DEVELOPMENT OF NEW ANALYTICAL METHODS FOR COMBINED PHARMACEUTICAL FORMULATIONS BY DIFFERENCE AND DERIVATIVE SPECTROSCOPY

THESIS

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By

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CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT OF NEW ANALYTICAL METHODS FOR COMBINED PHARMACEUTICAL FORMULATIONS BY DIFFERENCE AND DERIVATIVE SPECTROSCOPY**" and submitted by **C.V.N. PRASAD**, ID. No. **93PHXF406** for award of **Ph.D. Degree** of the Institute, embodies original work done by him under my supervision.

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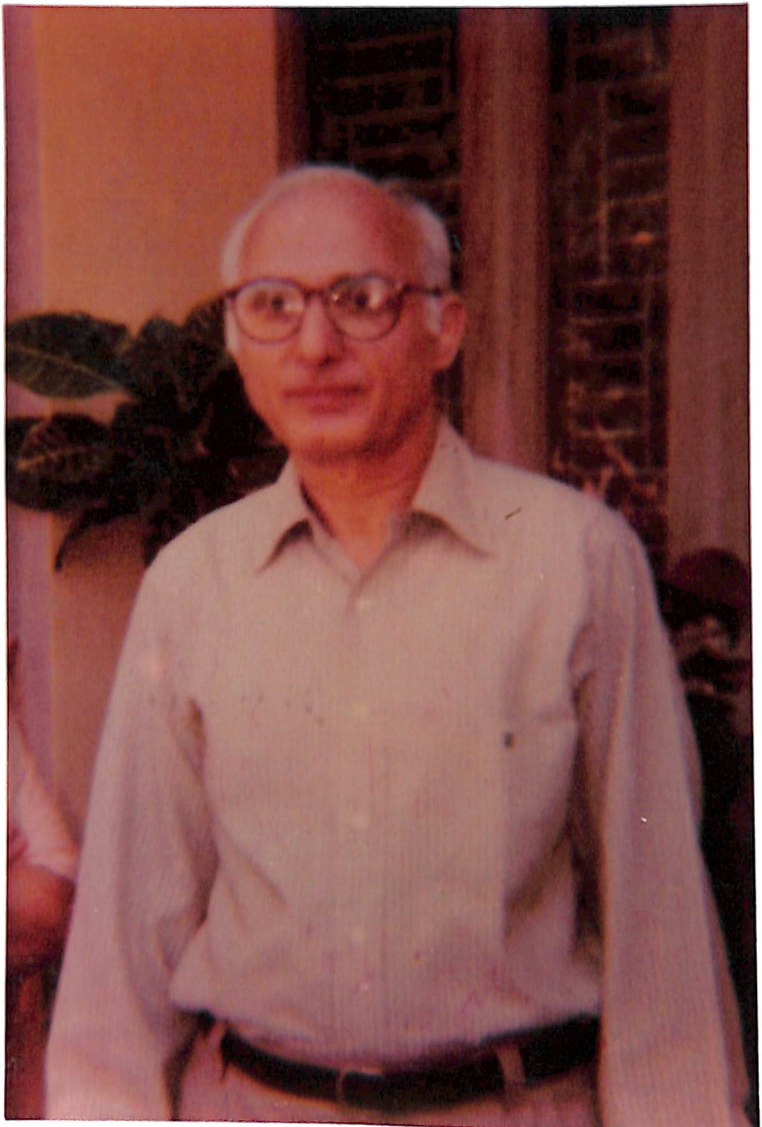

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SUMMARY

Drug substances are often delivered as formulations and thus combined with inert substances like additives to facilitate the formulation, stability of drug (s) and also for their acceptability by the patient. The drug formulations usually contain more than one drug substance to attain the better therapeutic effect. These formulations are produced frequently as a batch of few thousands and the number of samples finally tested represent a small quantity of the total production. Hence, there is a need for proper assay method to test these dosage forms meant for clinical use. The official monographs describe various assay methods for the determination of pure drug substances and for their formulations as single constituent, and a very few procedures for combined formulations. Thus, analysis of drugs in combined formulation is always a challenge to pharmaceutical chemist. Therefore, there has always been great significance attached with those methods, which involves the simultaneous determination of drug substances from the combined preparations.

The analytical techniques based on chromatographic separations like HPLC, GC and HPTLC have become popular in the estimation of drug substances from the marketed formulations. However, these popular methods demand lot of time, cost and technical expertise in achieving better results. Thus, the objective of this thesis work was to propose new analytical methods for combined formulations based on the principles of UV spectroscopy, which are simple, precise, cost effective and does not demand any extra skill from the analyst.

The various UV spectroscopic techniques used in this thesis for the analytical method development were difference, derivative and derivative-difference spectroscopy.

The drug formulations analysed based on the principles of difference spectroscopy were

a. Metronidazole and nalidixic acid tablets
b. Metronidazole and diloxamide furoate tablets

Similarly, the various drug combinations attempted using derivative spectroscopy were

a. Tinidazole and furazolidone tablets
b. Tinidazole and clotrimazole tablets
c. Tinidazole and norfloxacin tablets
d. Tinidazole, furazolidone and diloxamide furoate tablets
e. Metoprolol tartrate and hydrochlorothiazide tablets
f. Propranolol hydrochloride and hydrochlorothiazide tablets
g. Atenolol and amlodipine besylate tablets
h. Amlodipine besylate and enalapril maleate tablets
i. Atenolol, amiloride hydrochloride and hydrochlorothiazide tablets & capsules
j. Haloperidol and trihexyphenidyl hydrochloride tablets

At the end, the above two proposed techniques have been combined to resolve the overlapping spectra and analyse the drug mixture in

a. Phenobarbitone and phenytoin sodium tablets.

The above proposed methods were found to be accurate, simple and precise. The developed methods were validated according to the procedures described in U.S.P. 23 and also the detailed statistical treatment of analytical data was reported. The proposed methods will be useful for routine analysis of drug substances from their combined preparations.

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Preamble

Drugs have been used for centuries in the treatment of various diseases. They may be from plant, animal or synthetic source. These chemical substances must be transformed into a suitable formulation, acceptable to the patient. To transform into such suitable dosage form, it is often necessary to combine with a number of inert substances like excipients or adjuvants before administration by a suitable route.

It is believed that the therapeutic response of a drug is an attribute of its pharmacological activity. However, as on date the variations in therapeutic response were observed when the same drug is administered as different dosage forms or similar dosage form produced by different manufacturers. This could be due to the variations in physico-chemical properties of the drug, the effect of various excipients present in the dosage forms and the method of formulation. The excipients are added in a dosage form to fulfill various requirements like the uniformity of composition, ease of formulation, the physico-chemical stability and to ensure patient acceptability. Though, the excipients are considered to be inert in nature, still they can influence the stability, rate of release of drugs from the dosage forms and their subsequent absorption. Hence all these factors can influence the therapeutic action. In general, the prescription of a drug to a patient by physician is primarily based on adequate therapeutic performance with less toxic effects rather than on oraganoleptic characteristics, cost and availability in attractive packing etc. Hence, the quality of a dosage form becomes more critical since all drug products directly affect the health of the consumer who lacks knowledge to identify its quality unlike any other commodity.

In this era of competition, the number of drug products enter into market with a similar composition is increasing yearly. The conditions under which they are being made by various pharmaceutical firms are of great concern. Since, they are intended

for clinical use, there should be a proper quality consciousness to be built in during the manufacturing stage.

The official standards for pharmaceutical chemicals and formulated products are available in monographs and are designed primarily to set permissive limits of tolerance for the product at the time it reaches the patient. However, the conformance to compendial standards as the sole basis for judging the quality of a final product can be grossly misleading. A compendial monograph could never cover all possibilities that might adversely effect the quality of a product. The difficulties lie in part as the final dosage forms are frequently produced in batches of hundreds or even million in number. The number of samples assayed at the end of process is not likely to represent whole production. Hence, to produce a quality final product, much depends on the practising standards, testing methodology and the currently followed quality control program of a manufacturing company.

In pharmaceutical analysis, as in most other analytical fields, the major problem is not in the assay procedure itself but in the task of separating or purifying the active ingredient(s) of interest from impurities and other substances that can interfere with accuracy of the analytical results. The necessary degree of purification is often determined on the basis of whether or not other substances present will interfere in anyway with the chosen method of analysis. In a method for a single, pure drug substance, separation or purification methods are not usually necessary as they can be analysed directly by titration or some other suitable method. However, the application of any analytical method to a pharmaceutical dosage form having one or more with its fillers, binders, stabilisers and other additives often requires a more careful preparation of the sample, including extensive pre-analysis study, purification or separation.

In the early years, separating the components of interest in a sample used to be carried out mostly by precipitation, extraction or distillation. In some situations the

separated components were treated with reagents and could be recognised by the yielded colour, optical activities etc. Whereas, the amount of analytes was used to be determined by gravimetric or titrimetric methods. The classical methods for separating and determining analytes still find use in many laboratories in their revised form, especially, the volumetric methods have been revised to reflect the development of improved methods such as complexometric or potentiometric titrations. But the extent of their general application is decreasing with the advent of technology.

In the 1950's, the methods for separation and determination of chemical species like absorption and emission spectrophotometry, chromatography, electrode potential, conductivity etc. were introduced. But, the application of above techniques could not be exploited till late seventies due to lack of reliable and simple instrumentation. The modern instruments are equipped with an enormous array of tools for carrying out analysis and detecting them at microgram to nanogram level.

The application of spectrophotometry in its various forms like ultraviolet-visible (UV-VIS), infrared (IR), fluorimetry, nuclear magnetic resonance (NMR) and mass spectrophotometry (MS), have created an impact on the direction of pharmaceutical analysis and the product quality assurance in the early eighties. The UV-VIS spectroscopy has always had an important role in drug analysis for both qualitative and quantitative purpose. However, the development of quantitative chromatographic techniques like high performance liquid chromatography (HPLC), gas chromatography (GC) and high performance thin layer chromatography (HPTLC) methods and their wide spread usage has come in the way of importance of UV-VIS spectrophotometry and it's application in the field of quantitative drug analysis in the last decade. But, these chromatographic techniques demand lot of time, expertise and cost in their operation. On the other hand, several new developments in spectrophotometry such as the use of diode-array detectors (DAD), UV detectors in HPLC, difference, derivative techniques and chemometric methods

like multiple linear regression analysis (MLRA), principal component regression (PCR), partial least squares (PLS) and multi-component analysis (MA) have become available to the drug analysts. As a result, spectrophotometry is still a valuable technique in various areas of drug analysis.

In general, the final selection of method depends on many factors like the extent of accuracy, precision, possible interference from other components of a mixture, the sample size, the concentration range of analyte, the physico-chemical properties of sample matrix and finally the number of samples to be analysed. Other factors like speed, ease, skill, cost of equipment and the sample cost should also be considered before the selection of method.

1.2 SCOPE AND OBJECTIVE OF THE WORK

The objective of the present thesis is to develop new analytical methods for the simultaneous estimation of drugs in combined formulations by using UV-VIS spectroscopy based techniques namely difference and derivative spectroscopy. These techniques have been selected mainly due to their potential and phenomenal success in resolving complex multi-component systems of pharmaceutical interest in recent years. There are several assay procedures available in monographs for determination of single drug constituent of a formulation.

For pure drug substances, the U.S.P. [1] recommended a titrimetric (e.g.: aspirin, dicyclomine HCl and thiabendazole), potentiometric titration (e.g.: diazepam, dipyridamole and ketoconazole) or HPLC (e.g.: alprazolam, azithromycin and ciprofloxacin HCl) method but for drug formulations HPLC method has been recommended (e.g.: aspirin tablets, diazepam tablets injection and alprazolam tablets). Whereas, the B.P. [2] and I.P. [3] prescribed a potentiometric titration (e.g.: econazole nitrate, metoclopramide HCl and penicillin-G potassium) or a titrimetric (e.g.: aspirin, flurbiprofen, mefenamic acid and levodopa) method for pure drug substances and ultraviolet spectrophotometric (e.g.: diazepam tablets, metoclopramide HCl tablets and flurbiprofen tablets) or titrimetric (e.g.: aspirin tablets and mefenamic acid capsules) method for the drug formulations. The B.P. 1993 also suggested a second-derivative ultraviolet spectrophotometric method for purity determination of vehicles (e.g.: benzene content in ethanol and isopropyl alcohol) and for the drug formulations (e.g.: fluphenazine HCl tablets, perphenazine tablets and procyclidine HCl tablets/injection). The above three monographs also prescribed a similar assay method other than a chromatographic method for drug formulations like a titrimetric method for ascorbic acid tablets, codeine phosphate tablets/injection and ethambutol HCl tablets. Also, an ultraviolet spectrophotometric method for chlorpheniramine maleate tablets/injection, chlorpromazine HCl tablets/injection, indomethacin capsules/suppositories, methyldopa tablets, pentazocine HCl tablets and pyrazinamide tablets was recommended by these

monographs. The U.S.P. 23 also recommended assay methods for the simultaneous determination of combined drug formulations (e.g.: acetaminophen - aspirin tablets, diphenhydramine HCl - pseudoephedrine HCl capsules, spironolactone - hydrochlorothiazide tablets, sulphamethoxazole - trimethoprim tablets and levonorgestrel - ethinylestradiol tablets). Whereas no such methods have been recommended for combined drugs in B.P. 1993 (except for carbidopa - levodopa tablets) and in I.P. 1996 (except for carbidopa - levodopa tablets, pyrimethamine - sulphadoxine tablets). The assay procedures described in various monographs for combined preparations are not adequate to meet with growing number of commercial drug formulations. Therefore, there is an imperative need for the development of suitable assay procedures for simultaneous estimation of drug combinations.

In this thesis work, an attempt has been made to develop simultaneous assay procedures of drug combinations for which suitable monographic procedures are not available (except for hydrochlorothiazide - propranolol HCl prescribed in U.S.P. 23). Thus, the developed new methods based on difference and derivative spectroscopy will be practically useful for simultaneous estimation of drugs from their respective dosage forms. In accordance with the above view, the following experimental design has been planned.

The experimental design deals with the following set of drug combinations:

1. The difference spectroscopic assay method was developed for
   a. Metronidazole and nalidixic acid tablets
   b. Metronidazole and diloxanide furoate tablets

2. The derivative spectroscopic assay method was developed for
   a. Tinidazole and furazolidone tablets
   b. Tinidazole and clotrimazole tablets
   c. Tinidazole and norfloxacin tablets
   d. Tinidazole, furazolidone and diloxanide furoate tablets

e. Metoprolol tartrate and hydrochlorothiazide tablets

f. Propranolol hydrochloride and hydrochlorothiazide tablets

g. Atenolol, amiloride hydrochloride and hydrochlorothiazide tablets & capsules

h. Atenolol and amlodipine besylate tablets

i. Amlodipine besylate and enalapril maleate tablets

j. Haloperidol and trihexyphenidyl hydrochloride tablets

3. A new analytical method has been developed with the combination of both difference and derivative spectroscopy for the drug combination of

a. Phenobarbitone and phenytoin sodium

Furthermore, the methods have been developed for the determination of active ingredient(s) of the drug formulations, which were not official in U.S.P. 23, B.P. 1993 and in I.P. 1996 (except for propranolol hydrochloride - hydrochlorothiazide and metoprolol tartrate - hydrochlorothiazide). Since the potency definition (limits) for the investigated drug combinations are not available in above three monographs (except for propranolol hydrochloride - hydrochlorothiazide and metoprolol tartrate - hydrochlorothiazide), the limits mentioned for the drugs as single components in tablets or capsules (except amlodipine for which amount claimed on label has been considered) have been used to interpret the results of the analysis.

The experimental part deals with the description of methodology, results and discussion of the new methods of analysis developed for the simultaneous estimation of the various drug combinations.

All the spectrophotometric methods of estimations were carried out by linear calibration curves prepared with separate standards. The linearity of the calibration curves has been demonstrated with the help of the regression analysis as well as various statistical tests like Student's *t*-test and analysis of variance (ANOVA). The

instrument was calibrated as per the official [2,3] and other reported [4] methods. The validation of developed methods was conducted as per the official procedures described in U.S.P. 23.

CHAPTER 2

DIFFERENCE SPECTROSCOPY

2. DIFFERENCE SPECTROSCOPY

2.1 Introduction

The electronic absorption spectrophotometry is perhaps the most widely used technique, both officially and unofficially, for the quantitative assay of drugs in dosage forms. The quantitation of single drug in pure as well as in dosage forms can be carried out directly through Beer's Law [5], if no other absorbing material is present to interfere or if the interfering material can be removed. In general, most of the spectrophotometric assay procedures include a prior separation or isolation step to avoid any possible interference from the excipients, which may contribute to the total absorbance at the wavelength of estimation. However, the direct spectrophotometric determination of drugs in multi-component dosage forms is often complicated by spectral overlap and interferences from formulation matrix known as background or irrelevant absorption. This background often consists of relatively featureless absorption that increases in intensity towards shorter wavelength.

Several techniques have been devised to compensate for background absorption such as three-point correction method [5], solving two simultaneous equations [6,7] and/or using absorbance ratios at certain wavelengths [8,9]. However, during the application of these methods [5-9] the presence of spectral interferences and/or spectral overlap would certainly leads to erroneous result [10]. The other approaches aimed at solving this problem have been employed, includes orthogonal functions [10-12], graphical or linear plot method [13], difference spectrophotometry [14], derivative spectrophotometry [15] and newer chemometric methods like principal component regression (PCR) [16,17], simplex [17], partial least squares (PLS) [17,18], multi-component analysis (MA) [17,18] and multiple linear regression analysis (MLRA) [17,19,20].

In double beam spectrophotometry, a reference solution is scanned simultaneously with the sample solution to compensate for any .opacity of cell, solvent or added

reagents. Ideally, the reference and sample solution should be identical in all respects except for the presence of the analyte. In practice, when analysing pharmaceutical samples, there is always uncertainty concerning interfering materials which may have accompanied the sample and remain uncompensated by the reference solution.

Difference spectrophotometry is a method for compensating the presence of additives in a drug sample which otherwise interferes with the spectrum of drug being determined. The technique involves the measurement of the absorbance difference (ΔA) at a defined wavelength between two samples in which physical or chemical properties of one drug would have been changed. It was assumed that the above change would not affect the spectral property of interfering material. Thus, difference spectrophotometry utilises an ideal reference solution by employing an aliquot of the sample solution itself as a reference adjusted by change in pH or other parameter. The reference and sample solution also contains both drug substances being analysed including the additives at exactly the same concentrations. The variations in the spectrum of the sample, if any, caused by pH or any other variations will be recorded by the instrument as a characteristic of difference spectrum of the sample. If the other materials present are unaffected by the change in conditions, their contribution to the total absorbance of each solution will be identical and their effects will be cancelled. In general, the spectral changes are induced by simple aqueous acids and alkalis to cause a reversible ionization of groups directly conjugated to a chromophore. Since many drugs are weak acids or weak bases, their state of ionization and absorptivity depend on the pH of the solution. Therefore, it is important that the acid and base solutions be selected such that both are at least two pH units away on either side from pK_a of drug, when the pH values are closer to pK_a, small changes in pH may cause appreciable changes in the difference spectrum. Hence, the selection of buffers has to be appropriate and has been critically reviewed for barbiturate solutions [14] and for amphoteric drug substances like pyridoxine and sulphathiazole [14]. While in most instances of difference spectra involve pH effects, sometimes, spectral changes may also results from factors other than ionization. These will permit sample and reference

solution to be prepared at identical pH with an improved likelihood for cancellation of interferences. The above concept had helped in identifying drug products against their oxidised [14,21] and reduced samples [14,22-24] after a suitable treatment. There are also reports available for the estimation of primary amine with formation of Schiff's base in presence of a secondary amine [14].

The principal advantage of difference spectrophotometry lies in its potential for the cancellation of interferences. The existence of isosbestic points (i.e. the wavelength at which the absorption of the solutions are equal) provides a useful test for such cancellation and can be used to analyse mixtures without interference from each other. Such a determination without interference from the other drug in a binary mixture will be possible at the isosbestic points, provided the isosbestic point of one drug lies at or near the maximum of the difference spectrum of the other drug. Even in the absence of such fortuitous juxtaposition of the isosbestic points of the difference spectra, the spectra may still be used for determination provided the absorbance of one drug is linear with concentration at the isosbestic point of other drug and vice versa. In contrast, a difference spectra method for single component dosage forms are usually simple and lie in the identification of a convenient analytical wavelength at which a maximum amplitude difference will be observed. Therefore, this wavelength may be used for quantitative determinations in exactly the same manner that simple absorbance is used in conventional spectra.

The difference spectrophotometry has been proved to be useful as an analytical technique for the assay of single or binary mixture of pharmaceutical dosage forms by elimination of specific interference from degradation products [25,26], co-formulated drugs [14,27-29] and from the formulation matrix [14]. This particular usefulness of the technique has been extensively reviewed by many workers in the past [14,30,31].

The review of literature shows that various categories of drugs have been successfully estimated from their drug formulations by difference spectrophotometry. The various

reports for single drug preparations include analgesics & anti-inflammatory [32-36], antibiotics [22,27,28,37-39], antihypertensives diuretics [25,40,41], antipsychotics [42-44], vitamins [29,45] and others [23,24,26,46-50]. Similarly, the various reported categories of combined drug preparations include analgesics & anti-inflammatory [51-56], sulfonamides [57-59], antidiarrhoeals [60-62], antipsychotics [63-66] and others [21,67-69]. The summary of this literature review was presented in Appendix C.

The basis of inducing spectral changes in above drug substances include use of simple diluted aqueous acids like hydrochloric acid (HCl), sulfuric acid (H_2SO_4) and solutions of sodium hydroxide (NaOH). In few instances, the drug substances have been treated with solutions like zinc [22,23] and germanium oxide (GeO_2) [64].

The method of determination of drug in above reported preparations is mostly at the wavelength of maximum amplitude difference for single drug products and the isosbestic point procedure for combined drug formulations. The literature review also suggested that this technique is not only suitable for the estimation of drugs in their solid dosage forms but also useful in estimating from other dosage forms like ophthalmic solutions [22], injections [32], nasal drops [47] and infusions [56].

In addition to above, difference spectrophotometry can be used for quality control purposes, whenever the interfering material is well defined an appropriate dilution of a suitable reference solution can be used in the reference cell. However, the difference absorbance is susceptible to systemic errors when there is uncertainty in the concentration of interfering materials in the samples to be assayed. This error increases in proportion relative to the ratio between molar absorptivity of the interference and to that of the drug [70].

2.2 Design of the experiment

This section describes the estimation of two different drug combinations as pure admixtures and dosage forms by zero-order difference spectrophotometry. The basic experimental approach followed for the work using this technique was same throughout.

All the reported work in this section was carried out using JASCO model 7800 double beam UV-VIS spectrophotometer with 1 cm quartz cells. A scan speed of 480 nm min and a bandwidth of 3 nm were maintained. Ordinate maximum and minimum were adjusted according to the amplitudes of measured values. The instrument was calibrated before the development of each method according to the procedures described in official monographs [2,3] and the results obtained were reported in Appendix A.

The stepwise experimental approach is:

a. Preparation of standard solutions of pure drugs with selected acid and alkali buffers to obtain equimolar solutions with appropriate concentration range to maintain the desired formulation ratio.
b. Recording of the difference spectrum of pure drugs by placing the acidic solutions in the reference compartment and their alkali solutions in the sample compartment.
c. Identification of the suitable isosbestic points for both the drugs at which the estimation of other drug would be possible from their standard solutions.
d. Examining the linear proportionality of absorbance values with different concentrations of drug solutions at the possible isosbestic wavelengths identified in step " c".
e. Preparation of different series of solutions from the respective stock solutions of pure drugs for the construction of the calibration curve. The series of solutions were always prepared as a representative of both pure drugs and mixtures. The comparison of regression equations between pure drug solutions with that of the

mixtures was done to establish the selectivity of chosen wavelength for the determination.

f. To establish an ideal concentration range for drugs in combination and to study possible interferences, if any, between mixture components, an interaction study with mixture solutions was carried out at the identified wavelengths in step " e". This range was also required to fulfill the compliance of Beer's law and to meet the proportion of drugs in commercial formulations, which ought to be estimated by the method.

g. Validation of the developed method according to the procedures described in U.S.P. 23.

h. Application of the developed method to commercial preparations for the suitability in routine analysis.

i. Finally, the statistical analysis of data obtained for standard solutions and the commercial preparations of each combination was done and the details were discussed under the respective experimental section. The various mathematical expressions used for the statistical treatment of data were presented in Appendix D.

2.3 SIMULTANEOUS QUANTITATIVE DETERMINATION OF METRONIDAZOLE AND NALIDIXIC ACID IN TABLETS BY DIFFERENCE SPECTROPHOTOMETRY

The combination of metronidazole (MDZ) and nalidixic acid (NA) as a tablet is being widely used for diarrhoea and dysentery of mixed infective origin. The reported methods for the estimation of MDZ as single preparation includes ultraviolet [23], polarographic [71-73], colorimetric [74,75], GC [76], HPLC [77,78] and ultraviolet derivative spectrophotometric method [79]. There are also reports available for MDZ in combination with other drugs [62, 80-92]. In particular, the reports related to MDZ in combination of NA employed HPTLC [80], multi-component analysis (MA) [81] and HPLC method [82]. Whereas, the literature reports available for the estimation of NA as single constituent includes TLC [93], HPLC [94], fluorimetry [95] and ultraviolet method [96]. However, the official monographs described titrimetric [1], potentiometric titration method [2,3] for pure MDZ, and HPLC [1], titrimetric [2,3] and ultraviolet method [3] for its determination from various available dosage forms as a single preparation. Similarly, the official monographs described titrimetric [1], potentiometric titration method [2,3] for pure NA and ultraviolet method [1-3] for its determination from different dosage forms. This section of the thesis comprises the details of the successful design of a zero-order difference spectrophotometric method for the estimation of these drugs from its pure admixtures and in tablet preparations.

Materials and Reagents

Metronidazole (Gufic Ltd., India) and nalidixic acid (Win-Medicare, India) were obtained as gift samples. Hydrochloric acid (Qualigens, India), sodium hydroxide (Qualigens, India) and methanol (Qualigens, India) used were all of analytical grade.

Standard Solutions

The stock solution of pure MDZ and NA were prepared by dissolving 25 mg of each of the pure drug separately in 50 ml of methanol. Appropriate volume aliquots of MDZ and NA solutions were transferred separately into 10 ml volumetric flasks in duplicate. The volumes were made up with 0.1M HCl and 0.1M NaOH to give a series of equimolar solutions containing 5-25 µg ml MDZ (Table 2.1 and Series A in Table 2.2) and NA (Table 2.1 and Series C in Table 2.2). Similarly, two series of 10 ml each equimolar solutions of mixtures of MDZ and NA in 0.1M HCl and 0.1M NaOH were prepared from the stock solutions. The first series contained a constant concentration of NA (15 µg ml) and a varying concentration of 5-25 µg ml of MDZ (Table 2.1 and Series B in Table 2.2). While, the second series contained a constant concentration of MDZ (10 µg ml) and a varying concentration of 5-25 µg ml of NA (Table 2.1 and Series D in Table 2.2).

Interaction Study

Two separate series of mixture solutions of 10 ml each of MDZ and NA in 0.1M HCl and 0.1M NaOH were prepared from fresh stock solutions according to the procedures mentioned above. The first series contained a constant concentration of NA (15 µg/ml) and a varying concentration of MDZ (5-35 µg/ml). Similarly, the second series contained a constant concentration of MDZ (10 µg/ml) and a varying concentration of NA (5-40 µg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate MDZ (10 µg/ml) and NA (15 µg/ml) standard and test samples with 0.1M HCl and 0.1M NaOH were prepared in duplicate from freshly prepared stock solutions according to the above mentioned procedures.

b. *Linearity*

Separate series of solutions of MDZ and NA containing 5-35 µg ml of each pure drug were prepared from the stock solutions meant for method validation.

c. *Specificity*

Series of five mixture solutions of each containing MDZ (10 µg ml) and NA (15 µg ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of each brand (AX, AY and AZ) were accurately weighed, powdered and a weight of powder equivalent to 10 mg of MDZ (and 15 mg of NA) was dissolved in 50 ml of methanol by thorough mixing and diluted to volume in a 50 ml volumetric flask. The samples were filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded in each instance. Appropriate volume aliquots of each filtrate were diluted with 0.1M HCl and 0.1M NaOH to obtain equimolar solutions containing approximately 10 µg ml of MDZ and 15 µg ml of NA.

Procedure

The normal absorbance as well as the absorbance difference of acidic and equimolar alkali solutions of pure drug and samples were measured in the range of 260-350 nm by placing the acidic solutions in the reference compartment and the alkali solutions in the sample compartment. The absorbance difference of the analytes at 292.0 and 325.2 nm was corrected for the absorbance difference, if any, between 0.1M NaOH solution and 0.1M HCl at these wavelengths.

Results and Discussion

The normal spectra of MDZ and NA in both acidic and alkaline solutions were shown in Figures 2.1 and 2.2. It was evident from their spectra recorded in individual acidic (Spectra "**a**" in Figures 2.1 and 2.2) and alkali solutions (Spectra "**b**" in Figures 2.1 and 2.2) that both the pure drugs were overlapping in the range of spectral measurement. As mentioned in the introduction of this section, the earlier

conventional methods [5-9] could not be considered for their simultaneous estimation due to the appreciable absorbance of one drug at or near the wavelength maximum of the other drug and vice versa. Therefore, a zero-order difference spectrophotometric method was considered due to the spectral shifts observed for both the drugs in alkaline media. This shift in wavelength maximum was found to be more in the case of MDZ than compared to NA. The recorded difference absorption spectrum of MDZ and NA solutions resulted in producing isosbestic points at 292.0 nm for MDZ (Figures 2.1 and 2.3) and at 270.6 and 325.2 nm for NA (Figures 2.2 and 2.3). It can also be observed from Figure 2.3 that the recorded mixture spectra of drug solutions showed an appreciable absorbance difference (ΔA) at the isosbestic point of other drug. This fortuitous juxtaposition of the isosbestic points helped in their successful determination from combined preparations and in eliminating the interference from matrix components.

The absorbance value of the difference spectrum at 325.2 nm was considered over the absorbance value at 270.6 nm for MDZ due to a greater ΔA at former wavelength and that of NA at 292.0 nm have been used for the determination of the drugs from their mixtures. It was mainly assumed that the absorbance contribution from other component of drug mixture was negligible, because of their cancellation of individual absorbances at these wavelengths. (Figure 2.3). The proportionality of ΔA values to concentration of drug solutions were examined by measuring a series of pure drug (Table 2.1, Series A and C of Table 2.2) and mixture solutions (Table 2.1, Series B and D of Table 2.2) at the above selected wavelengths. The calculated regression equations based on the ΔA values (Table 2.1) of MDZ and NA solutions were presented in Table 2.2. The spectra obtained for mixture solutions (Series B and D of Table 2.2) were shown in Figures 2.4 and 2.5 respectively. The presence of distinct isosbestic points at 292.0 nm (Figure 2.4), and at 325.2 and 270.6 nm (Figure 2.5) suggested no interferences in the estimation of MDZ and NA. It was also proved that absorbance values were proportional to their concentrations in mixtures.

The mutual independence of the analytical signals of MDZ and NA at the above selected wavelengths was confirmed by a careful mixture interaction study. It was evident from reported results (Figure 2.6) that the varying concentrations of NA (5-40 μg ml) solutions did not interfere up to 30 μg ml in the estimation of MDZ (10 μg ml) at 325.2 nm (Figure 2.6A). Similarly, the varying concentrations of MDZ (5-35 μg ml) did not interfere up to 25 μg ml in the estimation of NA (15 μg ml) at 292.0 nm (Figure 2.6B). Hence, the earlier proposed concentration range for pure drugs and mixtures were ideal and their accurate determinations can be achieved at the suggested concentration ratio (i.e. 10 μg ml for MDZ and 15 μg/ml for NA).

The statistical analysis of the data obtained for pure drug solutions and their admixtures suggested that the small standard deviation values associated with the determinations (Table 2.1) indicated the high level of precision of the proposed method and the non-interference of one drug in the absorption measurement of other drug. Further, the precision of the method was also evident from the lesser values of standard error and coefficient of variation (also known as relative standard deviation, RSD). The percentage ratio of the residuals indicated a random scatter in case of both the pure drug solutions and their admixtures (Table 2.1). The calculated F-values (F_{Calc}) of test for non-linearity [97] were less than the critical value (F_{Crit}) at 5% significance level (Table 2.1) and thus suggested that measured values did not deviate significantly from the best-fit line.

The regression equations of the pure drug solutions and those of mixtures were similar (Table 2.2). This similarity and the correlation coefficient values for all four series of solutions indicated the non-interference of one drug in the estimation of the other. A one-way ANOVA test [98] was performed based on the separate linear calibration graph constructed with three replicates per point. The values considered were the lowest and highest variation observed from the mean absorbance value of each pure drug concentration during the replicate measurement of standard solutions (Table 2.1).

It was evident from reported results that, the calculated F-values (F_{calc}) were less than that of critical value (F_{crit}) at 5% significance level (Tables 2.3 and 2.4) and suggested that calibration line presents homoscedasticity (standard residuals have a uniform variance).

The reported slope values without intercept on the ordinate at 95% confidence limits [98] suggested that the calibration lines of NA solutions did not deviate from the origin as the above obtained values lie within the confidence limits (Table 2.2). While, the above reported slope values for MDZ solutions fail to meet the 95% confidence limits. However, the satisfactory confidence limits of slope were obtained at 99.8% for MDZ pure drug (Slope: 3.90E-02 to 4.17E-02) and for its mixture solutions at 99.5% (Slope: 3.88E-02 to 4.16E-02).

Further, the calculated Student's *t*-test [98] values (t_{calc}) were reported to be far larger than the critical value (t_{crit}) obtained at 5% significance level suggesting the positive correlation between AA values and its concentrations (Table 2.2). The precision of the fit by regression equations was also confirmed from the standard error values of intercept, slope and the estimate [98].

The developed method was validated according to the procedures described in U.S.P. 23 and the results obtained were shown in Table 2.5. The reported limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A and C of Table 2.2.

The percentage recoveries obtained from the pure drug admixtures and commercial products were reported in Table 2.6. The results indicated a mean recovery of 100.04% and 99.69% for pure MDZ and NA respectively. Whereas the commercial formulations showed a mean recovery value of 99.62 to 100.40% for MDZ and 99.00 to 99.70% for NA respectively. These values were conforming to the limits of

U.S.P. 23, B.P. 1993 and I.P.1996 for MDZ and NA in pure form and as well in its tablets (Appendix B). The calculated F-values (F_{Calc}) of a one-way ANOVA test [98] for both MDZ and NA were less than the critical value (F_{Crit}) at 5% level indicates no significant difference between the mean recovery values of commercial preparations and the pure admixtures (Table 2.6). Therefore, the non-interference from the formulation matrix in the assay of the drugs from commercial preparations was established.

The drug solutions were protected from light throughout the study and the solutions were stable for a period of 2 hrs in presence of 0.1M HCl and 0.1M NaOH. The reported pK_a values of MDZ and NA were 2.5 and 6.0 respectively [99]. Therefore, the pH of 0.1M HCl (pH ≈ 1.0) and that of 0.1M NaOH (pH ≈ 13.0) were at least 1.5 pH units away from the pK_a values of drugs. Therefore, small variations in pH of the solvents did not lead to appreciable changes in the absorbance values.

Thus the proposed method of determination of MDZ and NA was found to be accurate and precise, and easier compared to other reported methods [80-82]. Therefore, the rapidity of the proposed method makes it useful in routine analysis for the simultaneous estimation of these drugs from combined formulations.

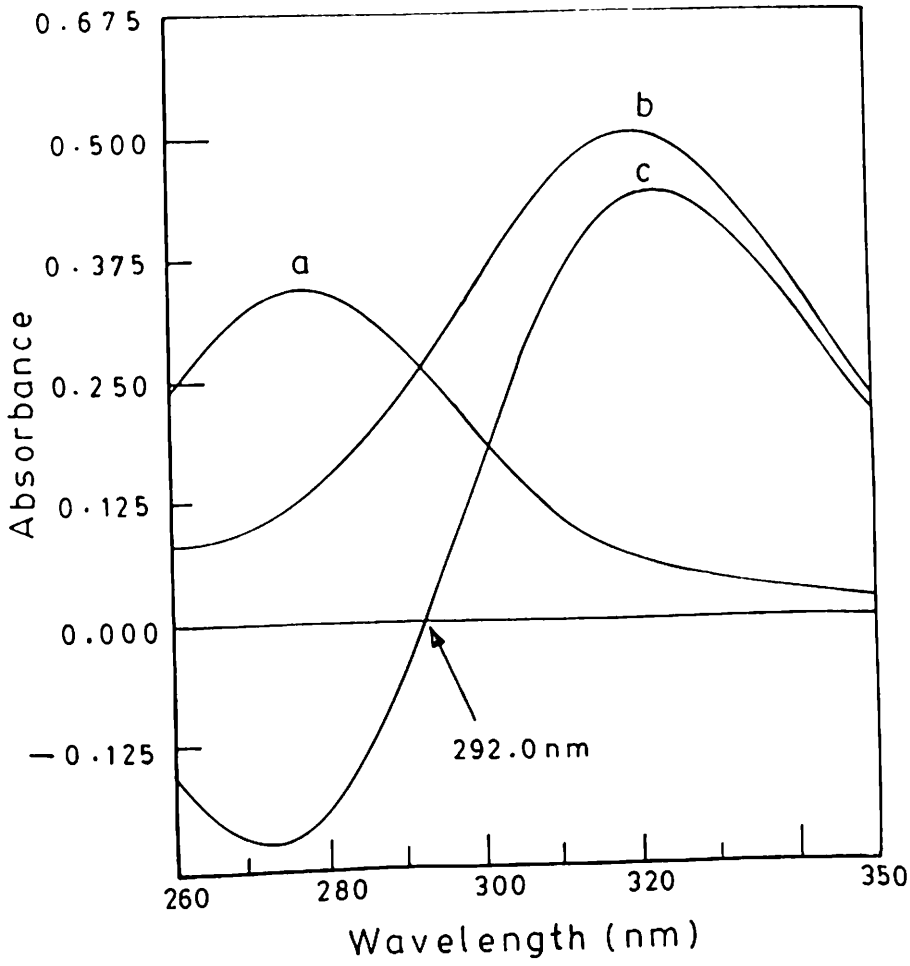

Figure 2.1 Absorption spectra of metronidazole (10 µg/ml) in (a) 0.1M HCl, (b) 0.1M NaOH and (c) their difference spectra.

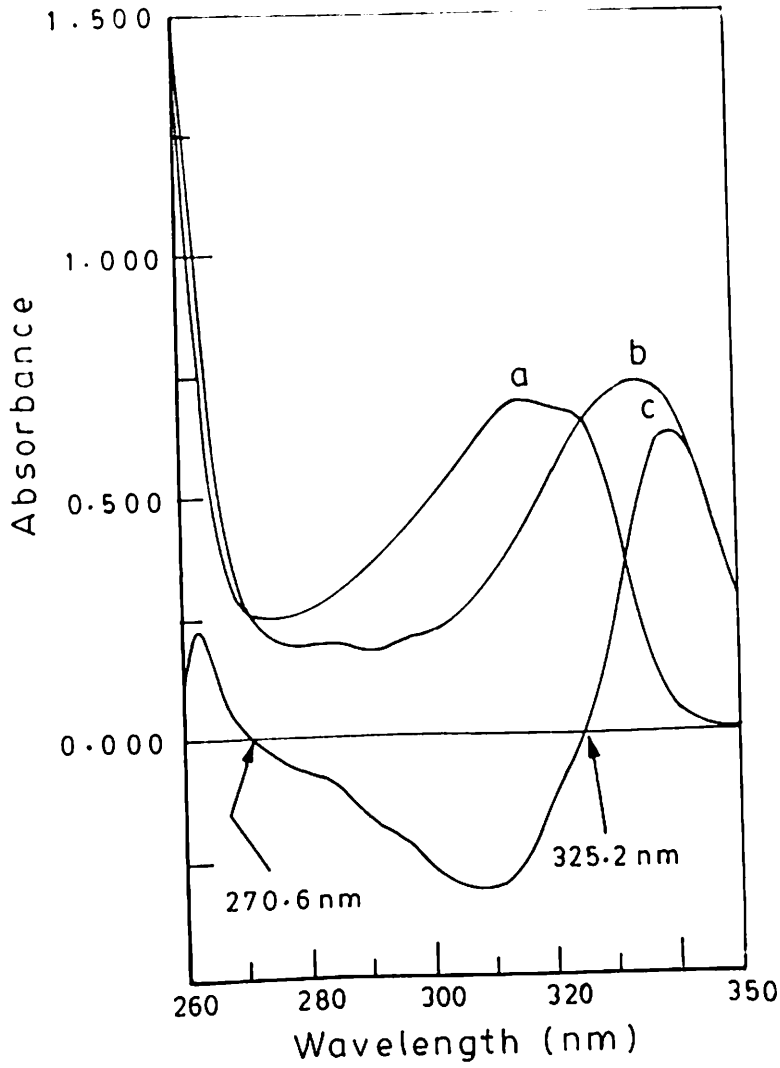

Figure 2.2 Absorption spectra of nalidixic acid (15 µg/ml) in (a) 0.1M HCl, (b) 0.1M NaOH and (c) their difference spectra.

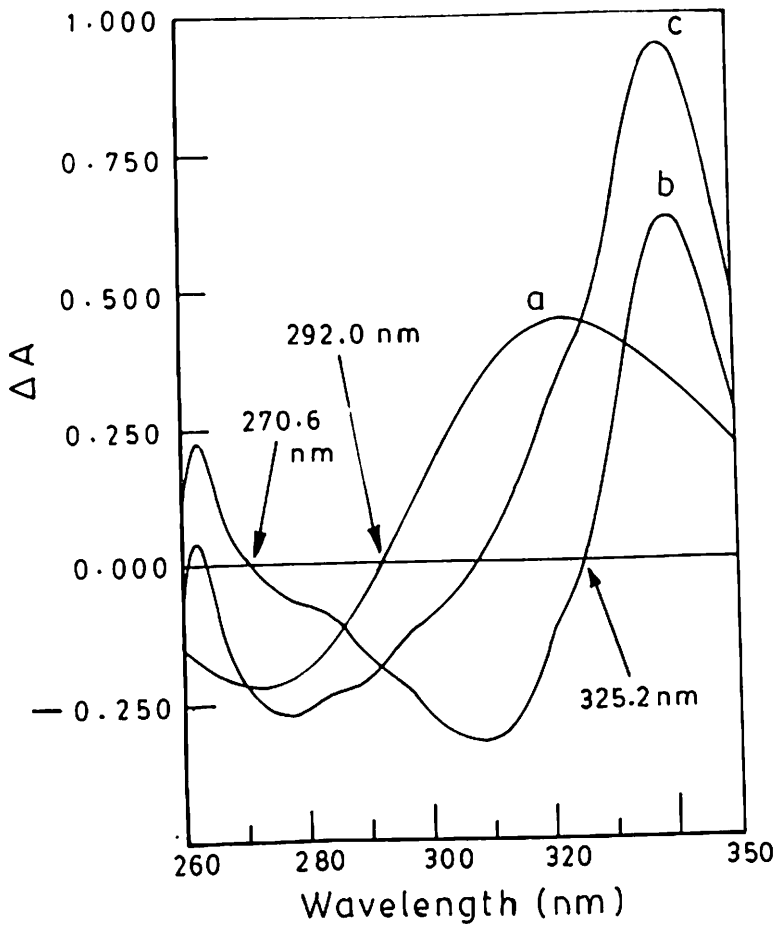

Figure 2.3 **Difference spectra of (a) metronidazole (10 µg/ml), (b) nalidixic acid (15 µg/ml) and (c) their mixture in 0.1M HCl Vs 0.1M NaOH.**

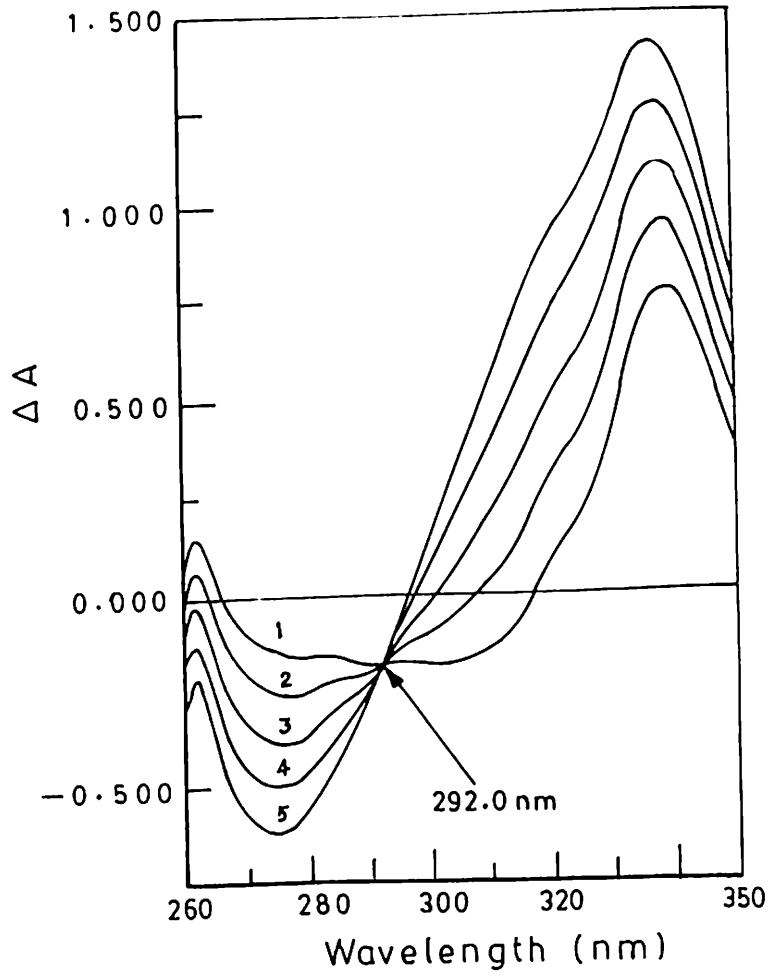

Figure 2.4 **Difference spectra of metronidazole (5, 10, 15, 20 and 25 µg/ml) and nalidixic acid (15 µg/ml) in 0.1M HCl Vs 0.1M NaOH in curves 1-5, respectively.**

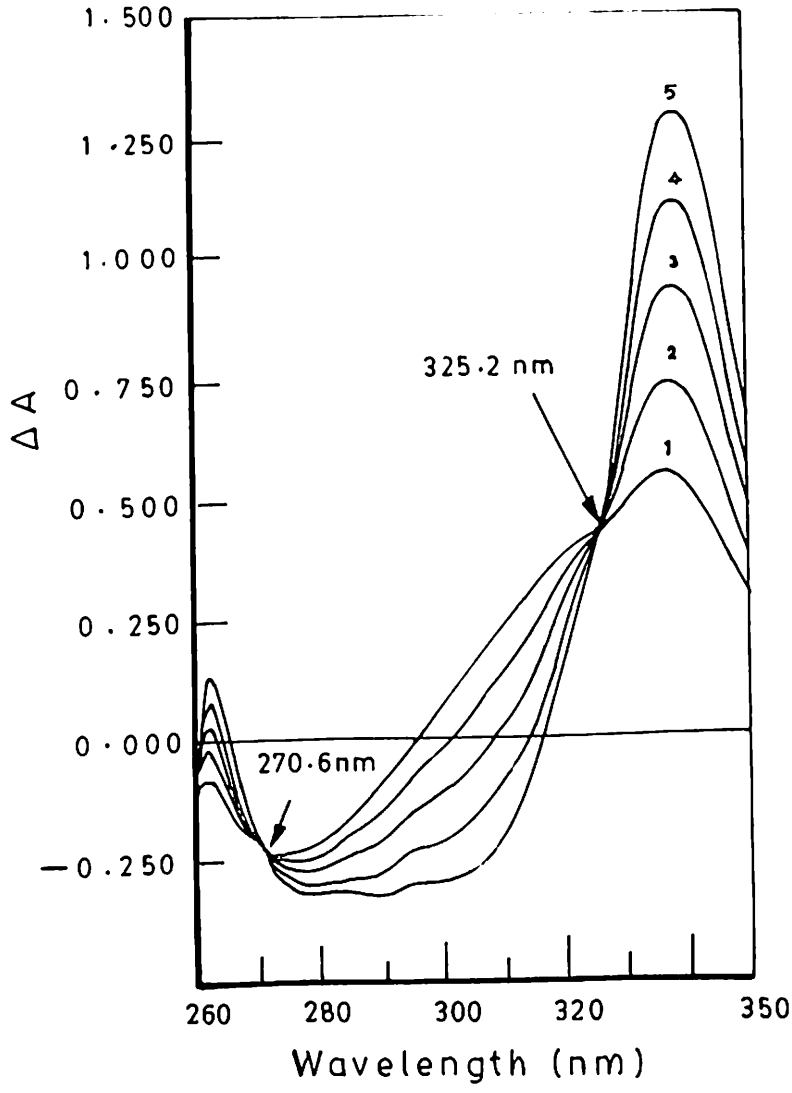

Figure 2.5 Difference spectra of nalidixic acid (5, 10, 15, 20 and 25 µg/ml) and metronidazole (10 µg/ml) in 0.1M HCl Vs 0.1M NaOH in curves 1-5, respectively.

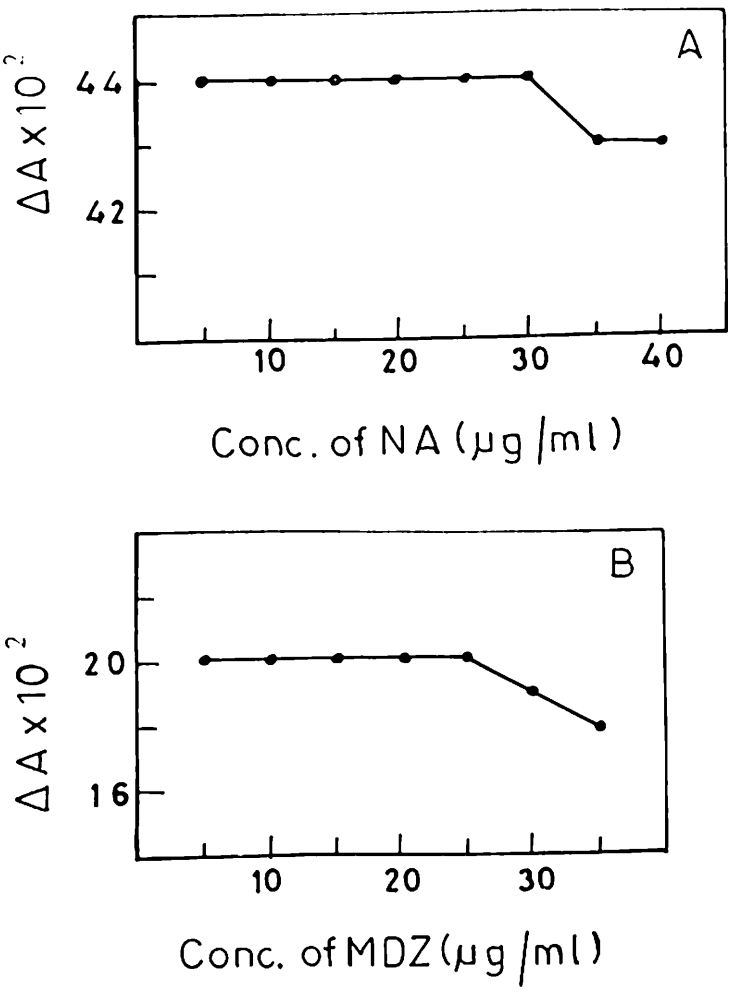

Figure 2.6 **Interaction graph for (A) metronidazole (10 µg/ml) in mixture with nalidixic acid (at 325.2 nm) and (B) nalidixic acid (15 µg/ml) in mixture with metronidazole (at 292.0 nm) in 0.1M HCl Vs 0.1M NaOH.**

Table 2.1: Selectivity of the method for the simultaneous determination of MDZ and NA in standard solutions by difference spectrophotometry

Composition of the solution (µg ml)		Mean absorbance [a] value (ΔA) (MDZ at 325.2 nm, NA at 292.0 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
MDZ	NA					Calc	Crit [b]
5	0	0.241 ± 0.003	1.30	0.0009	99.46	0.42	3.86
10	0	0.440 ± 0.003	0.86	0.0012	100.45	0.28	
15	0	0.643 ± 0.004	0.60	0.0012	100.12	0.28	
20	0	0.847 ± 0.005	0.58	0.0015	99.72	0.17	
25	0	1.047 ± 0.004	0.42	0.0014	100.08	0.20	
5	15	0.242 ± 0.002	1.12	0.0008	99.93	1.22	
10	15	0.441 ± 0.003	0.80	0.0012	100.57	0.72	
15	15	0.646 ± 0.002	0.36	0.0023	99.72	1.67	
20	15	0.849 ± 0.003	0.33	0.0009	99.62	1.10	
25	15	1.045 ± 0.003	0.21	0.0006	100.25	1.85	
0	5	0.068 ± 0.001	2.38	0.0005	99.85	2.16	3.86
0	10	0.135 ± 0.003	2.13	0.0009	98.62	0.68	
0	15	0.195 ± 0.002	1.25	0.0007	101.79	0.95	
0	20	0.264 ± 0.004	1.60	0.0013	99.60	0.31	
0	25	0.329 ± 0.004	1.22	0.0012	99.84	0.35	
10	5	0.068 ± 0.002	2.47	0.0005	99.73	3.23	
10	10	0.135 ± 0.003	2.28	0.0009	98.55	0.96	
10	15	0.194 ± 0.002	1.03	0.0006	102.24	2.28	
10	20	0.266 ± 0.003	1.15	0.0009	99.20	0.96	
10	25	0.329 ± 0.003	1.12	0.011	99.96	0.66	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 2.2: Regression analysis for the determination of MDZ and NA in standard solutions by difference spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 325.2 nm for MDZ at 292.0 nm for NA)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t*-test for correlation	
	MDZ	NA			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	5 - 25	0	Y = 4.03E-02.X + 3.84E-02	0.9999	2.12E-03	1.28E-04	2.03E-03	3.16E-02, 4.51E-02	3.99E-02, 4.07E-02	4.14E-02	122	3.18
Series B	5 - 25	15	Y = 4.03E-02.X + 4.09E-02	0.9999	3.14E-03	1.90E-04	3.00E-03	3.09E-02, 5.09 E-02	3.96E-02, 4.09E-02	4.14E-02	122	
Series C	0	5 - 25	Y = 1.30E-02.X + 2.55E-03	0.9998	2.49E-03	1.51E-04	2.38E-03	-5.39E-03, 1.05E-02	1.25E-04, 1.35E-02	1.31E-02	87	
Series D	10	5 - 25	Y = 1.31E-02.X + 2.34E-03	0.9997	3.17E-03	1.91E-04	3.02E-03	-7.68E-03, 1.24E-02	1.24E-02, 1.37E-02	1.31E-02	71	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 2.3: One-way ANOVA test for linearity of pure MDZ solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.2249	1.2249	0.217	3.71
Lack of fit	3	2.72E-05	9.07E-06		
Within line	10	4.18E-04	4.18E-05		
Total	14	1.2253			

*: at P = 0.05 level of significance.

Table 2.4: One-way ANOVA test for linearity of pure NA solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	0.1285	0.1285	0.541	3.71
Lack of fit	3	3.69E-05	1.23E-05		
Within line	10	2.27E-04	2.27E-05		
Total	14	0.1288			

*: at P = 0.05 level of significance.

Table 2.5: Validation report for the determination of MDZ and NA in standard solutions by difference spectrophotometry

Analytical parameter	Results	
	MDZ (325.2 nm)	NA (292.0 nm)
Accuracy (%)	100.02 ± 0.55	99.79 ± 0.58
Precision (%)	99.31 99.69 100.00 100.45 100.68 RSD: 0.55	98.97 99.48 100.00 100.00 100.51 RSD: 0.58
Specificity	A 10 µg/ml of MDZ and 15 µg/ml of NA mixture solution will show an absorbance value (ΔA) of 0.441 ± 0.003	A 15 µg/ml of NA and 10 µg/ml of MDZ mixture solution will show an absorbance value (ΔA) of 0.194 ± 0.002
LOD (µg/ml)	0.15	0.55
LOQ (µg/ml)	0.51	1.83
Linearity (µg/ml)	5 - 35	5 - 35
Ruggedness (%)	100.02 ± 0.55	99.79 ± 0.58

Table 2.6: Results of the assay of pure drug admixtures and commercial formulations of MDZ and NA by difference spectrophotometry

Sample	Label Claim (mg/tab.)		Recovery (%) [a]		F-test for Mean Recovery		
					Calc		Crit [b]
	MDZ	NA	MDZ (325.2 nm)	NA (292.0 nm)	MDZ	NA	(MDZ / NA)
Pure drug admixture	-	-	100.04 ± 0.43	99.69 ± 0.58	1.47	1.06	3.24
Brand AX	200	300	99.62 ± 0.48	99.20 ± 0.75			
Brand AY	200	300	100.40 ± 0.61	99.70 ± 0.90			
Brand AZ	200	300	100.22 ± 0.85	99.00 ± 0.79			

[a] : Mean and standard deviation for five determinations.
[b] : Theoretical value of F(3,16) based on one-way ANOVA test at P = 0.05 level of significance.

2.4 SIMULTANEOUS QUANTITATIVE DETERMINATION OF METRONIDAZOLE AND DILOXANIDE FUROATE IN TABLETS BY DIFFERENCE SPECTROPHOTOMETRY

The combination of metronidazole (MDZ) and diloxanide fuorate (DF) as a tablet is being widely used for acute and chronic amoebiasis and giardiasis. The official monographs described titrimetric [1], potentiometric titration method [2,3] for MDZ as pure drug and HPLC [1], titrimetric [2,3] and ultraviolet method [3] for its determination from various available dosage forms as single preparation. Similarly, the official monographs described a potentiometric titration method [2,3] for DF as pure drug and ultraviolet method [2,3] for its determination from its available solid dosage forms. As mentioned in previous experiment, there are various reported methods for MDZ as a single preparation [23, 71-79] and in combination of other drugs [62, 80-92]. In particular, the reports related to DF combination employed spectrophotometric [83,84] and HPLC methods [85,86]. The literature reports for individual assay of DF from its dosage forms include ultraviolet [26,100,101], colorimetric [102,103] and GC [104] methods. This section of the thesis describes the details of the successful design of a zero-order difference spectrophotometric method for the estimation of these drugs from its pure admixtures and in tablet preparations.

Materials and Reagents

Metronidazole and diloxanide furoate both were obtained as gift samples from Rhone-Poulenc, India. Hydrochloric acid (Qualigens, India), sodium hydroxide (Qualigens, India) and methanol (Qualigens, India) used were of analytical grade.

Standard Solutions

The stock solutions of pure MDZ and DF were prepared by dissolving 25 mg of each of the pure drug separately in 50 ml of methanol. Appropriate volume aliquots of MDZ and DF were transferred separately into 10 ml volumetric flasks in duplicate. The volumes were made up with 0.1M HCl and 0.1M NaOH to give a series of

equimolar solutions containing 5-25 µg/ml of MDZ (Table 2.7 and Series A in Table 2.8) and 5-15 µg/ml DF (Table 2.7 and Series C in Table 2.8). Similarly, two series of equimolar solutions of mixtures of 10 ml MDZ and DF in 0.1M HCl and 0.1M NaOH were prepared using the stock solutions. The first series contained a constant concentration of DF (12.5 µg/ml) and a varying concentration of 5-25 µg/ml of MDZ (Table 2.7 and Series B in Table 2.8). While, the second series contained a constant concentration of MDZ (10 µg/ml) and a varying concentration of 5-15 µg/ml of DF (Table 2.7 and Series D in Table 2.8).

Interaction Study

Two separate series of mixture solutions of 10 ml each of MDZ and DF in 0.1M HCl and 0.1M NaOH were prepared from fresh stock solutions according to the procedures mentioned above. The first series contained a constant concentration of DF (12.5 µg/ml) and a varying concentration of MDZ (5-35 µg/ml). Similarly, the second series contained a constant concentration of MDZ (10 µg/ml) and a varying concentration of DF (5-35 µg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate solutions of MDZ (10 µg/ml) and DF (12.5 µg/ml) standard and test samples were prepared with 0.1M HCl and 0.1M NaOH from freshly prepared stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of MDZ and DF containing 5-30 µg/ml of each pure drug were prepared from the above stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing MDZ (10 µg/ml) and DF (12.5 µg/ml) were prepared from the above stock solutions meant for method validation.

Sample Preparations

Twenty tablets of each brand (BX, BY and BZ) were accurately weighed, powdered and a weight of powder equivalent to 10 mg of MDZ (and 12.5 mg of DF) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The samples were filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded in each instance. Appropriate volume aliquots of each filtrate were diluted with 0.1M HCl and 0.1M NaOH to obtain equimolar solutions containing approximately 10 µg ml of MDZ and 12.5 µg/ml of DF.

Procedure

The normal absorbance as well as the absorbance difference (ΔA) of acidic and equimolar alkali solutions of pure drug and samples were measured in the range of 230-350 nm by placing the acidic solutions in the reference compartment and the alkali solutions in the sample compartment. The absorbance difference of the analytes at 283.4 and 292.0 nm was corrected for the absorbance difference if any, between 0.1M NaOH solution and 0.1M HCl at these wavelengths.

Results and Discussion

The normal spectra of MDZ and DF in both acidic and alkaline solutions were shown in Figures 2.7 and 2.8. It was clear from the recorded individual spectra in acidic (Spectra "**a**" in Figures 2.7 and 2.8) and alkali solutions (Spectra "**b**" in Figures 2.7 and 2.8) that the overlapping of pure drugs was more in acidic solutions than in alkali solutions. However, the simultaneous estimation of drugs in presence of either of the above buffer solution could not be achieved with the earlier mentioned methods [5-9] due to significant absorption of one drug at or near the wavelength maximum of the other drug and vice versa. Therefore, a zero-order difference spectrophotometeric method was considered due to the spectral changes of pure drugs caused by the alkali solutions. Although, the observed wavelength shift in case of DF solutions was not significant but the recorded difference absorption spectrum of MDZ and DF solutions

have produced isosbestic points at 292.0 nm for MDZ (Figures 2.7 and 2.9), and at 253.4 and 283.4 nm for DF (Figures 2.8 and 2.9). It can also be observed from Figure 2.9 that the recorded mixture spectra of drug solutions showed an appreciable ΔA at the isosbestic point of other drug. This fortuitous juxtaposition of the isosbestic points facilitated the successful determination from combined preparations and in eliminating the interference from matrix components.

Thus for quantitative estimation, the absorbance values of the difference spectrum of MDZ at 283.4 nm was considered over the absorbance value at 253.4 nm due to greater absorbance difference at former wavelength. Whereas, the absorption value at 292.0 nm was considered for the determination of DF from the combined mixtures. This selection of wavelengths was done mainly due to the absorbance contribution from other component of drug mixture was considered to be negligible at these isosbestic points (Figure 2.9).

The proportionality of ΔA values to concentration of drug solutions were determined by measuring a series of pure drug (Table 2.7, Series A and C of Table 2.8) and mixture solutions (Table 2.7, Series B and D of Table 2.8) at the above selected wavelengths. The regression equations calculated with measured ΔA values of MDZ and DF solutions were reported in Table 2.8. The spectra obtained from such mixture solutions (Series B and D of Table 2.8) were shown in Figures 2.10 and 2.11 respectively. The presence of distinct isosbestic points at 292.0 nm (Figure 2.10), and at 253.4 and 283.4 nm (Figure 2.11) suggested that no interferences in the estimation of MDZ and DF. It was also proved that absorbance values were proportional to their concentrations in drug mixtures.

The mutual independence of the analytical signals of MDZ and DF at the selected wavelengths was confirmed by a mixture interaction study. It was evident from reported results (Figure 2.12) that the varying concentrations of DF up to 20 μg/ml did

not interfere in the estimation of MDZ (10 µg/ml) at 283.4 nm (Figure 2.12A). Similarly, the varying concentrations of MDZ did not interfere up to 25 µg/ml in the estimation of DF (12.5 µg ml) at 292.0 nm (Figure 2.12B). Hence, the earlier proposed concentration range for pure drugs and mixtures (Table 2.7) were ideal and their accurate determinations can be achieved at the suggested concentration ratio (i.e. 10 µg ml of MDZ and 12.5 µg ml of DF).

The reported statistical analysis of the data obtained for pure drug solutions and their admixtures suggested that, the standard deviation values associated with the determinations were small including the coefficient of variation and the standard error (Table 2.7). Thus, it demonstrated the precision of the proposed method. The percentage ratio of residuals indicated a random scatter of points in case of both pure drug solutions and their admixtures (Table 2.7). However, the reported values of F-test for non-linearity [97] were found to be less than the critical value at 5% significance level suggested the linearity over the concentration range.

The regression equations of the pure drug solutions and those of mixtures were similar (Table 2.8). The non-interference of one drug in the estimation of the other drug was supported by the obtained correlation coefficient values for different series of drug solutions (Table 2.8). A one-way ANOVA test [98] was performed based on the separate linear calibration graph constructed with three replicates per point. These values included the lowest and highest variation observed from the mean value of each pure drug concentration during the replicate measurements (Table 2.7). It was evident from the reported results that the calculated F-values were less than that of critical value at 5% significance level (Tables 2.9 and 2.10) suggested that calibration line presents homoscedasticity.

The reported slope values for MDZ solutions with zero intercept on the ordinate fall within the 95% confidence limits of slope and thus demonstrated that the obtained

calibration lines did not deviate from the origin. Whereas, the reported slope values of DF solutions with zero intercept on the ordinate fails to lie within the 95% confidence limits. However, the satisfactory confidence limits of slope were identified at 99.9% for pure drug (Slope: 9.06E-03 to 1.08E-02) and for mixture solutions at 99.8% (Slope: 9.08E-03 to 1.08E-02). A Student's *t*-test for correlation at 5% significance level showed that the calculated values were far larger than the critical value obtained from Student '*t*' tables [98] and thus confirmed the existence of strong positive correlation between measured values and concentrations of both drugs. The precision of the fit by regression equations was also confirmed from the standard error of intercept, slope and the estimate.

The developed method was validated according to the procedures described in U.S.P. 23 and the results obtained were shown in Table 2.11. The reported limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A and C of Table 2.8. The drug solutions were protected from light throughout the study and the solutions were stable for a period of 2 hrs in presence of 0.1M HCl and 0.1M NaOH.

The percentage recovery values obtained from the pure drug admixtures and from the commercial products were reported in Table 2.12. The results indicated a mean recovery of 99.99% and 100.05% for MDZ and DF respectively. Whereas, the commercial formulations showed a mean recovery values of 99.85 to 99.92% for MDZ and 99.91 to 100.09% for DF. These values were within the limits specified by U.S.P. 23, B.P. 1993 and I.P. 1996 for MD and DF as a single preparation (Appendix B). The calculated F-values of one-way ANOVA test [98] for both MDZ and DF were less than the critical value at 5% level indicates no significant differences in the results achieved between the commercial preparations and the admixtures (Table 2.12). Therefore, the non-interference from formulation matrix in the assay of the drugs from commercial preparations was established.

Thus, the proposed method of determination for MDZ and DF was found to be accurate and precise. The simplicity and rapidity of the proposed method over the other reported methods [83-86] makes it useful in routine analysis for their simultaneous determination from available commercial preparations.

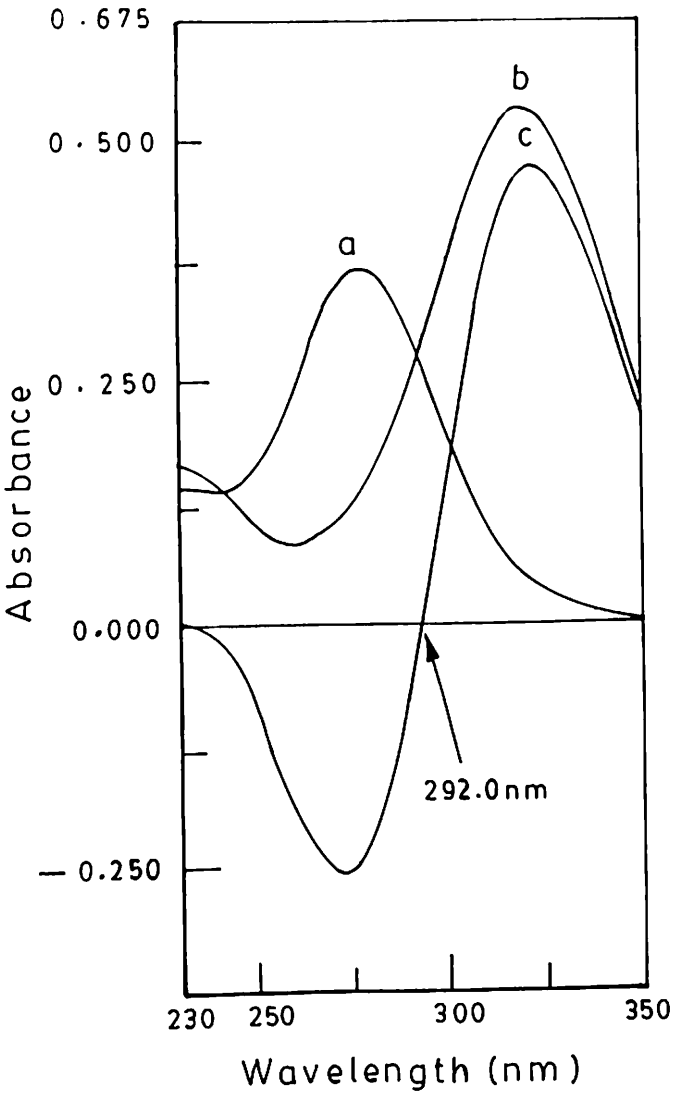

Figure 2.7 Absorption spectra of metronidazole (10 µg/ml) in (a) 0.1M HCl, (b) 0.1M NaOH and (c) their difference spectra.

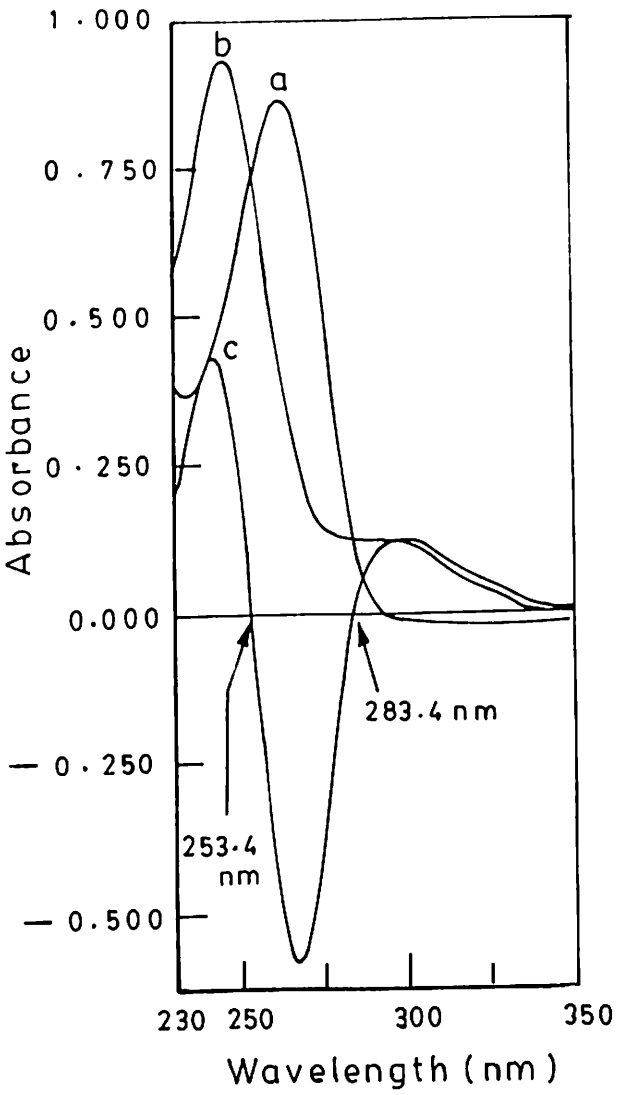

Figure 2.8 Absorption spectra of diloxanide furoate (12.5 µg/ml) in (a) 0.1M HCl, (b) 0.1M NaOH and (c) their difference spectra.

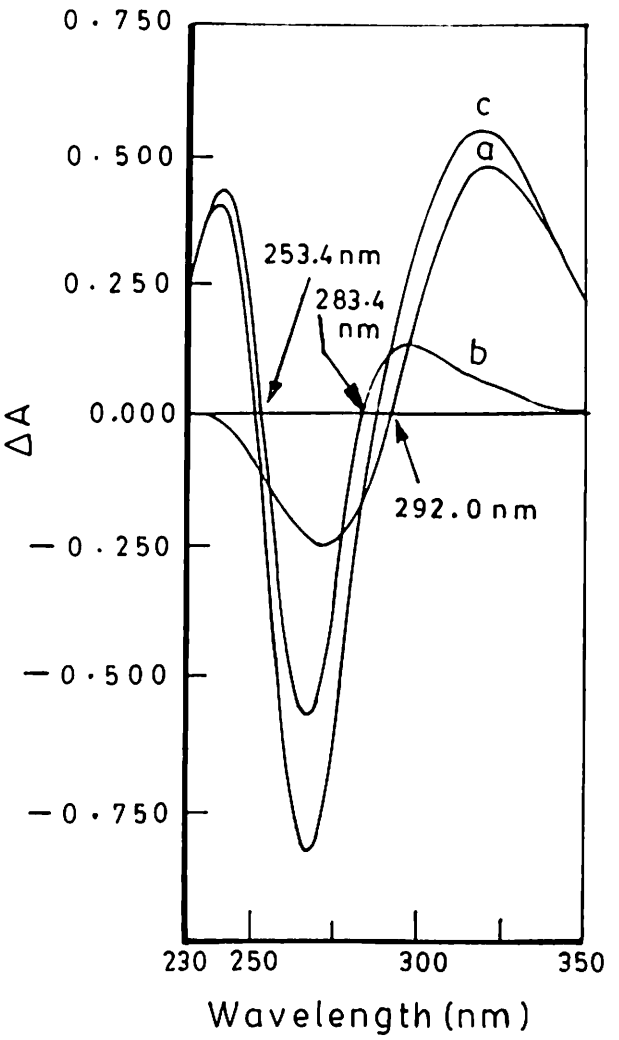

Figure 2.9 Difference spectra of (a) metronidazole (10 µg/ml), (b) diloxanide furoate (12.5 µg/ml) and (c) their mixture in 0.1M HCl Vs 0.1M NaOH.

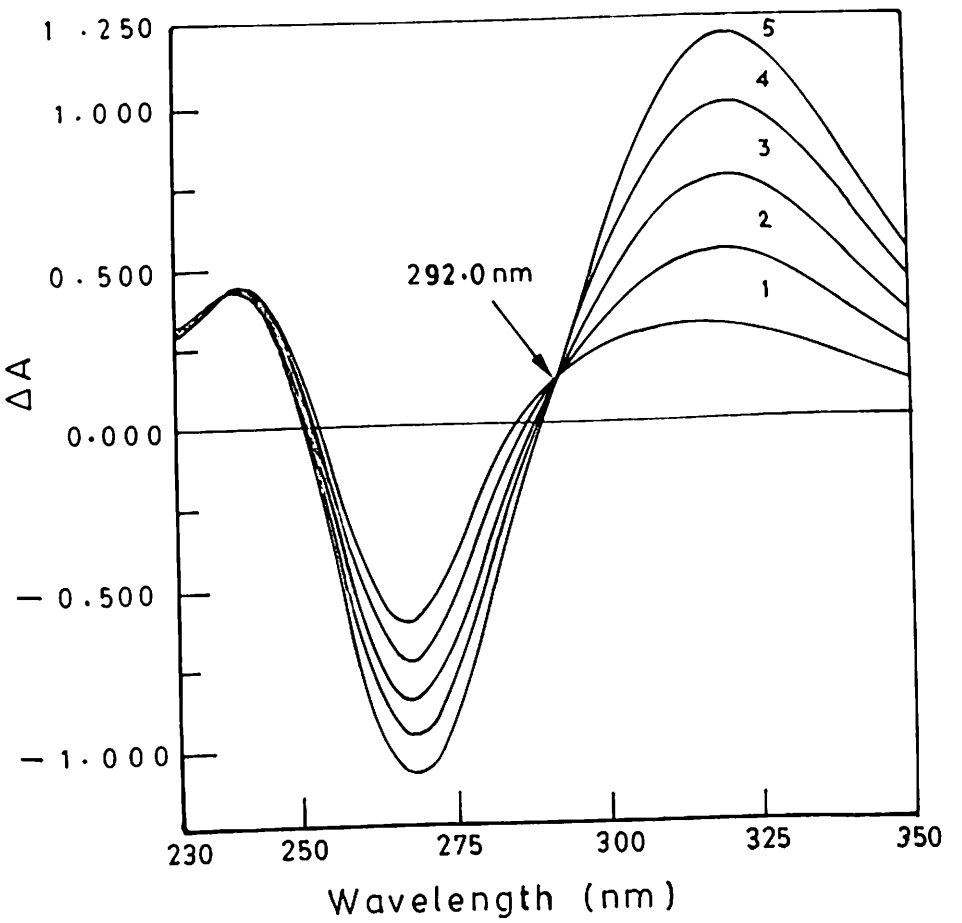

Figure 2.10 Difference spectra of metronidazole (5, 10, 15, 20 and 25 µg/ml) and diloxanide furoate (12.5 µg/ml) in 0.1M HCl Vs 0.1M NaOH in curves1-5, respectively.

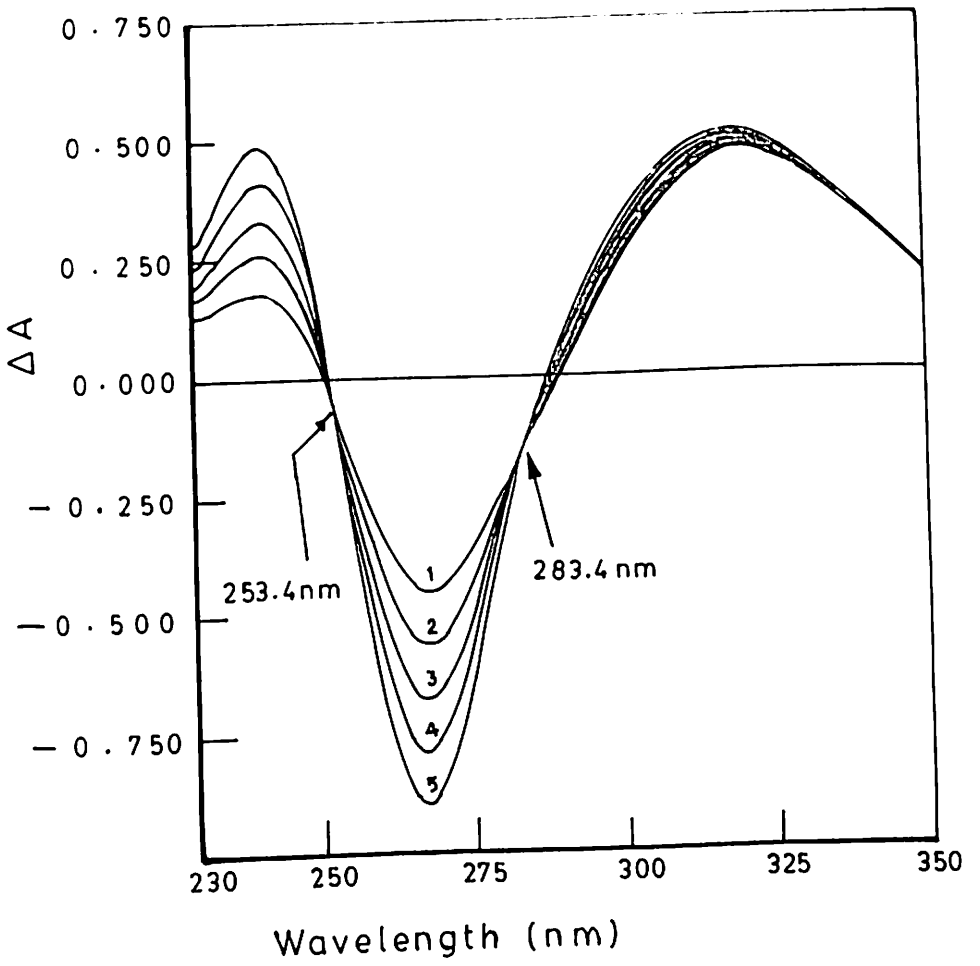

Figure 2.11 Difference spectra of diloxanide furoate (5, 7.5, 10, 12.5 and 15 µg/ml) and metronidazole (10 µg/ml) in 0.1M HCl Vs 0.1M NaOH in curves 1-5, respectively.

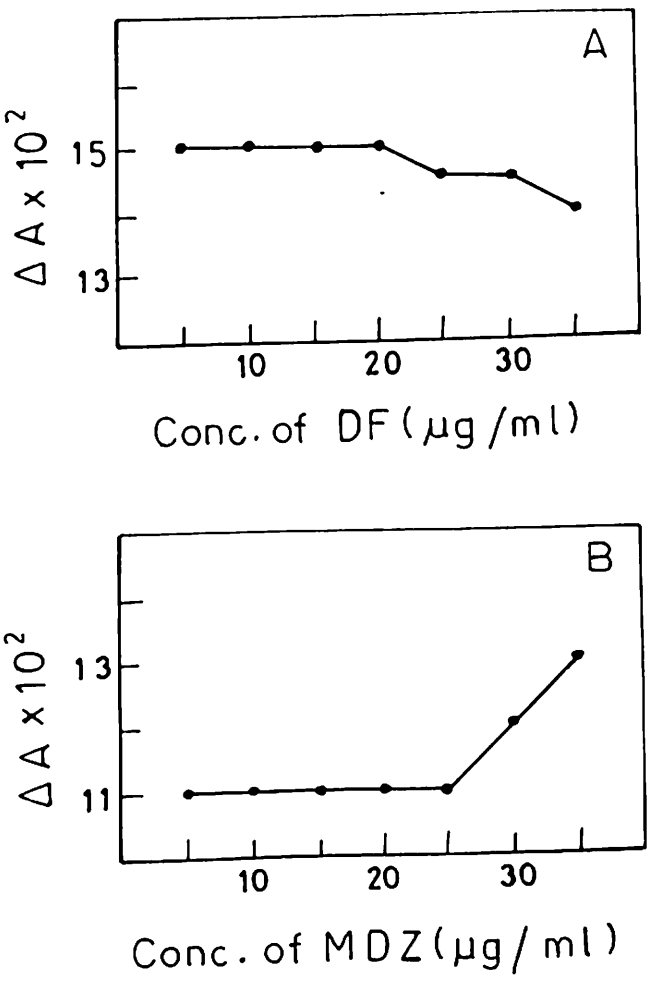

Figure 2.12 Interaction graphs for (A) metronidazole (10 µg/ml) in mixture with diloxanide furoate (at 283.4 nm) and (B) diloxanide furoate (12.5 µg/ml) in mixture with metronidazole (at 292.0 nm) in 0.1M HCl Vs 0.1M NaOH.

Table 2.7: Selectivity of the method for the simultaneous determination of MDZ and DF in standard solutions by difference spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (ΔA) (MDZ at 283.4 nm, DF at 292.0 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
MDZ	DF					Calc	Crit [b]
5	0	0.080 ± 0.002	2.59	0.0006	103.49	1.55	3.86
10	0	0.157 ± 0.001	1.08	0.0005	98.13	2.28	
15	0	0.228 ± 0.003	1.42	0.0010	99.35	0.63	
20	0	0.297 ± 0.001	0.57	0.0005	100.19	2.27	
25	0	0.369 ± 0.005	1.37	0.0016	100.28	0.26	
5	12.5	0.079 ± 0.002	2.52	0.0006	103.43	3.47	
10	12.5	0.156 ± 0.003	2.17	0.0017	98.18	1.20	
15	12.5	0.228 ± 0.003	1.34	0.0009	98.60	1.48	
20	12.5	0.292 ± 0.004	1.42	0.0013	101.35	0.80	
25	12.5	0.368 ± 0.003	0.88	0.0010	99.81	1.32	
0	5.0	0.035 ± 0.001	2.31	0.0002	98.88	0.25	3.86
0	7.5	0.059 ± 0.001	1.71	0.0003	100.62	0.16	
0	10.0	0.084 ± 0.001	1.39	0.0003	100.43	0.12	
0	12.5	0.110 ± 0.002	1.88	0.0006	99.74	0.03	
0	15.0	0.134 ± 0.003	2.34	0.0009	99.95	0.01	
10	5.0	0.035 ± 0.001	2.02	0.0002	101.42	0.83	
10	7.5	0.061 ± 0.001	2.37	0.0004	98.58	0.20	
10	10.0	0.085 ± 0.001	1.90	0.0005	100.49	0.16	
10	12.5	0.110 ± 0.001	1.76	0.0006	99.79	0.11	
10	15.0	0.135 ± 0.002	1.74	0.0007	100.13	0.07	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 2.8: Regression analysis for the determination of MDZ and DF in standard solutions by difference spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 283.4 nm for MDZ at 292.0 nm for DF)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* - test for correlation	
	MDZ	DF			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	5 -25	0	Y = 1.43E-02.X + 1.10E-02	0.9998	2.72E-03	1.64E-04	2.59E-03	2.51E-03, 1.98E-03	1.38E-02, 1.48E-02	1.46E-02	87	3.18
Series B	5 -25	12.5	Y = 1.43E-02.X + 1.07E-02	0.9996	3.91E-03	2.36E-04	3.73E-03	-1.77E-03, 2.31E-02	1.35E-02, 1.50E-02	1.46E-02	61	
Series C	0	5 -15	Y = 9.98E-03.X - 1.51E-02	0.9999	5.53E-04	5.22E-05	4.12E-04	-1.69E-02, -1.34E-02	9.82E-03, 1.01E-02	9.12E-03	122	
Series D	10	5 -15	Y = 9.92E-03.X - 1.37E-02	0.9999	8.74E-04	8.24E-05	6.53E-04	-1.65E-02, -1.09E-04	9.66E-03, 1.02E-02	9.14E-03	122	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 2.9: One-way ANOVA test for linearity of pure MDZ solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	0.1562	0.1562	0.964	3.71
Lack of fit	3	6.51E-05	2.17E-05		
Within line	10	2.25E-04	2.25E-05		
Total	14	0.1565			

*: at P = 0.05 level of significance.

Table 2.10: One-way ANOVA test for linearity of pure DF solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	0.0186	0.0186	0.109	3.71
Lack of fit	3	2.26E-06	7.55E-07		
Within line	10	6.91E-05	6.91E-06		
Total	14	0.0187			

*: at P = 0.05 level of significance.

Table 2.11: Validation report for the determination of MDZ and DF in standard solutions by difference spectrophotometry

Analytical parameter	Results	
	MDZ (283.4 nm)	DF (292.0 nm)
Accuracy (%)	100.44 ± 0.91	100.12 ± 0.53
Precision (%)	99.50 100.00 100.00 100.90 101.80 RSD: 0.91	99.62 99.62 100.25 100.25 100.88 RSD: 0.53
Specificity	A 10 µg/ml of MDZ and 12.5 µg/ml DF mixture solution will show an absorbance value (ΔA) of 0.156 ± 0.0034	A 12.5 µg/ml of DF and 10 µg/ml MDZ mixture solution will show an absorbance value (ΔA) of 0.110 ± 0.0019
LOD (µg/ml)	0.54	0.12
LOQ (µg/ml)	1.81	0.41
Linearity (µg/ml)	5 - 30	5 - 30
Ruggedness (%)	100.44 ± 0.91	100.12 ± 0.53

Table 2.12: Results of the assay of pure drug admixtures and commercial formulations of MDZ and DF by difference spectrophotometry

Sample	Label Claim (mg/tab.)		Recovery (%) [a]		F-test for Mean Recovery		
					Calc		Crit [b]
	MDZ	DF	MDZ (283.4 nm)	DF (292.0 nm)	MDZ	DF	(MDZ / DF)
Pure drug admixture	-	-	99.99 ± 0.63	100.05 ± 0.98	0.09	2.88	3.24
Brand BX	200	250	99.85 ± 0.55	99.91 ± 0.69			
Brand BY	200	250	99.85 ± 0.48	98.96 ± 0.21			
Brand BZ	400	500	99.92 ± 0.30	100.09 ± 0.69			

[a] : Mean and standard deviation for five determinations.

[b] : Theoretical value of F(3,16) based on one-way ANOVA test at P = 0.05 level of significance.

CHAPTER 3

DERIVATIVE SPECTROSCOPY

3. DERIVATIVE SPECTROSCOPY

3.1 Introduction

As mentioned in the introduction of difference spectroscopy the variable and non-specific spectral interferences have been treated by number of mathematical equations and graphical techniques, ranging from very simple to tedious and lengthy procedures according to the shape of irrelevant absorption spectrum. An irrelevant absorption in a spectrophotometric measurement always involves an observed distortion in the absorption curve due to the differences in absorbing impurity between batches and from general contamination during the drug manufacture. Therefore, the attention should be directed to mathematical methods of characterising absorption curves to eliminate the effects of all irrelevant absorption.

The conventional methods like simultaneous equations [6,7], absorbancy ratio [8,9] and graphical or linear plot [13] for resolving binary mixtures cannot be used because of their inherent limitations in eliminating such background interference. These methods demands certain preconditions like mixture peaks should be well separated [6,7], the superimposed spectra of two substances should show an isoabsorptive point [8,9] and the adherence to Beer's Law and additivity of absorbance [13]. The Mortan-Stubb's correction procedure [5] for irrelevant absorption uses only three points on absorption curve and assumes that such interference is linear over the range of wavelengths selected. It has been reported that irrelevant absorption is a quadratic function of wavelength [105]. Later on, a method based on orthogonal functions was proposed by Glenn [10] on the assumption that such irrelevant absorption curve can have a similar or different shape compared to the absorption curve of the substance being examined. Abdine et al [11], who investigated the applicability of above method [10] to spectrophotometric analysis of drugs in tablets acknowledged that great care must be taken in the choice of assay parameters like the selection of proper polynomial, number of points, wavelength range and intervals to discount the irrelevant absorption.

Although, difference spectrophotometry is supposed to take care of interference from formulation matrix, but it may not be useful when the interferants change their spectral pattern under the conditions used for the recording of the difference spectra (such as change of pH). It is reported that the presence of excipients in large quantities such as lactose, dibasic calcium phosphate in the final dilution may sometimes lead to interference in difference spectrophotometry due to their different spectral shapes in acidic and basic solutions [106]. This would have been cancelled automatically in the conventional method. The irrelevant absorption from various tablet excipients like lactose, starch, gelatin, talc and magnesium stearate in acidic solutions were studied in the ultraviolet region [11] and were found to be minimal except for lactose. Hence under these circumstances this technique cannot be used with accuracy and precision. However, such interference from matrix components can be avoided by a prior extraction of drug substances into a suitable solvent before their final dilution.

The requirements of a suitable analytical method are many, but certainly selectivity must be counted as the most important. The widely used measurement techniques by analytical chemists lack such inherent selectivity to allow straightforward application to highly complex materials. The separation procedures involving techniques such as chromatography etc. are useful and indeed essential for such cases. But for the reasons of simplicity, speed and cost, a direct approach will be desirable. Thus, there has always been interest in techniques that can improve the selectivity of measurement by other ways. The derivative spectrophotometry, is one such conceptually simple technique among those methods [15].

Derivative spectrophotometry offers a convenient solution to a number of well defined analytical problems such as resolution of multi-component systems, elimination of interference from sample turbidity, matrix background and enhancement of spectral details [15,107-117]. Although, it was introduced more than forty years ago [118-121] but it has been accepted only hesitantly because of the initial lack of reasonably priced instrumentation and original limitation to the second derivative [118-120]. However,

in recent years, the introduction of electronic differentiation by a computer interfaced with the spectrophotometer leads to the instant generation of derivative spectra with an increased sensitivity and precision.

In general, the differentiation of a curve or of its mathematical function is simply an estimation of the slope over the whole region. In the same way, it is possible to differentiate a spectrum after the curve is fitted with numeric algorithms. In derivative technique the absorbance (A) of a sample is differentiated with respect to wavelength (λ) to generate the first, second or higher order derivatives. For quantitative analysis, if Beer's law is obeyed for the normal spectrum (i.e. $A = \varepsilon bc$).

Then the equation [122] can be shown as

$$\frac{d^n A}{d\lambda^n} = \frac{d^n \varepsilon}{d\lambda^n} \cdot bc$$

where A : absorbance
ε : molar absorptivity (lt / mol. cm)
b : cell path length (cm)
c : concentration of the analyte (mol./ lt)

A similar type of equation for second-derivative spectrophotometry was reported in B.P. 1993 and I.P. 1996. An absorption band, also called an analytical band, can be more accurately described by approximation formulas. Gaussian functions are well suited for describing UV-VIS bands [123,124]. Then, the absorbance A of the band at wavelength λ is given by

$$A_\lambda = A_{max}\, e^{-Cx^2}$$

Where A_{max} : absorbance at wavelength maximum (λ_{max})
C : constant
x : ($\lambda - \lambda_{max}$)

Differentiating the above equation with respect to x leads to the following expressions

$$\frac{dA_\lambda}{d\lambda} = d^1 = (-2)\,Cx \,.\, A_\lambda$$

$$\frac{d^2A_\lambda}{d\lambda^2} = d^2 = 2C(2Cx^2 - 1) \,.\, A_\lambda$$

$$\frac{d^3A_\lambda}{d\lambda^3} = d^3 = (-4)C^2 x\,(2Cx^2 - 3) \,.\, A_\lambda$$

$$\frac{d^4A_\lambda}{d\lambda^4} = d^4 = 4C^2\,(4\,C^2x^2 - 12Cx^2 + 3) \,.\, A_\lambda$$

The value of constant C can be obtained by the half width of band using the following two equations.

$$C_{FWHM} = \frac{4 \ln 2}{FWHM}$$

$$C_\sigma = \frac{2}{\sigma^2}$$

where FWHM = full width at half maximum amplitude

σ = width of the band between the points of inflection.

The results of the differentiation of simple analytical bands are represented graphically in Figure 3.1. It is to be noted from figures that

i. The maximum of fundamental curve in odd-order derivative corresponds to a passage through zero and in even-order derivatives it corresponds to an extremum value of either a minimum or maximum. In other words, the basic maximum always produces a positive extremum in 4^{th}, 8^{th} and $4n^{th}$ derivative and a negative extremum (minimum) in 2^{nd}, 6^{th} and $(4n-2)^{th}$ derivative (where n = 1,2,3 ..... in both cases).

ii. Inflections on the fundamental curve lead to odd-order extrema and to passages through zero in even-order derivatives.

iii. With increasing order of the derivatives, the number of extrema exceeds that of the fundamental curve because each inflection gives an additional extremum by differentiation. In the n^{th} derivative one maximum produces n + 1 extrema.

iv. With increasing derivative order, the sharpness of the bands increases and σ, as well as FWHM, becomes smaller.

The actual position at which a derivative curve will pass through axis or else generate a maxima or minima was suggested by Morrey [125].

In practice, the differentiation discriminates against broad bands, emphasizing sharper features to an extent that increases with increasing derivative order. For Gaussian bands, the amplitude D of the n^{th} derivative is related to the n^{th} power of the inverse of bandwidth of the normal spectrum [126] as

$$Dn \propto \frac{1}{FWHM^n}$$

An inconvenience of the derivative technique is that the signal to noise ratio (SNR) becomes worse for progressively higher orders. The SNR of derivative signals depends on the shape of the spectrum and has been evaluated for different bands [127]. In Gaussian bands, if we consider an SNR of 1 in the normal spectrum, the SNR is given by $2.02/M$, $3.26/M^2$, $8.10/M^3$ and $17.8/M^4$ for first, second, third and

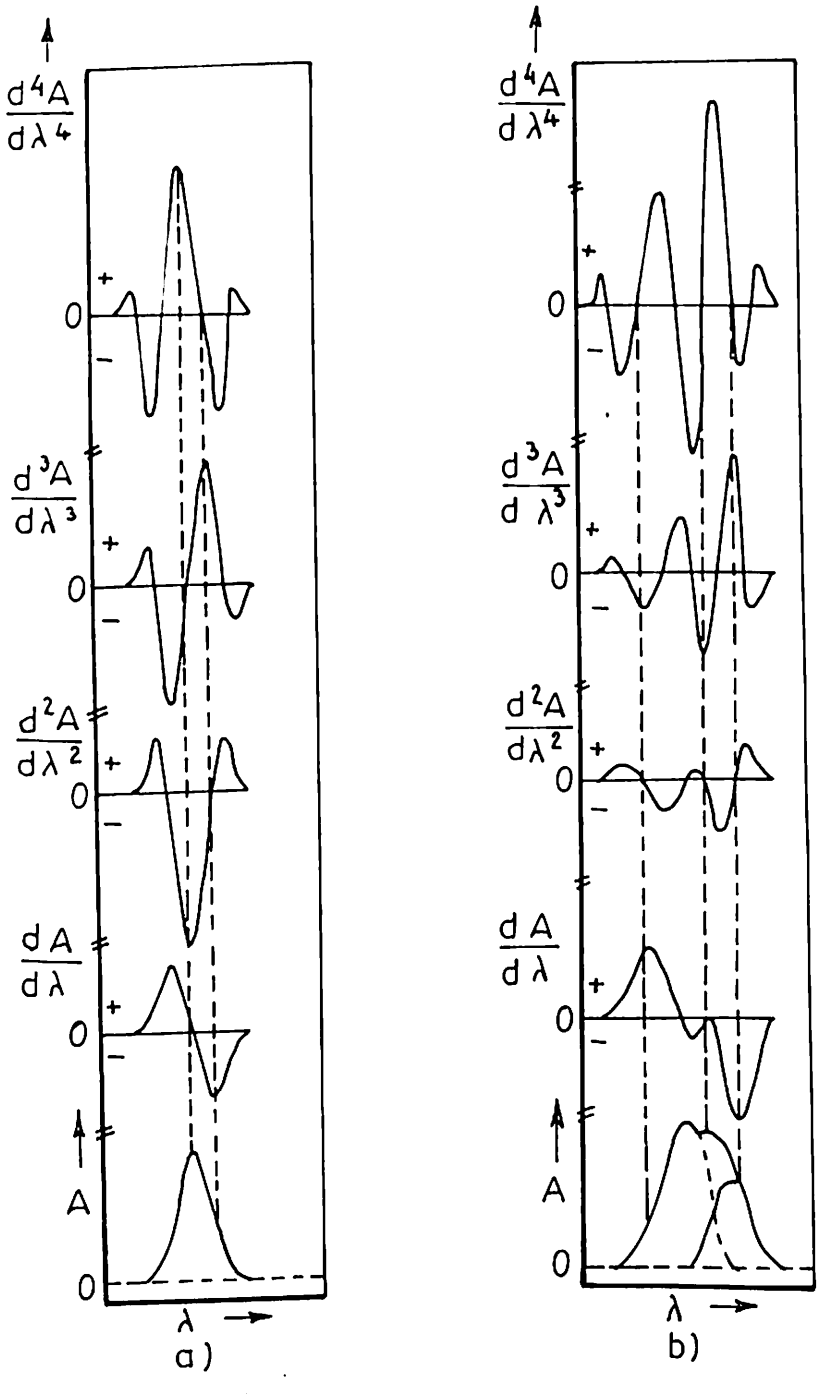

Figure 3.1 **Differentiation of Computed Gaussian analytical bands: a) Fundamental curve and first-to fourth-order derivatives; b) Fundamental curve and first-to fourth-order derivatives of two superposed Gaussian bands.**

fourth derivative respectively where M is the number of points in peak full width at half maximum (FWHM).

In general, the differentiation of signals can never result in more information than is present in the original bands, but it is possible to bring desired information to the fore and to eliminate unwanted background by assuming a polynomial of n^{th} power is zero after (n + 1) differentiating steps [4] like

$$U^{0} = az^{4} + bz^{3} + cz^{2} + dz + e$$
$$U^{I} = 4az^{3} + 3bz^{2} + 2cz + d$$
$$U^{II} = 12az^{2} + 6bz + 2c$$
$$U^{III} = 24az + 6b$$
$$U^{IV} = 24a$$
$$U^{V} = 0$$

Extending these polynomials U^{0} to U^{n} to the earlier derivative expressions for analytical band.

$$D0 = d^{0} + U^{0}$$
$$D1 = d^{1} + U^{1}$$
$$\vdots$$
$$Dn = d^{n} + U^{n}$$

If U^{n} is zero then

$$Dn = d^{n}$$

Thus, differentiation can considerably improve the detection sensitivity of masked weaker bands by eliminating disturbing background absorption (e.g. in turbid solutions or opaque samples) [4].

The derivative curves generated from a normal spectrum are evaluated in many ways for quantitative purposes. These methods (also known as graphical methods) include

i. Peak-peak method (Figure 3.2a)
ii. Peak-peak ratio method (Figure 3.2a)
iii. Peak-tangent method (Figure 3.2b)
iv. Peak-zero method (Figure 3.2c and d)

The peak-peak (PP) method is most commonly used in quantitative multi-component analysis [15] to estimate concentrations of known substances. In derivatives of absorption curves the distance from a maximum to a minimum is generally directly proportional to the concentration of the substances. This method can be applied satisfactorily to the curves processed in even-order derivatives, as the peak positions are proportional to concentrations.

The peak-peak ratio (PPR) method is based on the ratios between pairs of neighboring peaks (P_1/ P_2 in Figure 3.2a). The ratio of the heights P_1 and P_2 will be constant as long as the ratio of components do not vary, even though the absolute concentrations are different and also modified either due to mixture components or background interference in pure solutions. Since the ratio obtained is a characteristic quantity of a substance it enables the analyst to estimate the varying concentrations of a substance by keeping the other components as constant. It is useful when searching for small differences in complicated derivative spectra [15].

In the peak-tangent (PT) method a common tangent is drawn to two neighbouring maxima or minima and the distance to the intermediate extremum value is measured parallel to the ordinate (t_1, t_2, t_3 in Figure 3.2b). This method can be applied satisfactorily if a linear background is present [128] but most of the time it is better to check whether a higher order can give better results.

The peak-zero (PZ) method of evaluation is the most widely used in quantitative analysis. The vertical distance Z from the zero line is measured (Figure 3.2c), which is proportional to the absolute value of the derivative [110] and also known as peak-baseline (PB) method. It is suitable for derivatives which have nearly symmetrical signals with respect to the abscissa and if individual curves overlap in a manner where by one of the signals passes through zero at the maximum of other drug (Figure 3.2d). This later technique is popularly known as zero-crossing point (ZCP) method [110].

Though, the zero-crossing derivative is a more rapid, simple for the assay of two-component mixtures and ideal in terms of eliminating systematic errors, but it suffers from sensitivity to small changes in the position of the interfering band compared to other discussed methods [110] in situations where the ideal zero-crossing point will be absent for quantitation. In such cases, a non-mathematical method known as compensation technique was proposed [128,129] for the detection and elimination of unwanted absorption during spectrophotometric analysis. The irrelevant absorption curve is assumed to possess the simplest shape and none of the characteristics of pure compound.

In two-component analysis, the compensation method involves a comparison of several difference spectra [mixture (m) - reference (r)] using different concentrations of a reference solutions where $m = C_x + C_y$, and C_x and C_y are concentrations of pure drug X and Y. If C_r for compound X is introduced in to reference cell, the absorption characteristics of the mixture gradually approach that of compound Y as C_x increases and finally coincides with the absorption curve of compound Y at the balance point, for which $C_r = C_x$. By analogy, C_y can be found by repeating the same steps using C_r for compound Y in the reference cell.

However, in two-component analysis if the two absorption curves of the analyte compounds (X and Y) are greatly overlapping or in the analysis of a minor component

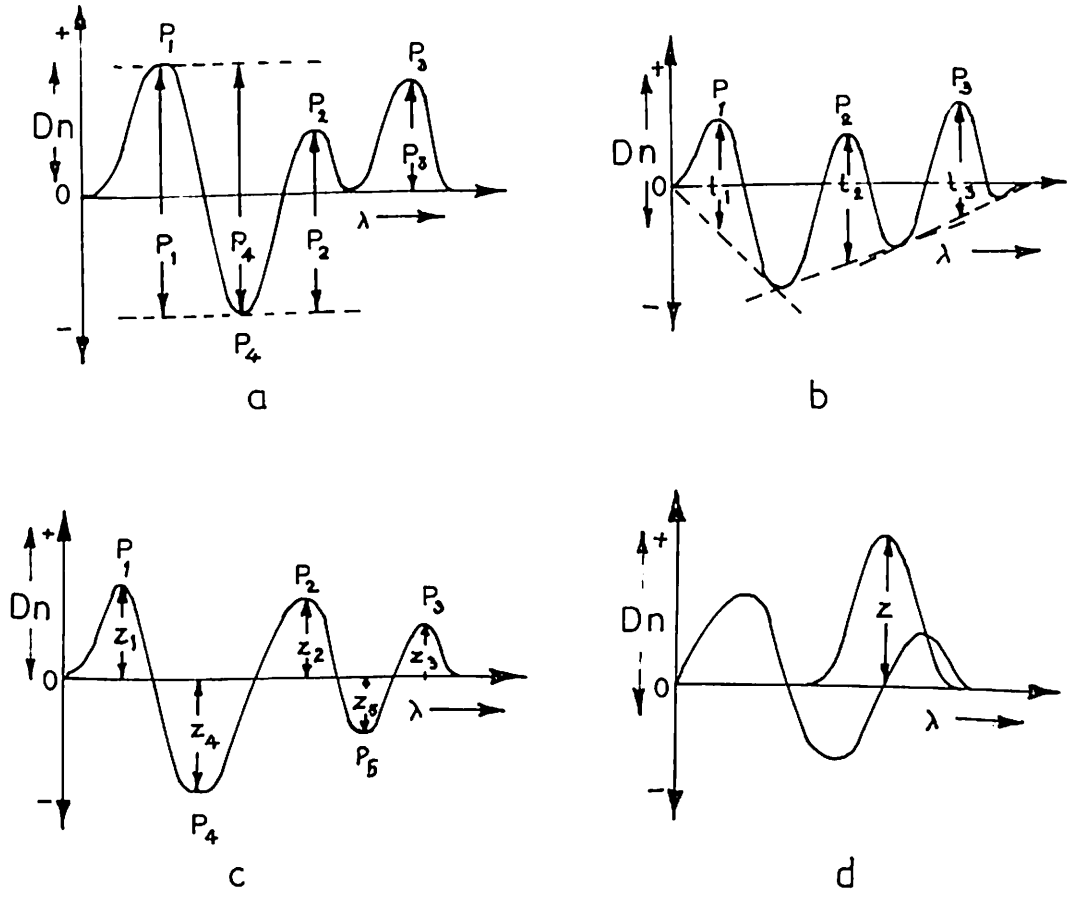

Figure 3.2 Different methods of evaluation of derivative spectra. a) Peak-peak method; b) Peak-tangent method; c) Peak-baseline method and d) Zero-crossing point method.

in the presence of a major component, evaluation of balance point will be difficult and erroneous results are expected. The accuracy of the method depends on the evaluation of the balance point. Therefore, in order to eliminate the personal bias in the detection of balance point during two-component analysis, the ratios of derivative measurements were to be considered as purity indices during spectrophotometric analysis [129]. Thus, the concentrations at which the ratios will be identical are called as the balance point. A graphical method was also suggested by same authors for above such situations on the basis of ratios in identifying the balance point [129] and thus, the technique is known as ratio-compensation (RC) method.

In some cases, where the zero-crossing technique is not preferred in two-component analysis due to loss of sensitivity, a ratio-spectra (RS) method was used [18,19, 130-133]. Where, the spectrum of one drug standard solution will be divided, wavelength by wavelength with a constant concentration of other drug solution as divisor and the ratio-spectra thus obtained are then differentiated with respect to wavelength. The derivative values corresponding to maximum or minimum were considered for the estimation of pure drug and the estimation of other drug was done by analogous procedure.

There were also few special methods available for evaluation of derivative spectra like additive and subtractive [4], multiplicative [4], log A derivative [4], differentiation-integration [4], extended peak-peak Ratio [15], half-wave graphical illustration [125] and side-peak-side ratio [134]. However, they are of little value in routine quantitative analysis.

There are numerous methods for generating derivatives of electric signals. The various methods used earlier were graphical methods [4] electro-mechanical systems [15], modulation systems [15], subtraction of delayed spectra [15] and dual-wavelength spectrophotometry [135-137]. The principal disadvantage of above methods is that the higher derivatives cannot be obtained directly. The analog differential methods

[15] were successful in overcoming the earlier limitations and also has advantage like the higher derivatives can be obtained relatively in simple manner. However, the resolution of the derivative spectra depends not only on slit width but also to a large extent on the rate of wavelength scan and on differentiation time constant. The position of extrema will shift with change in scan speed.

In the recent years, the digital differentiation methods [15] dominated over analog methods owing to the evolution of computers and because of the trend to digitalise the data. Here, the output signal of the spectrophotometer is fed to an analog-digital converter (AD converter) and the signals at each given wavelength are accumulated. The accumulated numerical data will be processed with the help of different algorithms [4] for digital differentiation. The Savitzky-Golay (SG) polynomial [138] is probably the most frequently used digital algorithm for generating derivatives of spectra and other curves produced by various signal sources. It is based on moving average computations where the best mean square fit of a set of (2m+1) consecutive values (m is number of equidistant ordinate values) are used for the polynomial of degree "n" and then numerically differentiated. The greatest advantage of digital method is that scan speed does not shift the position of the extrema, but the absolute values of the computed derivative data are small. Therefore, these must be multiplied by a factor of hundred or more to obtain useful signal heights but also with enhanced noise.

Thus, the digital differentiation process has created a great impact on spectrophotometric method of analysis in the last ten years. The derivative spectrophotometry represents an easy approach to the problem of resolving spectral overlap in pharmaceutical analysis. It has been successfully used for the analysis of pharmaceutical dosage forms and moreover, for the determination of several drugs alone or in combination by eliminating matrix interferences [114].

The review of reported literature shows that a large number of drug products either alone or in combination of other drugs were estimated using this technique. But, the number of single drug preparations [79,132,139-156] thus estimated were less compared to the two-component systems.

Thus the reported combined drug preparations represent different categories of drugs. Which are mainly analgesics & anti-inflammatory [157-169], antibiotics [133, 170-182], sulfonamides [129-131,183-190], antidiarrhoeals [191,192], antifungals [193-195], antihistamines [196-204], antihypertensives/diuretics [129,205-215], antipsychotics [129, 216-226], steroids [227-230], vitamins [231-235] and others [236-240]. The above reports suggest that the drug estimation from various dosage forms like suppositories [139,166], suspensions [144], ophthalmic solutions [145,150,153], ointments [154], injections [168,173,229] and aerosols [199] was also made possible with this technique apart from popular solid dosage forms.

The basis for quantitative determination involves mostly the principles of zero-crossing technique but also other techniques like ratio-spectra [130-133, 174,209,231,238], ratio-compensation [129], peak-baseline [129,139,141,190] and peak-peak ratio [129,142,172,198,213] were employed in their determination from various dosage forms. The review also suggested that derivative spectrophotometric technique can also be extended for resolving three [133,178,195,197,231] or more [232,233] components of drug mixtures.

There are also useful reports for identification and quantitation of drugs in presence of their related impurities [241-245], degradation products [246-251] and from the biological samples [252-255]. This particular UV-VIS derivative technique was also recommended for the study of other related topics of pharmaceutical interest like determination of dissolution rate [256,257], stability and degradation kinetics [257-259], determination of partition coefficient [260], biological absorption of drugs [261,262], drug-protein binding [263], molecular interactions of drugs [264] and

influence of colours used in pharmaceutical products [265]. The derivative technique has found application not only in UV-VIS spectrophotometry but also in other spectroscopic techniques like infrared [107], atomic absorption [266], flame emission [267] and also in spectrofluorimetry [141,268-284]. Clearly, the derivative spectrofluorimetry alone [141,268-282] or combined with synchronous scanning [273,283,284] has helped in resolving the overlapping of those mixtures which could not be achieved by UV methods especially in dealing with few formulations [141,268-275], impurities [276,277] and biological samples [278-284] have been reported. The summary of this literature review was presented in Appendix C.

The other major fields of non spectroscopic application of the derivative technique are in different chromatographic separation methods like thin layer [134,285], high performance [286-289], gas [290] and other methods like polarography [291-293] and thermography [294]. This technique has been used mainly to improve the separation of unsatisfactory peaks, to identify the collected fractions and to resolve the fine structures in polarograms and thermograms.

The applications of derivative spectrophotometry were also found to be significant in other areas like biochemical, environmental and forensic analysis [4] etc. The theory and practical applications of this techniques in various areas have been reviewed by many authors in the past [15,107-117].

The inception of computers in the analytical laboratory has fostered the development of newer chemometric methods for the analysis of pharmaceutical preparations yielding highly overlapped peaks. By using a multiple linear regression procedure these methods determine the concentration that best fits an over-dimensional equation system. This is in turn established by measuring the absorbance of a given sample at a larger number of wavelengths than that of analytes to be determined. These methods include principal component regression (PCR) [16,17], simplex [17], partial least

square (PLS) [17,18], multiple linear regression analysis (MLRA) [17,19,20] and multi-component analysis (MA) [17,18,20].

Recently, there are many reports available with successful application of these methods for resolving multiple components of a drug mixture from their derivative curves. These methods include PCR [16], MLRA [165,209,221,233] and MA [183,185,197,200,208].

Though, these methods need specific software but they are much more expeditious and can be applied to a large number of analytes in the sample. In addition, the method like partial least squares (PLS) does not require a prior knowledge of pure spectra of all the components in the mixture and takes account of interactions in the calibration matrix as well [16].

It is to be noted that chemometrics unlike other simple methods is only useful, where information is not obvious at first sight and can be obtained from the data. Hence, if the method is improperly used, it can lead to different conclusions than the expected. Therefore, a great deal of understanding of the nature of the problem and the questions to be asked is essential prior to using chemometric methods [295]. But, also the validation of a chemometric method often needs more work than validation of an univariant method [296] and thus, it lacks wide acceptability in routine analysis. On the other hand, graphical methods are much easier to implement and saves time and cost.

Based on the above mentioned reasons, a simple derivative spectrophotometric method was used as a tool for the development of new analytical methods.

3.2 Design of the experiment

The basic experimental approach adopted for the work carried out using derivative technique was same. The various formulations used for the method development include either a two-component mixture or a three-component mixture available in tablet or in capsule form.

All the selected drug combinations could not be estimated through difference spectrophotometry. This was mainly due to the incompatibility (e.g. lack of solubility or stability) of either one or all drugs in combination in any of the standard acid or alkaline buffer to induce changes in spectral difference and also due to lack of significant changes in the difference spectrum even though they are compatible in selected buffer systems. Hence, a derivative spectrophotometric method was preferred.

All the reported work in this section was carried out using JASCO model 7800 UV-VIS spectrophotometer with 1 cm quartz cells. A scan speed of 480 nm/min and a bandwidth of 3 nm were maintained. Ordinate maximum and minimum were adjusted according to the amplitudes of measured values. The instrument was calibrated (including for derivative spectra) before development of each method according to the procedures described in official monographs [2,3] and also to methods suggested by Talsky [4]. The results obtained were reported in Appendix A.

The stepwise experimental approach is:

a. Preparation of standard solution of the pure drugs at an appropriate concentration range to obtain the desired formulation ratio.
b. Selection of appropriate derivative order based on ideal point's selection to generate a derivative spectrum by Savitzky-Golay algorithm [138] and also to check the requirement of any smoothing process of derivative curves.
c. Examining the linear proportionality of absorbance values with different concentrations of drug solutions at the possible wavelengths of estimation.

d. Preparation of different series of the solutions from the respective stock solutions of pure drugs for the construction of the calibration curve. The series of solutions were always prepared as a representative of both pure drugs and mixtures. The comparison of the regression equations between pure drug solutions with that of the mixture was done to establish the selectivity of chosen wavelength for the determination.

e. To establish an ideal concentration range for drugs in combination and to study possible interferences, if any, between mixture components, an interaction study with mixture solutions was carried out at the identified wavelengths in step "c". This range was also required to fulfill the compliance of Beer's law and to meet the proportion of drugs in commercial formulations, which ought to be estimated by the method.

f. Validation of the developed method according to the procedures described in U.S.P. 23.

g. Application of the developed method to commercial preparation(s) for the suitability in routine analysis.

h. Finally, the statistical analysis of data obtained for standard solutions and the commercial preparations of each combination was done and the obtained details were discussed under the respective experimental section. The various mathematical expressions used for the statistical treatment of data were presented in Appendix D.

As discussed in introduction to the technique, a zero-crossing point of one drug has always been chosen as the wavelength for the estimation of the other and vice versa. But in few instances, the lack of a suitable zero-crossing point (for diloxanide furoate in combination of tinidazole and furazolidone), the lack of appreciable absorption at the zero-crossing wavelengths (for trihexyphenidyl HCl in combination of haloperidol), the closeness or identical zero-crossing points for the combined drugs (in case of metoprolol and hydrochlorothiazide) have compelled to change the method of estimation to either ratio-compensation or peak-baseline for their estimation.

In addition, wherever possible, the estimations were carried out at all available or ideal zero-crossing points (ZCP) and were compared among themselves and also with other preferred methods like ratio-compensation (RC) and peak-baseline (PB) method to study the selectivity within and between the methods.

3.3 SIMULTANEOUS DETERMINATION OF TINIDAZOLE AND FURAZOLIDONE IN TABLET PREPARATIONS BY FIRST- AND SECOND-DERIVATIVE SPECTROPHOTOMETRY

The combination of tinidazole (TD) and furazolidone (FD) in the form of tablet preparations is widely used for diarrhoea, amoebiasis and giardiasis. The I.P. 1996 described a titrimetric and ultraviolet method for the individual assay of TD as pure drug and from its tablet preparations respectively. While, the U.S.P. 23 and I.P. 1996 suggest an ultraviolet method for determination of FD as a pure drug and as well from its formulations. But B.P. 1993 prescribed ultraviolet method only for the pure drug. There are also reports available for the individual assay of TD by colorimetric [297-299], polarographic [300], ultraviolet [301,302] and HPTLC method [303,304] and for the individual assay of FD by ultraviolet [23], polarographic [305], colorimetric [306] and TLC method [307]. There are also few reports available for assay of TD [60,61,191,308-320] and FD [62, 321-323] in respective combination of other drugs and also combined together from its dosage forms [308-310]. The combined reports of these two drugs employed polarographic [308], HPLC [309], HPTLC [309] and multi-component analysis (MA) methods [310] for their determination. The objective of this work was to demonstrate two derivative spectrophotometric methods for the simultaneous determination of these drugs without prior separation from the combined preparations.

Materials and Reagents

Tinidazole (Gufic Ltd., India) and furazolidone (Rhone Poulnec, India) were obtained as gift samples. Dimethyl formamide (DMF; Qualigens, India) of analytical grade was used.

Standard Solutions

The stock solutions of TD and FD were prepared by dissolving 5 mg of each pure drug in 10 and 25 ml of DMF, respectively. Appropriate amounts of the stock solutions were transferred separately into 10 ml of volume flasks. The volumes were made up with DMF to give a series of solutions containing 10-20 µg/ml of TD and 3-7 µg/ml of FD, respectively. A three series of 10 ml mixtures of TD and FD in DMF were also prepared from the stock solutions. The first series contained a constant concentration of FD (5 µg ml) and a varying concentration of TD (10-20 µg/ml). Similarly, the second series contained a constant concentration of TD (15 µg/ml) and a varying concentration of FD (3-7 µg/ml). The final series contained a constant concentration of TD (15 µg/ml) and FD (5 µg/ml).

Interaction Study

Two separate series of mixture solutions were prepared from fresh stock solutions according to the procedure mentioned above. The first series contained a constant concentration of TD (15 µg/ml) and a varying concentration of FD (2.5-20 µg/ml). While, the second series contained a constant concentration of FD (5 µg/ml) and a varying concentration of TD (5- 40 µg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate TD (15 µg/ml) and FD (5 µg/ml) standard and test samples were prepared from fresh stock solutions according to above mentioned procedures.

b. ***Linearity***

Separate series of solutions of TD and FD of each containing 5-30 µg/ml and 2-20 µg/ml respectively were prepared from the stock solution meant for method validation.

c. *Specificity*

Series of five mixture solutions of each containing TD (15 µg/ml) and FD (5 µg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of TD in combination with FD (Brand CX) were accurately weighed, well powdered and a weight of the powder equivalent to 15 mg of TD (and 5 mg of FD) was dissolved in DMF by thorough mixing and made up to volume in a 50 ml volumetric flask. The sample was filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with DMF to obtain samples having a concentration of 15 µg/ml of TD and 5 µg/ml of FD.

Procedure

Zero-crossing point (ZCP) method

The absorbances of sample and standard solutions of TD and FD were recorded from 275-400 nm against a blank solution. The first-derivative absorption spectra for each set of solutions were subsequently recorded using the Savitzky-Golay parameter [138] of Δ = 10 points (Δ represents the width of the boundary over which the derivative is calculated) and no smoothing was necessary. The solutions were measured at the zero-crossing wavelengths of the other drug.

Ratio-compensation (RC) method

The sample cell contained the mixture of 15 µg/ml of TD and 5 µg/ml of FD and the reference cell contained a series of standard solutions (TD/FD) with different concentrations. The first- and second-derivative spectra were recorded from 275-400 nm using Δ = 10 and 30 points [138] respectively in each instance. Similarly, the first- and second-derivative spectra of pure drug solutions were also recorded against a blank solution. Different ratios, such as wavelength maxima to wavelength

minima or vice versa (as mentioned in Tables 3.2 and 3.3) for the pure drugs and the mixture were calculated. At the exact balance point the concentration of one of the analyte components of a mixture become equals to that of the reference solution and therefore the calculated ratios at such point should be equal to that of pure drug other than in the reference cell. The procedure was repeated to obtain the second analyte concentration from the sample and standard mixture.

Results and Discussion

The zero-order spectra of pure drug combinations were found to be overlapping (Figure 3.3). The traditional Vierordt method [5] involves the use of two simultaneous equations was attempted, but the results were not satisfactory due to significant contribution of absorbance from other components of the drug mixture. The other procedures [6-13] for the simultaneous analysis of mixtures could not be selected, because many of them require special attention in selecting the assay parameters. The previously adopted pH-induced differential spectrophotometeric method could not be applied due to lack of compatibility of FD in presence of such buffer systems. For these reasons, it was more convenient and simple to resolve the problem of closely overlapping spectra by making use of the first- and second-derivative spectra of the mixture (Figures 3.4 and 3.5).

In general, the common methods of evaluation of a derivative spectra for quantitative purposes include the graphical measurements like peak-peak (PP) [15] and peak-baseline (PB)[15] and zero-crossing measurements [110]. However, the success of a measurement depends on its sum of systematic and random errors. As mentioned earlier [110], the zero-crossing method would be ideal in terms of systematic error, but was more sensitive compared with graphical measurements, to small drifts of the band of the other component. In this instance, the poor resolution of both first- and second-derivative spectra (i.e. absence of separated peaks) of TD and FD prevent the correct use of graphical measurements and these methods fail in terms of systematic

errors, unlike zero-crossing methods. For these reasons, the zero-crossing method was compared with a ratio-compensation (RC) method [128,129] described earlier in the introduction of this chapter, to avoid the influence of such errors in the measurement.

The recorded first- and second-derivative spectra of TD and FD were shown in Figures 3.4 and 3.5. In the first-derivative mode, the zero-crossing point (ZCP) of TD was observed at 321.0 nm and the ZCP's of FD were found at 307.2 and 372.0 nm respectively. In case of second-derivative, the ZCP's of TD were occurred at 299.4 and 343.2 nm, and for FD at 287.0 and 340.0 nm. The ZCP's of first-derivative were distinctly separated over the second-derivative points and thus selected for the estimation of pure drugs from their mixtures. The first derivative ZCP's considered for their determination were at 321.0 nm for FD (i.e. ZCP of TD) and at 307.2 and 372.0 nm for TD (i.e. ZCP of FD). Hence, the measurements made at these selected wavelengths would be function of the only component of interest.

The proportionality of derivative absorbance values to the concentrations of TD and FD were found by measuring a series of pure drug (Table 3.1, Series A and C of Table 3.4) and mixture solutions (Table 3.1, Series B and D of Table 3.4) at the above selected wavelengths. The calculated regression equations based on measured derivative values (Table 3.1) of TD and FD solutions were reported in Table 3.4. The spectra obtained from such mixture solutions (Series B and D of Table 3.4) were shown in Figures 3.6 and 3.7. The presence of distinct isosbestic points at 321.0 nm (Figure 3.6), and at 307.2 and 372.0 nm (Figure 3.7) suggested that there were no interferences in the estimation of the individual components. It was also evident that absorbance values were proportional to their concentrations in drug mixtures. The mean absorbance values and the corresponding regression equations for TD measured at 372.0 nm were not shown in Tables 3.1, 3.4, 3.5 and 3.7 respectively to make the things simple. However, the assay values obtained at 372.0 nm were reported in the Table 3.8. The mutual independence of the absorbance value of TD and FD at their

wavelength(s) of estimation was determined by a careful mixture interaction study. It can be observed from reported results (Figure 3.8) that the varying concentrations of FD (2.5-20 μg ml) did not interfere up to 10 μg/ml in the estimation of TD (15 μg/ml) at 307.2 nm (Figure 3.8A). Similarly, the varying concentrations of TD (5-40 μg/ml) did not show any interference up to 25 μg/ml in the estimation of FD (5 μg/ml) at 321.0 nm (Figure 3.8B). Thus, the proposed concentration range for pure drugs and the mixture (Table 3.1) were ideal and the accurate determination of the two drugs would be possible at the suggested concentration ratio (i.e. 15 μg/ml of TD and 5 μg ml of FD).

In ratio-compensation method, the first- and second-derivative spectra were recorded for each reference solution of the analyte components and also against the mixture solution to achieve the balance point. Thereafter, the different ratios (as mentioned in Tables 3.2 and 3.3) were calculated between wavelength maxima to wavelength minima or vice versa from recorded derivative spectra. The mean ratio absorbance values thus obtained for pure drugs and mixture solutions at the balance point were reported in Tables 3.2 and 3.3.

It was clear from the statistical analysis of the data obtained for pure drug solutions and their admixtures that, the standard deviation associated with determinations, coefficient of variation and the standard error of values were reasonably small (Table 3.1). This demonstrated the precision of the ZCP method and the negligible interference of one drug in the absorption measurement of the other drug. The percentage ratio of residuals showed a deviation of within 1% (Table 3.1). However, the calculated values of F-test for non-linearity [97] were less than the theoretical value at 5% significance level and thus demonstrated that the calibration points did not deviate significantly from the best-fit line.

Similarly, the ratio values reported in Tables 3.2 and 3.3 showed that the ratios obtained for mixture solution at balance point were almost similar to that of mean value obtained for pure drug solutions. The standard deviation, standard error and coefficient of variation associated with ratios were also same and thus proved the existence of non-interference in the mixture solutions.

The regression equations of the pure drug solutions and those of mixtures were similar (Table 3.4) including the obtained correlation coefficients, which suggested the non-interference of one drug in the estimation of the other. A one-way ANOVA test [98] was performed based on the separate linear calibration graph constructed with three replicates per point including the lowest and highest variation observed from the mean absorption value of each pure drug concentration during the replicate measurements (Table 3.1). The calculated F-values were less than that of critical value at 5% significance level suggested that calibration line present homoscedasticity (Tables 3.5 and 3.6).

The slope values obtained for TD and FD solutions by facilitating their regression equations to pass through the origin with the help of an additional calibration point (0,0) have failed to confine within the 95% confidence limits of slope. This contrast was observed due to lack of significant change in absorbance value with change of concentration (only a third decimal change was noticed). However, the satisfactory confidence limits were identified at 97.5% (Slope: 1.52E-03 to 1.71E-03) and 99.8% (Slope: 1.54E-03 to 1.72E-03) for pure drug and mixture solutions of TD respectively. Similarly, the satisfactory confidence limits for FD observed to be at 99.9% for pure drug (Slope: 1.88E-03 to 2.23E-03) and 99.5% for mixture (Slope: 1.88E-03 to 2.21E-03). Further, the obtained values of Student's *t*-test for correlation [98] were highly significant at 5% level (Table 3.4) which suggested a strong positive correlation between measurement values and concentrations. The precision of the fit by regression

was also confirmed from the reported small values of standard error involved with intercept, slope and the estimate [98].

The developed method was validated according to the procedures described in U.S.P. 23 and the results obtained were reported in Table 3.7. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A and C of Table 3.4.

The percentage recovery values determined for standard solutions and a commercial product by both zero-crossing point (ZCP) and ratio-compensation (RC) methods were reported in Table 3.8. The mean recovery values obtained by both the methods for TD solutions as pure drug were 99.67 to 100.06% and for FD were 99.77 to 99.97%. Thus the limits of official monographs for TD and FD were met (Appendix B). The commercial formulation showed a mean recovery of 99.49 to 99.85% for TD and for FD were 99.71 to 100.19%. A two-way ANOVA test [98] performed at 5% level suggested that there were no significant differences between the two methods and also within the method, during the recovery of both drugs from commercial formulation and standard mixtures. Hence, it confirmed the non-interference from formulation matrix in the assay of the drugs in commercial preparation and these methods take in to account all sources of errors discussed earlier.

The above findings substantiate the usefulness of derivative spectrophotometry for the analysis of mixtures of the two drugs tested, either in pure form or in tablets. As the choice of products available with this particular combination was limited, the reported method could not be extended for many commercial products other than the reported. However, the results presented here must be considered as incentive to any such findings. The standard solutions prepared were found to be stable for 3 hrs in presence of DMF.

Thus, the proposed derivative methods were found to be accurate and precise, and easier compared to the reported methods [308-310]. Therefore, the rapidity of the proposed method makes it convenient in routine analysis for their estimation in combined formulation.

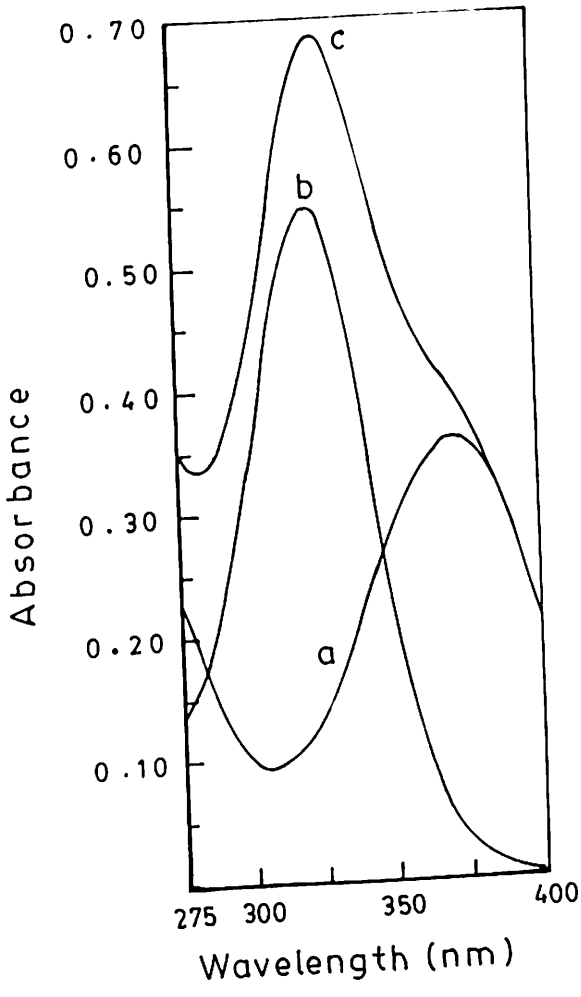

Figure 3.3 **Absorption spectra of (a) furazolidone (5 μg/ml), (b) tinidazole (15 μg/ml) and (c) their mixture in dimethyl formamide.**

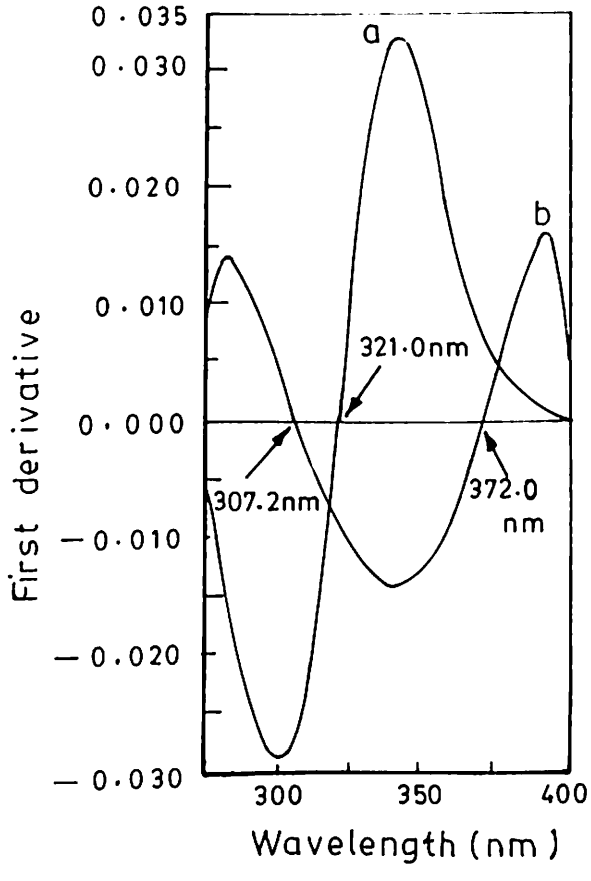

Figure 3.4 First-derivative spectra of (a) tinidazole (15 µg/ml) and (b) furazolidone (5 µg/ml) in dimethyl formamide.

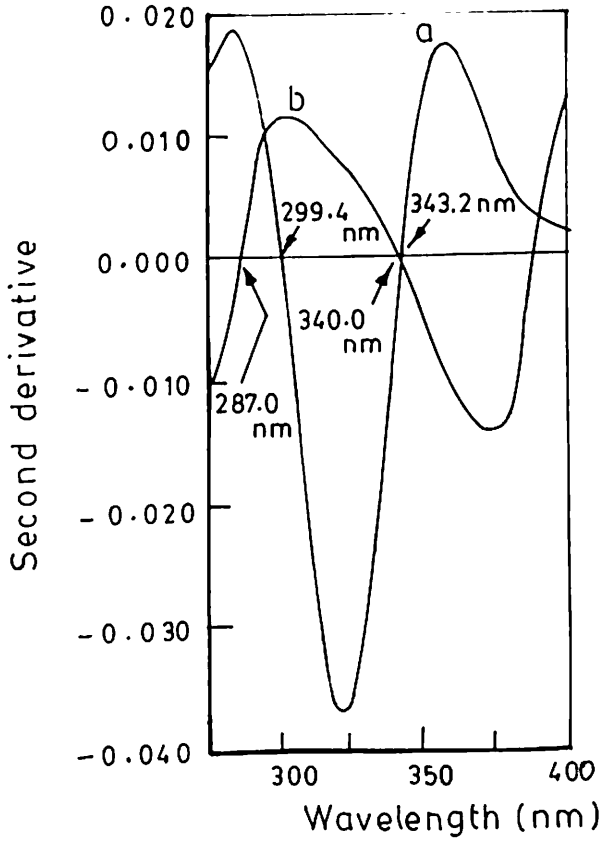

Figure 3.5 Second-derivative spectra of (a) tinidazole (15 µg/ml) and (b) furazolidone (5 µg/ml) in dimethyl formamide.

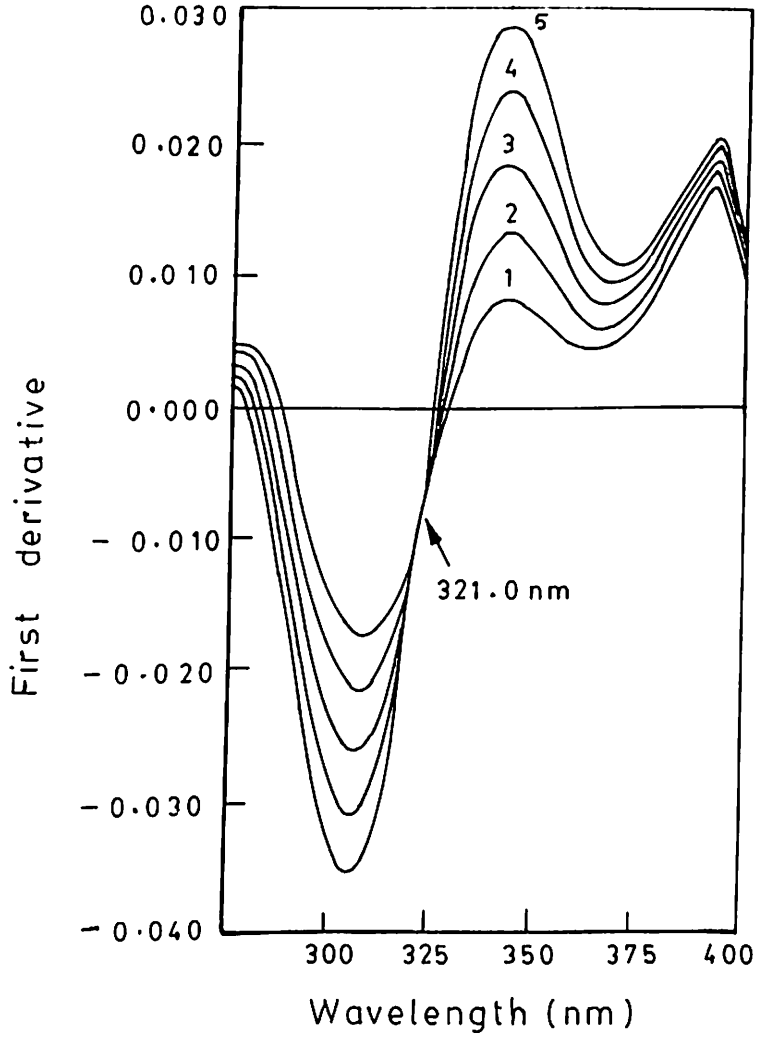

Figure 3.6 First-derivative spectra of tinidazole (10, 12.5, 15, 17.5 and 20 µg/ml) and furazolidone (5 µg/ml) in dimethyl formamide in curves 1-5, respectively.

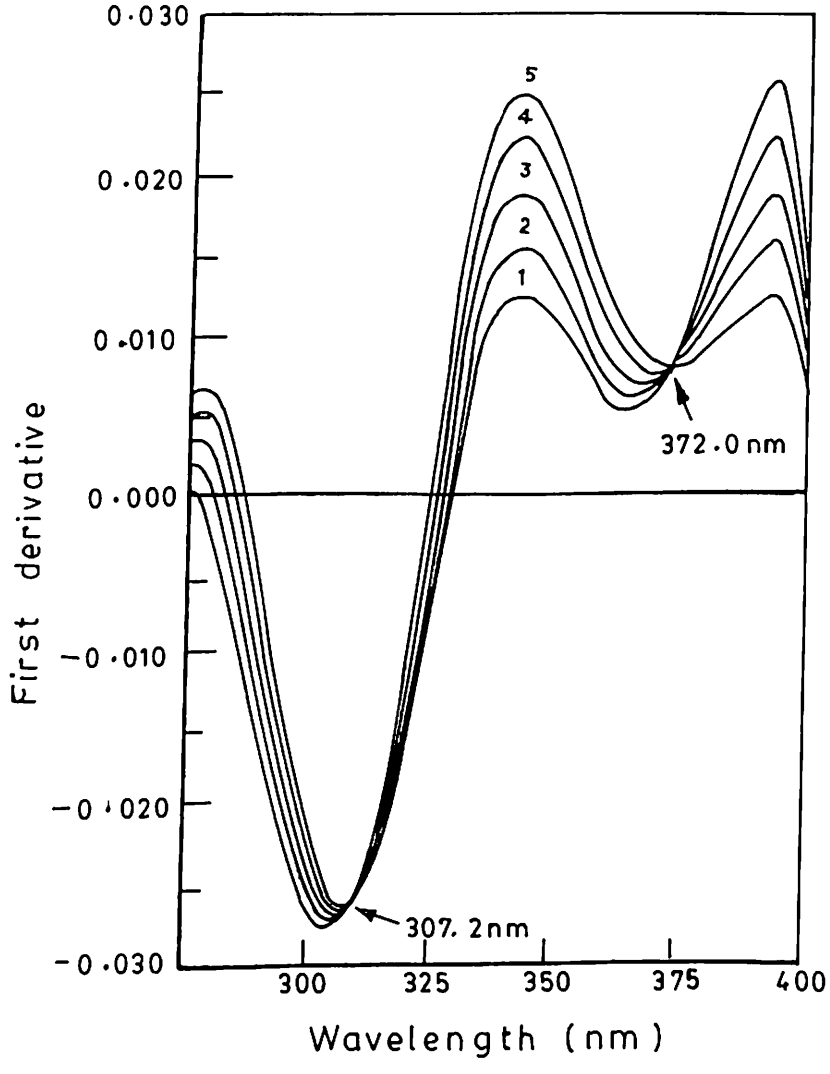

Figure 3.7 First-derivative spectra of furazolidone (3, 4, 5, 6 and 7 µg/ml) and tinidazole (15 µg/ml) in dimethyl formamide in curves 1-5, respectively.

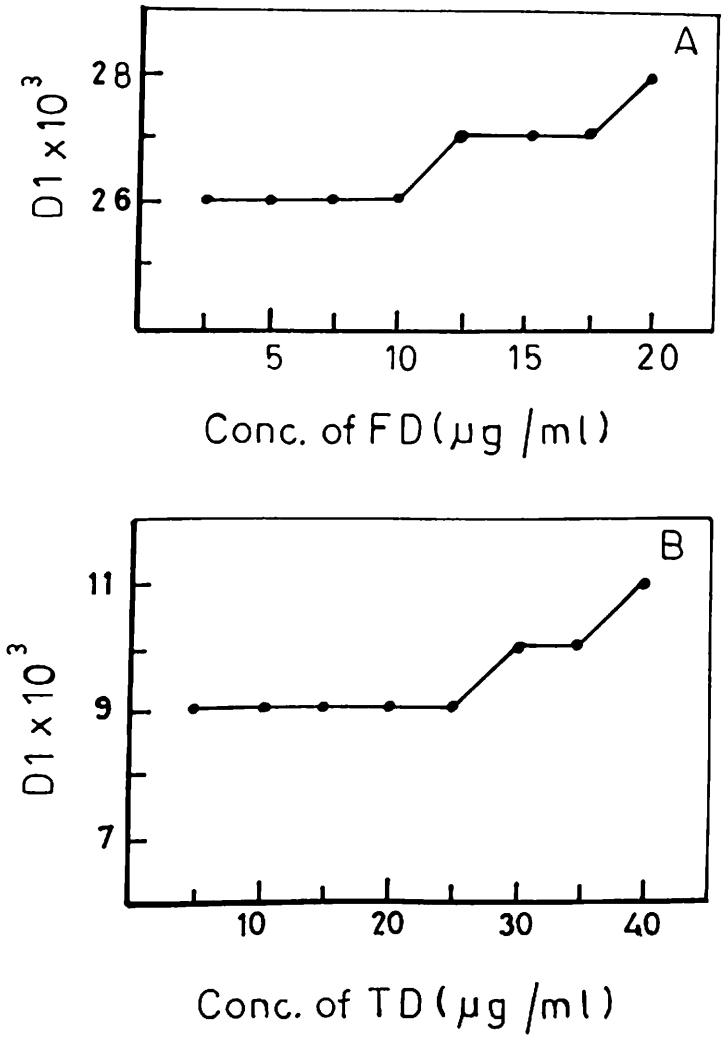

Figure 3.8 First-derivative interaction graphs for (A) tinidazole (15 µg/ml) in mixture with furazolidone (at 307.2 nm) and (B) furazolidone (5 µg/ml) in mixture with tinidazole (at 321.0 nm) in dimethyl formamide.

Table 3.1: Selectivity of the method for the simultaneous determination of TD and FD in standard solutions by first-derivative spectrophotometry

Composition of the solution (μg ml)		Mean absorbance [a] value (D1) (TD at 307.2 nm, FD at 321.0 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
TD	FD					Calc	Crit [b]
10.0	0	0.0179 ± 0.0005	2.59	0.0001	100.89	0.15	3.86
12.5	0	0.0223 ± 0.0005	2.16	0.0001	99.10	0.14	
15.0	0	0.0261 ± 0.0005	2.17	0.0002	100.15	0.10	
17.5	0	0.0303 ± 0.0005	1.59	0.0001	99.60	0.13	
20.0	0	0.0341 ± 0.0007	2.16	0.0002	100.35	0.05	
10.0	5	0.0184 ± 0.0002	1.48	0.0001	99.90	0.01	
12.5	5	0.0222 ± 0.0004	1.99	0.0001	100.03	0.01	
15.0	5	0.0262 ± 0.0005	1.85	0.0001	100.16	0.01	
17.5	5	0.0303 ± 0.0004	1.40	0.0001	99.89	0.01	
20.0	5	0.0343 ± 0.0004	1.28	0.0001	100.00	0.01	
0	3	0.0049 ± 0.0001	2.89	0.0001	99.35	0.08	3.86
0	4	0.0069 ± 0.0002	2.84	0.0001	100.71	0.05	
0	5	0.0090 ± 0.0003	2.88	0.0001	100.11	0.03	
0	6	0.0109 ± 0.0003	2.54	0.0001	99.64	0.02	
0	7	0.0130 ± 0.0004	2.77	0.0001	100.09	0.02	
15.0	3	0.0049 ± 0.0001	2.80	0.0001	100.20	0.27	
15.0	4	0.0069 ± 0.0002	2.80	0.0001	100.05	0.13	
15.0	5	0.0091 ± 0.0003	2.85	0.0001	99.21	0.08	
15.0	6	0.0109 ± 0.0003	2.76	0.0001	100.84	0.05	
15.0	7	0.0131 ± 0.0003	1.91	0.0001	99.74	0.08	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 3.2: Selectivity of the method for the determination of TD in presence of FD in standard solutions by first- and second-derivative ratio-compensation method

Composition of solution (μg/ml)		Ratio	Mean ratio [a] absorbance value	Standard error	Coefficient of variation (%)
TD	FD				
10.0	0	D1(301) / D1(342)	0.833± 0.004	0.0012	0.45
12.5	0		0.834± 0.008	0.0026	1.00
15.0	0		0.832± 0.004	0.0014	0.53
17.5	0		0.833± 0.005	0.0016	0.62
20.0	0		0.834± 0.007	0.0022	0.85
-	-		0.833± 0.001 *	0.0005	0.12
15.0	5		0.833± 0.004	0.0012	0.44
10.0	0	D2(359) / D2(323)	0.471± 0.008	0.0025	1.71
12.5	0		0.474± 0.006	0.0020	1.36
15.0	0		0.469 ± 0.006	0.0018	1.20
17.5	0		0.471 ± 0.005	0.0017	1.12
20.0	0		0.472 ± 0.005	0.0015	1.02
-	-		0.471 ± 0.002 *	0.0008	0.36
15.0	5		0.472 ± 0.002	0.0007	0.43
10.0	0	D2(359) / D1(342)	0.503 ± 0.009	0.0029	1.83
12.5	0		0.501 ± 0.005	0.0016	1.02
15.0	0		0.501 ± 0.009	0.0031	1.97
17.5	0		0.504 ± 0.006	0.0020	1.26
20.0	0		0.503 ± 0.001	0.0037	2.31
-	-		0.502 ± 0.002 *	0.0008	0.31
15.0	5		0.502 ± 0.008	0.0023	1.45

[a] : Average of ten replicate determinations with standard deviation.

* : Average ratio of pure drug solutions.

Table 3.3: Selectivity of the method for the determination of FD in presence of TD in standard solutions by first- and second-derivative ratio-compensation method.

Composition of solution (µg/ml)		Ratio	Mean ratio [a] absorbance value	Standard error	Coefficient of variation (%)
FD	TD				
3	0	D1(342) / D1(393)	0.823± 0.007	0.0023	0.88
4	0		0.822± 0.008	0.0026	1.02
5	0		0.821± 0.009	0.0031	1.19
6	0		0.824± 0.009	0.0027	1.04
7	0		0.822± 0.009	0.0029	1.11
-	-		0.822± 0.001 *	0.0005	0.12
5	15		0.822± 0.004	0.0012	0.46
3	0	D2(302) / D2(373)	0.786± 0.009	0.0028	1.12
4	0		0.785± 0.009	0.0029	1.17
5	0		0.784± 0.010	0.0034	1.35
6	0		0.785± 0.009	0.0029	1.18
7	0		0.786± 0.011	0.0036	1.46
-	-		0.785± 0.001 *	0.0004	0.12
5	15		0.785± 0.002	0.0007	0.28
3	0	D2(302) / D1(393)	0.664 ± 0.015	0.0047	2.25
4	0		0.667 ± 0.011	0.0035	1.65
5	0		0.663 ± 0.013	0.0041	1.93
6	0		0.665 ± 0.013	0.0041	1.98
7	0		0.663 ± 0.013	0.0042	2.02
-	-		0.664 ± 0.017 *	0.0008	0.26
5	15		0.665 ± 0.004	0.0012	0.60

[a] : Average of ten replicate determinations with standard deviation.

* : Average ratio of pure drug solutions.

Table 3.4: Regression analysis for the determination of TD and FD in standard solutions by first-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 307.2 nm for TD at 321.0 nm for FD)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t*-test for correlation	
	TD	FD			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	10 - 20	0	Y = 1.62E-03.X + 1.90E-03	0.9997	3.50E-04	2.27E-05	1.79E-04	7.86E-04, 3.01E-03	1.54E-03, 1.60E-03	1.71E-03	70	3.18
Series B	10 - 20	5	Y = 1.62E-03.X + 1.90E-03	0.9999	6.42E-05	4.17E-06	3.28E-05	1.69E-03, 2.10E-03	1.61E-03, 1.64E-03	1.72E-03	122	
Series C	0	3 - 7	Y = 2.05E-03.X - 1.24E-03	0.9999	6.83E-05	1.31E-05	4.16E-05	-1.46E-03, -1.03E-03	2.01E-03, 2.09E-03	1.88E-03	122	
Series D	15	3 - 7	Y = 2.04E-03.X - 1.23E-03	0.9998	1.16E-04	2.23E-05	7.05E-05	-1.60E-03, -8.63E-04	1.97E-03, 2.12E-03	1.88E-03	87	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 3.5: One-way ANOVA test for linearity of pure TD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	4.80E-04	4.80E-04	0.316	3.71
Lack of fit	3	5.76E-07	1.92E-07		
Within line	10	6.07E-06	6.07E-07		
Total	14	4.87E-04			

*: at P = 0.05 level of significance.

Table 3.6: One-way ANOVA test for linearity of pure FD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.20E-04	1.20E-04	0.053	3.71
Lack of fit	3	3.24E-08	1.08E-08		
Within line	10	2.04E-06	2.04E-07		
Total	14	1.22E-04			

*: at P = 0.05 level of significance.

Table 3.7: Validation report for the determination of TD and FD in standard solutions by first-derivative spectrophotometry

Analytical parameter	Results	
	TD (307.2 nm)	FD (321.0 nm)
Accuracy (%)	100.18 ± 0.85	99.55 ± 0.61
Precision (%)	99.61 99.61 99.61 100.53 101.53 RSD: 0.85	98.88 98.88 100.00 100.00 100.00 RSD: 0.61
Specificity	A 15.0 µg/ml of TD and 5.0 µg/ml of FD mixture solution will show an absorbance value (D1) of 0.026 ± 0.0005	A 5.0 µg/ml of FD and 15.0 µg/ml of TD mixture solution will show an absorbance value (D1) of 0.009 ± 0.0003
LOD (µg/ml)	0.33	0.06
LOQ (µg/ml)	1.10	0.20
Linearity (µg/ml)	5 - 30	2 - 20
Ruggedness (%)	100.18 ± 0.85	99.55 ± 0.61

Table 3.8: Results of the assay of pure drug admixtures and commercial formulation of TD and FD by first- and second-derivative spectrophotometry

Drug Name	Label Claim (mg/ tab.)	Recovery (%) [a]					F-test for Mean Recovery			
		ZCP Method		RC Method			Calc		Crit [b]	
							Samples	Methods	Samples†	Methods‡
TD		**D1 (307.2 nm)**	**D1(372.0 nm)**	**D1(301) / D1(342)**	**D2(359) / D2(323)**	**D2(359) / D1(342)**				
Pure drug admixture	-	99.67 ± 1.01	99.67 ± 1.07	99.89 ± 0.31	100.06 ± 0.32	99.89 ± 0.53	3.46	2.13	7.71	6.39
Brand CX	300	99.78 ± 0.97	99.55 ± 0.85	99.49 ± 0.45	99.85 ± 0.39	99.76 ± 0.44				
FD		**D1(321.0 nm)**		**D1(342) / D1 (393)**	**D2(302) / D2(373)**	**D2 (302) / D1(393)**				
Pure drug admixture	-	99.77 ± 0.93		99.81 ± 0.33	99.97 ± 0.21	99.97 ± 0.41	6.9E-04	2.18	10.12	9.28
Brand CX	100	99.84 ± 0.92		99.71 ± 0.45	100.19 ± 0.91	99.76 ± 0.99				

[a]: Mean and standard deviation for six determinations.

[b]: Theoretical value of $F(1,4)^{\dagger}$, $F(4,4)^{\ddagger}$ for TD and $F(1,3)^{\dagger}$, $F(3,3)^{\ddagger}$ for FD based on two-way ANOVA test at P = 0.05 level of significance.

3.4 SIMULTANEOUS DETERMINATION OF TINIDAZOLE AND CLOTRIMAZOLE IN TABLET PREPARATIONS BY THIRD-DERIVATIVE SPECTROPHOTOMETRY

The combination of tinidazole (TD) and clotrimazole (CMZ) as a tablet preparation is widely used for vaginitis. As mentioned earlier, there are various reports available for determination of TD as a single preparation [297-304] and in combination of other drugs [60,61,191,308-320]. The report related to TD in combination with CMZ employed HPLC method for their determination from tablet preparation [311]. The reports available for the individual assay of CMZ includes colorimetric [324,325], TLC [326] and HPLC method [327]. The I.P. 1996 described a titrimetric method for tinidazole as pure drug and ultraviolet method for its determination from solid dosage forms. The method of determination for CMZ includes HPLC prescribed by U.S.P. 23 for pure drug and for its dosage forms. While, B.P. 1993 and I.P. 1996 both recommended titrimetric and HPLC method for its pure and dosage forms respectively. The objective of this thesis work was to demonstrate two derivative spectrophotometric methods for simultaneous determination of these drugs without prior separation from their laboratory mixtures and a commercial preparation.

Materials and Reagents

Tinidazole (Gufic Ltd., India) and clotrimazole (Franco-Indian, India) were obtained as gift samples. Sodium hydroxide (Qualigens, India) and methanol (Qualigens, India) of analytical grade were used.

Standard Solutions

The standard solutions of TD and CMZ were prepared separately by dissolving 5 mg of pure drug in 10 and 25 ml methanol, respectively. Appropriate amounts of the stock solution were transferred to 10 ml volumetric flasks. The volumes were made up with 0.01M NaOH to give a series of solutions containing 5-15 μg/ml of TD and 2-6 μg/ml of CMZ. A three series of 10 ml mixtures of TD and CMZ in 0.01M NaOH were

prepared from the stock solutions. The first series contained a constant concentration of CMZ (5 µg/ml) and a varying concentration of TD (5-15 µg/ml). Similarly, the second series contained a constant concentration of TD (12.5 µg/ml) and a varying concentration of CMZ (2-6 µg/ml). The final series contained a constant concentration of TD (12.5 µg/ml) and CMZ (5 µg/ml).

Interaction Study

Two separate series of mixture solutions were prepared from fresh stock solutions according to the procedures mentioned above. The first series contained a constant concentration of TD (12.5 µg/ml) and a varying concentration of CMZ (2-14 µg/ml). While, the second series contained a constant concentration of CMZ (5 µg/ml) and a varying concentration of TD (10-60 µg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate TD (12.5 µg/ml) and CMZ (5 µg/ml) standard and test samples were prepared from fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of TD (5-80 µg/ml) and CMZ (1-15 µg/ml) were prepared from the stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing TD (12.5 µg/ml) and CMZ (5 µg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of TD (Brand DX) in combination with CMZ were accurately weighed, well powdered, and a powder weight equivalent to 12.5 mg of TD (and 5 mg of CMZ) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The sample was filtered through Whatman filter paper No. 1. The

first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate was diluted with 0.01M NaOH to give samples with a concentration of 12.5 μg/ml TD and 5 μg ml CMZ.

Procedure

Zero-crossing point (ZCP) method

The absorbances of sample and standard solutions of TD and CMZ were recorded from 220-300 nm against a blank solution. The third-derivative absorption spectra for each set of solutions were subsequently recorded using Δ = 60 points [138] and thereafter the spectra were smoothened using Δ = 30 points [138] to improve signal to noise ratio. The solutions were measured at the zero-crossing wavelength of the other drug.

Ratio-compensation (RC) method

The sample cell contained the mixture of sample and standard solutions containing 12.5 μg/ml of TD and 5 μg/ml of CMZ and the reference cell contained a series of standard solutions (TD/CMZ) with different concentrations. The third-derivative spectra were recorded in each instance using appropriate set parameters mentioned in ZCP method. Similarly, the third-derivative spectra of pure drug solutions were recorded against a blank in each instance. Different ratios such as wavelength maxima to wavelength minima or vice versa (as mentioned in Table 3.10) for the pure drugs and the mixture were calculated. At the exact balance point the concentration of one of the analyte components of a mixture become equal to that of the reference solution and therefore the calculated ratio at such point should be equal to that of pure drug other than in the reference cell. The procedure was repeated to obtain the second analyte concentration from the sample and standard mixture.

Results and Discussion

The zero-order spectra of pure drug combinations were found to be overlapping (Figure 3.9). The absence of well defined wavelength maxima in the range of spectral measurement did not allow use of any of the conventional methods [5-13] discussed earlier. The previously adopted pH-induced differential spectrophotometric method could not be utilised due to incompatibility of CMZ in presence of acidic buffers. The alkaline solutions prepared were of a lower strength of 0.01M, as the drug was observed to degrade slowly in presence of higher alkaline solutions. Hence, a derivative spectrophotometric method was considered to resolve the overlapping. But such overlapping of spectra could not be resolved in first- and second-derivative mode. In addition, the spectra lack significant minima for graphical measurements and also ideal zero-crossing wavelengths in both derivative modes. Thus, a third-derivative spectra was considered to be essential for resolving such overlapping due to the presence of ZCP's (Figure 3.10). For the reasons discussed in previous experiment, the zero-crossing method [110] was also compared with a ratio-compensation method [128,129]. The third-derivative spectra were found to have ZCP's at 267.6 nm for TD and for CMZ at 246.0 nm (Figure 3.10). Thus, the zero-crossing points (ZCP's) considered for the determinations were at 246.0 and 267.6 nm for TD and CMZ respectively.

The proportionality of the derivative values with different concentrations of TD and CMZ were examined by measuring a series of pure drug (Table 3.9, Series A and C of Table 3.11) and mixture solutions (Table 3.9, Series B and D of Table 3.11) at the zero-crossing wavelength of the other drug. The regression equations obtained with the reported mean derivative absorbance values (Table 3.9) for pure drugs and their mixtures were shown in Table 3.11. The third-derivative spectra recorded for the mixture solutions (Series B and D of Table 3.11) were presented in Figures 3.11 and 3.12. The presence of distinct isosbestic point at 267.6 nm (Figure 3.11) and at 246.0 nm (Figure 3.12) suggested no interferences in the estimation of TD and CMZ.

It was also confirmed that absorbance values were proportional to their concentrations in drug mixtures. The mutual independence of the absorbance value of TD and CMZ at their wavelength of estimation was observed by a mixture interaction study. It was suggested from reported results (Figure 3.13) that the varying concentrations of CMZ (2-14 μg/ml) did not interfere up to 9 μg/ml in the estimation of TD (12.5 μg/ml) at 246.0 nm (Figure 3.13A). While, the solutions containing varying concentrations of TD (10-60 μg/ml) did not interfere up to 40 μg/ml in the estimation of CMZ (5 μg/ml) at 267.6 nm (Figure 3.13B). Thus, it proved the proposed concentration range for standard solutions was ideal and the accurate determination of the two drugs would be possible at the suggested concentration ratio (i.e. 12.5 μg/ml of TD and 5 μg ml of CMZ).

In ratio-compensation method, the third-derivative spectra for each pure drug solution was recorded against its blank solution and also against its mixture solution to identify the desired balance point. The different ratios calculated between wavelength maxima to wavelength minima or vice versa were reported in Table 3.10.

The statistical analysis of the analytical data achieved by zero-crossing method for standard solutions suggested that the reported mean derivative absorbance values obtained for the replicates together with standard deviation, coefficient of variation and standard error were relatively small and thus indicated the high precision of the method. The reported percentage ratio of residuals showed a deviation of within 1% (Table 3.9). However, the calculated values of F-test for non-linearity [97] were less than the theoretical value at 5% significance level demonstrated that the calibration points did not deviate significantly from the best-fit line.

Similarly, the mean ratio absorbance values reported in Table 3.10 showed that the ratios obtained for mixture solutions at balance point were similar to that of mean value obtained for pure drug solutions. The deviations reported with observed ratios

were relatively small and the coefficient of variation associated with readings was less than 1% indicating the good reproducibility of the proposed RC method.

The regression equations together with correlation coefficients, standard error associated with each intercept, slope and estimate were reported in Table 3.11. The similarity observed between regression equations of pure drugs and mixtures suggested that there were no interferences in the estimation of one drug in presence of the other. A one-way ANOVA test [98] was conducted by considering three replicates per calibration point. The values considered were the lowest and highest variation observed from the mean absorbance value of each concentration during the replicate measurement of pure drug solutions (Table 3.9). The reported F-values were less than the critical value at 5% significance level suggested that the variance was uniform throughout the calibration line (Tables 3.12 and 3.13).

The ideal confidence limits of slope with zero intercept on the ordinate for both pure drugs and mixtures was found at 99.8% (Slope values of 1.93E-03 to 2.07E-03 for TD solutions, 2.69E-03 to 3.36E-03 and 2.74E-03 to 3.27E-03 for CMZ pure and mixture, respectively) instead of the widely reported 95% confidence limits (Table 3.11). This particular contrast was mainly due to lack of significant change in absorbance values with concentrations (only a third decimal change was noticed). However, the calculated values of Student's *t*-test [98] being significantly higher than the theoretical value (Table 3.11) at 5% level suggested the positive correlation established between the measured values and concentrations.

The proposed method was validated according to the procedures specified by U.S.P. 23 and the results were reported in Table 3.14. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of the regression equations obtained in Series A and C of Table 3.11.

The percentage recovery values obtained for pure drug admixtures as well as for the commercial preparation achieved by both the methods were reported in Table 3.15. It was clear from the obtained values that they meet the official monograph requirements for these drugs as single constituent preparations (Appendix B). The reported values of a Student's *t*-test (two-sided) [98] did not exceed the theoretical value at 5% level indicates no significant differences within the methods (Table 3.15). Further, a one-way ANOVA test [98] confirmed that there were no significant differences between the methods in recovery of these drugs as the calculated F-values were reportedly less than the theoretical value at 5% significance level. The prepared standard solutions were stable for 3 hrs in 0.01M NaOH.

Thus, the proposed derivative methods were found to be accurate and precise, and easier compared to the reported method [311] Therefore, it makes convenient for the simultaneous estimation of these drugs in routine analysis.

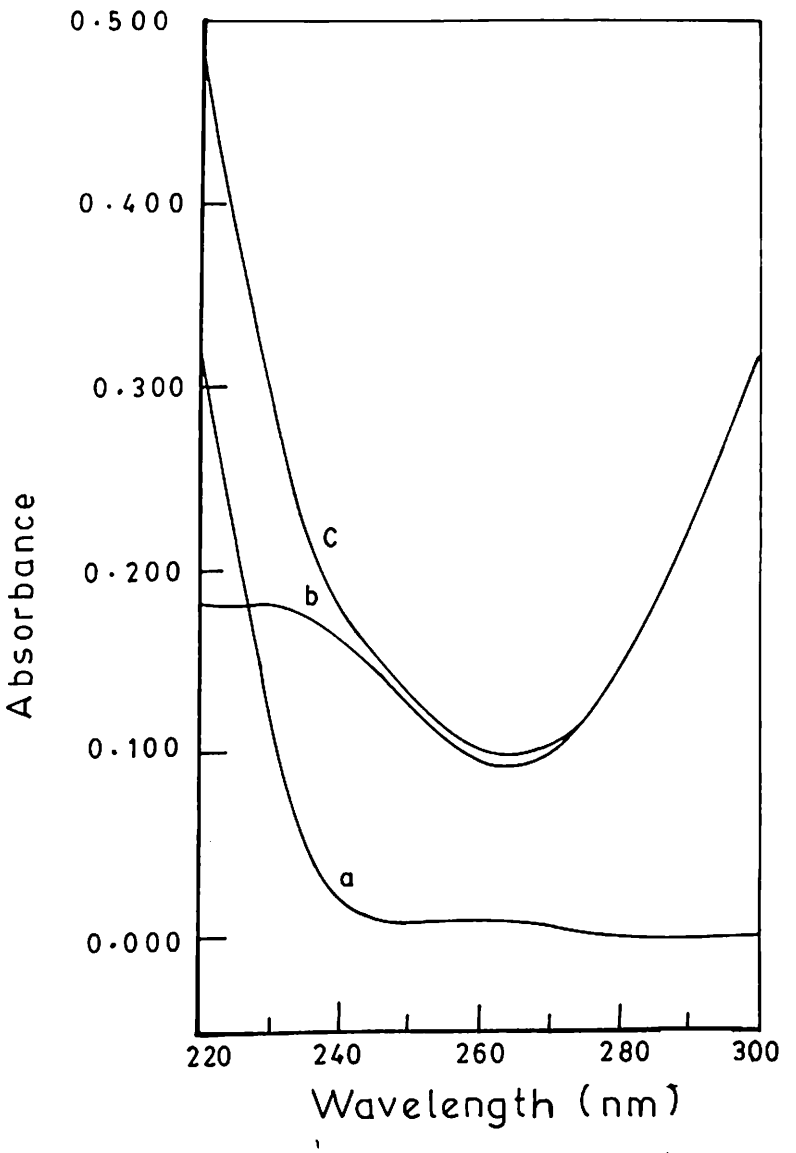

Figure 3.9 Absorption spectra of (a) clotrimazole (5 µg/ml), (b) tinidazole (12.5 µg/ml) and (c) their mixture in 0.01M NaOH.

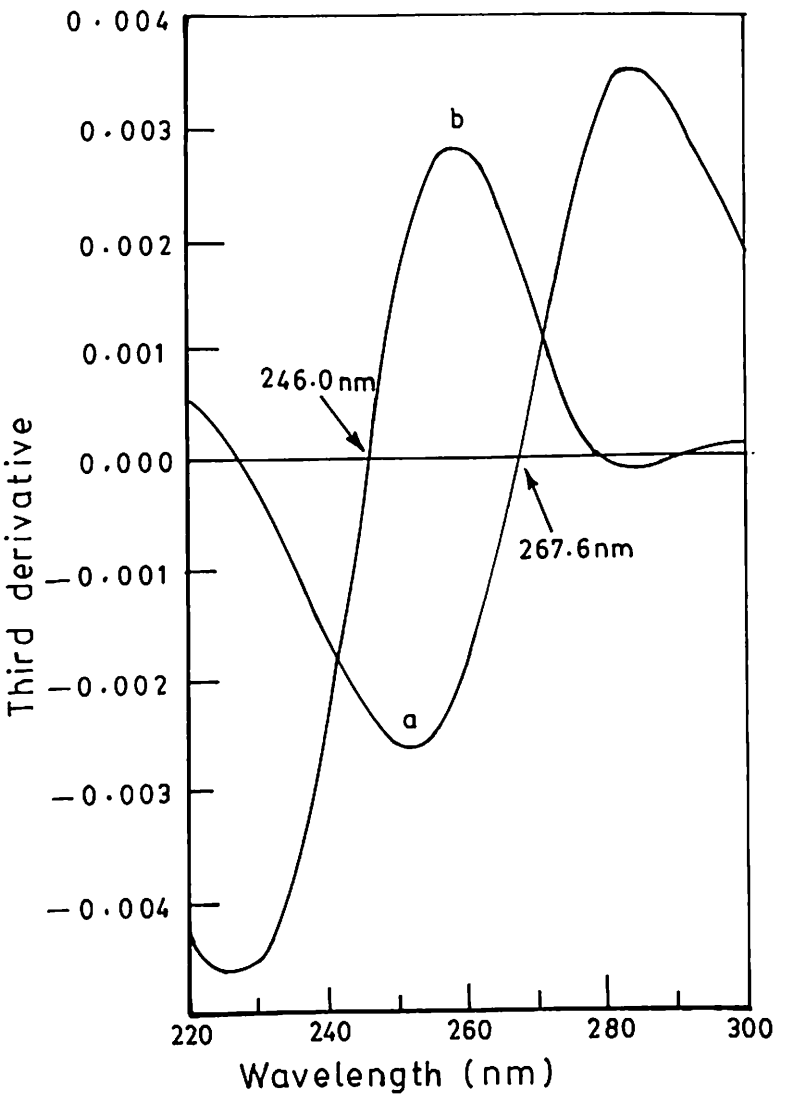

Figure 3.10 Third-derivative spectra of (a) tinidazole (12.5 µg/ml) and (b) clotrimazole (5 µg/ml) in 0.01M NaOH.

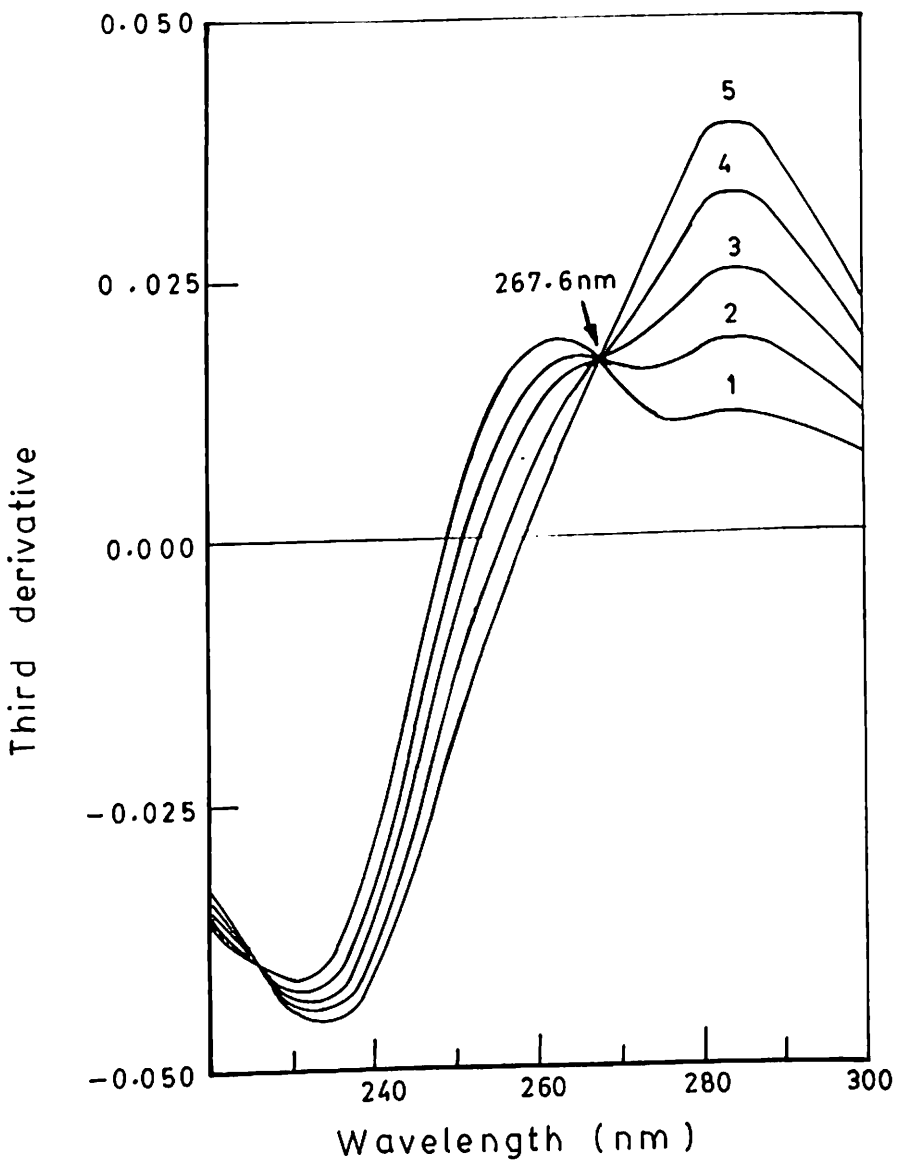

Figure 3.11 Third-derivative spectra of tinidazole (5, 7.5, 10, 12.5 and 15 μg/ml) and clotrimazole (5 μg/ml) in 0.01M NaOH in curves 1-5, respectively.

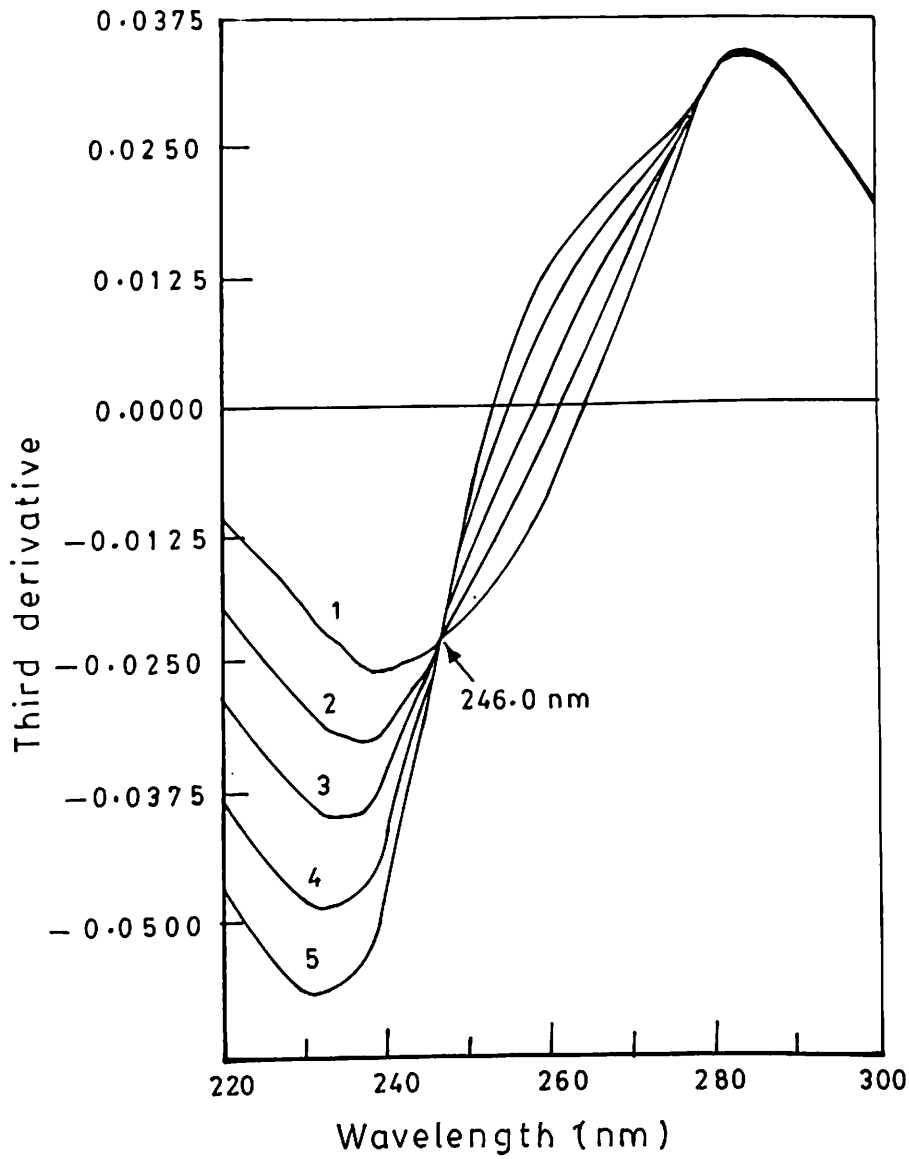

Figure 3.12 Third-derivative spectra of clotrimazole (2, 3, 4, 5 and 6 μg/ml) and tinidazole (12.5 μg/ml) in 0.01M NaOH in curves 1-5, respectively.

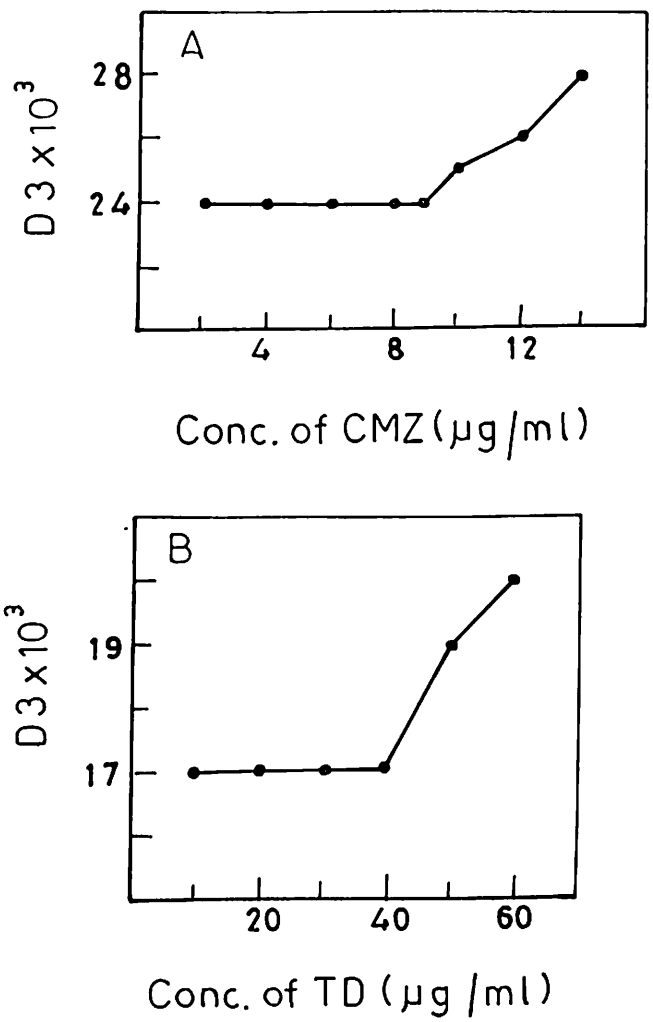

Figure 3.13 Third-derivative interaction graphs for (A) tinidazole (12.5 µg/ml) in mixture with clotrimazole (at 246.0 nm) and (B) clotrimazole (5 µg/ml) in mixture with tinidazole (at 267.6 nm) in 0.01M NaOH.

Table 3.9: Selectivity of the method for the simultaneous determination of TD and CMZ in standard solutions by third-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D3) (TD at 246.0 nm, CMZ at 267.6 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
TD	CMZ					Calc	Crit [b]
5.0	0	0.0089 ± 0.0001	1.39	0.0001	99.68	0.23	3.86
7.5	0	0.0139 ± 0.0002	1.65	0.0001	99.96	0.07	
10.0	0	0.0189 ± 0.0004	2.27	0.0001	100.15	0.02	
12.5	0	0.0239 ± 0.0003	1.28	0.0001	100.30	0.04	
15.0	0	0.0290 ± 0.0004	1.84	0.0001	99.77	0.01	
5.0	5	0.0089 ± 0.0002	2.78	0.0001	99.84	0.05	
7.5	5	0.0140 ± 0.0003	2.46	0.0001	99.73	0.02	
10.0	5	0.0189 ± 0.0004	2.14	0.0001	100.41	0.02	
12.5	5	0.0239 ± 0.0003	1.59	0.0001	100.05	0.02	
15.0	5	0.0290 ± 0.0004	1.38	0.0001	99.86	0.02	
0	2	0.0078 ± 0.0002	2.34	0.0001	100.36	0.32	3.86
0	3	0.0108 ± 0.0002	1.99	0.0001	100.21	0.23	
0	4	0.0139 ± 0.0002	2.09	0.0001	99.84	0.13	
0	5	0.0170 ± 0.0003	1.98	0.0001	99.19	0.09	
0	6	0.0198 ± 0.0004	1.93	0.0001	100.54	0.07	
12.5	2	0.0078 ± 0.0002	2.70	0.0001	100.48	0.13	
12.5	3	0.0108 ± 0.0002	2.61	0.0001	100.23	0.07	
12.5	4	0.0140 ± 0.0002	2.11	0.0001	99.30	0.06	
12.5	5	0.0169 ± 0.0003	2.10	0.0001	99.81	0.04	
12.5	6	0.0198 ± 0.0003	1.81	0.0001	100.33	0.04	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 3.10: Selectivity of the method for the determination of TD and CMZ in standard solutions by third-derivative ratio-compensation method

Composition of solution (μg/ml)		Mean ratio absorbance value [a]		Standard error	Coefficient of variation (%)
TD	CMZ	D3(252) / D3(285)	D3(258) / D3(229)		
5.0	0	0.786 ± 0.002	-	0.0008	0.31
7.5	0	0.787 ± 0.006	-	0.0019	0.79
10.0	0	0.786 ± 0.005	-	0.0016	0.66
12.5	0	0.787 ± 0.005	-	0.0014	0.57
15.0	0	0.788 ± 0.004	-	0.0012	0.51
-	-	0.787 ± 0.001 *	-	0.0004	0.13
12.5	5	0.787 ± 0.001	-	0.0004	0.16
0	2	-	0.596 ± 0.003	0.0011	0.58
0	3	-	0.596 ± 0.003	0.0008	0.46
0	4	-	0.597 ± 0.002	0.0007	0.38
0	5	-	0.597 ± 0.004	0.0014	0.75
0	6	-	0.596 ± 0.004	0.0014	0.75
-	-	-	0.596 ± 0.001 *	0.0003	0.10
12.5	5	-	0.597 ± 0.004	0.0011	0.61

[a] : Average of ten replicate determinations with standard deviation.

* : Average ratio of pure drug solutions.

Table 3.11: Regression analysis for the determination of TD and CMZ in standard solutions by third-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 246.0 nm for TD at 267.6 nm for CMZ)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t*-test for correlation	
	TD	CMZ			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	5 - 15	0	Y = 2.00E-03.X - 1.12E-03	0.9999	8.07E-05	7.61E-06	6.05E-05	-1.38E-03, -8.67E-04	1.98E-03, 2.03E-03	1.94E-03	122	3.18
Series B	5 - 15	5	Y = 2.00E-03.X - 1.04E-03	0.9999	7.53E-05	7.10E-06	5.63E-05	-1.28E-03, -7.96E-04	1.98E-03, 2.02E-03	1.94E-03	122	
Series C	0	2 - 6	Y = 3.02E-03.X - 1.77E-03	0.9998	1.39E-04	3.27E-05	1.03E-04	1.34E-03, 2.22E-03	2.92E-03, 3.13E-03	3.28E-03	87	
Series D	12.5	2 - 6	Y = 3.00E-03.X - 1.86E-03	0.9999	1.00E-04	2.37E-05	7.53E-05	1.54E-03, 2.18E-03	2.93E-03, 3.08E-03	3.27E-03	122	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 3.12: One-way ANOVA test for linearity of pure TD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit}*
Regression	1	7.56E-04	7.56E-04	0.086	3.71
Lack of fit	3	7.35E-08	2.45E-08		
Within line	10	2.86E-06	2.86E-07		
Total	14	7.59E-04			

*: at P = 0.05 level of significance.

Table 3.13: One-way ANOVA test for linearity of pure CMZ solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit}*
Regression	1	2.78E-04	2.78E-04	0.046	3.71
Lack of fit	3	3.30E-08	1.10E-08		
Within line	10	2.40E-06	2.40E-07		
Total	14	2.80E-04			

*: at P = 0.05 level of significance.

Table 3.14: Validation report for the determination of TD and CMZ in standard solutions by third-derivative spectrophotometry

Analytical parameter	Results	
	TD (246.0 nm)	CMZ (267.6 nm)
Accuracy (%)	99.91 ± 0.68	100.23 ± 0.78
Precision (%)	99.16 99.58 99.58 100.41 100.83 RSD: 0.68	99.41 99.41 100.58 100.58 101.17 RSD: 0.78
Specificity	A 12.5 µg/ml of TD and 5.0 µg/ml of CMZ mixture solution will show an absorbance value (D3) of 0.024 ± 0.0003	A 5.0 µg/ml of CMZ and 12.5 µg/ml of TD mixture solution will show an absorbance value (D3) of 0.017 ± 0.0003
LOD (µg/ml)	0.09	0.10
LOQ (µg/ml)	0.30	0.34
Linearity (µg/ml)	5 - 80	1 - 15
Ruggedness (%)	99.91 ± 0.68	100.23 ± 0.78

Table 3.15: Results of the assay of pure drug admixtures and commercial formulation of TD and CMZ by third-derivative spectrophotometry

Drug Name	Label Claim (mg/ tab.)	Recovery (%) [a]		Student *t*- test for Mean Recovery			F-test for Mean Recovery	
		ZCP Method	RC Method	Calc ZCP Method	Calc RC Method	Crit [b]	Calc	Crit [c]
TD		**D3(246.0 nm)**	**D3(252) / D3(285)**					
Pure drug admixture	-	99.78± 1.05	100.06 ± 0.24	0.34	1.91	2.23	0.53	3.10
Brand DX	500	99.60 ± 0.80	99.60 ± 0.53					
CMZ		**D3(267.6 nm)**	**D3(258) / D3(229)**					
Pure drug admixture	-	99.89 ±1.08	100.13 ± 0.49	0.53	0.81	2.23	0.51	3.10
Brand DX	200	99.58 ± 0.98	99.94 ± 0.31					

[a] : Mean and standard deviation for six determinations.

[b] : Theoretical value of '*t* (two-sided)' at P = 0.05 level of significance with 10 d.f.

[c] : Theoretical value of F(3,20) based on one-way ANOVA test at P = 0.05 level of significance.

3.5 SIMULTANEOUS DETERMINATION OF TINIDAZOLE AND NORFLOXACIN IN COMBINED TABLET PREPARATIONS BY FIRST-DERIVATIVE SPECTROPHOTOMETRY

The combination of tinidazole (TD) and norfloxacin (NF) as a tablet preparation is used for diarrhoea of mixed origin. As mentioned earlier, there are various reports available for quantitative determination of TD as single preparation [297-304] and in combination of other drugs [60,61,191,308-320]. In particular, the reports related to NF combination employed HPLC [312,313], HPTLC [314], colorimetric [315,316] and multi-component analysis (MA) methods [317]. There are also few reports available for the individual assay of NF, which includes colorimetric [328,329], ultraviolet [330-332], fluorimetric [333,334], polarographic [335,336] and HPLC method [337]. The I.P. 1996 described titrimetric and ultraviolet method for TD as pure drug and from its solid dosage form respectively. A potentiometric titration and HPLC method was recommended by both U.S.P. 23 and I.P. 1996 for NF as pure drug and its determination from solid dosage form respectively. The objective of this thesis work was to demonstrate two derivative spectrophotometric methods for the simultaneous determination of these drugs without prior separation from pure drug admixtures and combined preparations.

Materials and Reagents

Tinidazole (Gufic Ltd., India) and norfloxacin (Cipla, India) were obtained as gift samples. Sodium hydroxide (Qualigens, India) and dimethyl formamide (DMF; Qualigens, India) used were of analytical grade.

Standard Solutions

The stock solutions of TD and NF were prepared separately by dissolving 5 mg of each pure drug in 10 ml DMF. Appropriate amounts of the stock solution were transferred to 10 ml volumetric flasks. The volumes were made up with 0.01M NaOH to give a separate series of solutions containing 2.5-10 μg/ml of TD and NF. Further, a

three series of 10 ml mixtures of TD and NF in 0.01M NaOH were prepared from the stock solutions. The first series contained constant concentration of NF (5 µg/ml) and a varying concentration of TD (2.5-10 µg/ml). Similarly, the second series contained constant concentration of TD (7.5 µg/ml) and a varying concentration of NF (2.5-10 µg/ml). Finally, the third series contained a constant concentration of TD (7.5 µg/ml) and NF (5 µg/ml).

Interaction Study

Two series of mixture solutions were prepared from fresh stock solutions according to the procedures mentioned above. The first series contained a constant concentration of NF (5 µg/ml) and a varying concentration of TD (5-45 µg/ml). Similarly, the second series contained a constant concentration of TD (7.5 µg/ml) and a varying concentration of NF (5-40 µg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of both TD (7.5 µg/ml) and NF (5 µg/ml) were prepared from the fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of TD (2.5-30 µg/ml) and NF (2.5-35 µg/ml) were prepared from the stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing TD (7.5 µg/ml) and NF (5 µg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of each brand (EX and EY) containing TD and NF were accurately weighed, well powdered, and a powder weight equivalent to 7.5 mg of TD

(and 5.0 mg of NF) was dissolved in DMF by thorough mixing and made up to volume in a 50 ml volumetric flask. The samples were filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with 0.01M NaOH to give samples with a concentration of 7.5 μg/ml TD and 5 μg/ml NF.

Procedure

Zero-crossing point (ZCP) method

The absorbances of sample and standard solutions of TD and NF were recorded from 250-360 nm against a blank solution. The first-derivative spectra for each set of solutions were subsequently recorded using $\Delta = 20$ points [138] and no smoothing was necessary. The absorbances of the solutions were measured at the zero-crossing wavelength of the other drug.

Ratio-compensation (RC) method

The sample cell contained the mixture of sample and standard solutions containing TD (7.5 μg/ml) and NF (5 μg/ml) and the reference cell contained a series of standard solutions (TD/NF) with different concentrations. The first-derivative spectra were recorded in each instance using appropriate set parameters mentioned in ZCP method. Similarly, the first-derivative spectra of pure drugs were recorded against a blank in each instance. Different ratios such as wavelength maxima to wavelength minima or vice versa (as mentioned in Table 3.17) for the pure drugs and mixtures were calculated. As discussed earlier, at the balance point the concentration of one of the analyte components of a mixture becomes equal to that of reference cell and ratios calculated should be equal at such point. The procedure was repeated to obtain the second analyte concentration from the sample and standard mixture.

Results and Discussion

The normal spectra of TD and NF were found to be greatly overlapping (Figure 3.14) with prominent shoulder peaks in the spectra of NF, which made their estimation difficult with the help of methods discussed earlier [5-13]. The previously proposed pH-induced differential spectrophotometric method could not be used due to lack of stability of NF in acidic buffer system. Hence, a first-derivative spectrophotometric method was considered to resolve such overlapping associated with shoulder peaks (Figure 3.15).

Though, the first-derivative spectra obtained for TD (7.5 μg/ml) and NF (5 μg/ml) was found to have ZCP's at 266.8 and 317.4 nm for TD and at 300.2 and 326.8 nm for NF (Figure 3.15), but, the derivative spectrum was not fully resolved from the shoulder peaks. However, this problem could have been resolved easily by processing the recorded spectra in higher derivative modes. Instead, the ratio-compensation method [128,129] was considered and compared with zero-crossing method [110] to study the possible effect, if any, of shoulder in the measurement of standard and sample solutions. Thus, in ZCP method, the measurements of TD were made at 326.8 nm and NF at 317.4 nm as both were closer to the shoulder peak.

The proportional relationship between the measured amplitudes and concentrations were determined at the selected zero-crossing wavelengths by measuring a series of pure drug (Table 3.16, Series A and C of Table 3.18) and mixture solutions (Table 3.16, Series B and D of Table 3.18). The regression equations obtained with the reported mean derivative absorbance values (Table 3.16) for pure drugs and their mixture solutions were presented in Table 3.18. The first-derivative spectra recorded for the mixture solutions were presented in Figures 3.16 and 3.17. The presence of distinct isosbestic points at 266.8 nm and 317.4 nm (Figure 3.16) and at 300.2 and 326.8 nm (Figure 3.17) suggested no interferences including shoulder peak in the

estimation of TD and NF. Further, it was also proved that the measured values obey a proportional relationship with the concentrations of standard mixtures.

In addition, the mutual independence of the absorbance values of TD and NF at the selected wavelength of their estimation was confirmed by a mixture interaction study. The reported results (Figure 3.18) clearly demonstrated that TD solutions with varying concentrations (5-45 µg/ml) did not interfere up to 25 µg/ml in the estimation of NF (5 µg/ml) at 317.4 nm (Figure 3.18A). While, the solutions containing varying concentrations of NF (5-40 µg/ml) did not interfere up to 10 µg/ml in the estimation of TD (7.5 µg/ml) at 326.8 nm (Figure 3.18B). Thus, it suggested the earlier identified concentration ranges for TD and NF were ideal to carry out the accurate determinations.

In ratio-compensation method, the first-derivative spectra for each pure drug solution was recorded against its blank solution and also against its mixture solution to identify the desired balance point. The different ratios calculated between wavelength maxima to wavelength minima or vice versa were reported in Table 3.17.

From the statistical analysis of data achieved by ZCP method for standard solutions showed that, the mean derivative absorbance values of standard solutions together with standard deviation, coefficient of variation and standard error were small and therefore indicated the precision of the method (Table 3.16). The percentage ratio of residual values showed random scatter about the calibration line. But, the values obtained through F-test for non-linearity [97] suggested that such random scatter was not significant at 5% level and the calibration points were linear in the proposed concentration range. From Table 3.17, it can clearly be observed that the reported ratios for pure drug solutions and their mixtures were highly reproducible. The similarity between the mean absorbance ratio value of pure drug solutions and the

mixture solutions at the balance point suggested no interferences in the estimation of both the drugs by the proposed RC method.

The linear regression equations together with correlation coefficients, standard error associated with each intercept, slope and estimate were reported in Table 3.18. It was clear from the similarity of regression equations (Table 3.18: Series A-D) that there were no interferences in the estimation of one drug in presence of the other. A one-way ANOVA test [98] by considering three replicates per calibration point was performed. These values included the lowest and highest variation observed from the mean derivative absorbance value of each concentration during the replicate measurement of pure drug solutions. It was proved from the results reported in Tables 3.19 and 3.20 that variations associated with measurements were uniform throughout the calibration line.

The reported slope values (Table 3.18) without intercept on ordinate fall within the 95% confidence limits, which confirms the regression equations did not deviate from the origin. Further, the strong positive correlation between the measured values and concentrations was also confirmed at 5% level by the calculated values of Student's *t*-test for correlation which were highly significant (Table 3.18). The precision of the fit by regression was also confirmed from the standard error of each intercept, slope and estimate which found to be very small (Table 3.18).

The proposed method was validated according to the procedures described in U.S.P. 23 and the results obtained were reported in Table 3.21. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equation obtained in Series A and C of Table 3.18.

The percentage recovery values obtained for standard solutions and for the two commercial preparations (EX and EY) by both ZCP and RC methods were reported in

Table 3.22. It was clear from the recovery values that they meet the official requirements for single drug preparations of both the pure drugs (Appendix B). The reported F-values of a two-way ANOVA test [98] did not exceed the theoretical value at 5% level suggested that no significant deviations occurred between the methods and within the samples of a method (Table 3.22). The prepared solutions were stable for 3 hrs in 0.01M NaOH and protected from light throughout the study.

Thus the proposed derivative methods offer interesting possibilities for resolving the overlapping spectra. These methods were found to be precise and accurate. The simplicity and rapidity of these methods compared to other reported methods [312-317] makes it convenient for routine analysis.

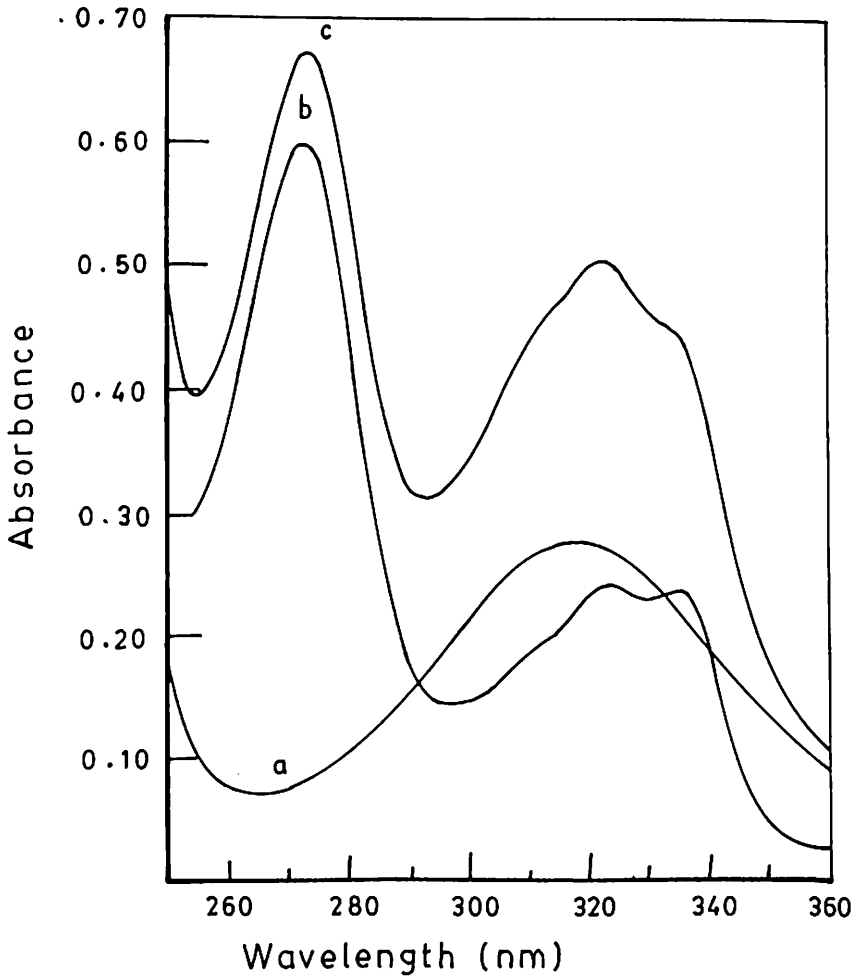

Figure 3.14 Absorption spectra of (a) tinidazole (7.5 µg/ml), (b) norfloxacin (5 µg/ml) and (c) their mixture in 0.01M NaOH.

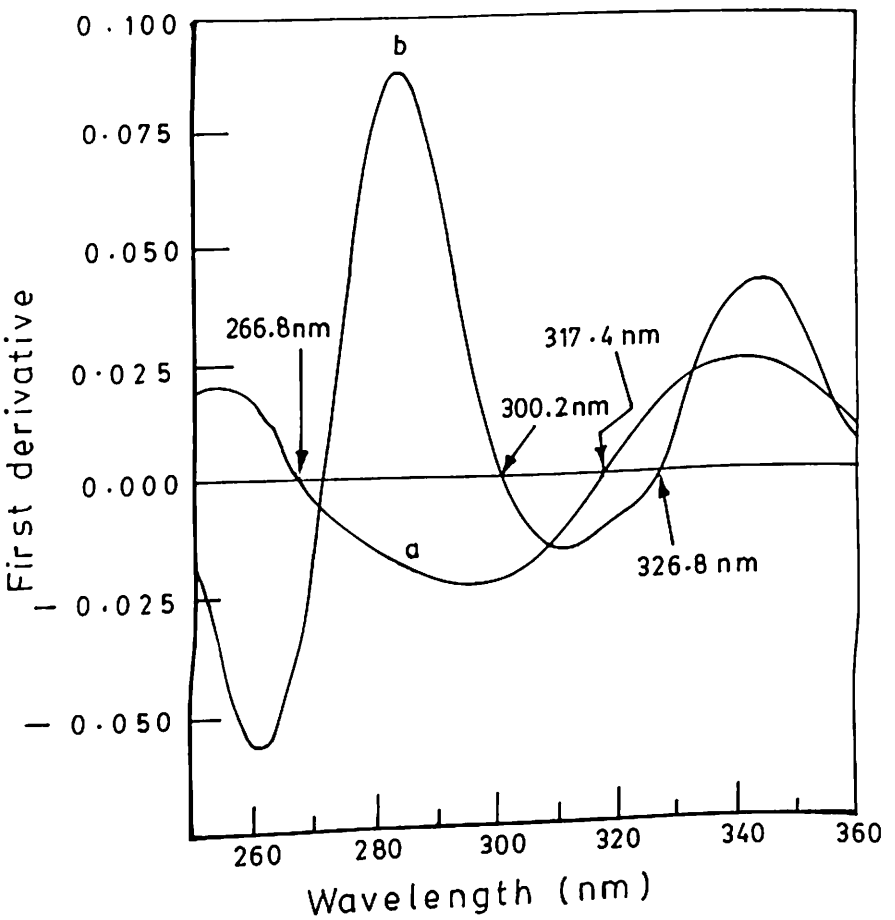

Figure 3.15 First-derivative spectra of (a) tinidazole (7.5 µg/ml) and (b) norfloxacin (5.0 µg/ml) in 0.01M NaOH.

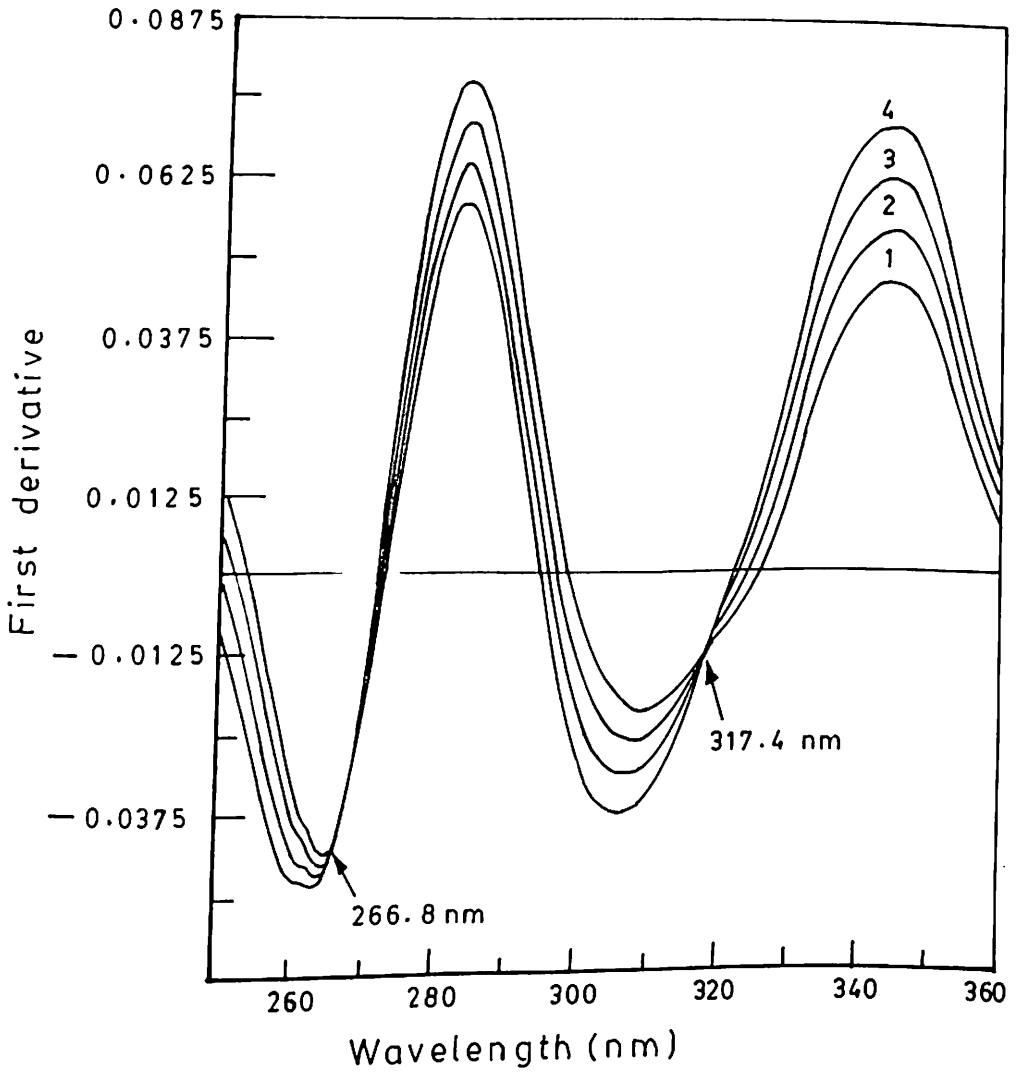

Figure 3.16 First-derivative spectra of tinidazole (2.5, 5, 7.5 and 10 µg/ml) and norfloxacin (5 µg/ml) in 0.01M NaOH in curves 1-4, respectively.

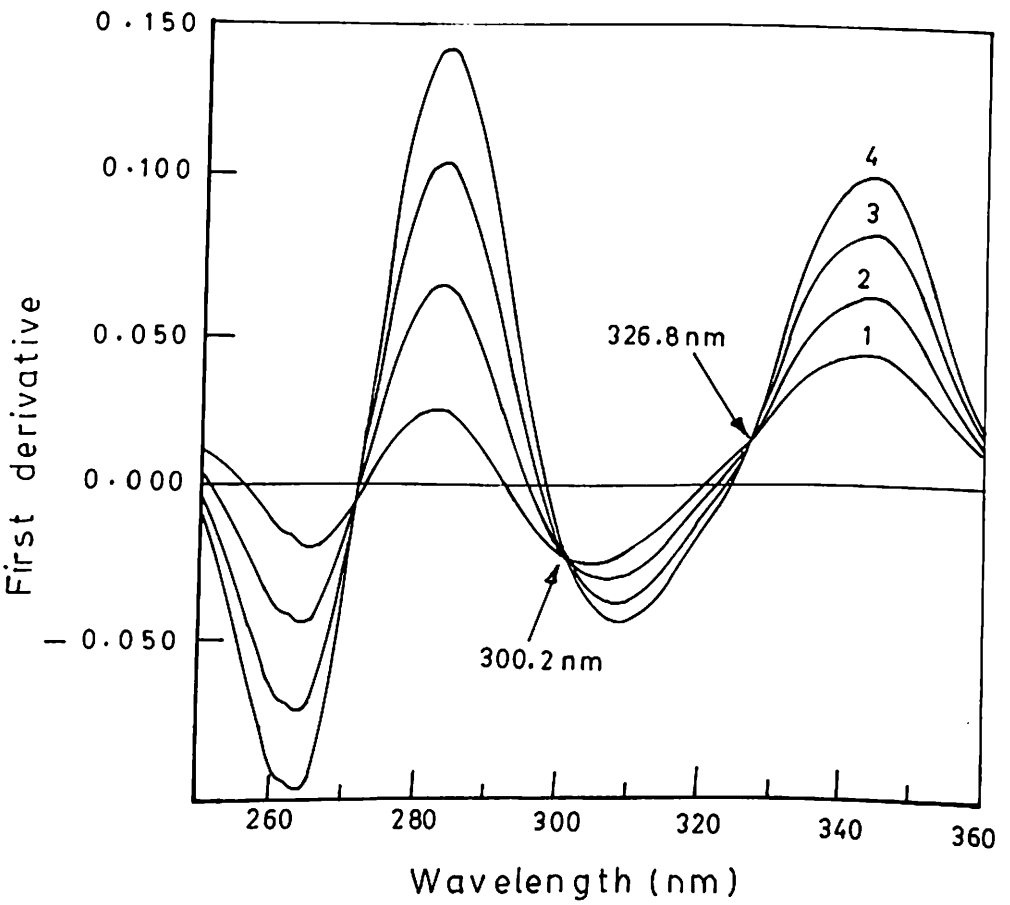

Figure 3.17 First-derivative spectra of norfloxacin (2.5, 5, 7.5 and 10 μg/ml) and tinidazole (7.5 μg/ml) in 0.01M NaOH in curves 1-4, respectively.

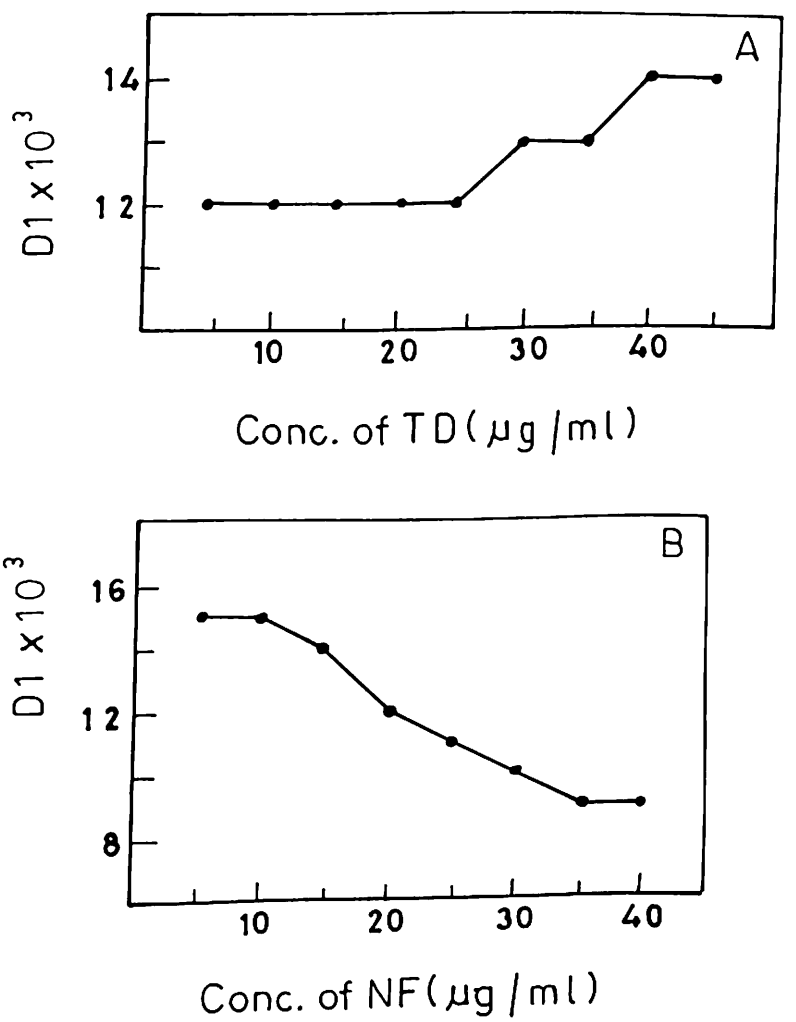

Figure 3.18 First-derivative interaction graphs for (A) norfloxacin (5 µg/ml) in mixture with tinidazole (at 317.4 nm) and (B) tinidazole (7.5 µg/ml) in mixture with norfloxacin (at 326.8 nm) in 0.01M NaOH.

Table 3.16: Selectivity of the method for the simultaneous determination of TD and NF in standard solutions by first-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D1) (TD at 326.8 nm, NF at 317.4 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
TD	NF					Calc	Crit [b]
2.5	0	0.0050 ± 0.0004	2.89	0.0001	100.64	0.11	4.26
5.0	0	0.0100 ± 0.0003	2.93	0.0001	99.44	0.03	
7.5	0	0.0149 ± 0.0003	2.58	0.0001	100.10	0.02	
10.0	0	0.0199 ± 0.0004	1.89	0.0004	100.04	0.02	
2.5	5.0	0.0051 ± 0.0002	2.98	0.0001	98.60	0.53	
5.0	5.0	0.0100 ± 0.0003	2.89	0.0001	101.32	0.15	
7.5	5.0	0.0151 ± 0.0004	2.34	0.0001	99.70	0.10	
10.0	5.0	0.0200 ± 0.0003	1.53	0.0001	99.94	0.13	
0	2.5	0.0062 ± 0.0002	2.98	0.0001	100.40	0.04	4.26
0	5.0	0.0122 ± 0.0002	2.11	0.0001	99.80	0.02	
0	7.5	0.0181 ± 0.0002	1.33	0.0001	99.86	0.02	
0	10.0	0.0241 ± 0.0003	1.18	0.0001	100.10	0.02	
7.5	2.5	0.0064 ± 0.0001	1.99	0.0001	100.94	0.37	
7.5	5.0	0.0125 ± 0.0001	1.27	0.0001	99.36	0.24	
7.5	7.5	0.0183 ± 0.0003	1.83	0.0001	99.89	0.05	
7.5	10.0	0.0242 ± 0.0002	1.08	0.0001	100.16	0.08	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(2,9) at P = 0.05 level of significance.

Table 3.17: Selectivity of the method for the determination of TD and NF in standard solutions by first-derivative ratio-compensation method

Composition of solution (µg/ml)		Mean ratio absorbance value [a]		Standard error	Coefficient of variation (%)
TD	NF	D1(342) / D1(296)	D1(311) / D1(344)		
2.5	0	1.092 ± 0.016	-	0.0051	1.47
5.0	0	1.084 ± 0.023	-	0.0074	2.17
7.5	0	1.089 ± 0.021	-	0.0068	1.97
10.0	0	1.087 ± 0.008	-	0.0026	0.76
-	-	1.088 ± 0.003 *	-	0.0016	0.30
7.5	5.0	1.089 ± 0.028	-	0.0088	2.57
0	2.5	-	0.422 ± 0.002	0.0005	0.38
0	5.0	-	0.423 ± 0.003	0.0012	0.88
0	7.5	-	0.424 ± 0.003	0.0008	0.58
0	10.0	-	0.426 ± 0.002	0.0005	0.38
-	-	-	0.424 ± 0.002 *	0.0008	0.37
7.5	5.0	-	0.424 ± 0.003	0.0009	0.70

[a] : Average of ten replicate determinations with standard deviation.

* : Average ratio of pure drug solutions.

Table 3.18: Regression analysis for the determination of TD and NF in standard solutions by first-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 326.8 nm for TD at 317.4 nm for NF)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* - test for correlation	
	TD	NF			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	2.5 -10	0	Y = 1.99E-03.X - 4.65E-09	0.9999	5.75E-05	8.40E-06	4.73E-05	-2.47E-04, 2.47E-04	1.95E-03, 2.03E-03	1.99E-03	100	4.30
Series B	2.5 -10	5	Y = 1.99E-03.X + 9.49E-05	0.9999	1.35E-04	1.98E-05	1.11E-04	-4.88E-04, 6.78E-04	1.91E-03, 2.08E-03	2.00E-03	100	
Series C	0	2.5 -10	Y = 2.38E-03.X + 2.75E-04	0.9999	4.33E-05	6.33E-06	3.53E-05	-2.60E-04, 6.56E-04	2.31E-03, 2.45E-03	2.40E-03	100	
Series D	7.5	2.5 -10	Y = 2.38E-03.X + 4.50E-04	0.9999	9.52E-05	1.39E-05	7.74E-05	-8.20E-06, 8.10E-04	2.33E-03, 2.44E-03	2.42E-03	100	

[a] : Based on four calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 2 d.f.

Table 3.19: One-way ANOVA test for linearity of pure TD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	3.75E-04	3.75E-04	1.74E-04	4.46
Lack of fit	2	9.33E-11	4.66E-11		
Within line	8	2.14E-06	2.67E-07		
Total	11	3.77E-04			

*: at P = 0.05 level of significance.

Table 3.20: One-way ANOVA test for linearity of pure NF solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	5.31E-04	5.31E-04	0.185	4.46
Lack of fit	2	4.35E-08	2.17E-08		
Within line	8	9.40E-07	1.17E-07		
Total	11	5.32E-04			

*: at P = 0.05 level of significance.

Table 3.21: Validation report for the determination of TD and NF in standard solutions by first-derivative spectrophotometry

Analytical parameter	Results	
	TD (326.8 nm)	NF (317.4 nm)
Accuracy (%)	99.91 ± 0.70	100.16 ± 0.67
Precision (%)	99.33 99.33 99.53 100.67 100.67 RSD: 0.70	99.20 100.00 100.00 100.80 100.80 RSD: 0.67
Specificity	A 7.5 µg/ml of TD and 5.0 µg/ml of NF mixture solution will show an absorbance value (D1) of 0.015 ± 0.0004	A 5.0 µg/ml of NF and 7.5 µg/ml of TD mixture solution will show an absorbance value (D1) of 0.012 ± 0.0001
LOD (µg/ml)	0.07	0.05
LOQ (µg/ml)	0.24	0.15
Linearity (µg/ml)	2.5 - 30	2.5 - 35
Ruggedness (%)	99.91 ± 0.70	100.16 ± 0.67

Table 3.22: Results of the assay of pure drug admixtures and commercial formulations of TD and NF by first-derivative spectrophotometry

Drug Name	Label Claim (mg/ tab.)	Recovery (%) [a]		F-test for Mean Recovery			
		ZCP Method	RC Method	Calc		Crit [b]	
				Samples	Methods	Samples [†]	Methods [‡]
TD		**D1(326.8 nm)**	**D1(342) / D1(296)**				
Pure drug admixture	-	99.88 ± 0.99	99.47 ± 0.65	0.16	0.49	19.0	18.5
Brand EX	600	99.77 ± 0.81	100.00 ± 1.72				
Brand EY	600	99.43 ± 0.89	100.50 ± 1.10				
NF		**D1(317.4 nm)**	**D1(311) / D1(344)**				
Pure drug admixture	-	100.26 ± 0.98	100.13 ± 0.65	4.23	11.31	19.0	18.5
Brand EX	400	100.13 ± 1.40	99.72 ± 0.46				
Brand EY	400	100.13 ± 1.08	99.87 ± 0.71				

[a]: Mean and standard deviation for six determinations.

[b]: Theoretical value of $F(2, 2)^{\dagger}$ and $F(1, 2)^{\ddagger}$ based on two-way ANOVA test at P = 0.05 level of significance.

3.6 SIMULTANEOUS DETERMINATION OF TINIDAZOLE, FURAZOLIDONE AND DILOXANIDE FUROATE IN COMBINED TABLET PREPARATIONS BY SECOND-DERIVATIVE SPECTROPHOTOMETRY

The combination of tinidazole (TD), furazolidone (FD) and diloxanide furoate (DF) in the form of tablet preparation is widely used for treating diarrhoea associated with bacterial growth, amoebiasis and giardiasis. The various reports available including official monographs for the estimation of these drugs either as single preparation [1-3,23,26,100-104,297-307] or in combination of other drugs [60-62,80-86,191, 308-310,318-320] were discussed in previous sections of this chapter. There are no reports available for the determination of these drugs from their combined preparations. Hence, the objective of this work was to propose a second-derivative spectrophotometric method for the simultaneous determination of these drugs without prior separation from pure drug mixtures and a combined formulation.

Materials and Reagents

Tinidazole (Griffon, India), furazolidone (Aristo, India) and diloxanide furoate (Aristo, India) were obtained as gift samples. Dimethyl formamide (DMF; Qualigens, India) of analytical grade was used.

Standard Solutions

The stock solutions of pure TD, FD and DF were prepared separately by dissolving 5 mg of each of pure drug in 10 ml of DMF. Appropriate volume aliqouts of the stock solutions were transferred to 10 ml volumetric flasks. The volumes were made up with distilled water to give a series of solutions containing TD (5-20 μg/ml), FD (2.5-10 μg/ml) and DF (7.5-15 μg/ml). Similarly, three series of 10 ml of each mixture were also prepared in distilled water from the stock solutions. The first series contained a constant concentration of FD (2.5 μg/ml), DF (12.5 μg/ml) and a varying

concentration of TD (5-20 μg/ml). The second series contained a constant concentration of DF (12.5 μg/ml), TD (10 μg/ml) and a varying concentration of FD (2.5-10 μg/ml). While, the third series contained a constant concentration of TD (10 μg/ml), FD (2.5 μg/ml) and a varying concentration of DF (7.5-15 μg/ml). Finally, a binary mixture solution containing TD (10 μg/ml) and FD (2.5 μg/ml) was also prepared.

Interaction Study

Three separate series of mixture solutions were also prepared from the fresh stock solutions by considering a mixture of two drugs with a constant concentration and the other with varying concentration as shown in Figure 3.24.

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of TD (10 μg/ml), FD (2.5 μg/ml) and DF (12.5 μg/ml) were prepared from fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of TD (5-50 μg/ml), FD (2.5-20 μg/ml) and DF (5-30 μg/ml) were prepared from the stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing TD (10 μg/ml), FD (2.5 μg/ml) and DF (12.5 μg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of the combined formulation (Brand FX) was accurately weighed, well powdered and a powder weight equivalent to 10 mg of TD (corresponds to 2.5 mg of FD and 12.5 mg DF) was dissolved in DMF by thorough mixing and made up to

volume in a 50 ml volumetric flask. The sample was filtered through Whatman filter paper No 1. The first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate was diluted with distilled water to give samples with a concentration of 10 μg/ml of TD and corresponding amounts of FD (2.5 μg/ml) and DF (12.5 μg/ml).

Procedure

Zero-crossing point (ZCP) method

The absorbances of sample and standard solutions were recorded from 260-450 nm against a blank solution. The second-derivative spectra for each set of solutions were subsequently recorded by using $\Delta = 25$ points [138] and the spectra were smoothened using $\Delta = 30$ points to eliminate the noise produced above 350 nm in the recorded spectrum of FD. The solutions were measured at the ZCP's of the other two drugs in each instance.

Ratio-compensation (RC) method

The sample cell contained the mixture of sample and standard solutions containing TD (10 μg/ml), FD (2.5 μg/ml) and DF (12.5 μg/ml). While, the reference cell contained a binary mixture solution having TD (10 μg/ml) and FD (2.5 μg/ml). The second-derivative spectrum was recorded in each instance using appropriate set parameters mentioned in ZCP method. A ratio calculated between wavelength maxima to wavelength minima was compared to that of a similar ratio calculated for pure drug solutions recorded against a blank solution.

Results and Discussion

The zero-order spectra of pure drugs were found to be overlapping (Figure 3.19) and thus making their simultaneous determination difficult with the earlier mentioned methods [5-13]. In addition, there were no successful reports available for quantitative determination of any three-component mixtures by above methods [5-12]. A graphical

plot method used earlier with the problems related to three-component mixtures was reported [13], but it was time consuming and less accurate. The pH-induced differential spectrophotometric method could not be used due to lack of literature support for handling the three-component mixtures. Hence, a second-derivative spectrophotometric method was considered for resolving the overlapping over its first-derivative spectra to facilitate the determination of DF along with TD and FD. It was observed during initial study that the first-derivative spectra have ideal ZCP's for the estimation of TD and FD, but lacks such a point for DF and also wavelength minima to assist its determination by any other reliable methods like ratio-compensation method. This particular draw back was overcome during its processing in second-derivative mode (Figure 3.20).

It can be observed from Figure 3.20 that DF does not show any appreciable absorbance above 320 nm, thus the problem has seized to a two-component mixture in the other region of spectral measurement. The various useful ZCP's observed for estimation were at 335.4 and 397.4 nm for TD, and at 340.8 nm for FD. In addition, FD estimation would also be possible at 420.0 nm as there was no interference from other two drugs due to their lack of absorbance at maximum wavelength of FD. To determine quantitatively TD and FD only those wavelengths at which greater absorbance values would be measured were considered. Thus, TD was estimated at 335.4 nm and FD at 420.0 nm. While the determination of DF was carried out by ratio-compensation method with a ratio calculated between wavelength maxima and wavelength minima of second-derivative spectra (Figure 3.20).

The proportional relationship between the measured values and concentrations were found at the selected wavelengths of estimation by measuring a series of pure drug (Table 3.23, Series A, C and E of Table 3.25) and mixture solutions (Table 3.23, Series B, D and F of Table 3.25). The regression equations obtained with mean derivative absorbance values (Table 3.23) for pure drugs and their mixtures were

presented in Table 3.25. The second-derivative spectra obtained for the mixture solutions were presented in Figures 3.21-3.23. The presence of distinct isosbestic point(s) at 340.8 and a constant absorption at 420.0 nm (Figure 3.21), and at 335.4 and 397.4 nm (Figure 3.22) followed by a constant absorption pattern after 320.0 nm (Figure 3.23) suggested no interference in the estimation of one drug in presence of other drugs. It was also confirmed that the measured values were proportional to the concentrations of standard mixtures.

It was evident from reported results of mixture interaction study (Figure 3.24) that the non-interference zone for varying concentrations of FD, TD and DF were existed up to 25.50 and 20 μg/ml respectively at 335.4 nm for FD and TD, and at 286.4 nm for DF (Figure 3.24). Thus, the earlier proposed concentration ranges for the standard and sample solutions were appropriate for their accurate determinations.

In RC method, the second-derivative spectrum of DF was recorded from its mixture solutions against a prepared binary mixture and also its pure drug spectrum against a blank solution. The ratios thus obtained with the above solutions were reported in Table 3.24.

A detailed statistical analysis of the data obtained by above methods for standard solutions suggested that, the mean derivative absorbance values of standard solutions and their related deviations were small including coefficient of variation and the standard error thus indicating the precision of the method (Table 3.23). The percentage ratio of residual values showed random scatter in some cases about the best-fit line (Table 3.23). But from the values obtained through F-test for non-linearity [97] suggested that such error was not significant at the 5% level. From Table 3.24, it had been proved that the calculated ratios were highly reproducible and suggested the method adaptability for the determination of DF in standard and sample solutions.

The linear regression equations together with correlation coefficients and standard error involved with each intercept, slope and estimate were reported in Table 3.25. The similarity observed between regression equations of pure drug and mixture solutions suggested no interferences in the estimation of one drug in presence of the others. The homogeneity of variance within calibration line was determined by a one-way ANOVA test [98] with three replicates per calibration point. The values included were the lowest and highest variation observed from the mean absorbance value of each pure drug concentration during the replicate measurement of standard solutions (Table 3.23). The reported F-values were not significant at 5% level and thus demonstrated the linearity (Tables 3.26-3.28).

The slope values without intercept on the ordinate were reported along with 95% confidence limits in Table 3.25. It had been observed that linear proportionality between measured values and concentrations existed with FD and DF solutions where the obtained slope values fall within the confidence limits. However, to observe above such relationship with TD solutions the satisfactory confidence limits were found at 98% for pure drug (Slope: 3.64E-04 to 4.49E-04) and for mixtures at 99% (Slope: 3.69E-04 to 4.44E-04). In addition, the obtained Student's *t*-test [98] values were highly significant compared to theoretical value at 5% level confirmed the positive correlation between them.

The proposed method was validated according to the procedures described in U.S.P. 23 and the results obtained were reported in Table 3.29. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A, C and E of Table 3.25.

The percentage recoveries obtained for standard solutions and a commercial preparation with the proposed method were reported in Table 3.30. It was clear that the obtained recovery values were satisfactory according to the limits of official

monographs for the individual assay of these drugs (Appendix B). As there were no other formulations of this combination available commercially than the reported, the proposed method was tried out with only one formulation. It was confirmed from the reported Student's *t*-test [98] values that there were no significant differences between the mean recoveries of standard solutions and commercial preparation at 5% level (Table 3.30). The prepared solutions were stable for 3 hrs in presence of distilled water.

Thus the above results demonstrate that the complex problem of quantitating mixtures of three components with overlapping spectra can be easily solved. The proposed second-derivative method of determination was accurate, simple and precise for the routine analysis of pharmaceutical dosage forms.

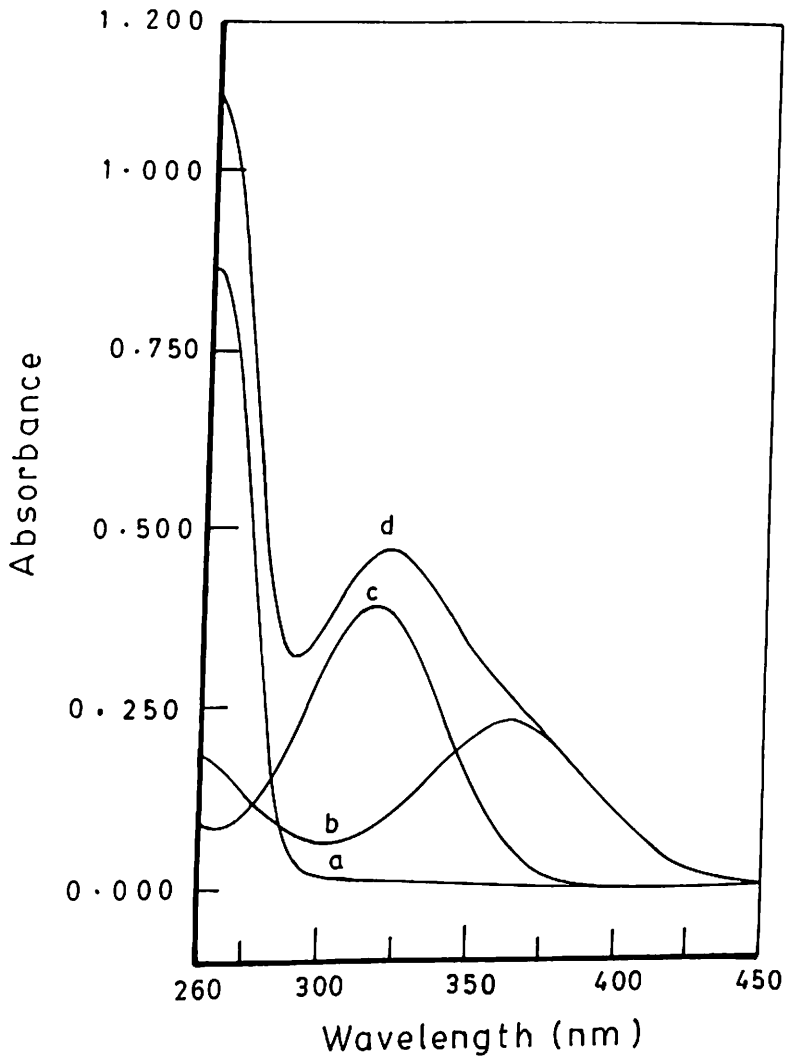

Figure 3.19 Absorption spectra of (a) diloxanide furoate (12.5 µg/ml), (b) furazolidone (2.5 µg/ml), (c) tinidazole (10 µg/ml) and (d) their mixture in distilled water.

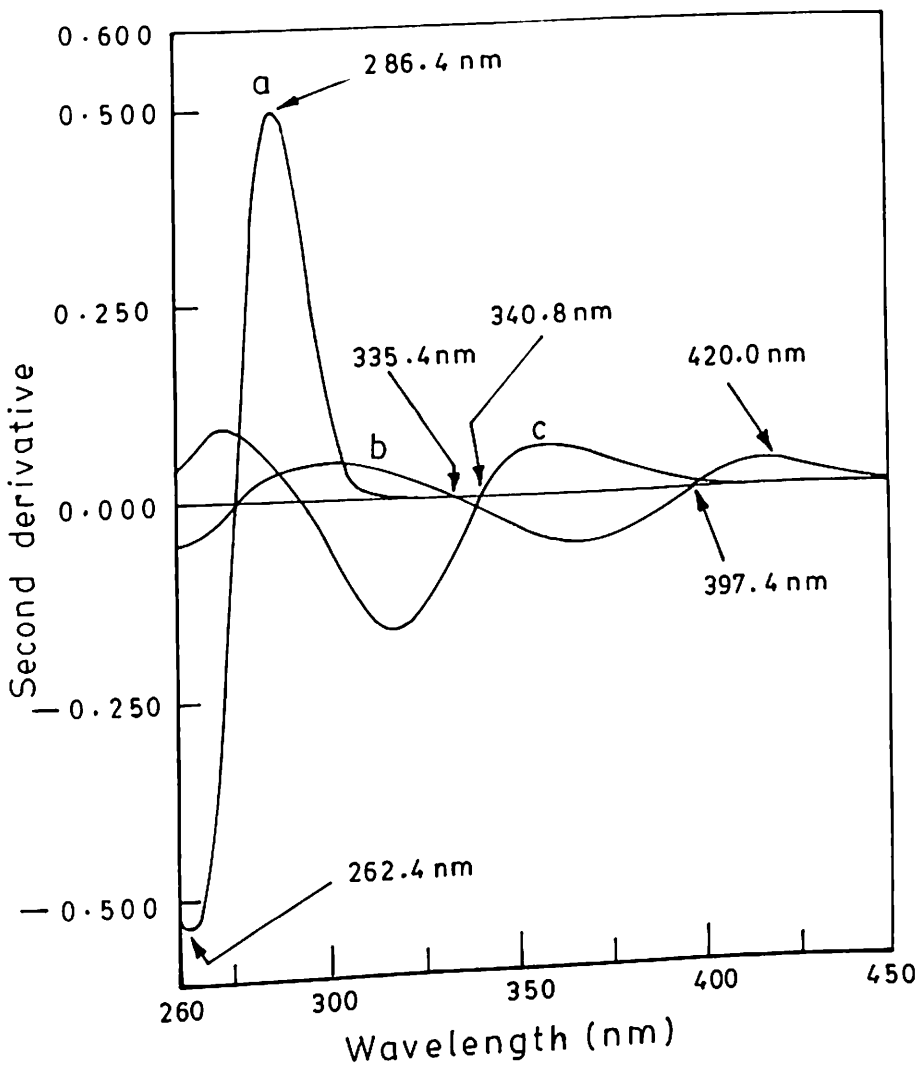

Figure 3.20 Second-derivative spectra of (a) diloxanide furoate (12.5 µg/ml), (b) furazolidone (2.5 µg/ml) and (c) tinidazole (10 µg/ml) in distilled water.

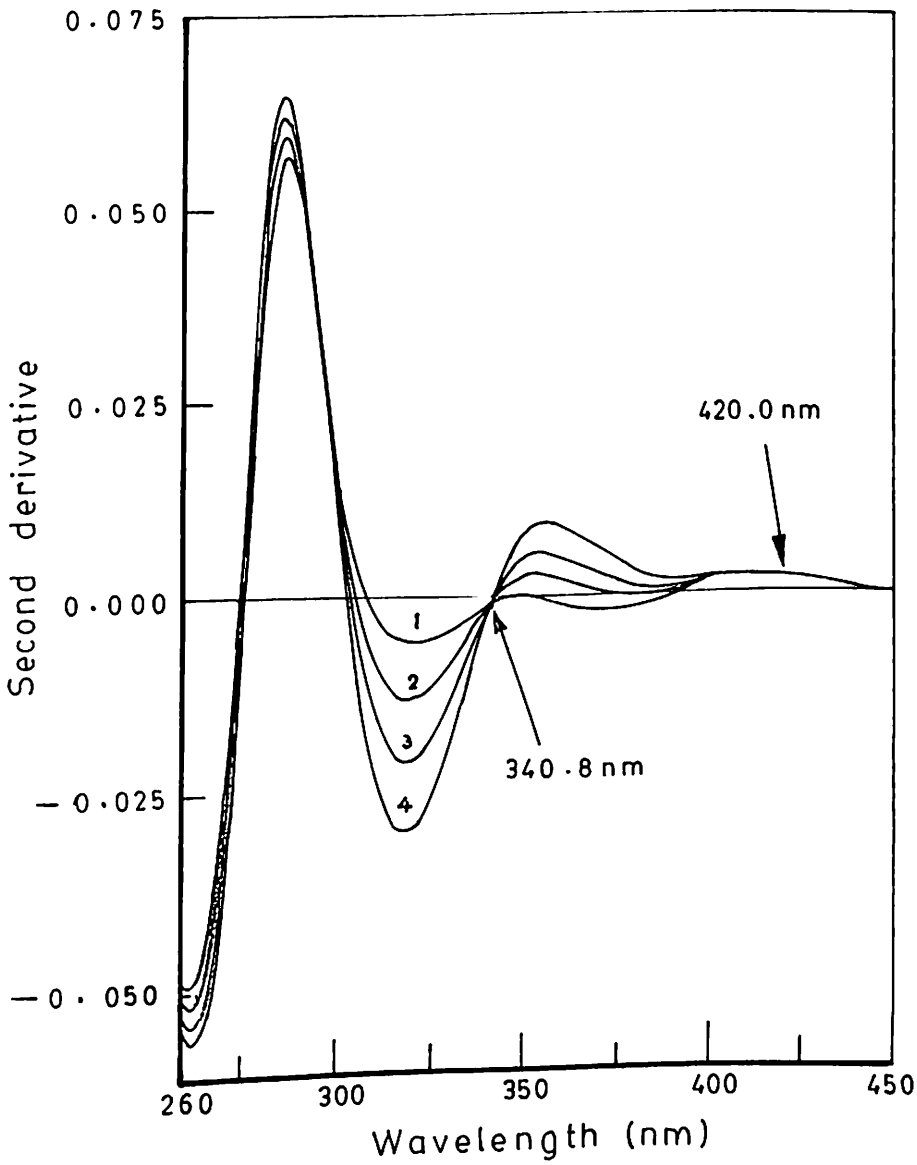

Figure 3.21 Second-derivative spectra of tinidazole (5, 10, 15 and 20 μg/ml in curves 1-4, respectively) with a constant concentration of furazolidone (2.5 μg/ml) and diloxanide furoate (12.5 μg/ml) in distilled water.

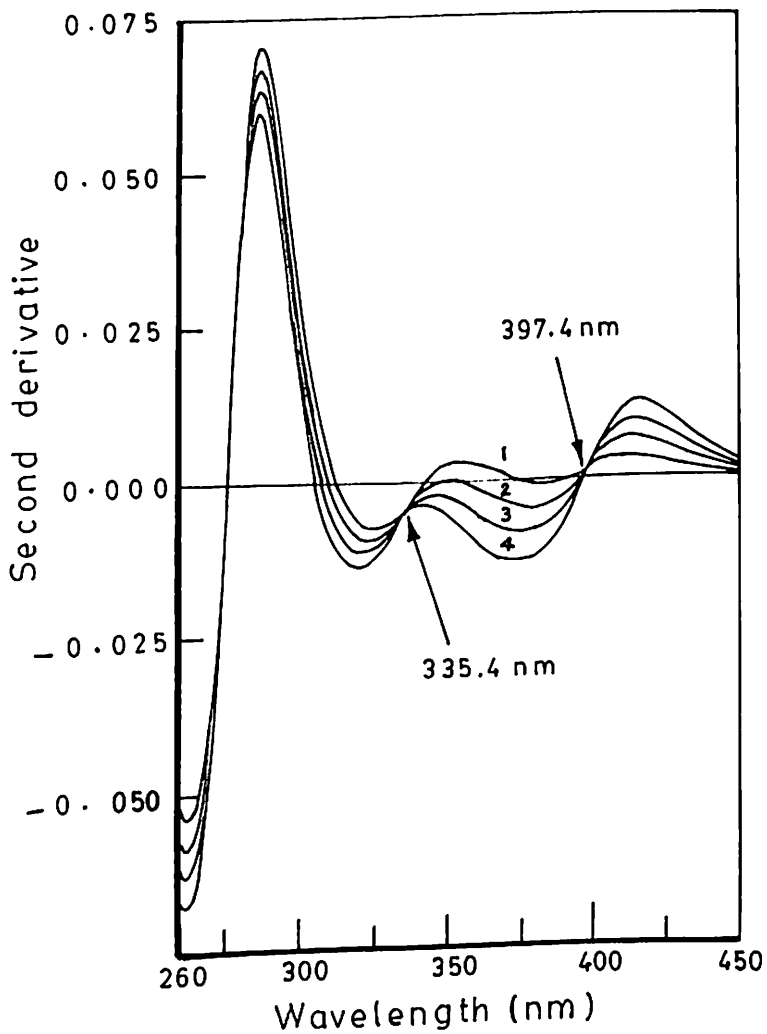

Figure 3.22 Second-derivative spectra of furazolidone (2.5, 5, 7.5 and 10 μg/ml in curves 1-4, respectively) with a constant concentration of tinidazole (10 μg/ml) and diloxanide furoate (12.5 μg/ml) in distilled water.

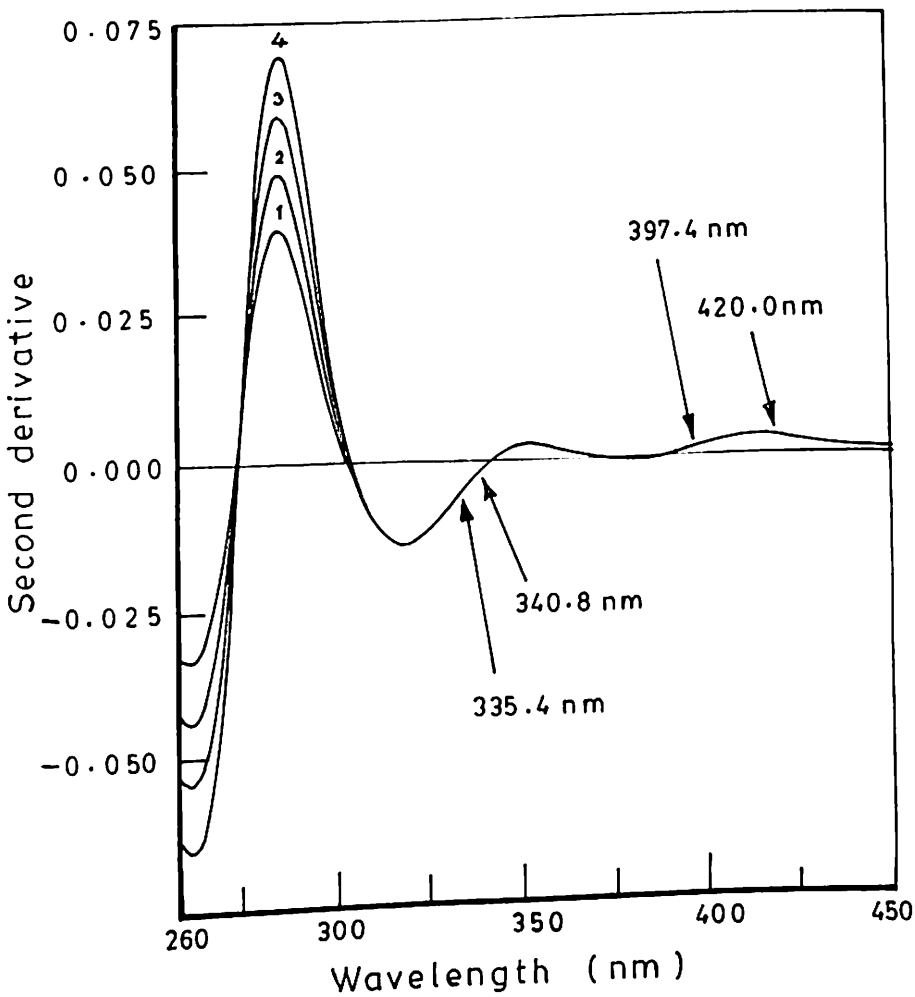

Figure 3.23 Second-derivative spectra of diloxanide furoate (7.5, 10, 12.5 and 15 µg/ml in curves 1-4, respectively) with a constant concentration of tinidazole (10 µg/ml) and furazolidone (2.5 µg/ml) in distilled water.

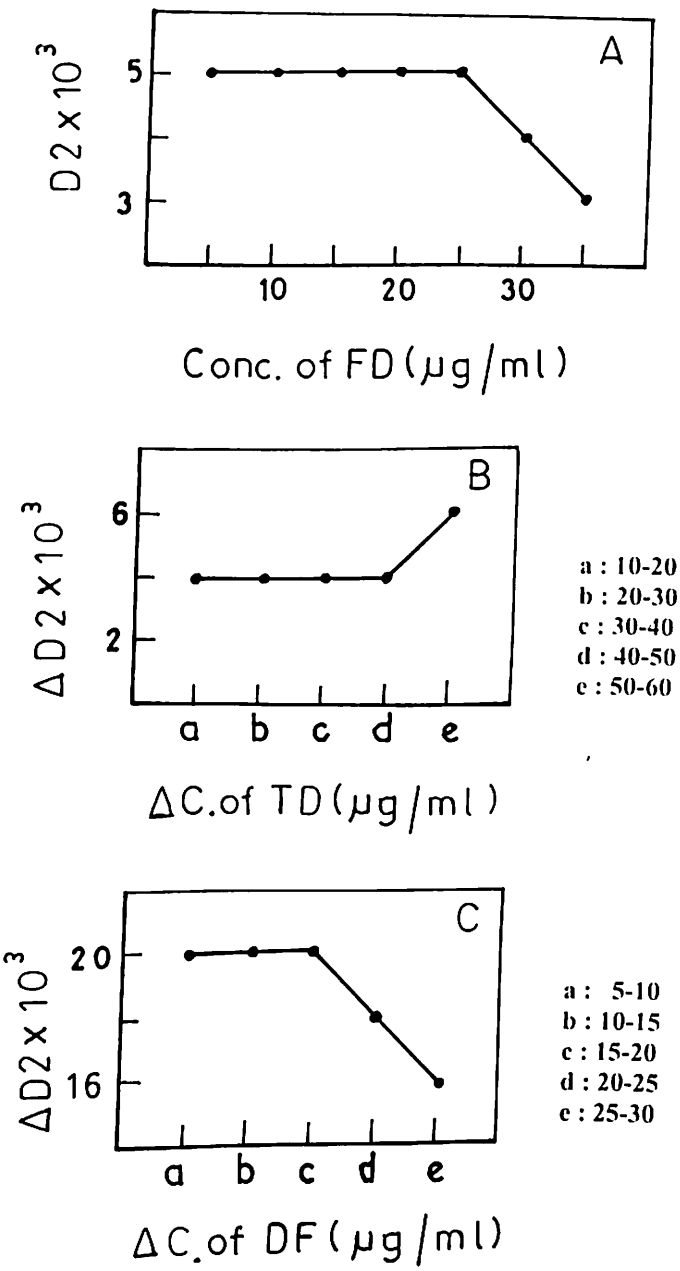

Figure 3.24 Second-derivative interaction graphs for (A) diloxanide furoate (12.5 µg/ml) and tinidazole (10 µg/ml) in mixture with furazolidone (at 335.4 nm), (B) furazolidone (2.5 µg/ml) and diloxanide furoate (12.5 µg/ml) in mixture with tinidazole (at 335.4 nm) and (C) tinidazole (10 µg/ml) and furazolidone (2.5 µg/ml) in mixture with diloxanide furoate (at 286.4 nm) in distilled water.

Table 3.23: Selectivity of the method for the simultaneous determination of TD, FD and DF in standard solutions by second-derivative spectrophotometry

Composition of the solution (µg/ml)			Mean Absorbance [a] value (D2) (TD at 335.4 nm, FD at 420.0 nm, DF at 286.4 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
TD	FD	DF					Calc	Crit [b]
5.0	0	0	0.0030 ± 0.0001	2.24	0.0001	100.87	1.04	4.26
10.0	0	0	0.0050 ± 0.0001	2.32	0.0001	99.99	0.34	
15.0	0	0	0.0071 ± 0.0001	0.98	0.0001	98.92	0.93	
20.0	0	0	0.0090 ± 0.0001	1.38	0.0001	100.57	0.29	
5.0	2.5	12.5	0.0029 ± 0.0001	2.59	0.0001	100.96	0.23	
10.0	2.5	12.5	0.0050 ± 0.0001	0.88	0.0001	99.29	0.67	
15.0	2.5	12.5	0.0070 ± 0.0001	1.54	0.0001	99.82	0.11	
20.0	2.5	12.5	0.0090 ± 0.0001	0.46	0.0001	100.22	0.76	
0	2.5	0	0.0029 ± 0.0001	1.17	0.0001	101.09	4.16	4.26
0	5.0	0	0.0059 ± 0.0001	2.18	0.0001	99.83	0.31	
0	7.5	0	0.0088 ± 0.0002	2.72	0.0001	99.12	0.09	
0	10.0	0	0.0116 ± 0.0002	2.35	0.0001	100.47	0.07	
10.0	2.5	12.5	0.0029 ± 0.0001	2.29	0.0001	100.18	0.27	
10.0	5.0	12.5	0.0058 ± 0.0001	1.95	0.0001	100.21	0.09	
10.0	7.5	12.5	0.0089 ± 0.0001	1.20	0.0001	99.54	0.10	
10.0	10.0	12.5	0.0118 ± 0.0002	1.63	0.0001	100.19	0.03	
0	0	7.5	0.0302 ± 0.0004	1.59	0.0001	100.10	0.01	4.26
0	0	10.0	0.0402 ± 0.0005	1.40	0.0001	99.89	0.01	
0	0	12.5	0.0500 ± 0.0004	0.75	0.0001	100.08	0.01	
0	0	15.0	0.0599 ± 0.0006	1.16	0.0002	100.02	0.01	
10.0	2.5	7.5	0.0298 ± 0.0005	1.70	0.0001	99.96	0.01	
10.0	2.5	10.0	0.0398 ± 0.0004	1.20	0.0001	100.07	0.01	
10.0	2.5	12.5	0.0499 ± 0.0008	1.66	0.0002	99.93	0.01	
10.0	2.5	15.0	0.0599 ± 0.0008	1.43	0.0002	100.01	0.01	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(2,9) at P = 0.05 level of significance.

Table 3.24: Selectivity of the method for the determination of DF in presence of TD and FD in standard solutions by second-derivative ratio-compensation method

Composition of solution (µg/ml)			Mean ratio absorbance value [a]	Standard error	Coefficient of variation (%)
DF	TD	FD	D2(286.4) / D2(262.4)		
7.5	0	0	0.942 ± 0.002	0.0008	0.25
10.0	0	0	0.941 ± 0.009	0.0030	1.03
12.5	0	0	0.944 ± 0.003	0.0008	0.26
15.0	0	0	0.944 ± 0.008	0.0025	0.84
-	-	-	0.943 ± 0.001 [•]	0.0007	0.14
12.5	10	2.5	0.944 ± 0.003	0.0010	0.33

[a] : Average of ten replicate determinations with standard deviation.

[•] : Average ratio of pure drug solutions.

Table 3.25: Regression analysis for the determination of TD, FD and DF in standard solutions by second-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)			Regression equations [a] (at 335.4 nm for TD at 420.0 nm for FD at 286.4 nm for DF)	Corr. coeff.	Standard error			95% Confidence interval		Slope without Intercept	Student *t* - test for correlation	
	TD	FD	DF			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	5 - 20	0	0	Y = 4.07E-04.X + 9.72E-04	0.9998	8.34E-05	6.09E-06	6.80E-05	6.14E-04, 1.33E-03	3.80E-04, 4.33E-04	4.46E-04	71	4.30
Series B	5 - 20	2.5	12.5	Y = 4.06E-04.X + 8.95E-04	0.9999	4.40E-05	3.21E-06	3.60E-05	7.05E-04, 1.08E-03	3.92E-04, 4.20E-04	4.42E-04	100	
Series C	0	2.5 - 10	0	Y = 1.16E-03.X + 1.15E-04	0.9998	8.74E-05	1.28E-05	7.14E-05	-2.61E-04, 4.91E-04	1.10E-03, 1.21E-03	1.16E-03	71	
Series D	10	2.5 - 10	12.5	Y = 1.19E-03.X - 5.40E-05	0.9999	4.27E-05	6. 23E-06	3.50E-05	-2.37E-04, 1.29E-04	1.16E-03, 1.21E-03	1.18E-03	100	
Series E	0	0	7.5 -15	Y = 3.97E-03.X + 4.60E-04	0.9999	6.52E-05	5.63E-06	3.79E-05	-1.00E-04, 1.02E-03	3.91E-03, 4.02E-03	3.99E-03	100	
Series F	10	2.5	7.5 -15	Y = 4.02E-03.X - 3.40E-04	0.9999	7.33E-05	3.15E-04	3.16E-05	-7.20E-04, 2.82E-05	3.98E-03, 4.05E-03	3.99E-03	100	

[a] : Based on four calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 2 d.f.

Table 3.26: One-way ANOVA test for linearity of pure TD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	6.24E-05	6.24E-05	0.337	4.46
Lack of fit	2	1.35E-08	6.78E-09		
Within line	8	1.61E-07	2.01E-08		
Total	11	6.26E-05			

*: at P = 0.05 level of significance.

Table 3.27: One-way ANOVA test for linearity of pure FD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.26E-04	1.26E-04	0.047	4.46
Lack of fit	2	5.01E-08	2.50E-09		
Within line	8	4.29E-07	5.36E-08		
Total	11	1.27E-04			

*: at P = 0.05 level of significance.

Table 3.28: One-way ANOVA test for linearity of pure DF solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.47E-03	1.47E-03	0.017	4.46
Lack of fit	2	2.36E-08	1.18E-08		
Within line	8	5.59E-06	6.99E-07		
Total	11	1.47E-03			

*: at P = 0.05 level of significance.

Table 3.29: Validation report for the determination of TD, FD and DF in standard solutions by second-derivative spectrophotometry

Analytical parameter	Results		
	TD (335.4 nm)	FD (420.0 nm)	DF (286.4/262.4 nm)
Accuracy (%)	100.00 ± 0.37	100.17 ± 0.53	100.04 ± 0.57
Precision (%)	99.60 99.60 100.20 100.20 100.40 RSD: 0.37	99.60 99.60 100.33 100.66 100.66 RSD: 0.53	99.60 99.60 100.00 100.00 101.00 RSD: 0.57
Specificity	A 10 µg/ml of TD, 12.5 µg/ml of DF and 2.5 µg/ml of FD mixture solution will show an absorbance value (D2) of 0.005 ± 0.001	A 2.5 µg/ml of FD, 10 µg/ml of TD and 12.5 µg/ml of DF mixture solution will show an absorbance value (D2) of 0.003 ± 0.0001	A 12.5 µg/ml of DF, 10 µg/ml of TD and 2.5 µg/ml of FD mixture solution will show an absorbance ratio of 0.944 ± 0.003 by RC method
LOD (µg/ml)	0.50	0.18	0.03
LOQ (µg/ml)	1.67	0.62	0.10
Linearity (µg/ml)	5 - 50	2.5 - 20	5 - 30
Ruggedness (%)	100.00 ± 0.37	100.17 ± 0.53	100.04 ± 0.57

Table 3.30: Results of the assay of pure drug admixtures and commercial formulation of TD, FD and DF by second-derivative spectrophotometry

Sample	Label Claim (mg/tab.)			Recovery (%) [a]			Student *t*-test for Mean Recovery			
	TD	FD	DF	TD (335.4 nm)	FD (420.0 nm)	DF (286.4 / 262.4)	Calc TD	Calc FD	Calc DF	Crit [b] (TD / FD / DF)
Pure drug admixture	-	-	-	100.03 ± 0.45	99.85 ± 0.60	100.11 ± 0.27	0.48	0.69	0.64	2.18
Brand FX	300	75	375	100.16 ± 0.54	100.09 ± 0.68	99.98 ± 0.49				

[a] : Mean and standard deviation for seven determinations.

[b] : Theoretical value of '*t* (two-sided)' at P = 0.05 level of significance with 12 d.f.

3.7 SIMULTANEOUS DETERMINATION OF METOPROLOL TARTRATE AND HYDROCHLOROTHIAZIDE IN COMBINED TABLET PREPARATIONS BY FIRST- AND SECOND-DERIVATIVE SPECTROPHOTOMETRY

The combination of metoprolol tartrate (MTP) with hydrochlorothiazide (HTZ) as a tablet is widely used to treat mild to moderate hypertension. The literature reports available for the individual assay of MTP includes colorimetric [338,339], TLC [340], ultraviolet [341] and a fluorimetric method [342]. Similarly, the various methods reported for HTZ includes colorimetric [343], ultraviolet [41,344,345] and HPLC [346]. There are many reports available for quantitative estimation of HTZ in combination of other drugs [1,129, 205-211,347-360] including MTP [347]. The last mentioned report [347] employed a direct ultraviolet method for their determination after separation. The official method for MTP prescribed a potentiometric titration [1-3] for pure drug, and HPLC [1] and ultraviolet [3] method for its dosage forms. The official method for HTZ prescribed HPLC [1] and potentiometric titration [2,3] method as pure drug and HPLC [1] and ultraviolet method [2,3] method for its solid dosage form. The U.S.P. 23 prescribed a separate HPLC method for the determination of both the drugs from its combined tablet preparation. The objective of this work was to demonstrate a first- and second-derivative ratio-compensation method for determination of these drugs without prior separation from its combined preparations.

Materials and Reagents

Metoprolol tartrate (Cipla, India) and hydrochlorothiazide (Lupin, India) were obtained as gift samples. Methanol (Qualigens, India) and sodium hydroxide (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solutions of pure drugs were prepared in methanol by dissolving 5 and 10 mg of HTZ and MTP respectively into separate 10 ml volumetric flasks.

Appropriate amounts of the stock solutions were transferred to 10 ml volumetric flasks. The volumes were diluted with 0.01M NaOH to give a series of solutions containing MTP (40-60 μg/ml) and HTZ (5-10 μg/ml). A three series of 10 ml mixture solutions in 0.01M NaOH were also prepared from the stock solutions. The first series contained a constant concentration of HTZ (6.25 μg/ml) and a varying concentration of MTP (40-60 μg/ml). Similarly, the second series contained a constant concentration of MTP (50 μg/ml) and a varying concentration of HTZ (5-10 μg/ml). Finally, the third series contained a constant concentration of MTP (50 μg/ml) and HTZ (6.25 μg/ml).

Interaction Study

Two separate series of mixture solutions were also prepared from fresh stock solutions according to the procedures mentioned above. The first series contained a constant concentration of HTZ (6.25 μg/ml) and a varying concentration of MTP (20-100 μg/ml). While, the second series contained a constant concentration of MTP (50 μg/ml) and a varying concentration of HTZ (5-40 μg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of MTP (50 μg/ml) and HTZ(6.25 μg/ml) were prepared from fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of MTP (5-70 μg/ml) and HTZ (5-20 μg/ml) were prepared from the stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing MTP (50 μg/ml) and HTZ (6.25 μg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of combined formulations (Brand GX) was accurately weighed, well powdered and a powder weight equivalent to 50 mg of MTP (and 6.25 mg of HTZ) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The sample was filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with 0.01M NaOH to give a concentration of 50 μg/ml of MTP and 6.25 μg/ml of HTZ.

Procedure

The sample cell contained the mixture of sample and standard solutions containing MTP (50 μg/ml) and HTZ (6.25 μg/ml) and the reference cell contained a series of standard solutions of pure MTP or HTZ. The first- and second-derivative spectra were recorded in each instance from 230-300 nm using Δ = 15 and 20 points [138] respectively. Similarly, the first- and second-derivative spectra of pure drug solutions against a blank solution were also recorded. Thereafter, different ratios between wavelength maxima to wavelength minima or vice versa in different derivative modes (as mentioned in Tables 3.32 and 3.33) were calculated for standard and sample solutions. The ratios thus obtained were compared at the balance point [128,129] discussed earlier in the introduction of this chapter.

Results and Discussion

The zero-order spectra of pure drug combinations were very similar and overlapping (Figure 3.25). The earlier discussed conventional methods [5-13] could not be proposed for resolving due to lack of separation of wavelength peaks. While, the pH-induced differential spectrophotometric method could not be adopted as the pure drug spectra of MTP from 230-300 nm looks alike in presence of acid and alkali buffer systems [361]. Thus, a first- and second-derivative spectrophotometric method was considered for resolving such close overlapping from their solutions. But, the

recorded spectra in first- and second-derivative mode exhibited either a complete overlap or same ZCP's for both the pure drugs (Figures 3.26 and 3.27). Therefore, ZCP method could not be considered as an option for their successful determination for being sensitive to small drifts of the band of the other component. As discussed earlier, the ratio-compensation method was found to be a suitable alternative for such situations. Hence, a ratio-compensation method was proposed for their simultaneous determination.

In this method, the first- and second-derivative spectra of standard and sample solutions were recorded against suitable solutions (as discussed in procedure). The different ratios like first-derivative minima to maxima, second-derivative maxima to minima and second-derivative to first-derivative maxima from the measured absorbance values (as reported in Tables 3.32 and 3.33) were calculated and were compared between pure drug solutions and mixtures obtained at the balance point (Tables 3.32 and 3.33).

The proportionality between measured amplitudes and concentrations were found at their first-derivative wavelength maxima by measuring a series of pure drug (Table 3.31, Series A and C of Table 3.34) and mixture solutions (Table 3.31, Series B and D of Table 3.34). The regression equations thus calculated with the measured values were reported in Table 3.34.

The mutual independence of analytical signals at first derivative zero-crossing wavelengths was demonstrated from the reported results of mixture interaction study (Figure 3.28). It was observed that MTP solutions with varying concentration (20-100 μg/ml) did not interfere up to 70 μg/ml in the estimation of HTZ (6.25 μg/ml) at 247.4 nm (Figure 3.28A). Similarly, the varying concentrations of HTZ (5-40 μg/ml) did not interfere up to 25 μg/ml in the estimation of MTP (50 μg/ml) at 249.4 nm (Figure 3.28B). Therefore, it suggested that the earlier proposed

concentration ranges for MTP and HTZ were ideal and accurate determination of mixture solution would be possible at 50 µg/ml of MTP and 6.25 µg/ml of HTZ.

The statistical analysis of data obtained by first-derivative method for standard solutions showed that the deviations associated with the measured derivative absorbance values were small including coefficient of variation and the standard error. Thus it suggested the precision of the method (Table 3.31). The percentage ratio of residuals showed a deviation of within 0.5% and demonstrated that the calibration points did not deviate significantly from the best-fit line. It was also confirmed from reported F-values of test for non-linearity [97] which were less than the critical value at 5% significance level (Table 3.31). The different ratios calculated for pure drug solutions and mixtures were reported in Tables 3.32 and 3.33. The identical ratios obtained in each instance suggested that there were no interferences in the measurement of one drug in presence of the other and thus indicated high reproducibility of the method. This can also be observed from the values of standard error and coefficient of variation (Tables 3.32 and 3.33).

The linear regression equations obtained with the mean absorbance values of standard solutions (Table 3.31) were shown in Table 3.34 and were similar. This similarity associated with correlation coefficients and the standard error involved with each intercept, slope and estimate suggested the precision of the fit. Thus, it demonstrated no interferences in the determination of one drug in presence of other. A one-way ANOVA test [98] was performed with three replicates per calibration point including the lowest and highest variation from the mean value of each concentration observed during the replicate measurements of the pure drug solutions. The uniformity of variance within the calibration lines of pure drugs was confirmed from the reported F-values at 5% significance level (Tables 3.35 and 3.36) and thus demonstrated the linearity.

It has been verified for MTP solutions that the reported slope values with zero intercept on the ordinate fall within the 95% confidence limits of slope confirms the calibration lines did not deviate from the origin. However, the percentage level of confidence limits for HTZ solutions had to be modified for above such relation as they failed to meet the widely reported 95% limits. The revised such new limits for pure drug were identified at 97.5% (Slope: 7.80E-03 to 8.13E-03) and for mixtures at 99.8% (Slope 7.82E-03 to 8.23E-03). The calculated values of Student's *t*-test [98] were relatively higher compared to theoretical values at 5% significance level demonstrated the strong positive correlation between derivative values and concentrations of pure drugs (Table 3.34).

The proposed method was validated according to the procedures described in U.S.P. 23 and the corresponding results were reported in Table 3.37. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A and C of Table 3.34.

The percentage recovery values obtained for standard solutions and a commercial preparation with the proposed method were reported in Table 3.38. The obtained mean recoveries were satisfactory according to the limits of official monographs (Appendix B) for their individual assay [1-3] and for the combination [1]. The reported F-values of a two-way ANOVA test [98] for samples suggested that there were no significant differences in the mean recoveries of samples and also between different absorbance ratios considered for their estimation at 5% significance level. (Table 3.38). The prepared solutions were stable for 3 hrs in 0.01M NaOH.

The proposed method was found to be accurate and reproducible in the absence of evaluation by zero-crossing method due to overlapping. Thus, the developed method can be useful and easier compared to the reported method [1,347] in routine analysis of pharmaceutical dosage forms without prior separation.

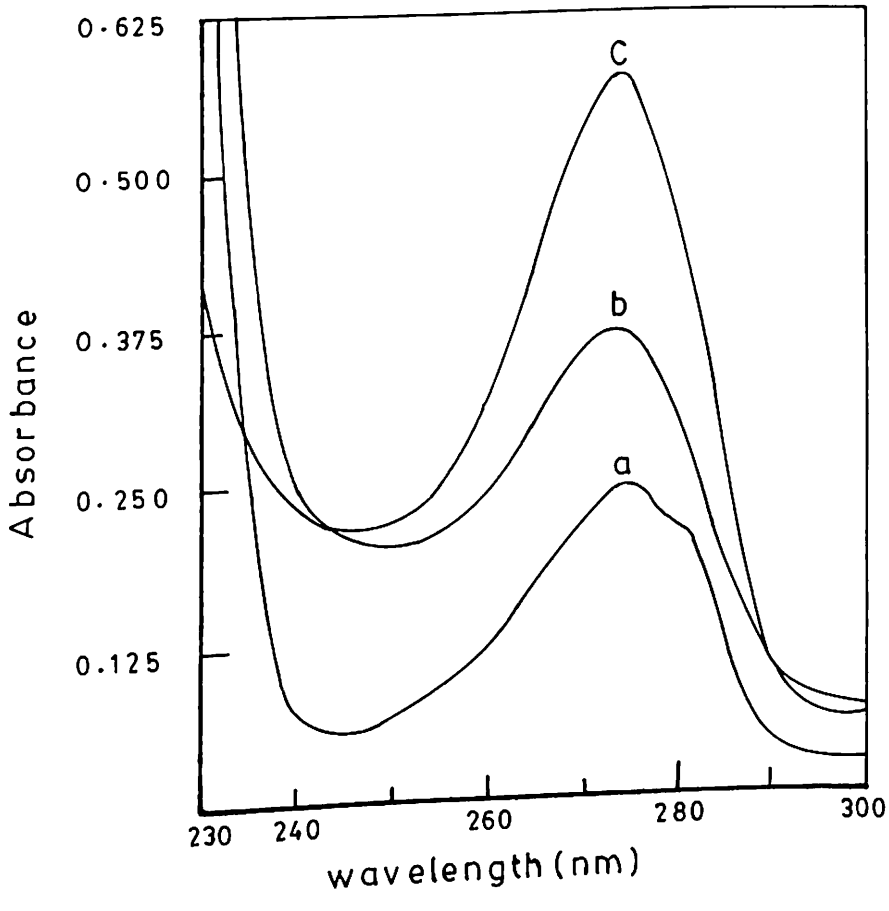

Figure 3.25 Absorption spectra of (a) metoprolol tartrate (50 µg/ml), (b) hydrochlorothiazide (6.25 µg/ml) and (c) their mixture in 0.01M NaOH.

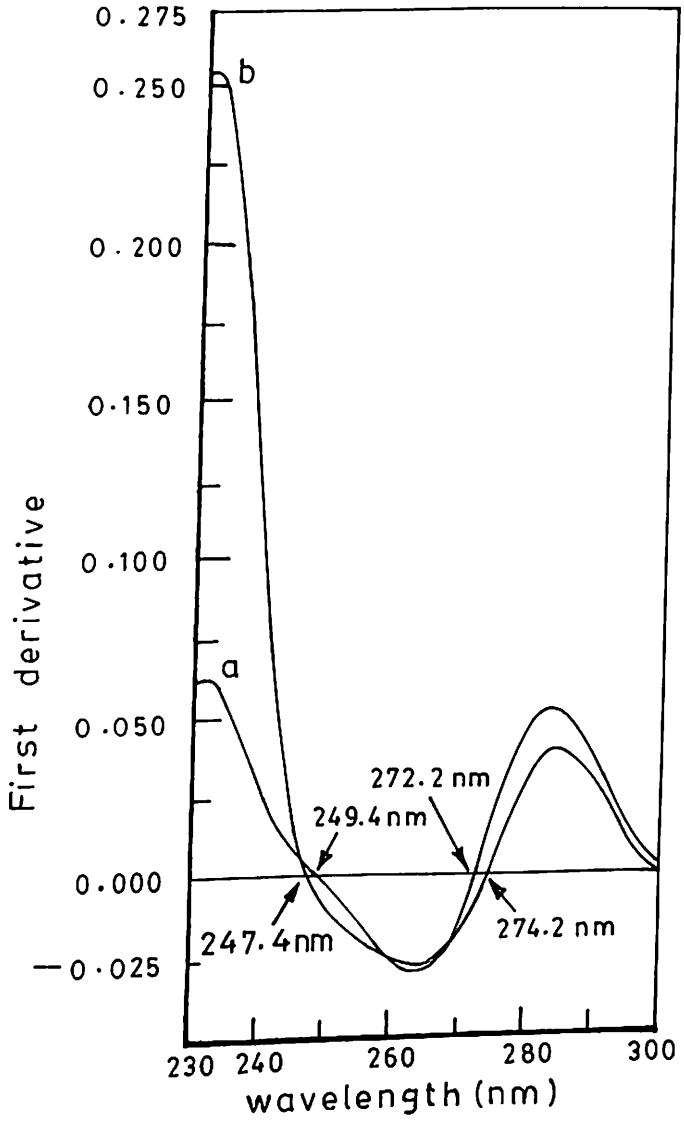

Figure 3.26 First-derivative spectra of (a) hydrochlorothiazide (6.25 µg/ml) and (b) metoprolol tartrate (50 µg/ml) in 0.01M NaOH.

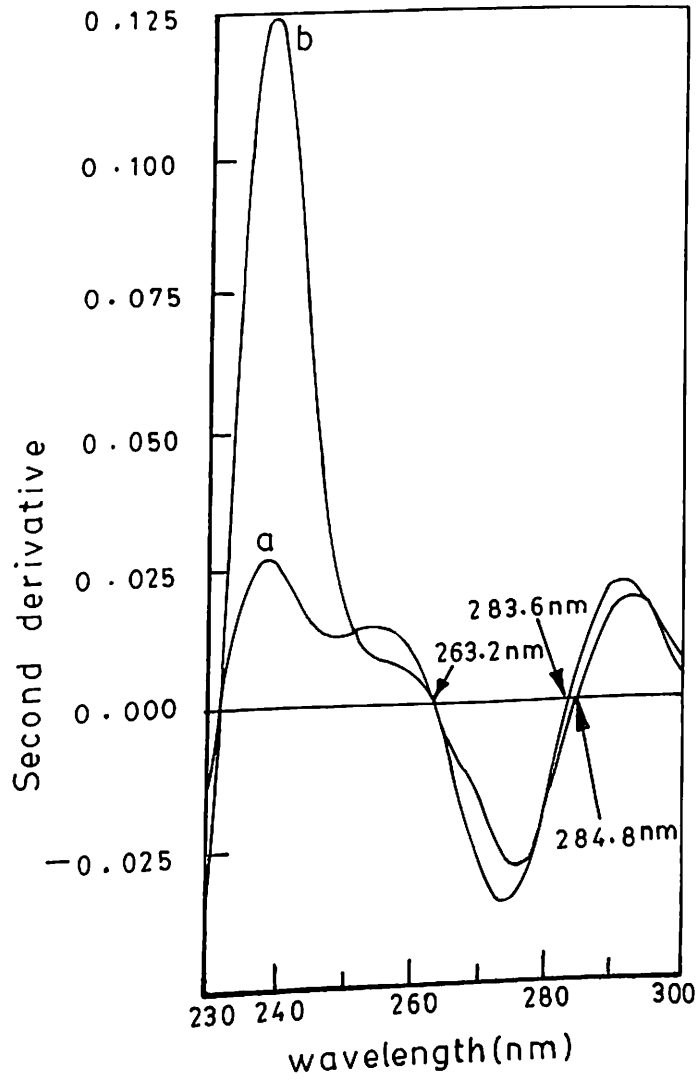

Figure 3.27 Second-derivative spectra of (a) hydrochlorothiazide (6.25 µg/ml) and (b) metoprolol tartrate (50 µg/ml) in 0.01M NaOH.

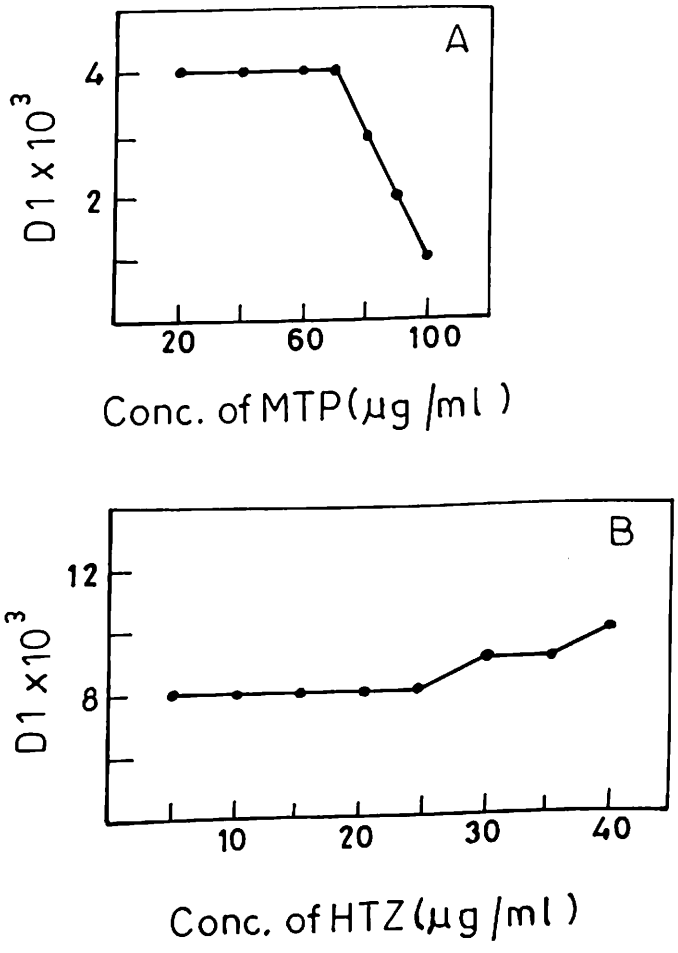

Figure 3.28 First-derivative interaction graphs for (A) hydrochlorothiazide (6.25 µg/ml) in mixture with metoprolol tartrate (at 247.4 nm) and (B) metoprolol tartrate (50 µg/ml) in mixture with hydrochlorothiazide (at 249.4 nm) in 0.01M NaOH.

Table 3.31: Selectivity of the method for the simultaneous determination of MTP and HTZ in standard solutions by first-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D1) (MTP and HTZ at 284.0 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
MTP	HTZ					Calc	Crit [b]
40	0	0.0319 ± 0.0004	1.26	0.0001	99.86	0.03	3.86
45	0	0.0359 ± 0.0003	0.78	0.0001	99.99	0.07	
50	0	0.0398 ± 0.0005	1.17	0.0001	100.28	0.03	
55	0	0.0439 ± 0.0006	1.55	0.0002	99.89	0.01	
60	0	0.0479 ± 0.0007	1.40	0.0002	99.95	0.01	
40	6.25	0.0320 ± 0.0005	1.62	0.0002	99.98	0.01	
45	6.25	0.0360 ± 0.0005	1.45	0.0002	100.05	0.01	
50	6.25	0.0400 ± 0.0006	1.52	0.0002	99.98	0.01	
55	6.25	0.0440 ± 0.0005	1.19	0.0002	99.94	0.01	
60	6.25	0.0480 ± 0.0007	1.48	0.0002	100.03	0.01	
0	5.00	0.0382 ± 0.0007	2.06	0.0002	100.02	0.04	3.86
0	6.25	0.0481 ± 0.0010	2.19	0.0003	100.25	0.02	
0	7.50	0.0583 ± 0.0007	1.16	0.0002	99.74	0.05	
0	8.75	0.0682 ± 0.0007	1.06	0.0002	99.84	0.04	
0	10.00	0.0779 ± 0.0009	1.22	0.0003	100.16	0.02	
50	5.00	0.0382 ± 0.0008	2.31	0.0003	99.80	0.01	
50	6.25	0.0481 ± 0.0009	1.88	0.0003	100.13	0.01	
50	7.50	0.0581 ± 0.0009	1.61	0.0003	100.08	0.01	
50	8.75	0.0682 ± 0.0012	1.81	0.0004	100.01	0.01	
50	10.00	0.0783 ± 0.0010	1.33	0.0003	99.94	0.01	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 3.32: Selectivity of the method for the determination of MTP in presence of HTZ in standard solutions by first- and second-derivative ratio-compensation method

Composition of solution (µg/ml)		Ratio	Mean ratio [a] absorbance value	Standard error	Coefficient of variation (%)
MTP	HTZ				
40	0	D1(264) / D1(284)	0.682± 0.010	0.0033	1.54
45	0		0.684± 0.006	0.0019	0.89
50	0		0.685± 0.006	0.0019	0.91
55	0		0.683± 0.008	0.0024	1.13
60	0		0.682± 0.009	0.0029	1.35
-	-		0.683± 0.001 *	0.0006	0.19
50	6.25		0.682± 0.005	0.0015	0.70
40	0	D2(294) / D2(276)	0.652± 0.007	0.0021	1.03
45	0		0.651± 0.007	0.0021	1.03
50	0		0.652± 0.008	0.0026	1.30
55	0		0.653± 0.007	0.0021	1.02
60	0		0.651± 0.006	0.0019	0.95
-	-		0.652± 0.001 *	0.0003	0.10
50	6.25		0.650± 0.007	0.0022	1.04
40	0	D2(294) / D1(284)	0.468 ± 0.003	0.0010	0.68
45	0		0.470 ± 0.004	0.0014	0.94
50	0		0.469 ± 0.017	0.0037	2.48
55	0		0.469 ± 0.009	0.0027	1.84
60	0		0.470 ± 0.004	0.0012	0.80
-	-		0.469 ± 0.001 *	0.0005	0.23
50	6.25		0.468 ± 0.010	0.0032	2.15

[a] : Average of ten replicate determinations with standard deviation.

* : Average ratio of pure drug solutions.

Table 3.33: Selectivity of the method for the determination of HTZ in presence of MTP in standard solutions by first- and second-derivative ratio-compensation method

Composition of solution (µg/ml)		Ratio	Mean ratio [a] absorbance value	Standard error	Coefficient of variation (%)
HTZ	MTP				
5.00	0	D1(263) / D1(284)	0.574± 0.007	0.0022	1.21
6.25	0		0.574± 0.004	0.0014	0.75
7.50	0		0.576± 0.008	0.0024	1.33
8.75	0		0.577± 0.009	0.0027	1.50
10.00	0		0.576± 0.009	0.0029	1.60
-	-		0.575± 0.001 *	0.0006	0.23
6.25	50		0.575± 0.007	0.0021	1.16
5.00	0	D2(291) / D2(274)	0.624± 0.004	0.0014	0.69
6.25	0		0.624± 0.003	0.0008	0.42
7.50	0		0.625± 0.003	0.0008	0.41
8.75	0		0.626± 0.003	0.0011	0.54
10.00	0		0.624± 0.003	0.0009	0.49
-	-		0.624± 0.001 *	0.0003	0.10
6.25	50		0.623± 0.007	0.0023	1.15
5.00	0	D2(291) / D1(284)	0.424 ± 0.009	0.0029	2.17
6.25	0		0.424 ± 0.005	0.0016	1.22
7.50	0		0.425 ± 0.005	0.0017	1.28
8.75	0		0.425 ± 0.005	0.0017	1.23
10.00	0		0.424 ± 0.005	0.0017	1.28
-	-		0.424 ± 0.001 *	0.0002	0.10
6.25	50		0.423 ± 0.006	0.0021	1.60

[a] : Average of ten replicate determinations with standard deviation.

* : Average ratio of pure drug solutions.

Table 3.34: Regression analysis for the determination of MTP and HTZ in standard solutions by first-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 284.0 nm for MTP and HTZ)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* - test for correlation	
	MTP	HTZ			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	40 - 60	0	Y = 8.00E-04.X - 1.58E-04	0.9999	2.41E-04	4.77E-06	7.52E-05	-9.23E-04, 6.08E-04	7.85E-04, 8.15E-04	7.97E-04	122	3.18
Series B	40 - 60	6.25	Y = 7.97E-04.X + 1.82E-04	0.9999	6.52E-05	1.29E-06	2.15E-05	-2.54E-05, 3.89E-04	7.92E-04, 8.00E-04	7.99E-04	122	
Series C	0	5 - 10	Y = 7.97E-03.X - 1.63E-03	0.9999	2.87E-04	3.73E-05	1.45E-04	-2.54E-03, -7.18E-04	7.84E-03, 8.09E-03	7.81E-03	122	
Series D	50	5 - 10	Y = 8.01E-03.X - 1.87E-03	0.9999	1.19E-04	1.54E-05	7.03E-05	-2.25E-03, -1.49E-03	7.96E-03, 8.06E-03	7.82E-03	122	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 3.35: One-way ANOVA test for linearity of pure MTP solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	4.71E-04	4.71E-04	0.092	3.71
Lack of fit	3	1.62E-07	5.42E-08		
Within line	10	5.87E-06	5.87E-07		
Total	14	4.77E-04			

*: at P = 0.05 level of significance.

Table 3.36: One-way ANOVA test for linearity of pure HTZ solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	3.00E-03	3.00E-03	0.066	3.71
Lack of fit	3	3.78E-07	1.26E-07		
Within line	10	1.89E-05	1.89E-06		
Total	14	3.02E-03			

*: at P = 0.05 level of significance.

Table 3.37: Validation report for the determination of MTP and HTZ in standard solutions by first-derivative spectrophotometry

Analytical parameter	Results	
	MTP D1(264 / 284 nm)	HTZ D1(263 / 284 nm)
Accuracy (%)	99.85 ± 0.65	100.03 ± 0.82
Precision (%)	99.25 99.25 99.75 100.25 100.75 RSD: 0.65	99.17 99.17 100.20 100.80 100.80 RSD: 0.82
Specificity	A 50 µg/ml of MTP and 6.25 µg/ml HTZ mixture solution will show an absorbance ratio of 0.682 ± 0.005 by RC method	A 6.25 µg/ml of HTZ and 50 µg/ml of MTP mixture solution will show an absorbance ratio of 0.575 ± 0.007 by RC method
LOD (µg/ml)	0.28	0.06
LOQ (µg/ml)	0.94	0.18
Linearity (µg/ml)	5 - 70	5 - 20
Ruggedness (%)	99.85 ± 0.65	100.03 ± 0.82

Table 3.38: Results of the assay of pure drug admixtures and commercial formulation of MTP and HTZ by first- and second-derivative spectrophotometry

Drug Name	Label Claim (mg/tab.)	Recovery (%) [a]			F-test for Mean Recovery			
					Calc		Crit [b]	
					Samples	Methods	Samples [†]	Methods [‡]
MTP		**D1(264) / D1(284)**	**D2(294) / D2(276)**	**D2(294) / D1(284)**				
Pure drug admixture	-	99.92 ± 047	99.74 ± 0.96	99.11 ± 0.98	2.26	4.09	18.5	19.0
Brand GX	100	99.58 ± 0.46	99.22 ± 0.53	99.18 ± 0.77				
HTZ		**D1(263) / D1(284)**	**D2(291) / D2 (274)**	**D2(291) / D1(284)**				
Pure drug admixture	-	99.71 ± 0.60	99.54 ± 0.51	99.84 ± 0.91	2.12	1.44	18.5	19.0
Brand GX	12.5	98.95 ± 1.13	99.46 ± 0.77	99.72 ± 0.92				

[a] : Mean and standard deviation for six determinations.

[b] : Theoretical value of $F(1, 2)^{\dagger}$ and $F(2, 2)^{\ddagger}$ based on two-way ANOVA test at $P = 0.05$ level of significance.

3.8 SIMULTANEOUS DETERMINATION OF PROPRANOLOL HYDROCHLORIDE AND HYDROCHLOROTHIAZIDE IN COMBINED FORMULATIONS BY FIRST- AND SECOND-DERIVATIVE SPECTROPHOTOMETRY

The combination of propranolol HCl (PP) and hydrochlorothiazide (HTZ) in the form of tablet is widely used to treat mild to moderate hypertension. There are many reports available for the individual assay of PP, which includes colorimetric [362-365], ultraviolet [366,367], fluorimetric [368] and HPLC method [369]. There are also reports available for PP in combination of other drugs [211,348,349,370] including HTZ [348,349]. The later mentioned two reports employed TLC [348] and direct ultraviolet method for their determination after separation [349]. The literature reports available for HTZ as a single preparation [41,343-346] and in combination of other drugs [205-211,272,347-360] were mentioned in previous section. The official monographs prescribed HPLC [1] and potentiometric titration [2,3] method for PP as pure drug, and HPLC [1] and ultraviolet [2,3] method for its assay from single drug preparations. While, the official methods for HTZ prescribed HPLC [1] and potentiometric titration [2,3] method as pure drug, and HPLC [1] and ultraviolet [2,3] method for its assay from solid dosage forms. The U.S.P. 23 also reported combined HPLC method for the estimation of these drugs from its dosage forms. The objective of this work was to propose a simple and rapid first- and second-derivative spectrophotometric method for the determination of these drugs in the presence of each other as well as the excipients.

Materials and Reagents

Propranolol HCl (Cipla, India) and hydrochlorothiazide (Lupin, India) were obtained as gift samples. Methanol (Qualigens, India) and sodium hydroxide (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solutions of pure drugs were prepared in methanol by dissolving 5 mg each of pure drug into separate 10 ml volumetric flasks. Appropriate amounts of the aliquots of stock solutions were transferred to 10 ml volumetric flasks. The volumes were diluted with 0.01M NaOH to give a series of solutions containing PP (5-15 μg/ml) and HTZ (5-10 μg/ml). A three series of 10 ml mixture solutions were also prepared from the stock solutions. The first series contained a constant concentration of HTZ (6.25 μg/ml) and a varying concentration of PP (5-15 μg/ml). The second series contained a constant concentration of PP (10 μg/ml) and a varying concentration of HTZ (5-10 μg/ml). The final series contained a constant concentration of PP (10.μg/ml) and HTZ (6.25 μg/ml).

Interaction Study

Two separate series of mixture solutions were also prepared from fresh stock solutions according to the above mentioned procedures. The first series contained a constant concentration of PP (10 μg/ml) and a varying concentration of HTZ (5-35 μg/ml). While, the second series contained a constant concentration of HTZ (6.25 μg/ml) and a varying concentration of PP (5-40 μg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of PP (10 μg/ml) and HTZ (6.25 μg/ml) were prepared from fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of PP (5-35 μg/ml) and HTZ (5-20 μg/ml) were prepared from the stock solutions meant for method validation.

c. *Specificity*

Series of five mixture solutions of each containing PP (10 μg/ml) and HTZ (6.25 μg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of combined formulation (Brand HX) was accurately weighed, well powdered and a powder weight equivalent to 10 mg of PP (and 6.25 mg of HTZ) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The sample was filtered through Whatman filter paper No. 1. The first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with 0.01M NaOH to give a concentration of 10 μg/ml of PP and 6.25 μg/ml of HTZ.

Procedure

Zero-crossing point (ZCP) method

The absorbances of sample and standard solutions were recorded from 230-300 nm against a blank solution. The second-derivative spectra for each set of solutions were subsequently recorded by using Δ = 20 points [138]. The solutions were measured at the zero-crossing wavelength of other drug in each instance.

Ratio-compensation (RC) method

The sample cell contained the mixture of sample and standard solutions containing PP (10 μg/ml) and HTZ (6.25 μg/ml), and the reference cell was replaced with successively varying concentrations of pure drugs. The first- and second-derivative spectra were recorded in each instance from 230-300 nm using Δ = 15 and 20 points [138] respectively. Similarly, the first- and second-derivative spectra of pure drug solutions against a blank solution were also recorded. Then, different ratios between wavelength maxima to wavelength minima or vice versa in different derivative modes (as mentioned in Tables 3.40 and 3.41) were calculated for standard and sample

solutions. The ratios thus obtained were compared at the balance point [128,129] as discussed earlier in the introduction of this chapter.

Results and Discussion

The zero-order spectra of pure drug combinations were overlapping (Figure 3.29). The earlier discussed conventional methods [5-13] could not be adopted, as they have not met the preliminary requirements due to no significant separation of wavelength peaks. The earlier proposed pH-induced differential spectrophotometric method could not be used, as there were no significant spectral changes induced by acidic and alkali solutions for PP in the range of spectral measurement. Therefore, a first- and second-derivative method was considered for resolving such overlapping between the two drugs. The zero-crossing points (ZCP's) identified in both derivative modes does not coincide exactly with the wavelength maxima or minima of the other drug (Figures 3.30 and 3.31). Thus, to avoid the influence of any errors in the measurements due to small drifts of the band of the other component, the ZCP method was compared with RC method to maintain the precision during the estimation of drug solutions.

The second-derivative ZCP's were considered for their determination over first-derivative points for no particular reason. The ZCP's found in the second-derivative mode were 263.2 and 283.6 nm for HTZ, and at 270.6 and 297.6 nm for PP. For the reasons of simplicity, only the readings observed at 283.6 nm for PP and at 270.6 nm for HTZ were reported in Tables 3.39 and 3.42-3.45. However, the percentage recoveries obtained for standard and sample solutions at all reported ZCP's were shown in Table 3.46.

The proportional relationship between derivative values and concentrations were determined in second-derivative mode by measuring a series of pure drug (Table 3.39, Series A and C of Table 3.42) and mixture solutions (Table 3.39, Series B and D of

Table 3.42). Thus obtained regression equations from the measured values (Table 3.39) were reported in Table 3.42. The spectra obtained for the mixture solutions were presented in Figures 3.32 and 3.33. It was interesting to note that all the lines converge to the zero-crossing wavelengths (i.e. distinct isosbestic point) of PP (Figure 3.32) and HTZ (Figure 3.33) that confirmed the non-interference of one drug in presence of the other. In addition, the measured heights were also proportional to their respective concentrations and the measured values have not shifted from their point of measurement.

The mutual independence of analytical signals at their wavelength of estimation was confirmed by mixture interaction study performed in first-derivative mode. This was done primarily to check the suitability of this derivative order for the determination from mixtures. It was demonstrated from reported results (Figure 3.34) that the varying concentration of HTZ (5-35 µg/ml) did not interfere up to 20 µg/ml in the estimation of PP (10 µg/ml) at 272.2 nm (Figure 3.34A). Similarly, the varying concentrations of PP (5-40 µg/ml) did not interfere up to 25 µg/ml in the estimation of HTZ (6.25 µg/ml) at 252.6 nm (Figure 3.34B). Thus, the proposed concentration ranges for standard solutions of PP and HTZ were ideal and accurate determinations would be possible by maintaining concentrations of PP at 10 µg/ml and for HTZ at 6.25 µg/ml.

In RC method, the first- and second-derivative spectra of standard and sample solutions were recorded against suitable solutions (as discussed in procedure). The different ratios like first-derivative minima to maxima, second-derivative maxima to minima and second-derivative to first-derivative maxima from the measured absorbance values (as reported in Tables 3.40 and 3.41) were compared between pure drug solutions and mixtures at the balance point (Tables 3.40 and 3.41).

The statistical analysis of data achieved in second-derivative mode suggested that, the mean derivative absorbance values together with standard deviation, coefficient of variation and the standard error were small (Table 3.39). Thus, it showed the precision of the method. The percentage ratio of residuals showed random scatter about the best-fit line. The calculated values of F-test for non-linearity [97] indicated that such random distribution was not significant at 5% level (Table 3.39) and thus established the linearity of calibration points in the proposed concentration range.

The different mean ratio absorbance values calculated for pure drug solutions and mixtures were reported in Tables 3.40 and 3.41. The identical ratios obtained in each instance suggested that there were no interferences in the measurement of one drug in presence of the other and also reproducibility of the method. This was clear from the values of standard error and coefficient of variation (Tables 3.40 and 3.41).

The linear regression equations generated with the mean absorbance values of standard solutions were shown in Tables 3.42 and were similar. This similarity associated with correlation coefficients and standard error involved with each intercept, slope and estimate suggested that there was no interference in the estimation of one drug in presence of the other. Further, the linearity was confirmed by performing a one-way ANOVA test [98] with three replicates per calibration point. The values considered were the lowest and highest variation observed from the mean absorbance values of each pure drug concentration during the replicate measurement. The reported F-values in Tables 3.43 and 3.44 at 5% significance level suggested that the variance was uniform throughout the line and thus the calibration lines were linear.

The reported slope values with zero intercept on ordinate for PP and HTZ solutions fall within the 95% confidence limits (Table 3.42) and thus demonstrated that the intercepts of regression equations did not deviate from the origin. Further, the calculated Student's *t*-test [98] values, which were highly significant at 5% level

suggested the positive correlation established between the measured values and concentrations.

The proposed method was validated according to the procedures described in U.S.P. 23 and the results obtained were reported in Table 3.45. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A and C of Table 3.42.

The percentage recovery values obtained for standard solutions and the commercial preparation by both zero-crossing and ratio-compensation method were reported in Table 3.46. It was clear from the reported recoveries that they meet the official requirements (Appendix B) for single and combined drug preparations of both the drugs. The reported values of a two-way ANOVA test [98] did not exceed the theoretical requirement at 5% level confirms that there were no significant differences between the samples of a method and between the estimations done by different methods (Table 3.46). The prepared solutions were stable for 3 hrs in 0.01M NaOH and were protected from light throughout the study.

Thus, the proposed derivative methods in either first- or second-order offer a great possibility for resolving the overlapping of spectra. The developed method was found to be accurate, simple and precise. The rapidity of these methods compared to reported methods [1,348,349] makes it convenient to adapt for routine analysis of the combined preparations.

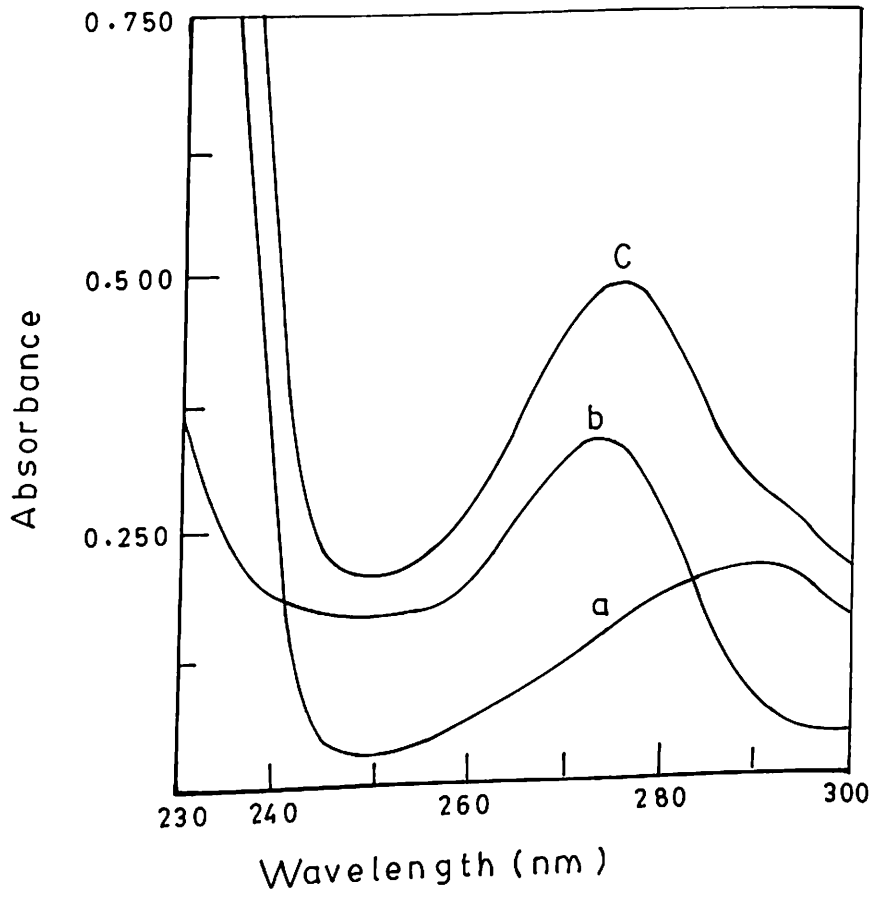

Figure 3.29 Absorption spectra of (a) propranolol HCl (10 µg/ml), (b) hydrochlorothiazide (6.25 µg/ml) and (c) their mixture in 0.01M NaOH.

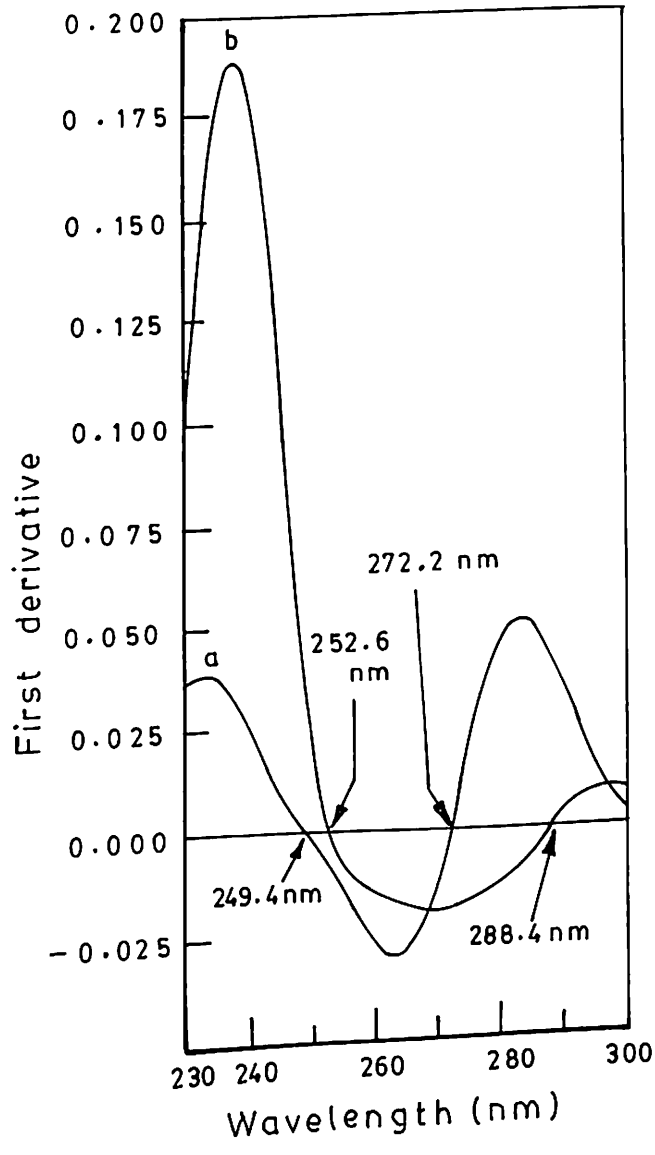

Figure 3.30 First-derivative spectra of (a) hydrochlorothiazide (6.25 µg/ml) and (b) propranolol HCl (10 µg/ml) in 0.01M NaOH.

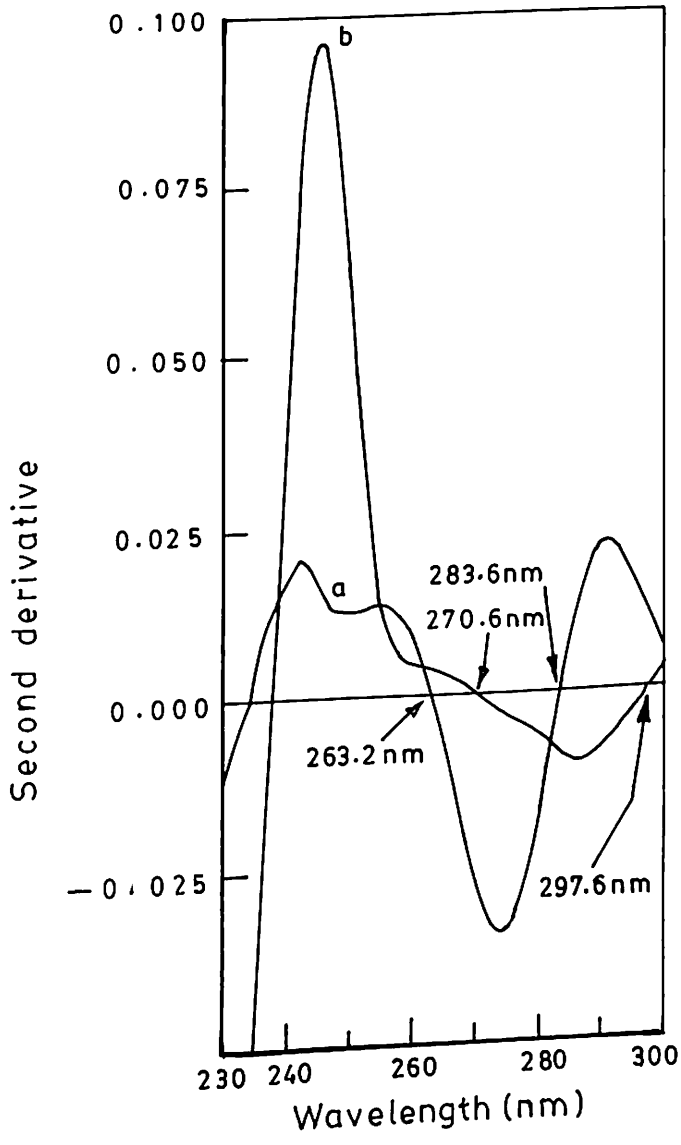

Figure 3.31 Second-derivative spectra of (a) hydrochlorothiazide (6.25 µg/ml) and (b) propranolol HCl (10 µg/ml) in 0.01M NaOH.

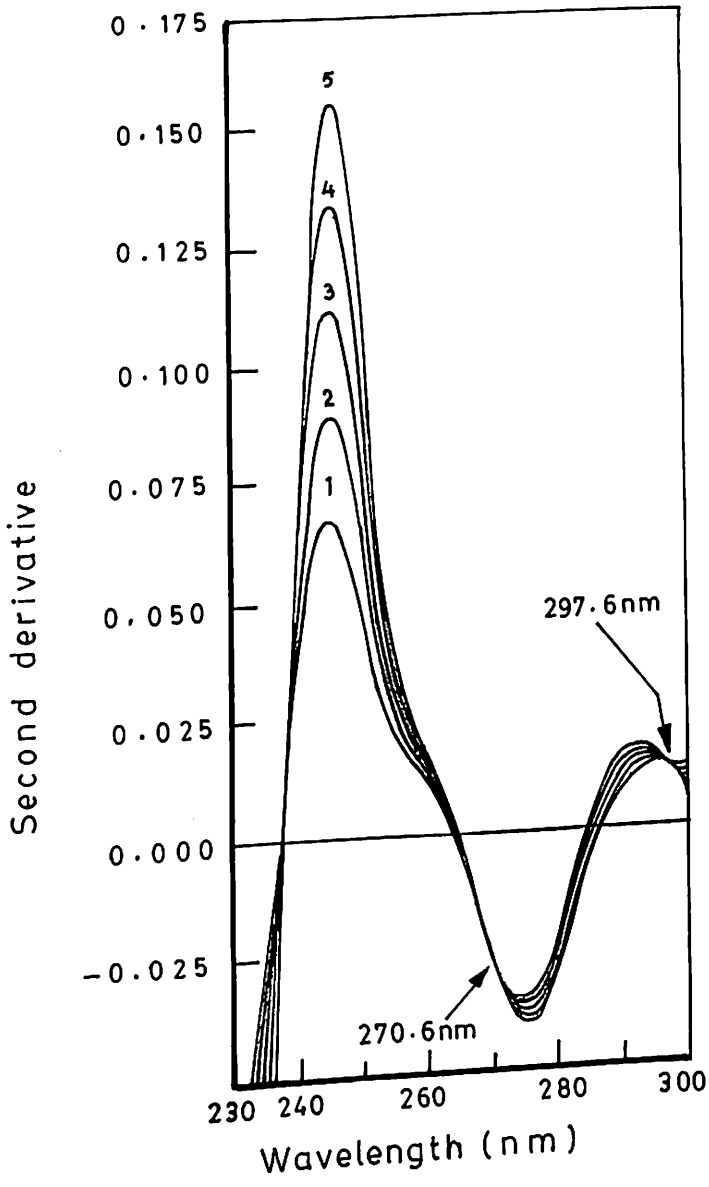

Figure 3.32 Second-derivative spectra of propranolol HCl (5, 7.5, 10, 12.5 and 15 μg/ml) and hydrochlorothiazide (6.25 μg/ml) in 0.01M NaOH in curves 1-5, respectively.

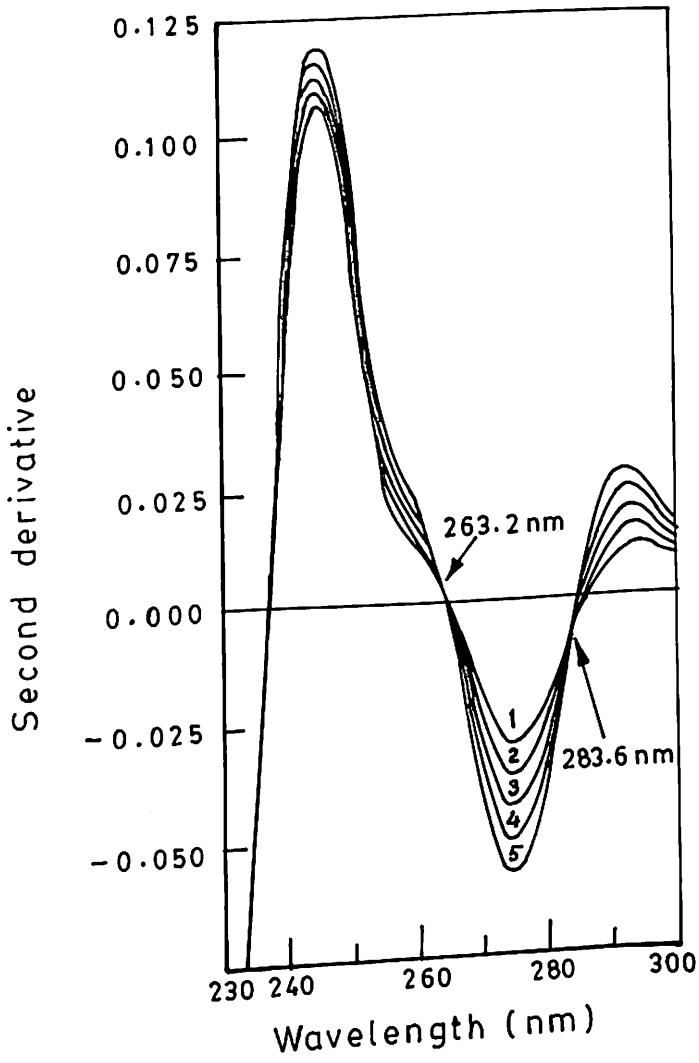

Figure 3.33 Second-derivative spectra of hydrochlorothiazide (5, 6.25, 7.5, 8.75 and 10 μg/ml)) and propranolol HCl (10 μg/ml) in 0.01M NaOH in curves 1-5, respectively.

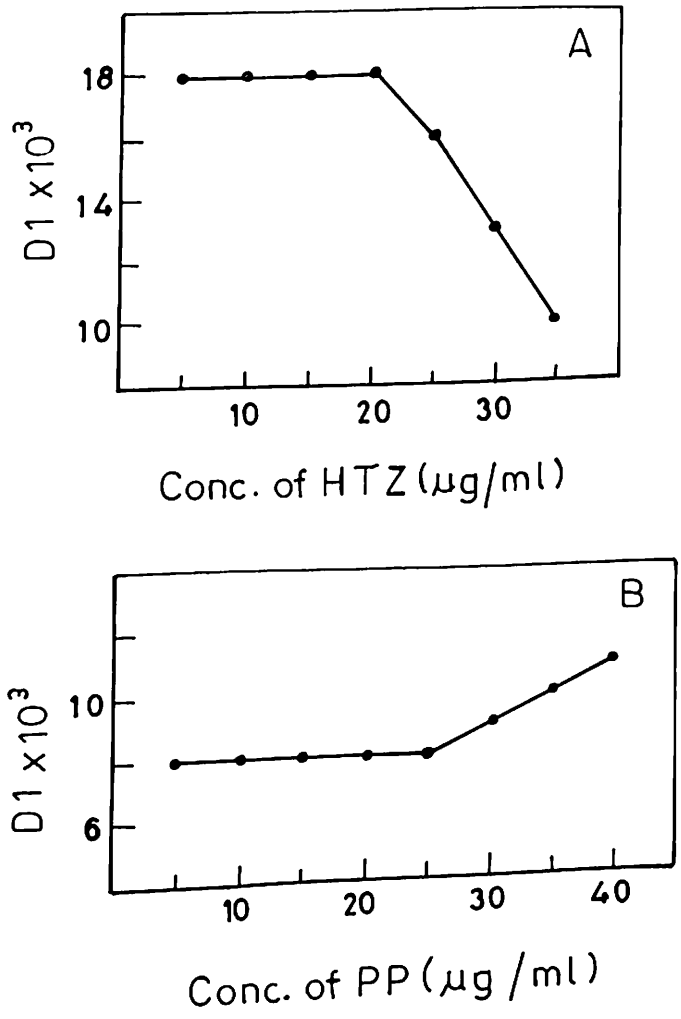

Figure 3.34 First-derivative interaction graphs for (A) propranolol HCl (10 µg/ml) in mixture with hydrochlorothiazide (at 272.2 nm) and (B) hydrochlorothiazide (6.25 µg/ml) in mixture with propranolol HCl (at 252.6 nm) in 0.01M NaOH.

Table 3.39: Selectivity of the method for the simultaneous determination of PP and HTZ in standard solutions by second-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D2) (PP at 283.6 nm, HTZ at 270.6 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
PP	HTZ					Calc	Crit [b]
5.0	0	0.0041 ± 0.0001	1.52	0.0001	99.15	0.46	3.86
7.5	0	0.0063 ± 0.0001	1.26	0.0001	100.32	0.29	
10.0	0	0.0085 ± 0.0001	1.56	0.0001	100.23	0.10	
12.5	0	0.0107 ± 0.0001	1.02	0.0003	100.36	0.16	
15.0	0	0.0129 ± 0.0002	1.51	0.0001	99.66	0.05	
5.0	6.25	0.0042 ± 0.0001	2.06	0.0001	98.82	0.93	
7.5	6.25	0.0063 ± 0.0001	1.86	0.0001	99.86	0.49	
10.0	6.25	0.0085 ± 0.0001	1.86	0.0001	100.93	0.27	
12.5	6.25	0.0107 ± 0.0002	1.58	0.0001	100.60	0.24	
15.0	6.25	0.0131 ± 0.0002	1.85	0.0001	99.34	0.11	
0	5.00	0.0220 ± 0.0003	1.65	0.0001	101.17	0.79	3.86
0	6.25	0.0285 ± 0.0006	2.31	0.0002	99.38	0.24	
0	7.50	0.0344 ± 0.0005	1.70	0.0001	99.72	0.30	
0	8.75	0.0406 ± 0.0005	1.38	0.0002	99.21	0.33	
0	10.00	0.0460 ± 0.0005	1.27	0.0002	100.71	0.31	
10.0	5.00	0.0220 ± 0.0004	2.02	0.0001	100.99	0.22	
10.0	6.25	0.0284 ± 0.0003	0.99	0.0001	99.18	0.54	
10.0	7.50	0.0342 ± 0.0002	0.73	0.0001	99.61	0.69	
10.0	8.75	0.0399 ± 0.0007	1.70	0.0002	100.22	0.09	
10.0	10.00	0.0459 ± 0.0004	0.88	0.0001	100.12	0.26	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 3.40: Selectivity of the method for the determination of PP in presence of HTZ in standard solutions by first- and second-derivative ratio-compensation method

Composition of solution (µg/ml)		Ratio	Mean ratio [a] absorbance value	Standard error	Coefficient of variation (%)
PP	HTZ				
5.0	0	D1(271) / D1(237)	0.101± 0.002	0.0006	1.78
7.5	0		0.101± 0.002	0.0005	1.49
10.0	0		0.101± 0.001	0.0004	1.26
12.5	0		0.101± 0.002	0.0005	1.39
15.0	0		0.103± 0.001	0.0003	0.89
-	-		0.101± 0.001 *	0.0004	0.78
10.0	6.25		0.102± 0.001	0.0003	0.94
5.0	0	D2(287) / D2(245.6)	0.109± 0.001	0.0004	1.18
7.5	0		0.110± 0.001	0.0004	0.59
10.0	0		0.110 ± 0.001	0.0001	0.34
12.5	0		0.109 ± 0.002	0.0005	1.43
15.0	0		0.109 ± 0.001	0.0004	1.22
-	-		0.109 ± 0.001 *	0.0002	0.37
10.0	6.25		0.109 ± 0.001	0.0004	1.18
5.0	0	D2(245.6) / D1(237)	0.508 ± 0.001	0.0013	0.81
7.5	0		0.509 ± 0.002	0.0007	0.46
10.0	0		0.509 ± 0.005	0.0014	0.89
12.5	0		0.510 ± 0.003	0.0010	0.64
15.0	0		0.510 ± 0.003	0.0011	0.68
-	-		0.509 ± 0.001 *	0.0005	0.19
10.0	6.25		0.509 ± 0.003	0.0009	0.60

[a]: Average of ten replicate determinations with standard deviation.

*: Average ratio of pure drug solutions.

Table 3.41: Selectivity of the method for the determination of HTZ in presence of PP in standard solutions by first- and second-derivative ratio-compensation method

Composition of solution (µg/ml)		Ratio	Mean ratio [a] absorbance value	Standard error	Coefficient of variation (%)
HTZ	PP				
5.00	0	D1(263) / D1(284)	0.574 ± 0.007	0.0022	1.21
6.25	0		0.574 ± 0.004	0.0014	0.75
7.50	0		0.576 ± 0.008	0.0024	1.33
8.75	0		0.577 ± 0.009	0.0027	1.50
10.00	0		0.576 ± 0.009	0.0029	1.60
-	-		0.575 ± 0.001 *	0.0006	0.23
6.25	10		0.575 ± 0.005	0.0015	0.84
5.00	0	D2(291) / D2(274)	0.624 ± 0.004	0.0014	0.69
6.25	0		0.624 ± 0.003	0.0008	0.42
7.50	0		0.625 ± 0.003	0.0008	0.41
8.75	0		0.626 ± 0.003	0.0011	0.54
10.00	0		0.624 ± 0.003	0.0009	0.49
-	-		0.624 ± 0.001 *	0.0003	0.10
6.25	10		0.622 ± 0.004	0.0011	0.58
5.00	0	D2(291) / D1(284)	0.424 ± 0.009	0.0029	2.17
6.25	0		0.424 ± 0.005	0.0016	1.22
7.50	0		0.425 ± 0.005	0.0017	1.28
8.75	0		0.425 ± 0.005	0.0017	1.23
10.00	0		0.424 ± 0.005	0.0017	1.28
-	-		0.424 ± 0.001 *	0.0002	0.10
6.25	10		0.422 ± 0.004	0.0013	1.00

[a]: Average of ten replicate determinations with standard deviation.

*: Average ratio of pure drug solutions.

Table 3.42: Regression analysis for the determination of PP and HTZ in standard solutions by second-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 283.6 nm for PP at 270.6 nm for HTZ)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* - test for correlation	
	PP	HTZ			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	5 - 15	0	Y = 8.81E-04.X - 2.89E-04	0.9999	5.81E-05	5.48E-06	4.30E-05	4.80E-04, 1.00E-04	8.63E-04, 8.98E-04	8.64E-04	122	3.18
Series B	5 - 15	6.25	Y = 8.94E-04.X - 4.03E-04	0.9998	1.09E-04	1.04E-05	8.19E-05	-7.70E-04, -4.10E-05	8.60E-04, 9.29E-04	8.71E-04	87	
Series C	0	5 - 10	Y = 4.80E-03.X - 1.75E-03	0.9996	6.31E-04	8.18E-05	3.25E-04	-3.76E-03, 2.53E-04	4.54E-03, 5.06E-03	4.63E-03	61	
Series D	10	5 - 10	Y = 4.76E-03.X - 1.58E-03	0.9998	4.03E-04	5.23E-05	2.08E-04	-2.87E-03, -2.99E-04	4.59E-03, 4.92E-03	4.60E-03	87	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 3.43: One-way ANOVA test for linearity of pure PP solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.45E-04	1.45E-04	0.053	3.71
Lack of fit	3	6.51E-09	2.17E-09		
Within line	10	4.12E-07	4.12E-08		
Total	14	1.45E-04			

*: at P = 0.05 level of significance.

Table 3.44: One-way ANOVA test for linearity of pure HTZ solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.08E-03	1.08E-03	0.422	3.71
Lack of fit	3	9.30E-07	3.10E-07		
Within line	10	7.34E-06	7.34E-07		
Total	14	1.09E-03			

*: at P = 0.05 level of significance.

Table 3.45: Validation report for the determination of PP and HTZ in standard solutions by second-derivative spectrophotometry

Analytical parameter	Results	
	PP (283.6 nm)	HTZ (270.6 nm)
Accuracy (%)	99.93 ± 0.58	99.77 ± 0.82
Precision (%)	99.33 99.33 100.00 100.50 100.50 RSD: 0.58	99.04 99.36 99.36 100.00 101.11 RSD: 0.82
Specificity	A 10 µg/ml of PP and 6.25 µg/ml of HTZ mixture solution will show an absorbance value (D2) of 0.008 ± 0.0001	A 6.25 µg/ml of HTZ and 10 µg/ml of PP mixture solution will show an absorbance value (D2) of 0.028 ± 0.0003
LOD (µg/ml)	0.15	0.21
LOQ (µg/ml)	0.49	0.68
Linearity (µg/ml)	5 - 35	5 - 20
Ruggedness (%)	99.93 ± 0.58	99.77 ± 0.82

Table 3.46: Results of the assay of pure drug admixtures and commercial formulation of PP and HTZ by first- and second-derivative spectrophotometry

Drug Name	Label Claim (mg/ tab.)	Recovery (%) [a]					F-test for Mean Recovery			
		ZCP Method		RC Method			Calc		Crit [b]	
							Samples	Methods	Samples[†]	Methods[‡]
PP		D2(263.2 nm)	D2 (283.6 nm)	D1(271) / D1 (237)	D2(287) / D2 (245.6)	D2(245.6) / D1(237)				
Pure drug admixture	-	99.63 ± 1.09	99.99 ± 0.71	100.26 ± 0.58	100.02 ± 0.67	99.86 ± 0.42				
Brand HX	40	99.70 ± 1.33	99.25 ± 0.97	99.78 ± 1.10	99.09 ± 0.68	100.06 ± 0.40	2.87	0.72	7.71	6.39
HTZ		D2(270.6 nm)	D2(297.6 nm)	D1(263) / D1(284)	D2(291) / D2 (274)	D2(291) / D1(284)				
Pure drug admixture	-	100.11 ± 0.48	99.73 ± 0.59	100.17 ± 0.49	99.76 ± 0.51	99.64 ± 0.45				
Brand HX	25	99.88 ± 0.89	99.73 ± 0.59	99.30 ± 1.41	100.13 ± 0.37	99.85 ± 0.64	0.23	0.28	7.71	6.39

[a] : Mean and standard deviation for six determinations.

[b] : Theoretical value of $F(1,4)^{\dagger}$ and $F(4,4)^{\ddagger}$ based on two-way ANOVA test at $P = 0.05$ level of significance.

3.9 SIMULTANEOUS DETERMINATION OF ATENOLOL AND AMLODIPINE BESYLATE IN COMBINED TABLET PREPARATIONS BY FIRST-DERIVATIVE SPECTROPHOTOMETRY

The combination of atenolol (ATL) and amlodipine besylate (AMD besylate) as a tablet preparation is widely used for treatment of hypertension and angina. There are various reports for the individual assay of ATL, which includes capillary zone electrophoresis [371], flow injection fluorimetry [372], ultraviolet [373], GC [374], and HPLC methods [375]. The various reports for estimation of ATL in combination of other drugs [214,215,350-352,376,377] including AMD [377] were also available. The last mentioned report employed a HPLC method for its determination. Similarly, the literature reports for individual assay of AMD includes HPLC [378,379] and colorimetric [380,381] method. There are also few reports for AMD in combination of other drugs [377, 382]. The B.P. 1993 and I.P. 1996 prescribe potentiometric titration method for ATL as pure drug and ultraviolet method for its determination from solid dosage forms. However, no such reports are available for AMD besylate in above three monographs. The objective of this work was to propose a first-derivative spectrophotometric method for estimation of these drugs from two combined tablet preparations without prior separation.

Materials and Reagents

Atenolol (Lupin, India) and amlodipine besylate (Torrent, India) were obtained as gift samples. Methanol (Qualigens, India) and hydrochloric acid (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solution of ATL was prepared by dissolving 5 mg of pure drug in 10 ml methanol. Appropriate amounts of the stock solution were transferred into 10 ml volumetric flasks and volumes were made up with 0.1M HCl to give a series of

solutions containing 15-30 μg/ml of ATL. Similarly, the stock solution of AMD was prepared by dissolving 6.93 mg of AMD besylate (equivalent to 5 mg of active AMD) in 10 ml methanol. Appropriate amounts of the stock solution were transferred to 10 ml volumetric flasks and volumes were made up with 0.1M HCl to give a series of solutions containing 2.5-10 μg/ml of AMD (present as AMD besylate). A two series of 10 ml mixture solutions were also prepared from the above stock solutions. The first-series contained a constant concentration of AMD (2.5 μg/ml) and a varying concentration of ATL (15-30 μg/ml). The second series contained a constant concentration of ATL (25 μg/ml) and a varying concentration of AMD (2.5-10 μg/ml).

Interaction Study

Two separate series of mixture solutions were also prepared from fresh stock solutions according to the above mentioned procedures. The first series contained a constant concentration of AMD (2.5 μg/ml) and a varying concentration of ATL (10-80 μg/ml). While, the second series contained a constant concentration of ATL (25 μg/ml) and a varying concentration of AMD (5-35 μg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of ATL (25 μg/ml) and AMD (2.5 μg/ml) were prepared from the fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of ATL (5-40 μg/ml) and AMD (1-60 μg/ml) were prepared from the stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing ATL (25 μg/ml) and AMD (2.5 μg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of each brand (IX and IY) were accurately weighed, well powdered and a powder weight equivalent to 25 mg of ATL (and 2.5 mg of AMD present as AMD besylate) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The samples were filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded. Appropriate volumes aliquots of filtrate were diluted with 0.1M HCl to give a concentration of 25 μg/ml of ATL and 2.5 μg/ml of AMD.

Procedure

The absorbances of sample and standard solutons of ATL and AMD were recorded from 220-300 nm against the blank solution. The first-derivative spectra for each set of ATL and AMD were subsequently recorded using $\Delta = 5$ points [138] and no smoothing was necessary. The absorbances of the solutions were measured at the zero-crossing wavelength of other drug in each instance.

Results and Discussion

The normal spectra of ATL and AMD were found to be overlapping (Figure 3.35). The methods discussed previously [5-13] could not be proposed as there was no significant separation of peaks in the range of measurement. The pH-induced difference spectrophotometric method [14] could not be adopted as a method of choice due to spectra of pure drugs looks similar in presence of acid and alkali buffer systems and thus it will not provide the required isosbestic points for the simultaneous determination. Hence, a first-derivative spectrophotometric method was adopted to resolve the minor component (AMD) in presence of major component (ATL) with spectral overlap. In addition, the zero-crossing points (ZCP's) of the pure drugs were nearer to the wavelength maxima of other drug, which facilitated their simultaneous determination (Figure 3.36).

The zero-crossing wavelengths considered for the determination from standard and sample solutions were at 239.0 nm for ATL, and at 250.0 and 273.4 nm for AMD. For simplicity, the measured values of AMD at 250.0 nm were only reported in tables related to method suitability (Tables 3.47, 3.48, 3.50 and 3.51). But, the recoveries obtained for mixture solutions at both the estimated wavelengths were reported in Table 3.52.

A series of pure drug (Table 3.47, Series A and C of Table 3.48) and mixture solutions (Table 3.47, Series B and D of Table 3.48) were examined at the zero-crossing wavelengths of the other drug to study the proportionality of derivative values to its concentrations. The regression equations obtained from such measured values (Table 3.47) were reported in Table 3.48. The derivative spectra obtained for mixture solutions demonstrated the presence of distinct isosbestic points at 250.0 and 273.4 nm (Figure 3.37) for ATL and at 239.0 nm (Figure 3.38) for AMD which suggested the non-interference of one drug in presence of other.

The mutual independence of analytical signals of standard solutions in the proposed concentration range was determined by a mixture interaction study. It was clear from the reported results (Figure 3.39) that the varying concentrations of ATL (10-80 μg/ml) did not interfere up to 65 μg/ml in the estimation of AMD (2.5 μg/ml) at 250.0 nm (Figure 3.39A). Similarly, the varying concentrations of AMD (5-35 μg/ml) did not interfere up to 20 μg/ml in the estimation of ATL (25 μg/ml) at 239.0 nm (Figure 3.39B). Thus, the earlier considered working concentration ranges for both drugs were ideal to obtain the accurate determinations.

The statistical analysis of data achieved by first-derivative method for standard solutions suggested that the mean derivative values along with standard deviation, coefficient of variation and the standard error were minimum and thus demonstrated that the proposed method was highly reproducible (Table 3.47). The percentage ratio

of residual values were found within 0.5% indicating that the calibration points were linear with the best-fit line in the proposed concentration range. This was confirmed also from the calculated F-values for non-linearity test [97] which were less than the critical value at 5% significance level (Table 3.47).

The linear calibration lines obtained for pure drugs and mixtures were similar. This was evident from the reported correlation coefficients and standard error involved with intercept, slope and the estimate (Table 3.48). A one-way ANOVA test [98] was also conducted with three replicate values per calibration point including the lowest and highest variation observed from the mean derivative values of each drug concentration during their replicate measurement. The calculated F-values were less than the theoretical value at 5% significance level (Tables 3.49 and 3.50) suggested the homogeneity of variance within the calibration lines.

The reported slope values without intercept on the ordinate were within the 95% confidence limits (Table 3.48) and thus demonstrated that regression equations of standard solutions did not deviate from the origin. In addition, the results of Student's *t*-test for correlation were also significant to indicate the positive correlation between measured values and concentrations.

The developed method was validated according to the procedures described in U.S.P. 23. The results of the study were reported in Table 3.51. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were obtained from the slope of regression equations (Series A and C of Table 3.48).

The percentage recoveries obtained with standard and sample solutions of ATL and AMD were reported in Table 3.52. The recoveries of ATL have met the official requirements (Appendix B). Whereas, the prescribed limits for AMD were not available in U.S.P. 23, B.P. 1993 and I.P. 1996, but the recoveries match with the

labeled claim. The calculated F-values of a one-way ANOVA test [98] were less than the critical value at 5% level and thus showed no significant differences between the recoveries of ATL solutions. Similarly, a two-way ANOVA test [98] for AMD solutions suggested that there were no differences between recoveries found at different zero-crossing wavelengths and also among the solutions measured at a particular ZCP (Table 3.52). The prepared solutions were stable for 3 hrs in 0.1M HCl.

Thus the proposed method succeeded in measuring the concentration of a minor component in a two-component mixture with overlapping spectra. The developed method was found to be accurate and precise. The rapidity and simplicity of the proposed method over reported method [377] makes it convenient for routine analysis of the combined preparations.

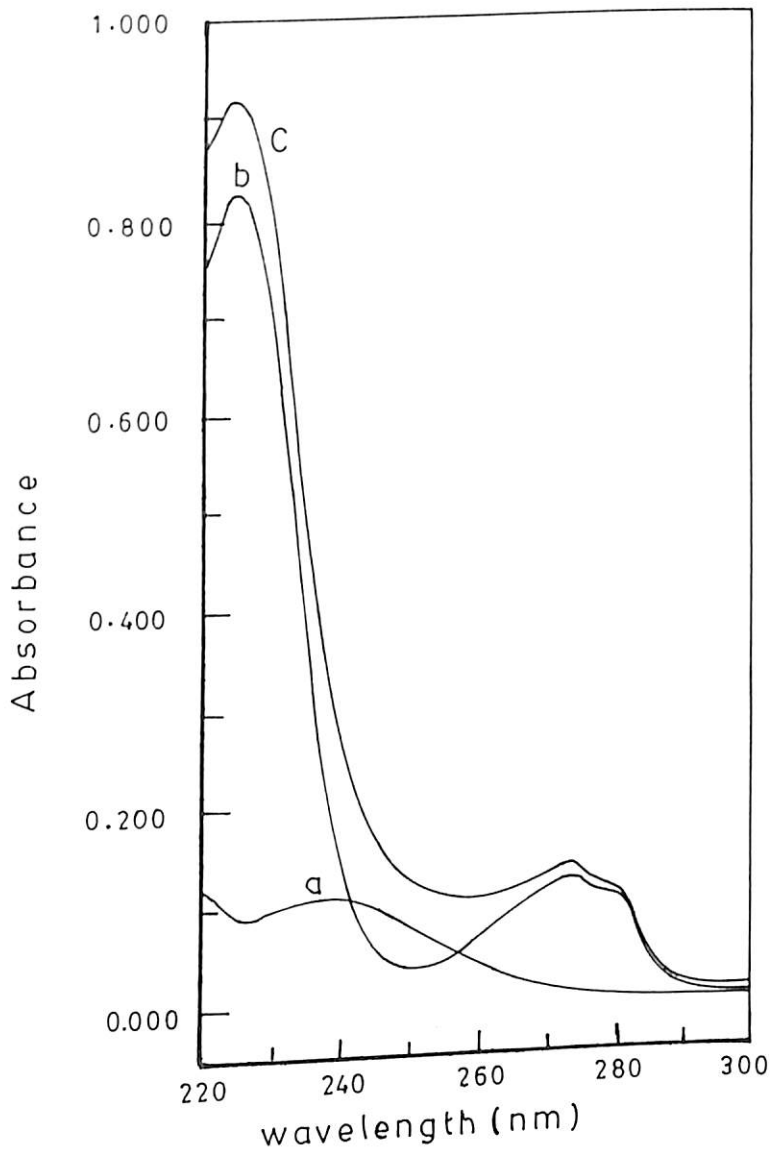

Figure 3.35 Absorption spectra of (a) amlodipine (2.5 µg/ml), (b) atenolol (25 µg/ml) and (c) their mixture in 0.1M HCl.

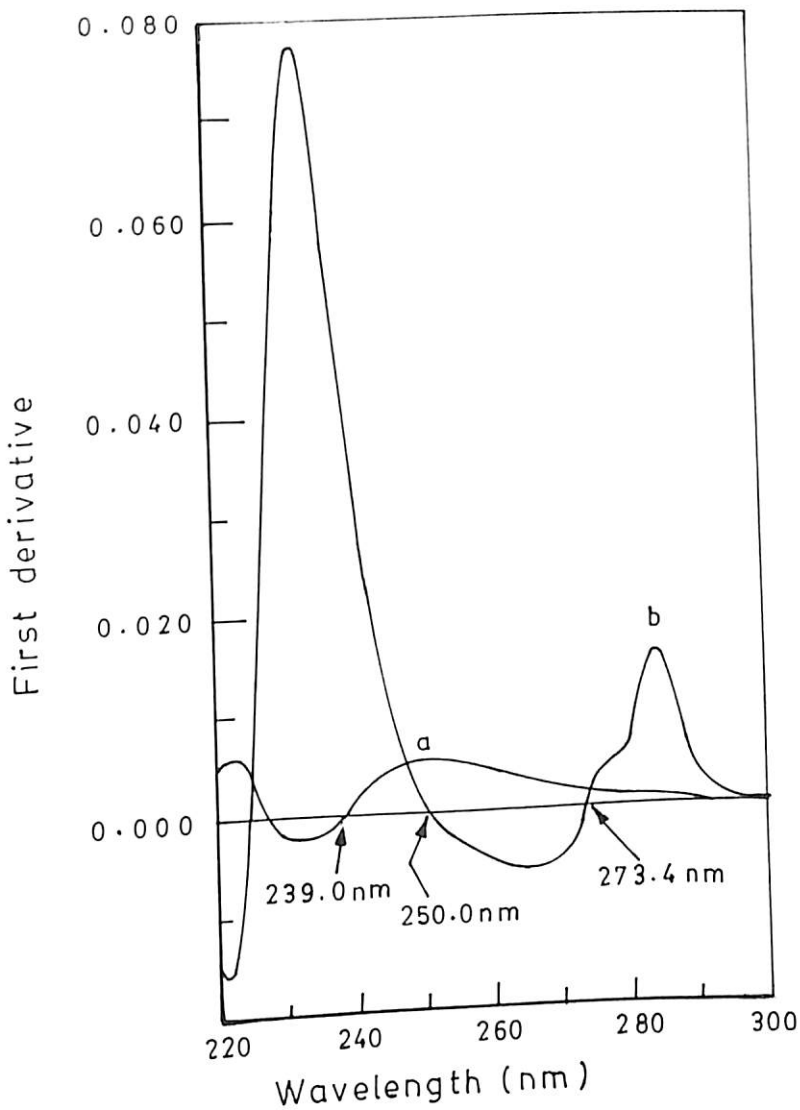

Figure 3.36 First-derivative spectra of (a) amlodipine (2.5 µg/ml) and (b) atenolol (25 µg/ml) in 0.1M HCl.

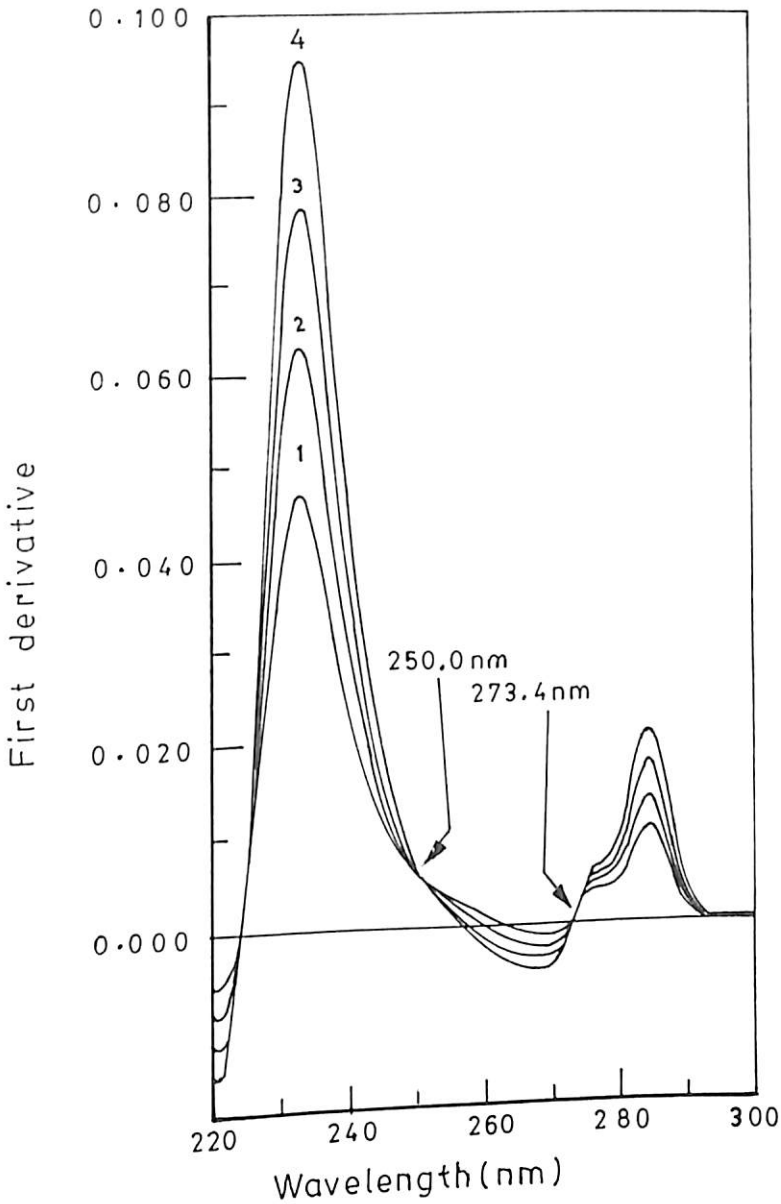

Figure 3.37 First-derivative spectra of atenolol (15, 20, 25 and 30 μg/ml) and amlodipine (2.5 μg/ml) in 0.1M HCl in curves 1-4, respectively.

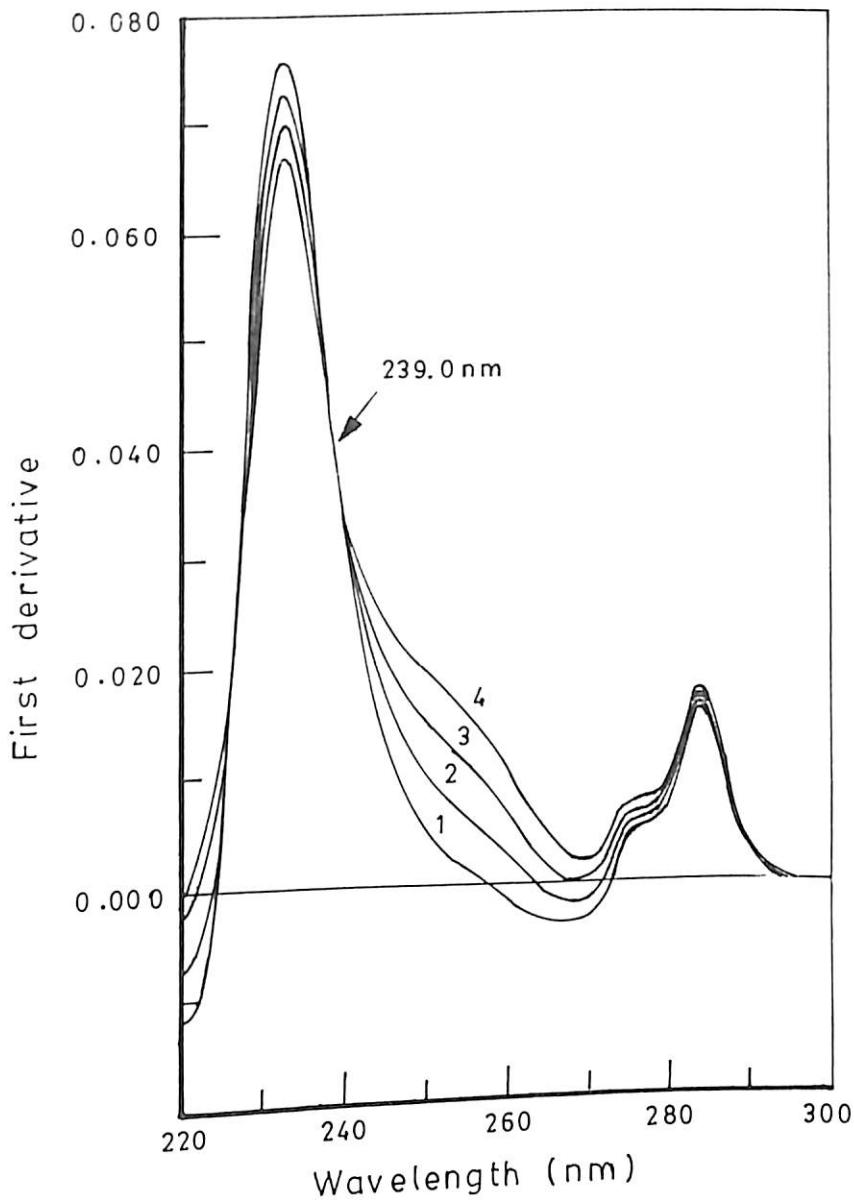

Figure 3.38 First-derivative spectra of amlodipine (2.5, 5, 7.5 and 10 μg/ml) and atenolol (25 μg/ml) in 0.1M HCl in curves 1-4, respectively.

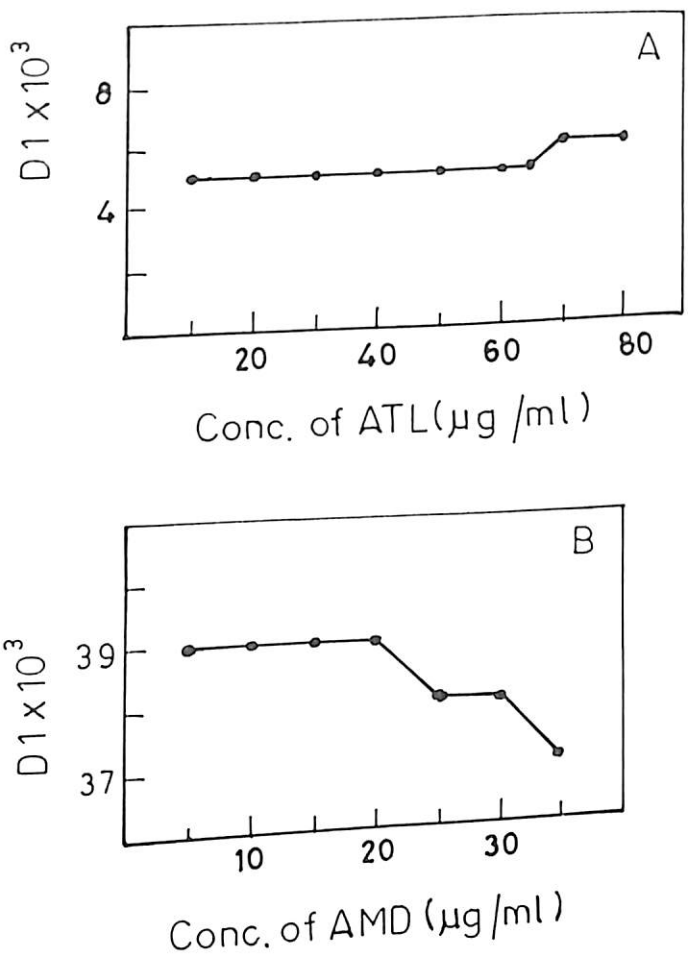

Figure 3.39 First-derivative interaction graphs for (A) amlodipine (2.5 µg/ml) in mixture with atenolol (at 250.0 nm) and (B) atenolol (25 µg/ml) in mixture with amlodipine (at 239.0 nm) in 0.1M HCl.

Table 3.47: Selectivity of the method for the simultaneous determination of ATL and AMD in standard solutions by first-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D1) (ATL at 239.0 nm, AMD at 250.0 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
ATL	AMD					Calc	Crit [b]
15	0	0.0233 ± 0.0006	2.37	0.0002	99.63	0.06	4.26
20	0	0.0311 ± 0.0009	2.56	0.0003	100.23	0.03	
25	0	0.0390 ± 0.0007	1.89	0.0002	100.30	0.03	
30	0	0.0472 ± 0.0001	2.40	0.0003	99.78	0.02	
15	2.5	0.0233 ± 0.0002	0.98	0.0001	99.53	0.40	
20	2.5	0.0310 ± 0.0004	1.48	0.0001	100.50	0.09	
25	2.5	0.0391 ± 0.0007	1.77	0.0002	99.99	0.04	
30	2.5	0.0470 ± 0.0008	1.70	0.0002	99.89	0.03	
0	2.5	0.0057 ± 0.0001	2.52	0.0001	99.12	0.26	4.26
0	5.0	0.0107 ± 0.0002	1.59	0.0001	100.42	0.19	
0	7.5	0.0156 ± 0.0003	2.04	0.0001	100.38	0.05	
0	10.0	0.0208 ± 0.0002	0.84	0.0001	99.73	0.18	
25	2.5	0.0057 ± 0.0001	2.57	0.0001	100.22	0.08	
25	5.0	0.0107 ± 0.0001	1.09	0.0001	100.05	0.14	
25	7.5	0.0158 ± 0.0004	2.40	0.0001	99.67	0.01	
25	10.0	0.0208 ± 0.0003	1.48	0.0001	100.15	0.02	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(2,9) at P = 0.05 level of significance.

Table 3.48: Regression analysis for the determination of ATL and AMD in standard solutions by first-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 239.0 nm for ATL at 250.0 nm for AMD)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* - test for correlation	
	ATL	AMD			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	15 -30	0	Y = 1.59E-03.X - 7.30E-04	0.9999	2.74E-04	1.18E-05	1.33E-04	-1.91E-03, 4.48E-04	1.54E-03, 1.64E-03	1.57E-03	100	4.30
Series B	15 -30	2.5	Y = 1.59E-03.X - 5.86E-04	0.9999	2.88E-04	1.24E-05	1.38E-04	-1.82E-03, 6.51E-04	1.53E-03, 1.64E-03	1.56E-03	100	
Series C	0	2.5 -10	Y = 2.00E-03.X - 6.25E-04	0.9999	9.19E-05	1.34E-05	7.46E-05	3.49E-05, 1.16E-03	1.93E-03, 2.10E-03	2.06E-03	100	
Series D	25	2.5 -10	Y = 2.00E-03.X - 7.10E-04	0.9999	5.47E-05	8.00E-06	4.37E-05	3.44E-04, 1.14E-03	1.94E-03, 2.10E-03	2.06E-03	100	

[a] : Based on four calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 2 d.f.

Table 3.49: One-way ANOVA test for linearity of pure ATL solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	9.45E-04	9.45E-04	0.087	4.46
Lack of fit	2	2.78E-07	1.39E-07		
Within line	8	1.28E-05	1.60E-06		
Total	11	9.58E-04			

*: at P = 0.05 level of significance.

Table 3.50: One-way ANOVA test for linearity of pure AMD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	3.75E-04	3.75E-04	0.614	4.46
Lack of fit	2	1.18E-07	5.92E-08		
Within line	8	7.71E-07	9.64E-08		
Total	11	3.76E-04			

*: at P = 0.05 level of significance.

Table 3.51: Validation report for the determination of ATL and AMD in standard solutions by first-derivative spectrophotometry

Analytical parameter	Results	
	ATL (239.0 nm)	AMD (250.0 nm)
Accuracy (%)	99.87 ± 0.76	99.73 ± 0.95
Precision (%)	99.23 99.23 99.61 100.25 101.02 RSD: 0.76	98.24 99.56 100.00 100.00 100.85 RSD: 0.95
Specificity	A 25 µg/ml of ATL and 2.5 µg/ml of AMD mixture solution will show an absorbance value (D1) of 0.039 ± 0.0007	A 2.5 µg/ml of AMD and 25 µg/ml of ATL mixture solution will show an absorbance value (D1) of 0.005 ± 0.0001
LOD (µg/ml)	0.25	0.11
LOQ (µg/ml)	0.84	0.37
Linearity (µg/ml)	5 - 40	1 - 60
Ruggedness (%)	99.87 ± 0.76	99.73 ± 0.95

Table 3.52: Results of the assay of pure drug admixtures and commercial formulations of ATL and AMD by first-derivative spectrophotometry

Drug Name	Label Claim (mg/tab.)	Recovery (%) [a]		F-test for Mean Recovery			
				Calc		Crit	
				Samples	Methods	Samples	Methods
ATL		D1(239.0 nm)					
Pure drug admixture	-	99.79 ± 0.85		2.69	-	3.89•	-
Brand IX	50	100.44 ± 0.44					
Brand IY	50	99.46 ± 0.66					
AMD		D1(250.0 nm)	D1(273.4 nm)				
Pure drug admixture	-	100.13 ± 0.63	98.94 ± 1.23	1.06	1.78	19.0†	18.5‡
Brand IX	5	99.99 ± 0.74	99.76 ± 0.22				
Brand IY	5	100.19 ± 0.83	100.17 ± 0.79				

[a] : Mean and standard deviation for five determinations.

• : Theoretical value of F(2,12) based on one-way ANOVA test at P = 0.05 level of significance.

†,‡ : Theoretical value of $F(2, 2)^{\dagger}$ and $F(1, 2)^{\ddagger}$ based on two-way ANOVA test at P = 0.05 level of significance.

3.10 SIMULTANEOUS DETERMINATION OF AMLODIPINE BESYLATE AND ENALAPRIL MALEATE IN COMBINED TABLET PREPARATIONS BY FIRST-DERIVATIVE SPECTROPHOTOMETRY

The combination of amlodipine besylate (AMD besylate) and enalapril maleate (EPM) in the form of tablet is widely used for the treatment of hypertension of mixed origin. There are various reports available for individual assay of AMD as single preparation [378-381] and in combination of other drugs [377,382] including the EPM [382]. The last mentioned report [382] employed a HPLC method for their determination. The literature reports available for the individual assay of EPM as pure drug includes ultraviolet [144,383], HPLC [384], IR [385] and colorimetric methods [386]. There are also reports available for estimation of EPM in combination of other drugs [210,353,387-389]. The U.S.P. 23 and I.P. 1996 both reported HPLC method for EPM as pure drug and from its solid dosage form. There are no such reports available for AMD in official monographs [1-3]. The objective of this work was to develop a simple and rapid first-derivative spectrophotometric method for determination of these drugs from combined tablet preparations.

Materials and Reagents

Amlodipine besylate (Torrent, India) and enalapril maleate (Nicholas, India) were obtained as gift samples. Methanol (Qualigens, India) and hydrochloric acid (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solutions of pure drugs were prepared separately by dissolving 5 mg of EPM and 6.93 mg of AMD besylate (equivalent to 5 mg of active AMD) in a 10 ml volumetric flasks and made up to volume with methanol. Appropriate volume aliquots of the stock solutions were transferred into 10 ml volumetric flask and volumes were made up with 0.1M HCl to give a series of solutions containing 5-15 µg/ml of AMD

(present as AMD besylate) and 10-20 µg/ml for EPM. Two series of 10 ml mixture solutions were also prepared from the stock solutions. The first series contained a constant concentration of EPM (10 µg/ml) and a varying concentration of AMD (5-15 µg/ml). Similarly, the second series contained a constant concentration of AMD (10 µg/ml) and a varying concentration of EPM (10-20 µg/ml).

Interaction Study

Two separate series of mixture solutions were also prepared from the fresh stock solutions according to the above mentioned procedures with each series contained a constant concentration of one drug (10 µg/ml) and a varying concentration of other drug (5-35 µg/ml).

Method Validation

a. *Precision and Accuracy*

Five separate standard and test solutions of AMD (10 µg/ml) and EPM (10 µg/ml) were prepared from the fresh stock solutions according to the above mentioned procedures.

b. *Linearity*

Separate series of solutions of AMD (2.5-25 µg/ml) and EPM (5-40 µg/ml) were prepared from the stock solutions meant for method validation.

c. *Specificity*

Series of five mixture solutions of each containing 10 µg/ml of AMD and EPM were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of commercial preparation (Brand JX) was accurately weighed, well powdered and a powder weight equivalent to 10 mg each of AMD and EPM was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The sample was filtered through Whatman filter paper No.1. The

first and last 5 ml of the filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with 0.1M HCl to give each a concentration of 10 μg/ml of AMD and EPM.

Procedure

The zero-order spectra of standard and sample solutions were recorded from 215-260 nm against the blank solution. The first-derivative spectra for each set of AMD and EPM solutions were subsequently recorded using Δ=10 points [138] and no smoothing was necessary. The absorbances of standard and sample solutions were measured at the selected wavelength of determinations in each instance.

Results and Discussion

The zero-order spectra of AMD and EPM were found to be overlapping (Figure 3.40). The methods discussed earlier [5-13] could not be proposed due to two reasons. The first being lack of significant wavelength maxima within the measured spectra of EPM in the proposed range of measurement and the other due to no spectral separation between measured pure drugs which were preliminary requirement for utilisation of above methods. The pH-induced differential spectrophotometric method could not be considered due to no significant differences between the recorded spectra of pure drugs in presence of acid and alkali buffer systems. Hence, a first-derivative spectrophotometric method was proposed to resolve such overlapping (Figure 3.41). The first-derivative processing of recorded spectra yielded zero-crossing points (ZCP's) only for AMD but not for the other drug. But, it can be observed that the absorption pattern of EPM becomes constant above 245.0 nm (Figure 3.41). This behaviour of spectra remains same in the proposed concentration range (10-20 μg/ml). Thus, the estimations of EPM solutions were performed at 226.6 nm over 239.0 nm due to greater absorbance value at former wavelength. Whereas, the estimations of AMD solutions were carried at its wavelength maxima of 251.0 nm as the interference

from other component of drug mixture was considered to be minimum and also remains constant at this wavelength (Figure 3.41).

A series of pure drug (Table 3.53, Series A and C of Table 3.54) and mixture solutions (Table 3.53, Series B and D of Table 3.54) were examined at the above suggested wavelengths to determine the proportionality existed between the measured derivative values and concentrations of drug solutions. The regression equations obtained from measured values (Table 3.53) were reported in Table 3.54. The first-derivative spectra obtained for the mixture solutions of pure drugs were showed in Figures 3.42 and 3.43. The presence of distinct isosbestic points at 226.6 and 239.0 nm, and followed by linearity at 251.0 nm showed that there were no interferences in the estimation of AMD in presence of EPM (Figure 3.42). Similarly, the constant absorption pattern at 251.0 nm and followed by linearity at 226.6 and 239.0 nm demonstrated the non-interference of AMD in the estimation of EPM (Figure 3.43).

As described in previous sections, a mixture interaction study was planned here also to study the mutual independence of analytical signals at their wavelengths of estimation. It was evident from reported results (Figure 3.44) that the varying concentrations of both AMD and EPM did not interfere in the estimation of other drug up to 25 μg/ml as shown in Figure 3.44. Thus, the chosen concentration ranges were ideal and accurate determinations were possible by maintaining a constant concentration of each 10 μg/ml for both AMD and EPM.

The statistical analysis of data obtained by first-derivative method for standard solutions demonstrated that the deviations associated with measured values like the standard deviation, coefficient of variation and the standard error were minimum and thus indicated the reproducibility of the proposed method (Table 3.53). The percentage ratio of residuals showed a random scatter of within 1% from the calibration line. The obtained values of F-test for non-linearity [97] suggested that such random scatter of

calibration points was not significant at 5% level which confirmed the linearity within the proposed range.

The linear calibration lines obtained for pure drugs and mixture solutions were reported in Table 3.54 and were similar. This similarity was also confirmed from the obtained correlation coefficients and the standard error involved with each intercept, slope, and the estimate. A one-way ANOVA test [98] was performed to confirm that there was homogeneity with variance throughout the calibration line. The test involved three replicates per calibration point includes the lowest and highest deviation observed from the mean value of each pure drug concentrations during their replicate measurements. The reported F-values were less significant at 5% level and thus strongly recommended the linearity within the proposed concentration range (Tables 3.55 and 3.56).

The reported slope value with zero intercept on the ordinate fall within the 95% confidence interval indicated that the intercept values did not deviate from the origin (Table 3.54). The reported Student's *t*-test values were highly significant at 5% level suggested the positive correlation between the measured values and concentrations of drugs (Table 3.54).

The proposed method was validated according to the procedures prescribed in U.S.P. 23 and thus obtained results were showed in Table 3.57. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated from the slope values of Series A and C of Table 3.54.

The percentage recoveries of standard and sample solutions of both drugs were reported in Table 3.58. The recovery values of EPM have met the monograph prescribed limits for pure drug and its solid dosage form (Appendix B). However, such limits were not available for AMD [1-3], but the obtained recoveries match well

with the labeled claim. The reported Student's *t*-test [98] values were not significant at 5% level (Table 3.58) confirmed that there were no significant differences between mean recoveries of standard and sample mixtures of both the drugs. The prepared drug solutions were stable for 3 hrs in presence of 0.1M HCl.

Thus proposed first-derivative method was found to be accurate, simple and precise. The rapidity of proposed method over reported method [382] makes it useful for their simultaneous determination from the combined preparations.

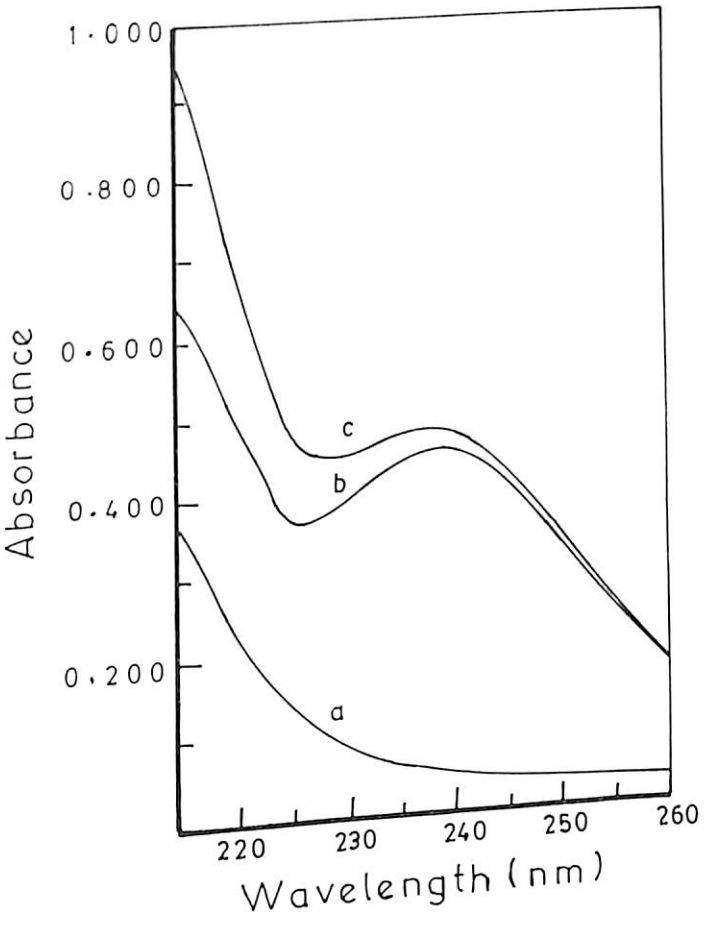

Figure 3.40 Absorption spectra of (a) enalapril maleate (10 µg/ml), (b) amlodipine (10 µg/ml) and (c) their mixture in 0.1 M HCl.

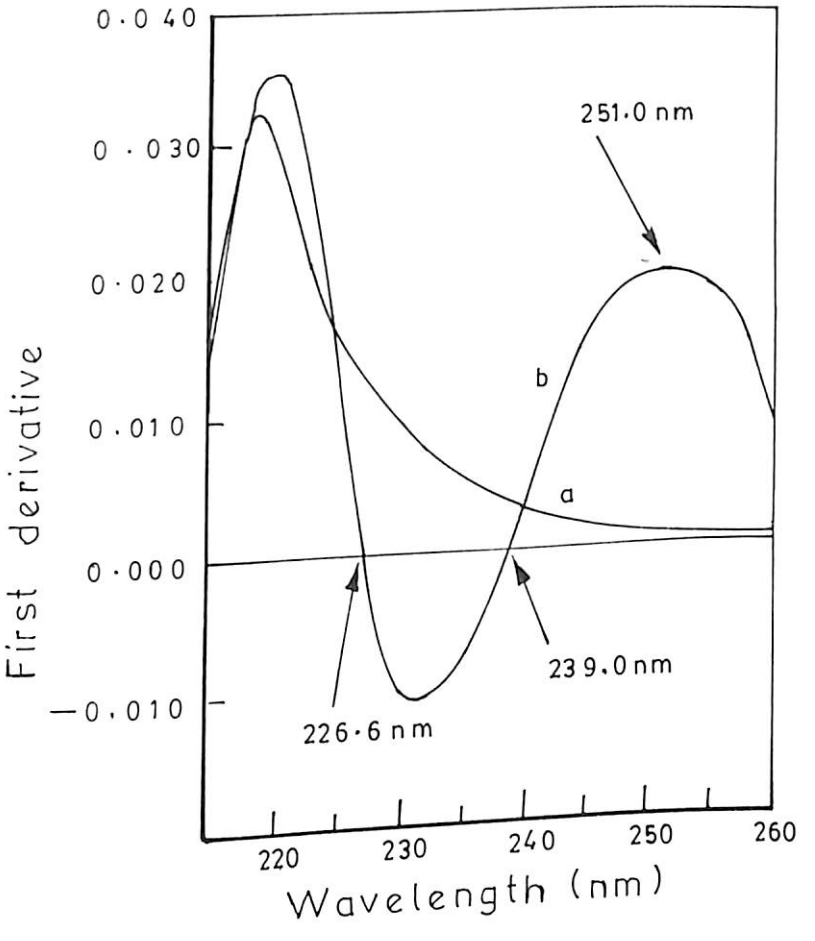

Figure 3.41 First-derivative spectra of (a) enalapril maleate (10 µg/ml) and (b) amlodipine (10 µg/ml) in 0.1M HCl.

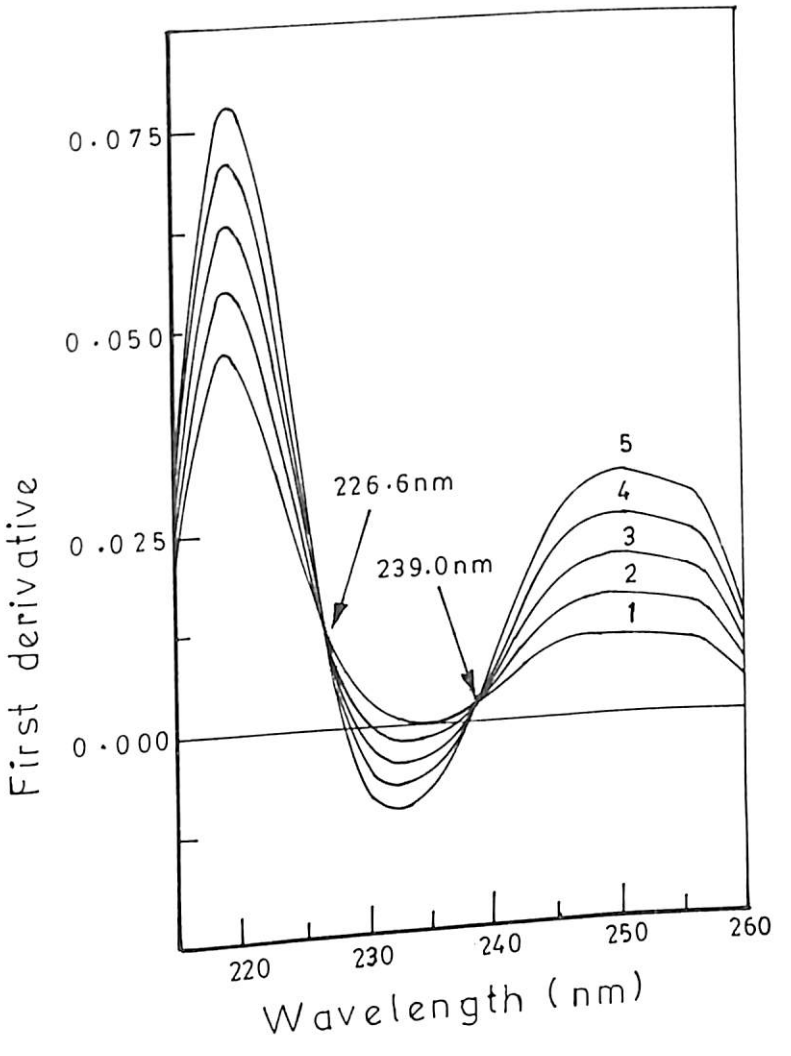

Figure 3.42 First-derivative spectra of amlodipine (5, 7.5, 10, 12.5 and 15 µg/ml) and enalapril maleate (10 µg/ml) in 0.1M HCl in curves 1-5, respectively.

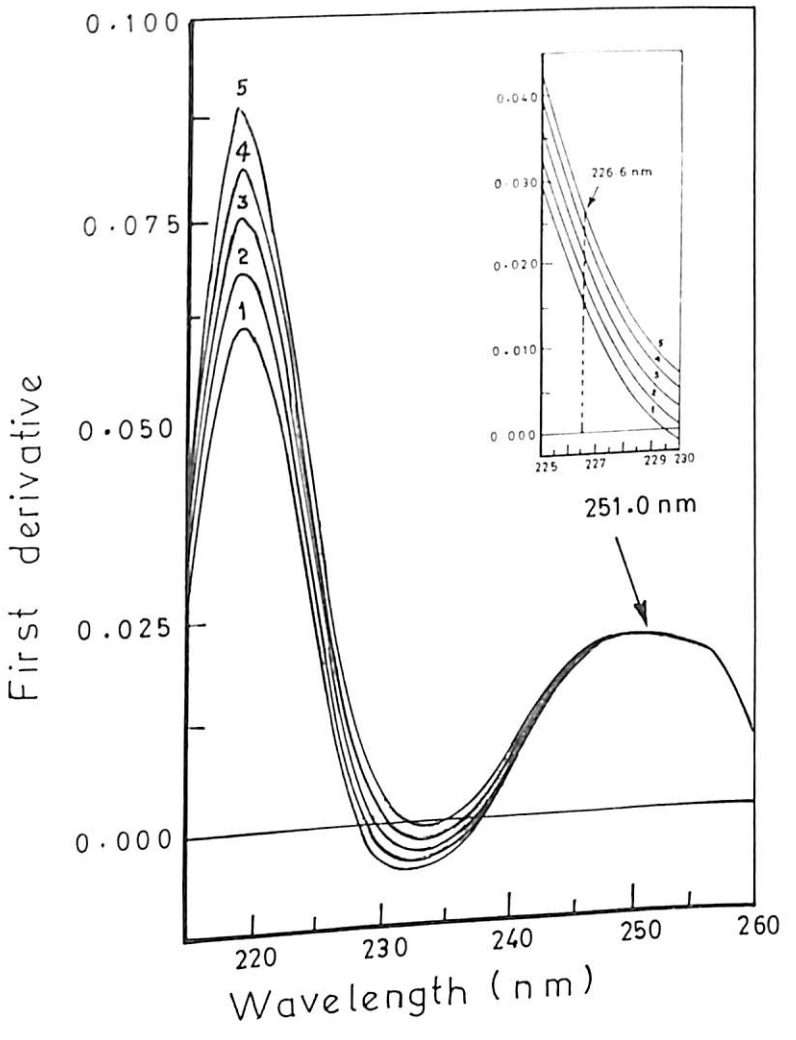

Figure 3.43 First-derivative spectra of enalapril maleate (10, 12.5, 15, 17.5 and 20 µg/ml) and amlodipine (10 µg/ml) in 0.1M HCl in curves 1-5, respectively.

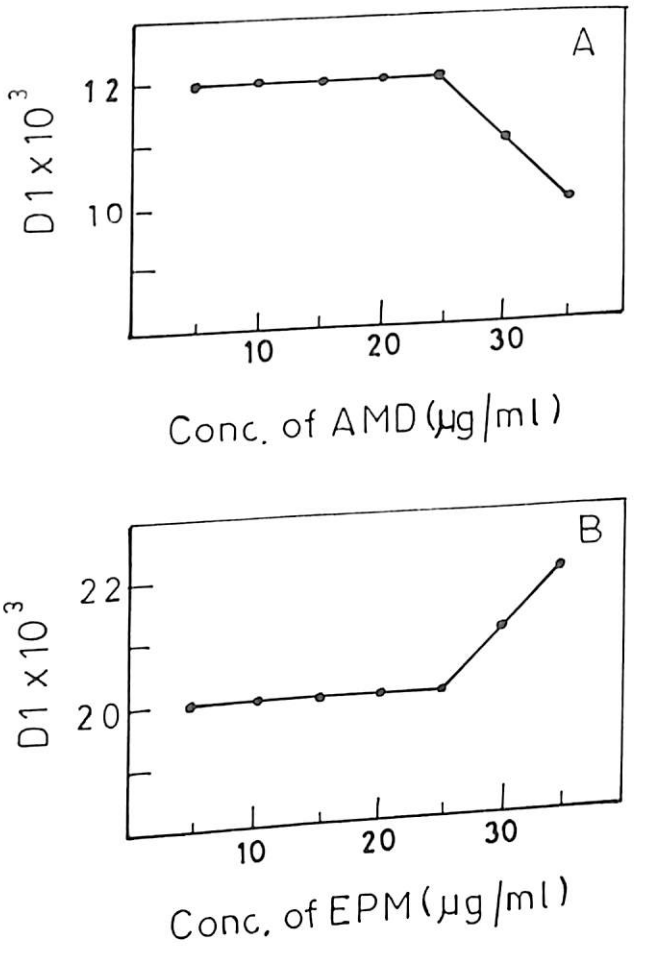

Figure 3.44 First-derivative interaction graphs for (A) enalapril maleate (10 µg/ml) in mixture with amlodipine (at 226.6 nm) and (B) amlodipine (10 µg/ml) in mixture with enalapril maleate (at 251.0 nm) in 0.1M HCl.

Table 3.53: Selectivity of the method for the simultaneous determination of AMD and EPM in standard solutions by first-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D1) (AMD at 251.0 nm, EPM at 226.6 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
AMD	EPM					Calc	Crit [b]
5.0	0	0.0099 ± 0.0002	2.18	0.0001	99.56	0.19	3.86
7.5	0	0.0148 ± 0.0002	1.60	0.0001	100.78	0.16	
10.0	0	0.0200 ± 0.0003	1.87	0.0001	99.78	0.07	
12.5	0	0.0251 ± 0.0005	2.14	0.0001	99.67	0.03	
15.0	0	0.0300 ± 0.0004	1.55	0.0001	100.18	0.04	
5.0	10.0	0.0105 ± 0.0002	2.61	0.0001	99.75	0.35	
7.5	10.0	0.0155 ± 0.0003	1.93	0.0001	100.38	0.30	
10.0	10.0	0.0207 ± 0.0004	2.00	0.0001	99.30	0.15	
12.5	10.0	0.0254 ± 0.0006	2.43	0.0002	100.83	0.07	
15.0	10.0	0.0308 ± 0.0006	2.03	0.0002	99.66	0.07	
0	10.0	0.0128 ± 0.0003	2.23	0.0001	99.21	0.13	3.86
0	12.5	0.0156 ± 0.0003	1.63	0.0001	100.38	0.16	
0	15.0	0.0185 ± 0.0004	2.08	0.0001	100.65	0.07	
0	17.5	0.0215 ± 0.0002	0.83	0.0001	99.91	0.33	
0	20.0	0.0246 ± 0.0003	1.31	0.0001	99.75	0.10	
10.0	10.0	0.0124 ± 0.0003	2.61	0.0001	100.26	0.21	
10.0	12.5	0.0154 ± 0.0003	2.56	0.0001	100.51	0.15	
10.0	15.0	0.0186 ± 0.0004	2.65	0.0001	99.45	0.09	
10.0	17.5	0.0217 ± 0.0006	2.89	0.0002	99.24	0.06	
10.0	20.0	0.0244 ± 0.0006	2.48	0.0001	100.63	0.06	

[a] : Average of ten determinations with standard deviation.
[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 3.54: Regression analysis for the determination of AMD and EPM in standard solutions by first-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 251.0 nm for AMD at 226.6 nm for EPM)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* - test for correlation	
	AMD	EPM			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	5 -15	0	Y = 2.02E-03.X - 2.44E-04	0.9999	1.29E-04	1.22E-05	9.57E-05	-6.55E-04, 1.66E-04	1.98E-03, 2.06E-03	2.01E-03	122	3.18
Series B	5 -15	10	Y = 2.02E-03.X + 3.62E-04	0.9998	2.19E-04	2.07E-05	1.64E-04	-3.36E-04, 1.06E-03	1.95E-03, 2.09E-03	2.04E-03	87	
Series C	0	10 -20	Y = 1.18E-03.X + 8.60E-04	0.9998	2.01E-04	1.30E-05	1.03E-04	2.22E-04, 1.50E-03	1.14E-03, 1.23E-03	1.23E-03	87	
Series D	10	10 -20	Y = 1.21E-03.X + 2.90E-04	0.9996	2.95E-04	1.91E-05	1.51E-04	-6.48E-04, 1.22E-03	1.15E-03, 1.28E-03	1.23E-03	61	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 3.55: One-way ANOVA test for linearity of pure AMD solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	7.77E-04	7.77E-04	0.064	3.71
Lack of fit	3	6.27E-08	2.09E-08		
Within line	10	3.27E-06	3.27E-07		
Total	14	7.80E-04			

* : at P = 0.05 level of significance.

Table 3.56: One-way ANOVA test for linearity of pure EPM solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	2.55E-04	2.55E-04	0.093	3.71
Lack of fit	3	5.46E-08	1.82E-08		
Within line	10	1.96E-06	1.96E-07		
Total	14	2.57E-04			

* : at P = 0.05 level of significance.

Table 3.57: Validation report for the determination of AMD and EPM in standard solutions by first-derivative spectrophotometry

Analytical parameter	Results	
	AMD (251.0 nm)	EPM (226.6 nm)
Accuracy (%)	100.20 ± 0.76	100.11 ± 0.63
Precision (%)	99.50 99.50 100.00 101.00 101.00 RSD: 0.76	99.68 99.68 100.00 100.00 101.20 RSD: 0.63
Specificity	A 10 µg/ml of both AMD and EPM mixture solution will show an absorbance value (D1) of 0.020 ± 0.0004	A 10 µg/ml of both EPM and AMD mixture solution will show an absorbance value (D1) of 0.012 ± 0.0003
LOD (µg/ml)	0.14	0.25
LOQ (µg/ml)	0.47	0.82
Linearity (µg/ml)	2.5 - 25	5 - 40
Ruggedness (%)	100.20 ± 0.76	100.11 ± 0.63

Table 3.58: Results of the assay of pure drug admixtures and commercial formulation of AMD and EPM by first-derivative spectrophotometry

Sample	Label Claim (mg/tab.)		Recovery (%) [a]		Student *t*-test for Mean Recovery		
					Calc		Crit [b]
	AMD	EPM	AMD (251.0 nm)	EPM (226.6 nm)	AMD	EPM	(AMD / EPM)
Pure drug admixture	-	-	99.92 ± 1.21	99.48 ± 1.45	0.40	0.83	2.23
Brand JX	5	5	100.15 ± 0.77	100.06 ± 0.92			

[a] : Mean and standard deviation for six determinations.
[b] : Theoretical value of '*t*(two-sided)' at P = 0.05 level of significance with 10 d.f.

3.11 SIMULTANEOUS DETERMINATION OF ATENOLOL, AMILORIDE HYDROCHLORIDE AND HYDROCHLOROTHIAZIDE IN COMBINED FORMULATIONS BY FIRST-DERIVATIVE SPECTROPHOTOMETRY

The combined formulation of atenolol (ATL), amiloride HCl (AMH) and hydrochlorothiazide (HTZ) is available in the form of a tablet or capsule, is widely used for moderate to severe hypertension not controlled by a single antihypertensive agent. The official monographs have prescribed the procedures for individual assay of ATL [2,3], AMH [1-3], HTZ [1-3] as well as AMH and HTZ combination [1]. The reported official methods of determination for AMH include titrimetric method [1] and potentiometric titration [2,3] for pure drug, and HPLC [1] and ultraviolet [2,3] method for the assay from its solid dosage form. The details of reported methods including official methods for HTZ and ATL as a pure drug [1-3,41,343-346,371-375] and in combination of other drugs [1,129,205-211,214,215,347-360,376,377] were discussed in earlier sections of this chapter. There are also various individual reports available for the assay of AMH by ultraviolet [390] and fluorimetry [391]. The various reports available for estimation of AMH in combination of other drugs [351,352,392,393] including ATL and HTZ [351,352]. The last two mentioned reports employed HPLC [351,352] and multi-component analysis (MA) method [352] for the determination of the three-component mixture. The objective of this present work was to propose a simple and rapid first-derivative spectrophotometric method for the quantitative determination of these drugs in the presence of each other as well as the excipients.

Materials and Reagents

Atenolol (Lupin, India), amiloride HCl (Hoechst, India) and hydrochlorothiazide (Lupin, India) were obtained as gift samples. Methanol (Qualigens, India) and hydrochloric acid (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solutions of pure ATL, AMH and HTZ were prepared by dissolving 5 mg of each of the pure drugs in 10 ml of methanol. Appropriate volume aliquots of ATL and HTZ were transferred into 10 ml volumetric flasks. The volumes were made up with 0.1M HCl to give a series of equimolar solutions containing 15-30 μg/ml of ATL and 7.5-15 μg/ml of HTZ. The dilutions of AMH were prepared by transferring 5 ml of above prepared stock solution into a 10 ml volumetric flask and diluted to volume with methanol to give a second stock solution. Appropriate volume aliquots of AMH were transferred from the second stock solution into 10 ml volumetric flask and volumes were made up with 0.1M HCl to give a series of solutions containing 1.25-5.0 μg/ml of AMH.

Similarly, three series of 10 ml mixtures of each were also prepared in 0.1M HCl from the stock solutions. The first series contained a constant concentration of HTZ (12.5 μg/ml), AMH (1.25 μg/ml) and a varying concentration of ATL (15-30 μg/ml). While, the second series contained a constant concentration of ATL (25 μg/ml), HTZ (12.5 μg/ml) and a varying concentration of AMH (1.25-5.0 μg/ml). Finally, the third series contained a constant concentration of ATL (25 μg/ml), AMH (1.25 μg/ml) and a varying concentration of HTZ (7.5-15 μg/ml).

Interaction Study

Three separate series of mixture solutions were also prepared from fresh stock solutions by considering a mixture of two drugs with a constant concentration and the other as the second component with a varying concentration as showed in Figure 3.50.

Method Validation

a. *Accuracy and Precision*

Five separate standard and test solutions of ATL (25 µg/ml), AMH (1.25 µg/ml) and HTZ (12.5 µg/ml) were prepared from the fresh the stock solutions according to the above mentioned procedures.

b. *Linearity*

Separate series of solutions of ATL (5-60 µg/ml), AMH (1.25-30 µg/ml) and HTZ (5-30 µg/ml) were prepared from the stock solutions meant for method validation.

c. *Specificity*

Series of five mixture solutions of each containing ATL (25 µg/ml), AMH (1.25 µg/ml) and HTZ (12.5 µg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets (or capsules) of commercial preparation (Brand KX and KY) were accurately weighed, well powdered (or mixed) and a powder weight equivalent to 25 mg of ATL (corresponds to 1.25 mg of AMH and 12.5 mg of HTZ) was dissolved in methanol by thorough mixing and diluted to volume in a 50 ml volumetric flask. The samples were filtered separately through Whatman filter paper No.1. The first and last 5 ml of each filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with 0.1M HCl to give samples with a concentration of 25 µg/ml of ATL and corresponding concentrations of 1.25 µg/ml of AMH and 12.5 µg/ml of HTZ.

Procedure

The absorbances of standard and sample solutions were measured in the range of 220-380 nm against a blank solution. The first-derivative spectra were recorded by using $\Delta = 15$ points [138] and no smoothing of spectrum was found necessary. The above prepared solutions were measured at the zero-crossing wavelengths of other two drugs.

Results and Discussion

The zero-order spectra of pure drugs were found to be overlapping (Figure 3.45) which makes their simultaneous determination difficult by the methods discussed earlier [5-13]. Apart from that, there were no successful reports available for handling any three-component mixtures for the quantitative determination by these methods [5-12]. A graphical plot method [13] was suggested earlier for the problems related to three-component mixtures, but such methods were time consuming and lack precision. The pH-induced difference spectrophotometric method could not be used due to lack of literature support for resolving three-component mixture and also as the solubility of pure AMH will decrease with an increase in pH of the solution [394]. Therefore, 0.1M HCl was used for diluting the pure drugs and mixture solutions. A first-derivative spectrophotometric method was considered for resolving the overlapping due to the presence of ideal zero-crossing points (ZCP's) for their simultaneous determination. The first-derivative wavelengths considered were 375.0 nm for AMH, 308.6 nm for HTZ and 243.6 nm for ATL (Figure 3.46). The negligible absorption of ATL and HTZ at a wavelength of 375.0 nm made it easier for the estimation of AMH. The wavelength 308.6 nm being a ZCP for ATL and AMH did not interfere in the measurement of HTZ at this wavelength. In a similar manner, a wavelength of 243.6 nm was considered for the determination of ATL being a ZCP for HTZ. At this wavelength, the AMH did not show any appreciable absorption at a selected concentration level of 1.25 μg/ml and thus its contribution to the absorption of the mixture was considered to be negligible (Figure 3.46).

The proportionality between the measured derivative values and corresponding concentrations of drugs were examined at the selected zero-crossing wavelengths by measuring a series of pure drug (Table 3.59, Series A, C and E of Table 3.60) and mixture solutions (Table 3.59, Series B, D and F of Table 3.60). The regression equations obtained with mean derivative absorbance values for pure drugs and their mixtures were showed in Table 3.60. The first-derivative curves obtained for the

above mixture solutions were presented in Figures 3.47-3.49. The presence of distinct isosbestic points (Figures 3.48 and 3.49), the constant derivative value at the zero-crossing wavelengths of drug in determination (Figures 3.47-3.49) and followed by linearity at the wavelength of its determination (Figures 3.47-3.49) showed that there were no interferences in the estimation of one drug in presence of other drugs and thus confirmed the proportional relationship between them.

The mixture interaction studies of above three mixtures were conducted at a single wavelength due to lack of absorbance shown by either one drug or more at other selected wavelengths of determination (Figure 3.46). It was observed from reported mixture interaction study that the non-interference region for varying concentrations of AMH, HTZ and ATL were existed up to 2.5, 15 and 40 µg/ml respectively at 243.6 nm (Figure 3.50). Thus, the earlier proposed concentration ranges for the standard and sample solutions were appropriate for their accurate determinations.

A detailed statistical analysis of the data obtained by above methods for standard solutions suggested that, the mean derivative absorbance values obtained for the replicates of standard solutions and their related deviations were small including the coefficient of variation and the standard error. Thus it indicated the precision of the method (Table 3.59). The percentage ratio of residuals showed a deviation of within 1% about the calibration line but from the values obtained through F-test for non-linearity [97] suggested that such deviation about the line was not significant at 5% level and thus demonstrated the linearity of calibration points.

The linear regression equations together with correlation coefficients and standard error involved with each intercept, slope and estimate were reported in Table 3.60. The similarity obtained between regression equations of pure drugs and mixture solutions suggested no interferences in the estimation of one drug in presence of other. The homogeneity of variance throughout the calibration line was studied by carrying

out a one-way ANOVA test [98] with three replicates per calibration point. The values included were the lowest and highest variation observed from the mean derivative value of each pure drug concentration during the replicate measurement of standard solutions (Table 3.59). The reported F-values suggested that variance seen within the calibration line was not significant at 5% level and thus showed the linearity within the proposed concentration range (Tables 3.61-3.63).

Further, the reported slope values without intercept on the ordinate for ATL and AMH solutions fall within 95% confidence limits, which suggested that the intercepts of these regression equations did not deviate from the origin (Table 3.60). However, such reported slope values for HTZ solutions required modification of 95% confidence limits to 99.8% for both pure drug (Slope: 3.23E-04 to 4.62E-04) and mixture (Slope: 3.22E-04 to 4.73E-04). The obtained values of Student's *t*-test [98] were relatively higher compared to the theoretical value at 5% significance level (Table 3.60) suggested the positive correlation between measurements and concentrations of all three drugs.

The proposed method was validated according to the procedures described in U.S.P. 23 and the results obtained were reported in Table 3.64. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated based on the slope of regression equations obtained in Series A, C and E of Table 3.60.

The percentage recovery values obtained for standard solutions and for commercial preparations with the proposed method were reported in Table 3.65. It was clearly evident from the recoveries that they have met the limits recommended by monographs [1-3] for the individual assay of these drugs (Appendix B). The obtained F-values of a one-way ANOVA test [98] were less than the critical value at 5% level suggested that there were no significant variations between the mean recovery values

of standard solutions and commercial preparations (Table 3.65). The prepared solutions were stable for 3 hrs in 0.1M HCl.

Thus, the above results demonstrated that the proposed derivative method could be successfully utilised for the simultaneous estimation of three-component commercial drug mixture without any interference. The developed method was found to be accurate and precise. The simplicity and rapidity of developed method compared to other reported methods [351,352] will make it convenient for the routine analysis of pharmaceutical dosage forms.

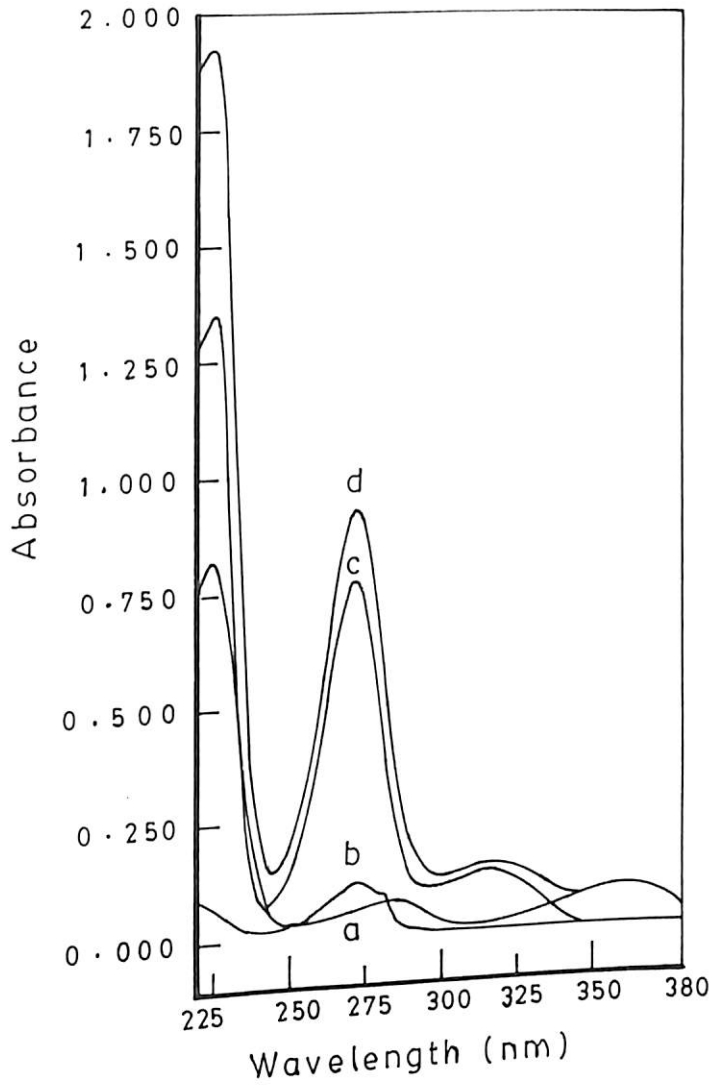

Figure 3.45 Absorption spectra of (a) amiloride HCl (1.25 µg/ml), (b) atenolol (25 µg/ml), (c) hydrochlorothiazide (12.5 µg/ml) and (d) their mixture in 0.1M HCl.

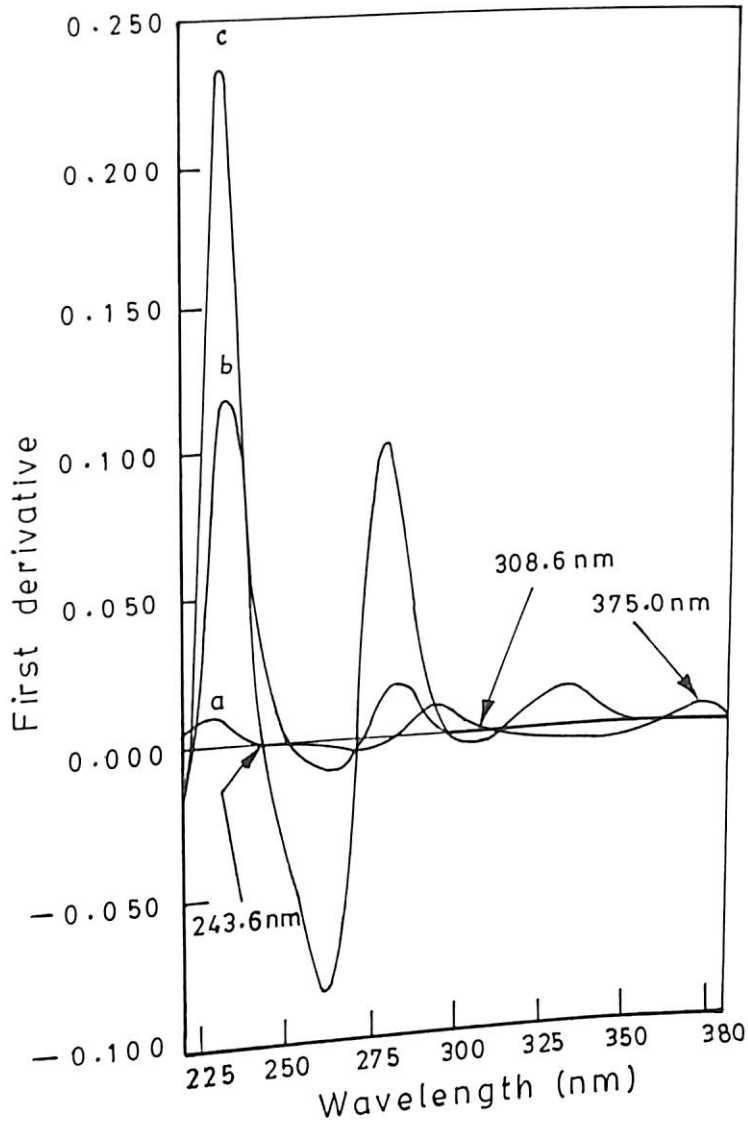

Figure 3.46 First-derivative spectra of (a) amiloride HCl (1.25 µg/ml), (b) atenolol (25 µg/ml) and (c) hydrochlorothiazide (12.5 µg/ml) in 0.1M HCl.

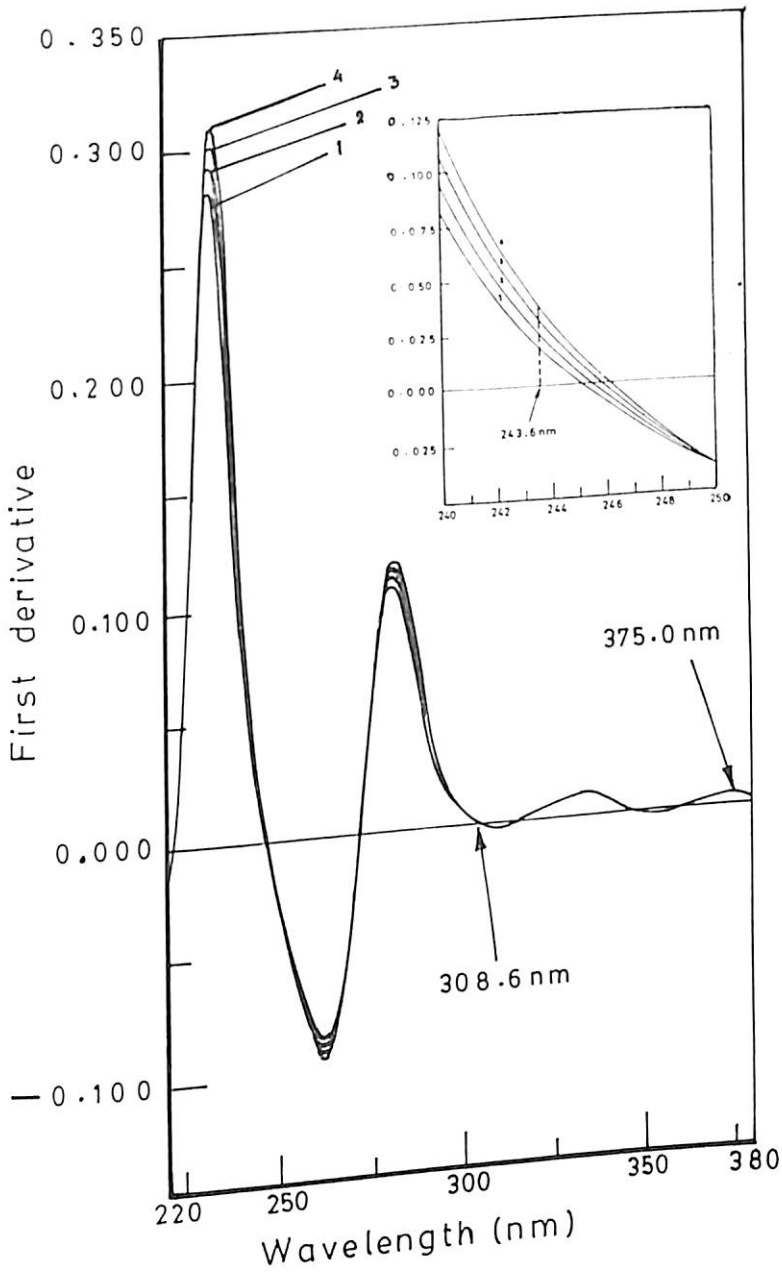

Figure 3.47 First-derivative spectra of atenolol (15, 20, 25 and 30 μg/ml; in curves 1-4, respectively) with a constant concentration of amiloride HCl (1.25 μg/ml) and hydrochlorothiazide (12.5 μg/ml) in 0.1M HCl.

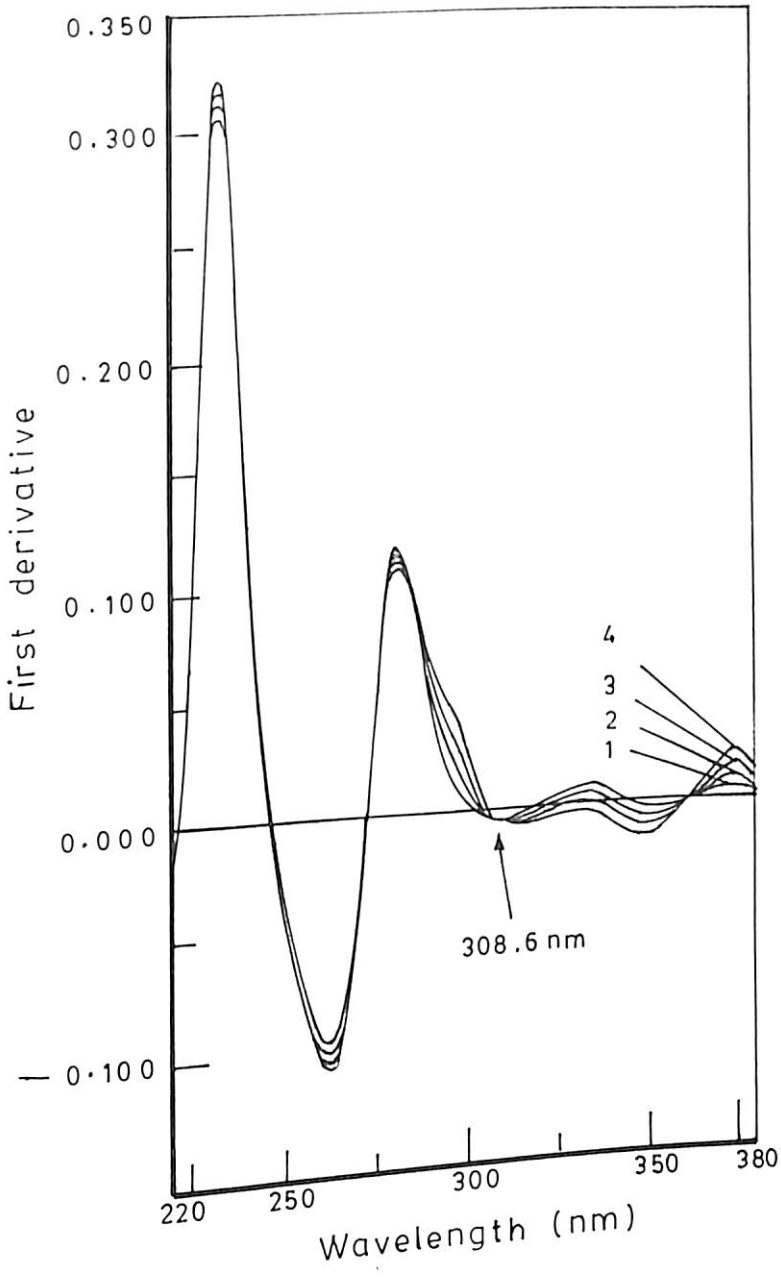

Figure 3.48 First-derivative spectra of amiloride HCl (1.25, 2.5, 3.75 and 5.0 μg/ml; in curves 1-4, respectively) with a constant concentration of atenolol (25 μg/ml) and hydrochlorothiazide (12.5 μg/ml) in 0.1M HCl.

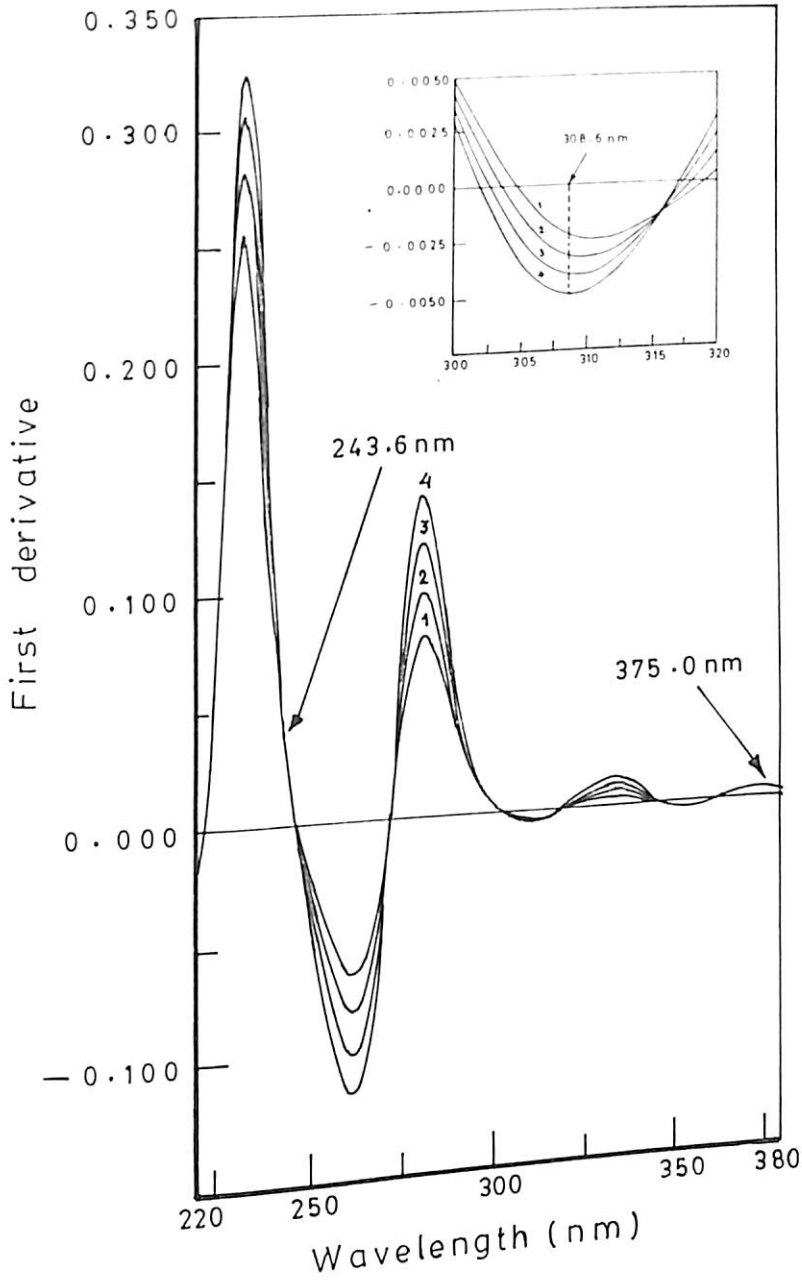

Figure 3.49 First-derivative spectra of hydrochlorothiazide (7.5, 10.0, 12.5 and 15.0 µg/ml; in curves 1-4, respectively) with a constant concentration of atenolol (25 µg/ml) and amiloride HCl (1.25 µg/ml) in 0.1M HCl.

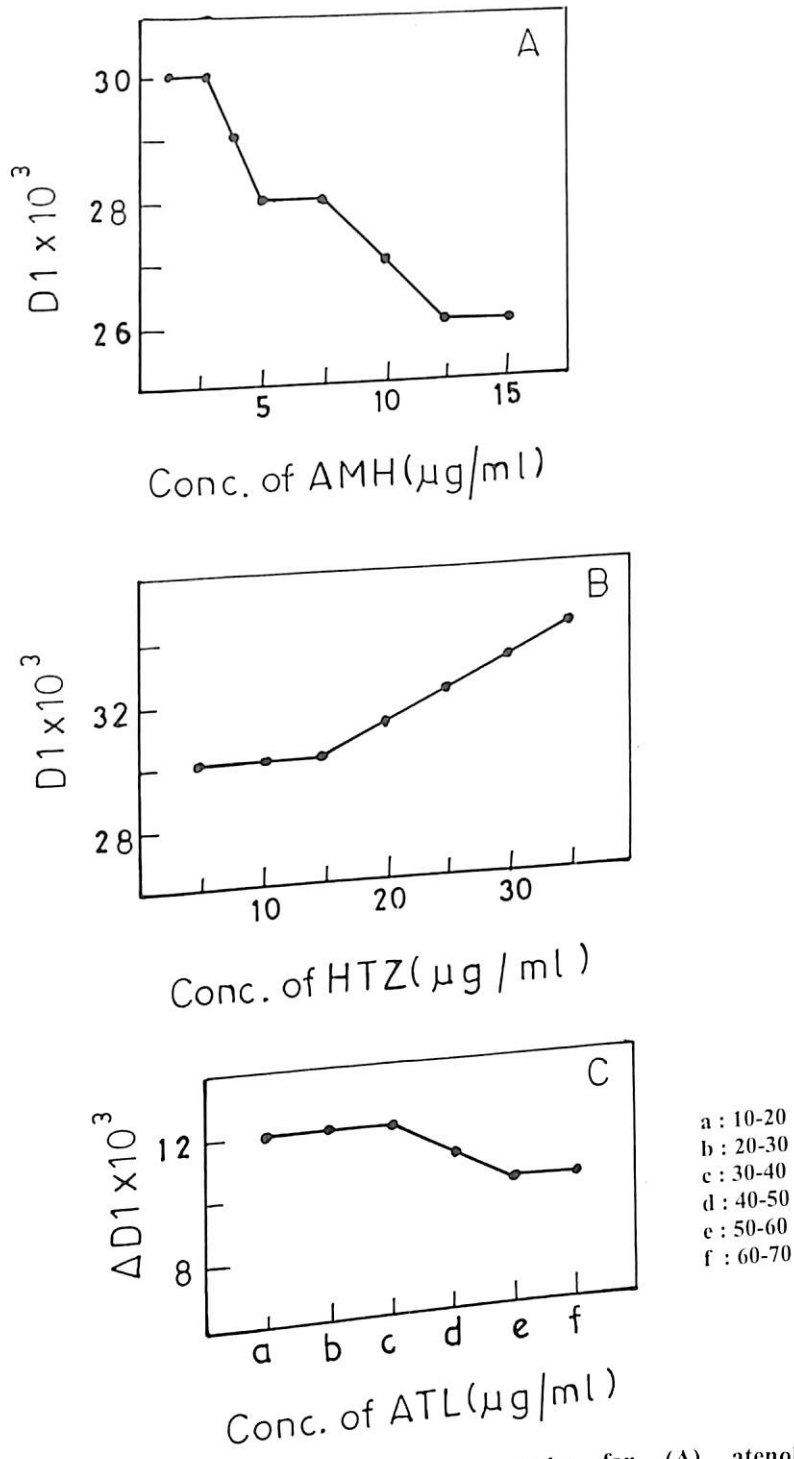

Figure 3.50 First-derivative interaction graphs for (A) atenolol (25 µg/ml) and hydrochlorothiazide (12.5 µg/ml) in mixture with amiloride HCl (at 243.6 nm), (B) atenolol (25 µg/ml) and amiloride HCl (1.25 µg/ml) in mixture with hydrochlorothiazide (at 243.6 nm) and (C) amiloride HCl (1.25 µg/ml) and hydrochlorothiazide (12.5 µg/ml) in mixture with atenolol (at 243.6 nm) in 0.1M HCl.

Table 3.59: Selectivity of the method for the simultaneous determination of ATL, AMH and HTZ in standard solutions by first-derivative spectrophotometry

Composition of the solution (µg/ml)			Mean absorbance [a] value (D1) (ATL at 243.6 nm, AMH at 375.0 nm, HTZ at 308.6 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
ATL	AMH	HTZ					Calc	Crit [b]
15	0	0	0.0180 ± 0.0003	1.59	0.0001	100.11	0.01	4.26
20	0	0	0.0241 ± 0.0005	1.94	0.0001	99.83	0.01	
25	0	0	0.0300 ± 0.0007	2.35	0.0002	100.06	0.01	
30	0	0	0.0361 ± 0.0007	1.96	0.0002	99.99	0.01	
15	1.25	12.5	0.0181 ± 0.0004	2.12	0.0001	99.82	0.01	
20	1.25	12.5	0.0240 ± 0.0003	1.35	0.0001	100.15	0.02	
25	1.25	12.5	0.0300 ± 0.0005	1.67	0.0001	100.08	0.01	
30	1.25	12.5	0.0361 ± 0.0006	1.69	0.0002	99.92	0.01	
0	1.25	0	0.0050 ± 0.0001	1.44	0.0001	100.65	0.39	4.26
0	2.50	0	0.0100 ± 0.0002	1.98	0.0001	99.65	0.05	
0	3.75	0	0.0150 ± 0.0002	1.55	0.0001	99.81	0.04	
0	5.00	0	0.0199 ± 0.0003	1.44	0.0001	100.15	0.02	
25	1.25	12.5	0.0050 ± 0.0001	0.47	0.0001	100.62	3.06	
25	2.50	12.5	0.0101 ± 0.0001	1.45	0.0001	99.64	0.08	
25	3.75	12.5	0.0151 ± 0.0001	0.87	0.0001	99.85	0.10	
25	5.00	12.5	0.0200 ± 0.0004	1.89	0.0001	100.13	0.01	
0	0	7.5	0.0019 ± 0.0001	0.61	0.0001	99.40	1.63	4.26
0	0	10.0	0.0029 ± 0.0001	1.99	0.0001	100.58	0.07	
0	0	12.5	0.0039 ± 0.0001	2.34	0.0001	100.02	0.03	
0	0	15.0	0.0049 ± 0.0001	1.24	0.0001	99.87	0.06	
25	1.25	7.5	0.0019 ± 0.0001	2.65	0.0001	100.51	0.07	
25	1.25	10.0	0.0029 ± 0.0001	2.11	0.0001	99.48	0.05	
25	1.25	12.5	0.0039 ± 0.0001	1.87	0.0001	100.00	0.03	
25	1.25	15.0	0.0049 ± 0.0001	1.54	0.0001	100.09	0.03	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(2,9) at P = 0.05 level of significance.

Table 3.60: Regression analysis for the determination of ATL, AMH and HTZ in standard solutions by first-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)			Regression equations [a] (at 243.6 nm for ATL at 375.0 nm for AMH at 308.6 nm for HTZ)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t* –test for correlation	
	ATL	AMH	HTZ			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	15-30	0	0	Y = 1.21E-03.X - 8.99E-05	0.9999	7.21E-05	3.11E-06	3.46E-05	-3.99E-04, 2.20E-04	1.19E-03, 1.22E-04	1.20E-03	100	4.30
Series B	15-30	1.25	12.5	Y = 1.20E-03.X + 3.39E-05	0.9999	8.65E-05	3.73E-06	4.29E-05	-3.38E-04, 4.05E-04	1.18E-03, 1.22E-03	1.20E-03	100	
Series C	0	1.25-5.0	0	Y = 3.99E-03.X + 6.35E-05	0.9999	5.56E-05	1.62E-05	4.50E-05	-1.75E-04, 3.02E-04	3.92E-03, 4.06E-03	4.00E-03	100	
Series D	25	1.25-5.0	12.5	Y = 4.00E-03.X + 3.35E-05	0.9999	5.03E-05	1.47E-05	4.14E-05	-1.83E-04, 2.50E-04	3.93E-03, 4.06E-03	4.00E-03	100	
Series E	0	0	7.5 -15	Y = 3.93E-04.X - 9.99E-04	0.9999	3.16E-05	2.73E-05	1.53E-05	-1.14E-03, -8.63E-04	3.81E-04, 4.05E-04	3.25E-04	100	
Series F	25	1.25	7.5 -15	Y = 3.98E-04.X - 1.03E-03	0.9999	2.78E-05	2.40E-06	1.34E-05	-1.15E-03, -9.09E-04	3.87E-04, 4.08E-04	3.28E-04	100	

[a] : Based on four calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 2 d.f.

Table 3.61: One-way ANOVA test for linearity of pure ATL solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	5.49E-04	5.49E-04	0.478	4.46
Lack of fit	2	6.78E-07	3.39E-07		
Within line	8	5.67E-06	7.09E-07		
Total	11	5.55E-04			

*: at P = 0.05 level of significance.

Table 3.62: One-way ANOVA test for linearity of pure AMH solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	3.72E-04	3.72E-04	0.174	4.46
Lack of fit	2	3.36E-08	1.68E-08		
Within line	8	7.73E-07	9.66E-08		
Total	11	3.73E-04			

*: at P = 0.05 level of significance.

Table 3.63: One-way ANOVA test for linearity of pure HTZ solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum squares (MS)	F_{Calc}	F_{Crit} *
Regression	01	1.45E-05	1.45E-05	0.116	4.46
Lack of fit	02	2.03E-09	1.01E-09		
Within line	08	6.99E-08	8.74E-09		
Total	11	1.46E-05			

* : at P = 0.05 level of significance.

Table 3.64: Validation report for the determination of ATL, AMH and HTZ in standard solutions by first-derivative spectrophotometry

Analytical parameter	Results		
	ATL (243.6 nm)	AMH (375.0 nm)	HTZ (308.6 nm)
Accuracy (%)	100.26 ± 0.58	100.16 ± 0.52	100.14 ± 0.46
Precision (%)	99.67 99.67 100.33 100.65 100.98 RSD: 0.58	99.60 99.79 100.00 100.60 100.80 RSD: 0.52	99.74 99.74 100.00 100.42 100.79 RSD: 0.46
Specificity	A 25 µg/ml of ATL, 1.25 µg/ml of AMH and 12.5 µg/ml of HTZ mixture solution will show an absorbance value (D1) of 0.030 ± 0.0001	A 1.25 µg/ml of AMH, 25 µg/ml of ATL and 12.5 µg/ml of HTZ mixture solution will show an absorbance value (D1) of 0.005 ± 0.0001	A 12.5 µg/ml of HTZ, 1.25 µg/ml of AMH and 25 µg/ml of ATL mixture solution will show an absorbance value (D1) of 0.004 ± 0.0001
LOD (µg/ml)	0.09	0.04	0.12
LOQ (µg/ml)	0.29	0.12	0.39
Linearity (µg/ml)	5 - 60	1.25 - 30	5 - 30
Ruggedness (%)	100.26 ± 0.58	100.16 ± 0.52	100.14 ± 0.46

Table 3.65: Results of the assay of pure drug admixtures and commercial formulations of ATL, AMH and HTZ by first-derivative spectrophotometry

Sample	Label Claim (mg per tab./caps.)			Recovery (%) [a]			F-test for Mean Recovery			
	ATL	AMH	HTZ	ATL (243.6 nm)	AMH (375.0 nm)	HTZ (308.6 nm)	ATL	Calc AMH	HTZ	Crit [b] (ATL / AMH / HTZ)
Pure drug admixture	-	-	-	99.77 ± 0.58	100.10 ± 0.54	99.50 ± 0.92	1.05	0.47	3.41	3.68
Brand KX (tab.)	50	2.5	25	99.11 ±1.13	100.20 ± 0.43	100.33 ± 0.38				
Brand KY (caps.)	50	2.5	25	99.05 ±1.06	100.35 ± 0.33	100.21 ± 0.27				

[a] : Mean and standard deviation for six determinations.

[b] : Theoretical value of F(2,15) based on one-way ANOVA test at P = 0.05 level of significance.

3.12 SIMULTANEOUS DETERMINATION OF HALOPERIDOL AND TRIHEXYPHENIDYL HYDROCHLORIDE IN COMBINED TABLET PREPARATIONS BY SECOND-DERIVATIVE SPECTROPHOTOMETRY

The combined tablet preparation of haloperidol (HPL) and trihexyphenidyl HCl (TPL) is widely used for schizophrenia and psychosis. The official monographs [1-3] prescribed the procedure for individual assay of HPL and TPL. The official assay procedure for HPL pure drug is titrimetric method recommended by U.S.P. 23, B.P. 1993 and I.P. 1996 and HPLC method for its tablet dosage forms. The U.S.P. 23 suggested HPLC method for both TPL pure drug and its dosage forms. Whereas, the B.P. 1993 and I.P. 1996 recommended a titrimetric method for TPL as pure drug and HPLC method for its dosage forms. The various reports available for the individual assay of HPL includes colorimetric [395], ultraviolet [396,397] and HPLC [398]. There are various reports available for estimation of HPL in combination of other drugs [399-401] including TPL [399,400]. The later two reports employed HPLC [399] and HPTLC methods [400] for their determination. The literature reports for TPL assay includes colorimetric [402,403], fluorimetric [404] and ultraviolet method [405]. The objective of this work was to propose a second-derivative spectrophotometric method for estimation of these drugs without prior separation from combined formulations.

Materials and Reagents

Haloperidol and trihexyphenidyl HCl both were obtained as gift samples from Torrent, India. Methanol (Qualigens, India) and hydrochloric acid (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solutions of HPL and TPL were prepared separately by dissolving 5 mg of each pure drug in 10 ml methanol. Appropriate amounts of the stock solution were transferred to 10 ml volumetric flasks. The volumes were made up with 0.1M HCl to

give a series of solutions containing 7.5-15 μg/ml of HPL and 5-20 μg/ml of TPL, respectively. A three series of 10 ml mixture solutions were prepared from the above stock solutions. The first series contained a constant concentration of TPL (5 μg/ml) and a varying concentration of HPL (7.5-15 μg/ml). The second series contained a constant concentration of HPL (12.5 μg/ml) and a varying concentration of TPL (5-20 μg/ml). In the final series, fifteen mixture solutions of each containing a constant concentration of HPL (12.5 μg/ml) and TPL (5 μg/ml) were also prepared.

Interaction Study

Two separate series of mixture solutions were also prepared from fresh stock solutions according to the above mentioned procedures. The first series contained a constant concentration of HPL (12.5 μg/ml) and a varying concentration of TPL (5-35 μg/ml). Similarly, the second series contained a constant concentration of TPL (5 μg/ml) and a varying concentration of HPL (5-40 μg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of HPL (12.5 μg/ml) and TPL (5 μg/ml) were prepared from fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of each containing 5-30 μg/ml of HPL and TPL were prepared from the stock solutions meant for method validation.

c. ***Specificity***

Series of five mixture solutions of each containing HPL (12.5 μg/ml) and TPL (5 μg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of each commercial preparation (Brand LX and LY) were accurately weighed, well powdered and a weight of each powder equivalent to 5 mg TPL (and 12.5 mg of HPL) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The samples were filtered through Whatman filter paper No.1. The first and last 5 ml of filtrate were discarded. Appropriate volume aliquots of filtrate were diluted with 0.1M HCl to give a concentration of 12.5 µg/ml HPL and 5 µg/ml of TPL.

Procedure

The absorbances of sample and standard solutions of HPL and TPL were recorded from 205-280 nm against the blank solution. The second-derivative absorption spectra for each set of solutions were subsequently recorded using $\Delta = 25$ points [138] and no smoothing was necessary. The absorbances of the solutions were measured at the zero-crossing wavelength of other drug. The final series of mixture solutions were used to measure the absorbances at 226.6 nm and also at the zero-crossing points (ZCP's) of other drug.

Results and Discussion

The zero-order spectra of HPL and TPL were found to be overlapping (Figure 3.51). Although, the estimation of HPL would have easily been possible from the zero-order spectra above 240.0 nm, but the aim of the study was to propose a simultaneous method for their determination. Hence, the earlier discussed conventional methods [5-13] could not be proposed, as there was no significant separation of peaks in the range of measurement. Similarly, the pH-induced differential spectrophotometric method could not be used due to lack of stability of TPL in alkali solutions. Therefore, a derivative spectrophotometric method was considered for resolving the minor component (TPL) in presence of major component (HPL) with spectral overlap. However, the first-derivative mode could not offer the desired ZCP's for their

determination, but such problem was overcome with processing of curves in second-derivative mode. The second-derivative process of curves had offered ZCP's for both the drugs along with wavelength maxima and minima, but the initial experiments with ratio-compensation (RC) method suggested a large interference from the major component of a mixture in the determination of the minor component at the balance point. The second-derivative spectra of pure drugs resulted in ZCP's at 241.2 and 261.4 nm for HPL and at 265.4 nm for TPL (Figure 3.52). It was observed that the second-derivative absorbance values of TPL at the ZCP's of HPL (i.e. 241.2 and 261.4 nm) were small for their determination from the mixtures (Figure 3.52). Thus, the peak-baseline (PB) distances of TPL and HPL from standard and sample mixtures were also considered at 226.6 nm and compared with the ZCP's determinations.

The proportionality of the derivative values to different concentrations of pure drugs were examined at the above selected wavelength of determination by measuring a series of pure drug (Table 3.66, Series A and C of Table 3.67) and mixture solutions (Table 3.66, Series B and D of Table 3.67). The regression equations obtained from such measured values were reported in Table 3.67. The derivative spectra obtained with the standard mixtures were showed in Figures 3.53 and 3.54. The presence of distinct isosbestic points at 241.2 and 261.4 nm (Figure 3.53) and a constant absorption pattern after 240.0 nm (Figure 3.54) followed by linearity at 226.6 nm (Figures 3.53 and 3.54) suggested that there was no interference in the estimation of the mixture of HPL and TPL.

The mutual independence of analytical signals of standard solutions in the proposed concentration range was observed by a mixture interaction study. It was clear from reported results that the varying concentration of TPL (5-35 μg/ml) did not interfere up to 20 μg/ml in the estimation of HPL (12.5 μg/ml) at 226.6 nm (Figure 3.55A). Similarly, the varying concentrations of HPL (5-40 μg/ml) did not interfere up to 30 μg/ml in the estimation of TPL (5 μg/ml) at 265.4 nm (Figure 3.55B). Thus, the

proposed concentration ranges for standard solutions of both drugs were ideal and accurate determinations were also possible at the proposed concentration ratio (i.e. 12.5 μg/ml for HPL and 5 μg/ml for TPL).

The statistical analysis of data achieved by second-derivative spectra for standard solutions showed that the mean derivative values along with standard deviation, coefficient of variation and the standard error were small which suggested the high reproducibility of the method (Table 3.66). While, the reported percentage ratio of residuals showed a deviation of within 1% of the best-fit line, but the calculated F-values for non-linearity test [97] suggested the deviations observed with the calibration points were not significant at 5% level thus indicating that the calibration points were linear (Table 3.66).

The linear calibration lines obtained for pure drugs and mixtures were similar (Table 3.67). This similarity was also proved from the reported correlation coefficients and the standard error involved with each intercept, slope and estimate. A one-way ANOVA test [98] was conducted with three replicates per calibration point including the lowest and highest variation observed from the mean absorbance of each concentration during the replicate measurement of pure drug solutions. It was evident from the reported values (Tables 3.68 and 3.69) that the variance observed with the calibration line was uniform and thus it proved the linearity within the proposed concentration range.

The slope values with zero intercept on ordinate were obtained by facilitating the regression equations of pure drugs to pass through origin with the help of an additional calibration point (0,0). Thus reported slope values (Table 3.67) were found to lie within the 95% confidence limits to suggest that the obtained intercepts of regression equations did not deviate from the origin. In addition, a Student's *t*-test [98] for correlation indicated that the reported values were relatively higher and significant

than the theoretical value at 5% level. Thus, it suggested the positive correlation between measured values and concentrations (Table 3.67).

The developed method was validated according to the procedures described in U.S.P. 23. The results of such study were reported in Table 3.70. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were calculated from the slope of regression equations obtained for Series A and C of Table 3.67.

The percentage recoveries obtained with pure admixture and sample preparations of HPL and TPL were reported in Table 3.71. The recovery values fairly meet the official requirements for individual assay of these two drugs from dosage forms (Appendix B). The reported F-values of a two-way ANOVA test [98] were less than the critical value at 5% level indicated that there were no significant differences between the recoveries obtained by ZCP and PB method and also between the samples of a method (Table 3.71). The prepared solutions were stable for 3 hrs in 0.1M HCl.

Thus the above findings substantiate the usefulness of the proposed method in measuring the concentration of a minor component in a two-component mixture with overlapping spectra. The developed method was found to be accurate and precise, and easier compared to the reported methods [399,400]. Therefore, the rapidity of these methods makes it adaptable for routine analysis of the combined formulations.

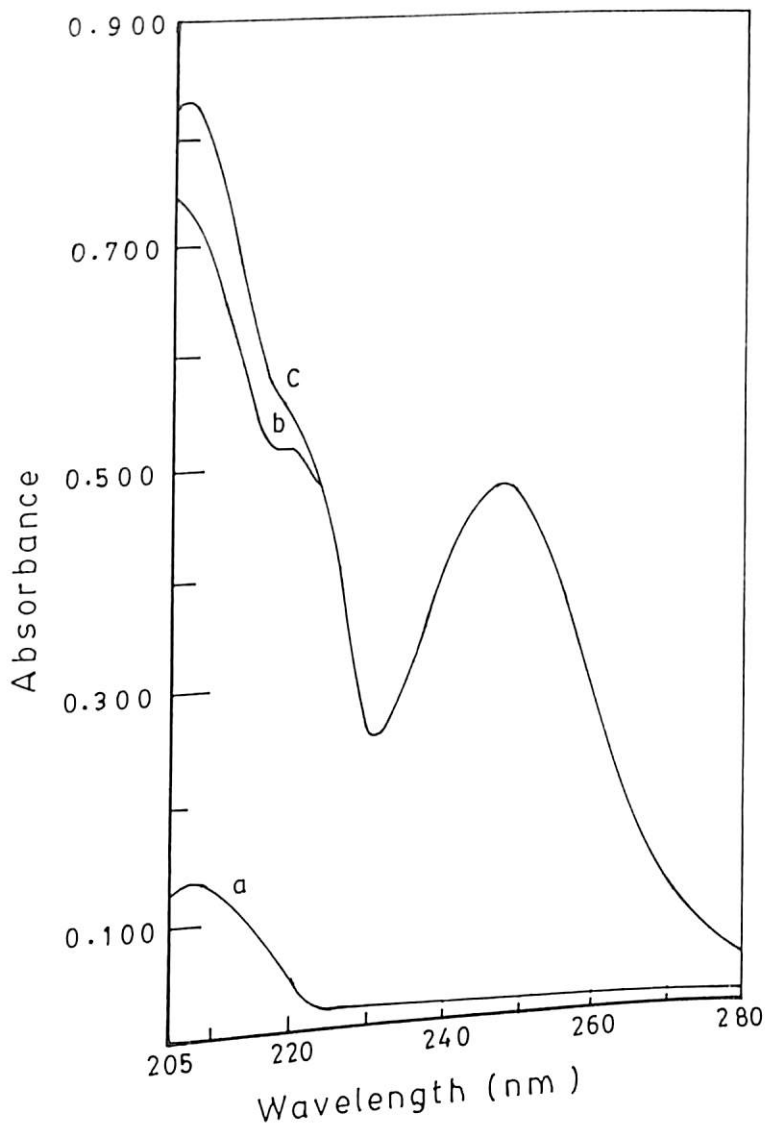

Figure 3.51 Absorption spectra of (a) trihexyphenidyl HCl (5 µg/ml), (b) haloperidol (12.5 µg/ml) and (c) their mixture in 0.1M HCl.

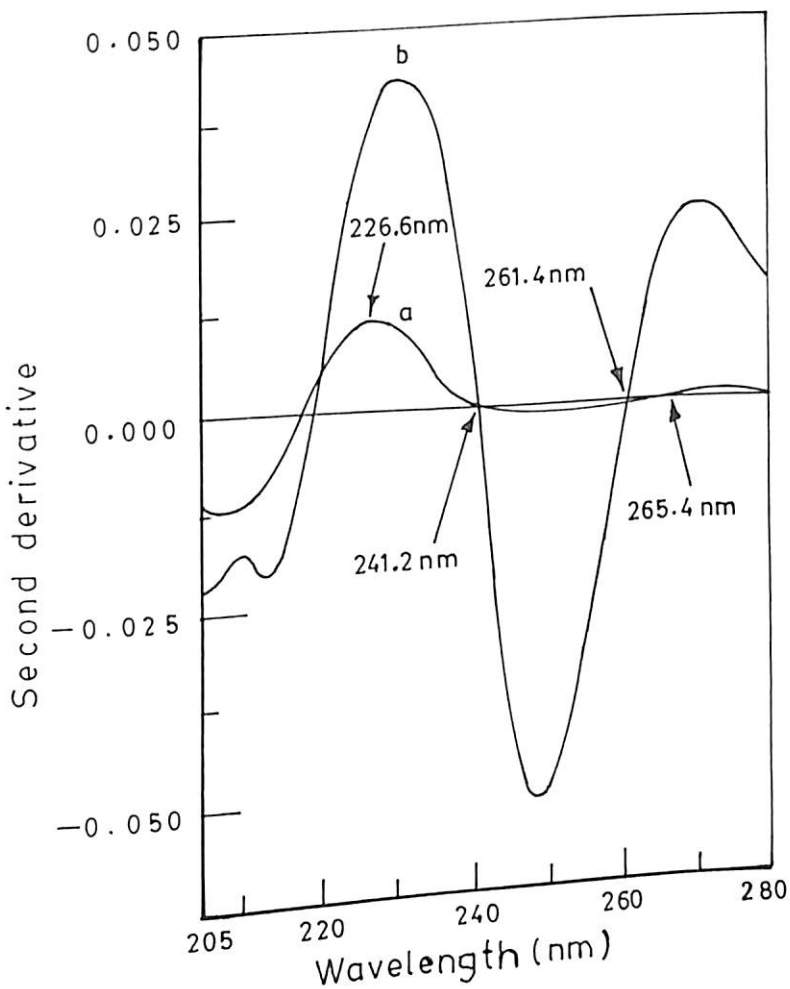

Figure 3.52 Second-derivative spectra of (a) trihexyphenidyl HCl (5 μg/ml) and (b) haloperidol (12.5 μg/ml) in 0.1M HCl.

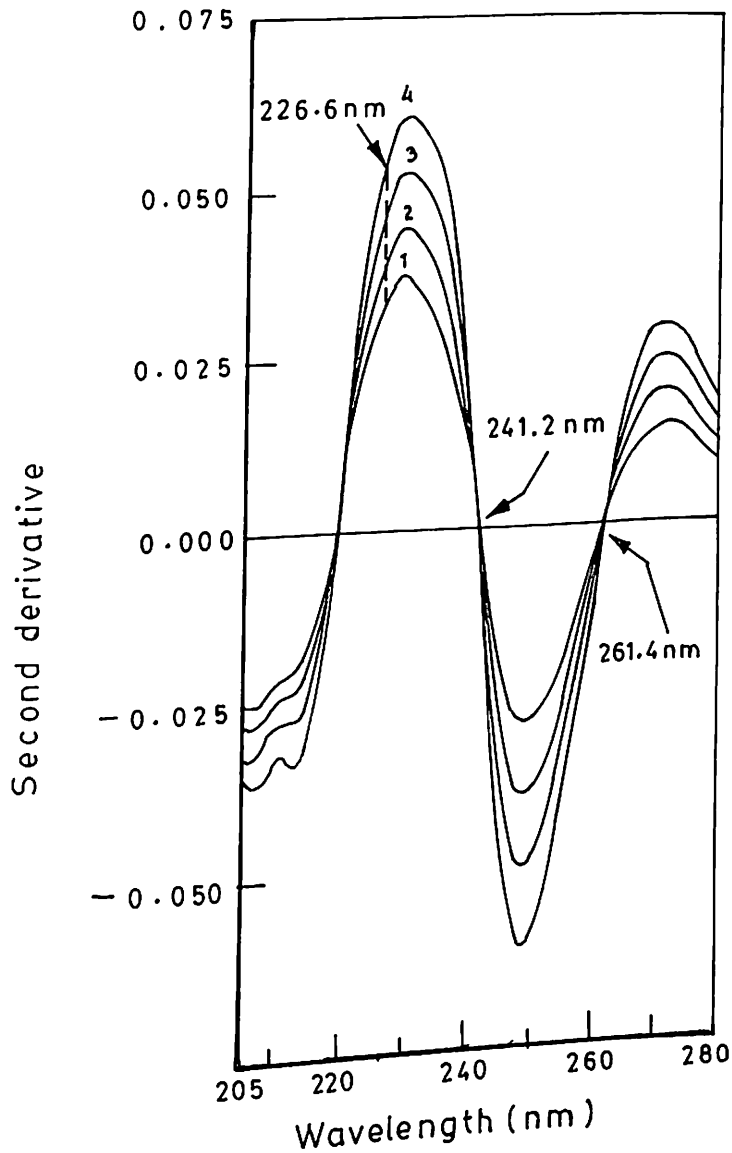

Figure 3.53 Second-derivative spectra of haloperidol (7.5, 10, 12.5 and 15 µg/ml) and trihexyphenidyl HCl (5 µg/ml) in 0.1M HCl in curves 1-4, respectively.

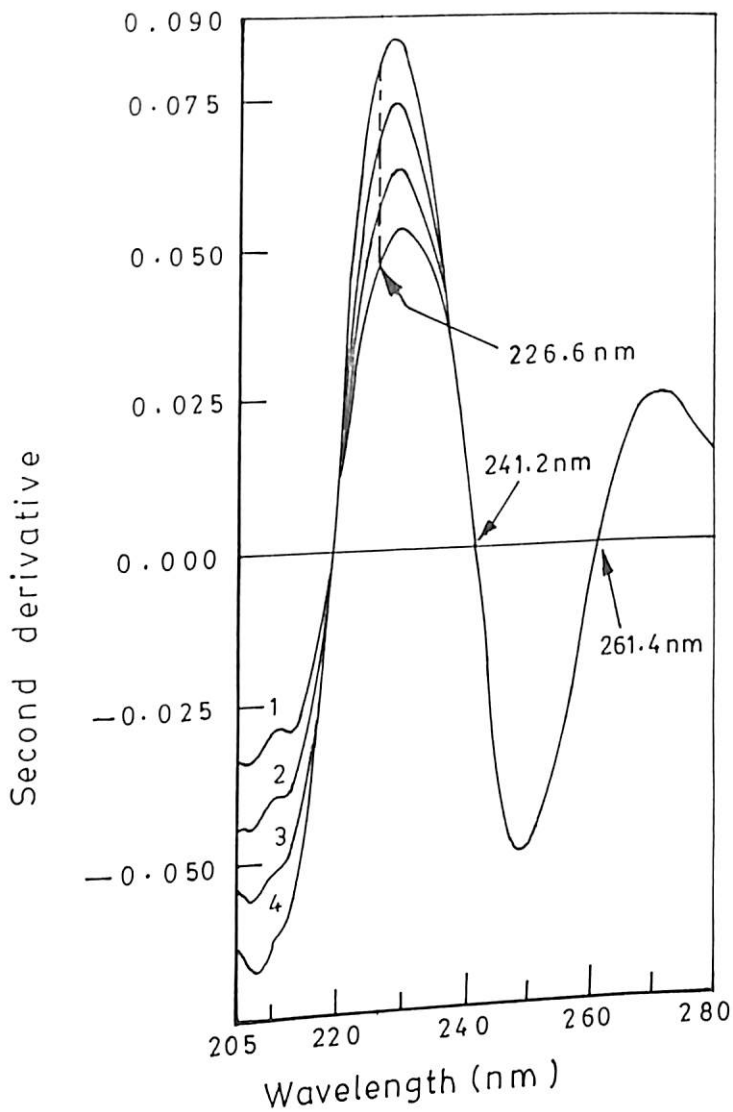

Figure 3.54 Second-derivative spectra of trihexyphenidyl HCl (5, 10, 15 and 20 μg/ml) and haloperidol (12.5 μg/ml) in 0.1M HCl in curves 1-4, respectively.

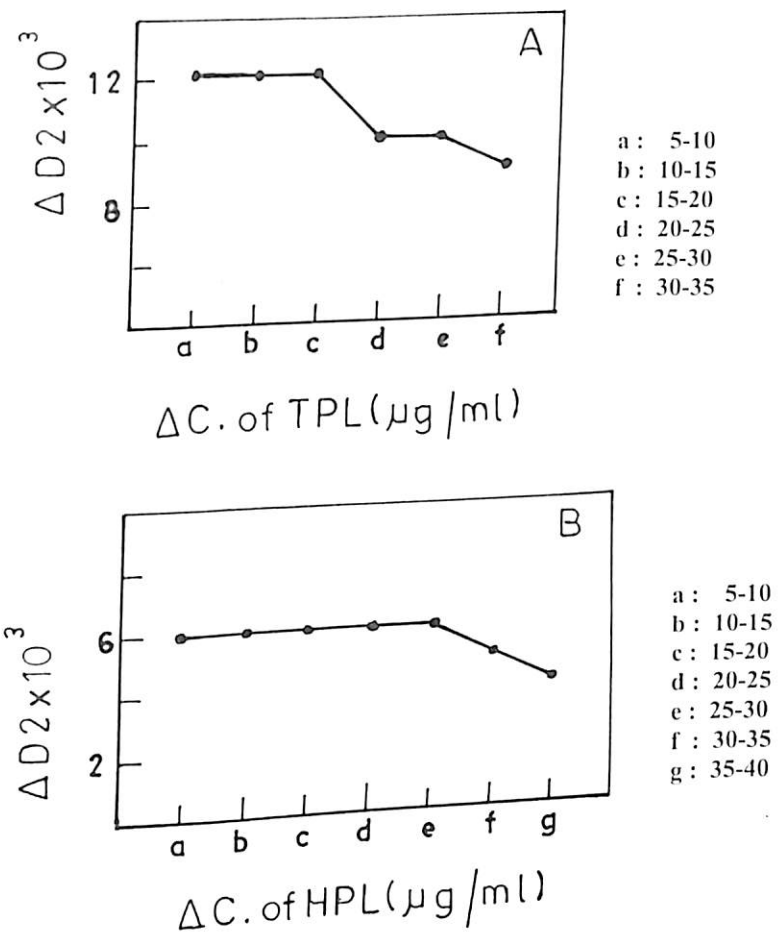

Figure 3.55 Second-derivative interaction graphs for (A) haloperidol (12.5 μg/ml) in mixture with trihexyphenidyl HCl (at 226.6 nm) and (B) trihexyphenidyl HCl (5 μg/ml) in mixture with haloperidol (at 265.4 nm) in 0.1M HCl.

Table 3.66: Selectivity of the method for the simultaneous determination of HPL and TPL in standard solutions by second-derivative spectrophotometry

Composition of the solution (µg/ml)		Mean absorbance [a] value (D2) (HPL at 265.4 nm, TPL at 226.6 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
HPL	TPL					Calc	Crit [b]
7.5	0	0.0094 ± 0.0001	2.06	0.0001	100.81	0.43	4.26
10.0	0	0.0125 ± 0.0002	1.76	0.0001	99.55	0.33	
12.5	0	0.0156 ± 0.0002	1.66	0.0001	99.23	0.24	
15.0	0	0.0184 ± 0.0003	2.10	0.0001	100.53	0.11	
7.5	5	0.0093 ± 0.0002	1.97	0.0001	100.80	0.28	
10.0	5	0.0124 ± 0.0002	1.61	0.0001	99.22	0.24	
12.5	5	0.0153 ± 0.0003	1.91	0.0001	99.78	0.11	
15.0	5	0.0181 ± 0.0003	2.00	0.0001	100.29	0.07	
0	5	0.0131 ± 0.0002	1.70	0.0001	100.74	0.80	4.26
0	10	0.0252 ± 0.0002	0.97	0.0001	99.85	0.67	
0	15	0.0374 ± 0.0005	1.49	0.0002	99.43	0.13	
0	20	0.0490 ± 0.0007	1.45	0.0002	100.31	0.08	
12.5	5	0.0128 ± 0.0003	2.52	0.0001	100.88	0.20	
12.5	10	0.0250 ± 0.0003	1.54	0.0001	99.38	0.14	
12.5	15	0.0368 ± 0.0007	2.09	0.0002	99.94	0.04	
12.5	20	0.0486 ± 0.0007	1.60	0.0002	100.14	0.04	

[a] : Average of ten determinations with standard deviation.

[b] : Theoretical value of F(2,9) at P = 0.05 level of significance.

Table 3.67: Regression analysis for the determination of HPL and TPL in standard solutions by second-derivative spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 265.4 nm for HPL at 226.6 nm for TPL)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t*-test for correlation	
	HPL	TPL			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	7.5 -15	0	Y = 1.20E-03.X + 4.86E-04	0.9996	2.65E-04	2.29E-05	1.28E-04	-6.54E-04, 1.63E-03	1.10E-03, 1.30E-03	1.23E-03	50	4.30
Series B	7.5 -15	5	Y = 1.18E-03.X + 4.86E-04	0.9997	2.01E-04	1.74E-05	9.68E-05	-3.80E-04, 1.35E-03	1.10E-03, 1.25E-03	1.21E-03	71	
Series C	0	5 -20	Y = 2.39E-03.X + 1.25E-03	0.9999	2.45E-04	1.79E-05	2.01E-04	-5.20E-05, 2.55E-03	2.28E-03, 2.49E-03	2.44E-03	100	
Series D	12.5	5 -20	Y = 2.38E-03.X + 9.79E-04	0.9999	1.78E-04	1.30E-05	1.45E-04	-1.30E-04, 2.13E-03	2.31E-03, 2.47E-03	2.43E-03	100	

[a] : Based on four calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 2 d.f.

Table 3.68: One-way ANOVA test for linearity of pure HPL solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	1.32E-04	1.32E-04	0.535	4.46
Lack of fit	2	1.68E-07	8.41E-08		
Within line	8	1.26E-06	1.57E-07		
Total	11	1.33E-04			

*: at P = 0.05 level of significance.

Table 3.69: One-way ANOVA test for linearity of pure TPL solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{crit} *
Regression	1	2.14E-03	2.14E-03	0.021	4.46
Lack of fit	2	2.37E-06	1.19E-08		
Within line	8	4.48E-06	5.60E-07		
Total	11	2.15E-03			

*: at P = 0.05 level of significance.

Table 3.70: Validation report for the determination of HPL and TPL in standard solutions by second-derivative spectrophotometry

Analytical parameter	Results	
	HPL (265.4 nm)	TPL (226.6 nm)
Accuracy (%)	99.94 ± 0.70	100.30 ± 0.87
Precision (%)	99.23 99.23 100.00 100.63 100.63 RSD: 0.70	99.23 100.00 100.00 100.76 101.52 RSD: 0.87
Specificity	A 12.5 µg/ml of HPL and 5.0 µg/ml of TPL mixture solution will show an absorbance value (D2) of 0.015 ± 0.0003	A 5.0 µg/ml of TPL and 12.5 µg/ml of HPL mixture solution will show an absorbance value (D2) of 0.046 ± 0.0002
LOD (µg/ml)	0.32	0.25
LOQ (µg/ml)	1.07	0.84
Linearity (µg/ml)	5 - 30	5 - 30
Ruggedness (%)	99.94 ± 0.70	100.30 ± 0.87

Table 3.71: Results of the assay of pure drug admixtures and commercial formulations of HPL and TPL by second-derivative spectrophotometry

Drug Name	Label Claim (mg/tab.)	Recovery (%) [a]		F-test for Mean Recovery			
		ZCP Method	PB Method	Calc		Crit [b]	
				Samples	Methods	Samples [†]	Methods [‡]
HPL		**D2(265.4 nm)**	**D2(226.6 nm)**				
Pure drug admixture	-	99.99 ± 0.91	100.11 ± 0.95				
Brand LX	5	99.72 ± 1.10	100.31 ± 0.69	0.02	0.15	19.0	18.5
Brand LY	5	100.18 ± 1.02	99.80 ± 0.51				
TPL		**D2(241.2 nm)**	**D2(261.4 nm)**	**D2(226.6 nm)**			
Pure drug admixture	-	99.71 ± 1.38	99.71 ± 1.38	100.11 ± 0.95			
Brand LX	2	100.00 ± 1.15	100.28 ± 1.38	100.31 ± 0.69	1.39	0.10	6.94	6.94
Brand LY	2	100.28 ± 1.38	100.28 ± 1.38	99.80 ± 0.51				

[a] : Mean and standard deviation for seven determinations.

[b] : Theoretical value of $F(2, 2)^{\dagger}$, $F(1, 2)^{\ddagger}$ for HPL and $F(2,4)^{\dagger,\ddagger}$ for TPL based on two-way ANOVA test at P = 0.05 level of significance.

CHAPTER 4

DERIVATIVE-DIFFERENCE SPECTROSCOPY

4. DERIVATIVE-DIFFERENCE SPECTROSCOPY

4.1 Introduction

As mentioned in difference spectroscopy, the presence of fortuitous juxtaposition of isosbestic points will serve as criteria for successful application of this technique to quantitatively determine the combined drug mixtures. However, there can be instances in which the recorded difference spectra of drugs between the two selected inducing agents for spectral changes like acid and alkali buffers will not show such characteristic isosbestic points. It is due to their identical display of small spectral changes in presence of those buffers. It was reported in those situations, the derivative method can be successfully combined with difference spectroscopy to resolve such overlapping of mixture components and these combined methods are known as derivative-difference spectroscopy [406-414].

The combined derivative-difference technique offers a distinct advantage over both the individual techniques by successfully eliminating the background interference from formulation matrix due to the refinement of spectra at two different stages. Hence, the possible interference displayed by the large amounts of tablet diluents like lactose and dibasic calcium phosphate in presence of any spectral change inducing agents can also be eliminated by this combined technique [106]. Though, the derivative technique alone can handle such problem very easily, but sometimes they require derivatives of higher orders in eliminating such interferences which may also results in encouraging the noise.

Thus, this combined technique should serve as an added advantage over both the techniques in eliminating the background interference of those mixtures, which shows alterations in their absorption pattern in presence of any spectral change inducing agents. The available literature reports indicate that this combined technique can also be useful for the quantitative determination of various drug substances either alone [37,406-411] or in combination of other drugs [215,412-414] from their dosage forms. The summary of this literature review was presented in Appendix C.

Therefore, this combined technique has been chosen as method of choice for resolving the overlapping spectra of phenobarbitone and phenytoin sodium from their combined dosage forms.

4.2 Design of the experiment

The work reported in this section was carried out using JASCO model 7800 UV-VIS spectrophotometer with 1 cm quartz cells. A scan speed of 480 nm/min and a bandwidth of 3 nm were maintained. Ordinate maximum and minimum were adjusted according to the amplitudes of measured derivative-difference values. The instrument was calibrated (including for derivative spectra) according to the procedures described in official monographs [2,3] and also to methods suggested by Talsky [4]. The results obtained were reported in Appendix A.

The stepwise experimental approach is:

a. Preparation of standard solutions of the pure drugs with the selected acid and alkali buffers to obtain equimolar solutions with appropriate concentration range to maintain the desired formulation ratio.
b. Recording the difference spectrum of pure drugs by placing the acidic solutions in the reference compartment and their alkali solutions in the sample compartment.
c. Recording of the derivative spectra from the one obtained in step "b" by choosing an appropriate derivative order and number of points (Δ) and order for processing by Savitzky-Golay algorithm [138] and also to check the necessity of any smoothing process of derivative curves.
d. Examining the linear proportionality of absorbance values with different concentrations of drug solutions at the possible wavelengths of estimation.
e. A similar procedure described in difference and derivative technique will be followed to identify the linear concentration range, to study the possible mixture interferences, if any, and to establish the selectivity of the method at chosen wavelengths for their determination from standard solutions.

f. Validation of the developed method according to the procedures described in U.S.P. 23.

g. Extending the developed method to commercial preparations available in the market to study the suitability of the method for routine analysis.

h. Finally, the statistical analysis of data obtained for standard solutions and the commercial preparations was done and the details were discussed in the experimental section. The various mathematical expressions used for the statistical treatment of data were presented in Appendix D.

4.3 SIMULTANEOUS DETERMINATION OF PHENOBARBITONE AND PHENYTOIN SODIUM IN COMBINED TABLET PREPARATIONS BY SECOND-DERIVATIVE DIFFERENCE SPECTROPHOTOMETRY

The combination of phenobarbitone (PBT) and phenytoin sodium (DPH) in the form of tablet preparation is widely used for major epilepsy and psychomotor seizures. The official monographs [1-3] have prescribed individual assay procedure for PBT and DPH. The method of determination of PBT includes HPLC prescribed by U.S.P. 23 for pure drug and for its dosage forms. While, the B.P. 1993 and I.P. 1996 both recommended a titrimetric and extraction cum gravimetric method for its pure and dosage forms respectively. Similarly, the monographs suggested HPLC [1] and potentiometric titration method [2,3] for DPH as pure drug, and HPLC [1] and titrimetric method [2,3] for its dosage forms. There are various literature reports available for the analysis of PBT as single preparation and that includes ultraviolet [415], titrimetric [416] and HPLC method [417], and also in combination of other drugs [66,129,216,217,413,418-421] including the DPH [129,419-421]. Whereas, the reports available for DPH as single preparation included ultraviolet [422-424], polarographic [425,426] and colorimetric method [427]. In particular, the reports related to the combination of these two drugs employed derivative ratio-compensation method [129], orthogonal functions [419], titrimetric [420] and potentiometric titration [421]. The objective of this work was to propose a second-derivative difference spectrophotometric method for the determination of PBT and DPH in the presence of each other as well as the excipients.

Materials and Reagents

Phenobarbitone and phenytoin sodium both were obtained as gift samples from Anglo-French, India. Hydrochloric acid (Qualigens, India), sodium hydroxide (Qualigens, India) and methanol (Qualigens, India) of analytical grade were used.

Standard Solutions

The stock solutions of pure PBT and DPH were prepared by dissolving 25 mg each of the pure drugs in 50 ml of methanol. Appropriate volume aliquots of the stock solution were transferred to 25 ml volumetric flasks in duplicate. The volumes were made up with 0.01M HCl and 0.01M NaOH to give a series of equimolar solutions containing 7.5-25 µg/ml of both PBT and DPH. Similarly, three series of 10 ml each of equimolar solutions of mixtures of PBT and DPH in 0.01M HCl and 0.01M NaOH were also prepared by using the stock solutions. The first series contained a constant concentration of PBT (7.5 µg/ml) and a varying concentration of DPH (7.5-25 µg/ml). The second and third series contained a constant concentration of DPH (15 and 25 µg/ml respectively) and a varying concentration of PBT (7.5-25 µg/ml).

Interaction Study

Two separate series of mixture solutions were also prepared from fresh stock solutions according to the above mentioned procedures. The first series contained a constant concentration of DPH (25 µg/ml) and a varying concentration of PBT (5-40 µg/ml). While, the second series contained a constant concentration of PBT (7.5 µg/ml) and a varying concentration of DPH (5-50 µg/ml).

Method Validation

a. ***Accuracy and Precision***

Five separate standard and test solutions of PBT (7.5 µg/ml) and DPH (15 µg/ml) were prepared from the fresh stock solutions according to the above mentioned procedures.

b. ***Linearity***

Separate series of solutions of PBT (5-40 µg/ml) and DPH (5-50 µg/ml) were prepared from the stock solutions meant for method validation.

c. *Specificity*

Series of five mixture solutions of each containing PBT (7.5 µg/ml) and DPH (15 µg/ml) were prepared from the stock solutions meant for method validation.

Sample Preparation

Twenty tablets of each commercial preparation (Brand MX and MY) were accurately weighed, well powdered and weight of the powder equivalent to 7.5 mg of PBT (and 15 or 25 mg of DPH) was dissolved in methanol by thorough mixing and made up to volume in a 50 ml volumetric flask. The samples were filtered through Whatman filter paper No.1. The first and last 5 ml of the filtrate were discarded. The sample solution of 25 ml each in 0.01M HCl and 0.01M NaOH were prepared by using appropriate aliquots of the filtrate to obtain a concentration of 7.5 µg/ml PBT and corresponding amount of DPH (15 or 25 µg/ml depending on the chosen preparation).

Procedure

The difference spectra between the 0.01M HCl and 0.01M NaOH solutions of pure drugs and sample were recorded from 230-275 nm by placing the acidic solution in the reference compartment and the alkali solution in the sample compartment. A second-derivative spectrum of each of the difference curves was subsequently recorded using Δ = 10 points [138] and the spectra were smoothened by 10 points [138]. The solutions were measured at 244.8 and 252.8 nm for PBT and DPH, respectively.

Results and Discussion

The zero-order spectra of pure PBT and DPH either in acid or in alkali buffers were very similar (Figure 4.1). It was evident that the recorded spectra lack significant wavelength maxima except with alkali solution of PBT. The conventional methods like simultaneous equations [6,7], orthogonal functions [10-12] and derivative ratio-compensation method [128,129] were tried earlier. But, the recoveries obtained

with sample solutions were not satisfactory except with orthogonal functions [419] and others [420,421]. The reasons for the failure of above methods could be attributed to the lack of fulfillment of method requirements by both pure drugs either in acidic or in alkali buffer systems. Although, the reported methods [419-421] gave encouraging results for the two drugs but they require special attention in selecting the assay parameters. Whereas, the recorded difference spectrum of pure drugs alone failed to produce the required isosbestic points (Figure 4.2), but showed significant alteration. It was also observed during the initial study in first and second-derivative mode that, the utilisation of derivative technique had failed to generate the required zero-crossing points (ZCP's) for DPH in first-order. In addition, the measured derivative values were very low and filled with noise at the ZCP's of other drug in second-order. Thus, the above findings have led to combine these two individual techniques for the determination of PBT and DPH in standard and sample solutions due to the presence of ZCP's in such recorded spectrum (Figure 4.3). The second-derivative difference curves were relatively better for the estimation of drugs compared with similar curves in first-derivative mode. In the latter, the ZCP of DPH had fallen close to the 230 nm, which was the boundary limit of selected wavelength range. Hence, the second-derivative difference curves were considered. The optimum working wavelengths considered for the determination of both drugs were 244.8 nm for PBT and at 252.8 nm for DPH (Figure 4.3).

The proportionality between measured derivative-difference values and concentrations were found by measuring a series of pure drugs (Table 4.1, Series A and D of Table 4.2) and mixture solutions (Table 4.1, Series B, C and E of Table 4.2) at the above selected wavelengths. The regression equations obtained from the measured values were reported in Table 4.2. The second-derivative difference spectra obtained with the standard mixtures (Series B, C and E of Table 4.2) were showed in Figures 4.4-4.6. The presence of distinct isosbestic point at 252.8 nm (Figures 4.4 and

4.5) and at 244.8 nm (Figure 4.6) suggested that there was no interference in the simultaneous estimation of both PBT and DPH.

The mutual independence of analytical signals of standard solutions during their estimation at the selected wavelengths was confirmed from a mixture interaction study. It was clear from the reported results (Figure 4.7) that the varying concentrations of PBT (5-40 μg/ml) did not interfere up to 20 μg/ml in the estimation of DPH (25 μg/ml) at 252.8 nm (Figure 4.7A). Similarly, the varying concentrations of DPH (5-50 μg/ml) did not interfere up to 25 μg/ml in the estimation of PBT (7.5 μg/ml) at 244.8 nm (Figure 4.7B). Thus, the proposed concentration range for standard solutions (Table 4.1) was optimum and accurate determination would be possible at 7.5 μg/ml of PBT and 15 or 25 μg/ml of DPH.

The detailed statistical analysis of data obtained by the proposed method for standard solutions demonstrated that the deviation values associated with the mean absorbance values (ΔD2) were small and thus indicated the precision of the proposed method. This was also confirmed from the obtained coefficient of variation (except for few concentrations where values were greater than 2.5%) and the reported standard error in measuring each concentration. The percentage ratio of residuals showed a random scatter about the best-fit line. However, such random scatter was observed to be not significant from the calculated F-values for non-linearity [97] at 5% level and thus suggested the linearity of calibration points.

The regression lines obtained for pure drugs and mixtures were similar (Table 4.2). This similarity was further supported from the obtained correlation coefficients and the standard error involved with each intercept, slope and estimate. A one-way ANOVA test [99] was performed with three replicates per calibration point including the lowest and highest variation observed from the mean absorbance of each concentration during the replicate measurements of pure drug solutions. The reported

F-values (Tables 4.3 and 4.4) were not significant at 5% level and thus demonstrated the linearity over the concentration range proposed.

The reported slope values without intercept on the ordinate for regression equations fall within 95% confidence interval which confirmed that the intercept values did not deviate significantly from the origin (Table 4.2). In addition, Student's *t*-test [98] values were highly significant at 5% level and thus indicated the positive correlation between measured values and concentrations (Table 4.2).

The developed method was validated according to the procedures described in U.S.P. 23 and the results were showed in Table 4.5. The limit of detection (LOD) and limit of quantitation (LOQ) [1,97] were determined from the slope of regression equations obtained in Series A and D of Table 4.2.

The percentage recoveries obtained with pure drug admixtures and commercial formulations were reported in Table 4.6. The recovery values have met the limits of official monographs for both the pure drugs and as well for their single drug formulations (Appendix B). The reported results of a Student's *t*-test (two-sided) [98] confirmed that there were no significant differences between the mean recoveries of standard and sample solutions at 5% level (Table 4.6). The prepared solutions were stable for 3 hrs in presence of 0.01M HCl and 0.01M NaOH. The reported pK_a values of PBT and DPH were 7.60 and 8.30 respectively (Appendix B) and hence the pH of 0.01M HCl (pH ≈ 2.0) and 0.01M NaOH (pH ≈ 12.0) were at least 2.0 pH units away from the pK_a values of drugs. Therefore, small variations in pH of the solvents did not lead to appreciable changes in the absorbance values [14].

Thus, the proposed method offers great advantage over other reported methods [129,419-421] in eliminating the interference from formulation matrix and also in resolving the overlapping spectra of the combined drug preparation. The developed

method was found to be accurate, precise and simple. Therefore, this combination of difference and derivative techniques provide a great help in routine analysis of two-component drug mixtures.

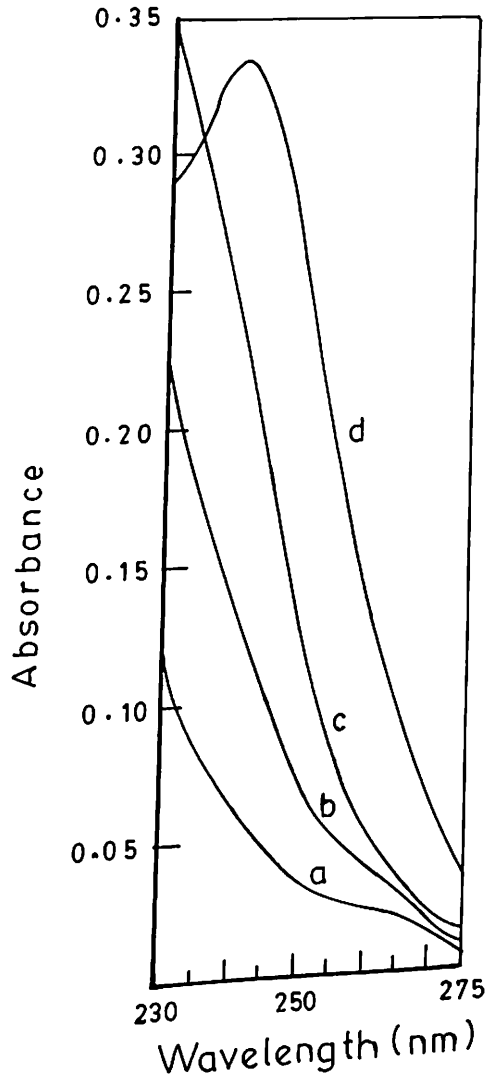

Figure 4.1 Absorption spectra of (a) phenobarbitone (7.5 µg/ml), (b) phenytoin sodium (15 µg/ml) in 0.01M HCl and (c) phenytoin sodium (15 µg/ml), (d) phenobarbitone (7.5 µg/ml) in 0.01M NaOH, respectively.

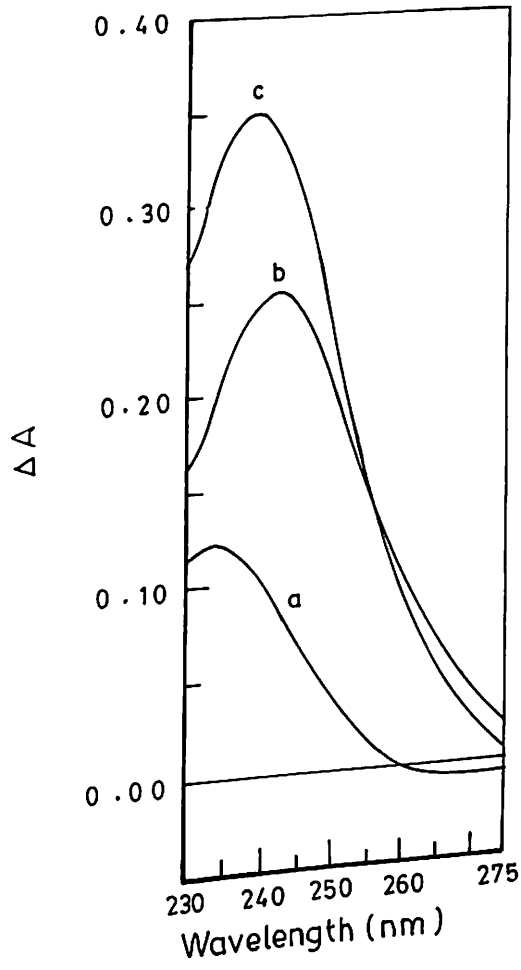

Figure 4.2 Difference spectra of (a) phenobarbitone (7.5 µg/ml), (b) phenytoin sodium (15 µg/ml) and (c) their mixture in 0.01M HCl Vs 0.01M NaOH.

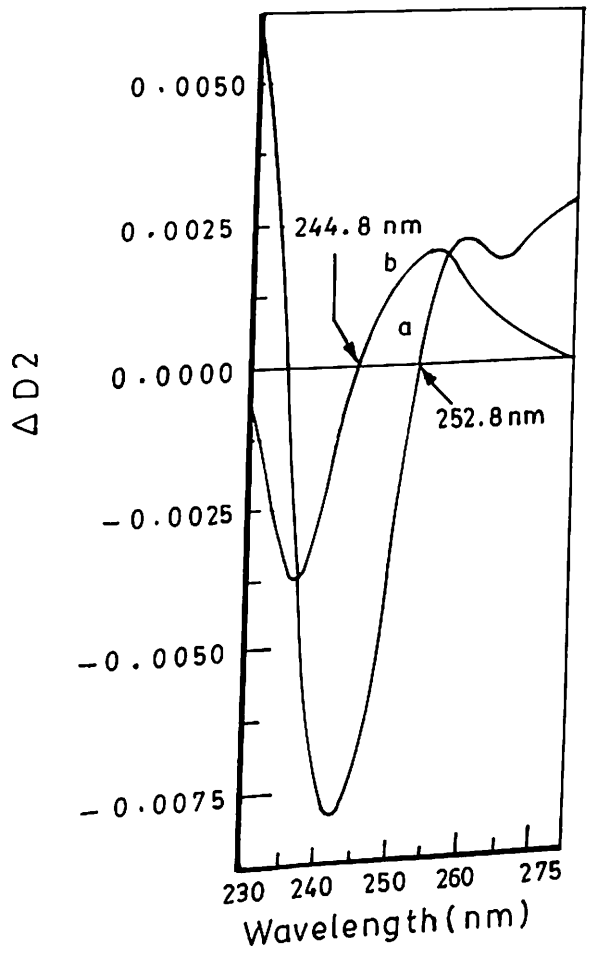

Figure 4.3 Second-derivative difference spectra of (a) phenobarbitone (7.5 µg/ml) and (b) phenytoin sodium (15 µg/ml) in 0.01M HCl Vs 0.01M NaOH.

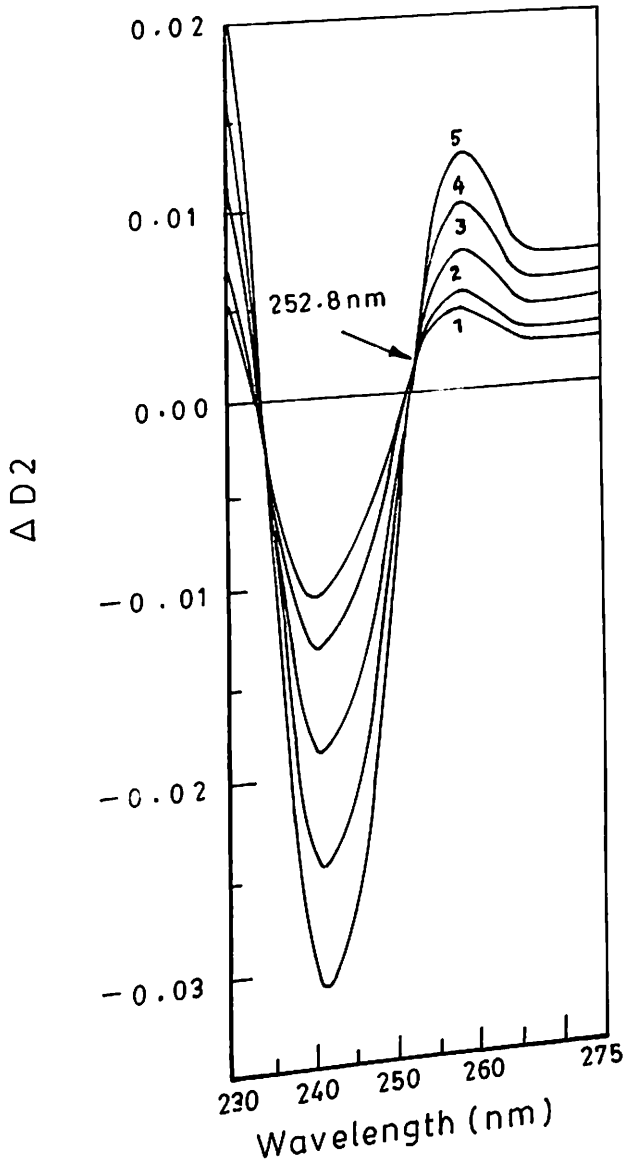

Figure 4.4 **Second-derivative difference spectra of (a) phenobarbitone (7.5, 10, 15, 20 and 25 μg/ml) and phenytoin sodium (15 μg/ml) in 0.01M HCl Vs 0.01M NaOH in curves 1-5, respectively.**

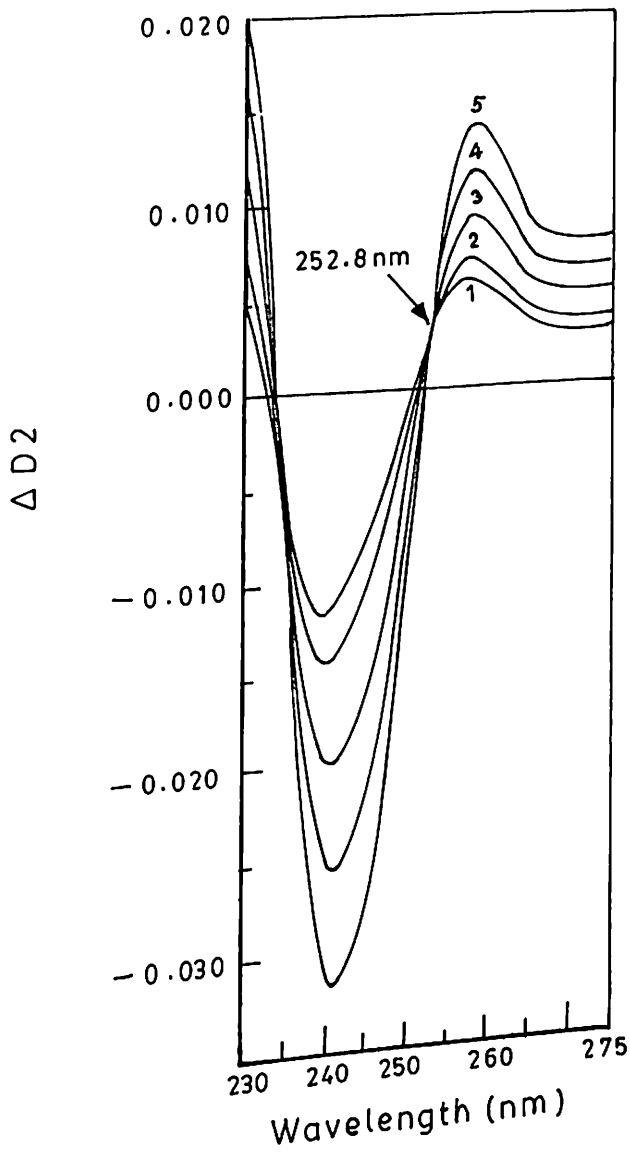

Figure 4.5 Second-derivative difference spectra of phenobarbitone (7.5, 10, 15, 20 and 25 µg/ml) and phenytoin sodium (25 µg/ml) in 0.01M HCl Vs 0.01M NaOH in curves 1-5, respectively.

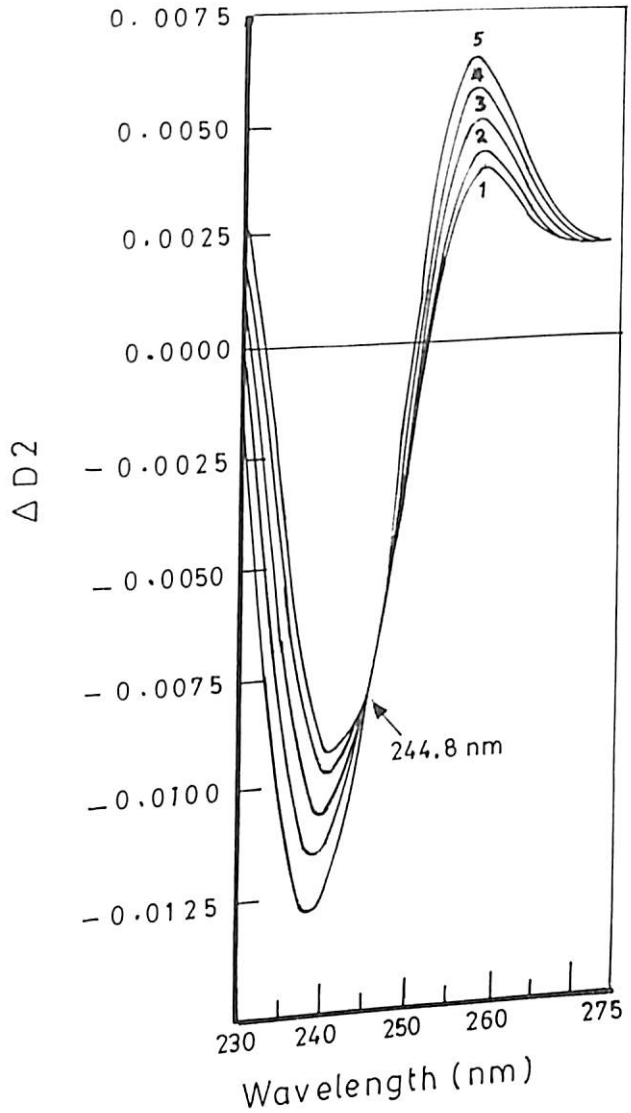

Figure 4.6 Second-derivative difference spectra of phenytoin sodium (7.5, 10, 15, 20 and 25 µg/ml) and phenobarbitone (7.5 µg/ml) in 0.01M HCl Vs 0.01M NaOH in curves 1-5, respectively.

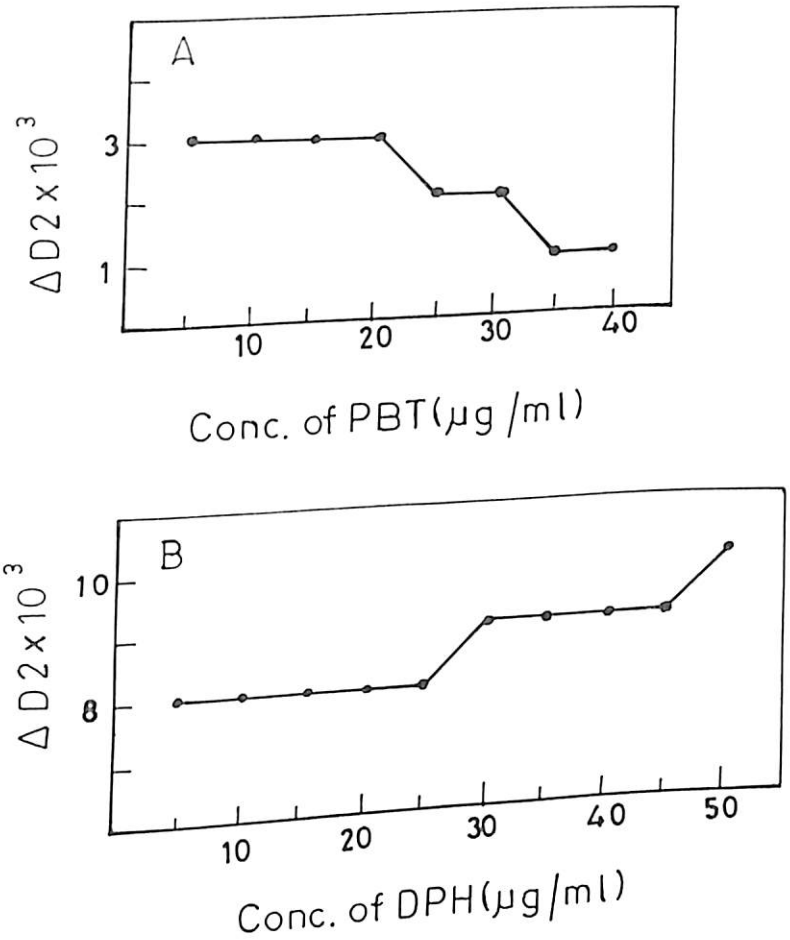

Figure 4.7 Second-derivative difference interaction graphs for (A) phenytoin sodium (25 μg/ml) in mixture with phenobarbitone (at 252.8 nm) and (B) phenobarbitone (7.5 μg/ml) in mixture with phenytoin sodium (at 244.8 nm) in 0.01M HCl Vs 0.01M NaOH.

Table 4.1: Selectivity of the method for the simultaneous determination of PBT and DPH in standard solutions by second-derivative difference spectrophotometry

Composition of the solution (μg/ml)		Mean absorbance [a] value (ΔD2) (PBT at 244.8 nm, DPH at 252.8 nm)	Coefficient of variation (%)	Standard error	Ratio of residual (%)	F-test for non-linearity	
PBT	DPH					Calc	Crit [b]
7.5	0	0.0078 ± 0.0001	1.38	0.0001	98.43	2.43	3.86
10	0	0.0102 ± 0.0001	1.39	0.0001	99.95	1.37	
15.0	0	0.0153 ± 0.0002	1.10	0.0001	100.97	0.99	
20.0	0	0.0205 ± 0.0002	1.07	0.0001	100.69	0.58	
25.0	0	0.0260 ± 0.0003	1.20	0.0001	99.36	0.29	
7.5	15.0	0.0077 ± 0.0001	1.60	0.0001	100.25	0.42	
10.0	15.0	0.0103 ± 0.0001	1.00	0.0001	100.18	0.59	
15.0	15.0	0.0156 ± 0.0001	1.04	0.0001	99.33	0.24	
20.0	15.0	0.0206 ± 0.0002	1.03	0.0001	100.40	0.14	
25.0	15.0	0.0259 ± 0.0002	0.66	0.0001	99.93	0.21	
7.5	25.0	0.0078 ± 0.0001	1.38	0.0001	99.33	1.85	
10.0	25.0	0.0102 ± 0.0001	1.66	0.0001	100.70	0.74	
15.0	25.0	0.0156 ± 0.0003	1.93	0.0001	99.29	0.23	
20.0	25.0	0.0205 ± 0.0002	0.98	0.0001	100.91	0.52	
25.0	25.0	0.0260 ± 0.0003	1.36	0.0001	99.62	0.17	
0	7.5	0.0008 ± 0.0001	2.95	0.0001	99.95	1.40	3.86
0	10.0	0.0011 ± 0.0001	2.99	0.0001	101.90	0.75	
0	15.0	0.0017 ± 0.0001	2.78	0.0001	99.18	0.35	
0	20.0	0.0023 ± 0.0001	1.90	0.0001	98.60	0.40	
0	25.0	0.0029 ± 0.0001	1.76	0.0001	100.91	0.31	
7.5	7.5	0.0008 ± 0.0001	2.79	0.0001	99.18	0.35	
7.5	10.0	0.0011 ± 0.0001	2.41	0.0001	101.42	0.25	
7.5	15.0	0.0017 ± 0.0001	2.64	0.0001	99.65	0.08	
7.5	20.0	0.0023 ± 0.0001	1.52	0.0001	99.51	0.13	
7.5	25.0	0.0029 ± 0.0001	1.81	0.0001	100.28	0.06	

[a] : Average of ten determinations with standard deviation.
[b] : Theoretical value of F(3,9) at P = 0.05 level of significance.

Table 4.2: Regression analysis for the determination of PBT and DPH in standard solutions by second-derivative difference spectrophotometry

Sample	Composition of solutions (µg/ml)		Regression equations [a] (at 244.8 nm for PBT at 252.8 nm for DPH)	Corr. coeff.	Standard error			95% Confidence interval		Slope without intercept	Student *t*-test for correlation	
	PBT	DPH			Intercept	Slope	Estimate	Intercept	Slope		Calc	Crit [b]
Series A	7.5 -25	0	Y = 1.04E-03.X - 1.51E-04	0.9998	1.96E-04	1.17E-05	1.67E-04	-7.75E-04, 4.72E-04	1.00E-03, 1.08E-03	1.03E-03	87	3.18
Series B	7.5 -25	15	Y = 1.04E-03.X - 9.72E-05	0.9999	9.26E-05	5.52E-06	7.93E-05	-3.92E-04, 1.97E-04	1.02E-03, 1.06E-03	1.04E-03	122	
Series C	7.5 -25	25	Y = 1.04E-03.X - 7.27E-05	0.9998	1.71E-05	1.02E-05	1.46E-04	-6.16E-04, 4.71E-04	1.00E-03, 1.07E-03	1.04E-03	87	
Series D	0	7.5 -25	Y = 1.20E-04.X - 9.38E-05	0.9996	3.31E-05	1.98E-06	2.83E-05	-1.99E-04, 1.17E-05	1.14E-04, 1.27E-04	1.17E-04	61	
Series E	7.5	7.5 -25	Y = 1.22E-04.X - 1.25E-04	0.9999	1.53E-05	9.10E-07	1.31E-05	-1.79E-04, -6.00E-05	1.18E-04, 1.25E-04	1.18E-04	122	

[a] : Based on five calibration values; X = Concentration of drug in µg/ml.

[b] : Theoretical value of '*t*' at P = 0.05 level of significance with 3 d.f.

Table 4.3: One-way ANOVA test for linearity of pure PBT solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	6.63E-04	6.63E-04	0.687	3.71
Lack of fit	3	1.82E-07	6.08E-08		
Within line	10	8.84E-07	8.84E-07		
Total	14	6.64E-04			

*: at P = 0.05 level of significance.

Table 4.4: One-way ANOVA test for linearity of pure DPH solutions

Source of variation	Degrees of freedom	Sum of squares (SS)	Mean sum of squares (MS)	F_{Calc}	F_{Crit} *
Regression	1	9.06E-06	9.06E-06	0.640	3.71
Lack of fit	3	8.43E-09	2.81E-09		
Within line	10	4.39E-08	4.39E-09		
Total	14	9.11E-06			

*: at P = 0.05 level of significance.

Table 4.5: Validation report for the determination of PBT and DPH in standard solutions by second-derivative difference spectrophotometry

Analytical parameter	Results	
	PBT (244.8 nm)	DPH (252.8 nm)
Accuracy (%)	100.30 ± 0.53	99.99 ± 0.81
Precision (%)	99.74 100.00 100.25 100.38 101.15 RSD: 0.53	98.84 99.42 100.57 100.57 100.57 RSD: 0.81
Specificity	A 7.5 µg/ml of PBT and 15.0 µg/ml of DPH mixture solution will show an absorbance value (ΔD2) of 0.0077 ± 0.0001	A 15.0 µg/ml of DPH and 7.5 µg/ml of PBT mixture solution will show an absorbance value (ΔD2) of 0.0017 ± 0.0001
LOD (µg/ml)	0.23	0.71
LOQ (µg/ml)	0.76	2.36
Linearity (µg/ml)	5 - 40	5 - 50
Ruggedness (%)	100.30 ± 0.53	99.99 ± 0.81

Table 4.6: Results of the assay of pure drug admixtures and commercial formulations of PBT and DPH by second-derivative difference spectrophotometry

Sample	Label Claim (mg/tab.)		Recovery (%) [a]		Student *t*-test for Mean Recovery		
					Calc		Crit [b]
	PBT	DPH	PBT (244.8 nm)	DPH (252.8 nm)	PBT	DPH	(PBT / DPH)
Pure drug admixture (1:2)	-	-	99.66 ± 0.64	99.76 ± 0.78	0.65	0.28	2.31
Brand MX	50	100	99.94 ± 0.71	99.65 ± 0.32			
Pure drug admixture (1:3.33)	-	-	100.21 ± 0.61	99.93 ± 0.70	0.55	0.30	2.31
Brand MY	30	100	100.38 ± 0.35	99.79 ± 0.70			

[a] : Mean and standard deviation for five determinations.
[b] : Theoretical value of '*t*(two-sided)' at P = 0.05 level of significance with 8 d.f.

CHAPTER 5

CONCLUSIONS

5. CONCLUSIONS

The most common problem often seen in the analysis of drug formulations is usually the interference from the matrix constituents. An ideally designed simultaneous estimation method should be able to eliminate the interferences of additives and other drug(s) of the formulation. The methods proposed in this report have been designed with this objective and achieved the same. The following are the conclusions drawn from the results obtained.

1. The results of difference, derivative and derivative-difference spectroscopic methods for the simultaneous estimation of drugs in combined formulations were found to be accurate and precise. This was evident from the statistical analysis and method validation. Thus, in view of the validity of these methods and accuracy of the results, it can be suggested that these techniques are quite useful for routine analysis on par with the existing chromatographic techniques.

2. Results of analysis of all the combined formulations reported in this thesis by using above techniques have been found to meet the official monograph requirements.

3. It has been observed that the need of separation or extraction procedure to eliminate additives of formulation matrix did not arise and the interference from such additives was found to be absent.

4. The estimation of individual drug in presence of other drug(s) of a formulation has been achieved successfully without any need of separation.

5. The results of present study also lead to suggest the following observations:

   A. **Difference spectroscopy**

   - This particular method of determination is relatively simple and easier compared to other two proposed techniques namely derivative and derivative-difference spectroscopy.

- The method has been proved to be a potential technique in eliminating the matrix interferences over derivative technique (where the technique will encourage the noise in the elimination process or curves will be filled with more noise) provided they do not undergo spectral alterations in presence of chosen buffer systems.
- The method could not be extended to other drug combinations except the two metronidazole combinations due to their lack of stability, solubility and/or lack of significant spectral changes with the treatment of different buffers.

B. Derivative spectroscopy

- The method is relatively a potential technique in overcoming the problems often seen in difference technique.
- The method is proved to be useful, especially,
  - **i.** in resolving the drug mixtures in presence of shoulder peaks (as found in tinidazole - norfloxacin, metoprolol tartrate - hydrochlorothiazide and atenolol - amlodipine besylate combinations).
  - **ii.** in resolving the greatly overlapped peaks (as observed in tinidazole - furazolidone, tinidazole - norfloxacin, tinidazole - furazolidone - diloxanide furoate, metoprolol tartrate - hydrochlorothiazide, propranolol HCl - hydrochlorothiazide, atenolol - amlodipine besylate and atenolol - amiloride HCl - hydrochlorothiazide combinations).
  - **iii.** in resolving the minor component with lack of significant absorption in presence of a major component (as seen in tinidazole - clotrimazole, amlodipine besylate - enalapril maleate and haloperidol - trihexyphenidyl HCl combinations).

C. Derivative-difference spectroscopy

- This combined technique is very useful in situations where the difference and derivative technique have failed individually, in handling the problems like matrix interference, overlapping and resolving the drug substances with similar

spectral features (as noticed in phenobarbitone - phenytoin sodium combination).

6. The following are the salient features of the study:

- The proposed derivative methods for tinidazole - furazolidone - diloxanide furoate and metoprolol tartrate - hydrochlorothiazide are the first successful reports so far available for their simultaneous determination including the chromatographic methods. Thus, the methods should be very useful in routine analysis.
- The proposed derivative-difference method for phenobarbitone - phenytoin sodium is very unique and also the most successful report available till date for this particular combination compared to other reported methods. Therefore, the proposed method should considered to be of a great value in their simultaneous determination.
- The proposed simultaneous spectrophotometric methods for metronidazole - nalidixic acid, tinidazole - furazolidone, tinidazole - norfloxacin and atenolol - amiloride HCl - hydrochlorothiazide have great advantage over other spectrophotometric methods reported recently like multi-component analysis (MA) in terms of the simplicity, reproducibility and transparency for wide spread usage in routine analysis.
- The developed methods for the combined formulations like metronidazole - diloxanide furaote, tinidazole - clotrimazole, tinidazole - furazolidone - diloxanide furoate, metoprolol tartrate - hydrochlorothiazide, propranolol HCl - hydrochlorothiazide, atenolol - amlodipine besylate, amlodipine besylate - enalapril maleate and haloperidol - trihexyphenidyl HCl, with the help of difference, derivative and derivative-difference are the only procedures available till now for their simultaneous determination.

However, the following facts are ought to be kept in mind during the adaptation of method for analysis.

A. Difference spectroscopy

- The drugs should be compatible in both acidic and alkali buffer system.
- The slight change in buffer strength at the time of preparation can shift the isosbestic point.
- This technique will eliminate only the interference of substances that do not undergo spectral alteration due to changes in the conditions (like pH). In such a situation, the derivative technique may be useful.

B. Derivative spectroscopy

- The spectral shape and its amplitude depend on the conditions such as scan speed, spectral bandwidth and value of data interval.
- The reported set parameters for derivative processing should not be used as such for the estimation of drugs. Instead, the method should be validated in the particular instrument.
- The method of digital differentiation and smoothing algorithm employed in the instrument for generating derivative spectra should be known prior to derivative processing.
- Smoothing always involves a compromise between resolution, noise (SNR) and distortion of the signal. Hence, the smoothing process of a curve should be used as and when necessary. It is advisable to consider few data points several times rather than many points at a time.
- It is preferable to quantitate the mixture in as low order as possible with resolved shoulders and inflection points. Since, higher orders are to be met with more noise.

- The main disadvantages of the zero-crossing point method are the risk of small drifts of the working wavelengths (practically eliminated with recent spectrophotometers).
- Generally, in zero-crossing method, the working wavelengths do not fall in correspondence of peaks of derivative spectrum. This may be particularly dangerous when the slope of the spectrum is very high with consequent loss of accuracy and precision. Incidentally, in the present case the above circumstances did not occur.
- The ratio-compensation method is also a reliable one. However, the high ratio between the components of estimation can influence the determination of drugs at the selected wavelength maxima or minima. Therefore, the method requires an accurate investigation of such variables. This method may be interesting and useful alternative in particular cases where the popular zero-crossing technique gives less sensitive or inaccurate results.

The above limitations are also to be kept in mind during the method determination with the help of **derivative-difference spectroscopy**.

By considering analysis time, ease of operation and derivative capability of the modern instruments, the reported methods are of great utility in the routine pharmaceutical analysis. Since B.P. 1993 recommended ultraviolet-derivative spectrophotometric procedure for the individual assay of few drug formulations, it is reasonable to expect a widespread application of derivative technique in future for routine analysis.

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APPENDIX A

CALIBRATION OF UV-VIS SPECTROPHOTOMETER

The analytical work reported in this thesis was carried out by using JASCO model 7800 double beam UV-VIS spectrophotometer.

It is indeed necessary to calibrate the instrument before devising any analytical technique to identify the systemic errors and to study the quality of the spectrum produced. Thus the utilised spectrophotometer was calibrated according to the procedures recommended in B.P. 1993 [2], I.P. 1996 [3] and also to the unofficial methods suggested by Talsky [4]. The test methodology and the results obtained along with corresponding spectra (wherever necessary) were discussed below.

The suitable settings of the instrument for calibration were a scan speed of 240 nm/min and a bandwidth of 3 nm. Quartz cells of 1 cm thickness were used. Ordinate maxima and minima were adjusted according to the magnitude of measured values in each case.

A.I: Official methods (Recommended by B.P. 1993 and I.P. 1996)

i. Control of Absorbances

Principle

To check the absorbance value of potassium dichromate solution at the wavelengths indicated in Table A.1 and the corresponding molar extinction coefficient value, A(1%, 1 cm) and comparison with the permitted limits.

Procedure

A 0.0025% W/V solution of potassium dichromate was prepared in distilled water and its absorbance was recorded at the prescribed wavelengths with distilled water in the reference cell.

Results

The observed absorbances confirmed to the reported values and were given in Table A.1.

ii. Limit of stray light

Principle

The amount of stray light will be detected at particular wavelength with suitable filters or solutions. For example the absorbance of a 1.2% W/V aqueous solution of potassium chloride with a path length of 1 cm should be more than 2 at 200 nm when compared with distilled water as reference liquid.

Procedure

A 1.2% W/V solution of potassium chloride was prepared in distilled water. The absorbance of the solution was recorded from 200-230 nm against distilled water as reference.

Result

The absorption value was found to be greater than 2.0 at 200 nm.

iii. Resolution

Principle

When a spectrum of 0.02% V/V solution of toluene in hexane is recorded, the ratio of the absorbance at the maximum at 269 nm to that of minimum at 266 nm should be at least 1.5.

Procedure

The spectrum of 0.02% V/V solution of toluene in hexane was recorded from 255-275 nm with hexane as reference.

Result

The ratio of greater than 1.5 was observed between absorption at 269 nm to that of 266 nm.

iv. Resolution of second-derivative spectrum

Principle

The measured second-derivative spectrum of a 0.020% V/V solution of toluene in methanol using methanol in reference cell in the range of 255-275 nm should show a small negative extremum (or trough) located between two large negative extrema (or trough) at about 261 nm and at 268 nm.

Procedure

A 0.020% V/V solution of toluene in methanol was prepared and the second-order spectrum was recorded in the range of 255-275 nm with methanol in the reference cell, using Savitzky-Golay parameter [138] of $\Delta = 1$ point.

Result

The observed second-order spectrum showed the presence of a small negative extremum between two large negative extrema (Figure. A.1).

A.II: Unofficial methods (suggested by Talsky, [4])

The methods were mainly proposed to study the quality of spectra produced by the instrument using various standard chemical substances. These various chemical substances, based on the type of spectra produced during their measurement in UV-VIS region, were classified into four types and these along with their reported and observed wavelength maximum (λ_{max}) were given in Table A.2.

The various tests performed with the instrument to study the production quality of derivative curves by utilising above mentioned substances of each category (Table A.2) were discussed below.

Type I

The spectra of these substances expected to show an asymmetric Guassian curve. The derivative module should be able to resolve the signal into two main peaks in fourth-derivative mode.

Procedure

A 400 mg of nickel chloride hexahydrate ($NiCl_2$, $6H_2O$) was dissolved in 10 ml of distilled water and the absorbance was recorded from 320-500 nm with distilled water in the reference cell. The fourth-derivative spectra was recorded by using Δ = 10 points [138]. The smoothing of the spectrum was done for five times by using Δ = 25 points [138] in each time.

Result

The obtained zero-order spectrum having the necessary features of asymmetric Gaussian curve was shown in Figure A.2a. The fourth-derivative spectrum thus obtained was able to resolve the spectra into two main peaks and was given in Figure A.2b.

Type II

The spectrum of these substances should be of Gaussian curve superposed by an underlying exponential function. The differentiation module should be able to eliminate this by higher order differentiation.

Procedure

A 1 mg of potassium iodide was dissolved in 100 ml of distilled water and the spectrum was recorded from 200-260 nm. The first-derivative spectrum was obtained with Δ = 10 points [138]. No smoothing was found necessary.

Result

The obtained zero-order spectra having the necessary features was shown in Figure A.3a. The first-derivative curve obtained was able to resolve the superposition of the Gaussian curve by an underlying exponential function and was showed in Figure A.3b

Type III

The normal spectrum is expected to show a distinct shoulder on the side of a peak. The differential module should be able to resolve the shoulder on the side of the peak.

Procedure

A methylene blue solution was prepared by dissolving of 1 mg in 100 ml of distilled water and the absorbance was recorded from 500-700 nm. The fourth-derivative of the recorded spectrum was obtained with Δ = 60 points [138] and the smoothing of spectrum was carried out with Δ = 20 points [138].

Result

The zero-order spectrum with a distinct shoulder peak was shown in Figure A.4a. The fourth-derivative of the recorded spectrum was able to resolve the shoulder on the side of the peak and was shown in Figure A.4b.

Type IV

The normal spectrum of these substances expected to show small irregularities. The second-derivative module should be able to resolve the small irregularities noticed in the fundamental spectrum.

Procedure

A 2.5 mg of potassium permanganate was dissolved in 50 ml of distilled water and the zero-order spectrum was recorded from 430-610 nm. The second-derivative spectrum was obtained by setting Δ = 10 points [138] and no smoothing was necessary.

Result

The irregularities observed in the fundamental spectrum were showed in Figure A.5a and were successfully resolved through second-derivative processing. The obtained resolved spectrum was showed in Figure A.5b and indicated that the second-derivative mode was optimum.

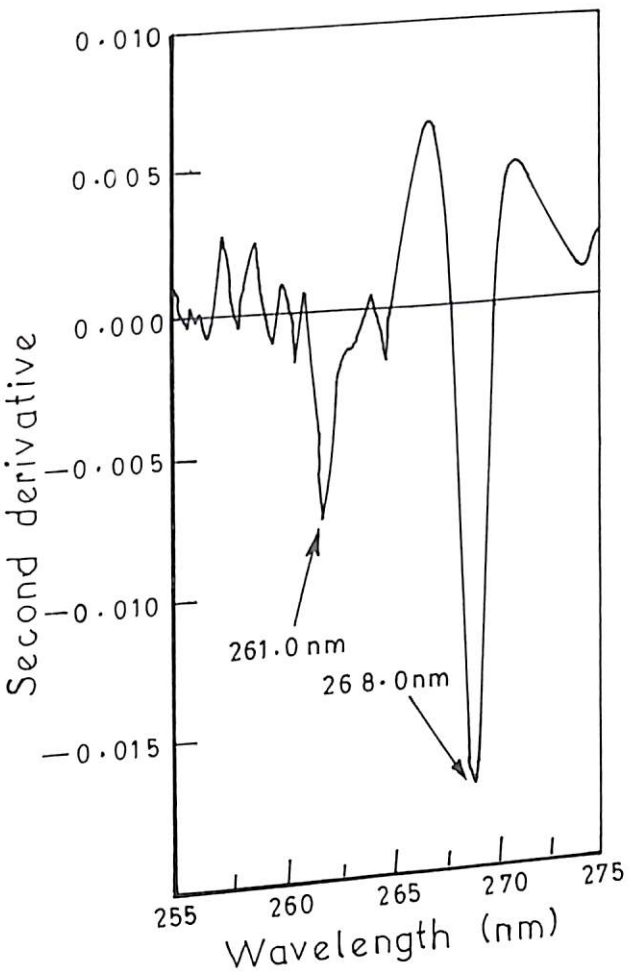


Figure A.1 Second-derivative spectrum of 0.020% v/v solution of toluene in methanol.

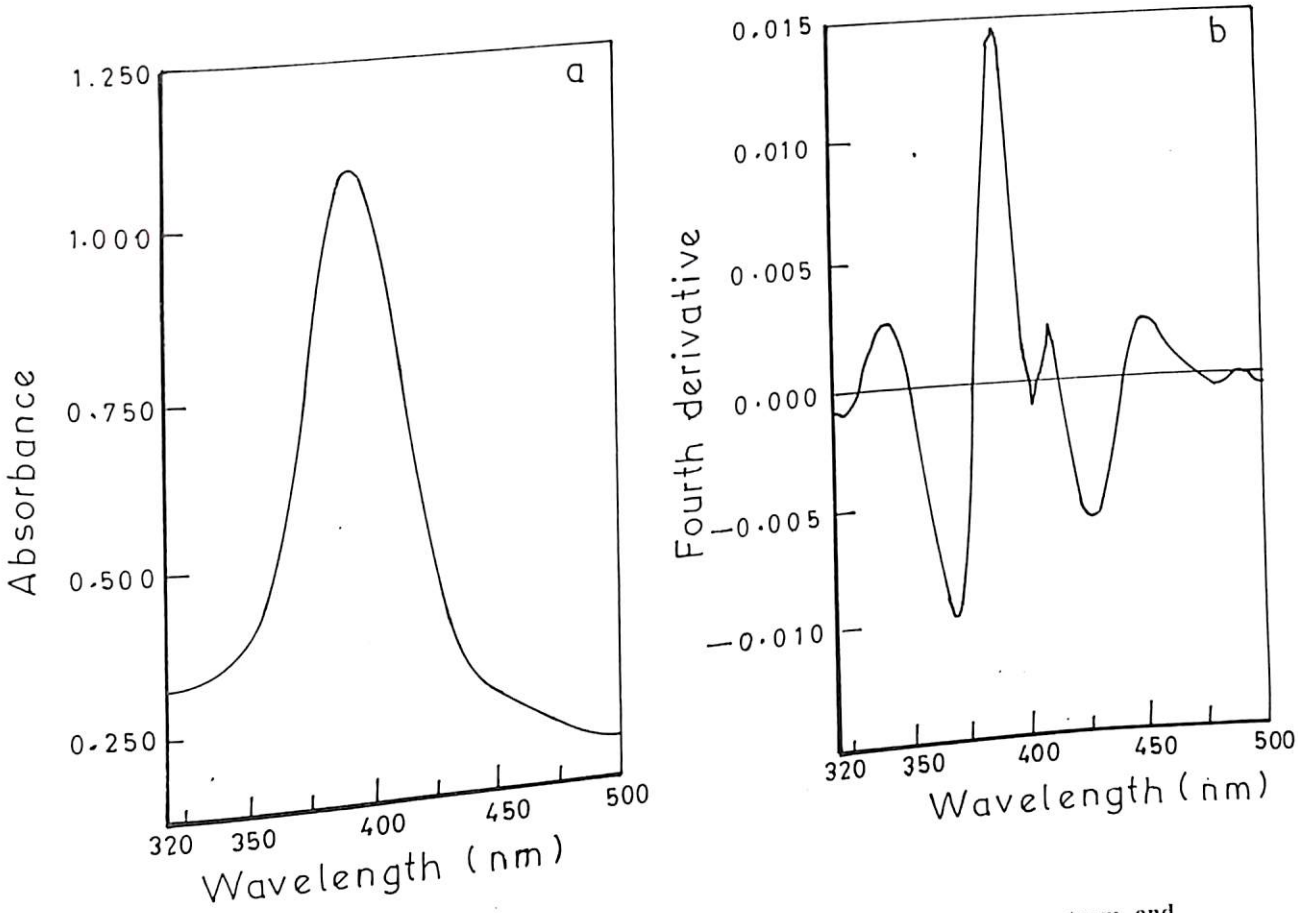


Figure A.2 Nickel chloride (Conc. 40 g/lt): (a) fundamental spectrum and (b) fourth-derivative spectrum in distilled water.

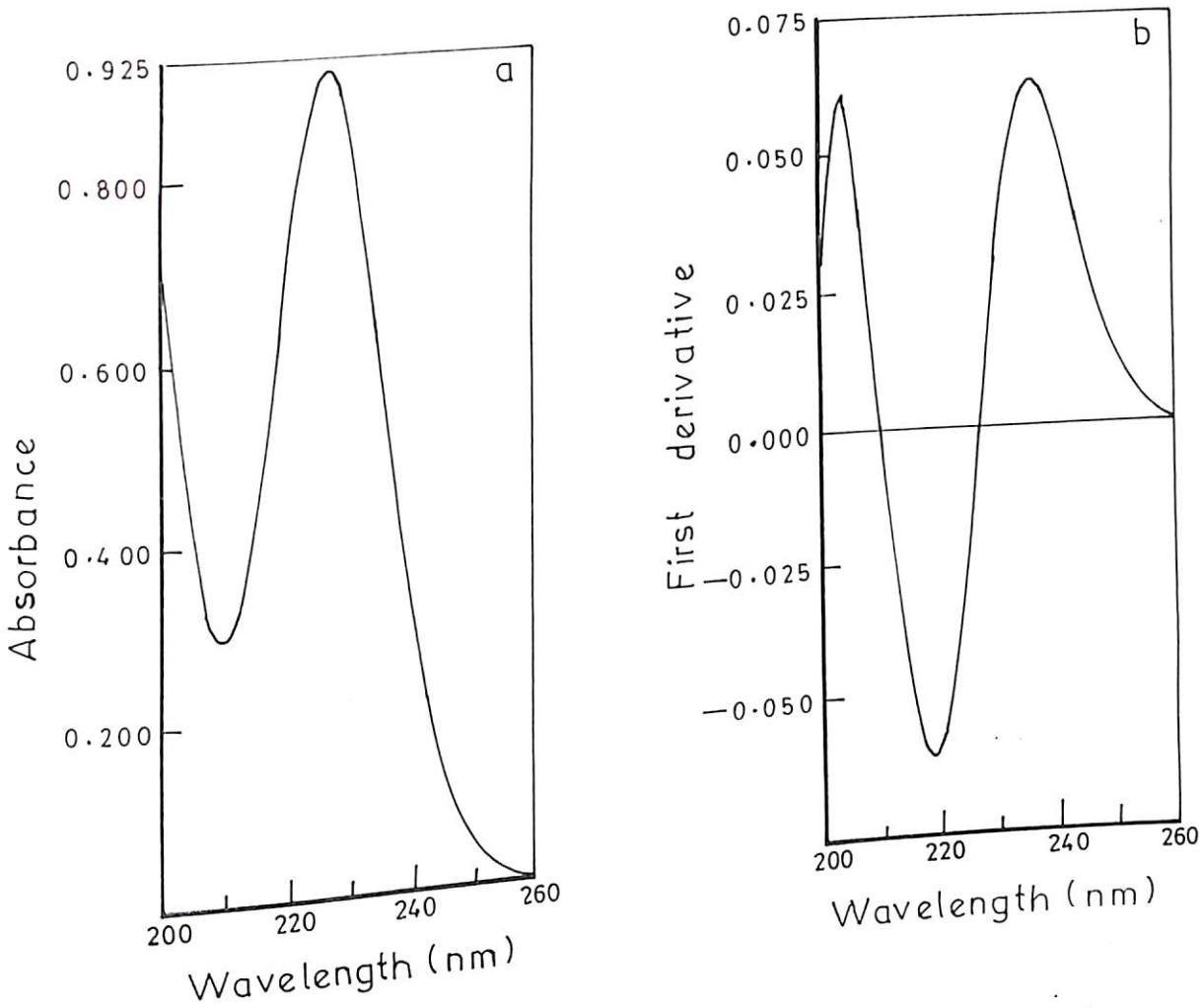


Figure A.3 Potassium iodide (Conc. 0.01 g/lt): (a) fundamental spectrum and (b) first-derivative spectrum in distilled water.

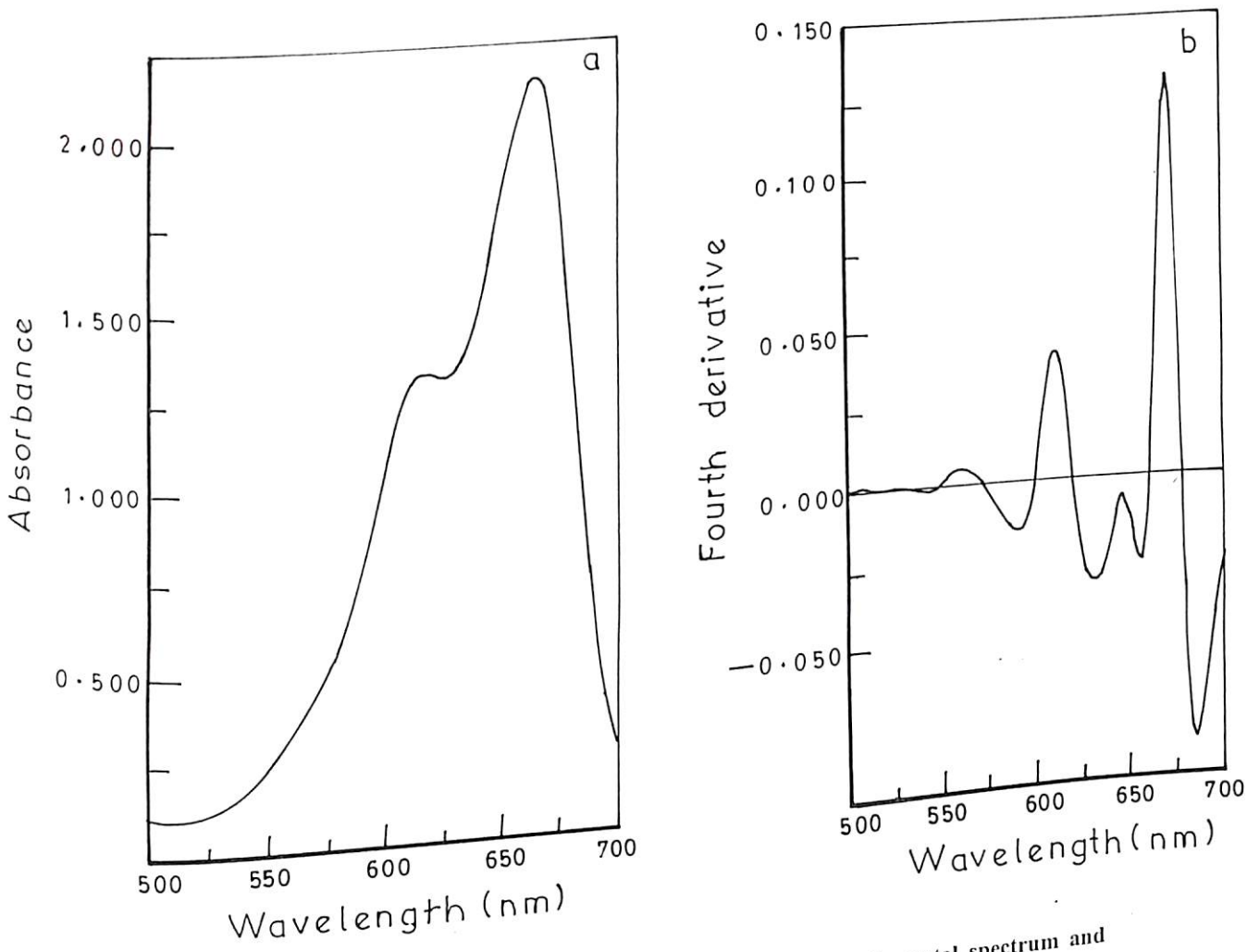


Figure A.4 Methylene blue (Conc. 0.01 g/lt): (a) fundamental spectrum and (b) fourth-derivative spectrum in distilled water.

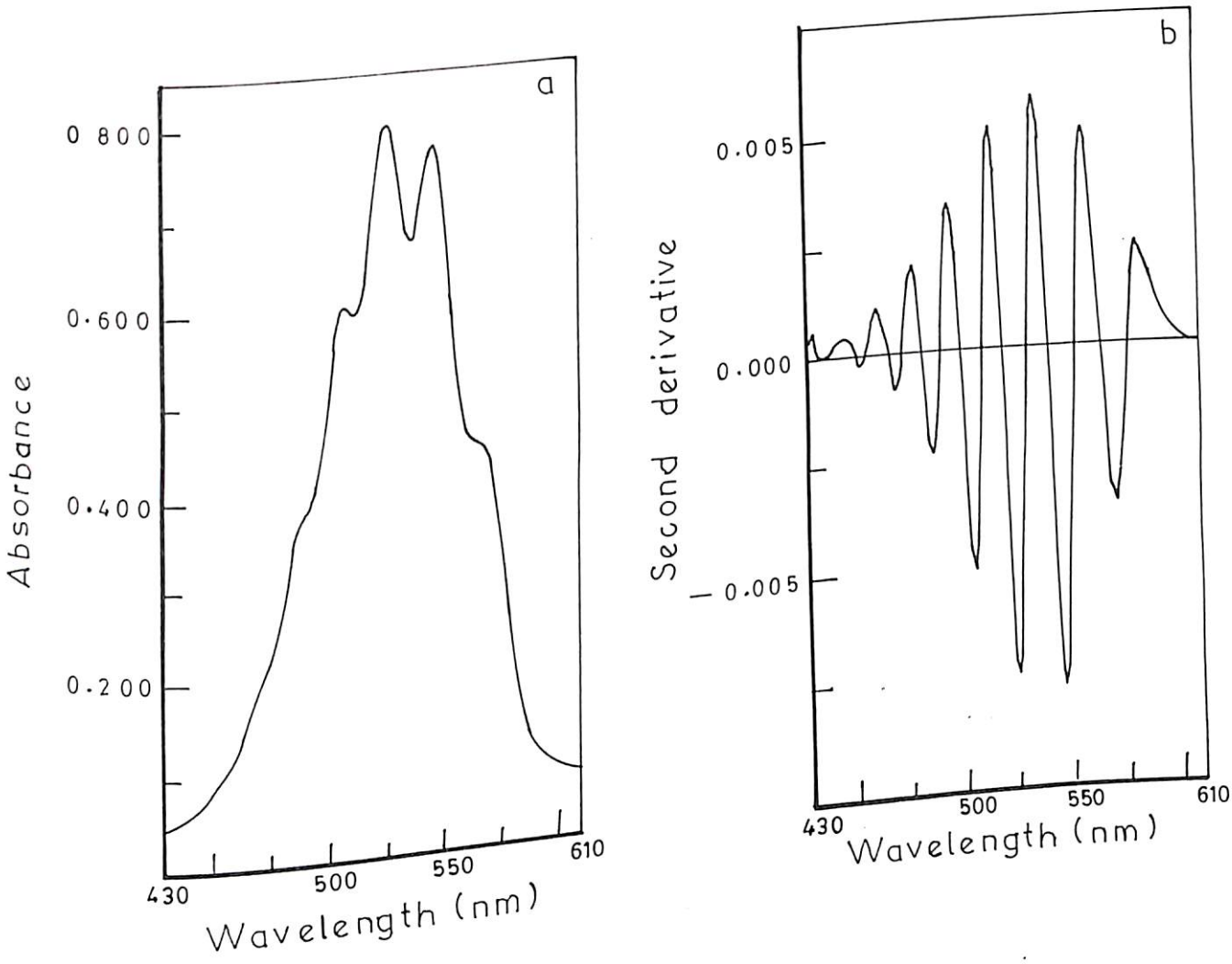


Figure A.5 Potassium permanganate (Conc. 0.05 g/lt): (a) fundamental spectrum and (b) second-derivative spectrum in distilled water.

Table A.1: Test for control of absorbances with potassium dichromate solution

Wavelength (nm)	Recommended value A (1%, 1 cm)	Permitted Max. tolerance	Observed Values	
			Absorbance (0.0025% W/V)	A (1%, 1 cm)*
235	124.5	122.9-126.2	0.312	125.0 ± 0.57
257	144.0	142.4-145.7	0.362	144.3 ± 0.44
313	48.6	47.0-50.3	0.123	48.8 ± 0.21
350	106.6	104.9-108.2	0.270	106.6 ± 0.50

*: Average of thirteen determinations.

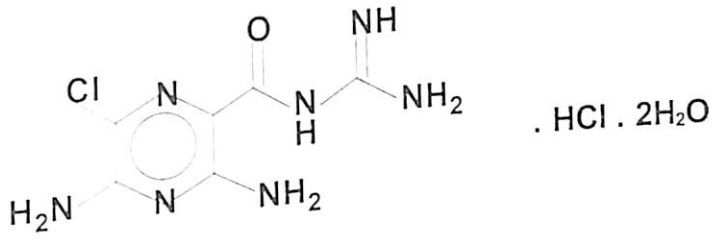
Table A.2: Classification of substances qualified for standard spectra of various typical shapes

Type	Substance	Conc. (g/lt)	λ-region (nm)	λ_{max}	
				Reported	Observed
I	$NiCl_2.6H_2O$	40.0	320-500	394	394
	$CuSO_4.5H_2O$	20.0	550-890	810	810
II	KI	0.01	200-260	226	226
	Congo Red	0.01	400-620	497	497
III	Methylene Blue	0.01	500-700	664	664
	$NiCl_2.6H_2O$	40.0	550-800	715	715
IV	$KMnO_4$	0.05	430-610	525	525
	BSA	1.0	240-370	277	277

APPENDIX B

ANALYTICAL PROFILE OF DRUGS

Drug Profile B.1: Amiloride Hydrochloride

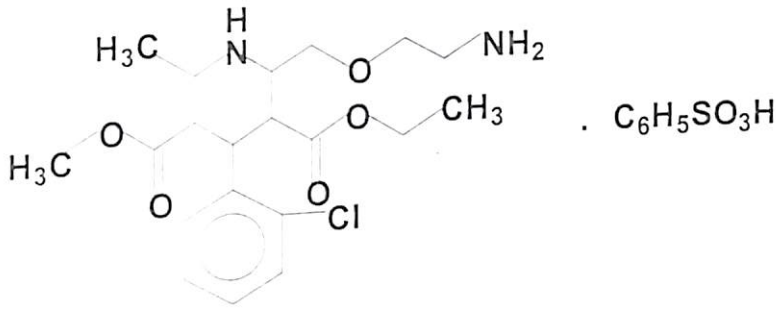


Chemical Name	:	N-amidino-3,5-diamino-6-chloropyrazine-2-carboxamide hydrochloride dihydrate
Emperical Formula	:	$C_6H_8ClN_7O$, HCl, $2H_2O$
Molecular Weight	:	302.1
Melting Point	:	285 – 288°C [430]
pK_a	:	8.70 [99]
Description	:	A pale yellow to greenish yellow powder
Solubility	:	Freely soluble in dimethylsulfoxide; slightly soluble in water and in ethanol (95%); practically insoluble in chloroform and ether.

Official Method of Analysis

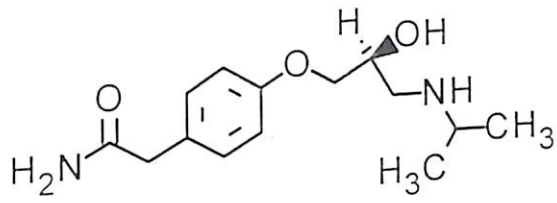
a. Pure Drug	:	Titrimetry [U.S.P. 23] Potentiometry [B.P. 1993, I.P. 1996]
Assay Limits	:	98.0-101.0% [U.S.P. 23, B.P. 1993, I.P.1996]
b. Dosage Forms	:	HPLC [U.S.P. 23] UV [B.P.1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23, B.P.1993, I.P. 1996]

Drug Profile B.2: Amlodipine Besylate



Chemical Name	:	2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3, 5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzene sulphonate [430]
Empeircal Formula	:	$C_{20}H_{25}Cl\ N_2O_5.\ C_6H_5SO_3H$
Molecular Weight	:	566.88
Description	:	A white, crystalline powder; odourless
Solubility	:	Freely soluble in chloroform, dichloromethane; soluble in methanol, alcohol (95%); slightly soluble in water, acetone; insoluble in ether.
Official Method of Analysis	:	Not available in U.S.P. 23, B.P. 1993, I.P. 1996

Drug Profile B.3: Atenolol

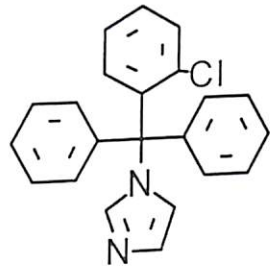


Chemical Name	:	(RS)-4-(2-hydroxy-3-isopropylamino propoxy) phenylacetamide
Emperical Formula	:	$C_{14}H_{22}N_2O_3$
Molecular Weight	:	266.3
Melting Point	:	152-155°C [3]
pK_a	:	9.60 [99]
Description	:	A white or almost white powder
Solubility	:	Sparingly soluble in water; soluble in absolute ethanol; slightly soluble in dichloromethane; practically insoluble in ether.

Official Method of Analysis

a. Pure Drug	:	Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	99.0-101.0% [B.P.1993, I.P. 1996]
b. Dosage Forms	:	UV [B.P.1993, I.P. 1996]
Assay Limits	:	92.5-107.5% [B.P.1993, I.P. 1996]

Drug Profile B.4: Clotrimazole

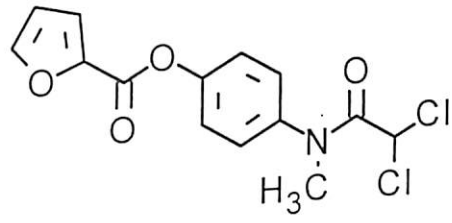


Chemical Name	:	1-(2-chlorotrityl) imidazole
Emperical Formula	:	$C_{22}H_{17}ClN_2$
Molecular Weight	:	344.8
Melting Point	:	141-145°C [2]
pK_a	:	4.70 [428]
Description	:	A white or pale yellow, crystalline powder
Solubility	:	Practically insoluble in water; freely soluble in acetone, chloroform, methanol and in ethanol (95%); slightly soluble in ether.

Official Method of Analysis

a. Pure Drug	:	HPLC [U.S.P. 23] Titrimetry [B.P.1993, I.P. 1996]
Assay Limits	:	98.0-102.0% [U.S.P. 23, I.P. 1996] 98.5-100.5% [B.P.1993]
b. Dosage Forms	:	HPLC [U.S.P. 23, B.P. 1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23] 95.0-105.0% [B.P.1993, I.P. 1996]

Drug Profile B.5: Diloxanide Furoate

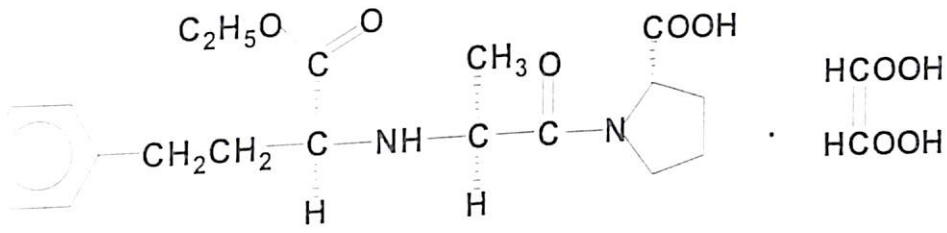


Chemical Name	:	4-(N-methyl-2, 2-dichloroacetamido) phenyl 2-furoate
Emperical Formula	:	$C_{14}H_{11}Cl_2NO_4$
Molecular Weight	:	328.2
Melting Point	:	114-116°C [2]
Description	:	A white, crystalline powder; odourless
Solubility	:	Very slightly soluble in water; freely soluble in chloroform; slightly soluble in ethanol (95%) and in ether.

Official Method of Analysis

a. Pure Drug	:	Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	98.0-102.0% [B.P.1993, I.P. 1996]
b. Dosage Forms	:	UV [B.P.1993, I.P. 1996]
Assay Limits	:	95.0-105.0% [B.P.1993, I.P. 1996]

Drug Profile B.6: Enalapril Maleate



Chemical Name : N-[N-[(S)-1-ethoxycarbonyl-3-phenyl propyl]-L-alanyl]-L-proline hydrogen maleate

Emperical Formula : $C_{20}H_{28}N_2O_5 . C_4H_4O_4$

Molecular Weight : 492.53

Melting Point : 144°C [430]

pK_a : 3.0, 5.49 [99]

Description : Off-white, crystalline powder

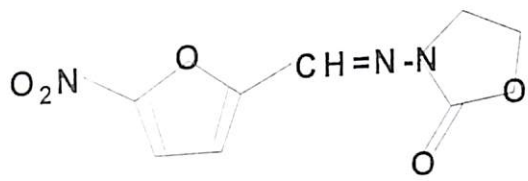
Solubility : Freely soluble in methanol and in dimethyl formamide; soluble in ethanol (95%); sparingly soluble in water.

Official Method of Analysis

a. Pure Drug : HPLC [U.S.P. 23, I.P. 1996]
Assay Limits : 98.0-102.0% [U.S.P. 23, I.P. 1996]

b. Dosage Forms : HPLC [U.S.P. 23, I.P. 1996]
Assay Limits : 90.0-110.% [U.S.P. 23, I.P. 1996]

Drug Profile B.7: Furazolidone

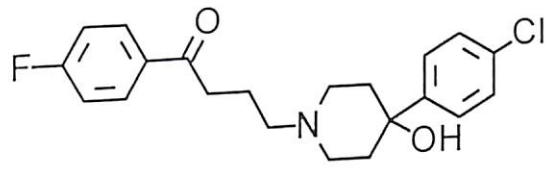


Chemical Name	:	3- (5-nitrofurfurylideneamino) oxazolidin -2-one
Emperical Formula	:	$C_8H_7N_3O_5$
Molecular Weight	:	225.2
Melting Point	:	256-257°C [430]
Description	:	A yellow, crystalline powder; odourless
Solubility	:	Very slightly soluble in water and ethanol (95%); slightly soluble in chloroform; practically insoluble in ether.

Official Method of Analysis

a. Pure Drug	:	UV [U.S.P. 23, B.P.1993, I.P. 1996]
Assay Limits	:	97.0-103.0% [U.S.P. 23, B.P.1993, I.P. 1996]
b. Dosage Forms	:	UV [U.S.P. 23, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23, I.P. 1996]

Drug Profile B.8: Haloperidol

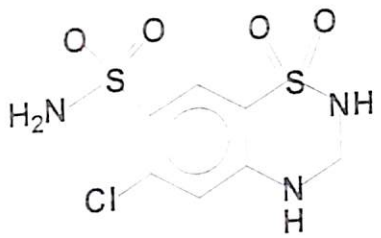


Chemical Name	:	4- [4-(4-chlorophenyl)-4-hydroxy piperidino]-4'-fluorobutyrophenone
Emperical Formula	:	$C_{21}H_{23}ClFNO_2$
Molecular Weight	:	375.9
Melting Point	:	147-152°C [1]
pK_a	:	8.3 [99]
Description	:	A white or slightly yellowish, amorphous or crystalline powder
Solubility	:	Practically insoluble in water; sparingly soluble in dichloromethane and in ethanol (95%); slightly soluble in ether; soluble in chloroform.

Official Method of Analysis

a. Pure Drug	:	Titrimetry [U.S.P. 23, B.P.1993, I.P. 1996]
Assay Limits	:	98.0-102.0% [U.S.P. 23] 99.0-101.0% [B.P. 1993, I.P.1996]
b. Dosage Forms	:	HPLC [U.S.P. 23, B.P.1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23, B.P.1993, I.P. 1996]

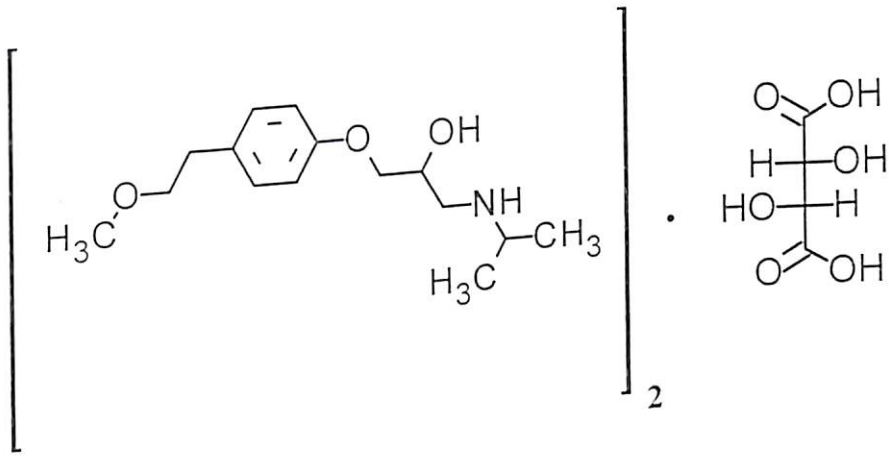
Drug Profile B.9: Hydrochlorothiazide



Chemical Name	:	6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide
Emperical Formula	:	$C_7H_8ClN_3O_4S_2$
Molecular Weight	:	297.7
Melting Point	:	273-275°C [430]
pK_a	:	7.0, 9.2 [99]
Description	:	A white, crystalline powder, odourless
Solubility	:	Very slightly soluble in water, soluble in acetone, sparingly soluble in ethanol (95%). It dissolves in dilute solution of alkali hydroxides.

Official Method of Analysis		
a. Pure Drug	:	HPLC [U.S.P. 23] Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	98.0-102.0% [U.S.P. 23, B.P.1993, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23] UV [B.P.1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23] 92.5-107.5% [B.P.1993, I.P. 1996]

Drug Profile B.10: Metoprolol tartrate

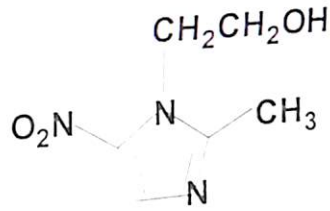


Chemical Name	:	(RS)-1-isopropylamino-3-p-(2-methoxyethyl) phenoxy propan-2-ol (2R,3R)-tartrate
Emperical Formula	:	$(C_{15}H_{25}NO_3)_2.C_4H_6O_6$
Molecular Weight	:	684.82
Melting Point	:	121-123°C [3]
pK_a	:	9.7 [99]
Description	:	White crystalline powder, virtually odourless
Solubility	:	Very soluble in water; soluble in alcohol and chloroform; practically insoluble in ether.

Official Method of Analysis

a. Pure Drug	:	Potentiometry [U.S.P. 23, B.P.1993, I.P. 1996]
Assay Limits	:	99.0-101.0% [U.S.P. 23, B.P.1993, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23], UV [I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23, I.P. 1996]

Drug Profile B.11: Metronidazole

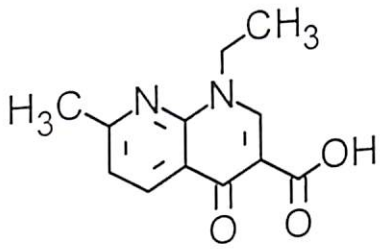


Chemical Name	:	2-(2-methyl-5-nitroimidazol-1-yl)-ethanol
Emperical Formula	:	$C_6H_9N_3O_3$
Molecular Weight	:	171.2
Melting Point	:	159-163°C [1]
pK_a	:	2.5 [99]
Description	:	A white or yellowish, crystalline powder
Solubility	:	Slightly soluble in water, acetone, dichloromethane and in ethanol (95%); very slightly soluble in ether.

Official Method of Analysis

a. Pure Drug	:	Titrimetry [U.S.P. 23] Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	99.0-101.0% [U.S.P. 23, B.P.1993, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23] Titrimetry [B.P.1993, I.P. 1996], UV [I.P. 1996]
Assay Limits (Tablets)	:	90.0-110.0% [U.S.P. 23] 95.0-105.0% [B.P.1993, I.P. 1996]

Drug Profile B.12: Nalidixic Acid

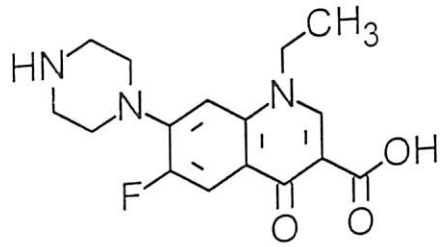


Chemical Name	:	1-ethyl-1, 4-dihydro-7-methyl-4-oxo-1, 8 naphthyridine-3-carboxylic acid
Emperical Formula	:	$C_{12}H_{12}N_2O_3$
Molecular Weight	:	232.2
Melting Point	:	225-231°C [1]
pK_a	:	6.0 [99]
Description	:	An almost white or pale yellow, crystalline powder
Solubilility	:	Practically insoluble in water; soluble in dichloromethane; slightly soluble in acetone and in ethanol(95%); very slightly soluble in ether. It dissolves in dilute solutions of alkali hydroxides.

Official Method of Analysis

a. Pure Drug	:	Titrimetry [U.S.P. 23] Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	99.0-101.0% [U.S.P. 23, B.P.1993, I.P. 1996]
b. Dosage Forms	:	UV [U.S.P. 23, B.P.1993, I.P. 1996]
Assay Limits	:	93.0-107.0% [U.S.P. 23] 95.0-105.0% [B.P.1993, I.P. 1996]

Drug Profile B.13: Norfloxacin

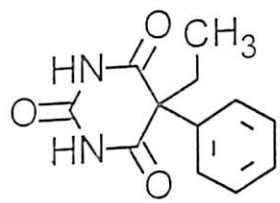


Chemical Name	:	1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl) -3-quinoline carboxylic acid
Emperical Formula	:	$C_{16}H_{18}FN_3O_3$
Molecular Weight	:	319.34
Melting Point	:	220-221°C [430]
pK_a	:	6.34, 8.75 [429]
Description	:	White to light yellow crystalline powder
Solubility	:	Freely soluble in glacial acetic acid, chloroform, acetone; slightly soluble in ethanol, methanol, less soluble in water; in soluble in ether.

Official Method of Analysis

a. Pure Drug	:	Potentiometry [U.S.P. 23, I.P. 1996]
Assay Limits	:	99.0-101.0% [U.S.P. 23, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23, I.P. 1996]

Drug Profile B.14: Phenobarbitone

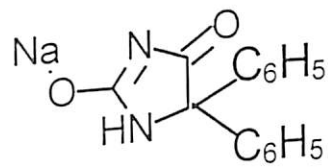


Chemical Name	:	5-ethyl-5-phenylbarbituric acid
Emperical Formula	:	$C_{12}H_{12}N_2O_3$
Molecular Weight	:	232.2
Melting Point	:	174-178°C [1]
pK_a	:	7.4 [99]
Description	:	Colourles crystals or a white, crystalline powder
Solubility	:	Very slightly soluble in water; freely soluble in ethanol (95%); soluble in ether; sparingly soluble in chloroform.

Official Method of Analysis

a. Pure Drug	:	HPLC [U.S.P. 23] Titrimetry [B.P.1993, I.P. 1996]
Assay Limits	:	98.0-101.0% [U.S.P. 23] 99.0-101.0% [B.P.1993, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23] Extraction cum gravimetric [B.P.1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23] 92.5-107.5% [B.P.1993, I.P. 1996]

Drug Profile B.15: Phenytoin Sodium

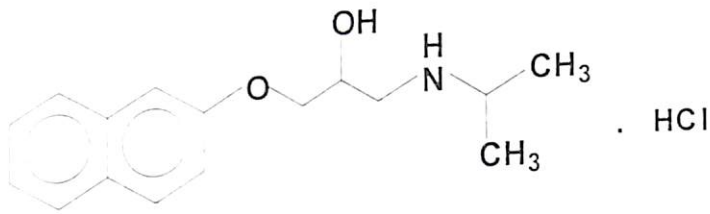


Chemical Name	:	5,5-diphenylimidazolidone-2,4-dione sodium
Emperical Formula	:	$C_{15}H_{11}N_2NaO_2$
Molecular Weight	:	274.3
pK_a	:	8.30 [99]
Description	:	A white, crystalline powder; slightly hygroscopic
Solubility	:	Soluble in water and ethanol (95%); practically insoluble in dichloromethane and in ether.

Official Method of Analysis

a. Pure Drug	:	HPLC [U.S.P. 23] Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	98.5-100.5% [U.S.P. 23, B.P.1993] 98.0-101.0% [I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23] Titrimetry [B.P.1993, I.P. 1996]
Assay Limits	:	95.0-105.0% [U.S.P. 23, B.P. 1993] 90.0-110.0% [I.P. 1996]

Drug Profile B.16: Propranolol Hydrochloride

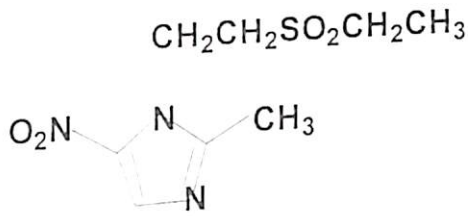


Chemical Name	:	(RS)-1-isopropylamino-3-(1-naphthyloxy) propan-2-ol hydrochloride
Emperical Formula	:	$C_{16}H_{21}NO_2$, HCl
Molecular Weight	:	295.8
Melting Point	:	163-166°C [2]
pK_a	:	9.45 [99]
Description	:	White or almost white powder, odourless
Solubility	:	Soluble in water and in ethanol (95%); slightly soluble in chloroform; practically insoluble in ether.

Official Method of Analysis

a. Pure Drug	:	HPLC [U.S.P. 23] Potentiometry [B.P.1993, I.P. 1996]
Assay Limits	:	98.0-101.5% [U.S.P. 23] 99.0-101.0% [B.P.1993, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23] UV [B.P.1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23] 92.5-107.5% [B.P.1993, I.P. 1996]

Drug Profile B.17: Tinidazole

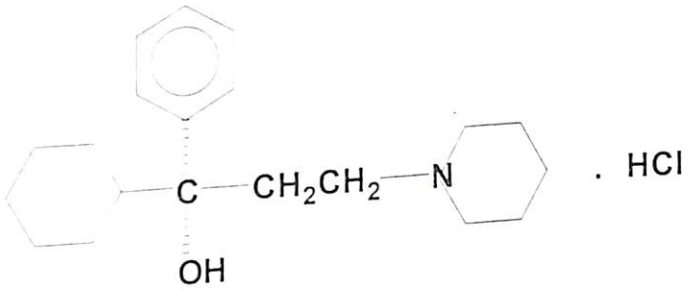


Chemical Name	:	1-[2- (ethylsulphonyl) ethyl]-2-methyl-5-nitroimidazole
Emperical Formula	:	$C_8H_{13}N_3O_4S$
Molecular Weight	:	247.26
Melting Point	:	125-128°C [3]
pK_a	:	1.82 [99]
Description	:	Pale yellow, crystalline powder; slight and characteristic odour
Solubility	:	Sparingly soluble in water; slightly soluble in ethanol (95%), in chloroform and in ether.

Official Method of Analysis

a. Pure Drug	:	Titrimetry [I.P. 1996]
Assay Limits	:	98.0-100.5% [I.P. 1996]
b. Dosage Forms	:	UV [I.P. 1996]
Assay Limits	:	95.0-105.0% [I.P. 1996]

Drug Profile B.18: Trihexyphenidyl hydrochloride



Chemical Name	:	(RS)-1-cyclohexyl-1-phenyl-3-piperidinopropan-1-ol hydrochloride
Emperical Formula	:	$C_{20}H_{31}NO$, HCl
Molecular Weight	:	337.9
Melting Point	:	247-253°C [430]
Description	:	A white or creamy white, crystalline powder, odourless
Solubility	:	Slightly soluble in water; soluble in chloroform, in ethanol (95%) and in methanol.

Official Method of Analysis

a. Pure Drug	:	HPLC [U.S.P. 23] Titrimetry [B.P.1993, I.P. 1996]
Assay Limits	:	98.0-102.0% [U.S.P. 23] 98.0-101.0% [B.P.1993, I.P. 1996]
b. Dosage Forms	:	HPLC [U.S.P. 23, B.P.1993, I.P. 1996]
Assay Limits	:	90.0-110.0% [U.S.P. 23, B.P.1993, I.P. 1996]

APPENDIX C

SUMMARY OF REPORTED LITERATURE ON THE USED TECHNIQUES

I. *DRUGS ANALYSED BY DIFFERENCE SPECTROPHOTOMETRY*

TABLE C.1: SINGLE PREPARATIONS

S.NO	DRUG NAME	SPECTRAL CHANGE INDUCING AGENTS	REF. NO
	I. Analgesics & anti-inflammatory		
1	Piroxicam	0.5M HCl Vs 0.1M NaOH	32
2	Indomethacin	Meth. 0.1M HCl Vs 0.1M NaOH	33
3	Phenylbutazone	0.01M HCl Vs pH 7.0	34
4	Oxyphenbutazone	-	35
5	Morphine	-	36
	II. Antibiotics		
6	Chloramphenicol	0.1M HCl Vs Zn treated HCl	22
7	Trimethoprim	0.04M H_2SO_4 Vs 0.08M NaOH 0.01M NaOH Vs H_2O	27 28
8	Tetracyclines	0.01M H_2SO_4 Vs 0.01M NaOH	37
9	Oxytetracyclines	0.01M H_2SO_4 Vs 0.01M NaOH	37
10	Rifamycin	pH 4.63 Vs $NaNO_2$	38
11	Ciprofloxacin HCl	0.01M HCl Vs 0.01M NaOH	39
	III. Antihypertensives/diuretics		
12	Nifedipine	-	25
13	Ethacrynic acid	H_2O Vs Phos. buffer.	40
14	Hydrochlorothiazide	-	41
	IV. Antipsychotics		
15	Nitrazepam	1.0M HCl Vs 1.0M NaOH	42
16	Diazepam	Ethanolic 0.5% H_2S0_4 Vs Ethanol	43
17	Chlorpromazine	H_2O Vs H_2O + acetic acid + H_2O_2	44

Table C.1 Contd.: Difference Spectrophotometry - Single preparations

	V. Vitamins		
18	Vitamin B_6	Phos. buffer pH 7.0 Vs 0.1M HCl	29
19	Vitamin C	Phos. buffer pH 6.2 Vs 0.1M HCl	45
	VI. Miscellaneous		
20	Furazolidone	Reduced Vs Non-reduced	23
21	Nitrofurantoin	Reduced Vs Non-reduced	23
22	Nitrazepam	Reduced Vs Non-reduced	23
23	Metronidazole	Reduced Vs Non-reduced	23
24	Isoniazid	Reduced Vs Non-reduced	24
25	Diloxanide furoate	Drug Vs Degraded product	26
26	Amidopyrine	0.1M HCl Vs Phos. buffer pH 7.0	46
27	Chlorhexidine	-	47
28	Mercaptopurine	-	48
29	Theophylline	pH 7.0 Vs pH 9.0	49
30	Salbutamol sulphate	pH 2.0 Vs pH 2.0 Drug soln.	50

TABLE C.2: DIFFERENCE SPECTROPHOTOMETRY - COMBINED PREPARATIONS

S.NO	DRUG NAME(S)	SPECTRAL CHANGE INDUCING AGENTS	REF. NO
	I. Analgesics & anti-inflammatory		
1	Paracetamol - Chlorzoxazone	0.02M NaOH Vs 0.02M HCl	51
2	Paracetamol - Aspirin	0.2M NaOH Vs NaOH + H_5BO_3 + KCl	52
		-	53
3	Paracetamol - Analgin	0.1M HCl Vs 0.1M NaOH	54
4	Paracetamol - Oxyphenbutazone	-	53
5	Oxyphenbutazone - Analgin	0.01M HCl Vs 0.01M NaOH	55
6	Mefenamic acid - Flufenamic acid	0.01M NaOH Vs 0.05M H_2SO_4	56
7	Amidopyrine - Phenacetin		
	II. Sulfonamides		
8	Sulphaguanidine - Sulphadimidine	0.1M HCl Vs 0.1M Na_2CO_3	57
9	Sulphamethaxazole - Trimethoprim	0.1N H_2SO_4 Vs 0.1N NaOH	58
		0.1M HCl Vs 0.1M NaOH	59
	III. Antidiarrhoeals		
10	Tinidazole - Diloxanide furoate	0.1M HCl Vs 0.1M NaOH	60
		-	61
11	Metronidazole - Furazolidone	0.1M HCl Vs Meth. 0.1M HCl	62
	IV. Antipsychotics		
12	Levodopa - beserazide	pH 7.0 Vs pH 7.0 + 0.1M boric acid	63
		GeO_2 + Na_2HPO_4 Vs Na_2HPO_4	64
13	Phenylbutazone - Salicylamide	H_2O Vs 0.1M HCl	65
		H_2O Vs 0.1M NaOH	66
14	Phenobarbitone - Theophylline	-	
	V. Miscellaneous		
15	Phenylpropanolamine HCl - Guaiphenesin	Oxidised Vs Unoxidised	21
16	Phenylpropanolamine HCl - Dextramethorphan HBr	Oxidised Vs Unoxidised	21
17	Salbutamol sulphate - Bromhexine HCl	0.1M HCl Vs 0.1M NaOH	67
18	Allopurinol - Flucytosine	0.1M HCl Vs 0.1M NaOH	68
19	Terbutaline sulphate - Orciprenaline sulphate	Treated HCl Vs NaOH	69

II. *DRUGS ANALYSED BY DERIVATIVE SPECTROPHOTOMETRY*

TABLE C.3: SINGLE PREPARATIONS

S.NO	DRUG NAME	METHOD	Dn	TECHNIQUE*	REF. NO
1	Metronidazole	UV	1	PB	79
2	Sulphamethizole	UV	1	RS	132
3	Ketoprofen	UV	2	PB	139
4	Diclofenac	UV	1	-	140
5	Loperamide	UV	2	PB	141
		FLU	2	PB	
6	Tetracyclines	UV	2	PP	142
7	Sulphathiazole HCl	UV	1	ZCP	143
8	Enalapril maleate	UV	4	-	144
9	Timolol maleate	UV	1	PB	145
10	Salbutamol sulphate	UV	1	PB	146
11	Famotidine	UV	2	PP	147
12	Albendazole	UV	1	PB	148
13	Bromhexine HCl	UV	2	PB	149
14	Cortisone acetate	UV	1	PB	150
15	Tamoxifen citrate	UV	1	PB	151
16	Indomethacin	UV	1&2	PP	152
17	Naproxen	UV	1&2	PP	152
18	Ibuprofen	UV	1&2	PB	152
19	Sulphacetamide sodium	UV	1	PB	153
20	Triamcinole acetonide	UV	1	PB	154
21	Clemastine fumarate	UV	2	PB	155
22	Saccharin	UV	2&4	PP	156
23	Sulphamerazine	FLU	1	PB	268
24	Folic acid	FLU	1	PB	269

*: ZCP: Zero-crossing point; PP: Peak-peak; PB: Peak-baseline; RS: Ratio-spectra.

TABLE C.4: DERIVATIVE SPECTROPHOTOMETRY- COMBINED PREPARATIONS

S.NO	DRUG NAME(S)	METHOD	Dn	TECHNIQUE*	REF. NO
	I. Analgesics & anti-inflammatory				
1	Paracetamol - Mefenamic acid	UV	1	ZCP	157
2	Paracetamol - Chlorzoxazone	UV	2	ZCP	158
3	Paracetamol - Orphenadrine HCl	UV	2	ZCP	158
4	Paracetamol - Aspirin	UV	1	-	159
5	Paracetamol - Chlormezanone	UV	1	ZCP	160
6	Paracetamol - Caffeine	UV	1	ZCP	161
7	Paracetamol - Methocarbamol	UV	2	ZCP	162
8	Paracetamol - Indomethacin	UV	1&2	ZCP	163
9	Paracetamol - Codeine	UV	1	ZCP	164
10	Paracetamol - Ibuprofen	UV	2	ZCP	166
11	Aspirin - Caffeine	UV	2	MLRA	165
12	Aspirin - Salicylic acid	UV FLU	2 2	- ZCP	166 270
13	Heroin - Morphine	UV	2	ZCP&PP	167
14	Analgin - Lidocaine HCl	UV	1	ZCP	168
15	Cocaine - Lidocaine	UV	2	PP	169
16	Cocaine - Procaine	UV	2	ZCP&PP	169
17	Cocaine - Tetracaine	UV	2	PP	169
18	Cocaine - Benzocaine	UV	2	ZCP&PP	169
	II. Antibiotics/sulfonamides				
19	Sulphamethazine - Sulphathiazole	UV	1	ZCP&RS	130
20	Sulphadiazine - Trimethoprim	UV	-	RS	131
21	Cepharadine - Clavulanic acid	UV	1	RS	170
22	Cefsulodin - Clavulanic acid	UV	1	ZCP	171
23	Cephradine - Arginine(L)	UV	2	ZCP&PP	172
24	Cefoperazone - Sulbactam	UV	1&2	ZCP	173
25	Ceftriaone - Streptomycin	UV	2&3	ZCP&RS	174
26	Cephalothin - Cephaloridine	UV	1&2	ZCP	175
27	Cefapirin Sod. - Cefuroxime sod.	UV	1&2	ZCP	176
28	Procaine Penicillin G - Penicillin G	UV	2	ZCP	177
29	Procaine Penicillin G - Penicillin G sod. - Dihydrostreptomycin sulph.	UV UV	1&3 3	RS ZCP	133 178
30	Ampicillin sod - Dicloxacillin sod.	UV	2	ZCP	179
31	Ampicillin - Cloxacillin	UV	2	ZCP	180

Table C.4 Contd.: Derivative Spectrophotometry – Combined preparations

32	Amoxycillin - Dicloxacillin	UV	1	ZCP	181
33	Amoxycillin - Cephalexin	UV	2	ZCP	182
34	Sulfamethaxazole - Trimethoprim	UV	1&2	RC	129
		UV	1	ZCP&MA	183
		UV	-	RS	184
35	Sulphamethazole - Trimethoprim - 1,4 Dioxane	UV	-	MA	185
36	Sulphamethazole - Trimethoprim - Phenazopyridine HCl	UV	3	PB	186
37	Sulphamethazole - Trimethoprim	UV	2	MA	187
38	Sulphathiazole - Sulphanilamide	UV	3&4	ZCP	188
39	Sulphacetamide - Sulphadimidine	UV	1	ZCP	189
40	Sulphathiazole - Sulphanilamide - Sulphadiazine	UV	1,3&5	PB	190
41	Oxytetracycline - Riboflavine	FLU	1	PB	271
	III. Antidiarrhoeals				
42	Diloxanide furoate - Tinidazole	UV	2	ZCP	191
43	Furaltadone - Chloramphenicol	UV	1&2	ZCP&RS	192
	IV. Antifungals				
44	Miconazole - Metronidazole	UV	1	ZCP&RS	193
45	Miconazole - Econazole	UV	2&3	ZCP	194
46	Clotrimazole - Azidamfenicol - Dexamethasone	UV	1&2	ZCP	195
	V. Antihistamines				
47	Dextromethorphan - Guaiphenesin	UV	1&2	ZCP	196
48	Dextromethorphan HBr - Chlorpheniramine maleate - Pseudoephedrine HCl	UV	2	MA	197
49	Diphenhydramine HCl - Naphazoline HCl	UV	1&2	PP	198
50	Isoprenaline HCl - Guaiphenesin	UV	2	ZCP	199
51	Cloprenaline HCl - Bromhexine HCl-Decloxizine HCl	UV	1	MA	200
52	Phenyl Propanolamine HCl - Carbinoxamine maleate	UV	1	ZCP	201
53	Theophylline - Guaiphenesin	UV	3	ZCP	202

Table C.4 Contd.: Derivative Spectrophotometry – Combined preparations

54	Pseudoephedrine HCl - Ibuprofen	UV	1	-	203
55	Promethazine HCl - Paracetamol	UV	2	ZCP	204
	VI. Antihypertensives /diuretics				
56	Hydrochlorothiazide - Cilazapril	UV	1	ZCP	205
57	Hydrochlorothiazide - Captopril	UV	-	-	206
		UV	1&2	ZCP	207
58	Hydrochlorothiazide - Amiloride HCl	UV	2	MA	208
		UV	-	RS&MLRA	209
59	Hydrochlorothiazide - Enalapril maleate	UV	1	ZCP	210
60	Hydrochlorothiazide - Spironolactone	UV	1,2	PP,RC&PB	129
		UV	1,2	ZCP	207
61	Hydrochlorothiazide - Hydralazine HCl	UV	2	ZCP	211
		FLU	1	ZCP	272
62	Spironolactone - Frusemide	UV	1&2	ZCP	207
63	Propranolol HCl - Hydralazine HCl	UV	2	ZCP	211
64	Clopamide - Pindolol	UV	1&2	ZCP	212
65	Polythiazide - Prazosin	UV	2	ZCP&PP	213
66	Atenolol - Chlorthiazide	UV	4	-	214
67	Atenolol - Nifedipine	UV	2	ZCP	215
	VII. Antipsychotics				
68	Phenobarbitone - Phenytoin sod.	UV	1&2	RC	129
69	Phenobarbital - Verapamil HCl	UV	1	ZCP	216
70	Phenobarbitone - Acepifylline	UV	1	ZCP	217
71	Amitriptylline - Chlordiazepoxide	UV	1&2	ZCP	218
72	Amitriptylline - Perphenazine	UV	1&2	ZCP	218
73	Amitryptylline - Imipramine	UV	1&2	ZCP	219
74	Trifluoperazine - Isopropamide	UV	1&2	ZCP	218
75	Trifluperazine HCl - Tranylcypromine sulphate	UV	1&4	ZCP	220
76	Caffeine - Amidopyrine	UV	1	MLRA	221
77	Caffeine - Pethidine - Amphetamine	UV	1&2	-	222
78	Diazepam - Imipramine HCl	UV	2	ZCP	223
79	Diazepam - Otilonium bromide	UV	1&2	RS	224

Table C.4 Contd.: Derivative Spectrophotometry – Combined preparations

80	Meclozine HCl - Pyridoxine HCl - Nicotinic acid	UV	1	ZCP	225
81	Phenoprobamate - Paracetamol	UV	1	ZCP	226
	VIII. Steroids				
82	Ethinyl estradiol - Levonorgestrel	UV	1	ZCP	227
83	Ethinyl estradiol - Norgestrel	UV	1	ZCP	228
84	Oestradiol benzoate - Progesterone	UV	2	ZCP	229
85	Oestradiol valverate - Testosterone ethanoate	UV	2	PP	230
86	Norethisterone - Norgestrel	UV	2	PP	230
	IX. Vitamins				
87	Vitamin B_1 - Vit B_6 - Vit B_{12}	UV	1&3	RS	231
88	Vitamin B_1 - Vit B_6 - Vit B_{12} - Uridine 5'-triphosphate	UV	2	ZCP & PP	232
89	Vitamin B_1 - Vit B_2 - Vit B_3 - Vit B_6 - Vit C	UV	1&3	MLRA	233
90	Ascorbic acid - Menadione sod. biphos.	UV	2	ZCP	234
91	Vitamin A- Vitamin E	UV	3	PP	235
92	Pyridoxal - Pyridoxamine	FLU Sync.FLU	1 1	ZCP ZCP	273
93	Vitamin B_1 - VitB_2 - VitB_6	FLU	2	PP	274
	X. Miscellaneous				
94	Strychnine - Brucine	UV	1	ZCP	236
95	Nitrofurantoin - Phenazopyridine	UV	1&2	ZCP & RS	237
96	Sulphaquinoxalonine - Sulphamethazine - Pyrimethamine	UV	1&4	ZCP & RS	238
97	Methylene Blue - Hexamine - Resorcinol	UV	1	ZCP	239
98	Rifampicin - Isoniazid - Pyrizinamide	UV	1	ZCP	240
99	Quinidine - Cinchonine	FLU	1	ZCP	275

*: ZCP: Zero-crossing point; PP: Peak-peak; PB: Peak-baseline; MA: Multi-component analysis; RS: Ratio-spectra; MLRA: Multiple linear regression analysis; RC: Ratio-compensation.

TABLE C.5: DRUG ESTIMATION IN PRESENCE OF IMPURITY BY DERIVATIVE SPECTROPHOTOMETRY

S.NO	DRUG AND IMPURITY	METHOD	Dn	TECHNIQUE*	REF. NO
1	Chlorpromazine & Sulphoxide	UV FLU	3 2	PP PP	241 276
2	Paracetamol & 4-Aminophenol	UV	3		242
3	Piroxicam & 2-Aminopyridine	UV	2	PP	243
4	Indomethacin & 5 Methoxy-2-methyl indole-3-acetic acid	UV	3	-	244
5	Ciprofloxacin & Ciprofloxacinamide, -N,N'-bis hydroxy methyl Ciprofloxacinamide	UV	4	ZCP	245
6	Phenothiazines & Sulphoxide	FLU	2	-	277

*: ZCP: Zero-crossing point; PP: Peak-peak

TABLE C.6: DRUG ESTIMATION IN PRESENCE OF DEGRADATION PRODUCTS BY DERIVATIVE SPECTROPHOTOMETRY

S.NO	DRUG NAME	METHOD	Dn	TECHNIQUE*	REF. NO
1	Cephalosporins	UV	1&2	PB	246
2	Sulpha drugs	UV	1	PB	247
3	Glibenclamide	UV	1&2	PP	248
4	Mebeverine HCl	UV	1&2	PP	248
5	Clopamide	UV	1&2	PP	248
6	Thiazide diuretics	UV	1	PP	249
7	Nitrendipine	UV	-	-	250
8	Phenylbutazone	UV	2&3	PP	251

*: PP: Peak-peak; PB: Peak-baseline.

TABLE C.7: DERIVATIVE SPECTROPHOTOMETRY IN BIOLOGICAL SAMPLES

S.NO	DRUG NAME(S)	METHOD	Dn	TECHNIQUE*	REF. NO
	I. Single preparations				
1	Paracetamol	UV	1	PB	252
2	Nitrazepam	UV	4&5	PB	253
3	Clonazepam	UV	4&6	PB	253
4	Pyridoxamine	FLU	1	PB	278
	II. Combined preparations				
5	Methaqualone - diazepam	UV	2	-	254
6	Cephalexin - cephradine	UV	1	-	255
		FLU	2	ZCP	279
7	Salicylic acid - Diffusinal	FLU	2	PP	280
8	Salicylic acid - naproxen	FLU	2	ZCP	281
9	Naproxen & its metabolite	FLU	2	PP	282
10	Aspirin & its metabolite	Sync. FLU	1&2	ZCP	283
11	Epinephrine - norepinephrine	Sync. FLU	2	ZCP	284

*: ZCP: Zero-crossing point; PP: Peak-peak; PB: Peak-baseline

TABLE C.8: UV-VIS DERIVATIVE SPECTROPHOTOMETRY – OTHER APPLICATIONS

S.NO	DRUG NAME(S)	NAME OF STUDY	Dn	TECHNIQUE*	REF. NO
1	Aspirin	Dissolution rate	1&2	PB	256
2	Acetazolamide	Dissolution rate & kinetic study	1	-	257
3	Norfloxacin	Stability	-	-	258
4	Indomethacin	Degradation kinetics	4	-	259
5	Chlorpromazine - Promazine	Partition coefficient	2	PB	260
6	Naphazoline - Oxprenolol	Percutaneous absorption	2	-	261
7	Piroxicam - Ranitidine	GI absorption	1	-	262
8	Rifampicin	Protein binding	1	-	263
9	Benzyl penicillins	Molecular interactions	2&4	PB	264
10	Methadone	Identification in presence of Tartrazine and Green S	2&4	-	265

*: PB: Peak-baseline.

III. *DRUGS ANALYSED BY DERIVATIVE-DIFFERENCE SPECTROPHOTOMETRY*

TABLE C.9: SINGLE AND COMBINED PREPARATIONS

S.NO	DRUG NAME(S)	SPECTRAL CHANGE INDUCING AGENTS	Dn	TECHNIQUE*	REF. NO
	I. Single preparations				
1	Oxytetracyclines	0.01M H_2SO_4 Vs 0.01M NaOH	1	PP	37
2	Tetracyclines	0.01M H_2SO_4 Vs 0.01M NaOH	1	PB	37
3	Chlorprothixene	Oxidised Vs Unoxidised	-	PB	406
4	Thiothixane	Oxidised Vs Unoxidised	-	PB	406
5	Flupenthixol. diHCl	Oxidised Vs Unoxidised	-	PB	406
6	Captopril	NaOH Vs Meth. drug soln.	2	PP	407
7	Clomipramine HCl	0.001N NaOH Vs 0.001N H_2SO_4	-	-	408
8	Oestradiol dipropionate	Acid Vs Alkali	1	PP	409
9	Oestradiol Valerate	Acid Vs Alkali	1	PP	409
10	Oestradiol benzoate	Acid Vs Alkali	1	PP	409
11	Testosterone propionate	Acid Vs Alkali	1	PP	409
12	Progesterone	Acid Vs Alkali	1	PP	409
13	Chlorpheniramine maleate	Meth. 0.1M HCl Vs Methanol	2	PB	410
14	Ethinyl Oestradiol	Methanol Vs Meth.NaOH	1&2	PB	411
	II. Combined preparations				
15	Aspirin - Dipyridamole	0.1M HCl Vs 0.1M NaOH	2	ZCP	215
16	Triprolidine - Pseudoephedrine - Dextromethorphan	0.1N H_2SO_4 Vs 0.1N NaOH	2&4	PB	412
17	Oxazepam - Dipyridamole	0.1N H_2SO_4 Vs 0.05N $Na_2B_4O_7$	1&2	PB	413
18	Phenobarbitone - Dipyridamole	0.1N H_2SO_4 Vs 0.1N NaOH	1&2	PB	413
19	Dipyridamole - Etilefrine	-	-	-	414

*: ZCP: Zero-crossing point; PP: Peak-peak; PB: Peak-baseline.

APPENDIX D

MATHEMATICAL EXPRESSIONS USED IN STATISTICAL TREATMENT OF ANALYTICAL DATA

Mean (Y):

The total sum of absorption value divided by the number of replicates of a concentration

$$\overline{Y} = \frac{\sum_{i=1}^{N} Y_i}{N}$$

$\overline{Y}$ = Mean of absorbance values; Y_i = Absorbance value;
N = No. of dilutions

Standard deviation (S):

The sum of squares of the differences of each value from the mean divided by one less than the number of replicates

$$S = \sqrt{\frac{\sum (Y - \overline{Y})^2}{N - 1}}$$

Y = absorbance of a dilution; $\overline{Y}$ = mean absorbance value; N = No. of dilutions

Coefficient of variation (C.V):

This is the relative measure of dispersion values and also known as relative standard deviation (RSD). The expression for its determination is

$$C.V\% = \frac{S}{\overline{Y}} \times 100$$

S = Standard deviation; $\overline{Y}$ = Mean of absorbance values

Standard error (S_Y):

It is a measure of the variability of the mean

$$S_{\bar{Y}} = \frac{S}{\sqrt{N}}$$

Linear regression equation (Method of least squares):

This equation helps in identifying the exact relationship between concentrations and measured values, may be linear or non-linear. The linear regression equation can be expressed as two different straight lines. One line is regression line Y (absorbance) on X (concentration) and another line is regression line X on Y. The line used to estimate values of Y knowing the values of X is called as regression line Y on X and the line used to estimate values of X is called regression line X on Y. In least squares method, we minimise sums of squares of errors. The appropriate equation of a line is in the form of $Y = a + bX$. The slope (b) values of regression line Y on X will be designated as b_{YX} and similarly, the slope values of regression line X on Y will be shown as b_{XY}.

$$b_{YX} = \frac{\sum(X-\bar{X})(Y-\bar{Y})}{\sum(X-\bar{X})^2}$$

X = Concentration of drug solution; $\bar{X}$ = Mean concentration of the drug
Y = Absorbance of chosen drug solution; $\bar{Y}$ = Mean absorbance of drug solutions

Slope without intercept (b):

This value is obtained by forcing the regression line through the origin with a introduction of point (0,0) and the slope value helps in understanding the proportional relationship between measured values and concentrations

$$b = \frac{\sum XY}{\sum X^2}$$

Standard error of estimate (S_{YX}):

It is the measure of dispersion of the standard points relative to the regression line. The appropriate expression used to obtain S_{YX} is

$$S_{YX} = \sqrt{\frac{\sum_{i=1}^{N} d_i^2}{(N-2)}}$$

Where $d_i^2 = (Y_{calc} - Y)^2$; Y_{calc} = Predicted value from the regression line
Y = Actual value observed for a particular concentration
N = Number of dilutions

Standard error of slope (S_b) and intercept (S_a):

These two values are calculated to study the amount of error involved in the determination of slope (S_b) and intercept (S_a) due to random scatter of points about the regression line. The appropriate expression to obtain S_b and S_a

$$S_b = \frac{S_{YX}}{\sqrt{\sum (X - \bar{X})^2}}$$

$$S_a = S_{YX}\left(\sqrt{\frac{1}{N} + \frac{\bar{X}^2}{\sum (X - \bar{X})^2}}\right)$$

S_{YX} = Standard error of estimate; N = Number of dilutions;
$\bar{X}$ = Mean value of concentrations; X = Concentrations of a chosen sample

Percentage ratio of residual (% RR):

This has been calculated to determine the random scatter of standard points about the regression line. The appropriate expression used for the determination is

$$\%RR = \frac{Y_{Cal}}{Y_o} \times 100$$

Y_{cal} = Predicted value from the regression line
Y_o = Actual value observed for a particular concentration

Confidence interval for slope and intercept:

The 95% confidence interval often used to confirm that the variations seen with the slope and intercept were well within the acceptable limits

$$\text{Slope} = b \pm t(S_b) = b \pm \frac{t(S_{YX})}{\sqrt{\sum (X - \bar{X})^2}}$$

$$\text{Intercept} = a \pm t(S_a) = a \pm t(S_{YX}) \left(\sqrt{\frac{1}{N} + \frac{\bar{X}^2}{\sum (X - \bar{X})^2}} \right)$$

S_a = Standard error of intercept; S_b = Standard error of slope
S_{YX} = Standard error of estimate; $\bar{X}$ = Mean value of observations
t(two-sided) = 't-value' from standard tables with a known degrees of freedom

F-test for non-linearity [97]:

This test gives a quantitative measure of the strength of evidence for non-linearity. If a linear relationship holds, standard deviation S_{YX} represents an estimate [with (N-2) degrees of freedom] of the sample population value σ will have a less value than the theoretical value at 5% level

$$F = \frac{S_{YX}^2}{\sigma^2}$$

S_{YX} = Standard error of estimate; σ = Standard deviation of sample population

Correlation coefficient (r):

The correlation coefficient is a quantitative measure of the relationship or correlation between two variables

$$r = \frac{N\sum XY - \sum X \sum Y}{\sqrt{\left[N\sum X^2 - \left(\sum X^2\right)\right]\left[N\sum Y^2 - \left(\sum Y^2\right)\right]}}$$

X = Concentation of a dilution; Y = Absorbance value of a dilution
N = No. of dilutions

Student's *t*-test for correlation:

This test is performed to see whether or not an observed correlation coefficient is real or due to chance

$$t_{N-2} = \frac{|r\sqrt{N-2}|}{\sqrt{1-r^2}}$$

t_{N-2} = "*t*-value" with (N-2) degrees of freedom at 5% level
r = Correlation coefficient

One-way ANOVA test:

This is a general method of analysing data from designed experiments, whose objective is to compare two or more group means. A one-way ANOVA test is used when we wish to test the equality of treatment means in experiments where two or more treatments are randomly assigned to different, independent experimental units. This one-way design separates the variance into two parts, that due to treatment differences (between) and that due to error within the treatment (within)

$$TSS = BSS + WSS$$

Where TSS = Total sum of squares
BSS = Between- treatments sum of squares
WSS = Within- treatment sum of squares

TSS can be obtained by using following equation

$$TSS = \sum Y^2 - \frac{(\sum Y)^2}{N}$$

$(\Sigma Y)^2/N$ is known as **correction term** (C.T), where $(\Sigma Y)^2$, the grand total of all of the observations squares, is divided by total number of observations (N).

BSS value can be obtained by using following equation

$$BSS = \sum \frac{T_i^2}{N_i} - C.T$$

T_i is the sum of observations and N_i is the number of observations in the treatment group i.

WSS can be obtained by using following equation

$$WSS = TSS - BSS$$

The degrees of freedom (d.f.) for various calculated terms

$$TSS = N_t - 1$$
$$BSS = N_c - 1$$
$$WSS = (N_t - 1) - (N_c - 1)$$

Where N_t = total no. of observations; N_c = No. of treatments

The F-value for the test was obtained by calculating

$$F = BMS / WMS$$

BMS = Between mean squares = BSS/d.f.
WMS = Within mean squares = WSS/d.f.

Two-way ANOVA test:

This test is useful when more than two treatments have to be compared treated under different conditions. The equation used to calculate different values is

$$TSS = CSS + RSS + ESS$$

TSS = Total sum of squares; CSS = Column sum of squares
RSS = Row sum of squares; ESS = Residual sum of squares

The above terms can be obtained by using following equations

$$TSS = \sum X^2 - C.T$$

$$ESS = TSS - CSS - RSS$$

$$CSS = \frac{\sum C_j^2}{R} - C.T$$

$$RSS = \frac{\sum R_i^2}{C} - C.T$$

Where C_j = total of column j; R = number of rows;
R_i = total of rows i; C = number of columns

The degree of freedom for various calculated terms is

$$TSS = N_t - 1$$
$$CSS = C - 1$$
$$RSS = R - 1$$
$$ESS = (C-1)(R-1)$$

The F-value is obtained for columns and rows by

$$F_{col} = CMS / EMS$$
$$F_{row} = RMS / EMS$$

CMS = Column mean square = CSS/ d.f.; **RMS** = Row mean square = RSS/d.f.
EMS = Residual mean square = ESS/d.f.

ANOVA test for linearity:

In general, a one-way ANOVA test was conducted to compare the variance due to deviations of Y from the fitted line (deviations from regression) to the variation due only to Y (the pooled error from Y replicates or the error within line).

The earlier mentioned equations in one-way ANOVA test for calculating various terms are remain same. However, the term BSS is further divided into two main components.

(a) the sum of squares(SS) due to the slope (regression SS) and

(b) the sum of squares (SS) due to deviations of the mean values (at each X) of Y from the fitted line (Lack of fit).

The equations used for calculating above two terms are

$$\text{Regression SS} = b^2 \Sigma (X-X)^2$$

$$\text{Lack of fit SS} = \text{BSS} - \text{Regression SS}$$

Where b = slope of the line

The F-value of the test was obtained by

$$F = \text{(Lack of fit MS) / (Within MS)}$$

Lack of fit MS = Lack of fit SS/d.f.; Within MS = Within SS/d.f.

Student's *t*-test for mean recoveries (*Two-independent groups test, variance unknown*):

This test is used to treat the means for two samples are equal

$$t = \frac{\left|\overline{X_1} - \overline{X_2}\right|}{S_p \sqrt{1/N_1 + 1/N_2}}$$

$$S_p^2 = \frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}$$

S_p = Pooled standard deviation; $\overline{X}_1$ & $\overline{X}_2$ = mean recoveries obtained from both groups
S1 & S2 = standard deviation of two samples; N1& N2 = Sample sizes for two groups

Limit of detection (LOD) [1,97]:

The LOD is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated, under the selected experimental conditions. Thus, limit tests merely substantiate that the analyte concentration is above or below a certain level and usually expressed in μg/ml. It can be calculated by following procedure

$$X_{LOD} = \frac{Y_{det} - a}{m}$$

Where Y_{det} = a + 3 × Standard error of estimate,
a = intercept; m = slope

Limit of quantitation (LOQ) [1,97]:

The LOQ is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions and expressed in μg/ml. It can be calculated by following procedure

$$X_{LOQ} = \frac{Y_{det} - a}{m}$$

Where Y_{det} = a + 10 × Standard error of estimate,
a = intercept; m = slope

The above mathematical expressions were obtained from "Pharmaceutical Statistics – Practical and Clinical Applications" by Sanford Bolton [98]. The reported statistical values of the experiments were also verified with software "Microsoft Excel 97 ".

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