SYNOPSIS

Pharmaceutical analysis plays a key role not only for the quality assurance of drugs but also to guarantee to the consumer a safer and reliable product. The major field of pharmaceutical analysis is in the medicinal chemical manufacturing industry. In all research on synthesis of new compounds, the analyst is an indispensable teammate of the synthetist. The analytical work required ranges from the standardized elemental analysis of organic compounds to highly specialized chemical or instrumental functional group determination. After the new drug has been produced on a laboratory scale, the next task of the analyst is to develop methods for process control, that is, for the analysis of intermediates. Such control must be maintained both at the pilot plant stage and during full-scale production of the drug.

The typical drug manufacturing company has a control division, which is organizationally independent of both production and research. The responsibility of this group is to certify to the company management that each manufactured lot of drug meets the applicable quality standards and then, and only then, to release it for distribution. The decision to release or reject the finished product is based upon the analytical data.

Little need be added concerning the work of pharmaceutical analyst in university and other non-commercial laboratories doing research in medicinal chemicals, since their activities are similar to those in drug industry research units. The same is true of consulting laboratories, except that these are also frequently called upon to render analytical assistance beyond the research phase of a new product and into the pilot plant or production stage.

Formulations containing a single drug or a combination of drugs for potentiating or complementing one another in therapy are increasing and in some cases there are no satisfactory methods available for the assay and quite often the reported procedures suffer from inherent
limitations. Therefore, there is a constant need for developing analytical methods which are simple, sensitive, rapid, accurate, precise, and that can be easily adapted by pharmaceutical industry.

The work presented in this thesis is concerned with the development of some new assay methods for six bioactive compounds, namely, salbutamol sulphate (SBS), an antibranchodilator; pefloxacin mesylate dihydrate (PFM), an antibiotic; captopril (CPT), an antihypertensive; famotidine (FMT), an antihistamine; albendazole (ALB), an anthelmentic; and Acyclovir (ACL), an antiviral agents. Owing to their low toxicity, high physiological activity, and versatile pharmacological action, are widely used in therapeutics. Because of their extensive therapeutic applications, their analysis has been the concern not only of the pharmaceutical analyst, but also of the pharmacologist. The pharmacopoeial procedures for the determination of some of these drugs in pure form use non-aqueous titrimetry and in formulations, generally involve multiple extraction steps followed by UV-spectrophotometry or high performance liquid chromatography (HPLC). Majority of the methods currently available for the determination of cited drugs in formulations are HPLC, TLC, HPTLC, GC, GC-MS, FIA-spectrofluorimetry, FIA-spectrophotometry, stripping voltammetry, capillary electrophoresis etc., all require highly sophisticated instruments which are expensive, and hence are not always readily available. Further, many of the above techniques including HPLC, TLC, HPTLC, GC, GC-MS and capillary electrophoresis are purely physical, giving room for a wide spread feeling that “the chemistry is going out of analytical chemistry”. To strike a balance, chemical and physical approaches have been blended together to develop some analytical methods based on titrimetry, spectrophotometry, and reaction rate measurements for the assay of the drugs cited. Using these methods, routine analysis can be performed without or with very simple instrumentation
resulting nevertheless in sensitive and accurate measurements with advantages of speed and simplicity. Realizing the importance of HPLC in pharmaceutical quality control, this technique has also been used for the assay of some of the cited drugs.

Keeping in view the needs of the pharmaceutical industry and the adequacies of titrimetric, spectrophotometric, HPLC and reaction rate methods, a modest attempt has been made to develop new methods for the assay of SBS, PFM, CPT, FMT, ALB and ACL, and this work is presented in this thesis.

The thesis entitled “NEW ANALYTICAL METHODS FOR THE ASSAY OF SOME BIOACTIVE COMPOUNDS” comprises of seven chapters. Each chapter is further divided into different sections, for proper classification of work and discussion. The Literature survey has been restricted to the methods dealing with the pharmaceuticals. Methods concerned with the analyses in body fluids will not be of great value to the pharmaceutical analyst; and hence not reviewed.

Chapter-I is introductory in nature and it gives a general account of bioactive compounds and importance of pharmaceutical analysis. At the end of this Chapter, the scope of the work is described.

Chapter-II deals with the titrimetric, the spectrophotometric and the HPLC methods for the assay of salbutamol sulphate (SBS). Chapter-II contains five sections. Section 2.0 is concerned with the presentation of the drug profile and the literature survey of the various methods developed so far for the determination of SBS. Section 2.1 describes the utility of bromate-bromide mixture and methyl orange as reagents for the assay of SBS. In titrimetry, the drug is titrated directly with bromate in the presence of a large excess of bromide and in sulphuric acid medium using methyl orange as indicator. Spectrophotometry is based on the
determination of surplus bromine by its bleaching action on methyl orange dye. The kinetic method depends on the linear relationship between concentration of the drug and time for oxidation and bromination as indicated by the bleaching of methyl orange acid colour. Titrimetry is applicable in 2-20 mg range. In spectrophotometry, Beer’s law is obeyed in 0.5-5.0 µg ml⁻¹ range while 5.0-25.0 µg ml⁻¹ concentrations can be determined by kinetic method.

Section 2.2 deals with the use of periodate as reagent for the determination of SBS by titrimetric method. In one of the method (Method A), SBS is quantitatively oxidised by periodate in buffer medium (pH 3) and subsequent determination of unreacted periodate by iodometric back titration. The other method (Method B) is based on the oxidation of SBS by periodate in buffer (pH 3) medium and subsequent determination of iodate formed by the reduction of periodate, iodometrically. The iodate so determined corresponds to the amount of SBS. The unreacted periodate was masked by molybdate. The use of Folin-Ciocalteu (F-C) reagent for the determination of SBS in pharmaceuticals is described in Section 2.3. In this method, the drug was reacted with F-C reagent in sodium carbonate medium and the resulting blue coloured chromogen was measured at 760 nm. In Section 2.4 iron(III)-ferricyanide was used as a reagent for the determination of SBS. The method is based on the reduction of iron(III) by the studied drug and subsequent interaction of iron(II) with ferricyanide to form Prussian blue. The product exhibits absorption maximum at 760 nm. Beer’s law is obeyed in the concentration range of 0.25- 3.00 µg ml⁻¹ for SBS. The molar absorptivity and Sandell sensitivity values are 2.37 × 10⁵ l mol⁻¹ cm⁻¹ and 3.34 ng cm⁻² for SBS. The limits of detection and quantification are reported.

Section 2.5 describes a simple high performance liquid chromatographic method for the determination of SBS in bulk drug and in formulations. Chromatographic separation was performed at ambient temperature on a reversed phase Hypersil ODS C₁₈ column (25 cm × 4.6
mm id) using a mobile phase consisting of acetonitrile – ammonium acetate buffer (pH 3.5)(50:50) at a flow rate of 1.0 ml min\(^{-1}\). The detector wavelength was set at 250 nm with a selectivity of 0.2 a.u.f.s. the method is applicable in the range of 2.61 to 212 \(\mu g \text{ ml}^{-1}\). the limit of detection was 1.33 \(\mu g \text{ ml}^{-1}\).

Chapter-III is concerned with the assay of pefloxacin mesylate dihydrate (PFM) by titrimetry, spectrophotometry and HPLC methods. It is divided into three sections. Section 3.0 contains the drug profile and literature survey of the methods for PFM. The use of neutralization titration method using sodium hydroxide as reagent in titrimetric and spectrophotometric determination of PFM is described in Section 3.1. In titrimetry, the drug solution is treated with a measured excess of sodium hydroxide followed by back titration of the residual base with hydrochloric acid using phenol red – bromothymol blue mixed indicator. Spectrophotometry involves treatment of a fixed amount of sodium hydroxide – phenol red mixture with varying amounts of drug, and measurement of decrease in absorbance of the dye at 560 nm. In titrimetry, a reaction stoichiometry of 1:1 was found in the quantification range of 4-20 mg of drug. Spectrophotometry allows determination of PFM in the range, 5-40 \(\mu g \text{ ml}^{-1}\). The molar absorptivity is \(5.91 \times 10^3 \text{ l mol}^{-1} \text{ cm}^{-1}\) and the Sandell sensitivity is 56.37 ng cm\(^{-2}\). Section 3.2 describes two spectrophotometric methods for the determination of PFM using bromophenol blue (BPB). One of the spectrophotometric methods is the extraction of PFM-BPB complex formed at pH 3.0 into chloroform layer and measurement of the absorbance at 420 nm. And the other method is the back extraction of the ion-pair complex formed at pH 3.0 to the aqueous buffer of pH 5.2 and measurement of the absorbance of the dye at 560 nm. Both methods are sensitive, accurate and precise and were applied for assay of PFM in tablets.
Assay of captopril (CPT) by titrimetric, potentiometric, conductometric and spectrophotometric is presented in Chapter IV. This chapter has two sections. As usual, Section 4.0 contains the drug profile and literature survey on the quantitative methods for CPT. The details of the neutralization titrimetric, potentiometric, conductometric and spectrophotometric methods are presented in Section 4.1. Three titrimetric methods reported for the determination of captopril are based on the direct titration of drug against standard sodium hydroxide with visual, potentiometric and conductometric end point detection. In visual titrimetry, phenol red-bromothymol blue mixed indicator was used to detect the end point. Visual and potentiometric titration methods are applicable over 2-20 mg range, whereas conductrometry is applied over the range of 5-20 mg. The indirect titrimetric and spectrophotometric methods also described in this section. In titrimetry, the drug solution is treated with a measured excess of sodium hydroxide followed by back titration of the residual base with hydrochloric acid using phenol red-bromothymol blue as mixed indicator. Spectrophotometry involves the treatment of a fixed amount of sodium hydroxide – phenol red mixture with varying amounts of drug and measurement of decrease in absorbance of the dye at 560 nm. In titrimetry, a reaction stoichiometry of 1:1 is found in the quantification range of 5-20 mg of drug. Spectrophotometry allows the determination of captopril in the range, 5-30 μg ml⁻¹ with an apparent molar absorptivity of 3.72 × 10³ l mol⁻¹ cm⁻¹ and a Sandell sensitivity of 58.32 ng cm². Section 4.2 describes titrimetric and spectrophotometric procedures for the estimation of captopril in pure drug and in dosage forms. The methods are based on the oxidation of the drug by a known excess of vanadate in acidic conditions followed by determination of the residual oxidant by titrimetry and spectrophotometry. Titrimetry involves the back titration of the unreacted vanadate with iron(II)ammonium sulphate using N-
phenylanthranilic acid indicator whereas in spectrophotometry unreacted vanadate is complexed with hydrogen peroxide and absorbance measured at 460 nm. For titrimetry, the reaction stoichiometry and the range of determination have been given. In spectrophotometric method, Beer’s law is obeyed over 0-350 μg ml⁻¹ with an apparent molar absorptivity of $0.28 \times 10^3$ l mol⁻¹ cm⁻¹ and a Sandell sensitivity of 0.80 μg ml⁻¹. The detection limit and the limit of quantification are 3.1 and 10.3 μg ml⁻¹, respectively.

Chapter 5.0 deals with the determination of famotidine (FMT), using titrimetric, spectrophotometric, reaction rate and HPLC techniques. This chapter is divided into five sections. Section 5.0 contains the drug profile and literature survey of the methods found in the literature for FMT. The use of bromate-bromide mixture and methyl orange as reagents has been extended to the titrimetric, spectrophotometric and kinetic methods for determination of FMT and the details are presented in Section 5.1. In titrimetry, the sample is treated with a measured excess of bromate in the presence of a large excess of bromide and in hydrochloric acid medium, and after the reaction is judged to be complete, the unreacted bromine is determined iodometrically. Spectrophotometry comprises of addition of a known excess of bromate-bromide mixture to the sample solution in hydrochloric acid medium followed by the estimation of surplus bromine by reacting it with a definite amount of methyl orange dye and measuring the absorbance at 520 nm. The reacted bromate amount corresponds to the sample content. Kinetic method in based on bromination – oxidation of famotidine by the bromine generated insitu by the addition of acid to bromate – bromide mixture. With small amounts of drug in solution, the rates of bromine generation and consumption are roughly equal. Under these conditions, bleaching of added methyl orange by bromine will occur only after the bromination – oxidation is complete. The time required for bleaching of the dye is measured
and related to sample concentration. The working conditions of the methods have been optimized. Titrimetry is applicable over 3-15 mg range whereas spectrophotometry permits the quantitation of famotidine in the concentration range of 0.50 – 2.25 μg ml\(^{-1}\) with an apparent molar absorptivity of 8.97×10\(^4\) 1 mol\(^{-1}\) cm\(^{-1}\) and Sandell sensitivity of 3.76 ng cm\(^{-2}\). The kinetic method allows the determination of 10-50 μg ml\(^{-1}\) of famotidine with reasonable accuracy and precision. The use of potassium iodate for the titrimetric and spectrophotometric determination of FMT are contained in Section 5.2. The titrimetric method (A) is based on the oxidation of the drug by a known excess of potassium iodate in acid medium and determination of the residual oxidant by iodometric back titration with thiosulphate. Three spectrophotometric procedures also involve the oxidation of the drug by iodate. One procedure (B) is based on iodometric determination of the excess iodate by an auxiliary reaction using variamine blue dye. The other two spectrophotometric procedures are based on the measurement of iodine formed in the redox reaction involving famotidine and iodate. In procedure C, iodine formed is oxidised to ICl\(_2^-\) in the presence of chloride ions and is used to iodinate 2,4-dichlorofluorescein dye and the iodinated dye is measured at 520 nm, whereas the iodine released is extracted into carbon tetrachloride and measured at 520 nm in method D. Two inexpensive methods, titrimetric and spectrophotometric, developed for the determination of famotidine in pure form and its dosage forms involving the use of chloramine-T as the oxidimetric reagent are contained in Section 5.3. In titrimetry, 3-15 mg sample is titrated directly with chloramine-T in hydrochloric acid medium to methyl orange end point. Spectrophotometric procedure is based on the oxidation of the drug by a measured excess of chloramine-T followed by the estimation of the unreacted oxidant with the well-known metol- primary aryl amine reaction that yields a characteristic red colour with the absorption maximum at 520 nm. The amount of chloramine-T used up corresponds to the
drug content. The analytical conditions of both methods were investigated and optimised. The molar-ratio of the titration reaction was found to be 1:2 (drug: oxidant) and the probable reaction scheme suggested in conformity with the stoichiometry. Spectrophotometry is valid in the concentration range of 0.0-40.00 µg ml\(^{-1}\) with an apparent molar absorptivity 2.78 \(\times\) 10\(^3\) \(\text{mol}\,\text{cm}^{-1}\) and Sandell sensitivity of 121.20 ng cm\(^{-2}\). Section 5.4 deals with the spectrophotometric determination of FMT using iron(III) and ferricyanide. The details of this method and its application to the assay of FMT in pharmaceuticals are contained in Section 5.4.

FMT was also determined by HPLC using a reversed phase Hypersil ODS C\(_{18}\) column (25 cm x 45 mm id) using a mobile phase consisting of acetonitrile-0.1% phosphoric acid (pH 3.0) (70:30) at a flow rate 1.0 ml min\(^{-1}\). The detector wavelength was at 279 nm with a sensitivity of 0.2 a.u.f.s. Quantification was made by measuring peak area. This work is described in Section 5.5. At the end of the chapter a concluding section is given which gives a critical assessment of the proposed methods for FMT.

Chapter 6.0 is devoted to titrimetric, spectrophotometric, kinetic and HPLC methods for the determination of albendazole (ALB) and has four sections. Section 6.0 gives a brief account of the drug profile and the literature survey of the various methods that have been developed for ALB. Section 6.1 describes the application of bromate-bromide mixture and methyl orange dye as reagents for the titrimetric, spectrophotometric and kinetic determination of FMT. The details of method development and method validation are similar to those described for SBS, and FMT. The description of a sensitive spectrophotometric method for ALB using potassium iodate is contained in Section 6.2. The basis of the method is similar to that described under FMT. Five methods (titrimetric and spectrophotometric) for the determination of ALB using chloramine-T as reagent are described in Section 6.3. Titrimetric procedure
(Method A) is based on the direct titration of the drug in hydrochloric acid medium with chloramine-T using methyl orange as indicator. In indirect titrimetric method (method B), a known excess of chloramine-T is added to an acidified solution of sample, and after a specified time, the residual oxidant is determined iodometrically. Spectrophotometric procedure (Method C) also involves the addition of a measured excess of chloramine-T in buffer medium of pH 2.70±0.1 and after the reaction is ensured to be complete, the surplus oxidant is determined by a well established colour reaction involving metol and primary arylamine that results in charge-transfer complex measurable at 520 nm. The other two spectrophotometric procedures involve treating the sample solution with a measured excess of chloramine-T in hydrochloric acid medium followed by estimation of the unreacted chloramine-T by reacting with fixed amount of either indigo carmine dye solution and measuring the absorbance at 610 nm (Method D) or with methyl orange solution and measuring the absorbance at 510 nm (Method E). The utility of HPLC for the quantification of ALB is described in Section 6.3. The separation and determination were achieved on a reverse phase Hypersil ODS 5 micron C18 column using a mobile phase consisting of 20 mM ammonium acetate (pH adjusted to 3.0): acetonitrile in the ratio 40:60 at a flow rate of 1.0 ml min\(^{-1}\) and UV-detection at 220 nm. Quantification was made by measuring peak area. The chapter concludes with a critical assessment of the proposed methods for ALB.

Chapter 7.0 deals with the titrimetric, spectrophotometric and HPLC methods for the assay of acyclovir (ACL). The chapter contains four sections. Section 7.0 as usual deals with drug profile and literature survey on the various methods available for ACL. Three simple methods using visual titrimetric, potentiometric and spectrophotometric techniques are described for the determination of ACL in pure form and in pharmaceutical formulations are
described in Section 7.1. The methods are based on the neutralisation reaction involving the primary amino group of the drug and acetous perchloric acid in acetic acid medium. In titrimetric methods, the titration was completed with visual or potentiometric end-point detection, crystal violet being used as the indicator in visual titration. In spectrophotometry, the drug is treated with a fixed amount of perchloric acid-crystal violet mixture and the increase in absorbance is measured at 570 nm and is related to drug concentration. Both titrimetric methods are applicable over 2-20 mg range of drug and the titration reaction follows a 1:1 stoichiometry. In spectrophotometry, Beer’s law is obeyed over the concentration range 5 – 55 μg ml⁻¹ with an apparent molar absorptivity and Sandell sensitivity of $1.78 \times 10^4$ l mol⁻¹ cm⁻¹ and 12.68 ng cm⁻², respectively. The limits of detection and quantification are calculated to be 1.696 and 5.654 μg ml⁻¹, respectively. Section 7.2 contains a simple and cost effective spectrophotometric method for the determination of ACL in bulk drug and in formulations. The method is based on the formation of blue coloured chromogen when the drug reacts with Folin – Ciocalteu (F-C) reagent in alkaline medium. The coloured species has an absorption maximum at 760 nm and obeys Beer’s law in the concentration range 50-450 μg ml⁻¹. The absorbance was found to increase linearly with increasing concentration of ACL which is corroborated by the calculated correlation coefficient value of 0.9998 (n=9). The apparent molar absorptivity and Sandell sensitivity were $1.65\times10^2$ l mol⁻¹ cm⁻¹ and 1.36 μg cm⁻², respectively. A spectrophotometric procedure for the estimation of ACL using iron(III)- ferricyanide reagent has been described in Section 7.3. The method is based on the reduction of iron (III) by the studied drug and subsequent interaction of iron (II) with ferricyanide to form Prussian blue. The product exhibits absorption maximum at 760nm. Beer’s law is obeyed in the concentration ranges of 25-200 μg ml⁻¹ for ACL. The molar absorptivity and Sandell sensitivity values are
9.07 \times 10^2 \text{ mol}^{-1} \text{ cm}^{-1} \text{ and } 248.28 \text{ ng cm}^{-2} \text{ for ACL. The HPLC determination of ACL in pharmaceuticals is described in Section 7.4. The chromatographic conditions comprised a reversed-phase C_{18} column (250 \times 4.6 \text{ mm i.d.}) with a mobile phase of acetonitrile- 20 \text{ m mol l}^{-1} \text{ aqueous ammonium acetate buffer of pH 4.5 (40:60). The flow rate was 0.8 ml min}^{-1} \text{ and UV detection was used at 250 nm. Calibration graph was linear from 1.98 - 59.4 \mu g \text{ ml}^{-1}. The chapter ends with the concluding remarks on the methods proposed in the present investigation.}

**Method development and Method validation**

The working conditions of all the developed methods were optimized by systematic investigation of several factors. In titrimetry, the reaction stoichiometry was evaluated and used for quantification purpose. Based on the reaction stoichiometry, the plausible reaction scheme is given wherever the author was confident of the nature of reaction. In all other cases, the presentation of the reaction scheme is avoided for want of clarity on the nature of reaction. The range of applicability (in milligram) is given for all the titrimetric methods. The linearity between the amount of drug and the titration end point was checked by calculating the correlation coefficient.

For all the spectrophotometric methods, the wavelength of maximum absorption, the Beer's law limits, the molar absorptivity and Sandell's sensitivity values are given. Detection and quantification limits calculated based on the standard deviation of response and the slope are also given. The regression equation relating the measured absorbance with the concentration of the drug and the corresponding regression coefficient are given for all the spectrophotometric methods.

The kinetic method developed for SBS, FMT and ALB is featured by the linear range, regression equation and regression coefficient.
For HPLC methods, in addition to the linear range, detection and quantification limits calculated based on signal to noise approach are also provided.

The accuracy and precision of all the developed methods were evaluated by seven replicate analyses performed on pure drug at three different levels (amounts or concentrations). The accuracy of the method was ascertained by calculating relative error (%), and the precision was determined by calculating the standard deviation and relative standard deviation (%). The range of error (%) for each of the levels (amount/concentration) is also reported.

The validity of the methods was ascertained by applying the methods to the determination of the drug content in pharmaceutical formulations. The same batch formulations were assayed by a reference method and the results were statistically evaluated by applying Student's t-test for accuracy and F-test for precision. This is done for every formulation investigated and the results are tabulated for each method.

The accuracy and validity of the method were further confirmed by performing the recovery tests via standard addition method.

**Publication of the work**

*Bulk of the work contained in the thesis has been published in reputed and refereed journals in the form of twenty one research articles and the publication of the remaining work is in different stages (refer list of publications).*