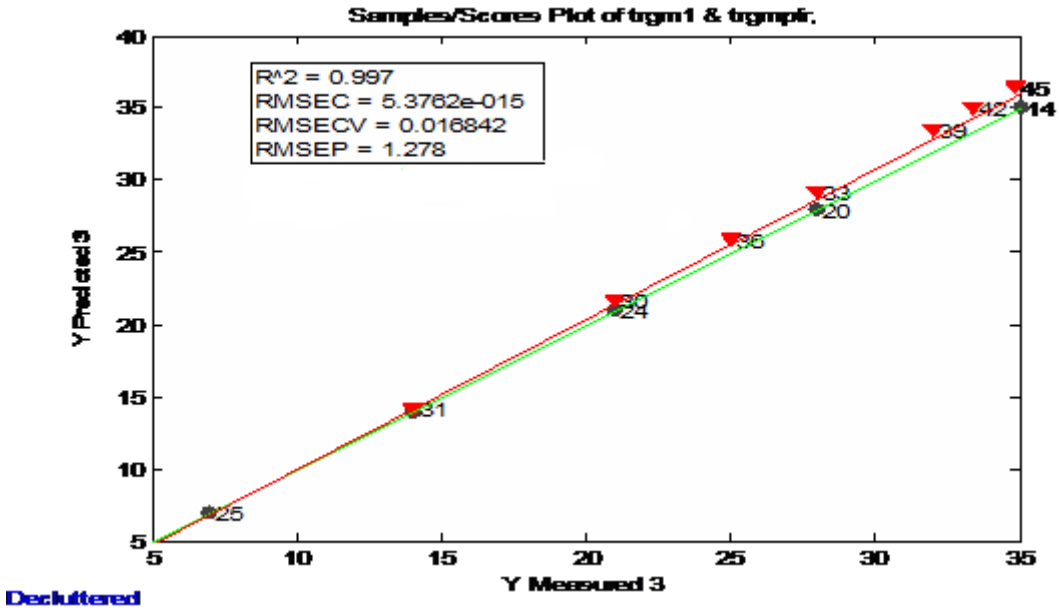
Graph.34 MLR Calibration and prediction curves of rosiglitazone maleate



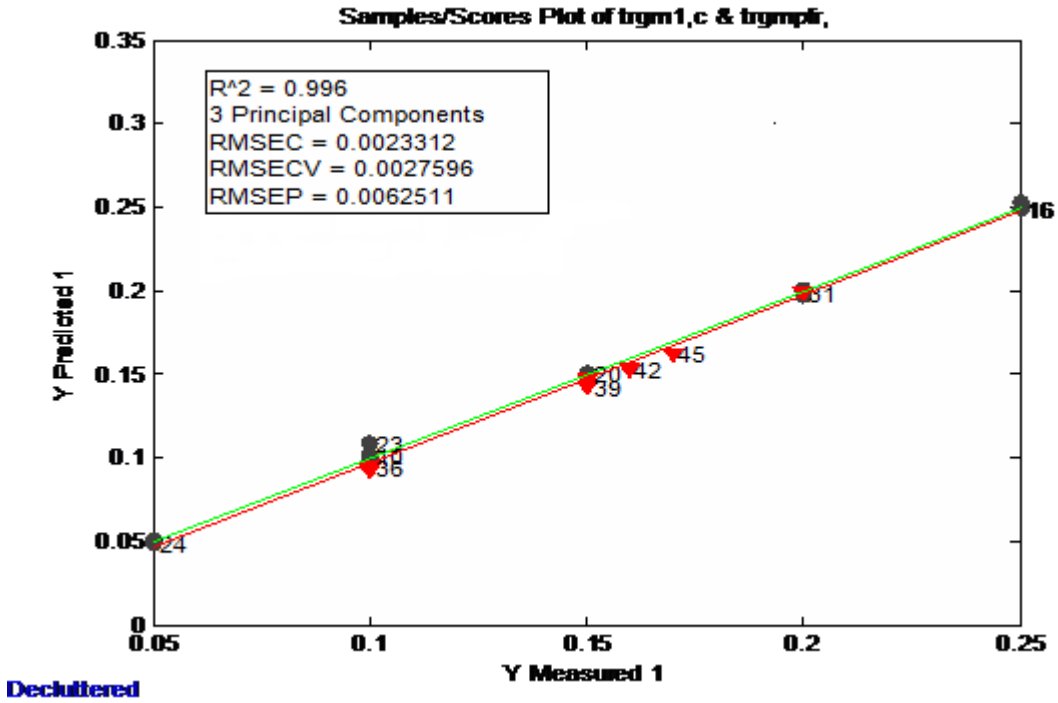
Graph.35 MLR Calibration and prediction curves of glibenclamide



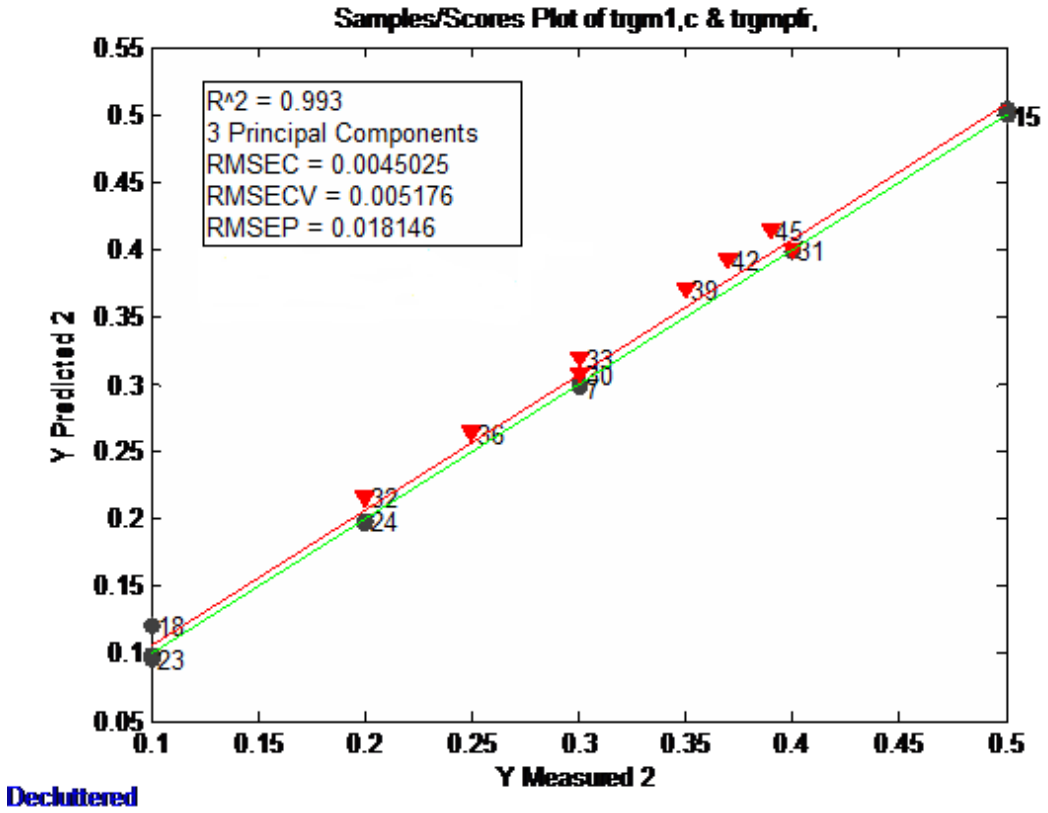
Graph.36 MLR Calibration and prediction curves of metformin hydrochloride



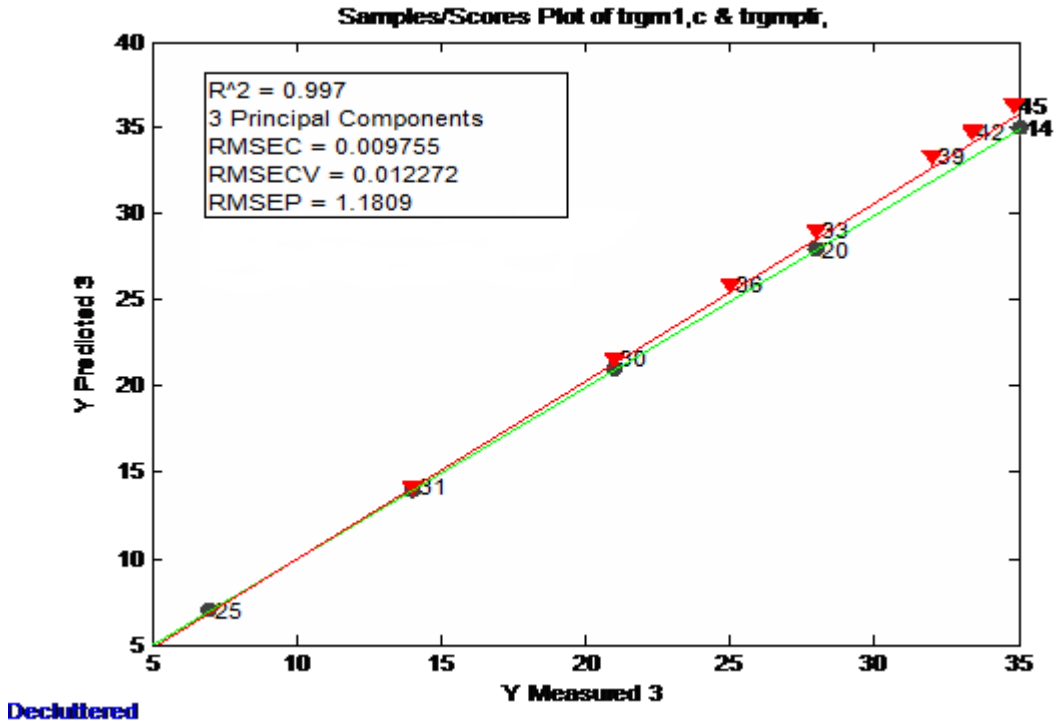
Graph.37 PCR Calibration and prediction curves of rosiglitazone maleate



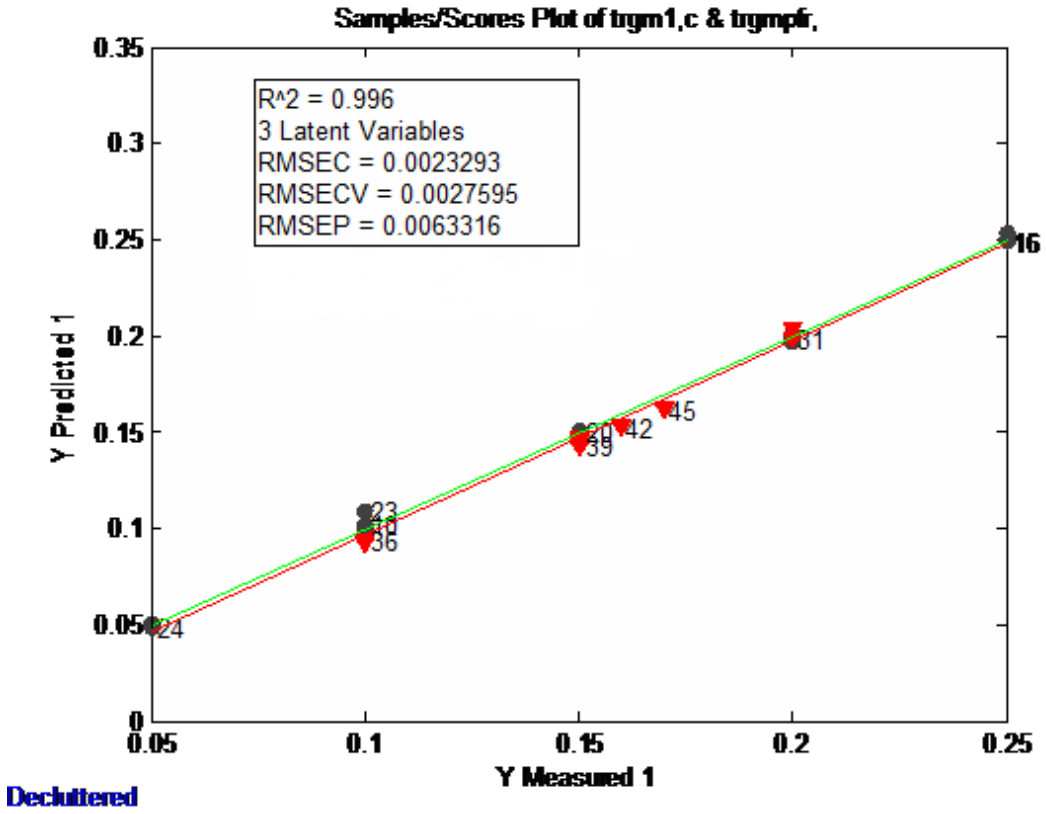
Graph.38 PCR Calibration and prediction curves of glibenclamide



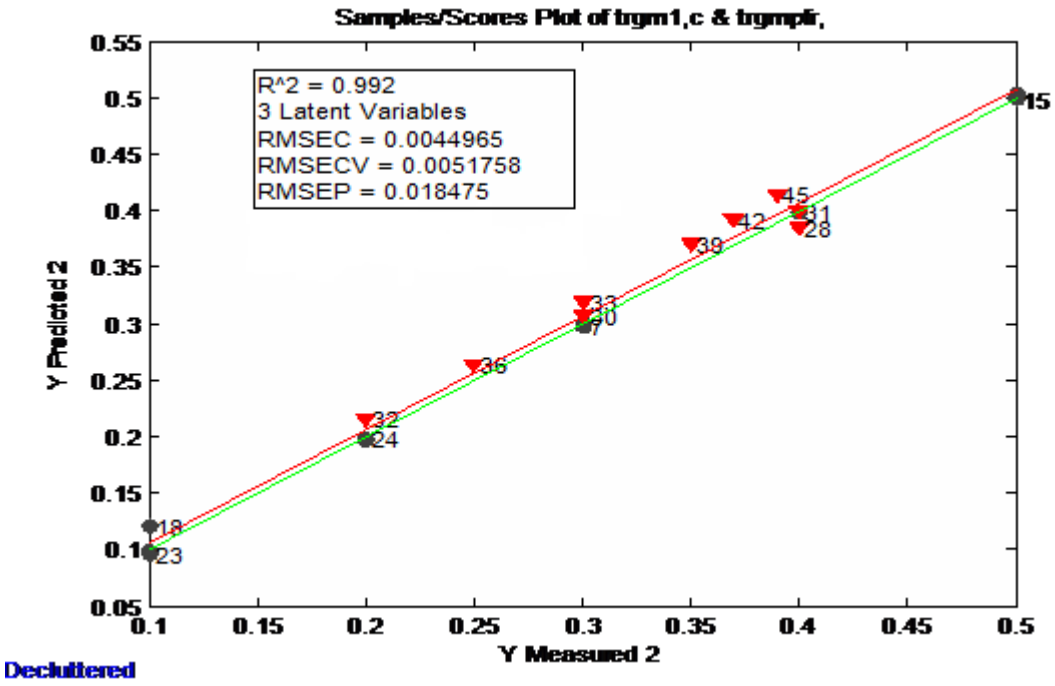
Graph.39 PCR Calibration and prediction curves of metformin hydrochloride



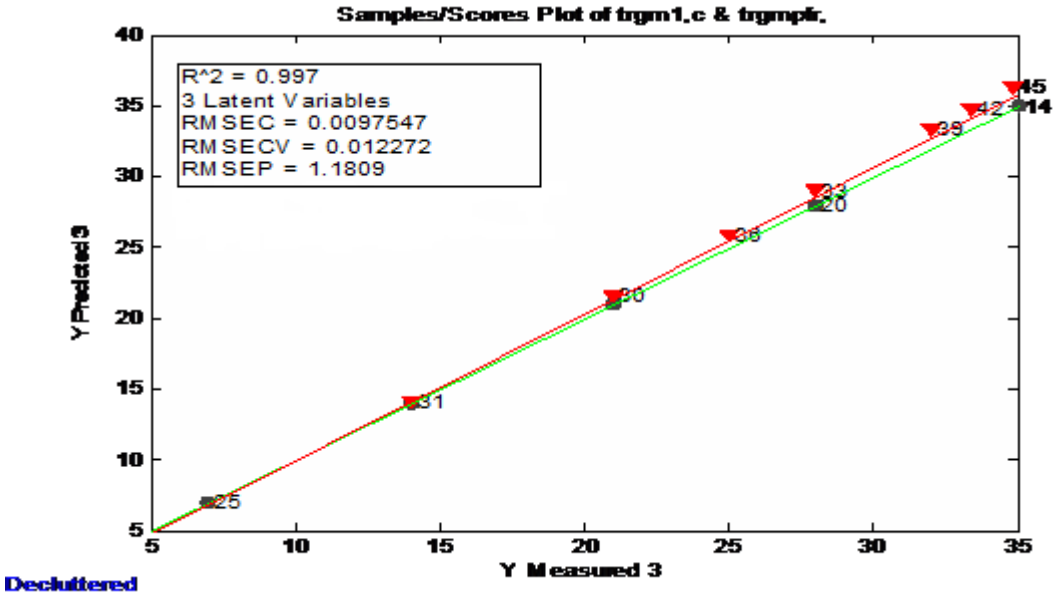
Graph.40 PLS Calibration and prediction curves of rosiglitazone maleate



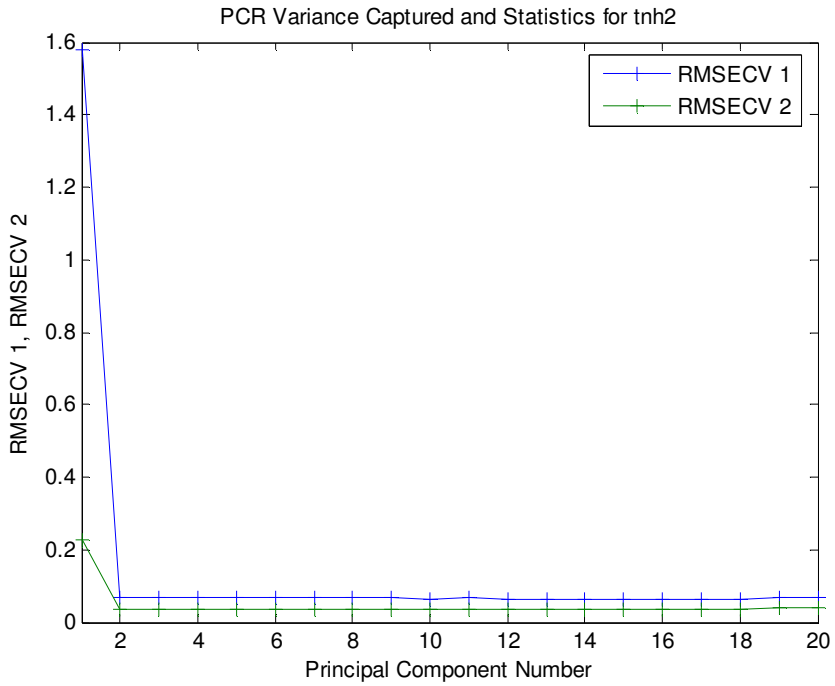
Graph.41 PLS Calibration and prediction curves of glibenclamide



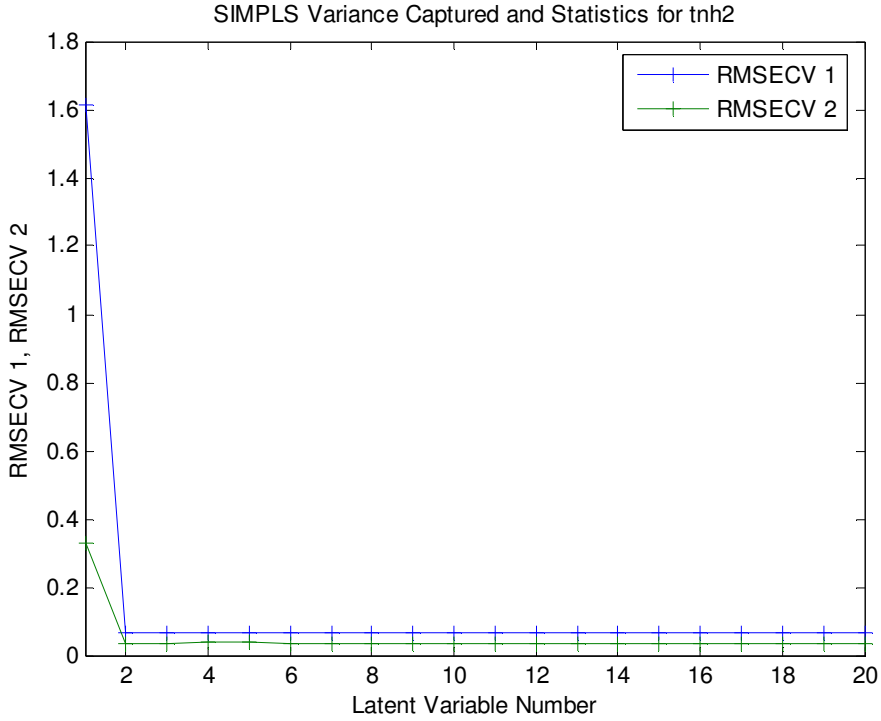
Graph.42 PLS Calibration and prediction curves of metformin hydrochloride



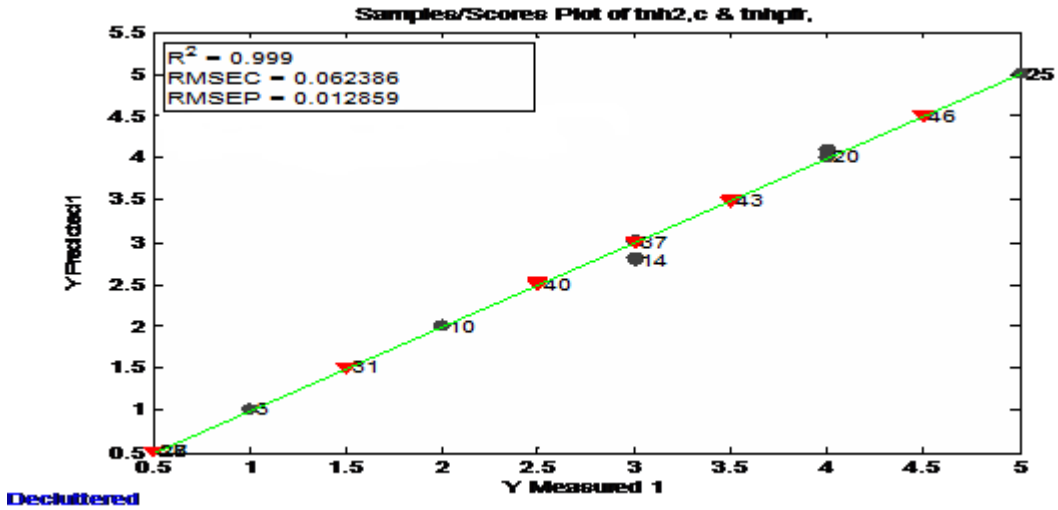
Graph.43 RMSECV values generated from calibration by PCR: nebivolol hydrochloride and hydrochlorothiazide



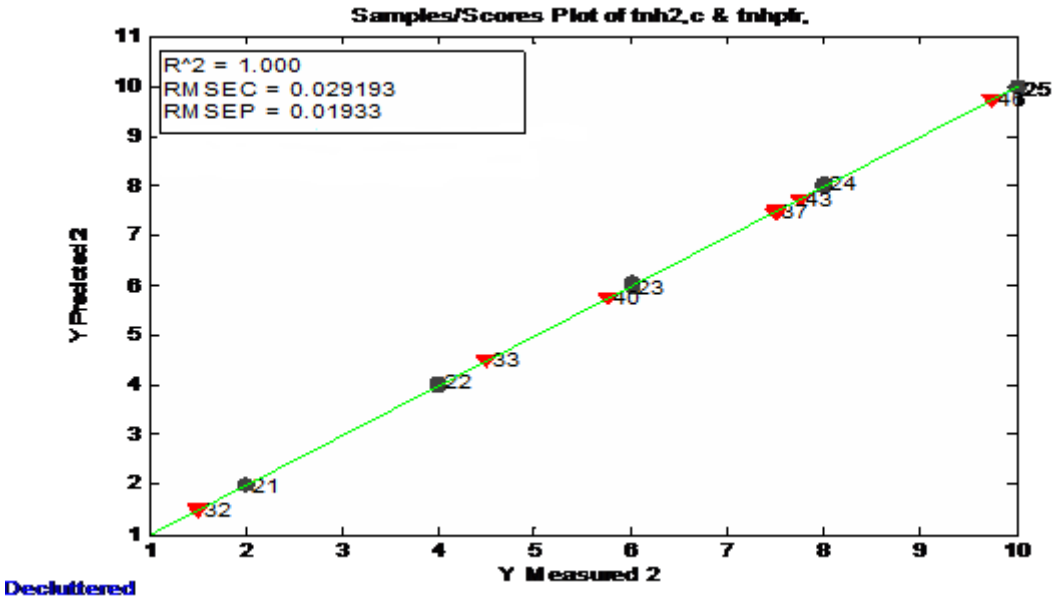
Graph.44 RMSECV values generated from calibration by PLS: nebivolol hydrochloride and hydrochlorothiazide



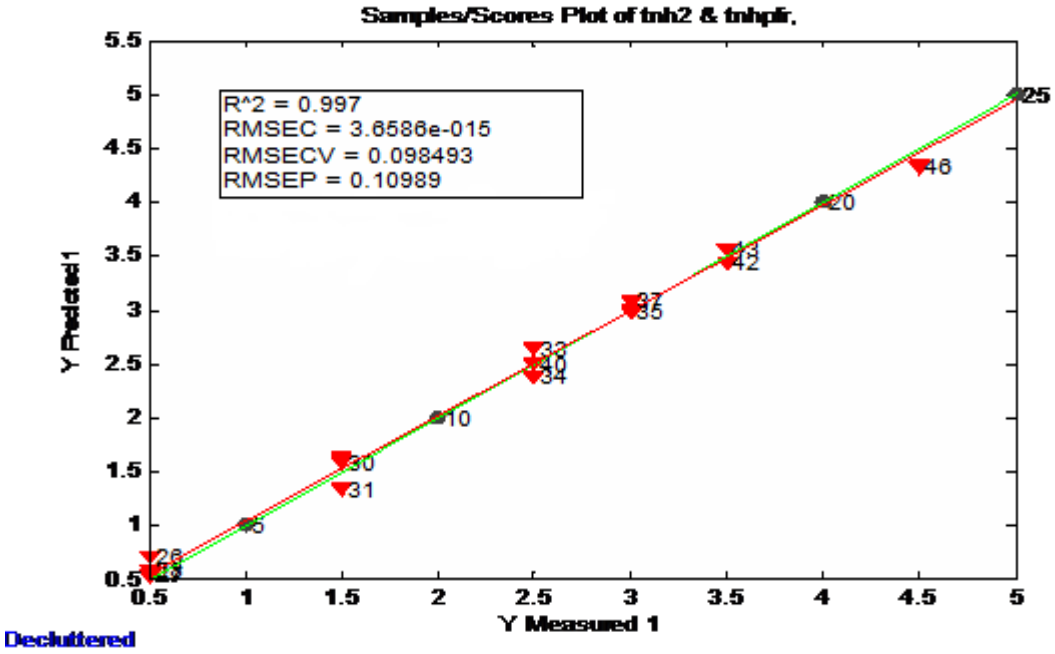
Graph.45 CLS Calibration and prediction curves of nebivolol hydrochloride



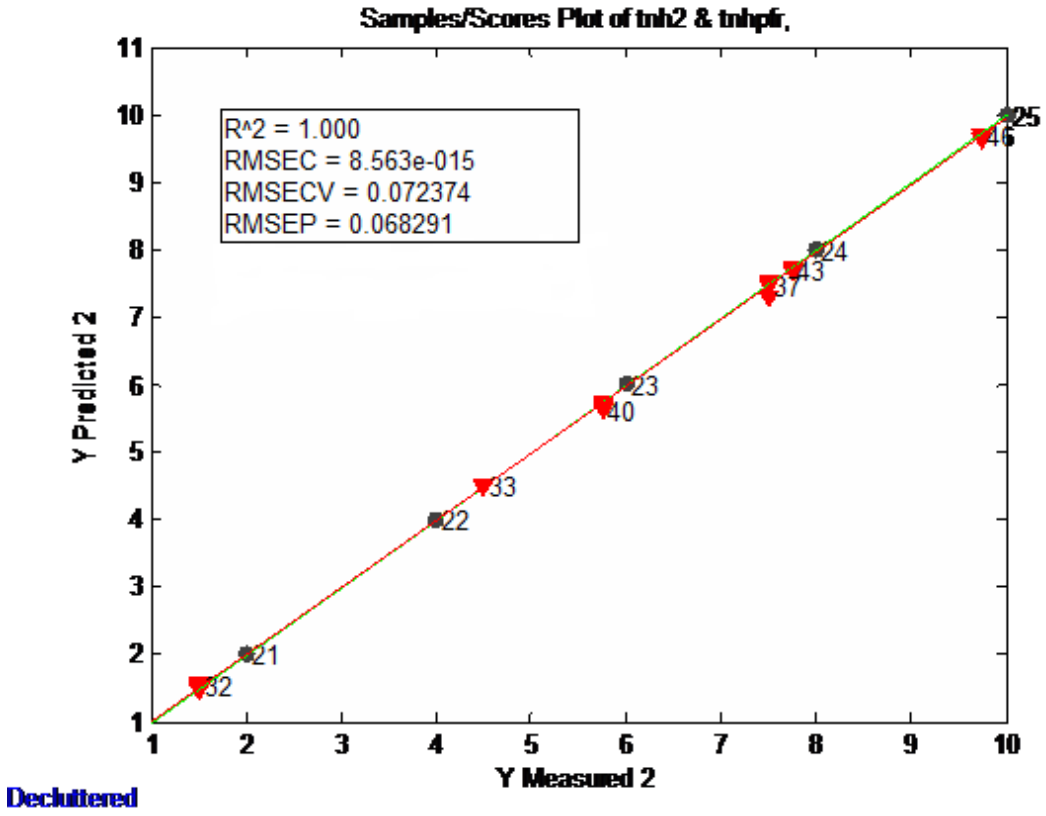
Graph.46 CLS Calibration and prediction curves of hydrochlorothiazide



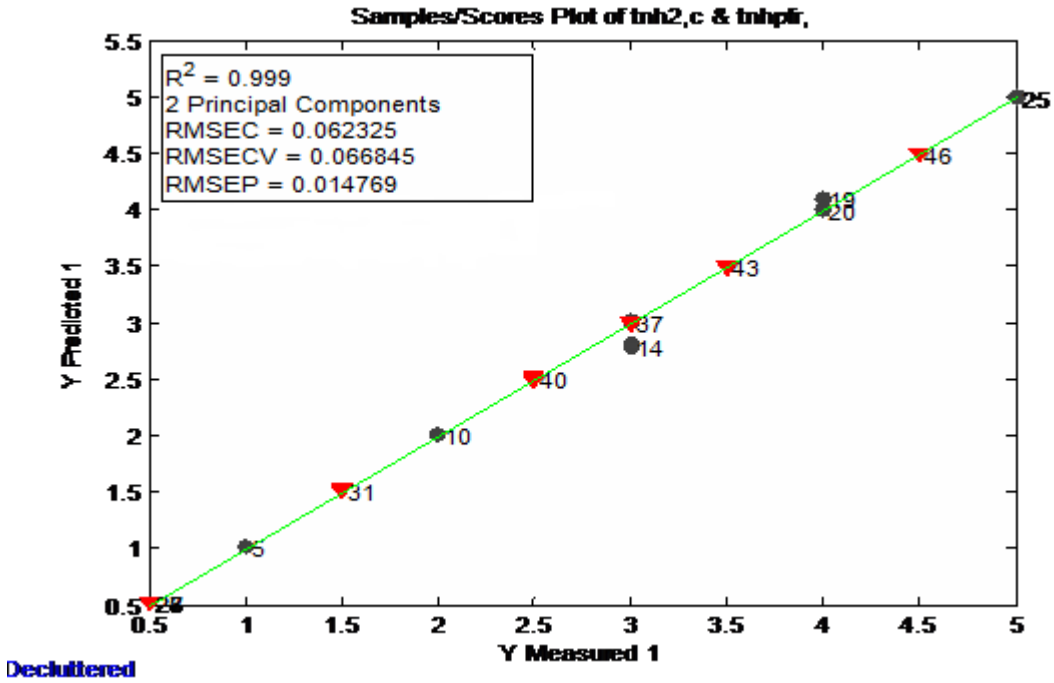
Graph.47 MLR Calibration and prediction curves of nebivolol hydrochloride



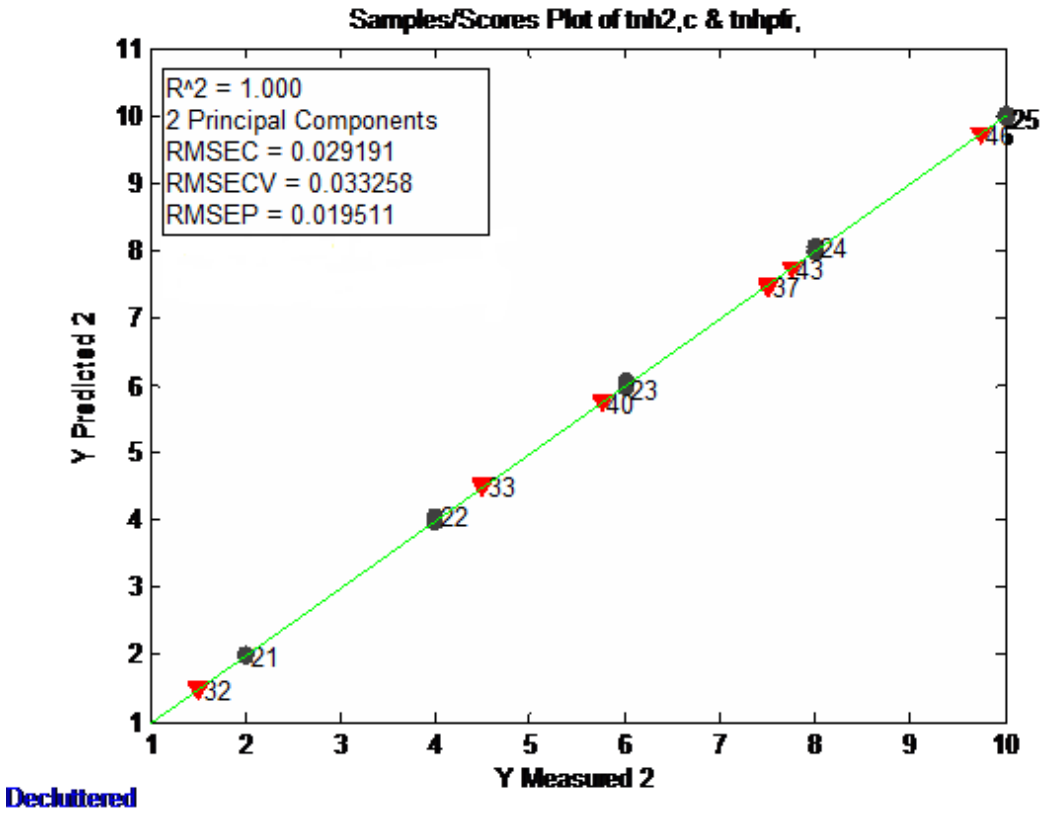
Graph.48 MLR Calibration and prediction curves of hydrochlorothiazide



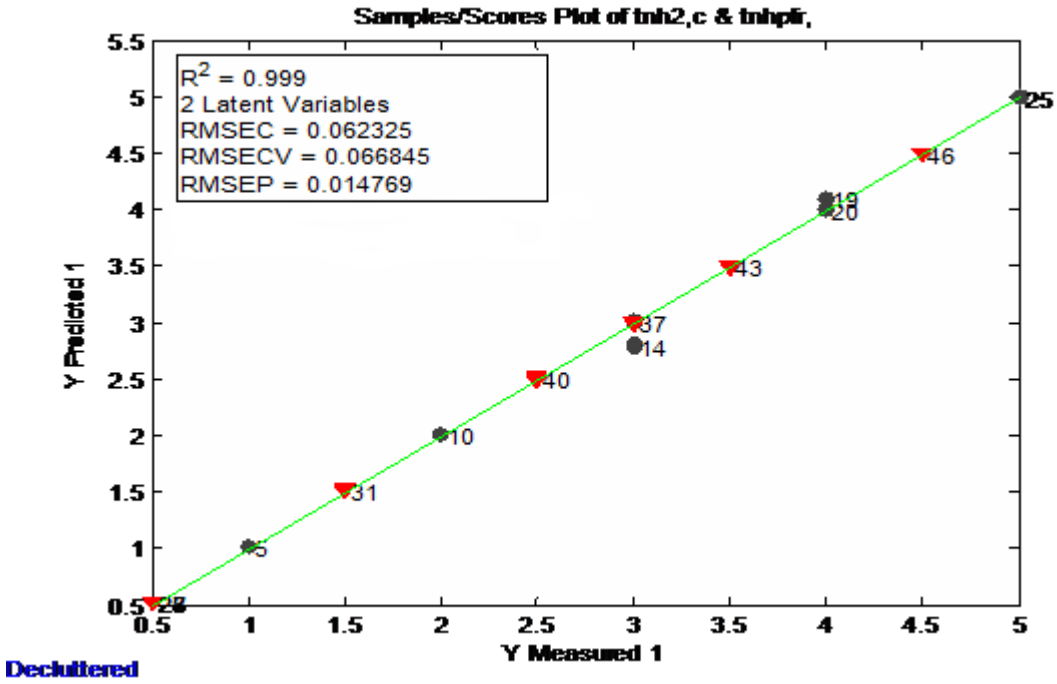
Graph.49 PCR Calibration and prediction curves of nebivolol hydrochloride



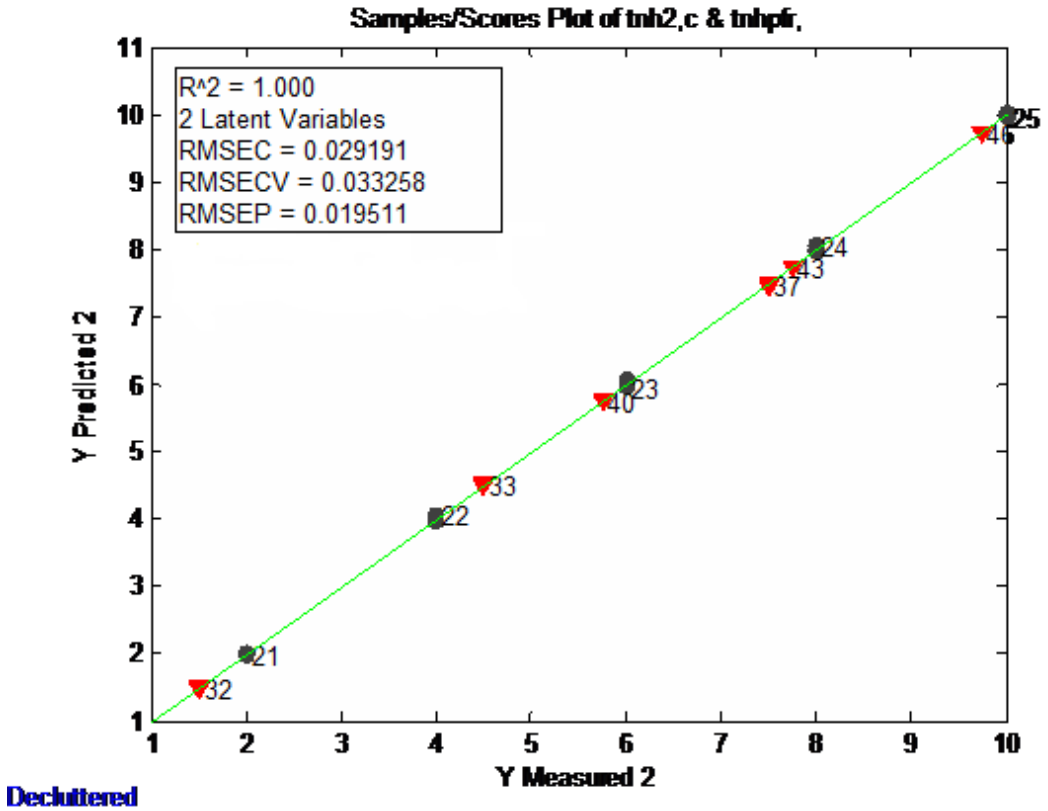
Graph.50 PCR Calibration and prediction curves of hydrochlorothiazide



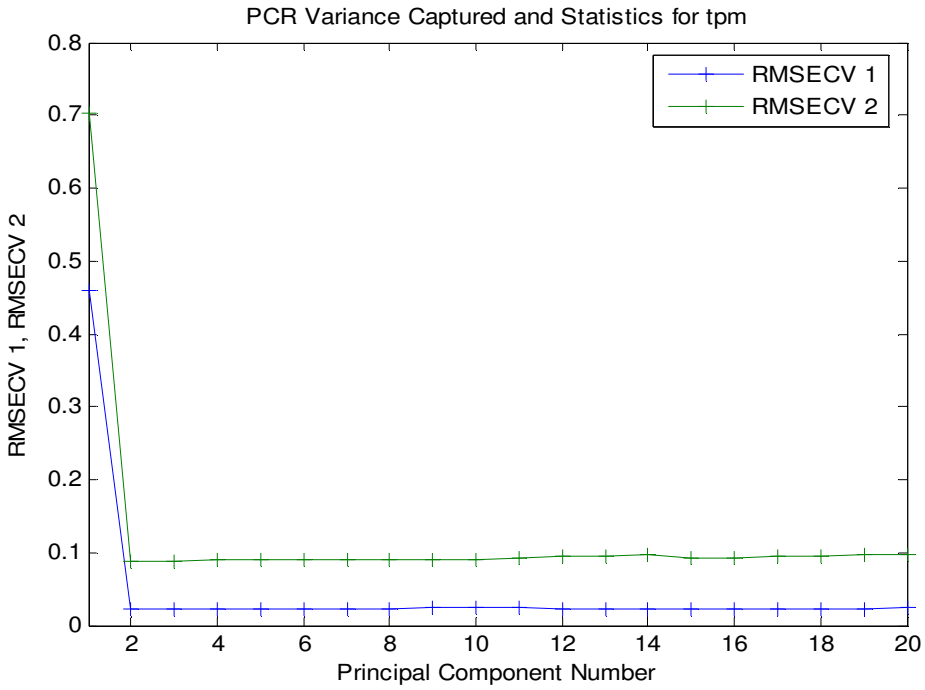
Graph.51 PLS Calibration and prediction curves of nebivolol hydrochloride



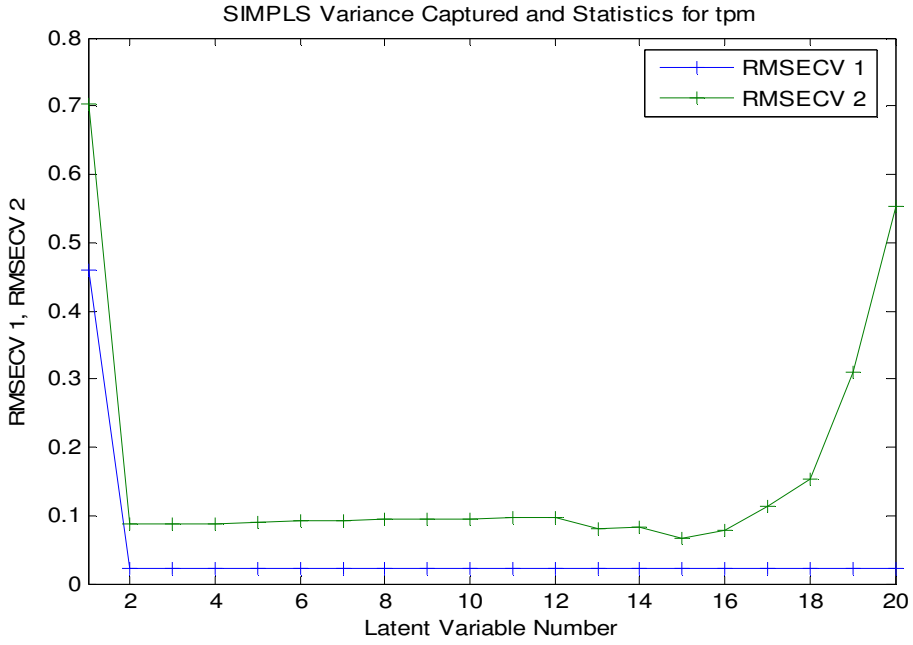
Graph.52 PLS Calibration and prediction curves of hydrochlorothiazide



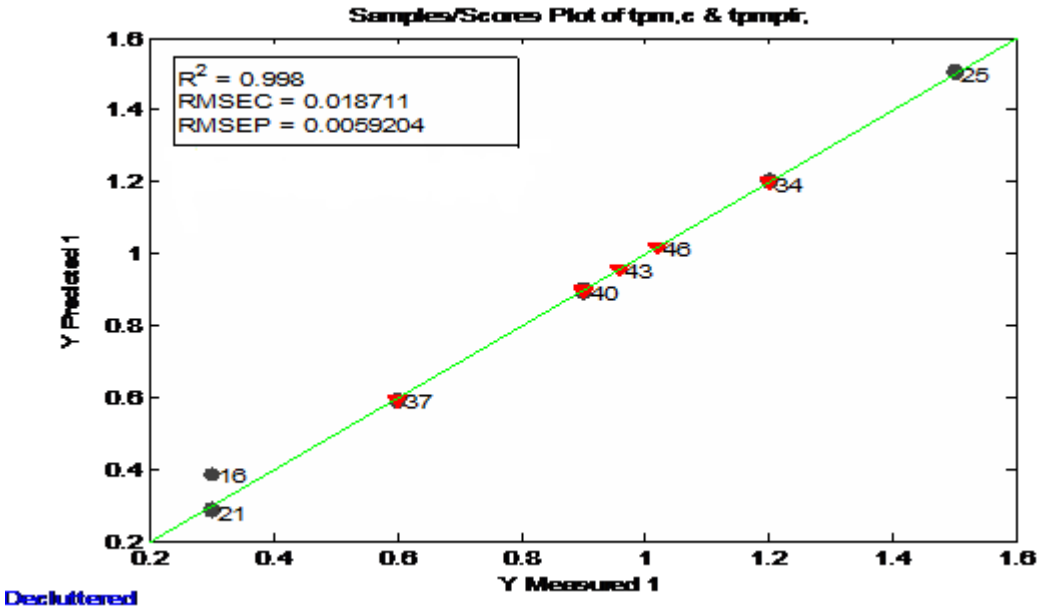
Graph.53 RMSECV values generated from calibration by PCR: pioglitazone hydrochloride and metformin hydrochloride



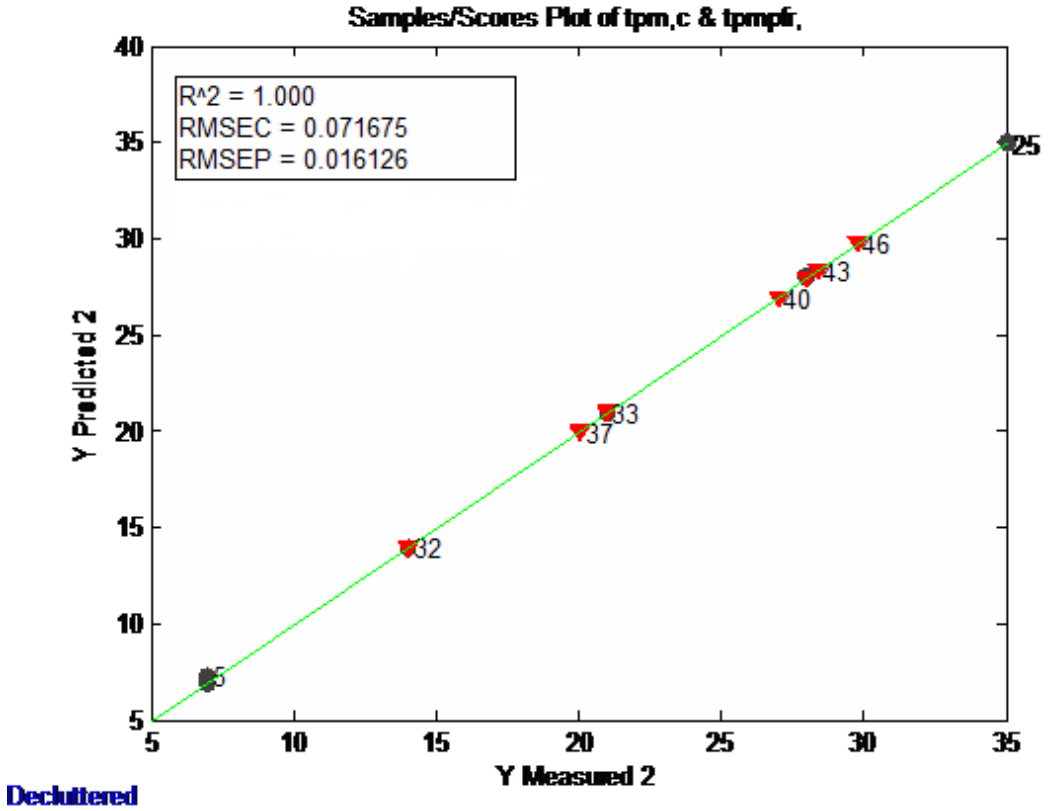
Graph.54 RMSECV values generated from calibration by PLS: pioglitazone hydrochloride and metformin hydrochloride



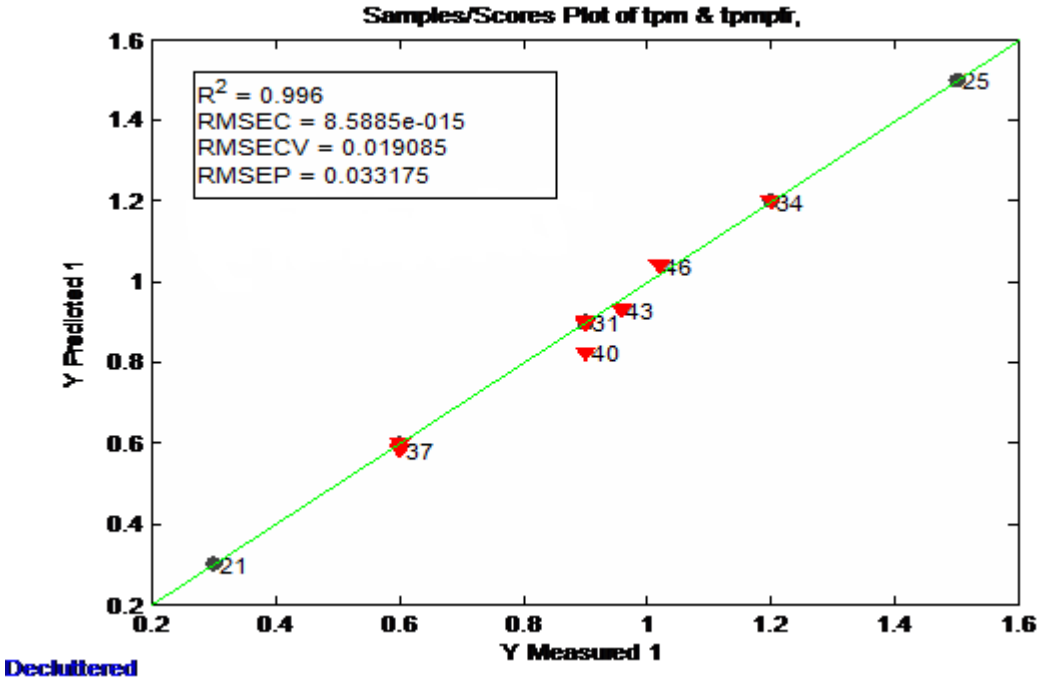
Graph.55 CLS Calibration and prediction curves of pioglitazone hydrochloride



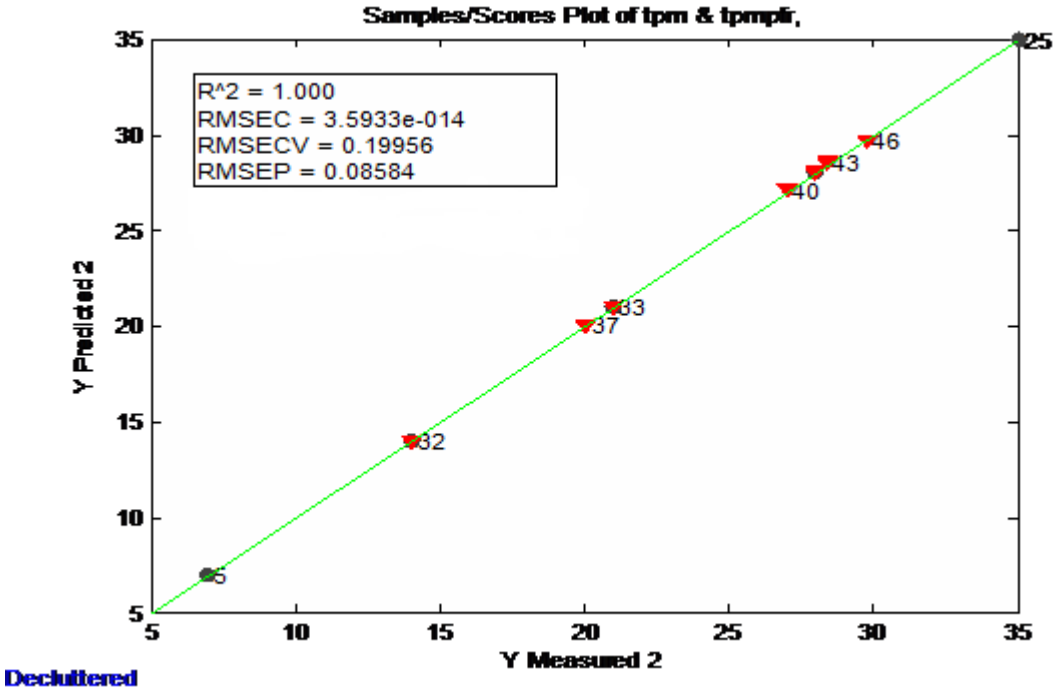
Graph.56 CLS Calibration and prediction curves of metformin hydrochloride



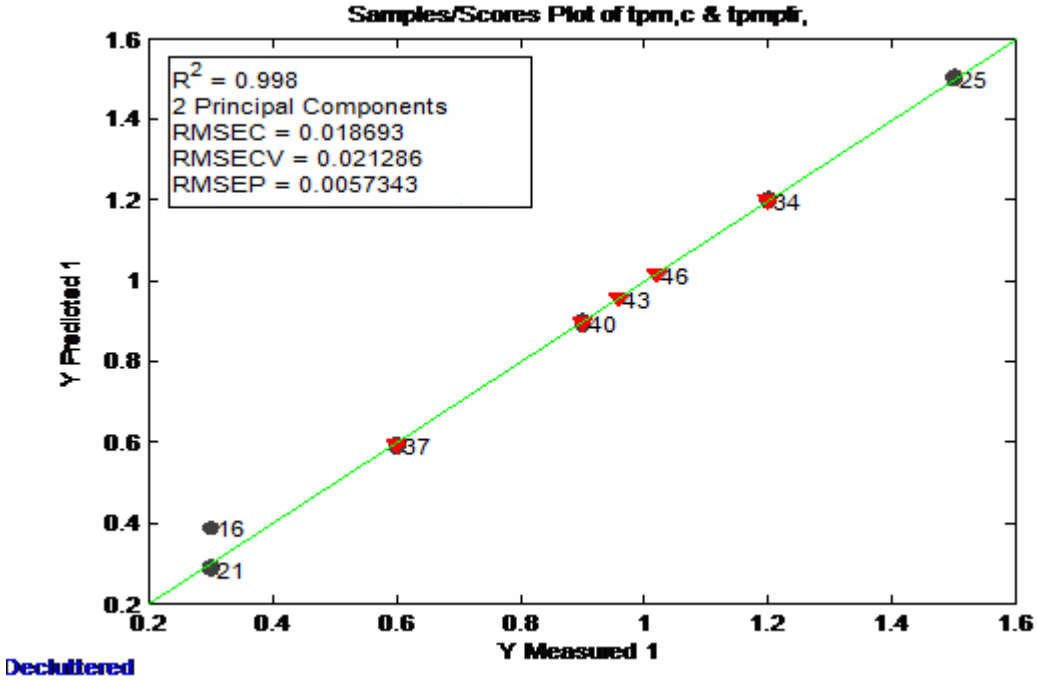
Graph.57 MLR Calibration and prediction curves of pioglitazone hydrochloride



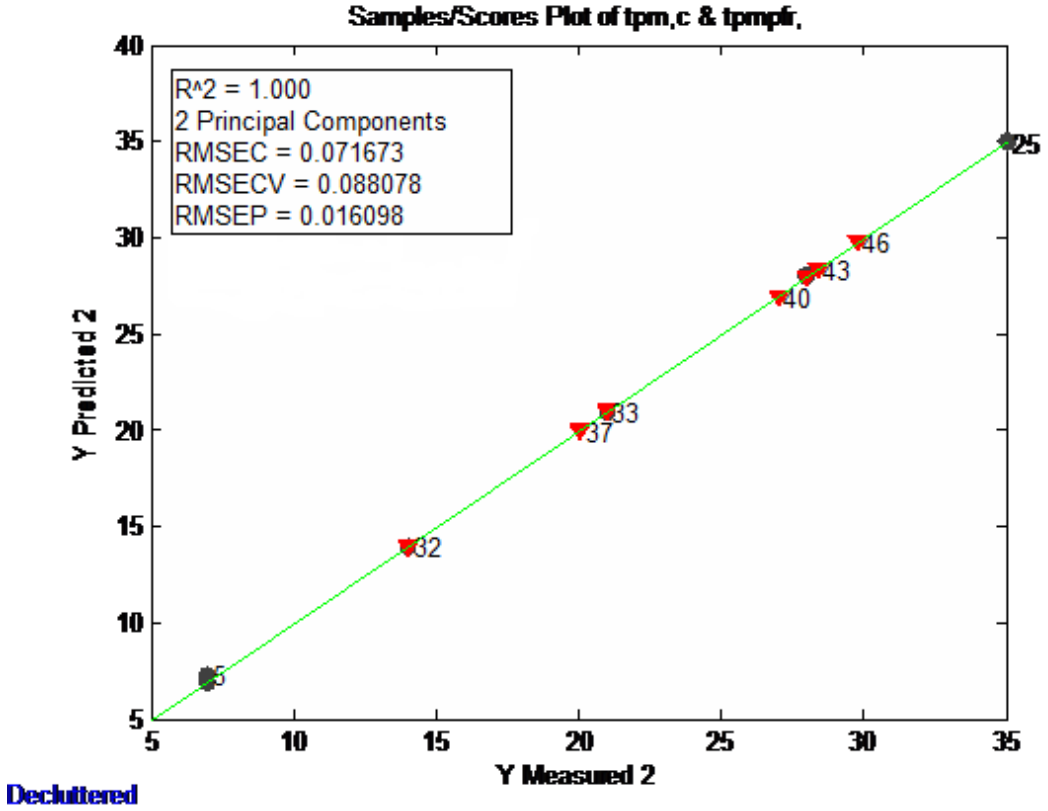
Graph.58 MLR Calibration and prediction curves of metformin hydrochloride



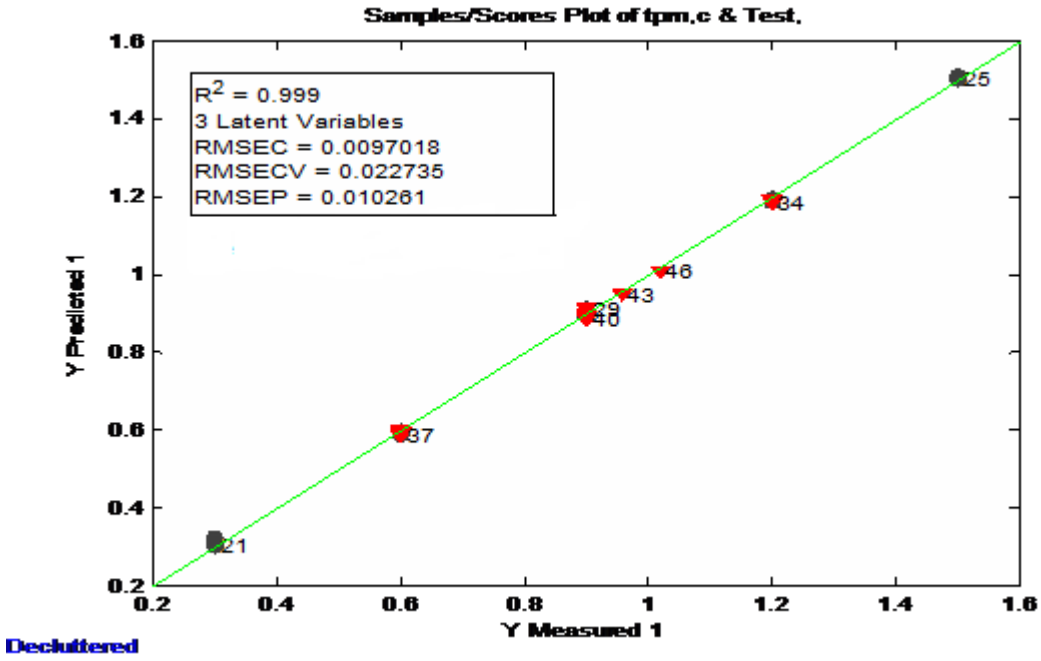
Graph.59 PCR Calibration and prediction curves of pioglitazone hydrochloride



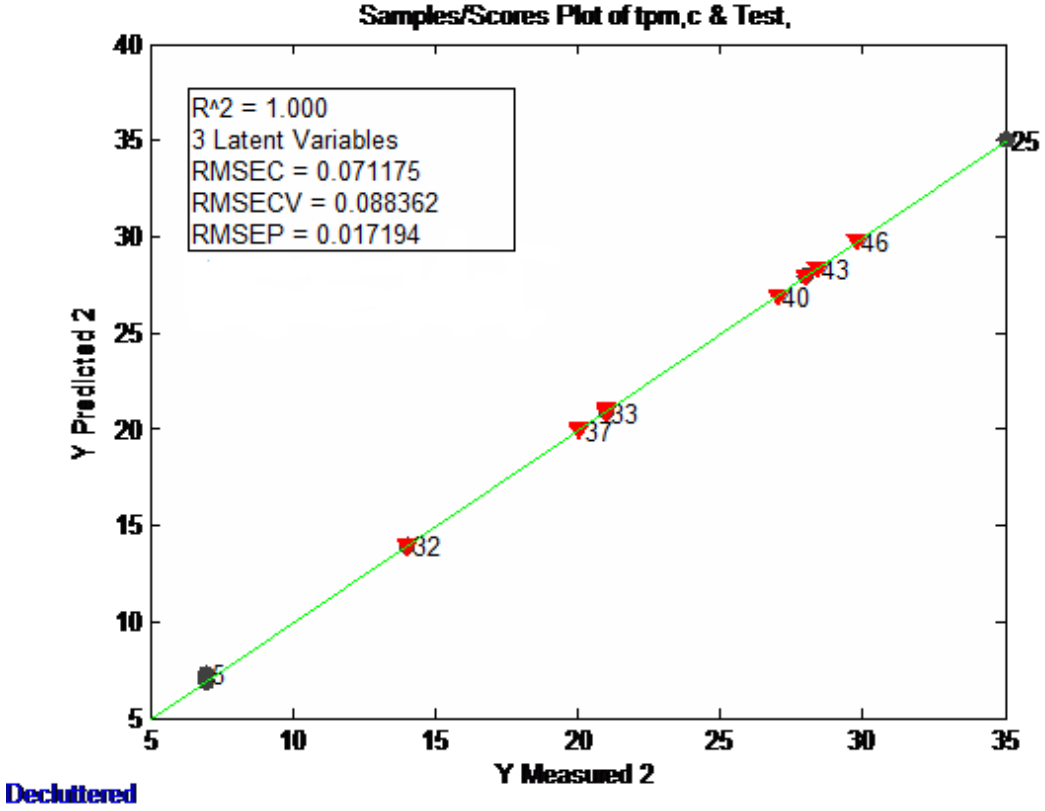
Graph.60 PCR Calibration and prediction curves of metformin hydrochloride



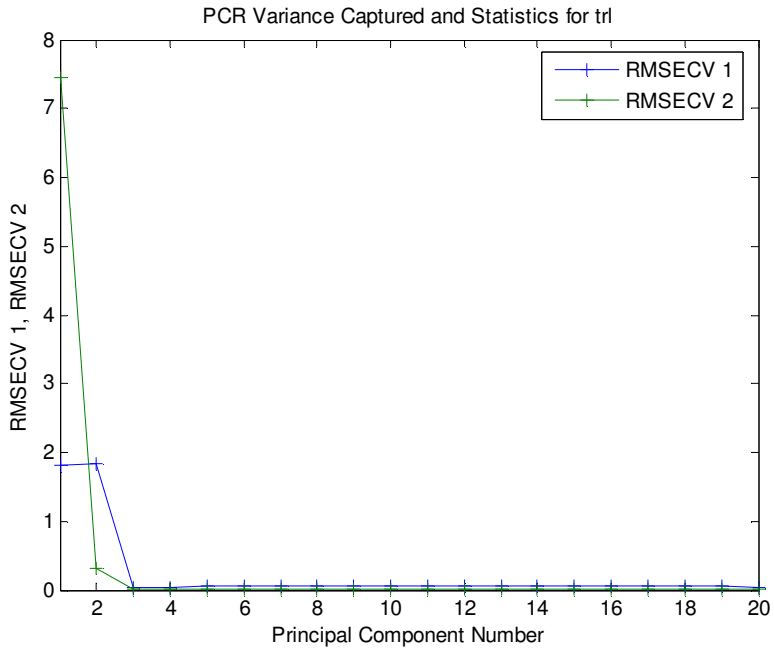
Graph.61 PLS Calibration and prediction curves of pioglitazone hydrochloride



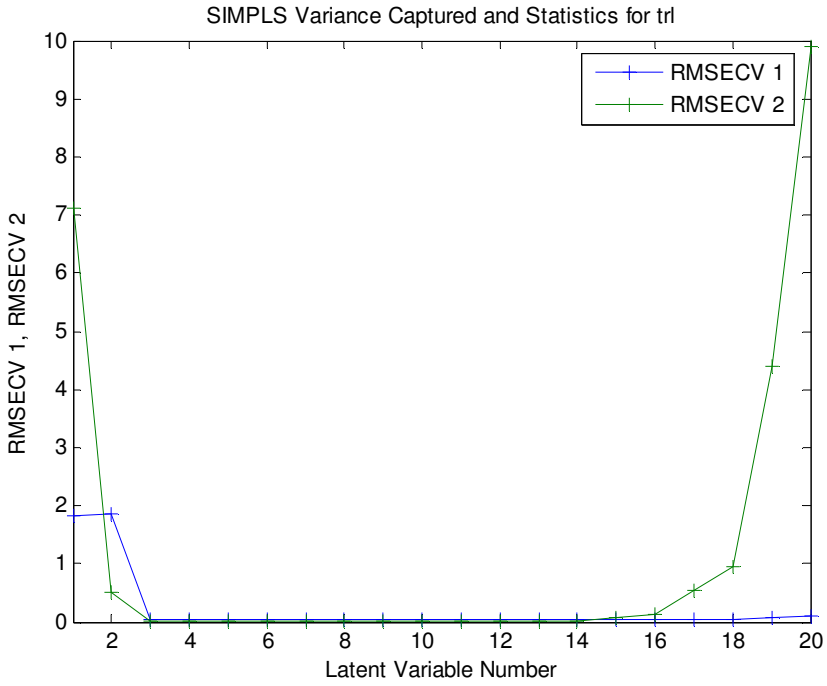
Graph.62 PLS Calibration and prediction curves of metformin hydrochloride



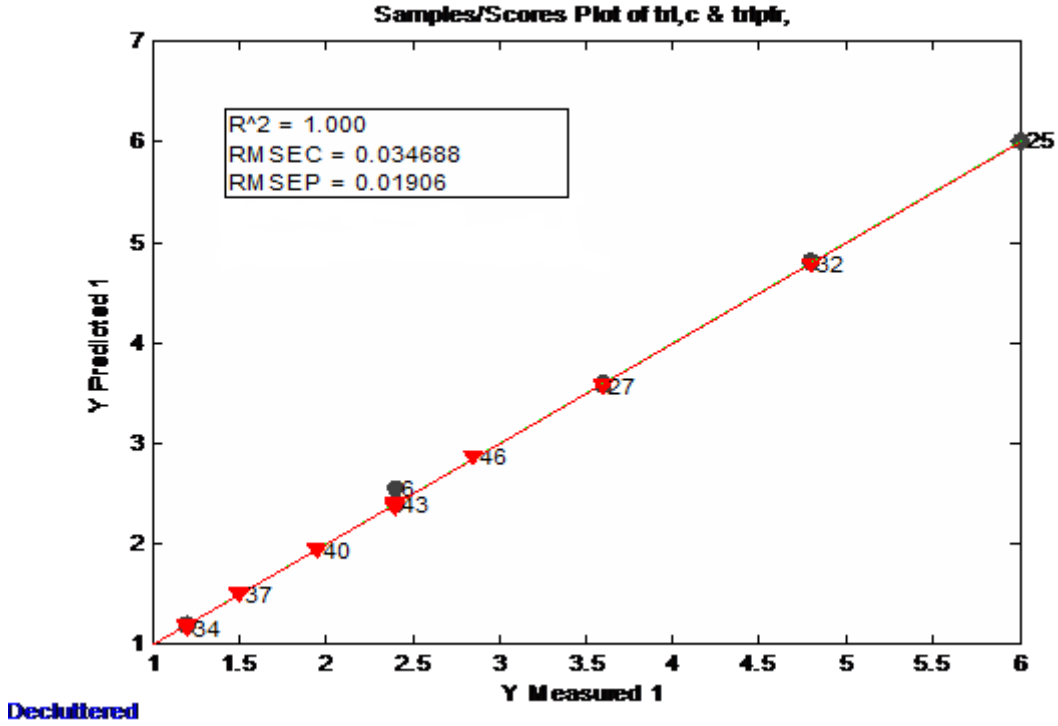
Graph.63 RMSECV values generated from calibration by PCR: losartan potassium and ramipril



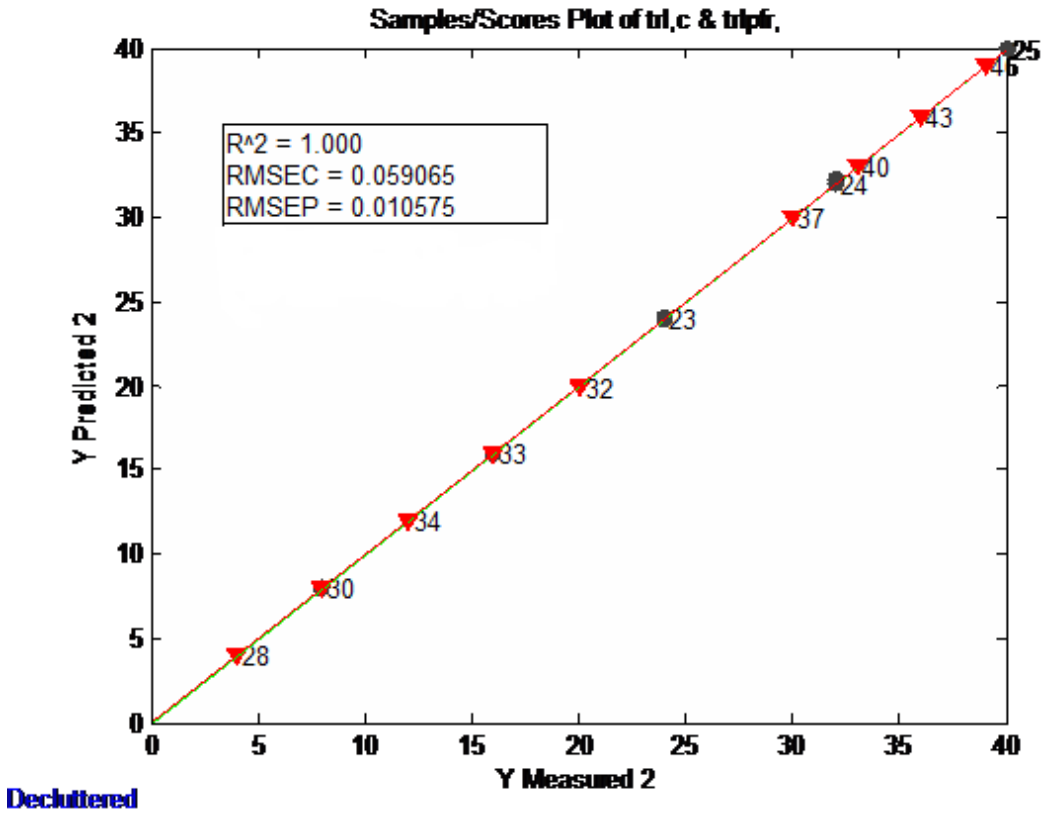
Graph.64 RMSECV values generated from calibration by PLS: losartan potassium and ramipril



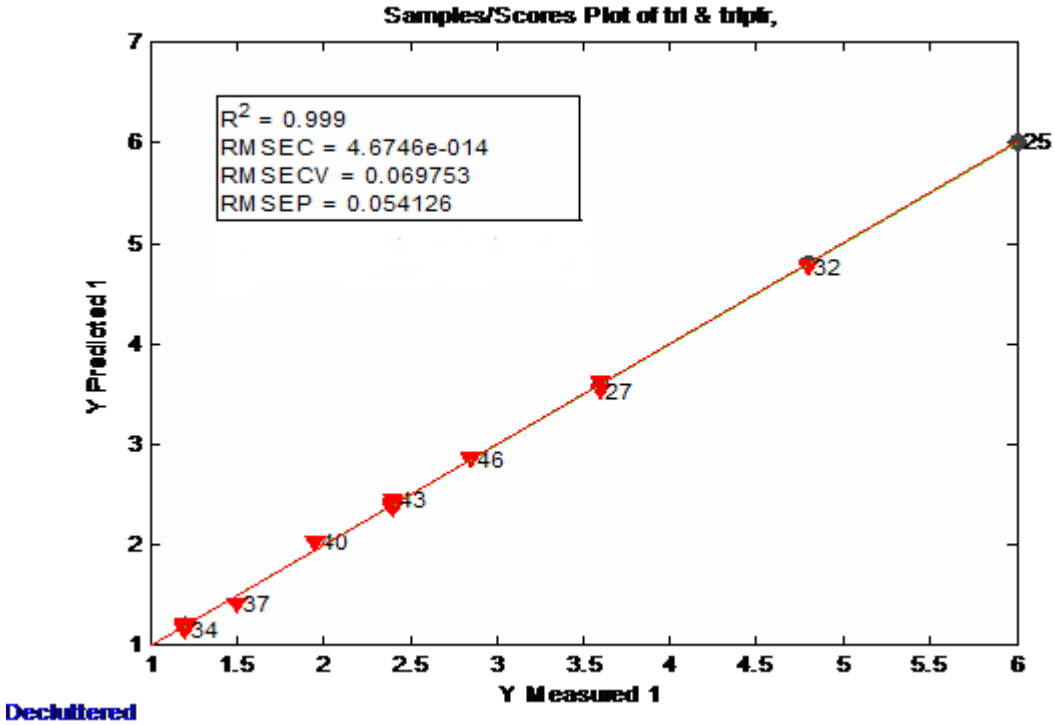
Graph.65 CLS Calibration and prediction curves of losartan potassium



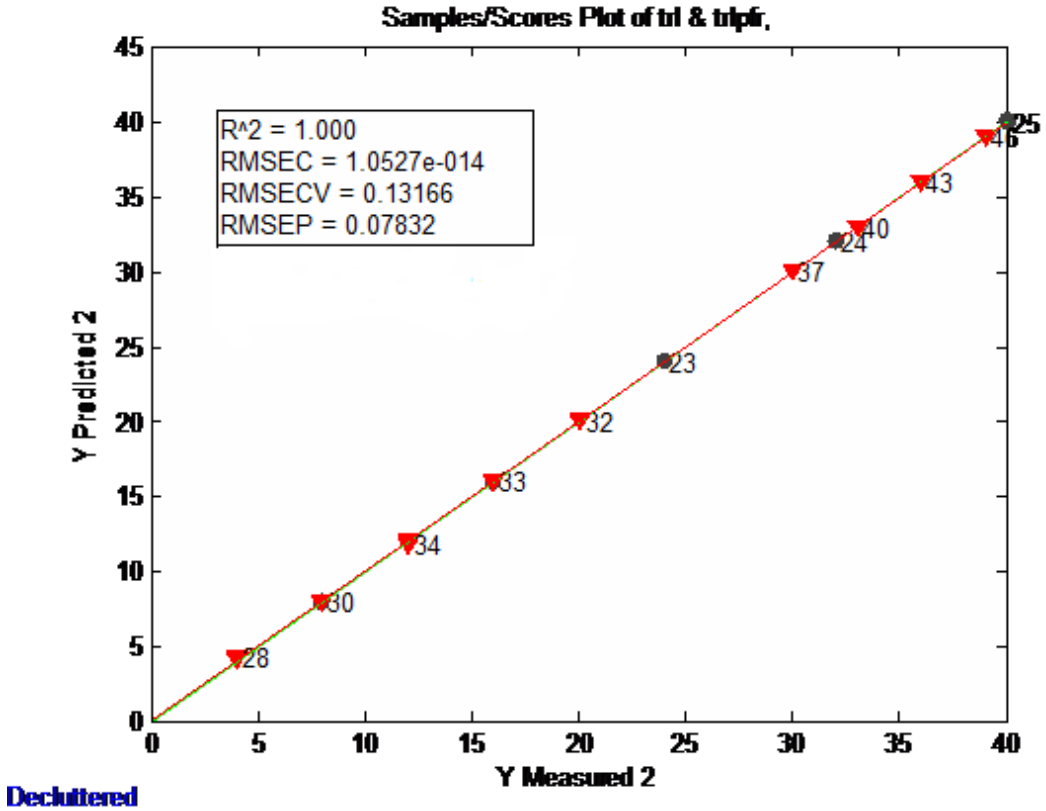
Graph.66 CLS Calibration and prediction curves of ramipril



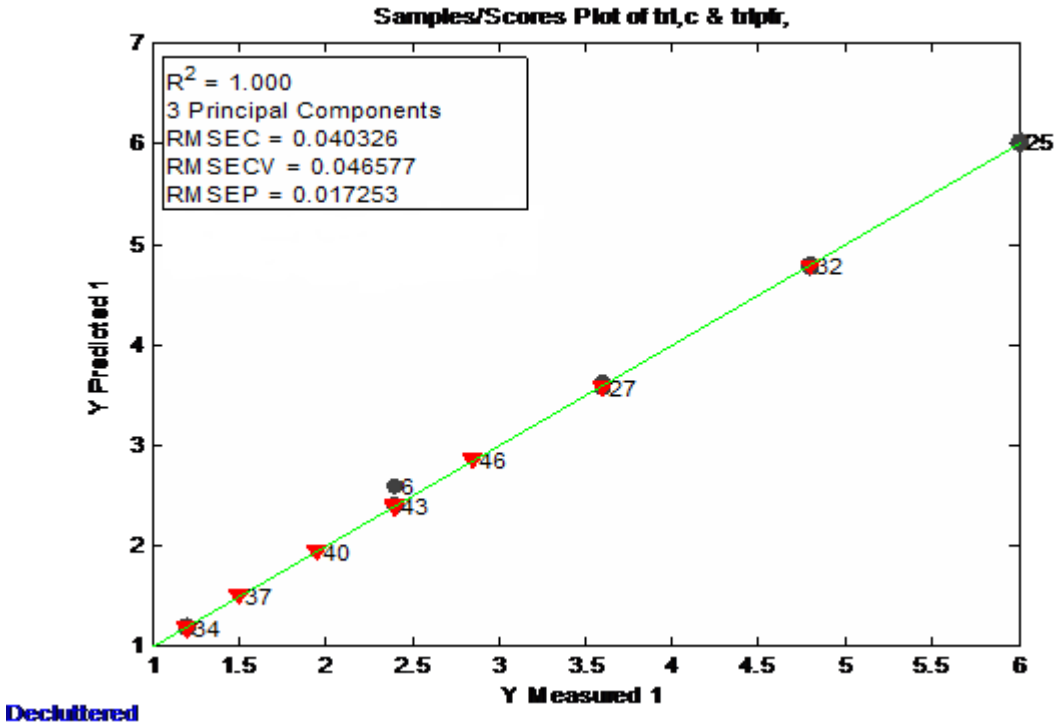
Graph.67 MLR Calibration and prediction curves of losartan potassium



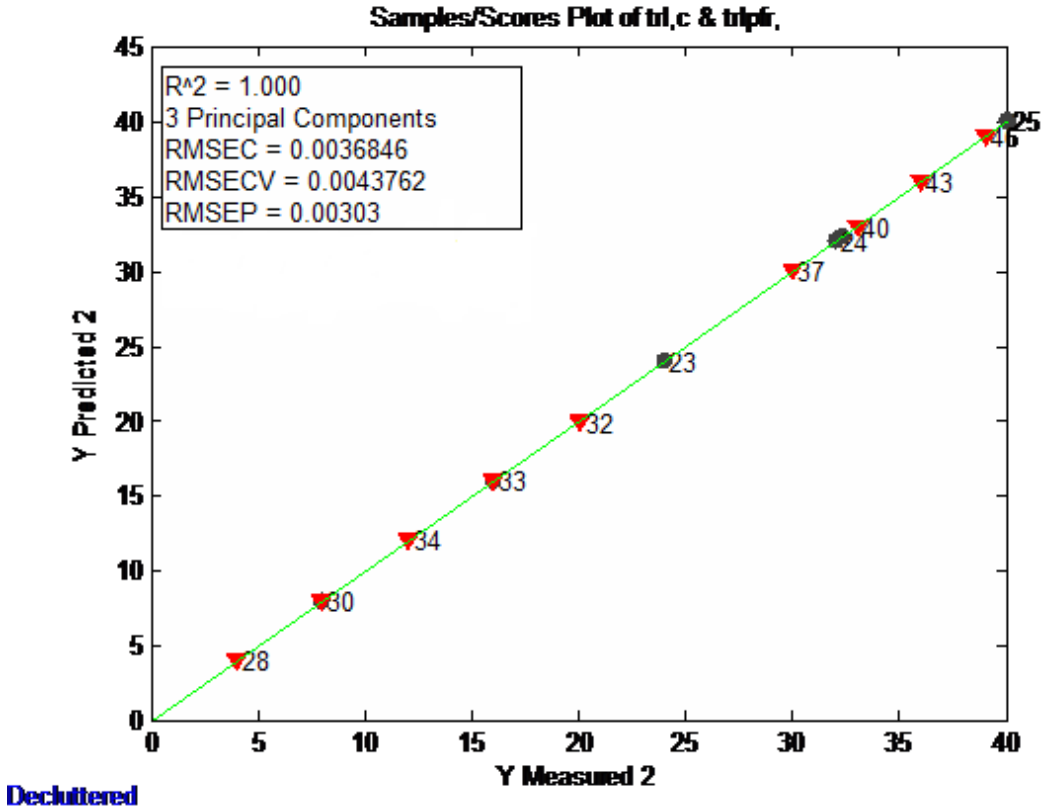
Graph.68 MLR Calibration and prediction curves of ramipril



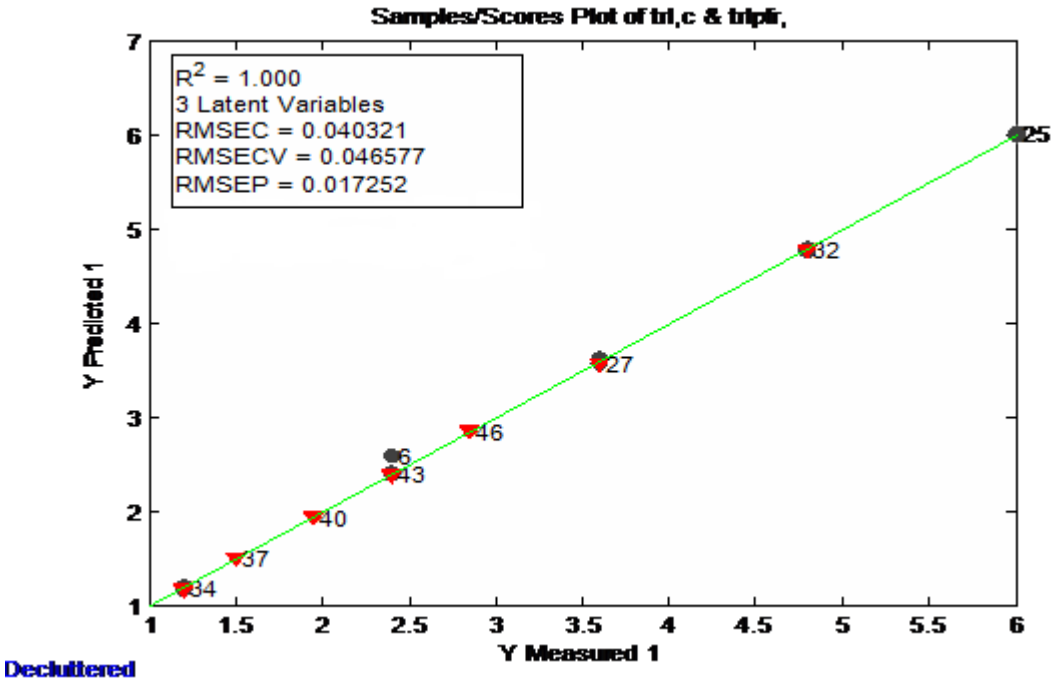
Graph.69 PCR Calibration and prediction curves of losartan potassium



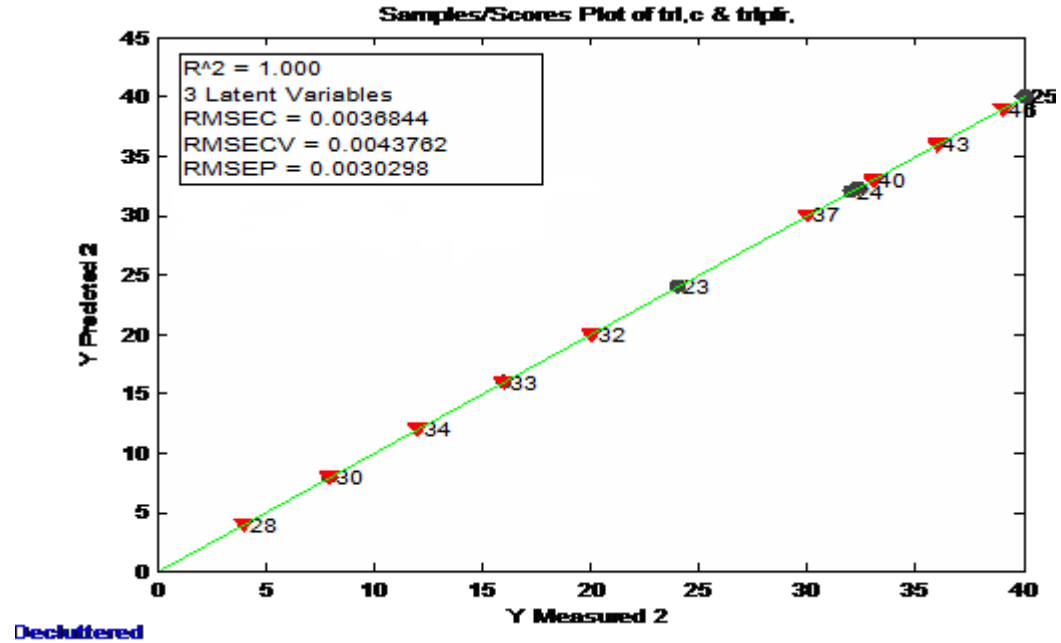
Graph.70 PCR Calibration and prediction curves of ramipril



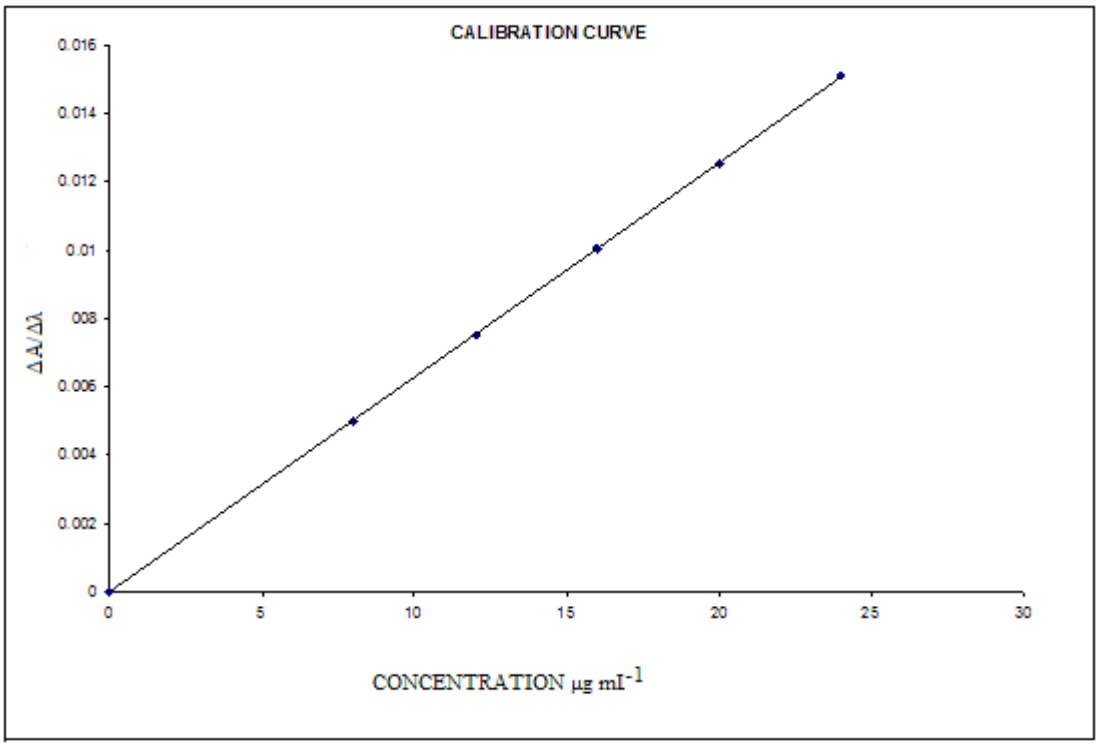
Graph.71 PLS Calibration and prediction curves of losartan potassium



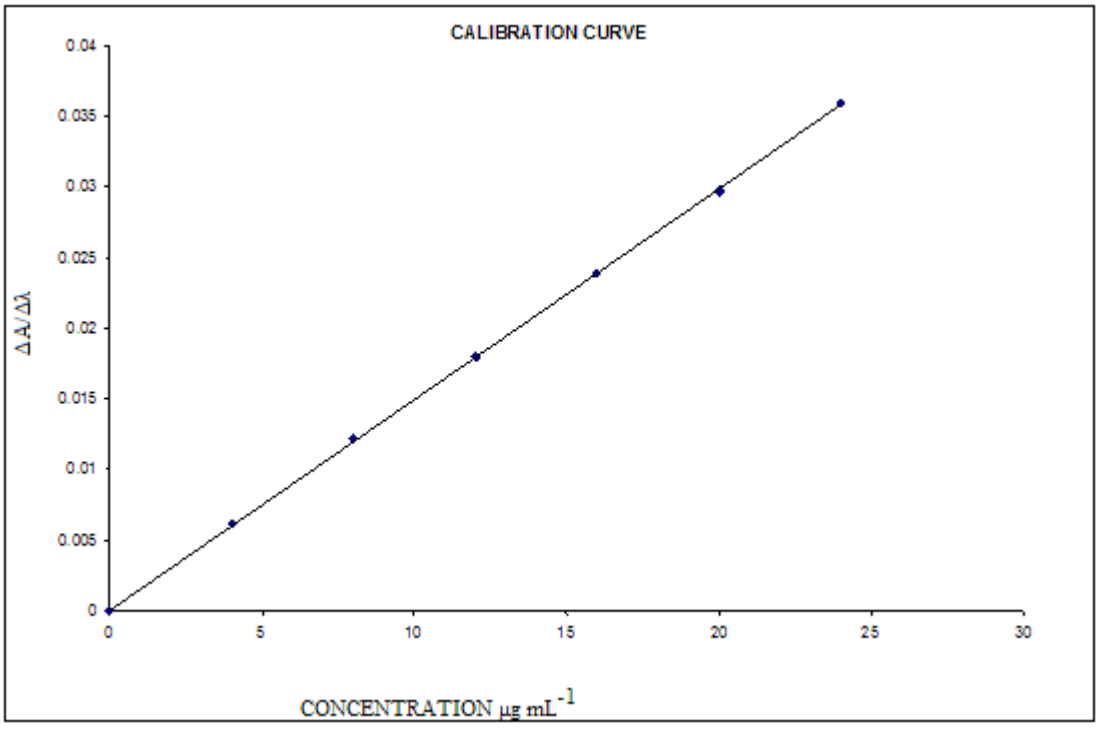
Graph.72 PLS Calibration and prediction curves of ramipril



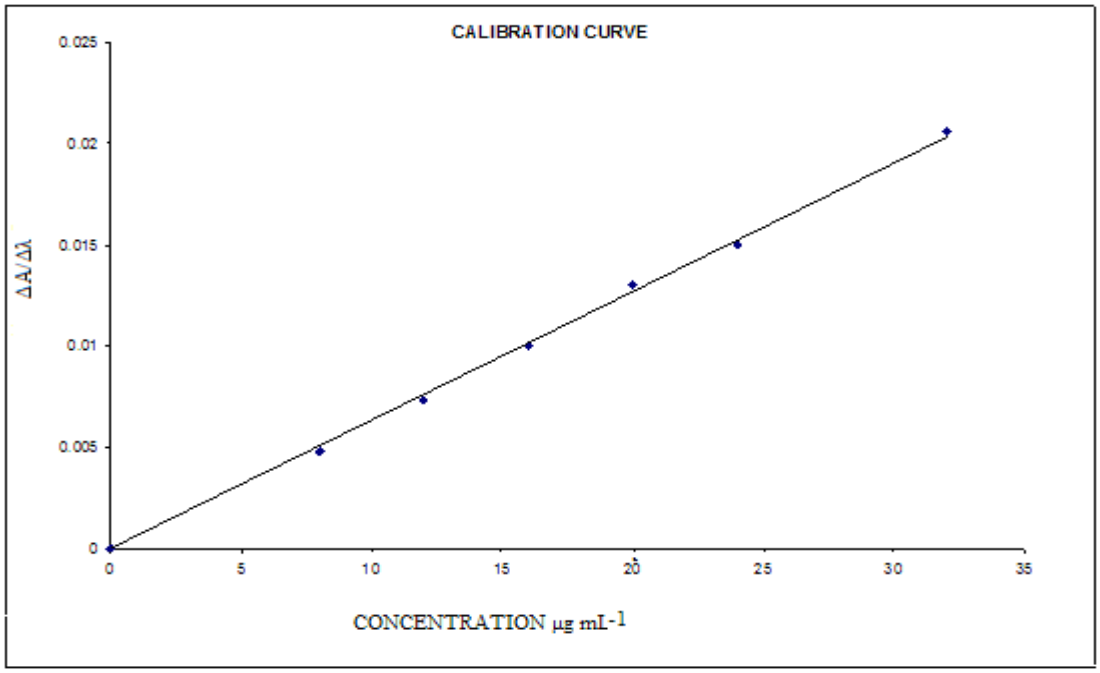
Graph.73 Calibration curve of atorvastatin calcium at 239nm by first order derivative spectroscopy



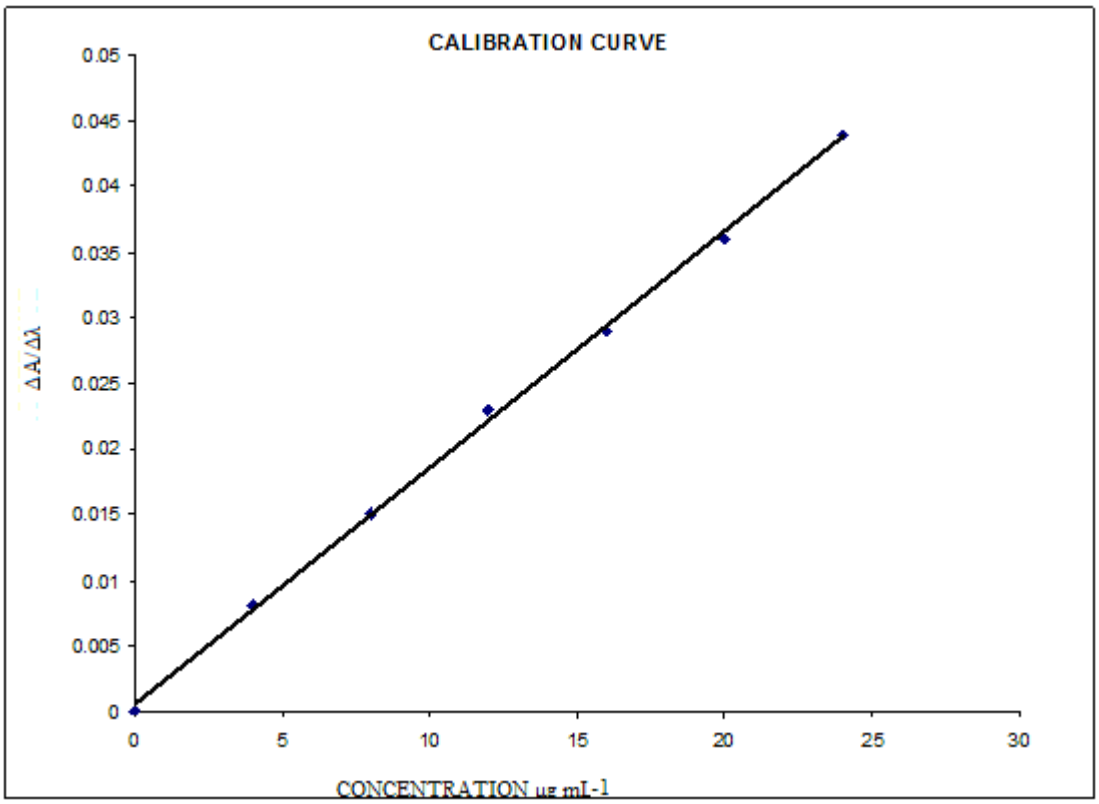
Graph.74 Calibration curve of fenofibrate at 246nm by first order derivative spectroscopy



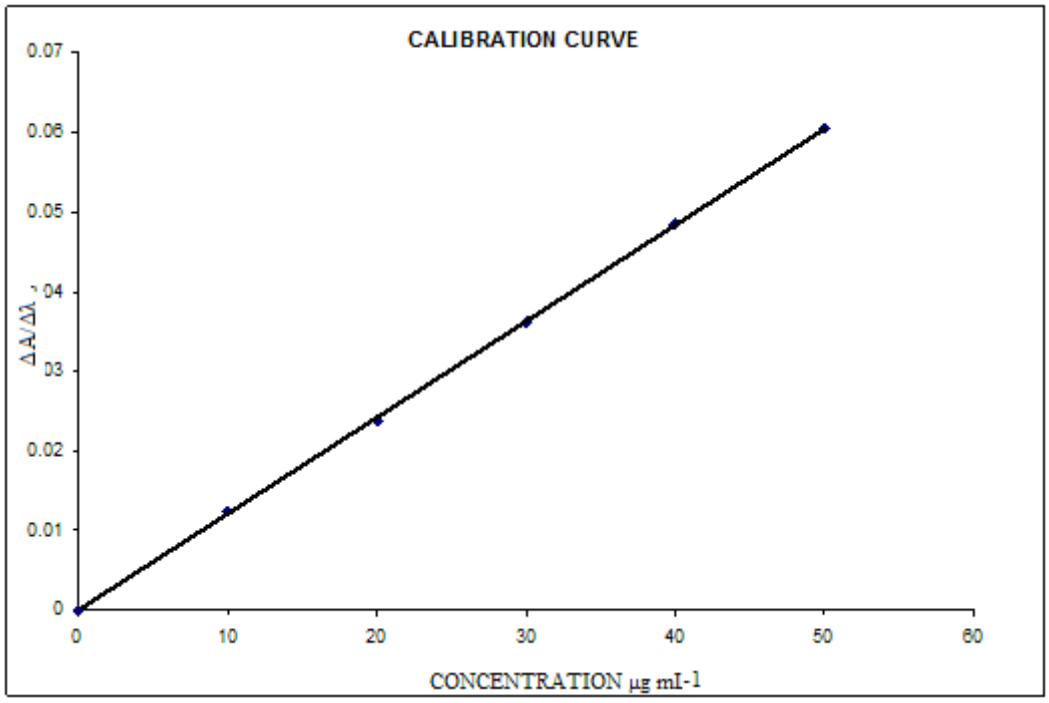
Graph.75 Calibration curve of atorvastatin calcium at 224nm by first order derivative spectroscopy



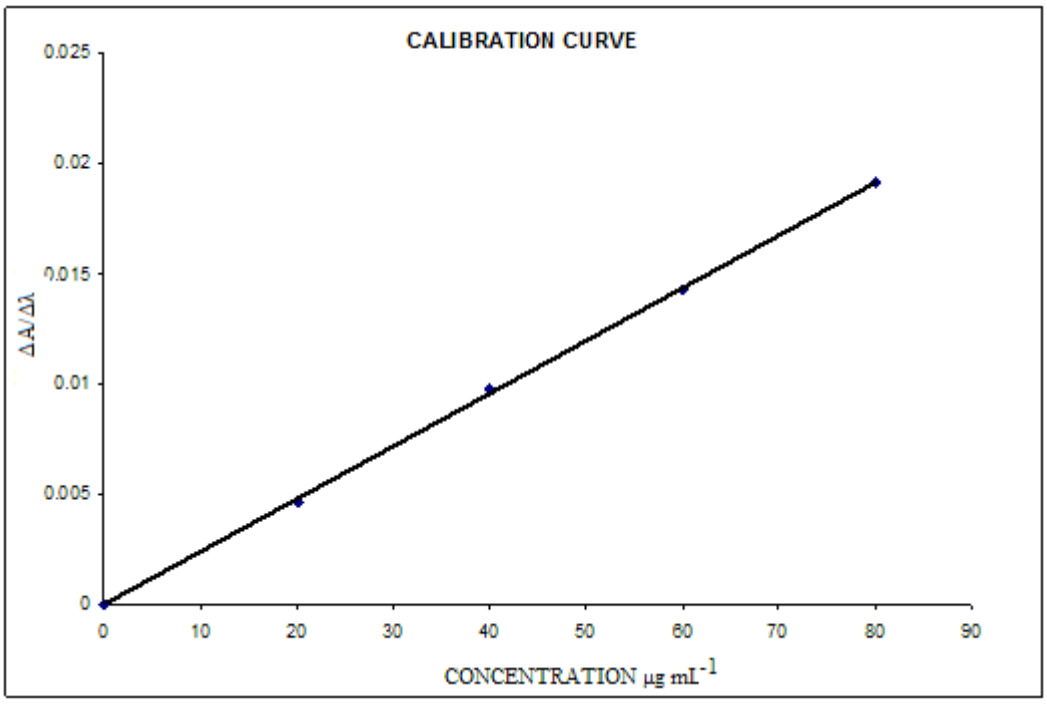
Graph.76 Calibration curve of ezetimibe at 233nm by first order derivative spectroscopy



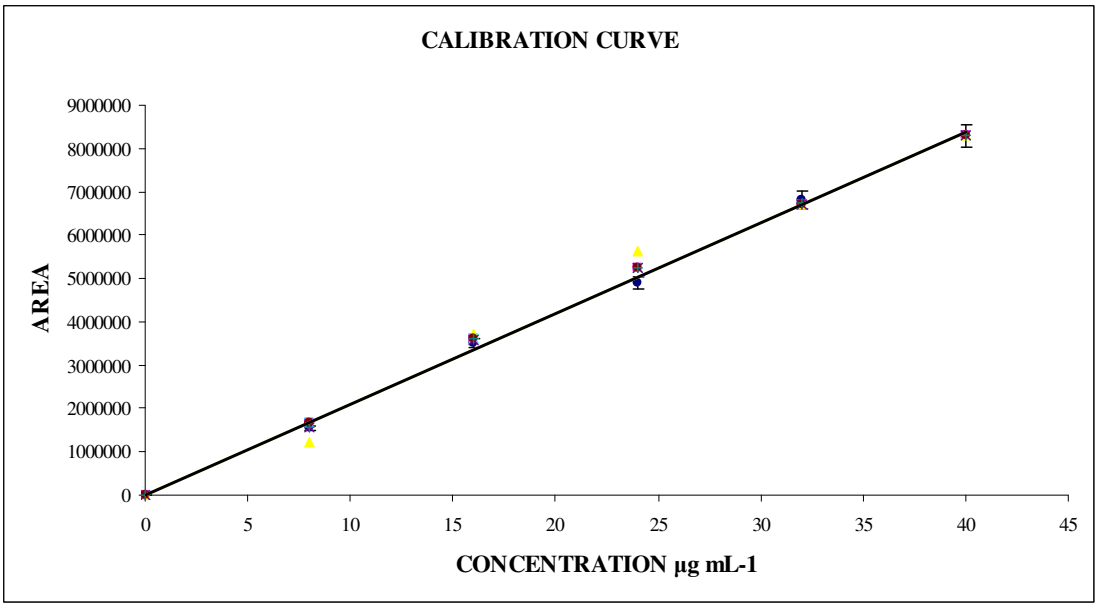
Graph.77 Calibration curve of of levofloxacin hemihydrate at 275.5nm by first order derivative spectroscopy



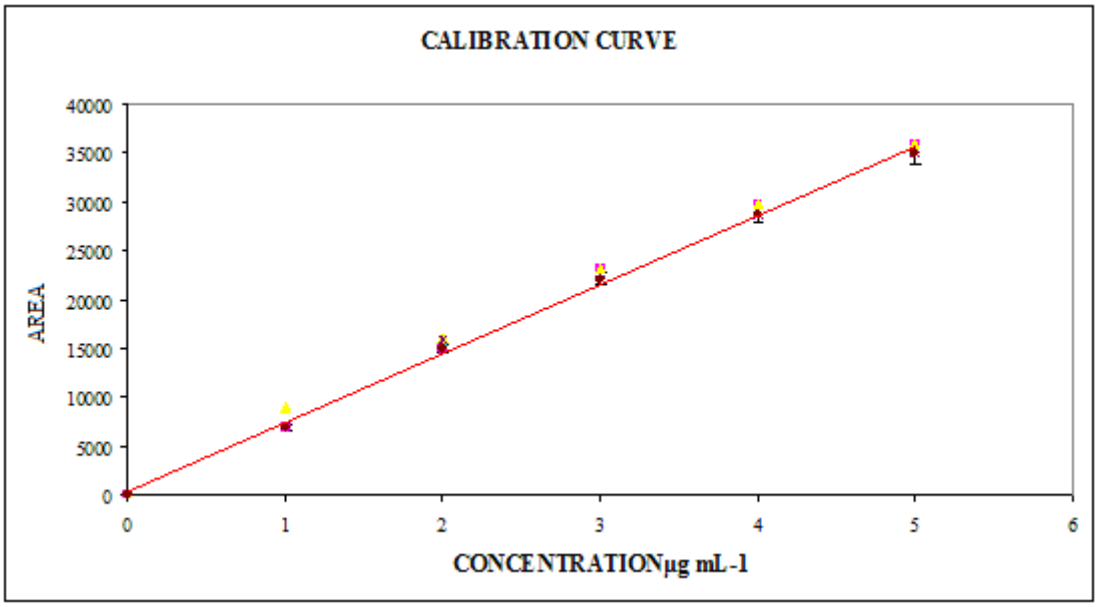
Graph.78 Calibration curve of ornidazole at 319nm by first order derivative spectroscopy



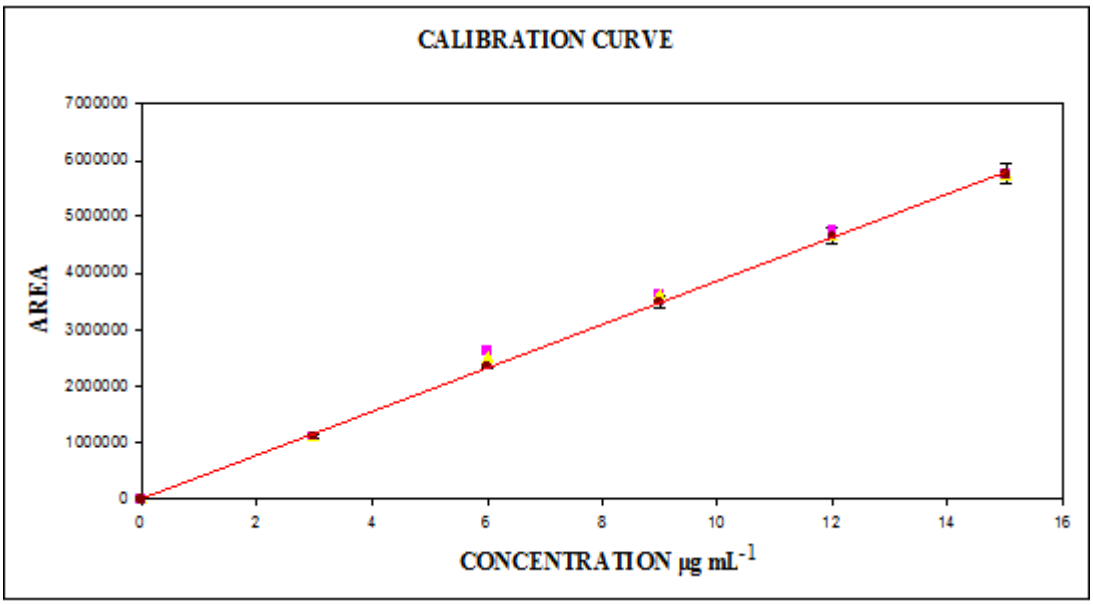
Graph.79 Calibration curve of losartan potassium by RP-HPLC



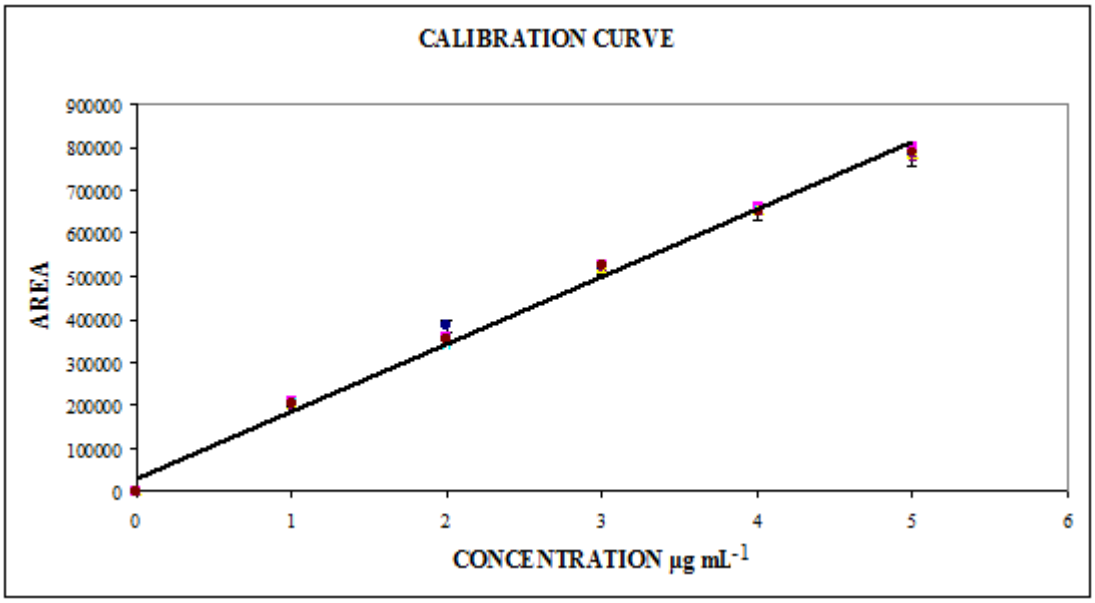
Graph.80 Calibration curve of amlodipine besylate by RP-HPLC



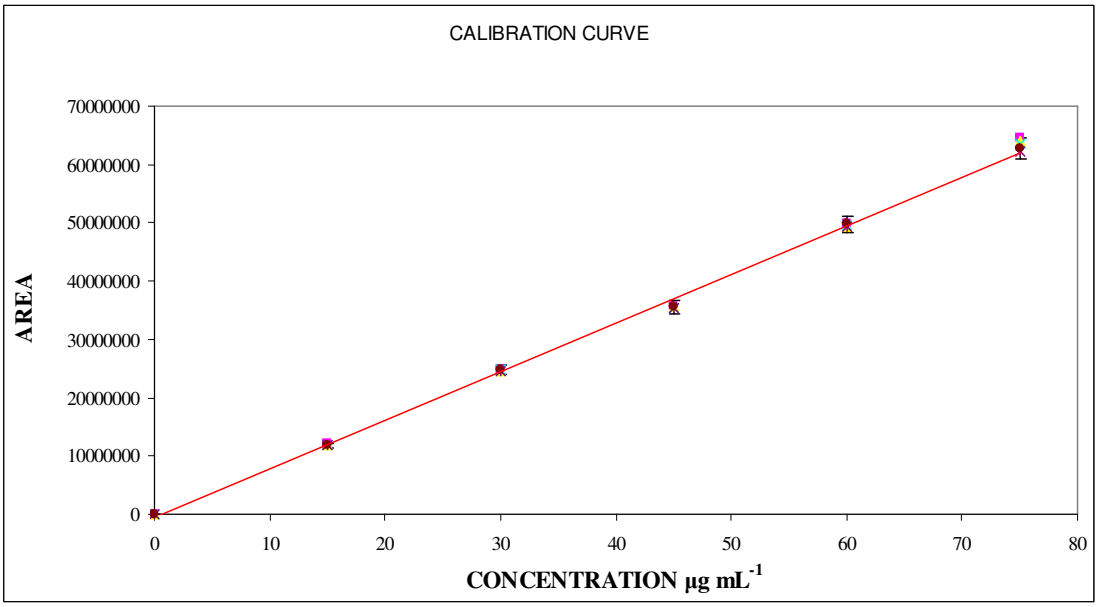
Graph.81 Calibration curve of hydrochlorothiazide by RP-HPLC



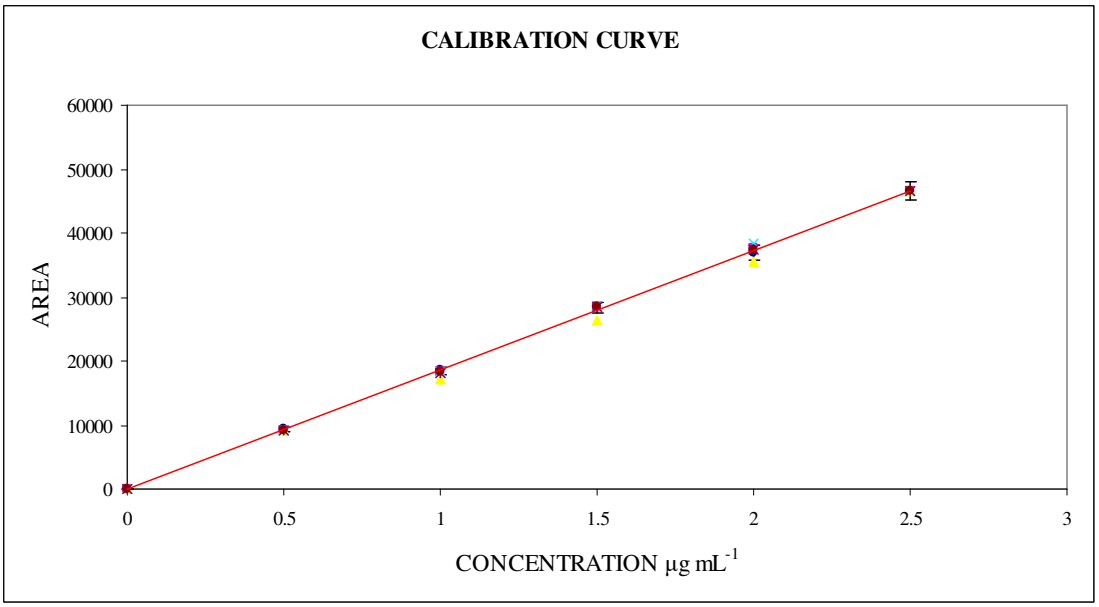
Graph.82 Calibration curve of atrovastatin calcium by RP-HPLC



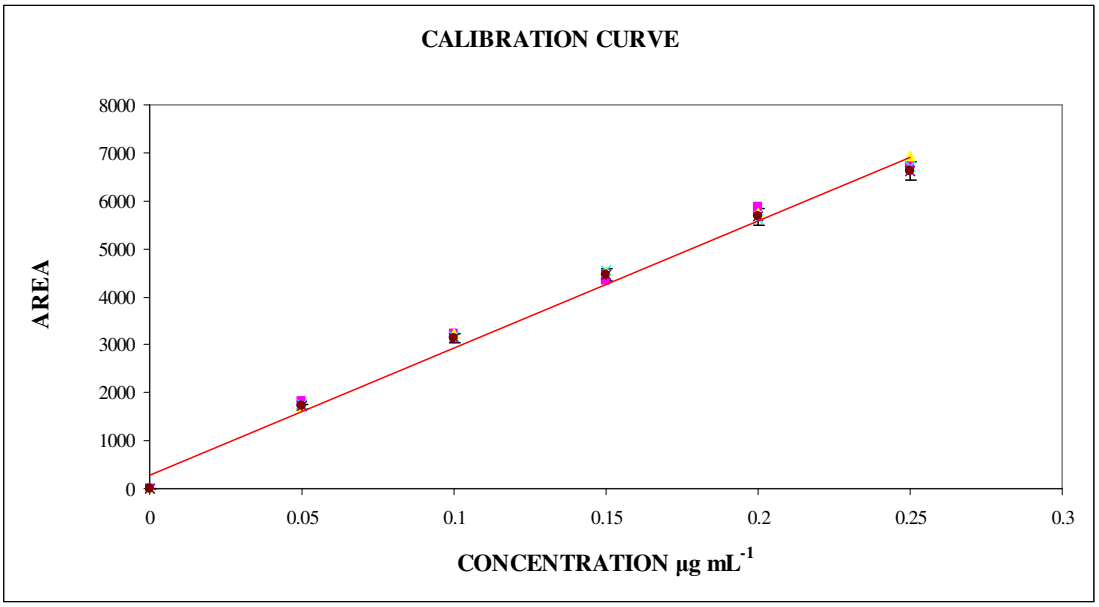
Graph.83 Calibration curve of fenofibrate by RP-HPLC



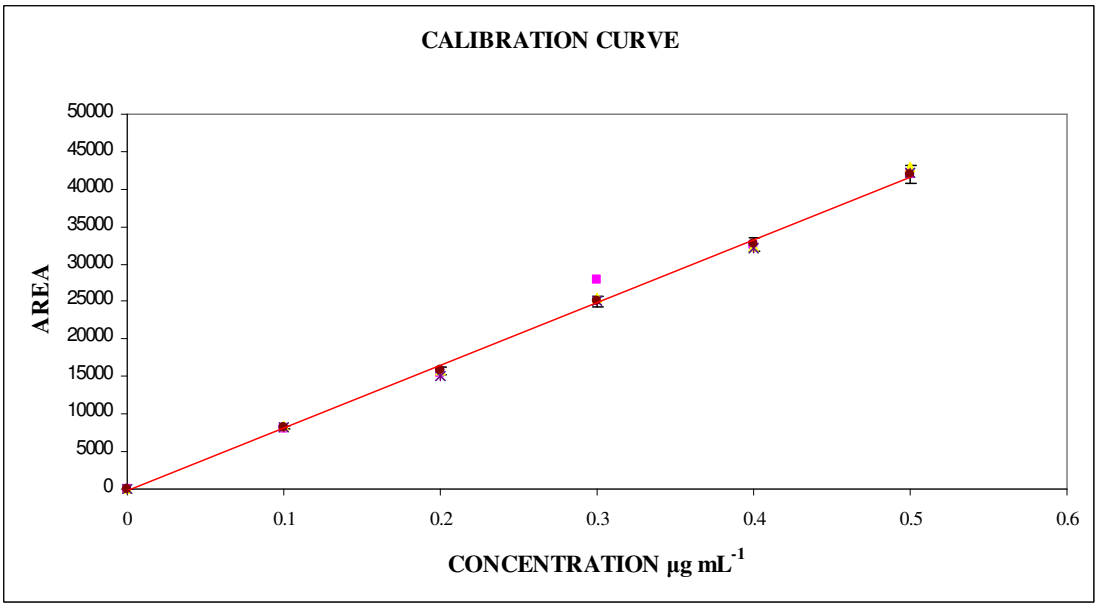
Graph.84 Calibration curve of folic acid by RP-HPLC



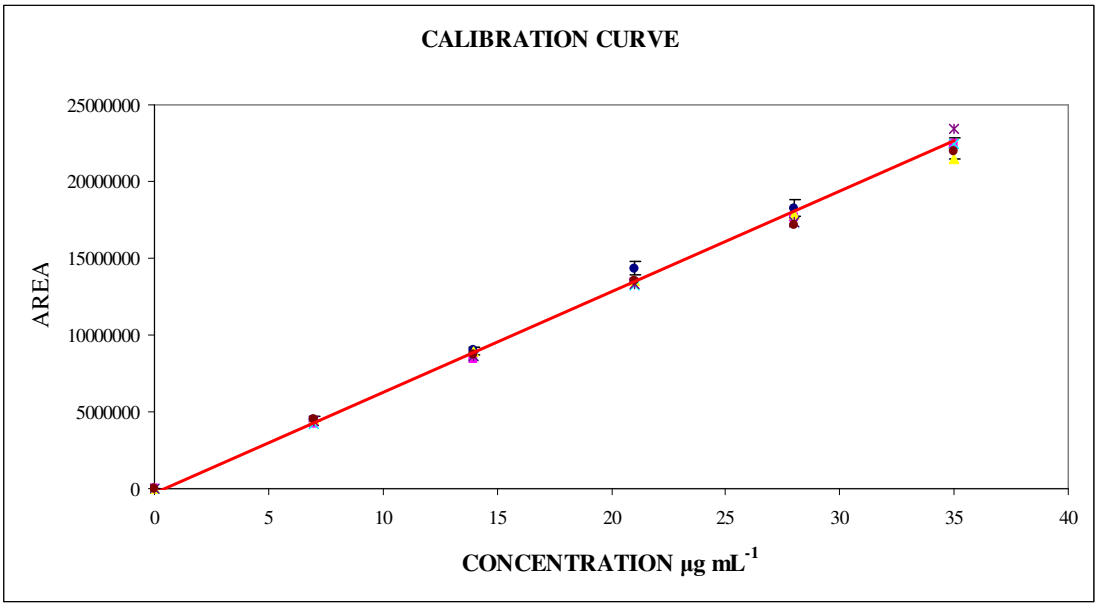
Graph.85 Calibration curve of rosiglitazone maleate by RP-HPLC



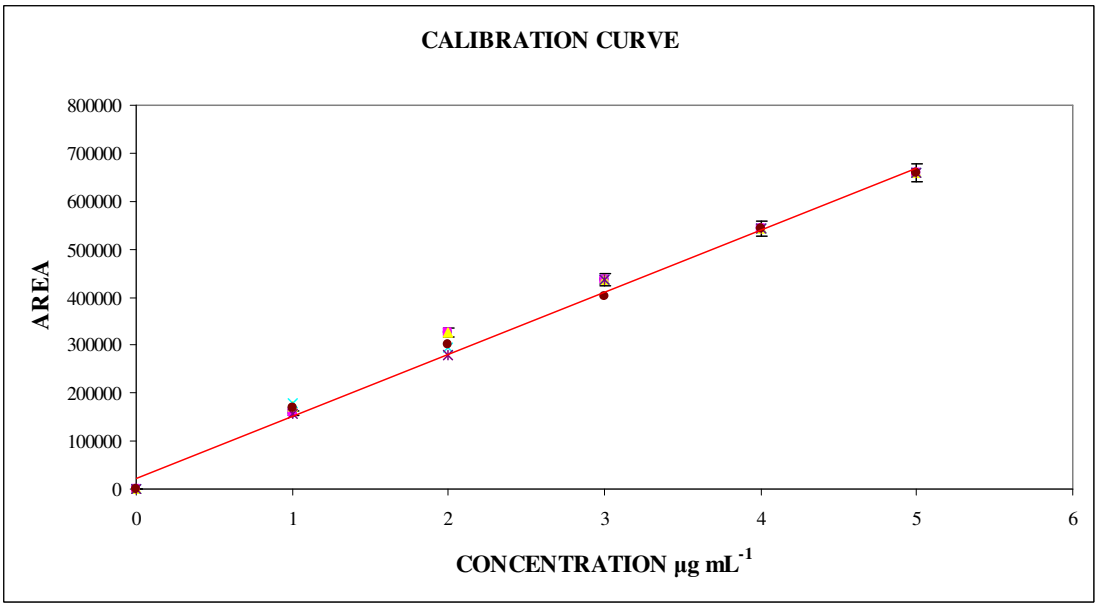
Graph.86 Calibration curve of glibenclamide by RP-HPLC



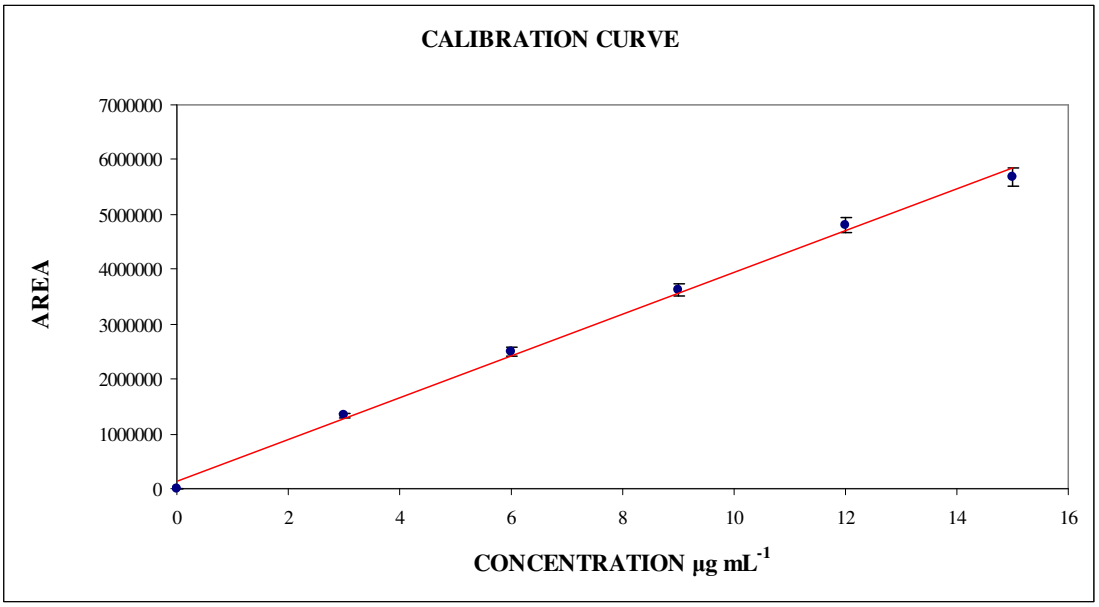
Graph.87 Calibration curve of metformin HCl by RP-HPLC



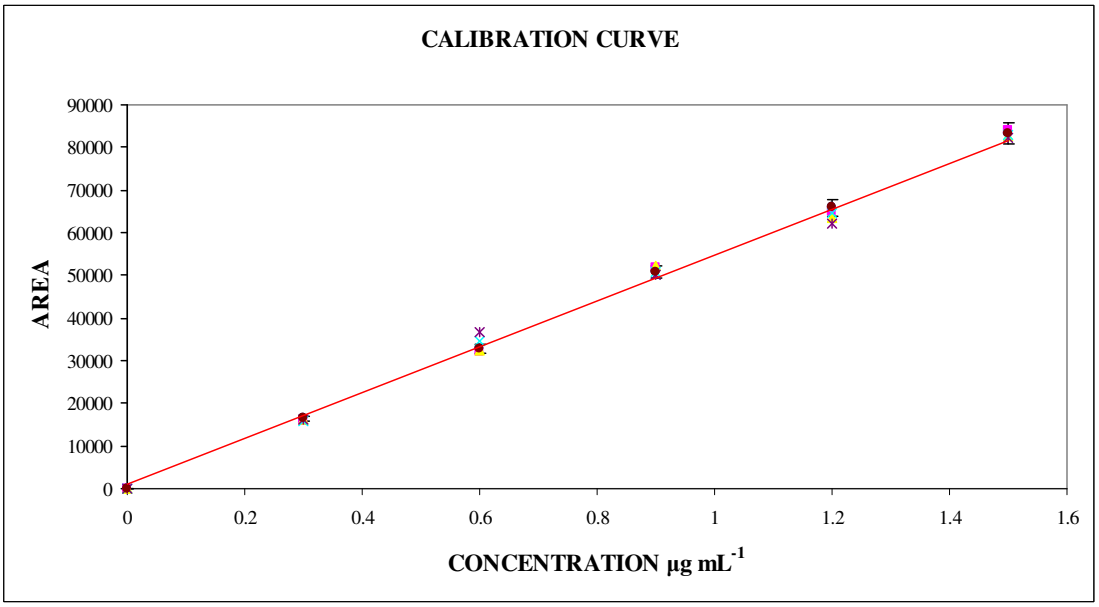
Graph.88 Calibration curve of nebivolol HCl by RP-HPLC



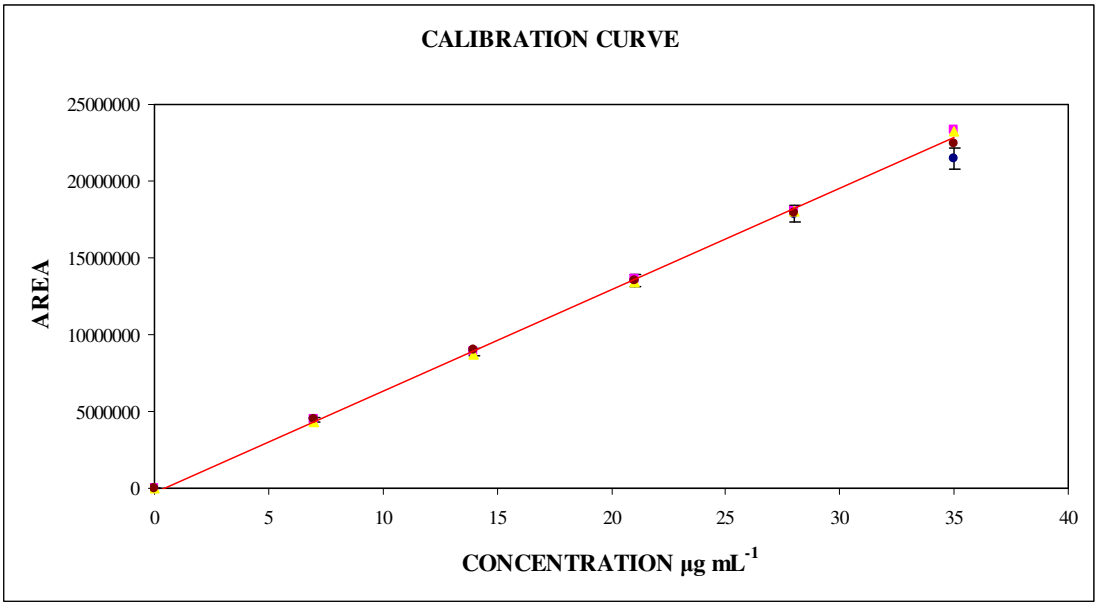
Graph.89 Calibration curve of hydrochlorothiazide by RP-HPLC



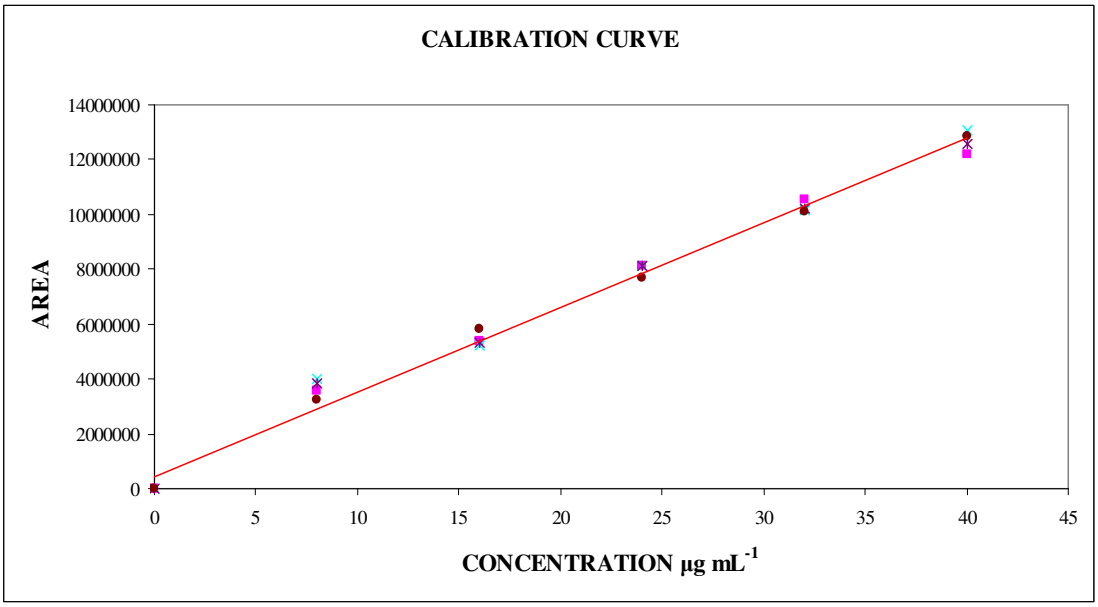
Graph.90 Calibration curve of pioglitazone HCl by RP-HPLC



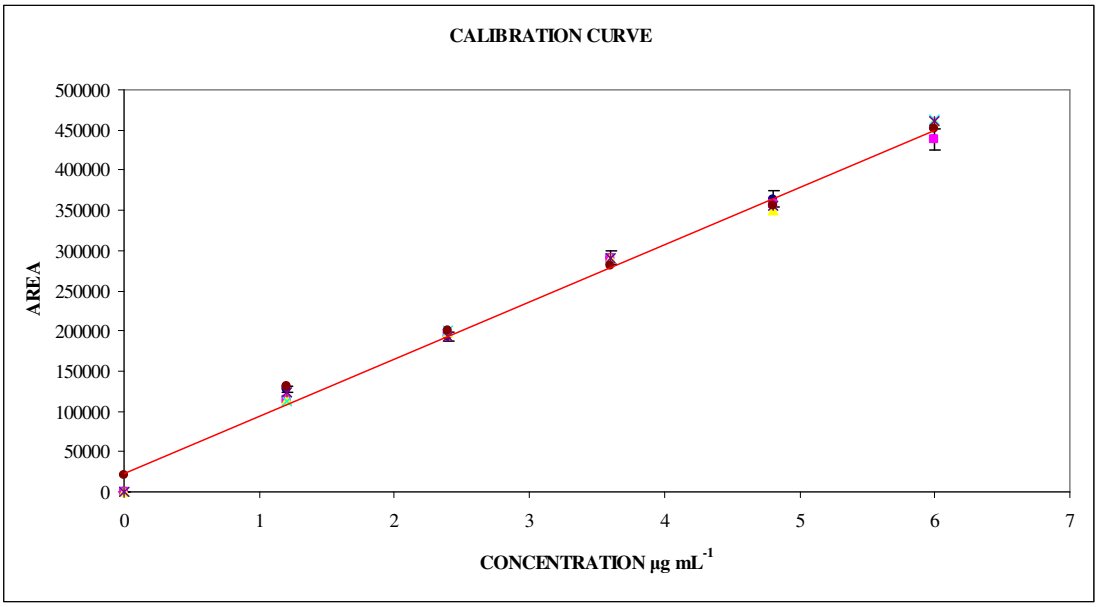
Graph.91 Calibration curve of metformin HCl by RP-HPLC



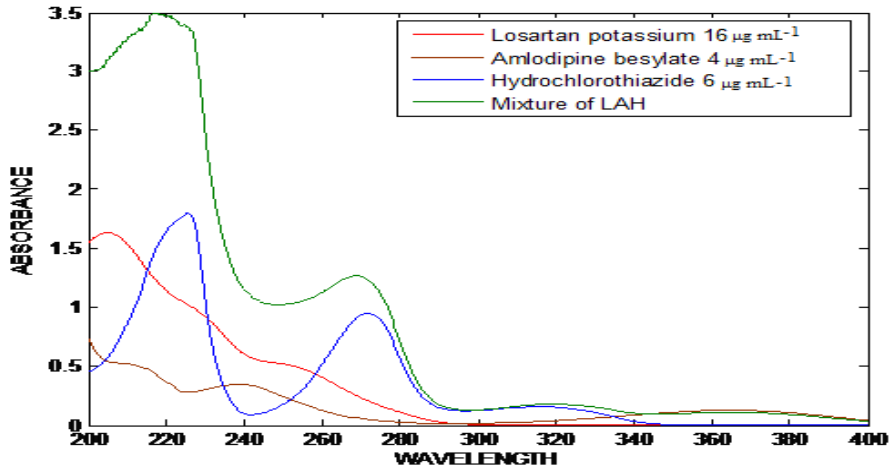
Graph.92 Calibration curve of losartan potassium by RP-HPLC



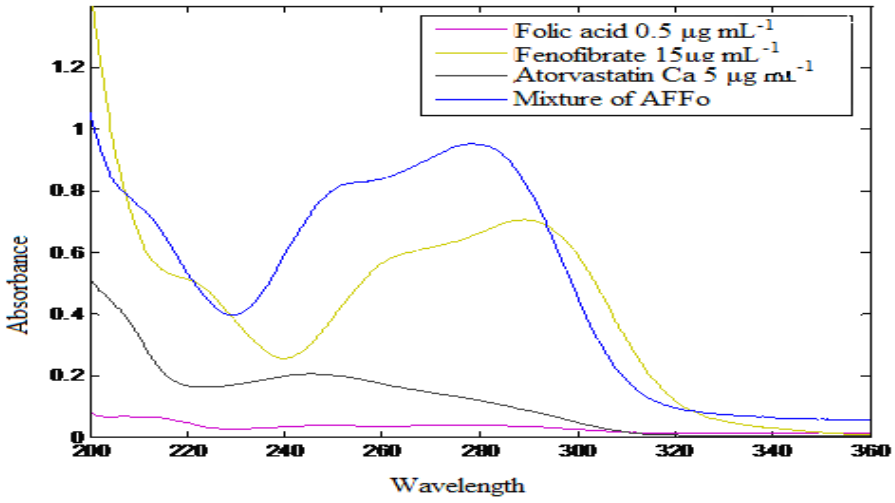
Graph.93 Calibration curve of ramipril by RP-HPLC



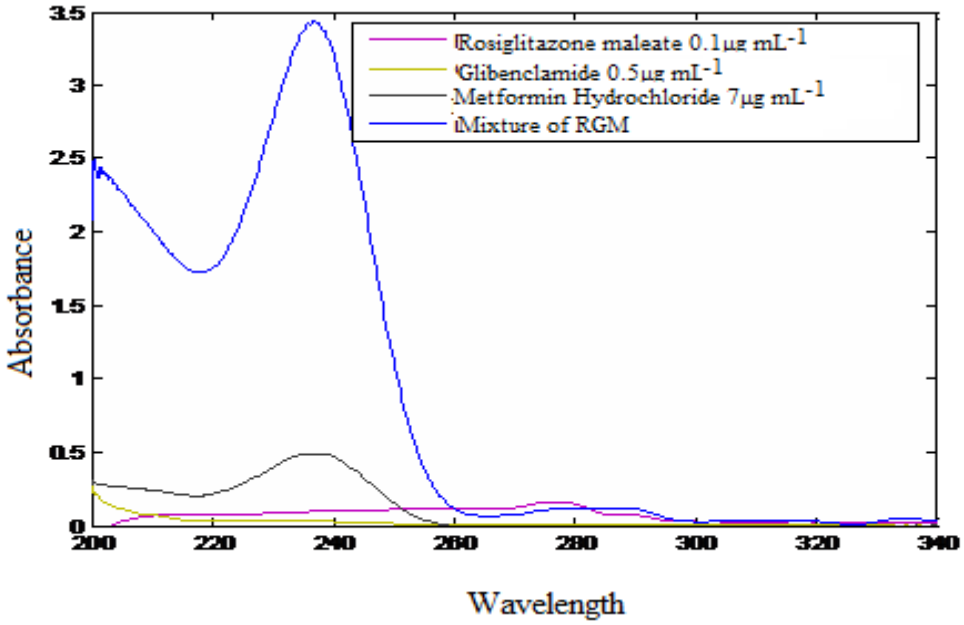
Spectrum.1 Overlapping spectrum of losartan potassium, amlodipine besylate and hydrochlorothiazide



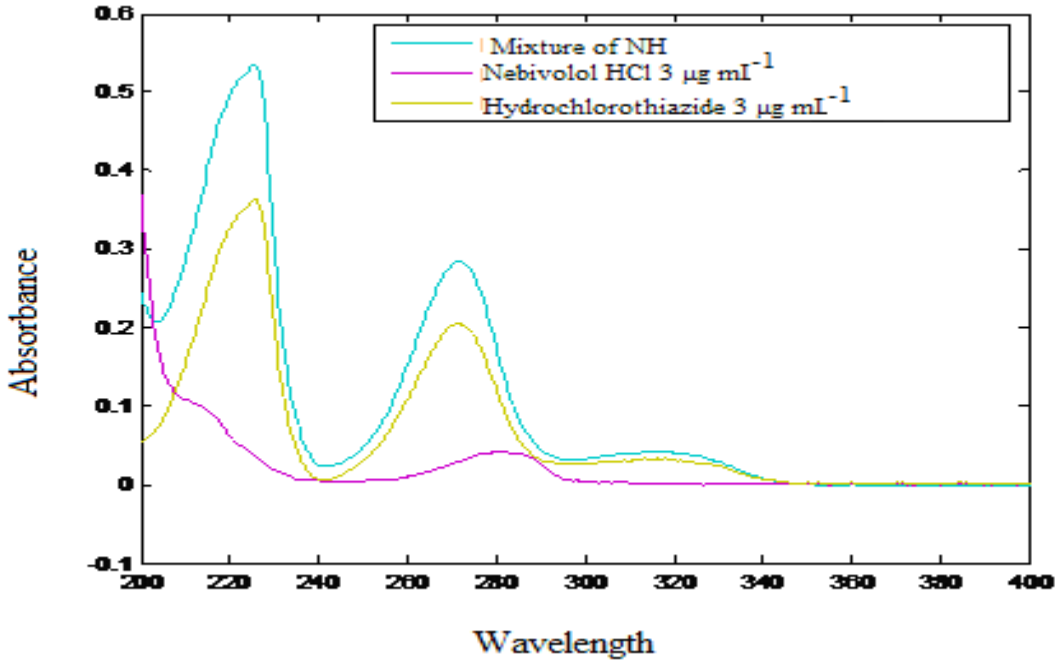
Spectrum. 2 Overlapping spectrum of atorvastatin calcium, fenofibrate and folic acid



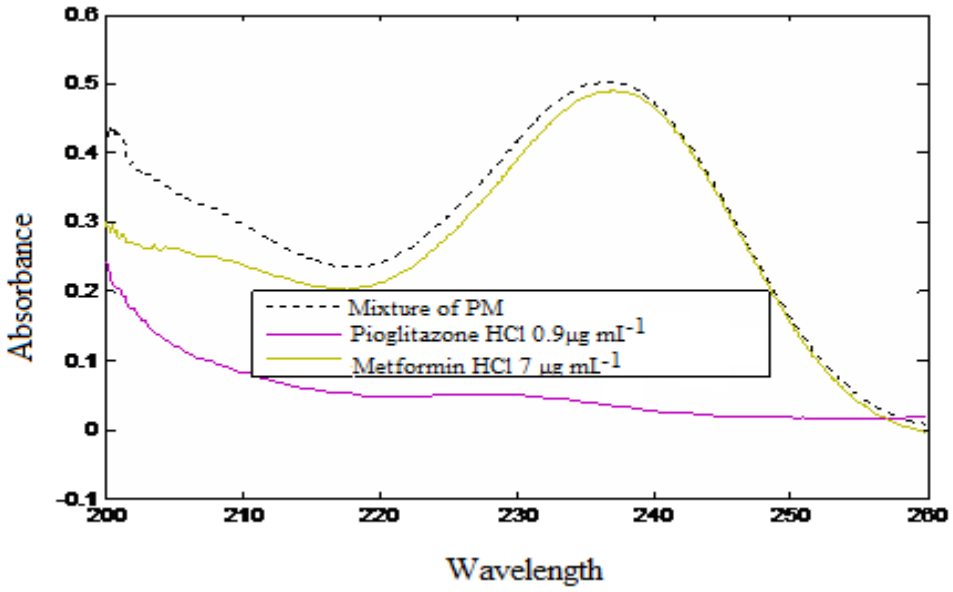
Spectrum. 3 Overlapping spectrum of rosiglitazone maleate, glibenclamide and metformin hydrochloride



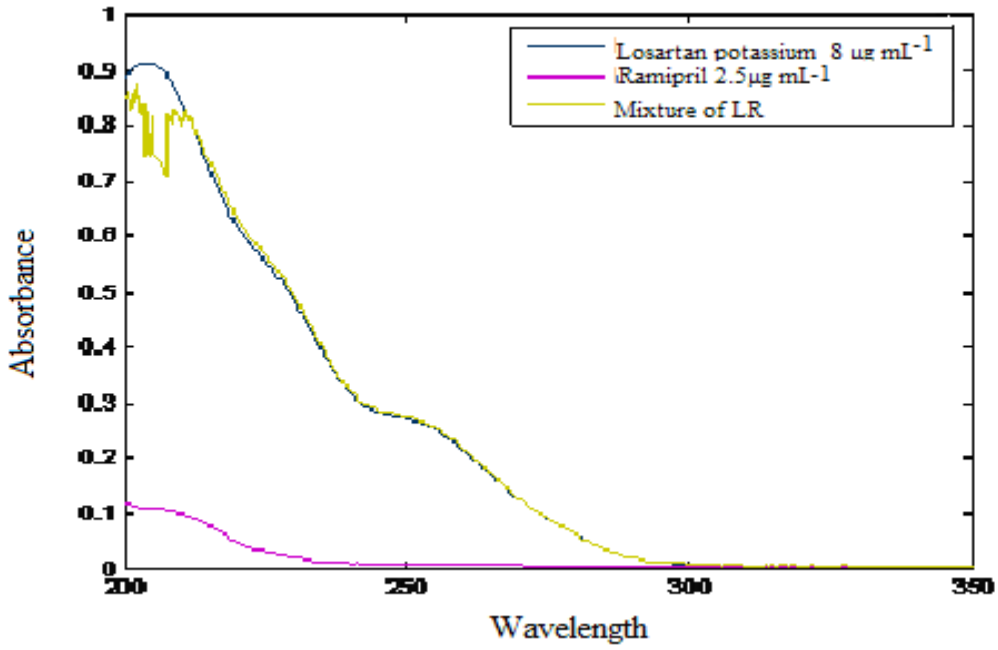
Spectrum.4 Overlapping spectrum of nebivolol hydrochloride and hydrochlorothiazide



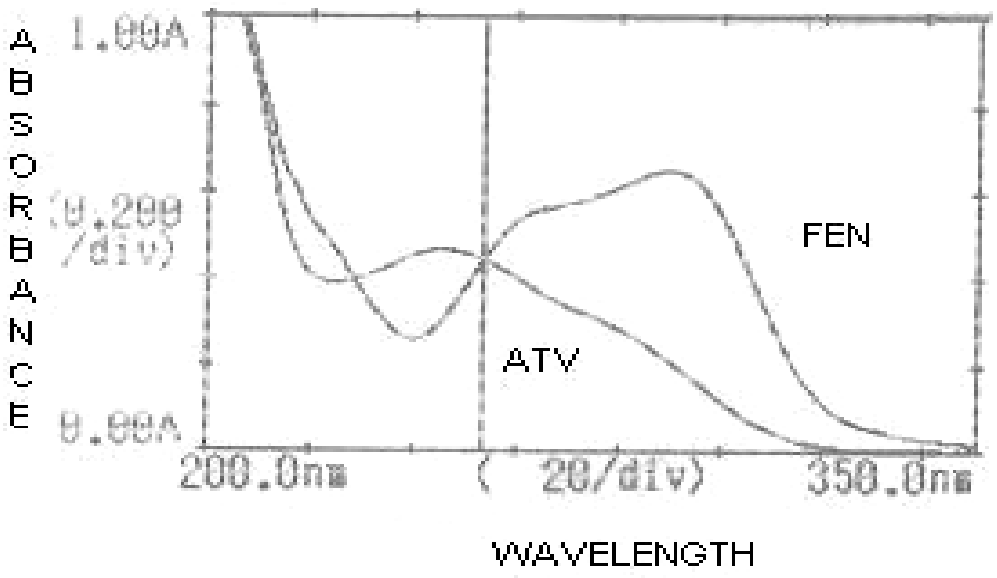
Spectrum.5 Overlapping spectrum of pioglitazone hydrochloride and metformin hydrochloride



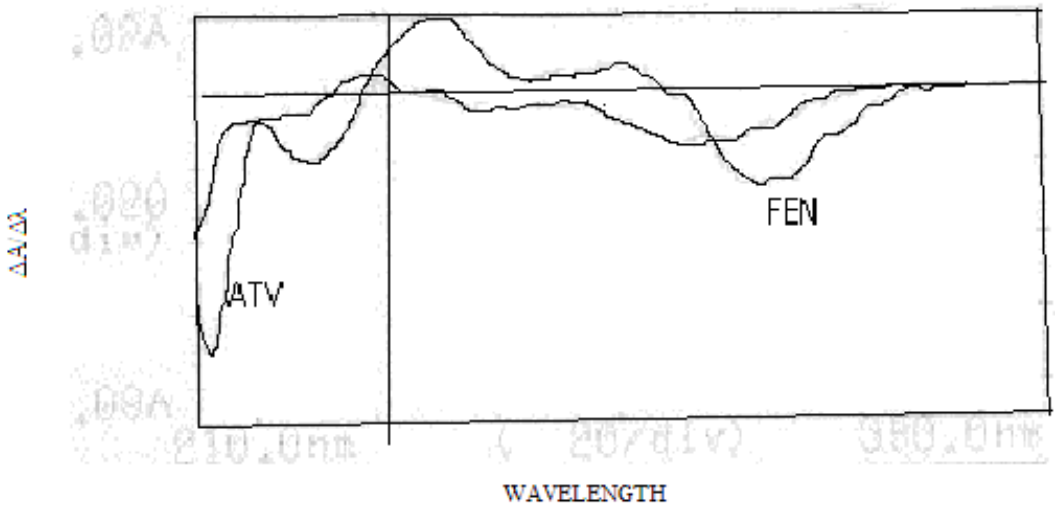
Spectrum.6 Overlapping spectrum of losartan potassium and ramipril



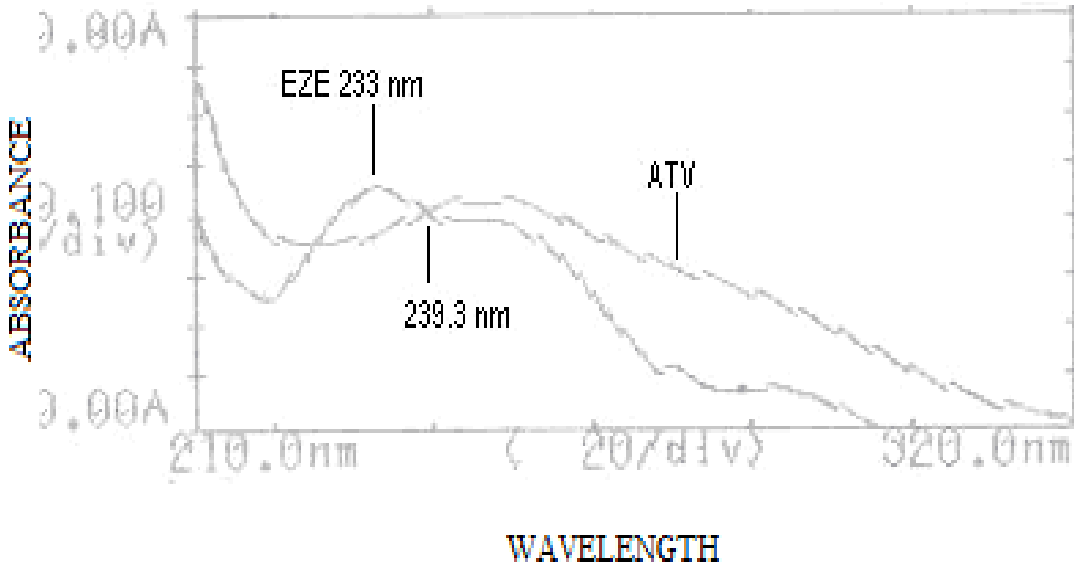
Spectrum.7 Zero order overlapping spectrum of atorvastatin calcium and fenofibrate



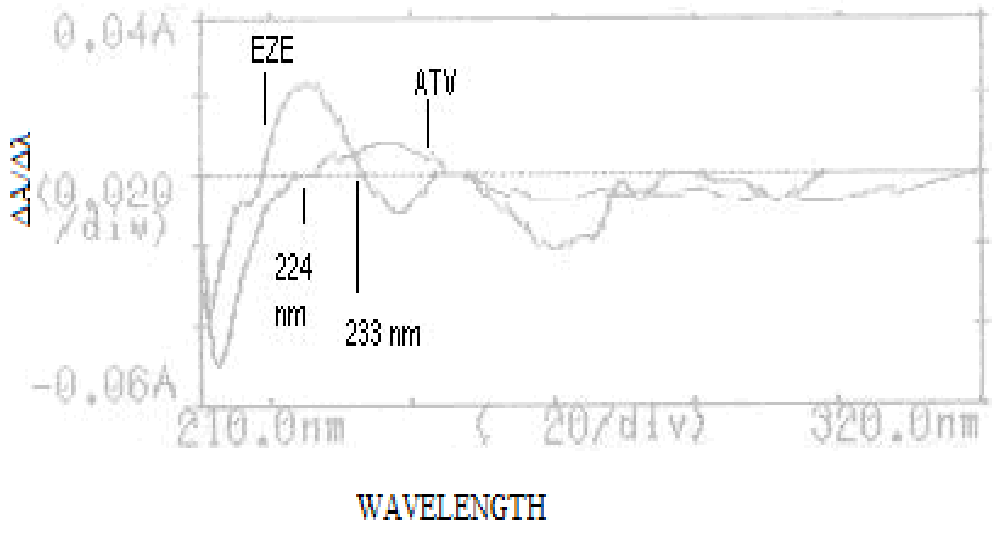
Spectrum.8 First order derivative overlapping spectrum of atorvastatin calcium and fenofibrate



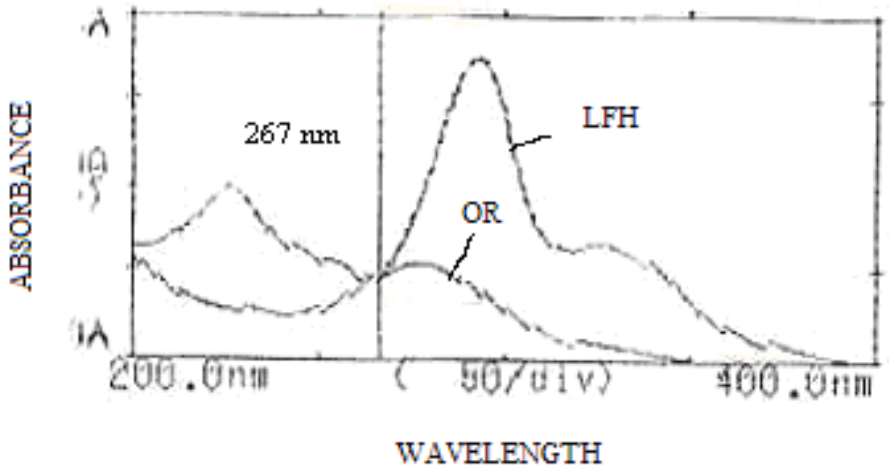
Spectrum.9 Zero order overlapping spectrum of atorvastatin calcium and ezetimibe



Spectrum.10 First order derivative overlapping spectrum of atorvastatin calcium and ezetimibe



Spectrum.11 Zero order overlapping spectrum of levofloxacin hemihydrate and ornidazole



Spectrum.12 First order derivative overlapping spectrum of levofloxacin hemihydrate and ornidazole

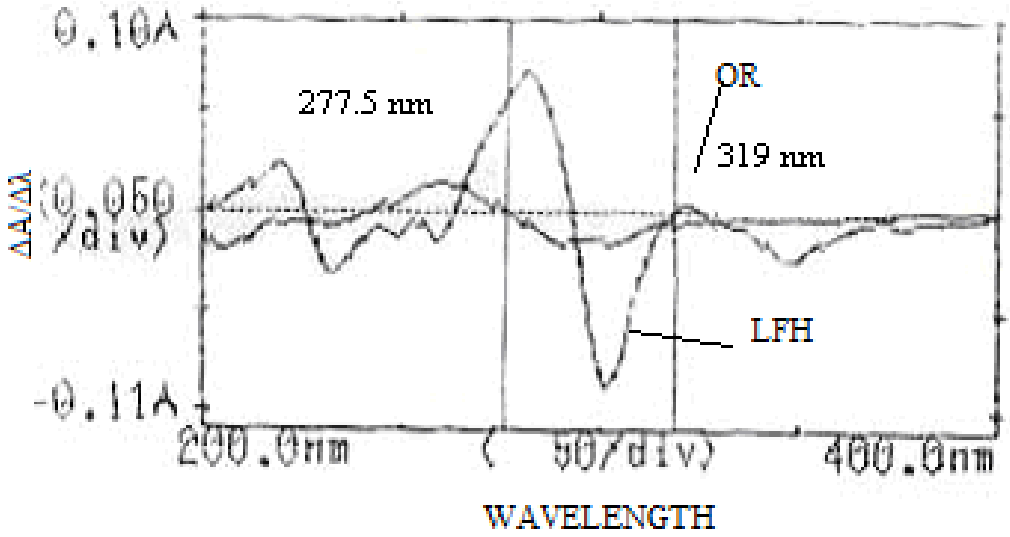


Table 1 Factorial design of the calibration set for triple combination

S.NO	COMPONENT A	COMPONENT B	COMPONENT C
1	0	0	0
2	0	-2	-2
3	-2	-2	+2
4	-2	+2	-1
5	+2	-1	+2
6	-1	+2	0
7	+2	0	-1
8	0	-1	-1
9	-1	-1	+1
10	-1	+1	+2
11	+1	+2	+1
12	+2	+1	0
13	+1	0	+2
14	0	+2	+2
15	+2	+2	-2
16	+2	-2	+1
17	-2	+1	-2
18	+1	-2	0
19	-2	0	+1
20	0	+1	+1
21	+1	+1	-1
22	+1	-1	-2
23	-1	-2	-1
24	-2	-1	0
25	-1	0	-2

Table 2 Factorial design of the calibration set for double combination

S.NO	COMPONENT A	COMPONENT B
1	-2	-1
2	-2	-2
3	-2	0
4	-2	+1
5	-2	+2
6	-1	-1
7	-1	-2
8	-1	0
9	-1	+1
10	-1	+2
11	0	-1
12	0	-2
13	0	0
14	0	+1
15	0	+2
16	+1	-1
17	+1	-2
18	+1	0
19	+1	+1
20	+1	+2
21	+2	-1
22	+2	-2
23	+2	0
24	+2	+1
25	+2	+2

Table 3 Composition of the calibration set for losartan potassium, amlodipine besylate and hydrochlorothiazide

Mixture	Concentration µg ml^{-1} Losartan potassium	Amlodipine besylate	Hydro chlorothiazide	Mixture	Concentration µg ml^{-1} Losartan potassium	Amlodipine besylate	Hydro chlorothiazide
1	24	3	9	14	24	5	15
2	24	1	3	15	40	5	3
3	8	1	15	16	40	1	12
4	8	5	6	17	8	4	3
5	40	2	15	18	32	1	9
6	16	5	9	19	8	3	12
7	40	3	6	20	24	4	12
8	24	2	6	21	32	4	6
9	16	2	12	22	32	2	3
10	16	4	15	23	16	1	6
11	32	5	12	24	8	2	9
12	40	4	9	25	16	3	3
13	32	3	15				

Table 4 Statistical parameters of chemometric methods for losartan potassium, amlodipine besylate and hydrochlorothiazide

Component	CLS	MLR	PCR	PLS		
	RMSECV	RMSECV	RMSEC	RMSECV	RMSEC	RMSECV
Losartan potassium	0.1647	0.2258	0.1647	0.1987	0.1647	0.1987
Amlodipine besylate	0.1183	0.2047	0.1183	0.1424	0.1183	0.1424
Hydrochlorothiazide	0.1823	0.2583	0.1823	0.2134	0.1823	0.2134

Table 5 Composition of physical, formulation and recovery set for losartan potassium, amlodipine besylate and hydrochlorothiazide

Physical µg ml^{-1}			Formulation µg ml^{-1}			Recovery µg ml^{-1}		
Losartan potassium	Amlodipine besylate	Hydrochlorothiazide	Losartan	Amlodipine	Hydrochlorothiazide	Losartan potassium	Amlodipine besylate	Hydrochlorothiazide
24.8	3.6	10.8	30	3	7.5	3	0.45	0.75
24.8	1.2	3.6	30	3	7.5	3	0.45	0.75
9.6	1.2	18	30	3	7.5	3	0.45	0.75
9.6	6	7.2	30	3	7.5	6	0.9	1.5
48	2.4	18	30	3	7.5	6	0.9	1.5
19.2	6	10.8	30	3	7.5	6	0.9	1.5
48	3.6	7.2	30	3	7.5	9	1.35	2.25
28.8	2.4	7.2	30	3	7.5	9	1.35	2.25
19.2	2.4	14.4	30	3	7.5	9	1.35	2.25

Table 6 Statistical parameters of chemometric methods for losartan potassium, amlodipine besylate and hydrochlorothiazide (Prediction)

Component	CLS		MLR		PCR		PLS	
	RMSEP	r	RMSEP	R	RMSEP	r	RMSEP	r
Losartan potassium	0.1264	1.0000	0.2062	1.0000	0.1250	1.0000	0.1250	1.0000
Amlodipine besylate	0.0908	0.9940	0.1940	0.9920	0.0856	0.9940	0.0856	0.9940
Hydrochlorothiazide	0.1110	0.9990	0.2328	0.9980	0.1120	0.9990	0.1120	0.9990

Table 7 Prediction results for losartan potassium, amlodipine besylate and hydrochlorothiazide from the validation (physical mixtures) samples by different chemometric methods

Component	CLS		MLR		PCR		PLS	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Losartan potassium	100.55	0.93	100.42	1.21	100.55	0.91	100.55	0.91
Amlodipine besylate	100.02	1.62	100.62	0.67	99.98	0.53	99.98	0.53
Hydrochlorothiazide	100.93	0.61	99.69	0.93	100.94	0.67	100.94	0.67

*Mean ± SD for 9 determinations.

Table 8 Prediction results of losartan potassium, amlodipine besylate and hydrochlorothiazide from the formulation samples by different chemometric methods

Method	Parameters	Losartan potassium	Amlodipine	Hydrochlorothiazide
CLS	Mean	100.02	100.39	101.28
	S.D	0.034	0.722	0.132
	%rsd	0.034	0.720	0.131
	SE	0.004	0.080	0.015
MLR	Mean	100.27	100.56	100.76
	S.D	0.078	1.018	1.172
	%rsd	0.078	1.013	1.163
	SE	0.009	0.113	0.130
PCR	Mean	100.01	100.37	101.32
	S.D	0.033	0.708	0.130
	%rsd	0.033	0.705	0.128
	SE	0.004	0.079	0.014
PLS	Mean	100.01	100.37	101.32
	S.D	0.033	0.708	0.130
	%rsd	0.033	0.705	0.128
	SE	0.004	0.079	0.014

*Mean ± SD for 9 determinations.

Table 9 Recovery studies of losartan potassium, amlodipine besylate and hydrochlorothiazide in formulation

	Losatan potassium			Amlodipine besylate			Hydrochlo rothiazide		
Method	Added µg ml^{-1}	Found µg ml^{-1}	*Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	*Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	*Recovery (%)
CLS	3	33.1	100.4	0.45	3.4	99.8	0.75	8.4	101.6
	6	36.0	100.0	0.9	3.9	99.2	1.5	9.1	100.6
	9	39.0	100.1	1.35	4.3	99.4	2.25	9.9	101.6
Mean			100.1			99.5			101.3
S.D			±0.22			±0.33			±0.64
MLR	3	33.0	100.1	0.45	3.5	101.3	0.75	8.4	102.2
	6	35.7	99.3	0.9	3.9	99.8	1.5	9.1	100.6
	9	39.1	100.2	1.35	4.4	100.5	2.25	9.8	100.3
Mean			99.9			100.5			101.0
S.D			±0.66			±0.98			±1.41
PCR	3	33.1	100.3	0.45	3.4	99.7	0.75	8.4	101.6
	6	36.0	100.0	0.9	3.9	99.0	1.5	9.1	100.6
	9	39.0	100.0	1.35	4.3	99.2	2.25	9.9	101.6
Mean			100.1			99.3			101.3
S.D			±0.22			±0.35			±0.63
PLS	3	33.1	100.3	0.45	3.4	99.7	0.75	8.4	101.6
	6	36.0	100.0	0.9	3.7	99.0	1.5	9.1	100.6
	9	39.0	100.0	1.35	4.3	99.2	2.25	9.9	101.6
Mean			100.1			99.3			101.3
S.D			±0.22			±0.35			±0.63

*Mean ± SD for 9 determinations.

Table 10 Composition of the calibration set for atorvastatin calcium, fenofibrate and folic acid

	Concentration µg ml^{-1}				Concentration µg ml^{-1}		
Mixture	Atorvastatin calcium	Fenofibrate	Folic acid	Mixture	Atorvastatin calcium	Fenofibrate	Folic acid
1	3.0	45.12	1.5	14	3.0	75.2	2.5
2	3.0	15.04	0.5	15	5.0	75.2	0.5
3	1.0	15.04	2.5	16	5.0	15.04	2.0
4	1.0	75.2	1.0	17	1.0	60.16	0.5
5	5.0	30.08	2.5	18	4.0	15.04	1.5
6	2.0	75.2	1.5	19	1.0	45.12	2.0
7	5.0	45.12	1.0	20	3.0	60.16	2.0
8	3.0	30.08	1.0	21	4.0	60.16	1.0
9	2.0	30.08	2.0	22	4.0	30.08	0.5
10	2.0	60.16	2.5	23	2.0	15.04	1.0
11	4.0	75.2	2.0	24	1.0	30.08	1.5
12	5.0	60.16	1.5	25	2.0	45.12	1.0
13	4.0	45.12	2.5				

Table 11 Statistical parameters of chemometric methods for atorvastatin calcium, fenofibrate and folic acid

	CLS	MLR	PCR	PLS		
Component	RMSECV	RMSECV	RMSEC	RMSECV	RMSEC	RMSECV
Atorvastatin calcium	0.0825	0.1074	0.0728	0.0843	0.0451	0.0626
Fenofibrate	0.6127	0.2600	0.0353	0.6166	0.1262	0.3436
Folic acid	0.0696	0.0917	0.0318	0.0698	0.1017	0.0318

Table 12 Composition of physical, formulation and recovery set for atorvastatin calcium, fenofibrate and folic acid

Physical µg ml^{-1}			Formulation µg ml^{-1}			Recovery µg ml^{-1}		
Atorvastatin calcium	Feno fibrate	Folic acid	Atorv astatin	Feno fibrate	Folic acid	Atorvastatin calcium	Feno fibrate	Folic acid
2.0	45.12	2.0	1.5625	25	0.78125	1	16	0.5
3.0	45.12	1.5	1.5625	25	0.78125	1	16	0.5
4.0	30.08	1.0	1.5625	25	0.78125	1	16	0.5
3.0	60.16	1.0	1.5625	25	0.78125	1.2	19.5	0.6
4.0	30.08	1.5	1.5625	25	0.78125	1.2	19.5	0.6
2.0	45.12	2.0	1.5625	25	0.78125	1.2	19.5	0.6
3.0	45.12	1.5	1.5625	25	0.78125	1.4	22.4	0.7
4.0	30.08	1.0	1.5625	25	0.78125	1.4	22.4	0.7
3.0	60.16	1.0	1.5625	25	0.78125	1.4	22.4	0.7

Table 13 Statistical parameters of chemometric methods for atorvastatin calcium, fenofibrate and folic acid (Prediction)

	CLS		MLR		PCR		PLS	
Component	RMSEP	r	RMSEP	R	RMSEP	r	RMSEP	r
Atorvastatin	0.0223	0.998	0.1059	1.0000	0.0451	0.9970	0.0426	0.9980
Fenofibrate	0.0081	1	0.3542	1.0000	0.1262	1.0000	0.1237	1.0000
Folic acid	0.0089	0.998	0.1436	1.0000	0.1017	0.9980	0.0101	0.9980

Table 14 Prediction results for atorvastatin calcium, fenofibrate and folic acid from the validation (physical mixtures) samples by different chemometric methods

	CLS		MLR		PCR		PLS	
Component	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Atorvastatin calcium	100.55	0.52	98.93	2.89	101.92	1.21	101.79	1.05
Fenofibrate	99.99	0.02	100.31	1.12	99.82	0.27	99.82	0.26
Folic acid	100.57	0.15	100.43	2.12	100.68	0.59	100.68	0.59

*Mean ± SD for 9 determinations.

Table 15 Prediction results of atorvastatin, fenofibrate and folic acid from the formulation samples by different chemometric methods

Method	Parameters	Atorvastatin	Fenofibrate	Folic acid
CLS	Mean	98.06	100.15	100.40
	S.D	1.543	0.217	0.578
	%rsd	1.573	0.217	0.576
	SE	0.171	0.024	0.064
MLR	Mean	98.77	99.55	103.25
	S.D	1.332	0.063	1.478
	%rsd	1.349	0.064	1.431
	SE	0.148	0.007	0.164
PCR	Mean	98.74	100.28	101.08
	S.D	0.518	0.484	0.269
	%rsd	0.525	0.482	0.266
	SE	0.058	0.054	0.030
PLS	Mean	99.18	100.28	101.08
	S.D	0.513	0.484	0.269
	%rsd	0.517	0.483	0.266
	SE	0.057	0.054	0.030

*Mean ± SD for 9 determinations.

Table 16 Recovery studies of atorvastatin calcium, fenofibrate and folic acid in formulation

	Atorvastatin calcium			Fenofibrate			Folic acid		
Method	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)
CLS	1	2.57	100.12	16	41.10	100.24	0.5	1.29	100.93
	1.2	2.78	100.67	19.5	44.52	100.03	0.6	1.39	100.63
	1.4	2.98	100.43	22.4	47.42	100.04	0.7	1.49	100.59
Mean			100.41			100.11			100.72
S.D			±0.449			±0.16			±0.57
MLR	1	2.52	98.35	16	41.08	100.20	0.5	1.29	100.93
	1.2	2.77	100.27	19.5	44.28	99.50	0.6	1.39	100.63
	1.4	2.997	101.15	22.4	47.298	99.79	0.7	1.49	100.59
Mean			99.92			99.83			100.72
S.D			±1.392			±0.47			±0.57
PCR	1	2.60	101.58	16	40.87	99.68	0.5	1.29	100.71
	1.2	2.77	100.28	19.5	44.45	99.89	0.6	1.39	100.62
	1.4	2.99	100.79	22.4	47.299	99.79	0.7	1.49	100.65
Mean			100.88			99.79			100.66
S.D			±1.107			±0.12			±0.12
PLS	1	2.60	101.54	16	40.87	99.69	0.5	1.29	100.70
	1.2	2.77	100.18	19.5	44.45	99.89	0.6	1.39	100.62
	1.4	2.98	100.64	22.4	47.30	99.79	0.7	1.49	100.64
Mean			100.79			99.79			100.66
S.D			±1.125			±0.12			±0.12

*Mean ± SD for 9 determinations.

Table 17 Composition of the calibration set for rosiglitazone maleate, glibenclamide and metformin hydrochloride

Mixture	Concentration µg ml^{-1} Rosiglitazone maleate	Gliben clamide	Metformin hydrochloride	Mixture	Concentration µg ml^{-1} Rosiglitazone maleate	Gliben clamide	Metformin hydrochloride
1	0.15	0.3	21	14	0.15	0.5	35
2	0.15	0.1	7	15	0.25	0.5	7
3	0.05	0.1	35	16	0.25	0.1	28
4	0.05	0.5	14	17	0.05	0.4	7
5	0.25	0.2	35	18	0.2	0.1	21
6	0.1	0.5	21	19	0.05	0.3	28
7	0.25	0.3	14	20	0.15	0.4	28
8	0.15	0.2	14	21	0.2	0.4	14
9	0.1	0.2	28	22	0.2	0.2	7
10	0.1	0.4	35	23	0.1	0.1	14
11	0.2	0.5	28	24	0.05	0.2	21
12	0.25	0.4	21	25	0.1	0.3	7
13	0.2	0.3	35				

Table 18 Statistical parameters of chemometric methods for rosiglitazone maleate, glibenclamide and metformin hydrochloride

Component	CLS	MLR	PCR	PLS		
	RMSECV	RMSECV	RMSEC	RMSECV	RMSEC	RMSECV
Rosiglitazone maleate	0.0024	0.0048	0.0023	0.0028	0.0023	0.0028
Glibenclamide	0.0044	0.0109	0.0045	0.0052	0.0045	0.0052
Metformin Hydrochloride	0.0112	0.0168	0.0098	0.0123	0.0098	0.0123

Table 19 Composition of physical, formulation and recovery set for rosiglitazone maleate, glibenclamide and metformin hydrochloride

Physical µg ml^{-1}			Formulation µg ml^{-1}			Recovery µg ml^{-1}		
Rosiglitazone maleate	Gliben clamide	Metformin hydrochloride	Rosi glitazone	Gliben clamide	Metformin	Rosiglitazone maleate	Gliben clamide	Metformin hydrochloride
0.1	0.2	28	0.1	0.25	25	0.05	0.1	7
0.15	0.3	21	0.1	0.25	25	0.05	0.1	7
0.2	0.4	14	0.1	0.25	25	0.05	0.1	7
0.1	0.2	28	0.1	0.25	25	0.06	0.12	8.4
0.15	0.3	21	0.1	0.25	25	0.06	0.12	8.4
0.2	0.4	14	0.1	0.25	25	0.06	0.12	8.4
0.1	0.2	28	0.1	0.25	25	0.07	0.14	9.8
0.1	0.3	28	0.1	0.25	25	0.07	0.14	9.8
0.1	0.25	25	0.1	0.25	25	0.07	0.14	9.8

Table 20 Statistical parameters of chemometric methods for rosiglitazone maleate, glibenclamide and metformin hydrochloride (Prediction)

	CLS		MLR		PCR		PLS	
Component	RMSEP	r	RMSEP	r	RMSEP	r	RMSEP	r
Rosiglitazone maleate	0.000479	0.999	0.0240	1.000	0.0063	0.999	0.0063	0.999
Glibenclamide	0.000732	0.999	0.3413	1.000	0.0181	0.999	0.0185	0.999
Metformin Hydrochloride	0.002177	1	1.2780	1.000	1.1809	1.000	1.1809	1.000

Table 21 Prediction results for rosiglitazone maleate, glibenclamide and metformin hydrochloride from the validation (physical mixtures) samples by different chemometric methods

	CLS		MLR		PCR		PLS	
Component	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Rosiglitazone maleate	99.49	0.27	100.21	1.44	98.79	1.45	98.79	1.45
Glibenclamide	99.69	0.25	99.30	0.73	100.54	1.63	100.54	1.63
Metformin hydrochloride	100.01	0.01	100.56	1.47	100.92	0.90	100.92	0.90

*Mean ± SD for 9 determinations.

Table 22 Prediction results of rosiglitazone maleate, glibenclamide and metformin hydrochloride from the formulation samples by different chemometric methods

Method	Parameters	Rosi glitazone	Gliben clamide	Metformin hydrochloride
CLS	Mean	99.40	99.50	99.96
	S.D	0.400	0.260	0.040
	%rsd	0.402	0.262	0.040
	SE	0.044	0.029	0.004
MLR	Mean	101.67	99.24	102.73
	S.D	0.577	0.349	2.016
	%rsd	0.568	0.351	1.962
	SE	0.064	0.039	0.224
PCR	Mean	98.67	100.27	100.15
	S.D	0.577	0.611	0.220
	%rsd	0.585	0.609	0.220
	SE	0.064	0.068	0.024
PLS	Mean	98.67	100.27	100.15
	S.D	0.577	0.611	0.220
	%rsd	0.585	0.609	0.220
	SE	0.064	0.068	0.024

*Mean ± SD for 9 determinations.

Table 23 Recovery studies of rosiglitazone maleate, glibenclamide and metformin hydrochloride in formulation

	Rosiglitazone maleate			Glibenclamide			Metformin hydrochloride		
Method	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)
CLS	0.05	0.149	99.07	0.1	0.35	99.76	7	32.00	100.03
	0.06	0.158	98.90	0.12	0.37	99.96	8.4	33.40	99.99
	0.07	0.168	98.92	0.14	0.39	99.62	9.8	34.80	99.99
Mean			98.96			99.78			100.00
S.D			±0.325			±0.305			±0.028
MLR	0.05	0.149	99.07	0.1	0.36	100.46	7	32.00	100.03
	0.06	0.158	98.71	0.12	0.37	99.64	8.4	33.40	99.99
	0.07	0.17	99.61	0.14	0.39	99.57	9.8	34.80	99.99
Mean			99.13			99.89			100.00
S.D			±0.702			±0.709			±0.028
PCR	0.05	0.15	100.67	0.1	0.35	100.38	7	32.02	100.06
	0.06	0.16	100.69	0.12	0.37	100.27	8.4	33.39	99.95
	0.07	0.17	100.78	0.14	0.39	100.18	9.8	34.90	100.39
Mean			100.71			100.28			100.14
S.D			±0.463			±0.197			±0.224
PLS	0.05	0.15	100.67	0.1	0.35	100.38	7	32.02	100.06
	0.06	0.16	100.69	0.12	0.37	100.27	8.4	33.39	99.95
	0.07	0.17	100.78	0.14	0.39	100.18	9.8	34.90	100.39
Mean			100.71			100.28			100.14
S.D			±0.463			±0.197			±0.224

*Mean ± SD for 9 determinations.

Table 24 Composition of the calibration set for nebivolol hydrochloride and hydrochlorothiazide

	Concentration µg ml^{-1}			Concentration µg ml^{-1}	
Mixture	Nebivolol hydrochloride	Hydrochlorothiazide	Mixture	Nebivolol hydrochloride	Hydrochlorothiazide
1	1	2	14	3	8
2	1	4	15	3	10
3	1	6	16	4	2
4	1	8	17	4	4
5	1	10	18	4	6
6	2	2	19	4	8
7	2	4	20	4	10
8	2	6	21	5	2
9	2	8	22	5	4
10	2	10	23	5	6
11	3	2	24	5	8
12	3	4	25	5	10
13	3	6			

Table 25 Statistical parameters of chemometric methods for nebivolol hydrochloride and hydrochlorothiazide

	CLS	MLR	PCR	PLS		
Component	RMSECV	RMSECV	RMSEC	RMSECV	RMSEC	RMSECV
Nebivolol Hydrochloride	0.0672	0.0985	0.0623	0.0668	0.0623	0.0668
Hydrochlorothiazide	0.0333	0.0724	0.0292	0.0333	0.0292	0.0333

Table 26 Composition of physical, formulation and recovery set for nebivolol hydrochloride and hydrochlorothiazide

Physical µg ml^{-1}		Formulation µg ml^{-1}		Recovery µg ml^{-1}	
Nebivolol hydrochloride	Hydro chlorothiazide	Nebivolol	Hydro chlorothiazide	Nebivolol hydrochloride	Hydro chlorothiazide
0.5	1.5	3	7.5	1	2
0.5	4.5	3	7.5	1	2
0.5	7.5	3	7.5	1	2
1.5	1.5	3	7.5	2	4
1.5	4.5	3	7.5	2	4
1.5	7.5	3	7.5	2	4
2.5	1.5	3	7.5	3	6
2.5	4.5	3	7.5	3	6
2.5	7.5	3	7.5	3	6

Table 27 Statistical parameters of chemometric methods for nebivolol hydrochloride and hydrochlorothiazide (Prediction)

	CLS		MLR		PCR		PLS	
Component	RMSEP	r	RMSEP	r	RMSEP	r	RMSEP	r
Nebivolol hydrochloride	0.0129	0.999	0.1099	0.9970	0.0148	0.9990	0.0148	0.9990
Hydrochlorothiazide	0.019	1	0.0683	1.0000	0.0195	1.0000	0.0195	1.0000

Table 28 Prediction results for nebivolol hydrochloride and hydrochlorothiazide from the validation (Physical mixtures) samples by different chemometric methods

Component	CLS		MLR		PCR		PLS	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Nebivolol hydrochloride	101.95	1.60	101.44	1.56	101.76	1.74	101.76	1.738
Hydrochlorothiazide	100.09	0.37	100.92	1.94	100.11	0.37	100.11	0.373

*Mean ± SD for 9 determinations.

Table 29 Prediction results of nebivolol and hydrochlorothiazide from the formulation samples by different chemometric methods

Method	Parameters	Nebivolol	Hydro chlorothiazide
CLS	Mean	100.08	99.57
	S.D	0.311	0.135
	%rsd	0.311	0.136
	SE	0.035	0.015
MLR	Mean	100.77	100.12
	S.D	1.937	0.351
	%rsd	1.924	0.350
	SE	0.215	0.039
PCR	Mean	100.08	99.57
	S.D	0.310	0.135
	%rsd	0.310	0.136
	SE	0.034	0.015
PLS	Mean	100.08	99.57
	S.D	0.310	0.135
	%rsd	0.310	0.136
	SE	0.034	0.015

*Mean ± SD for 9 determinations.

Table 30 Recovery studies of nebivolol hydrochloride and hydrochlorothiazide in formulation

	Nebivolol hydrochloride			Hydrochlorothiazide		
Method	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)
CLS	1	2.50	100.06	2	5.74	99.85
	2	3.50	100.01	4	7.73	99.78
	3	4.51	100.02	6	9.72	99.69
Mean			100.03			99.77
S.D			±0.188			±0.071
MLR	1	2.50	99.99	2	5.72	99.14
	2	3.57	100.58	4	7.74	99.51
	3	4.33	96.19	6	9.63	99.29
Mean			98.92			99.31
S.D			±2.383			±0.639
PCR	1	2.50	100.09	2	5.74	99.85
	2	3.50	99.98	4	7.73	99.78
	3	4.50	99.95	6	9.72	99.68
Mean			100.01			99.77
S.D			±0.198			±0.073
PLS	1	2.50	100.09	2	5.74	99.85
	2	3.50	99.98	4	7.73	99.78
	3	4.50	99.95	6	9.72	99.68
Mean			100.01			99.77
S.D			±0.198			±0.073

*Mean ± SD for 9 determinations.

Table 31 Composition of the calibration set for pioglitazone hydrochloride and metformin hydrochloride

	Concentration µg ml^{-1}			Concentration µg ml^{-1}	
Mixture	Pioglitazone hydrochloride	Metformin hydrochloride	Mixture	Pioglitazone hydrochloride	Metformin hydrochloride
1	0.3	7	14	1.2	21
2	0.6	7	15	1.5	21
3	0.9	7	16	0.3	28
4	1.2	7	17	0.6	28
5	1.5	7	18	0.9	28
6	0.3	14	19	1.2	28
7	0.6	14	20	1.5	28
8	0.9	14	21	0.3	35
9	1.2	14	22	0.6	35
10	1.5	14	23	0.9	35
11	0.3	21	24	1.2	35
12	0.6	21	25	1.5	35
13	0.9	21			

Table 32 Statistical parameters of chemometric methods for pioglitazone hydrochloride and metformin hydrochloride

	CLS	MLR	PCR	PLS		
Component	RMSECV	RMSECV	RMSEC	RMSECV	RMSEC	RMSECV
Pioglitazone hydrochloride	0.0215	0.0191	0.0187	0.0213	0.0097	0.0227
Metformin hydrochloride	0.0881	0.1996	0.0717	0.0881	0.0712	0.0884

Table 33 Composition of physical, formulation and recovery set for pioglitazon hydrochloride and metformin hydrochloride

Physical $\mu g\ ml^{-1}$		Formulation $\mu g\ ml^{-1}$		Recovery $\mu g\ ml^{-1}$	
Pioglitazone hydrochloride	Metformin hydrochloride	Piogli tazone	Metformin hydrochloride	Pioglitazone hydrochloride	Metformin hydrochloride
0.6	14	0.6	10	0.3	7
0.6	21	0.6	10	0.3	7
0.6	28	0.6	10	0.3	7
0.9	14	0.6	10	0.36	8.4
0.9	21	0.6	10	0.36	8.4
0.9	28	0.6	10	0.36	8.4
1.2	14	0.6	10	0.42	9.8
1.2	21	0.6	10	0.42	9.8
1.2	28	0.6	10	0.42	9.8

Table 34 Statistical parameters of chemometric methods for pioglitazon hydrochloride and metformin hydrochloride (Prediction)

	CLS		MLR		PCR		PLS	
Component	RMSEP	r	RMSEP	r	RMSEP	r	RMSEP	r
Pioglitazone hydrochloride	0.0059	0.998	0.0332	0.996	0.0057	0.998	0.0103	0.999
Metformin hydrochloride	0.0161	1	0.0858	1.000	0.0161	1.000	0.0172	1.000

*Mean ± SD for 9 determinations.

Table 35 Prediction results for pioglitazone and metformin hydrochloride from the validation (physical mixtures) samples by chemometric methods

Component	CLS		MLR		PCR		PLS	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Pioglitazone hydrochloride	99.41	0.65	100.37	1.111	99.43	0.59	99.43	0.59
Metformin hydrochloride	99.99	0.13	99.79	0.623	99.90	0.13	99.90	0.13

*Mean ± SD for 9 determinations.

Table 36 Prediction results pioglitazone hydrochloride and metformin hydrochloride from the formulation samples by different chemometric methods

Method	Parameters	Pioglitazone	Metformin hydrochloride
CLS	Mean	99.58	100.02
	S.D	0.864	0.029
	%rsd	0.868	0.029
	SE	0.096	0.003
MLR	Mean	99.01	100.24
	S.D	1.740	0.231
	%rsd	1.758	0.230
	SE	0.193	0.026
PCR	Mean	99.63	100.01
	S.D	0.821	0.031
	%rsd	0.824	0.031
	SE	0.091	0.003
PLS	Mean	99.63	100.01
	S.D	0.821	0.031
	%rsd	0.824	0.031
	SE	0.091	0.003

*Mean ± SD for 9 determinations.

Table 37 Recovery studies of pioglitazone hydrochloride and metformin hydrochloride in formulation

	Pioglitazone hydrochloride			Metformin hydrochloride		
Method	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)
CLS	0.3	0.90	99.79	7	26.997	100.01
	0.36	0.95	99.62	8.4	28.40	100.00
	0.42	1.02	99.83	9.8	30	100.22
Mean			99.75			100.08
S.D			±0.294			±0.224
MLR	0.3	0.90	97.12	7	27.12	100.31
	0.36	0.96	98.91	8.4	28.46	100.48
	0.42	1.02	100.62	9.8	29.80	99.84
Mean			98.88			100.21
S.D			±3.111			±0.345
PCR	0.3	0.895	99.81	7	26.997	99.99
	0.36	0.96	99.61	8.4	28.40	99.99
	0.42	1.02	99.69	9.8	29.80	100.01
Mean			99.71			99.99
S.D			±0.318			±0.015
PLS	0.3	0.89	99.10	7	26.99	99.99
	0.36	0.96	99.46	8.4	28.40	99.99
	0.42	1.01	98.97	9.8	29.79	99.99
Mean			99.18			99.99
S.D			±0.516			±0.014

*Mean ± SD for 9 determinations.

Table 38 Composition of the calibration set for Losartan potassium and Ramipril

	Concentration µg ml^{-1}			Concentration µg ml^{-1}	
Mixture	Ramipril	Losartan potassium	Mixture	Ramipril	Losartan potassium
1	1.2	8	14	3.6	32
2	1.2	16	15	3.6	40
3	1.2	24	16	4.8	8
4	1.2	32	17	4.8	16
5	1.2	40	18	4.8	24
6	2.4	8	19	4.8	32
7	2.4	16	20	4.8	40
8	2.4	24	21	6	8
9	2.4	32	22	6	16
10	2.4	40	23	6	24
11	3.6	8	24	6	32
12	3.6	16	25	6	40
13	3.6	24			

Table 39 Statistical parameters of chemometric methods for Losartan potassium and Ramipril

	CLS	MLR	PCR	PLS		
Component	RMSECV	RMSECV	RMSEC	RMSECV	RMSEC	RMSECV
Ramipril	0.0432	0.0698	0.0403	0.0466	0.0399	0.0463
Losartan potassium	0.0629	0.1317	0.0037	0.0044	0.0581	0.0636

Table 40 Composition of physical, formulation and recovery set for Losartan potassium and Ramipril

Physical $\mu g\ ml^{-1}$		Formulation $\mu g\ ml^{-1}$		Recovery $\mu g\ ml^{-1}$	
Ramipril	Losartan potassium	Rami Pril	Losartan potassium	Ramipril	losartan potassium
3.6	12	1.5	30	0.45	3
3.6	4	1.5	30	0.45	3
1.2	4	1.5	30	0.45	3
1.2	20	1.5	30	0.9	6
2.4	8	1.5	30	0.9	6
2.4	16	1.5	30	0.9	6
4.8	20	1.5	30	1.35	9
1.2	16	1.5	30	1.35	9
1.2	12	1.5	30	1.35	9

Table 41 Statistical parameters of chemometric methods for Losartan potassium and Ramipril (Prediction)

	CLS		MLR		PCR		PLS	
Component	RMSEP	r	RMSEP	r	RMSEP	r	RMSEP	r
Ramipril	0.0191	1	0.0541	0.999	0.0173	0.999	0.016	0.999
Losartan potassium	0.0106	1	0.0783	1.000	0.0030	1.000	0.0118	1.000

Table 42 Prediction results for Losartan potassium and Ramipril from the validation (physical mixtures) samples by different chemometric methods

	CLS		MLR		PCR		PLS	
Component	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
Ramipril	98.67	0.89	98.91	1.57	98.65	0.78	98.65	0.78
Losartan potassium	100.10	0.22	100.36	1.28	99.99	0.04	99.99	0.04

*Mean ± SD for 9 determinations.

Table 43 Prediction results of Losartan potassium and Ramipril from the formulation samples by different chemometric methods

Method	Parameters	Ramipril	Losartan potassium
CLS	Mean	100.49	100.01
	S.D	0.157	0.020
	%rsd	0.156	0.020
	SE	0.017	0.002
MLR	Mean	94.91	100.13
	S.D	1.539	0.179
	%rsd	1.621	0.179
	SE	0.171	0.020
PCR	Mean	99.77	100.14
	S.D	0.197	0.198
	%rsd	0.198	0.198
	SE	0.022	0.022
PLS	Mean	99.77	100.14
	S.D	0.197	0.198
	%rsd	0.198	0.198
	SE	0.022	0.022

*Mean ± SD for 9 determinations.

Table 44 Recovery studies of Losartan potassium and Ramipril in formulation

	Ramipril			Losartan potassium		
Method	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)	Added µg ml^{-1}	Found µg ml^{-1}	Recovery (%)
CLS	0.45	1.94	99.62	3	32.99	100.00
	0.9	2.40	100.13	6	35.99	99.98
	1.35	2.86	100.43	9	38.99	99.98
Mean			100.06			99.98
S.D			±0.392			±0.019
MLR	0.45	2.03	103.24	3	32.99	99.94
	0.9	2.46	101.53	6	35.97	99.99
	1.35	2.85	99.92	9	39.05	100.09
Mean			101.56			100.01
S.D			±1.677			±0.098
PCR	0.45	1.94	99.78	3	32.999	99.999
	0.9	2.39	99.99	6	36.02	100.02
	1.35	2.85	100.60	9	38.999	99.999
Mean			100.12			100.01
S.D			±0.656			±0.021
PLS	0.45	1.94	99.78	3	32.999	99.999
	0.9	2.39	99.99	6	36.02	100.02
	1.35	2.85	100.60	9	38.999	99.999
Mean			100.12			100.01
S.D			±0.656			±0.021

*Mean ± SD for 9 determinations.

Table 45 Optical regression characteristics for atorvastatin calcium, fenofibrate, atorvastatin calcium, ezetimibe, levofloxacin hemihydrate and ornidazole

*Parameters	Atorvastatin calcium 239 nm	Fenofibrate 246 nm	Atorvastatin calcium 233 nm	Ezetimibe 224 nm	Levofloxacin hemihydrate 277.5 nm	Ornidazole 319 nm
Beer's Law Limit (mg ml^{-1})	4 -24	4 -24	4 -24	4 -24	10 – 50	20 – 80
Molar absorptivity	350.32	540.47	736.81	819.33	773.47	53.44
Regression Equation:						
Slope	0.00063	0.0015	0.00062	0.00204	0.00195	0.00025
Intercept	-2.5E-05	8.85E-05	-0.0001	-0.0004	0.001659	0.00003
Correlation coefficient	0.9999	0.99986	0.9961	0.9993	0.9992	0.99987
Sandell's Sensitivity µg cm^{-2}/0.001A.U	1.5888	0.6755	1.6188	0.4922	0.6088	4.0603
Standard error of mean	7.05E-05	0.00022	0.00052	0.00073	0.001641	0.00014

* Average of Six determinations

Table 46 Results of analysis of formulation

S.No	Drug	Label Claim	*% Label claim found	*±S.D	*% RSD
1	Atorvastatin	10 mg	101.15	0.9964	0.9851
	Fenofibrate	160 mg	100.53	0.8488	0.8444
2	Atorvastatin	10 mg	102.01	1.2131	1.1892
	Ezetimibe	10 mg	101.09	1.8500	1.8310
3	Levofloxacin	250 mg	100.77	0.8364	0.8300
	Ornidazole	500 mg	100.77	0.7195	0.7135

*Each reading is an average of six determinations

Table 47 Results of recovery studies

S.No	Drug	% Recovery	*±S.D	% RSD*
1	Atorvastatin calcium	99.90	0.0370	0.8379
		99.90	0.9470	0.9480
		100.28	1.0320	1.0287
	Fenofibrate	99.86	0.9139	0.9150
		99.67	0.8215	0.8242
		100.03	0.8398	0.8395
2	Atorvastatin calcium	101.77	1.8670	1.8342
		102.03	1.3923	1.3646
		101.86	1.8839	1.8494
	Ezetimibe	99.93	1.2772	1.2781
		100.28	1.2153	1.2119
		99.55	1.8634	1.8719
3	Levofloxacin hemihydrate	100.28	0.4581	0.4568
		100.28	0.4319	0.4307
		100.33	0.3621	0.3609
	Ornidazole	99.46	1.6573	1.6663
		98.33	1.3975	1.4212
		99.79	1.1117	1.1140

*Each reading is an average of six determinations

Table 48 Results of intra day & inter day studies (n = 3)

S.NO	Parameters*	Atorvastatin calcium	Fenofibrate	Atorvastatin calcium	Ezetimibe	Levofloxacin hemihydrate	Ornidazole
Intra day							
1	% Amount found	100.7	100.56	100.12	101.51	102.14	100.41
2	%rsd	0.8387	0.5330	1.1300	1.062	0.6741	1.7332
Inter day							
1	% Amount found	100.63	100.74	100.98	100.09	101.79	100.63
2	%rsd	0.7753	0.6163	0.874	0.524	0.7952	1.5185

*Each reading is an average of six determinations

Table 49 System suitability parameters for losartan potassium, amlodipine besylate and hydrochlorothiazide

S.NO	Component	Rt	Theoretical plates	Capacity factor	Resolution factor	Asymmetry factor	Tailing factor
1	Losartan potassium	2.96	1819	1.96	1.88	1.08	1.06
2	Hydrochlorothiazide	4.11	28153	3.11		1.26	1.16
3	Amlodipine besylate	5.11	77369	4.11		0.77	0.86

*Each reading is an average of six determinations

Table 50 Optical and Regression characteristics of losartan potassium, amlodipine besylate and hydrochlorothiazide by RP-HPLC

S.NO	Parameters	Losartan potassium	Amlodipine besylate	Hydrochlorothiazide
1	λ_{max} nm	225	225	225
2	Beer's Law Limits (µg ml $^{-1}$)	8 - 40	1 - 5	3-15
	i) Slope	209391.5	7123.81	386729.8
	ii) Intercept	49137.89	440.58	71044.38
3	Correlation coefficient	0.9982	0.9987	0.9994
4	LOD (µg ml $^{-1}$)	0.7801	0.0495	0.0055
5	LOQ (µg ml $^{-1}$)	2.3641	0.1499	0.0168

*Each reading is an average of six determinations

Table 51 Assay of commercial formulation by RP-HPLC method for losartan potassium, amlodipine and hydrochlorothiazide

S.NO	Parameters*	Losartan potassium	Amlodipine	Hydrochlorothiazide
1	% Amount found	101.04	100.67	100.954
2	SD	1.1398	1.4543	0.4450
3	%rsd	1.1280	1.4446	0.4408

*Each reading is an average of six determinations

Table 52 Recovery study data by RP-HPLC method for losartan potassium, amlodipine besylate and hydrochlorothiazide

	Losartan potassium			Amlodipine besylate			Hydrochlorothiazide		
Method	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
HPLC	12	26.96	99.86	1.2	2.81	103.94	3	6.82	100.99
	15	30.405	101.35	1.5	3.08	102.8	3.75	7.66	102.17
	18	33.20	100.62	1.8	3.303	100.11	4.5	8.34	101.03
Mean			100.82			102.29			101.06
*S.D			0.5496			1.7429			0.7114
*%rsd			0.5451			1.7039			0.7039

*Each reading is an average of six determinations

Table 53 Precision by RP-HPLC method for losartan potassium, amlodipine besylate and hydrochlorothiazide

Intra day S.NO	Parameters*	Losartan potassium	Amlodipine besylate	Hydrochlorothiazide
1	% Amount found	100.95	100.29	101.24
2	SD	1.7639	1.1857	0.2489
3	%rsd	1.7474	1.1822	0.2459
Inter day				
1	% Amount found	101.14	100.38	100.67
2	SD	0.3284	0.3342	0.4336
3	%rsd	0.3247	0.3342	0.4307

*Each reading is an average of six determinations

Table 54 System suitability parameters for atorvastatin calcium, fenofibrate and folic acid

S.NO	Component	R_t	Theoretical plates	Capacity factor	Resolution factor	Asymmetry factor	Tailing factor
1	Folic acid	2.89	5011	1.89	3.05	1.5	1.19
2	Atorvastatin calcium	3.93	8405	2.93		1.67	1.38
3	Fenofibrate	7.98	3465	6.98		1.35	1.18

Table 55 Optical and Regression characteristics of atorvastatin calcium, fenofibrate and folic acid by RP-HPLC

S.NO	Parameters*	Atorvastatin calcium	Fenofibrate	Folic acid
1	λ_{max} nm	248	248	248
2	Beer's Law Limits (µg ml $^{-1}$)	1 - 5	15 - 75	0.5-2.5
	i) Slope	155419.8	838362.3	18678.64
	ii) Intercept	32188	-571954	-128.103
3	Correlation coefficient	0.9965	0.9992	0.9997
4	LOD (µg ml $^{-1}$)	0.0800	0.0330	0.0310
5	LOQ (µg ml $^{-1}$)	0.2423	0.1000	0.0941

Table 56 Assay of commercial formulation by RP-HPLC method for Atorvastatin, fenofibrate and folic acid

S.NO	Parameters*	Atorvastatin	Fenofibrate	Folic acid
1	% Amount found	100.68	100.21	99.81
2	SD	0.2297	0.1623	0.2734
3	%rsd	0.2282	0.1621	0.2739

*Each reading is an average of six determinations

Table 57 Recovery study data by RP-HPLC method for atorvastatin calcium, fenofibrate and folic acid

	Atorvastatin calcium			Fenofibrate			Folic acid		
Method	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
HPLC	1.6	2.63	101.95	24	39.47	101.99	0.8	1.3012	100.15
	2	3.018	100.9	30	45.48	101.59	1	1.5088	100.88
	2.4	3.404	100.19	36	51.48	101.32	1.2	1.7016	100.14
Mean			101.03			101.28			100.39
*S.D			0.7750			0.5291			0.3684
*%rsd			0.7671			0.5224			0.3669

*Each reading is an average of six determinations

Table 58 Precision by RP-HPLC method for atorvastatin calcium, fenofibrate and folic acid

Intra day				
S.NO	Parameters*	Atorvastatin calcium	Fenofibrate	Folic acid
1	% Amount found	100.82	100.33	99.95
2	SD	0.2698	0.1531	0.0111
3	%rsd	0.2676	0.1525	0.0111
Inter day				
1	% Amount found	100.54	100.09	99.67
2	SD	0.0206	0.0611	0.3541
3	%rsd	0.0205	0.061	0.3553

*Each reading is an average of six determinations

Table 59 System suitability parameters for rosiglitazone maleate, glibenclamide and metformin hydrochloride

S.NO	Component	R_t	Theoretical plates	Capacity factor	Resolution factor	Asymmetry factor	Tailing factor
1	Metformin hydrochloride	2.92	1670	1.92	3.23	1.23	1.12
2	Rosiglitazone maleate	5.25	18324	4.25		1.93	1.54
3	Glibenclamide	9.38	52790	8.3		1.39	1.15

Table 60 Optical and Regression characteristics of rosiglitazone maleate, glibenclamide and metformin hydrochloride by RP-HPLC

S.NO	Parameters*	Metformin hydrochloride	Rosiglitazone maleate	Glibenclamide
1	λ_{max} nm	230	230	230
2	Beer's Law Limits (µg ml $^{-1}$)	7 - 35	0.05 - 0.25	0.1-0.5
	i) Slope	636860.8	26714.38	83906.57
	ii) Intercept	-42901.3	291.119	-312.587
3	Correlation coefficient	0.9993	0.9959	0.9985
4	LOD (µg ml $^{-1}$)	1.0363	0.0465	0.0220
5	LOQ (µg ml $^{-1}$)	3.1402	0.1409	0.0666

*Each reading is an average of six determinations

Table 61 Assay of commercial formulation by RP-HPLC method for rosiglitazone, glibenclamide and metformin hydrochloride

S.NO	Parameters*	Metformin hydrochloride	Rosiglitazone	Glibenclamide
1	% Amount found	100.39	100.37	100.4
2	SD	0.3422	0.9858	0.2801
3	%rsd	0.3408	0.9822	0.2890

*Each reading is an average of six determinations

Table 62 Recovery study data by RP-HPLC method for rosiglitazone maleate, glibenclamide and metformin hydrochloride

	Metformin hydrochloride			Glibenclamide			Rosiglitazone maleate		
Method	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
HPLC	10	22.7	100.91	0.1	0.229	101.97	0.04	0.091	100.93
	12.5	25.26	101.04	0.125	0.25	100.03	0.05	0.101	101.63
	15	27.57	100.27	0.15	0.2795	101.64	0.06	0.111	101.23
Mean			100.70			100.89			101.03
*S.D			0.6053			0.8938			0.3589
*%rsd			0.6014			0.8858			0.3552

*Each reading is an average of six determinations

Table 63 Precision by RP-HPLC method for rosiglitazone maleate, glibenclamide and metformin hydrochloride

S.NO	Parameters*	Metformin hydrochloride	Rosiglitazone maleate	Glibenclamide
Intra day				
1	% Amount found	100.32	99.64	100.43
2	SD	0.5087	0.5907	0.2709
3	%rsd	0.5071	0.5928	0.2697
Inter day				
1	% Amount found	100.48	101.11	100.36
2	SD	0.1224	0.6721	0.3438
3	%rsd	0.1218	0.6647	0.3426

*Each reading is an average of six determinations

Table 64 System suitability parameters for nebivolol hydrochloride and hydrochlorothiazide

S.NO	Component	R_t	Theoretical plates	Capacity factor	Resolution factor	Asymmetry factor	Tailing factor
1	Hydrochlorothiazide	4.52	8512	3.52	13.67	1.3	1.2
2	Nebivololhydrochloride	19.12	38996	18.2		1.39	1.21

Table 65 optical and regression characteristics of nebivolol hydrochloride and hydrochlorothiazide by RP-HPLC

S.NO	Parameters*	Nebivolol hydrochloride	Hydrochlorothiazide
1	λ_{max} nm	240	240
2	Beer's Law Limits (µg ml $^{-1}$)	1 - 5	3 - 15
	i) Slope	129912.7	377480.5
	ii) Intercept	26462.75	192278.8
3	Correlation coefficient	0.9961	0.9971
4	LOD (µg ml $^{-1}$)	0.1562	0.0889
5	LOQ (µg ml $^{-1}$)	0.4734	0.2694

Table 66 Assay of commercial formulation by RP-HPLC method for nebivolol and hydrochlorothiazide

S.NO	Parameters*	Nebivolol	Hydrochlorothiazide
1	% Amount found	101.00	100.72
2	SD	0.3928	0.2594
3	%rsd	0.3884	0.2575

*Each reading is an average of six determinations

Table 67 Recovery study data by RP-HPLC method for nebivolol hydrochloride and hydrochlorothiazide

	Nebivolol Hydrochloride			Hydrochlorothiazide		
Method	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
HPLC	1.2	2.72	100.59	3	6.78	100.42
	1.5	3.02	100.63	3.75	7.65	101.95
	1.8	3.307	100.22	4.5	8.28	100.37
Mean			100.34			100.87
*S.D			0.2151			0.6934
*%rsd			0.2144			0.6873

*Each reading is an average of six determinations

Table 68 Precision by RP-HPLC method for nebivolol hydrochloride and hydrochlorothiazide

Intra day			
S.NO	Parameters*	Nebivolol hydrochloride	Hydrochlorothiazide
1	% Amount found	101.24	100.62
2	SD	0.4622	0.3094
3	%rsd	0.4565	0.3075
Inter day			
1	% Amount found	101	100.82
2	SD	0.3595	0.2098
3	%rsd	0.3559	0.2081

*Each reading is an average of six determinations

Table 69 System suitability parameters for metformin hydrochloride and pioglitazone hydrochloride

S.NO	Component	R_t	Theoretical plates	Capacity factor	Resolution factor	Asymmetry factor	Tailing factor
1	Metformin hydrochloride	2.81	1639	1.81	3.63	1.43	1.8
2	Pioglitazone hydrochloride	5.07	11661	4.07		1.09	1.76

Table 70 Optical and Regression characteristics of metformin hydrochloride and pioglitazone hydrochloride

S.NO	Parameters*	Metformin hydrochloride	Pioglitazone hydrochloride
1	λ_{max} nm	230	230
2	Beer's Law Limits (µg ml $^{-1}$)	7 - 35	0.3 – 1.5
	i) Slope	633749.9	55000.54
	ii) Intercept	45836.02	138.5397
3	Correlation coefficient	0.9988	0.9989
4	LOD (µg ml $^{-1}$)	1.3841	0.0498
5	LOQ (µg ml $^{-1}$)	4.1943	0.1509

*Each reading is an average of six determinations

Table 71 Assay of commercial formulation by RP-HPLC method for pioglitazone and metformin hydrochloride

S.NO	Parameters*	Metformin hydrochloride	Pioglitazone
1	% Amount found	100.46	101.19
2	SD	0.4297	1.0413
3	%rsd	0.4277	1.0290

*Each reading is an average of six determinations

Table 72 Recovery study data by RP-HPLC method for pioglitazone hydrochloride and metformin hydrochloride

Method	Metformin hydrochloride			Pioglitazone hydrochloride		
	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
HPLC	8	12.98	99.88	0.48	0.788	101.82
	10	15.03	100.19	0.6	0.91	100.83
	12	17.13	100.74	0.72	1.02	100.66
Mean			100.26			101.1
*S.D			0.4727			0.5737
*%rsd			0.4715			0.5674

*Each reading is an average of six determinations

Table 73 Precision by RP-HPLC method for pioglitazone hydrochloride and metformin hydrochloride

Intra day			
S.NO	Parameters*	Metformin hydrochloride	Pioglitazone hydrochloride
1	% Amount found	101.54	101.86
2	SD	0.4769	0.01272
3	%rsd	0.4697	0.01249
Inter day			
1	% Amount found	100.26	100.52
2	SD	0.0018	1.1636
3	%rsd	0.0018	1.5758

*Each reading is an average of six determinations

Table 74 System suitability parameters for losartan potassium and ramipril

S.NO	Component	R_t	Theoretical plates	Capacity factor	Resolution factor	Asymmetry factor	Tailing factor
1	Losartan potassium	4.6	3205	3.6	1.53	1.17	0.81
2	Ramipril	7.2	3009	6.2		1.63	1.32

Table 75 Optical and Regression characteristics of losartan potassium and ramipril

S.NO	Parameters*	Losartan potassium	Ramipril
1	λ_{max} nm	210	210
2	Beer's Law Limits (µg ml $^{-1}$)	8 - 40	1.2 – 6
	i) Slope	172307.4	122519.9
	ii) Intercept	-636077	-22186.8
3	Correlation coefficient	0.9935	0.9937
4	LOD (µg ml $^{-1}$)	2.2128	0.0546
5	LOQ (µg ml $^{-1}$)	6.7054	0.1656

*Each reading is an average of six determinations

Table 76 Assay of commercial formulation by RP-HPLC method for losartan potassium and ramipril

S.NO	Parameters*	Losartan potassium	Ramipril
1	% Amount found	100.83	100.50
2	SD	0.0194	0.4981
3	%rsd	0.0192	0.4956

*Each reading is an average of six determinations

Table 77 Recovery study data by RP-HPLC method for losartan potassium and ramipril

	Losartan potassium			Ramipril		
Method	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)	Added ($\mu g\ ml^{-1}$)	Found ($\mu g\ ml^{-1}$)	Recovery (%)
HPLC	12	27.39	101.45	0.6	1.359	100.71
	15	30.23	100.75	0.75	1.52	101.41
	18	33.24	100.74	0.9	1.652	100.15
Mean			100.98			100.76
*S.D			0.4398			0.5645
*%rsd			0.4356			0.5603

*Each reading is an average of six determinations

Table 78 Precision by RP-HPLC method for losartan potassium and ramipril

Intra day S.NO	Parameters*	Losartan potassium	Ramipril
1	% Amount found	100.83	100.50
2	SD	0.0194	0.4981
3	%rsd	0.0192	0.4956
Inter day			
1	% Amount found	100.84	100.67
2	SD	0.0075	0.6698
3	%rsd	0.0075	0.6653

*Each reading is an average of six determinations

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Original research paper

Simultaneous spectrophotometric determination of losartan potassium, amlodipine besilate and hydrochlorothiazide in pharmaceuticals by chemometric methods

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In the present work, four different spectrophotometric methods for simultaneous estimation of losartan potassium, amlodipine besilate and hydrochlorothiazide in raw materials and in formulations are described. Overlapped data was quantitatively resolved by using chemometric methods, classical least squares (CLS), multiple linear regression (MLR), principal component regression (PCR) and partial least squares (PLS). Calibrations were constructed using the absorption data matrix corresponding to the concentration data matrix, with measurements in the range of 230.5–350.4 nm ($\Delta\lambda$ = 0.1 nm) in their zero order spectra. The linearity range was found to be 8–40, 1–5 and 3–15 μg mL^{-1} for losartan potassium, amlodipine besilate and hydrochlorothiazide, respectively. The validity of the proposed methods was successfully assessed for analyses of drugs in the various prepared physical mixtures and in tablet formulations.

Keywords: losartan potassium, amlodipine besilate, hydrochlorothiazide, spectrophotometry, chemometry

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Losartan potassium (LOS), amlodipine besilate (AML) and hydrochlorothiazide (HYD) are drugs widely used for the treatment of hypertension and cardiovascular diseases in combined pharmaceutical preparations. Losartan potassium and its principal active metabolites block the vasoconstrictor and aldosterone-secreting effects of angiotensin II by selectively blocking the binding of angiotensin II to angiotensin II receptor type 1 (AT_1) receptor found in many tissues (vascular smooth muscle, adrenal gland). Amlodipine besilate inhibits the movement of calcium ions across the cell membrane into vascular smooth muscles and myocytes. Action is stronger in arterial resistance vessels causing peripheral vasodilatation and reduction in afterload. Hydrochlorothiazide inhibits the reabsorption of sodium and chloride at the beginning of the distal convo-

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luted tubule. It causes natriuretic effect mainly by decreasing sodium and chloride reabsorption in the cortical segment of the ascending limb of the loop of Henley by inhibition of a specific Na^+,Cl^- co-transporter (1).

A few estimations in body fluids, in bulk in combination with other drugs and in single dosage forms have been reported for losartan potassium, amlodipine besilate and hydrochlorothiazide such as HPLC (2–7), spectrophotometry (8), multivariate approach (9), multi-syringe chromatography (MSC) (10) and HPTLC (11, 12), ultra performance liquid chromatography (UPLC-MS) (13). All these drugs are available in combined tablet dosage forms, as antihypertensive agents. An extensive literature survey revealed that a number of methods are reported for the individual drugs but there is no report on simultaneous estimation of such a combination in physical mixtures or in pharmaceutical formulations by chemometric methods. The present article discusses the attempts made to develop simple, sensitive and reproducible methods for simultaneous estimation of these drugs in dosage forms.

Chemometric calibration techniques in spectral analysis are widely used in the quality control of drugs in mixtures and pharmaceutical formulations containing two or more drugs with overlapping spectra where separation procedures are not required in drug determination. We have also used these techniques for simultaneous analyses of mixtures (14–19).

In this study, four chemometric methods for spectral data processing are proposed for simultaneous determination of LOS, AML and HYD in their ternary mixtures and in tablets.

EXPERIMENTAL

Instrument and software

A Shimadzu (Japan) 2550-double beam spectrophotometer was used for all spectrophotometric measurements. Absorption spectra of the reference and test solutions were taken in 1-cm matched quartz cells over the range of 200–400 nm. Chemometric calculations on the resulting data were carried out with the PLS toolbox (Demover5.0) in MATLAB 7 (Math works).

Samples and solvents

Losartan potassium, amlodipine besilate and hydrochlorothiazide were kindly supplied by ATOZ India Ltd., India, and were certified to be 99.8, 99.6 and 99.9 % pure, respectively. The drugs were used without further purification. All the solvents used in spectrophotometric analysis were of analytical reagent grade. Trilopace tablets, batch number BF 70002 (Akums Drugs & Pharmaceuticals Ltd., India), which were claimed to contain 50 mg of losartan potassium USP, 5 mg of amlodipine besilate BP and 12.5 mg of hydrochlorothiazide IP, were used.

Standard solutions and mixtures

Stock solutions of LOS (1 mg mL^{-1}), AML (1 mg mL^{-1}) and HYD (2 mg mL^{-1}) in methanol were diluted with water to prepare the working solutions (0.16 mg mL^{-1}, 0.02 mg mL^{-1} and 0.06 mg mL^{-1}, respectively). The calibration set contained 25 and the prediction set 9 mixtures of calibration samples, so that the concentration of each drug in the resulting solutions was in its own linear dynamic range, as shown in Table 1. Furthermore, we demonstrated that in formulations LOS, AML and HYD range 1: 2.5: 10.

Tablet analysis

Twenty tablets were weighed accurately and powdered. An amount of the powder equivalent to 50 mg of AML, 125 mg of HYD and 500 mg of LOS was dissolved in 50 mL of methanol. The solution was ultrasonicated for 10 minutes. Then, the solution was filtered through Whatman filter paper No. 41. The filtrate (3 mL) was transferred into a 100 mL volumetric flask and made up to volume with Millipore water. Aliquots of these solutions were used in such a way that the concentration of each drug was within the range of the calibration matrix. The diluted solutions were analyzed six times. All the proposed chemometric methods were applied.

Chemometric methods

Classical least squares (CLS). – This method assumes Beer's law model with the absorbance at each frequency being proportional to the component concentration. In

Table I. Composition of the calibration set

Concentration (μg mL^{-1})				Concentration (μg mL^{-1})			
Mixture	LOS	AML	HYD	Mixture	LOS	AML	HYD
1	24	3	9	14	24	5	15
2	24	1	3	15	40	5	3
3	8	1	15	16	40	1	12
4	8	5	6	17	8	4	3
5	40	2	15	18	32	1	9
6	16	5	9	19	8	3	12
7	40	3	6	20	24	4	12
8	24	2	6	21	32	4	6
9	16	2	12	22	32	2	3
10	16	4	15	23	16	1	6
11	32	5	12	24	8	2	9
12	40	4	9	25	16	3	3
13	32	3	15				

LOS, AML, HYD – losartan potassium, amlodipine besilate, hydrochlorothiazide, respectively.

matrix notation, Beer's law model for m calibration standards containing l chemical components with the spectra of n digitized absorbances is given by:

$$A = C \times K + E_A \tag{1}$$

where A is the $m \times n$ matrix of calibration spectra, C is the $m \times l$ matrix of component concentration, K is the $l \times n$ matrix of absorptivity-path length products, and E_A is the $m \times n$ matrix of spectral errors. K then represents the matrix of pure component spectra at unit path length. The classical least squares solution according to Eq. (1) during calibration is:

$$\hat{K} = (C^T C)^{-1} C^T \times A \tag{2}$$

where $\hat{K}$ indicates the least-squares estimation of K.

Analysis based on spectrum a, of unknown component concentration (sample):

$$C_0 = (\hat{K}\hat{K}^T)^{-1} \hat{K} \times a \tag{3}$$

where C_0 is vector of predicted concentration and $\hat{K}^T$ is the transpose of matrix $\hat{K}$.

Multiple linear regressions (MLR). – If absorbance measurements for several solutions containing mixtures of the analytes are made in numbers exceeding the number of mixture components, then the system composed of the absorbance and concentration matrices will be overdimensioned and take the following matrix form:

$$A = KC \tag{4}$$

where A is the data absorbance calibration matrix, K is the matrix from which the proportionality constants are calculated from spectra for standard solutions of pure analytes and/or their mixtures, and C is the concentration matrix. The C prediction concentration matrix can be calculated from the following equation:

$$C = (K^T K)^{-1} K^T \tag{5}$$

where K' is the transpose of K and A is the absorbance matrix of unknown samples. Matrix K can be obtained in various ways. We calculated K values by *MLR* of the data for mixtures of analytes of known composition (20).

Principle component regression (PCR). – In the spectral work, the following steps can explain the fundamental concept of PCR. The original data obtained in absorbances (A) and concentrations (C) of analytes were reprocessed by mean-centring as A_0 and C_0, respectively. Using the ordinary linear regression:

$$C = a + b \times A \tag{6}$$

The coefficient b is: $b = P \times q$, where P is the matrix of eigenvectors and q is the C-loadings given by $q = D \times T^T \times A_0$. Here, T^T is the transpose of the score matrix T. D is a diagonal matrix having on components the inverse of the selected eigenvalues. Knowing b one can easily find a by using the formula $a = C_{mean} \times A^T_{mean} \times b$, where A^T_{mean} represents the transpose of the matrix having the entries of the mean absorbance values, and C_{mean} is the mean concentration of the calibration set.

Partial least squares (PLS). – In the UV-Vis spectra, the absorbance data (A) and concentration data (C) are mean centered to give the data matrix A_0 and vector C_0. The orthogonalized PLS algorithm has the following steps. The loading weight vector W has the following expression:

$$W = \frac{A_0^T C_0}{C_0^T C_0} \tag{7}$$

The scores and loadings are given by:

$$t_1 = \frac{A_0 W}{A_0^T t_1} \tag{8}$$

$$P_1 = t_1^T t_1 \tag{9}$$

$$q_1 = \frac{C_0^T t_1}{t_1^T t_1} \tag{10}$$

The matrix and vector of the residuals in A_0 and C_0 are:

$$A_1 = A_0 - t_1 P_1^T \tag{11}$$

$$C_1 = C_0 - t_1 q_1^T \tag{12}$$

From the general linear equation, the regression coefficients were calculated by:

$$b = W(P^T W)^{-1} q \tag{13}$$

$$a = C_{mean} - A^T_{meanb} \tag{14}$$

The built calibration equation is used for the estimation of the compounds in the samples (21).

RESULTS AND DISCUSSION

A calibration set was randomly prepared as mixtures of LOS, AML and HYD in their possible compositions by applying a multilevel multifactor design (22) (Table I). The UV absorbance data were obtained by measuring the absorbances in the region of 200–400 nm (Fig. 1). From this 230.5–350.4 nm wavelength was selected for construction

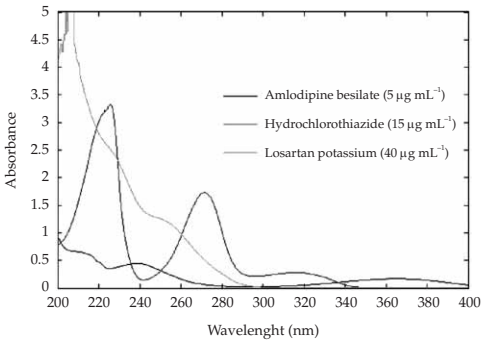

Fig. 1. Overlapping spectrum of LOS, AML and HYD.

Table II. Statistical parameters of chemometric methods in the calibration set

Method	Parameter	LOS	AML	HYD
CLS	RMSECV	0.1647	0.1183	0.1823
	RMSEP	0.1264	0.0908	0.1110
	R	1	0.9940	0.9990
MLR	RMSECV	0.2258	0.2047	0.2583
	RMSEP	0.2062	0.0908	0.2328
	R	1	0.9920	0.9980
PCR	RMSEC	0.1647	0.1183	0.1823
	RMSECV	0.1987	0.1424	0.2134
	RMSEP	0.1250	0.0856	0.1120
	R	1	0.9940	0.9990
PLS	RMSEC	0.1647	0.1183	0.1823
	RMSECV	0.1987	0.1424	0.2134
	RMSEP	0.1250	0.0856	0.1120
	R	1	0.9940	0.9990

CLS – classical least squares, MLR – multiple linear regression, PCR – principal component regression.
PLS – partial least squares, RMSEP – root mean square error of prediction, RMSECV – root mean square error of cross validation, RMSEC – root mean square error of calibration.
For other acronyms see Table I.

of the calibration model. The fit model was constructed by using the absorption data matrix corresponding to the concentration data matrix in CLS, MLR, PCR and PLS.

Before constructing the model, pre-processing (23) was carried out to reduce the effect of noise, improve the predictive ability of the model and simplify the model by making the data more normally distributed and the wavelength selection based on the best outcome for reduced error of spectral data. In Table II, *R* is defined as the correlation coefficient between constituent concentrations and shows the absorbance effects relating to the constituent of interest. Values obtained in the methods close to 1 mean no interference was coming from other constituents in the respective set of calibration mixtures.

The most commonly employed validation criterion is to divide the dataset into two subsets, a calibration set and a validation set. The calibration model is calculated using the calibration set. Then, the root mean square errors of calibration and validation, RMSEC – root mean square error of calibration and RMSECV – root mean square error of cross validation, are calculated by using the calibration model under investigation to predict the samples in the calibration set and validation set, respectively:

$$\mathrm{RMSEC} = \sqrt{\frac{(y - y_{\mathrm{pred}})^i (y - y_{\mathrm{pred}})}{m - 1}}$$

Selection of the optimum number of factors for PCR and PLS. – For PCR and PLS methods, 25 calibration spectra were used for the selection of the optimum number of factors by using the cross-validation with the leave-out-one technique. This allows modelling of the system with the optimum amount of information and avoidance of over-fitting or under-fitting. The cross-validation procedure consisted of systematically removing one of a group of training samples in turn and using only the remaining ones for the construction of latent variable factors and applied regression. The predicted concentrations were then compared with the actual ones for each of the calibration samples and the root mean square error of prediction (RMSEP) was calculated. The RMSEP was computed in the same manner each time, and then a new factor was added to the PCR and PLS model. The selected model was that with the smallest number of factors such that its

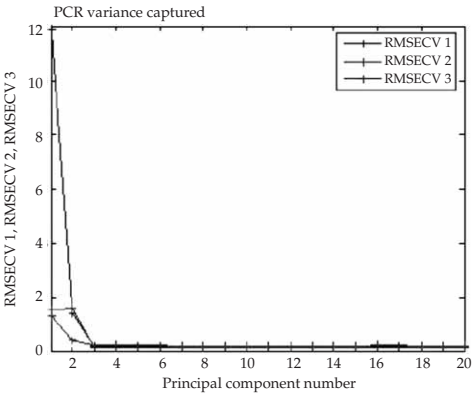

Fig. 2. Representation of RMSECV values generated from calibration by PCR: LOS, AML and HYD.

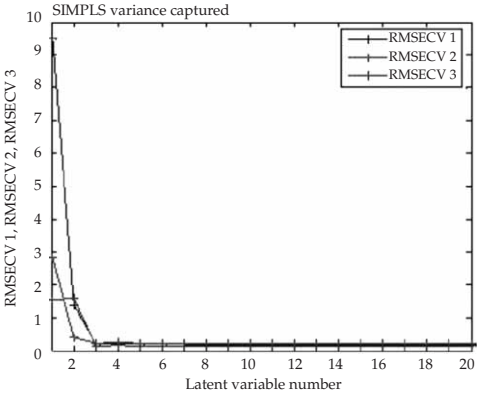

Fig. 3. Representation of RMSECV values generated from calibration by PLS: LOS, AML and HYD.

RMSECV values were not significantly greater than that for the model, which yielded the minimum RMSECV. A plot of RMSECV values against the number of components (Figs. 2 and 3) indicates that the latent variable factor 3 was optimum for PCR and PLS selected based on the RMSEC and RMSECV, respectively, for the estimation of the titled drugs. At the selected principal component of PCR and PLS, the concentrations of each sample was then predicted and compared with the known concentration and the RMSEP was calculated:

$$\text{RMSEC} = \sqrt{\sum_{i=y}^{N} \frac{(y_{\text{ipred}} - y_{\text{iref}})^2}{N}} \qquad \text{RMSEC} = \sqrt{\frac{(y - y_{pred})^i (y - y_{pred})}{m - 1}}$$

Table III. Composition of synthetic mixtures (formulation) and recovery set

Synthetic formulation (μg mL^{-1})[a]			Formulation (μg mL^{-1})[b]			Recovery (μg mL^{-1})[c]		
LOS	AML	HYD	LOS	AML	HYD	LOS	AML	HYD
24.8	3.6	10.8	30	3	7.5	3	0.45	0.75
24.8	1.2	3.6	30	3	7.5	3	0.45	0.75
9.6	1.2	18.0	30	3	7.5	3	0.45	0.75
9.6	6.0	7.2	30	3	7.5	6	0.90	1.50
48.0	2.4	18.0	30	3	7.5	6	0.90	1.50
19.2	6.0	10.8	30	3	7.5	6	0.90	1.50
48.0	3.6	7.2	30	3	7.5	9	1.35	2.25
28.8	2.4	7.2	30	3	7.5	9	1.35	2.25
19.2	2.4	14.4	30	3	7.5	9	1.35	2.25

[a] Synthetic mixture of LOS, AML and HYD (standards).
[b] Pharmaceutical formulation, tablet.
[c] Recovery – standard addition to pharmaceutical formulation..
For other acronyms see Table I.

Table IV. Prediction results for losartan, amlodipine and hydrochlorothiazide from the synthetic validation samples by different chemometric methods

Component	CLS		MLR		PCR		PLS	
	Mean ± SD[a]		Mean ± SD[a]		Mean ± SD[a]		Mean ± SD[a]	
LOS	100.6	0.9	100.4	1.2	100.6	0.9	100.6	0.9
AML	100.0	1.6	100.6	0.7	100.0	0.5	100.0	0.5
HYD	100.9	0.6	99.7	0.9	100.9	0.7	100.9	0.7

[a] Nine determinations.
For acronyms see Table I and II.

In order to test the proposed techniques, the validation set of synthetic mixtures (from standards) containing the three drugs (Table III) in variable ratios was carried out; the results are given in Table IV. The maximum values of the mean percent errors corresponding to CLS, MLR, and PCR and PLS for the same mixtures were completely acceptable because of their very small numerical values (below 0.2). Results of tablet analyses are shown in Table V.

Recovery and precision studies

To check the validity of the proposed methods, recovery studies were carried out by addition of the standard to the preanalyzed formulation. Results of recovery studies were found to be from 99.3 ± 0.3 to 101.3 ± 0.6 % (Table VI). Good precision of the method was indicated by RSD ranging 0.3–1.4 %.

Table V. Prediction results for losartan, amlodipine and hydrochlorothiazide in formulation samples by different chemometric methods

Method	Parameter[a]	LOS	AML	HYD
CLS	Mean (%)	100.02	100.39	101.28
	RSD (%)	0.034	0.720	0.131
MLR	Mean (%)	100.27	98.92	101.52
	RSD (%)	0.078	1.683	1.192
PCR	Mean (%)	100.01	100.36	101.32
	RSD (%)	0.033	0.705	0.128
PLS	Mean (%)	100.01	100.36	101.32
	RSD (%)	0.033	0.705	0.128

[a] Nine determinations.

Table VI. Recovery studies of LOS, AML and HYD in pharmaceutical formulations[a]

Method	LOS			AML			HYD		
	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)[b]	Added (μg mL^{-1})	Found (μg mL^{-1})	*Recovery (%)[b]	Added (μg mL^{-1})	Found (μg mL^{-1})	*Recovery (%)[b]
	3	33.1	100.4	0.5	3.4	99.8	0.8	8.4	101.6
CLS	6	36.0	100.0	0.9	3.9	99.2	1.5	9.1	100.6
	9	39.0	100.1	1.4	4.3	99.4	2.3	9.9	101.6
Mean			100.1			99.5			101.3
S.D			± 0.2			± 0.3			± 0.6
	3	33.0	100.1	0.5	3.5	101.3	0.8	8.4	102.2
MLR	6	35.7	99.3	0.9	3.9	99.9	1.5	9.1	100.6
	9	39.1	100.2	1.4	4.4	100.5	2.3	9.8	100.3
Mean			99.9			100.5			101.0
S.D			± 0.7			± 1.0			± 1.4
	3	33.1	100.3	0.5	3.4	99.8	0.8	8.4	101.7
PCR	6	36.0	100.0	0.9	3.9	99.0	1.5	9.1	100.6
	9	39.0	100.0	1.4	4.3	99.2	2.3	9.9	101.6
Mean			100.1			99.3			101.3
S.D			± 0.2			± 0.4			± 0.6
	3	33.1	100.3	0.5	3.4	99.7	0.8	8.4	101.7
PLS	6	36.0	100.0	0.9	3.9	99.0	1.5	9.1	100.6
	9	39.0	100.0	1.4	4.3	99.2	2.3	9.9	101.6
Mean			100.1			99.3			101.3
S.D			± 0.2			± 0.4			± 0.6

[a] Additions to 30 μg mL^{-1} LOS, 3 μg mL^{-1} AML and 7.5 μg mL^{-1} HYD in the formulation.
[b] Nine determinations.

CONCLUSIONS

Based on the results obtained in this work, the UV spectrophotometric method for simultaneous determination of losartan potassium, amlodipine besilate and hydrochlorothiazide in mixtures by multivariate calibration of synthetic and pharmaceutical samples is applicable. PLS and PCR using a calibration matrix constructed with absorption spectra were successfully applied to simultaneous analysis of these drugs in synthetic and pharmaceutical mixtures.

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S A Ž E T A K

Istodobno spektrofotometrijsko određivanje losartan kalija, amlodipin besilata i hidroklorotiazida u farmaceutskim pripravcima kemometrijskom metodom

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U radu su opisane četiri spektrofotometrijske metode za istodobno određivanje losartan kalija, amlodipin besilata i hidroklorotiazida u sirovinama i farmaceutskim pripravcima. Podaci koji su se preklapali kvantitativno su razlučeni kemometrijskim metodama, klasičnom metodom najmanjih kvadrata (CLS), multiplom linearnom regresijom (MLR), regresijom glavnih komponenata (PCR) te metodom parcijalnih najmanjih kvadrata (PLS). Kalibracije su provedene koristeći podatke o ovisnosti apsorpcije o koncentracijama, mjereći spektre nultog reda u rasponu 230,5–350,4 nm ($\Delta\lambda$ = 0,1 nm). Linearnost za losartan kalij bila je 8–40, za amlodipin besilat 1–5, a za hidroklorotiazid 3–15 μg mL^{-1}. Valjanost predloženih metoda uspješno je potvrđena analizom navedenih lijekova u različitim pripremljenim smjesama i tabletama.

Ključne riječi: losartan, amlodipin besilat, hidroklorotiazid, spektrofotometrija, kemometrija

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