CHAPTER 7

SUMMARY AND CONCLUSION

7.1 ANTIHYPERTENSIVES

Antihypertensive agents are now widely used for the treatment of various forms of hypertension. Antihypertensive agents in the form of monotherapy make simple treatment schedules possible, only a limited number of hypertensive drugs can be used in this way. These drugs are Beta blockers, diuretics, calcium antagonists and Angiotensin-Converting Enzyme (ACE) inhibitors. All other antihypertensive drugs cause sodium and water retention during chronic application. Thus, their blood pressure lowering action decreases or even totally disappears.

It is known several patients with hypertension require two or more antihypertensive drugs with complementary mechanisms of action to lower their blood pressure. The angiotensin II type 1- receptor antagonist (Irbesartan, Valsartan and Telmisartan) and the diuretic Hydrochlorothiazide are two antihypertensive agents that have a well recognized clinical efficacy. Their combination was shown in randomized, controlled trials to be more effective than each agent alone in lowering blood pressure, due to a dual and synergistic mechanism.

7.2 SPECTROPHOTOMETRIC METHODS

Spectrophotometric methods are the most commonly used techniques and continue to enjoy wide popularity. The common availability of the instrument, the simplicity of the procedures, speed, precision and accuracy of the technique still make the spectrophotometric methods more attractive. Derivative spectrophotometry in the UV-VIS region is a useful technique in
extracting qualitative and quantitative information from the overlapping bands of analytes and interferents. The main problem of spectrophotometric multi component analysis is the simultaneous determination of two or more active compounds in the same mixture without preliminary separation. Several spectrophotometric methods have been used for resolving mixture of compounds with overlapping spectra.

7.2.1 HPSAM for Binary Mixtures

The basis of this method has been explained for the spectrophotometric determination of two analytes with overlapping spectra. The requirements for the application of the method is to work at only two wavelengths, where the analytical signal due to one of the species (interferent) must be same and for the other one (analyte) should be different as much as possible. By plotting the analyte signals versus added analyte concentration, two straight lines are obtained that have the common point H which coordinates $(C_H, A_H)$; $C_H$ is the unknown analyte concentration and $A_H$ is the analytical signal due to the interferent species.

The method was developed for the following combinations

1. Irbesartan ($C_H$) and Hydrochlorothiazide ($A_H$)
2. Valsartan ($C_H$) and Hydrochlorothiazide ($A_H$)

In both the combinations, drug Hydrochlorothiazide was considered as an interferent and the wavelength selection was made from its spectrum. Wavelength selection was made in such a way that at the two wavelengths, the interferent is said to have same absorbance. Different mixtures were prepared to which known amount of the analyte was added (Irbesartan/Valsartan). The absorbance was measured at the selected wavelengths. A plot on the concentration of the analyte versus absorbance values at two wavelengths was made. Two straight lines were obtained and said to intersect at a point H, with the corresponding coordinates $C_H$ and $A_H$. Where $C_H$ corresponds to the concentration of the analyte and $A_H$ to the
absorbance of the interferent. The methods were validated for accuracy and precision. The suitability of the method was checked with marketed formulations. This method offers good selectivity, accuracy and precision and can be applied for simultaneous determination of wide range of Irbesartan/Valsratan and Hydrochlorothiazide concentrations. The high degree of reproducibility and valuable dynamic range as well as the simplicity are the advantages of the proposed method.

7.2.2 HPSAM for ternary mixtures

In this method one drug is considered as an analyte X and the other two as interferents Y and Z. The determination of the concentration of the analyte by HPSAM requires the selection of two wavelengths $\lambda_1$ and $\lambda_2$ at which the interferent species Y has the same absorbance. The known amount of X is successively then added to the mixture and the resulting absorbances were measured at the two selected wavelengths.

The contribution in absorbance value at the two selected wavelengths by the other interferent Z has to be corrected in order to obtain the concentrations of X and Y. A wavelength $\lambda_3$ was identified from the individual spectrum of all three drugs, where the interferent Z shows some absorbance and the other two (X and Y) are free from interference. The calibration graphs for Z were constructed at three selected wavelengths. The slope of these calibration graphs were used to obtain the corrected absorbance as mentioned below in equations (1) and (2).

$$A_{\text{Corr}, \lambda_1} = A_{\text{mix}, \lambda_1} - r_1 \times A_{\text{mix}, \lambda_3} \quad (1)$$

$$A_{\text{Corr}, \lambda_2} = A_{\text{mix}, \lambda_2} - r_2 \times A_{\text{mix}, \lambda_3} \quad (2)$$

Where $A_{\text{mix}, \lambda_1}$, $A_{\text{mix}, \lambda_2}$ and $A_{\text{mix}, \lambda_3}$ are the absorbances of sample (ternary mixture) at $\lambda_1$, $\lambda_2$ and $\lambda_3$ respectively. $A_{\text{Corr}, \lambda_1}$ and $A_{\text{Corr}, \lambda_2}$ are the net absorbances due to contribution of X and Y at $\lambda_1$ and $\lambda_2$ respectively, that are used for the construction of the HPSA graph. The values $r_1$ and $r_2$ are the
slope ratios of the drug Z calibration graphs and can be calculated by equations (3) and (4)

\[ r_1 = \frac{\text{Slope (in } \lambda_1 \text{)}}{\text{Slope (in } \lambda_3 \text{)}} \quad (3) \]

\[ r_2 = \frac{\text{Slope (in } \lambda_2 \text{)}}{\text{Slope (in } \lambda_3 \text{)}} \quad (4) \]

Thus, the method can be applied for simultaneous estimation three drugs with overlapping spectra without any prior separation.

Following combination of drugs were analysed by this method

1. Irbesartan (X), Hydrochlorothiazide (Y) and Telmisartan (Z)
2. Valsartan (X), Hydrochlorothiazide (Y) and Telmisartan (Z)
3. Ramipril (X), Hydrochlorothiazide (Y) and Telmisartan (Z)
4. Losartan (X), Hydrochlorothiazide (Y) and Amlodipine (Z)

All the methods include different processing steps like linearity of individual drugs, selection of suitable wavelengths and suitability of the method in different synthetic mixtures. The methods were validated for the reproducibility and accuracy results using different commercially available formulations. All the spectral measurements were made with Perkin Elmer UV –VIS Spectrophotometer (Lamda 25) and the graphs and calculations were made with the use of MS excel spread sheet. The good agreement between the results and known values indicate the successful applicability of the method for simultaneous determination of three drugs in formulations. This method overcomes the extraction process and hence the use of organic solvents. This study demonstrates that HPSAM can be useful in resolving overlapped spectra of ternary mixture than the traditional methods.

7.2.3 CHEMOMETRIC METHODS

Multivariate calibration methods have been applied for the analyses of multiple components. In these methods it is assumed that the series of mixtures for which the amount of each component and for which a series of
properties has been measured. The methods include Partial Least Squares (PLS) and Principal Component Regression (PCR).

PLS and PCR are calibration methods effective for the resolution of mixtures presenting serious spectral overlapping and non-linear absorbance additivity. The main advantage of a multivariate calibration when applied on spectral data is the high speed in processing both absorbance and concentration values. At the same time the errors in model calibration are minimized by using the absorbances of the full spectrum or selected wavelength regions.

The combined use of derivative spectrophotometry and chemometric techniques has demonstrated to be a highly convenient choice in the determination of multi-component matrices presenting serious spectral overlapping, thanks to their common potential ability to exploit minor spectral features. The main goal of the work was a comparison between the regression methods when they are applied on ordinary or derivative spectral data. The influence of derivative order from zero to two on the prediction ability of the methods were investigated.

The following combinations were analysed by PLS and PCR methods

**Binary mixtures**

1. Irbesartan and Hydrochlorothiazide
2. Valsartan and Hydrochlorothiazide
3. Telmisartan and Hydrochlorothiazide

The PLS and PCR models were developed using 151 data points for first two combinations and with 111 points for third combination. The pre-processing and cross validation steps were carried out for all the binary mixtures to get the optimum results.
**Ternary mixtures**

1. Irbesartan, Hydrochlorothiazide and Ramipril
2. Valsartan, Hydrochlorothiazide and Ramipril
3. Telmisartan, Hydrochlorothiazide and Ramipril
4. Trandolapril, Verapamil and Manidipine
5. Losartan, Hydrochlorothiazide and Amlodipine

The PLS and PCR models were calculated using 151 data points for all combinations except the fourth combination with 101 data points. The preprocessing and cross validation steps were carried out for all the mixtures to get low values of RMSEC and RMSEP.

The best assay results were obtained from the application of PLS and PCR to the second derivative spectra, being rich in useful information but bearing a limited signal noise. Very satisfactory results were obtained when the optimized models were applied to the analysis of synthetic mixtures and commercial drug formulations. According to these studies, multivariate calibration methods (PLS and PCR) coupled with derivative spectral data can be recommended as a very suitable choice to resolve severe overlapped absorption spectra of drug mixtures. This approach is simple in application, inexpensive and requires an easy treatment of the samples and provides reliable analytical results.

### 7.3 CHROMATOGRAPHIC METHODS

The most characteristic feature of the development in the pharmaceutical analysis in the past 25 years is that various forms of HPLC became the undoubtedly the most important method. The breakthrough of HPLC in compendial analysis of small organic molecules was extremely rapid. In the latest 29th revision of the United States Pharmacopoeia, HPLC is used for the assay of bulk materials of this type in about 45% of the monographs. This created an interest in developing a separation technique using HPLC for quantitative estimation of many drugs with minimal use of
organic solvents. Another effort was made to utilise the advantage of High Performance Thin Layer Chromatography, that a large number of samples can be analysed in a shorter time period. Unlike HPLC this method utilises less quantities of solvents, thus lowering the cost of analysis.

7.3.1 High Performance Liquid Chromatography (HPLC)

The HPLC (Shimadzu, Kyoto, Japan) instrument used was equipped with a model series LC-10 ADVP pump, Rheodyne 7725i injector with a 20µl loop and a SPD-10AVP UV-VIS detector. Separation and quantitation was made on a 150mm × 4.6 mm Phenomenex RP18 column with 5 µm particle size. The detection was made with UV detector and the data acquisition was performed using Winchrom software.

The chromatographic conditions were optimized especially the composition of the mobile phase through several trials to achieve good resolution and symmetric peak shapes of the analyte as well as short run time. It was found that a mixture of acetonitrile and water could achieve the purpose and was finally adopted as mobile phase in a gradient programme to estimate selected antihypertensive drugs. The gradient profile consisted of acetonitrile as mobile phase A and water as mobile phase B and the transition in the composition was made by a short gradient programme. In this gradient programme the mobile phase B composition changes from 30-45% between 0-6 min, 45-80% between 6-10 min and reduces back to 20% in the rest 8 min. The flow rate was maintained as 1ml/min and the total run time was 20 min for each sample injection.

The sample and standard solutions were introduced to the column by means of a 20µl loop and the chromatographic data were collected and processed with a computer system for data acquisition. Individual peaks were identified from retention time and concentrations were derived from the peak area for appropriate standard and sample solutions. Analysis was performed at room temperature.
Total chromatographic analysis time was 20 min with Telmisartan, Hydrochlorothiazide, Losartan, Irbesartan and Valsartan eluting with retention times 4.71, 5.68, 7.06, 9.56 and 15.39 min respectively. Calibration plots were linear over the range of 50-250 µg/ml for Hydrochlorothiazide and 25-125 µg/ml for other drugs. Recovery was in the range of 97-103% with the relative standard deviation of less than 2% for all the drugs. The high percentage recovery and low coefficient of variation confirm the suitability of the method for simultaneous analysis of all five antihypertensives.

The method was extended to the analysis of stated drugs from biological origin, human plasma. To one ml of the plasma known amount of all the drugs were added. Drugs were then extracted with subsequent addition of methanol as an extracting solvent. The combined extracts were evaporated to dryness. The residue obtained was reconstituted with a mixture of acetonitrile and water in the ratio 50:50 v/v. The drugs were quantified under the optimized chromatographic conditions and observed to have good reproducibility.

7.3.2 High Performance Thin Layer Chromatography (HPTLC)

Chromatography was performed on 20cm × 20 cm aluminium backed plates coated 0.2mm layers of silica gel 60 F_{254} (E.Merck, Germany). Samples were applied to the plates as 4mm bands, 5mm apart, by means of a Camag (Switzerland) Linomat V sample applicator fitted with a Camag microlitre syringe. Linear ascending development of the plates to a distance of 80 mm was performed with optimized mobile phase in a twin trough glass chamber previously saturated with mobile phase vapour for 20 min at room temperature (25^0C). After development the plate was scanned at the selected wavelength by means of Camag TLC scanner in absorbance mode, using the deuterium lamp. A variety of mobile phases were investigated to establish a suitable HPTLC method for analysis of the selected antihypertensive drugs. The suitability of the mobile phase was decided by study of the sensitivity of the assay, time required for analysis and the use of readily available solvents.
The retention factors were 0.35, 0.42, 0.54 and 0.60 for Ramipril, Telmisartan, Hydrochlorothiazide and Irbesartan respectively. The linear range was 500-2500 ng/spot for Hydrochlorothiazide and 250-1250 ng/spot for other drugs with the correlation coefficient greater than 0.9990. The method was validated and successfully used for the analysis of drugs in tablets.

7.4 CONCLUSION

Simultaneous multi-component analysis is an important issue in modern analytical chemistry. UV-Vis data usually contains non-specific data, which can be converted into useful information by multivariate calibration method. A comparative study of the use of PLS-1 and PCR methods for the estimation of binary and ternary mixtures were accomplished showing that these methods provide, with adequate software support, a clear example of the high resolving power of these techniques. The two chemometric methods reported allows the treatment of large amount of information on sample component concentrations and properties of a large number of samples that can be analysed in a relatively short time.

The combined advantages of speed, convenience and environmental friendliness have made chemometrics the preferred instrumental approach for the study. It was concluded that the two multivariate calibration methods were suitable to resolve overlapped absorption spectra of mixtures of antihypertensive drugs with satisfactory results and an ease treatment for the analyses of commercial samples.

The important characteristics of HPSAM include the simultaneous estimation of binary or ternary mixtures with high order of spectral overlapping and no extraction step was included thereby avoiding the use of organic solvents. The results showed the successful applicability of the method for simultaneous determination of antihypertensive agents because of
good selectivity, accuracy and precision. As the proposed HPSAM methods are sensitive than the previously reported spectrophotometric methods, can be employed for routine quality control analysis and a switch to the greener alternative may be justified.

Greener analytical separation technology has a dramatic and beneficial impact in the workplace, allowing more work to be performed with greatly reducing the amount of waste solvent associated with the collection of highly valued analytical HPLC data.

It is presumed that in the next few years there will be a continuing decline in the amount of solvent usage and waste generation associated with the analytical techniques. Hence an effort was made to develop a gradient elution technique to consume less solvent for simultaneous determination of five drugs and hence generate less waste than traditional HPLC.

The proposed HPTLC method offers simultaneous estimation of four drugs. The advantage of this method includes small amount of mobile phase requirement, speed of analysis and reduction in the cost per analysis. HPTLC does not suffer from pH restrictions and sample cleanup procedures. Several samples can be analysed simultaneously and the plates can be scanned several times at different wavelengths. Statistical analysis proved that the method is repeatable and selective for the analysis of selected antihypertensive drugs.

All the proposed methods can be extended for determination of degradation kinetics in stress conditions and in biological samples.