DEVELOPMENT OF NEW ANALYTICAL METHODS FOR SELECTED ANTIASTHMATIC, ANTIANGINAL, ANTIAMOEBIC, ANTIDEPRESSANT AND HYPNOTIC DRUGS

THESIS

Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

P.UMAPATHI

Under the supervision of

Dr.P.PARIMOO

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI (RAJASTHAN) INDIA

1996

TO MY PARENTS

TABLE OF CONTENTS

SECTION III

ACKNOWLEDGMENTS

I am indebted forever to my supervisor, Prof.P.Parimoo for his guidance, advice and support throughout the course of this work.

I sincerely thank Prof.S.Venkateswaran, Director, BITS, Pilani for allowing me to pursue my research work successfully.

I am deeply grateful to Prof.B.M.Mithal, Professor of Pharmacy and Deputy Director, BITS, Pilani for providing me an opportunity to pursue my Doctoral study at BITS.

I am deeply thankful to Prof.L.K. Maheswari, Dean, Research and Consultancy Division, BITS for his constant encouragement, suggestions and for providing the various facilities for completion of this work.

I am grateful to Dr.K.R.V. Subramanian, Dean, DLPD for providing me access to the computer resources.

My special thanks are due to Drs.R.N.Saha and Addepalli for their advice and encouragement.

I am deeply thankful to my colleagues, Mr.R.Jagdeesh Babu, Mr.R.Mahesh, Miss .A Bharathi, Mr.P.Srinivas, Mrs.Anita Srinivas, Mr.C.V.N. Prasad, Mr. Suresh Babu and Mr.Ravindar Babu for their multivarious helps without which this work would not have been completed.

I am indebted to Dr.S.C.Sivasubramaniam for his advice, suggestions, help and encouragement during this thesis work.

My deeply grateful to my friends, Mr.Ashok Premarajan and Mr.Jaisankar for their time, support and co-operation related to computational work.

I am deeply thankful to Prof.G.Sundar for his constant encouragement and advice.

I wish to thank all the non-teaching faculty of the Department of Pharmacy for their various helps during the course of this work.

My thanks are due to Mr.Raghuveer, Mr. Soni and Mr.Durasingh for their various helps and support during the course of this work.

I am grateful to M/s. Torrent Pharmaceuticals Ltd., Wockhardt Ltd., Cadila Laboratories Ltd., Cipla Ltd.,Eskayef Ltd., Croyden Chemical Works Ltd., and Themis Pharmaceuticals Ltd. for the gift samples of various pure drugs.

I am thankful to all my students who had participated in this project, especially Mr.K.S.Srinivasan, Mr. Arul, Mr. R. Prabhu, Mr. Abraham Kovoor, Mr. John Samuel, Mr.Shahjahan,Mr.Simu K Thomas, Mr. Vikas Agarwal and Mr. Vinod for their diligence and co-operation during the experiments.

Lastly, I wish to thank everyone who have helped me in the successful completion of this thesis work.

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE

PILANI RAJASTHAN

CERTIFICATE

This is to certify that the thesis entitled DEVELOPMENT OF NEW ANALYTICAL METHODS FOR SELECTED ANTIASTHMATIC, ANTIANGINAL, ANTIAMOEBIC, ANTIDEPRESSANT AND HYPNOTIC DRUGS and submitted by P.UMAPATHI, ID.No. 89PHXF017 for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor: P. Parimoo

Name: Dr.P.Parimoo
Designation: Professor

Date: 20th July 1996

SECTION I

A. INTRODUCTION

Drugs or medicaments are seldom presented for administration in their natural or pure states. Often these are formulations of one or more active ingredients with one or more therapeutically inert materials intentionally added as Pharmaceutical aid. The various pharmaceutical preparations that provide the manufacturing Pharmacist with the challenges of formulation and Physician with the choice of Pharmaceutical types are termed as Dosage Forms. The dosage form chosen for a particular drug is largely determined by the chemical and pharmacological properties of the drug itself. A slow acting drug may be formulated in various ways such that the Physician has a choice of route of administration, depending on his need. The dosage form may be a simple one or a complex drug delivery system. The complexity usually is not intentional, but rather is determined by the properties (such as high degree of uniformity, physiological availability and therapeutic effect) that are expected from or built into the dosage form. Thus the final composition of a formulation is based upon a rationale of providing a product possessing optimal biological properties and pharmaceutical elegance.

B. OBJECTIVE AND DEFINITION OF THE PROBLEM

The premier objective of a quality control programme in drug manufacturing is to ensure the professional user or consumer that every batch of product conforms to specifications and that each dose distributed will satisfy the claims made by the manufacturers in the label as well as meet all other legal requirements. Although in process quality control measures are a part of Good Manufacturing Practice, the quality control tests on each batch of product as per requirements of the Monograph are of paramount importance for a product of high quality.

Basically the quality of a medicinal product includes all those factors which contribute directly or indirectly to the safety, effectiveness and acceptability of the product. Generally pharmaceutical analysis comprises of those procedures necessary to determine identity, purity and amount of the constituents in the dosage form. In the case of complex drug formulations, it involves the determination of one or more substances simultaneously in the presence of other drugs as well as additives without actually destroying the chemical nature of the compound as such. Although the Monographical procedures are available, they are usually laborious and require skill and use of expensive analytical tools like Gas Liquid Chromatography and High Performance Liquid Chromatography.Since a good analytical procedure should be cheap, precise, accurate and give reproducible results, the development of such new analytical procedures for complex drug substances will be very useful in routine analytical procedures, where simple and quick methods are usually preferred.

The choice of analytical method depends sometimes on the range of concentration and percentage of the components to be determined.

Sometimes, the choice of method is determined by the composition of the substance and whether the determination is for a single constituent or for several components. The U.S.P. [1] describes the assay procedures for pure drugs as well as formulations which are mostly HPLC methods except for a few components eg. aluminium hydroxide in aspirin, codeine phosphate, alumina and magnesia tablet [1], reserpine and chlorthiazide (spectrophotometry), acetaminophen in acetaminophen and propoxyphene HCl tablets [1] whereas the British Pharma copoeia [2] describes titrimetric or spectrophotometric methods of assay for most of the drugs which have been investigated in this report. The Indian Pharmacopoeia [3] also describes titrimetric and spectrophotometric methods for the drugs investigated.

The objective of the present thesis has been to develop new analytical methods for the simultaneous estimation of drugs in complex formulations, especially tablets. From this perspective, it should be noted that the monographs of B.P 1993 and I.P. 1985 do not include procedures for many tablet preparations whereas U.S.P. 1995 does include the procedures for many multicomponent tablets. Even though such procedures are available for combinations, the number and type of various combinations available commercially in the market, especially in India, is much more than the procedures described in monographs.

Hence combinations for which such monographical procedures are not available have been chosen (except for theophylline, phenobarbitone and ephedrine HCl tablets) for investigation in this work so as to develop new methods which will be practically useful for the estimation of drugs in such combinations.

SECTION II

EXPERIMENTAL

A. GENERAL ASPECTS

The Section II of this thesis comprises of the description and results and discussion of the various new methods of analysis developed for the estimation of the following combination preparations of drugs:

Tablet Preparations

1. Salbutamol sulphate and Bromhexine HCl
2. Metronidazole and Di-iodohydroxyquinoline
3. Aspirin and Dipyridamole
4. Tinidazole and Diloxanide furoate
5. Theophylline, Phenobarbitone and Ephedrine resinate
6. Atenolol and Nifedipine
7. Imipramine Hydrochloride and Diazepam
8. Diphenhydramine Hydrochloride and Diazepam
9. Metronidazole and Furazolidone
10. Metronidazole and Nalidixic Acid

Liquid Preparation

Orciprenaline sulphate and Bromhexine HCl

The work has been presented on the basis of the techniques used (titrimetry, spectrofluorometry, infra-red and uv spectrophotometry).

The methods developed have been for the determination of the content of active ingredient [1] and none of the combinations investigated in the work (except theophylline, phenobarbitone and ephedrine resinate) are official in U.S.P. 23, B.P. 1993 and I.P. 1985. Since potency definition (limits) for the investigated drug combinations are not available in the U.S.P. 23 and B.P. 1993, the limits mentioned for the drugs as single components in tablets (wherever available) have been used to interpret the results of the analysis.

All the spectrophotometric methods of estimation were done with linear calibration curves prepared with separate standards [4]. All the curves used were linear so as to keep the number of standards required for the construction of the curve at an optimal number [4].

The rectilinearity of the calibration curves have been demonstrated with the help of the regression analysis as well as various statistical tests [5] .

Appendix I gives the Analytical Profile of the Drugs investigated and Appendix II lists the various research publications which have been published thus far based on this thesis.

1.00 NON-AQUEOUS TITRIMETRY

1.01 INTRODUCTION

Inorganic titrimetric analysis is almost invariably restricted to the use of water as the solvent medium for the simple reason that the substance being titrated is usually an electrolyte. The dielectric constant of water is such that it favours ionisation of the solutes. However, it is frequently necessary to titrate substances which are weak electrolytes like fatty acids. The so called strength of the acid is also a function of the ability of the solvent to promote the acidic nature of the acid and in this respect water is not an ideal solvent for the titration of weak aids or bases, since it can function either as an acid or as a base:

$$H_2O = H^+ + OH^-$$

A substance which does not ionise strongly to form hydrogen or hydroxyl ions produces a concentration of these ions which is not overwhelmingly in excess of their normal concentration in the solvent itself. Consequently it is difficult to determine accurately the slight excess of hydrogen ions present in the solution. Furthermore the acidic nature of the aqueous solvent tends to suppress the acid nature of the solute. It is possible, however, to select a solvent which is capable of enhancing the acidity of the solute, and in this case much more favourable conditions will exist for determining the solvated protons furnished by the weak acid.

The most widely used method for the determination of phenols involves bromination or acetylation. The fact that aromatic amines interfere with bromination methods and both amines and alcohols interfere with acetylation methods makes other procedures for phenol determination highly desirable [6] . Although phenols may be estimated by coupling methods, these reactions generally lack convenience and accuracy [6]. Titrations of phenols as acids is usually difficult owing to the weak acidic nature of phenols. Moss, Elliot and Hall [7] showed that phenols in the

solvent of anhydrous ethylenediamine behave as moderately strong acids and titrated phenols potentiometrically using sodium methoxide in ethylenediamine - ethanolamine as the titrant. Same procedure had been used by Katz and Glenn [8] with a recording device to improve the accuracy of end point determination. Fritz and Lisicki [9] titrated phenols potentiometrically in butylamine but found no successful visual indicator to perform the titration.

The various features required of a solvent to be suitable for non-aqueous titrimetry has been discussed by Fritz [10]. Dimethyl formamide is an odourless solvent in which many carboxylic acids as well as phenols behave as strong acids. It has good solvent powers for many acidic compounds and may be used in the titration of most acids except the weaker ones which require a more basic solvent. Its principal advantage lies in its ability to dissolve most acidic substances rather than in its enhancing of acidity and carboxylic acids, simple phenols as well as some enols and imides, behave as strong acids in this solvent. The acidic impurities, if any, in this solvent must be neutralised before titration [6]. Dimethylformamide is unstable in the presence of excess alkali if titrations are carried out too slowly [11] and hence the titrations must be done as quick as possible. The solvent is hygroscopic and should therefore not be exposed to the atmosphere for prolonged periods although there is no need for protection if the titration is carried out at normal speeds. But dimethylformamide used only for anhydrous compounds since the presence of water causes high results [12]. Ethylenediamine enhances the acid nature of many substances (including phenols) which are too feebly acidic to be determined by titration in any other medium [12]. The water content of solvent must be kept as low as possible although as much as 5% of moisture may be tolerated in the solvent when it is used for the titration of more strongly acidic substances [12]. If the moisture content is high, dehydration by distillation of the benzene azeotrope or distillation after treatment with sodium hydroxide and sodium metal may be done [12]. Although the titrimetric methods have been replaced , to a large extent, by

HPLC and spectrophotometric methods in U.S.P. 23 [1], still B.P. 1993 [2] and I.P. 1985 [3] have many official titrimetric methods. B.P. 1993 prescribes titrimetric procedure for aspirin, atenolol, bromhexine HCl, diazepam, diloxanide furoate, diphenhydramine HCl, dipyridamole, imipramine HCl, metronidazole, nifedipine, orciprenaline sulphate, pheno barbitone, salbutamol sulphate and theophylline out of which many are non-aqueous titrimetry [2]. Similarly, I.P.1985 has official titrimetric methods for di-iodohydroxyquinoline, diloxanide furoate, diphenhydramine HCl, metronidazole and theophylline [3]. The combination of metronidazole and di-iodohydroxyquinoline is being widely used for the treatment of amoebiasis and giardiasis. Chemically, metronidazole is 2-(2-Methyl-5-nitroimidazol-1-yl) ethanol and di-iodo hydroxyquinoline is 5,7-Di-iodoquin-8-ol. Thus this combination has one drug with an alcoholic hydroxyl group and the other with phenolic hydroxyl group. Similarly, the salbutamol sulphate is widely used as an anti-asthmatic and is chemically 2-tert-Butylamino-1-(4-hydroxy-3-hydroxy methyl phenyl) ethanol with a phenolic hydroxyl group. The drugs salbutamol sulphate and di-iodohydroxyquinoline which are phenolic in nature and are likely to behave as acids in ethylenediamine and dimethylformamide [6]. Phenols which have a negative group in the ortho or para position are stronger acids than unsubstituted phenols and naphthols. The -CHO, -COR, -COOR, $-CONH_2$ and $-NO_2$ group in the ortho or para position increases the acidity of phenols sufficiently to permit accurate titration in dimethylformamide using azo violet indicator [6]. Fritz and Keen [6] suggest that ortho-halogen substituted phenols can also be titrated by this procedure and report that a carboxyl group has no activating influence and may sometimes decrease the acidity of the phenol such as salicylic acid. The unsubstituted and alkyl or aryl substituted phenols and naphthols are reported to be too weakly acidic to be titrated using azo violet. Fritz and Keen recommend the use of ethylenediamine using o-nitroaniline indicator for such compounds [6]. Though polyhydric phenols may also be titrated in ethylenediamine, some polyhydric phenols such as resorcinol and

catechol are reported to give no definite end point, probably because of the weakly acidic nature of the second -OH group [6]. Although the titrimetric methods may be tried for phenols after taking into consideration the aforementioned facts, there are several phenols such as o- and p-aminophenol which cannot be titrated using a visual indicator due to the deep color formed during the titration [6].

Many methods have been reported for the estimation of di-iodohydroxyquinoline and salbutamol sulphate. The methods reported thus far for di-iodohydroxyquinoline include non-aqueous titrimetry of di-iodohydroxyquinoline alone by using acetic anhydride solvent with potentiometric end point [13], spectrocolorimetry using vanadyl complexes [14, 15], using 2,6-dichloroquinone chlorimide as chromogenic agent [16], using metal ions in dimethylformamide [17]. A spectrocolorimetric method using sodium nitrite [18] has also been described. The methods reported for the estimation of salbutamol sulphate include simple spectrophotometric estimation of salbutamol sulphate alone in tablets [19], zero, second and fourth order derivative spectrophotometry in synthetic admixtures of salbutamol sulphate and gelatin [20], derivative spectrophotometry of salbutamol sulphate in tablets [21,22], low frequency dielectric spectroscopy [23], zero-order spectrophotometric estimation of salbutamol sulphate and bromhexine hydrochloride in tablets [24], spectrofluorometric estimation in tablets [25] and high performance liquid chromatographic method for salbutamol sulphate alone with fluorescence detection [26] and with bromhexine hydrochloride in dosage forms [27].

The U.S.P. 23 [1] method for di-iodohydroxyquinoline in tablets is oxygen flask combustion (indirect iodimetry) method and that for salbutamol sulphate in tablets is HPLC. The B.P. 1993 [2] method for salbutamol sulphate in tablets is spectrophotometry whereas the I.P. 1985 methods of di-iodohydroxy quinoline and salbutamol sulphate are oxygen flask method (indirect iodimetry) and spectrophotometry [3] respectively.

1.02 Estimation of Salbutamol sulphate and Di-iodohydroxy quinoline by Non-aqueous Titrimetry

The estimations successfully developed in this thesis involving non-aqueous titrimetric procedures are:

1. The estimation of di-iodohydroxyquinoline in the presence of metronidazole in tablet preparations
2. The estimation of salbutamol sulphate in tablet preparations.

Indicators

a. o-Nitroaniline indicator: This solution was prepared by dissolving 0.15 gms of o-nitroaniline in 100ml of benzene.

b. Azoviolet indicator: This solution was prepared by dissolving azoviolet in 100 ml of benzene till a saturated solution was obtained.

The benzene used in these titrations was of A.R. grade. This was further dried by treatment with anhydrous calcium chloride [28].

Preparation of 0.1N sodium methoxide

One litre of 0.1N sodium methoxide was prepared by dissolving 4 gms of freshly cut sodium metal in a mixture of 20 ml of methanol and 50 ml of distilled dry benzene in the presence of nitrogen. The sodium metal was added in small pieces with constant stirring of the solution and the solution was cooled in ice-water [6]. The reaction was allowed to proceed for 45 minutes with stirring. Methanol was added till the solution became homogenous followed by benzene till the solutions become cloudy with stirring. This dilution procedure involving alternate addition of methanol and benzene was repeated until 1000ml of clear solution was obtained containing methanol and benzene in a

ratio of approximately 6:1. The solution was stored under an atmosphere of nitrogen.

Assay of Drugs

Assay of Salbutamol sulphate

Standardisation of 0.1N Sodium methoxide

Twenty five millilitres (25ml) of benzene-methanol (6:1) solvent was placed in a conical flask under an atmosphere of nitrogen gas. The acidic impurities of the solvent were neutralised by titration with 0.1N sodium methoxide using thymol blue to a blue end point. Sixty milligrams (60 mg) of accurately weighed benzoic acid was dissolved in the neutralised solvent and titrated against approximately 0.1N sodium methoxide to a blue end point. The titration was performed in presence of nitrogen gas to prevent contact with atmospheric carbon dioxide and the normality of the sodium methoxide solution was determined [12].

Titration of Salbutamol sulphate in ethylenediamine

Neutralisation of ethylenediamine

The procedure described by West [12] was adopted. Twenty millilitres (20 ml) of dried ethylenediamine [12] was placed in a conical flask provided with a stopper carrying openings for a nitrogen inlet, nitrogen exit and entry of the burette. Two to three drops of o-nitroaniline in benzene were added and the solvent was titrated against 0.1N sodium methoxide with a steady stream of nitrogen flowing through the flask to an orange red end point.

Salbutamol sulphate (100 mg) was accurately weighed, dissolved in the neutralised ethylenediamine and the solution was titrated with 0.1N sodium methoxide using o-nitroaniline to an orange red end point.

Assay of tablet samples

Thirty tablets of each brand were taken, weighed, powdered, mixed and a weight of the powder equivalent to 25 or 50 mg of salbutamol sulphate was dissolved in ethylenediamine and titrated against 0.1N sodium methoxide using o-nitro-aniline as indicator to an orange red end point.

Assay of di-iodohydroxyquinoline

Neutralisation of dimethylformamide

Twenty five millilitres (25 ml) of dimethylformamide was taken in a conical flask fitted with a stopper carrying openings for nitrogen inlet, exit and entry of the burette. Two to three drops of azoviolet in benzene were added after displacing the air inside with nitrogen, and the dimethylformamide was titrated with 0.1N sodium methoxide to a blue end point while allowing nitrogen to flow through the flask. About 200 mg of accurately weighed di-iodohydroxyquinoline was dissolved in this neutralised dimethylformamide and titrated against 0.1N sodium methoxide using azoviolet to a blue end point Similar titrations of di-iodohydroxyquinoline were performed with accurately weighed quantities of metronidazole to establish the non-interference of metronidazole in the estimation of di-iodohydroxyquinoline. The results obtained have been stated in table 1.

Twenty tablets of each brand [2] were taken, weighed, powdered, mixed and a weight of the powder equivalent to 200 or 400 mg of di-iodohydroxy quinoline was dissolved in dimethylformamide and titrated against 0.1N sodium methoxide using azoviolet to a blue end point (Table 2)

RESULTS AND DISCUSSION

The drug di-iodohydroxyquinoline is soluble in both dimethylformamide and ethylenediamine. Among the two solvents, dimethylformamide which is a comparatively odourless solvent and relatively pleasant to handle was chosen for the estimation of di-iodohydroxyquinoline for the following

reasons. Firstly, the solvent power of dimethylformamide was found to be adequate for the estimation of di-iodohydroxyquinoline using azoviolet as indicator. Secondly, the end point was sharp (yellow to bluish green) and metronidazole did not interfere in the assay i.e. the color change as well as the titre value for a specific amount of di-iodohydroxyquinoline in the presence and absence of metronidazole remained the same whereas metronidazole interfered in the end point determination when ethylenediamine was used. When di-iodohydroxyquinoline alone was titrated in ethylenediamine using o-nitroaniline as indicator, the color change was from yellow to orange red red whereas in the presence of metronidazole the color change was from yellow to dark red and the change was not sharp making the end point determination difficult. In addition, the titre value did not remain the same i.e. metronidazole interfered when ethylenediamine was used. Hence dimethylformamide with azoviolet as indicator was selected as the solvent for the estimation of di-iodohydroxyquinoline. Fortunately, the acidity of di-iodohydroxyquinoline is adequate to be titrated in dimethylformamide and does not require a more basic solvent such as ethylenediamine which would make the estimation of di-iodohydroxyquinoline in the presence of metronidazole impossible. Although o-nitroaniline too can be used as indicator in dimethylformamide the color change was not sharp and faded with time in dimethylformamide.

o-Nitroaniline used as 0.15% w/v solution in benzene gives a fairly sharp yellow to orange-red end point during titration of phenols in ethylenediamine. Since it is weaker acid than indicators such as azo violet, it can be used for the titration of acids which are too weak to be determined with indicators such as azo violet [6,12].

Although azoviolet changes colour in the titration of phenols and alkyl-substituted phenols, the color change was found to be gradual and premature for salbutamol sulphate in ethylenediamine whereas o-nitroaniline, which can be used in strongly basic solvents such as ethylenediamine for titration of phenols and alkyl substituted phenols [12]

gives a sharp end point of orange red with salbutamol sulphate . Hence o-nitroaniline has been used as an indicator for titration of salbutamol sulphate in ethylenediamine. In the case of salbutamol sulphate, the drug was not soluble in dimethylformamide and hence the ethylenediamine was chosen as the solvent. The drugs di-iodo hydroxyquinoline and salbutamol sulphate were easily soluble in dimethylformamide and ethylenediamine respectively and provided sharp end points. Since the di-iodohydroxyquinoline is being marketed as tablets in combination with metronidazole, an attempt was initially made to estimate metronidazole (which has an alcoholic -OH group) in the presence of di-iodohydroxyquinoline (which possesses a phenolic -OH group) using the concept of differential titration proposed by Fritz and Keen [6] for the titration of phenols in the presence of carboxylic acids. Fritz and Keen [6] have reported that ethylenediamine is a poor solvent for such differential titration since basic solvents tend to make all acids nearly the same strength. They suggest that ethylenediamine may be used for the combined determination of carboxylic acids and phenols using o-nitroaniline as indicator and carboxylic acid alone in the presence of phenols may be determined in acetone or acetonitrile using p-hydroxyazobenzene as indicator. Our attempt to estimate metronidazole in the presence of di-iodohydroxyquinoline using the above principle was not successful due to the difficulty in estimating metronidazole (which has an alcoholic -OH) in acetone due its weak acidic nature [6]. But the same principle of determining total phenolic and carboxylic content in ethylenediamine and only the carboxylic acid content in acetone has been successfully applied for the assay of paracetamol and ibuprofen in tablet preparations (Appendix 2). Thus di-iodohydroxyquinoline has been estimated in the presence of metronidazole in dimethylformamide (Tables 1 and 2) and salbutamol sulphate in ethylenediamine since it is not soluble in dimethylformamide. The low standard deviation values and co-efficient of variance presented in Table 1 clearly indicate that the presence of metronidazole does not affect the assay of di-iodohydroxyquinoline by the

Table 1. Results of Reproducibility and Precision of the Proposed Non-aqueous Titrimetric Method for Di-iodohydroxyquinoline and Salbutamol Sulphate

Sample (mg)			Titre Value[a] (ml)		% Recovered	
SAL	DIQ	MND	ETD	DMF	SAL	DIQ
25	–	–	1.33 ± 0.05	—	99.72	—
50	–	–	2.65 ± 0.05	—	99.35	—
0	200	0	—	5.84 ± 0.05	—	99.69
0	400	0	—	11.69 ± 0.07	—	99.77
0	200	200		5.85 ± 0.05	—	99.86
0	400	400	—	11.69 ± 0.06	—	99.77

[a] ml of 0.065N sodium methoxide; average of ten determinations

SAL - Salbutamol sulphate DIQ - Di-iodohydroxyquinoline

MND - Metronidazole ETD - Ethylenediamine

DMF - Dimethylformamide

Table 2. Results of Assay of Commereial Formulations by the Non-Aqueous Titrimetric Method

Sample (%)	Label Claim (mg per tablet)			% Recovered[a]		Coefficient of Variation	
	SAL	DIQ	MND	SAL	DIQ	SAL	DIQ
Brand A	2	—	—	99.45 ± 0.21	—	0.21	—
Brand B	2	—	—	99.35 ± 0.35	—	0.35	—
Brand C	2	—	—	99.95 ± 0.22	—	0.35	—
Brand D	—	325	250	——	99.55 ± 0.21	—	0.21
Brand E	—	325	250	——	100.55 ± 0.28	—	0.21

[a] Average of five determinations

SAL - Salbutamol sulphate MND - Metronidazole

DIQ - Di-iodohydroxyquinoline

proposed method. The assay results indicated that the amount of di-iodohydroxyquinoline as well as salbutamol sulphate in the tablets were within the limits prescribed by I.P. 1985 and U.S.P. 23.

The precision and accuracy of titrations in anhydrous solvents depend very largely on the sharpness of end point as in aqueous titrimetry. The proposed method using visual indicators shows distinct color change leading to a sharp end point and hence was found to be very suitable for the estimation of the drugs in tablets.

Calculation

Standardisation of 0.1N sodium methoxide

The sodium methoxide was standardised by using benzoic acid as the primary standard. Benzoic acid is a monoprotic acid of molecular weight 122.12. The reaction between sodium methoxide and benzoic acid is as follows:

$$C_6H_5COOH + CH_3ONa \rightarrow C_6H_5COONa + CH_3OH$$

Each 12.21 mg of C_6H_5COOH = 1 ml of 0.1N $NaOCH_3$

Normality of 0.1N $NaOCH_3$ was : 0.065N

Assay of salbutamol sulphate

Each 28.24 mg of $C_{13}H_{21}NO_3$,1/2 H_2SO_4 = 1 ml of 0.1N $NaOCH_3$

Assay of di-iodohydroxyquinoline

Each 39.70 mg of $C_9H_5I_2NO$ = 1 ml of 0.1N $NaOCH_3$

Percentage purity of the drugs were calculated by:

$$\frac{\text{Titre value} \times \text{equivalent weight factor} \times \text{normality of 0.1N } NaOCH_3 \times 100}{0.1 \times \text{weight of drug}}$$

2.00 INFRA-RED SPECTROPHOTOMETRY

1.01 INTRODUCTION

Quantitative infra-red spectrophotometry is based on the same principles as that of uv-visible spectrophotometry and has the advantage of possessing more number of bands to choose from when compared to the later. It may often be possible to select a fairly strong band for each component in a mixture such that little or no interference of one with another occurs [29]. This technique may be applied to quantitate substances which have similar chemical or physical properties in a mixture [1] but has not been widely used because of its inherent lack of accuracy. This inaccuracy on direct application of Beer-Lambert's Law may be due to the presence of scattered radiation, especially at high values of absorbance [29]. But in cases where all the components of a mixture are soluble in a solvent like chloroform to give high concentration solutions, the method may be worth trying since it is rapid. It may be advantageous for routine analysis of drugs in formulations such as tablets since the interference from excipients may be eliminated [29]. If Beer's Law is obeyed, the integrated areas of the absorption bands or peak heights in case of sharp bands may be used in calculations. The major difference between the uv-visible and infra-red spectrophotometry arises in terms of concentrations used. In case of solutions used in IR spectrophotometry for quantitative estimation, percentages of up to 10-15% w/v are common since all solvents have some absorption in one part or another of the infra-red region making very short pathlengths in the order of 0.025-0.1mm necessary [29].

The high concentration of solute makes the accurate cancellation of solvent absorption very difficult [29]. In addition, the use of solvent in a matched cell in the reference beam is not practical for infra-red work because it is generally not possible to obtain cells having identical transmission characteristics. The very short path lengths are difficult to reproduce and

the transmission characteristics of the window materials change gradually with use [30]. But errors may be reduced by applying a base-line techniqae which is based on the assumption that the absorption due to solvent (or a second component) is constant or varies linearly with frequency over the region of the absorption band [29-32].

The base line method involves selection of an absorption band of the substance under analysis which does not fall too close to the bands of other matrix components. The value of the incident radiant energy P_o is obtained by drawing a straight line tangent to the spectral absorption curve at the position of the sample's absorption band. The transmittance P_T is measured at the point of maximum [31].

The method of calculation has been illustrated in figure 1. `abc' is the recorded absorption of component A and `def' is the absorption caused by solvent and other components. The `agc' in the figure 1 connects either two minima `a' and `c' or two suitable wavelengths on each side of the band. The point `g' is obtained by dropping a line perpendicular to the zero transmittance line to meet `ac' at `g'. The extinction log P_o/P_T is calculated from the distances shown in the figure 1 [29]. Alternatively, the baseline method may be altered (when an instrument which can record the output in absorbance is available) so as to use a base line joining the points of lowest absorbance on the peak, preferably in reproducibly flat parts of the absorption line as in figure 2 [32]. The later technique [32] has been used for the estimation of aspirin and dipyridamole in this report since the infra-red spectrophotometer used was capable of recording an absorbance output. Many possible errors are eliminated by the base line method. All measurements are made at points on the spectrum which are sharply defined by the spectrum itself, thus eliminating the dependence on wavelength settings. Use of such ratios (log P_o/P_T) eliminated changes in instrument sensitivity, source intensity or changes in adjustment of the optical system [30].

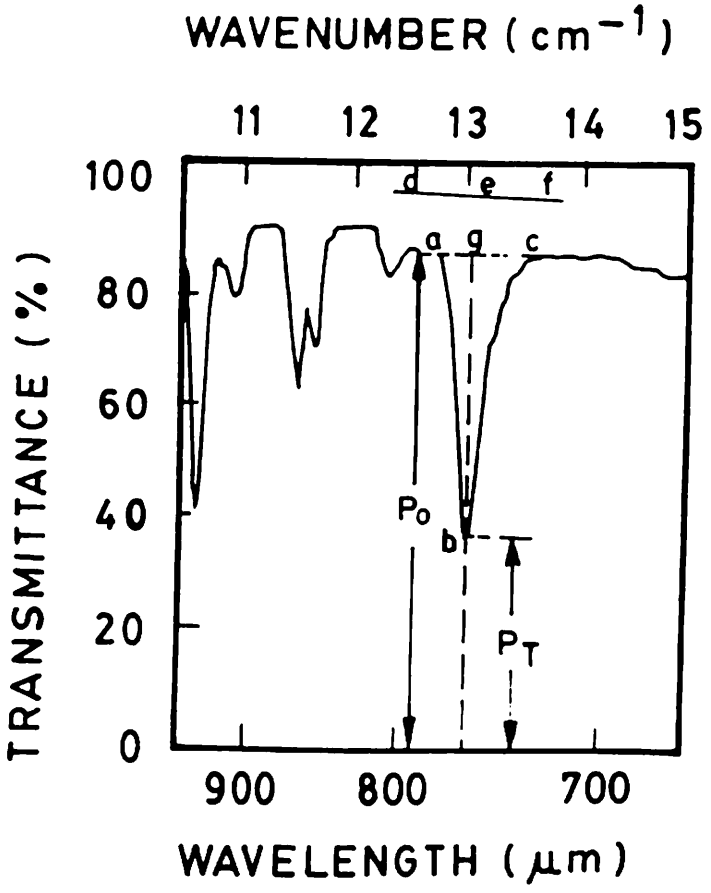


Fig 1. Infra-red spectrum showing application of base-line spectrum; spectrum recorded directly in transmittance (%) mode

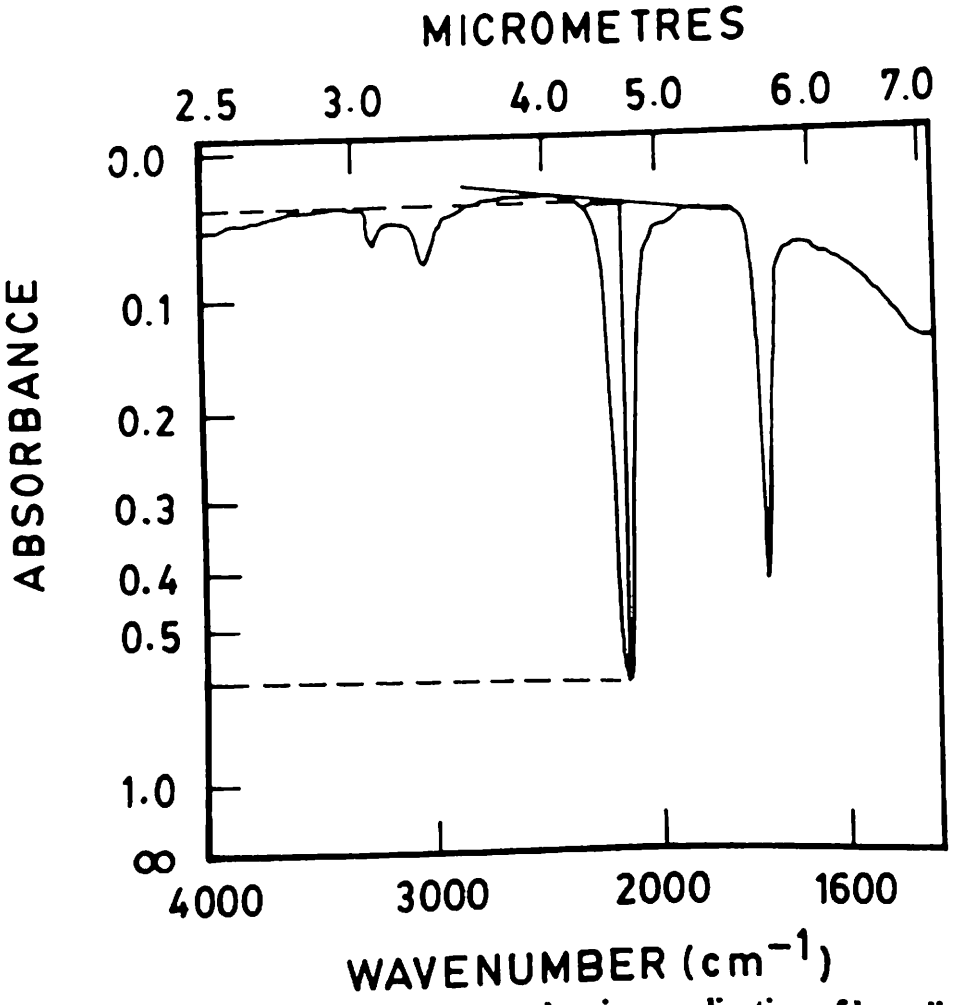


Fig 2. Infra-red spectrum showing application of base-line technique for quantitative estimation; spectrum recorded directly in absorbance mode

In case of mixtures where bands do interfere, simultaneous equations may be set up as in the case of uv-visible spectrophotometry [29] and sometimes, for the determination of small quantities of substances, present either as impurity or as solute in a preparation, compensation for absorption by the major component or solvent in the preparation may be achieved by introducing that component into the reference beam so as to produce a spectrum of the minor component alone [29]. The availability of sufficient energy for reliable results will normally be confirmed by the compliance of Beer's Law by the individual components at the chosen wavenumber [29].

The estimation successfully done in this report involving quantitative infra-red spectrophotometry is for aspirin and dipyridamole in pure admixtures as well as tablet preparations.

2.02 ESTIMATION OF ASPIRIN AND DIPYRIDAMOLE IN PURE ADMIXTURES AND IN TABLET PREPARATIONS BY QUANTITATIVE INFRA-RED SPECTROPHOTOMETRY

The use of drugs in combination for a particular therapeutic advantage makes the spectrophotometric analysis for the individual drugs in such dosage forms more complicated since usually the uv absorption spectra interfere with each other , at least to some extent resulting in the use of some kind of simultaneous estimation a must. The same is true in case of the combination of aspirin and dipyridamole in which direct estimation of one drug in the presence of the other by direct uv spectrophotometry will not be possible due to interference (Figure 3).Although dipyridamole shows appreciable absorption around the wavelength of 272 nm in 0.1N HCl and at 290nm in 0.1N NaOH when compared to aspirin, the proportion in which these drugs are present in commercial formulations (aspirin: dipyridamole = 2:3.75 or 2:2.5 or 2:1.5) may pose a problem for direct estimation of dipyridamole in the presence of aspirin as aspirin is likely to contribute some absorbance at these wavelengths. In addition, aspirin cannot be directly estimated in the presence of dipyridamole since the absorption of dipyridamole, when present along with aspirin in the proportions available as commercial formulations is comparatively high at the wavelengths of 230nm (in 0.1N HCl) and 290nm (in 0.1N NaOH) where aspirin exhibits maximum absorption. Hence the simultaneous estimation methods may have to be used for the estimation.

The combination of aspirin (O-Acetylsalicylic acid) and dipyridamole which is 2,2',2'',2'''[(4,8-Dipiperidinopyrimido [5,4-d] pyrimidine- 2,6-diyl)dinitrilo] tetra ethanol as tablets are being widely used as anti-anginal preparations.

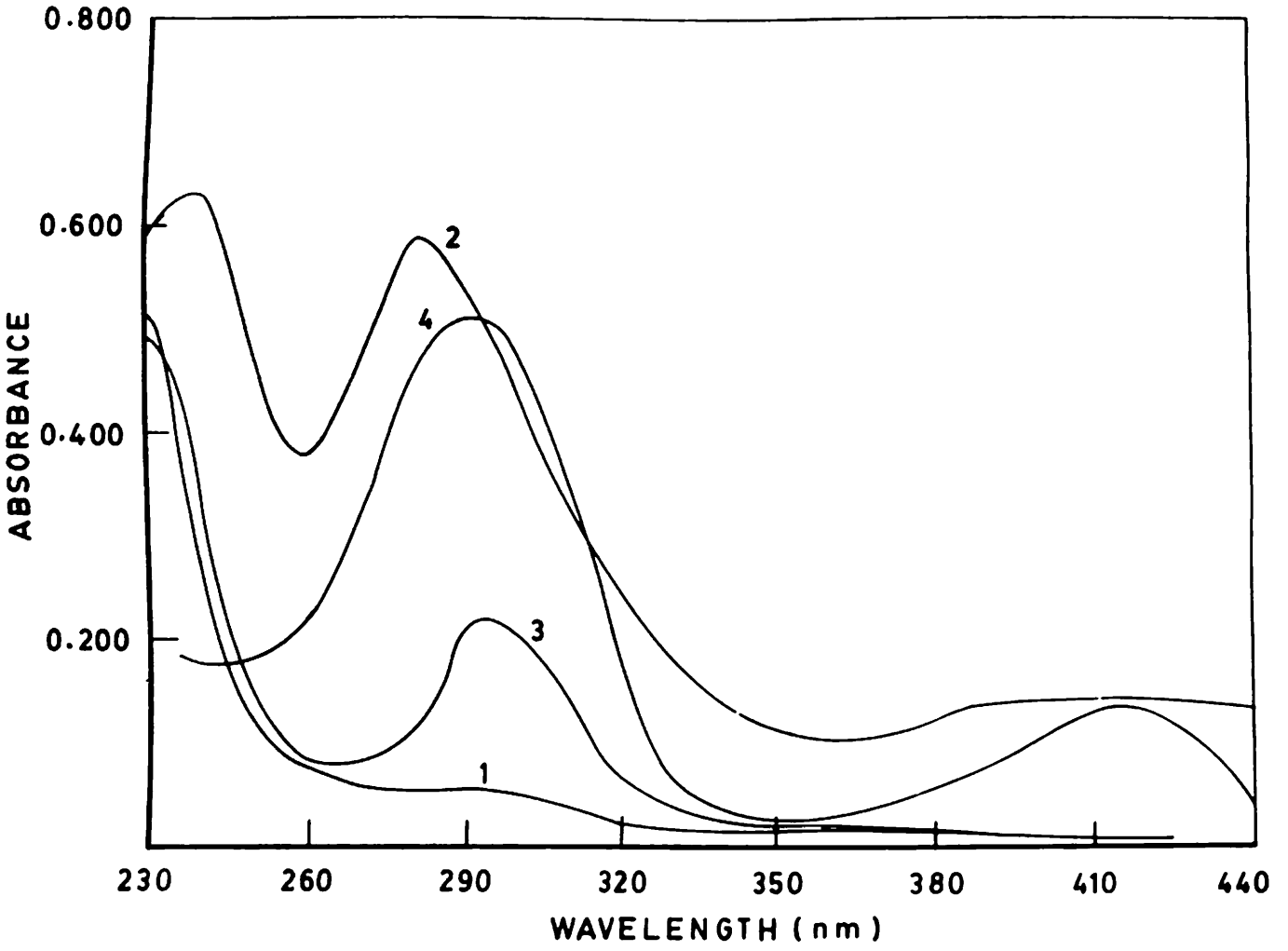


Fig 3. Normal absorption spectra of pure aspirin (10 mcg ml^{-1}) and dipyridamole (10 mcg ml^{-1}) in 0.1M HCl and 0.1M NaOH;Curves 1 & 2:Aspirin and dipyridamole in 0.1M HCl respectively; Curves 3 & 4:Aspirin and dipyridamole in 0.1M NaOH respectively

Analytical procedures reported thus far for the identification and quantitation of aspirin include titrimetry [33-38] involving titration of aspirin in methylisobutyl ketone with sodium methoxide [33], titration in tetrabutyl ammonium hydroxide [34], in dimethylformamide [35], in ethylenediamine [36] and in aqueous medium [37], differentiating non-aqueous titrimetry [38], microcalorimetry [39], spectrophotometric methods involving colorimetry [40, 41] ,derivative spectrophotometry in suppositories [42] and tablets [43-46], spectrofluorometric estimation of aspirin in the presence of salicylic acid in pharmaceutical preparations [41, 47-52], phosphorimetry [53], estimation by thin layer chromatography [54-58] and by gas liquid chromatography [59-62], estimation of aspirin in the presence of phenacetin and caffeine by nuclear magnetic resonance spectrophotometry [63] and estimation by HPLC in the presence of phenacetin and caffeine [64], in the presence of paracetamol and caffeine in tablets [65], in capsules in the presence of caffeine, dihydrocodeine bitartrate and promethazine hydrochloride [66] and in the presence of salicylic acid in pharmaceutical preparations [67,68]. The literature methods for estimation of dipyridamole include HPLC [68-72] and colorimetry using with thiocyanate-chromium (III) complexes [73]. The B.P. 1993 and I.P. 1985 prescribe titrimetric method and U.S.P. 23 HPLC method for aspirin tablets whereas the U.S.P. 23 and B.P. 1993 methods for dipyridamole tablets are HPLC and spectrophotometry respectively. The combination of aspirin with dipyridamole as tablets is not official in U.S.P. 23, B.P. 1993 and I.P. 1985.

In this thesis, various methods of simultaneous estimation, namely quantitative infra-red spectrophotometry, spectrofluorometry, linear plot, and second-order derivative difference spectrophotometric methods have been successfully designed for the estimation of these drugs. This section deals with details of the infra-red method and next with that of spectrofluorometry. The other methods have been described in section 5.

METHODS

Apparatus, Reagents and Equipment

1. *Chloroform A.C.S. - Spectrophotometric Grade (Aldrich Chemical Company, Inc., USA)*

2. *Aspirin U.S.P and dipyridamole U.S.P. were obtained as gift samples from M/s. Torrent Pharmaceuticals Ltd.*

3. *The infra-red spectra were recorded on a Jasco IR Report 100 and Perkin-Elmer 1310 IR automatic scanning spectrophotometer using calibrated sodium chloride cavity cells of path length 0.5mm with pure chloroform in the reference cell.*

Calibration of pathlength of the cavity cell

The method of counting interference fringes [32] was used for the calibration of the cavity cell. This method is based on the relationship between the pathlength of the cell and the peak to peak fringes. To calibrate the pathlength, the empty cell with parallel windows is placed in the spectrophotometer and scanned over a wavelength region. This scan produced an interference pattern as given in figure 4. The amplitude of the waveform may vary from 2% to 15% depending on the state of the windows [32]. The relationship between the pathlength of the cell,L and the peak to peak fringes is given by

$$L = \frac{n}{2(v-q)} \text{ cm} \qquad \text{(Equation I)}$$

where n is the number of complete peak to peak fringes between two maxima (or minima) at the frequencies of v and q.

In figure 4, the number of peak to peak fringes between the wave numbers of 1700 and 1600 cm^{-1} are ten. Hence the cell length as per equation (I) was

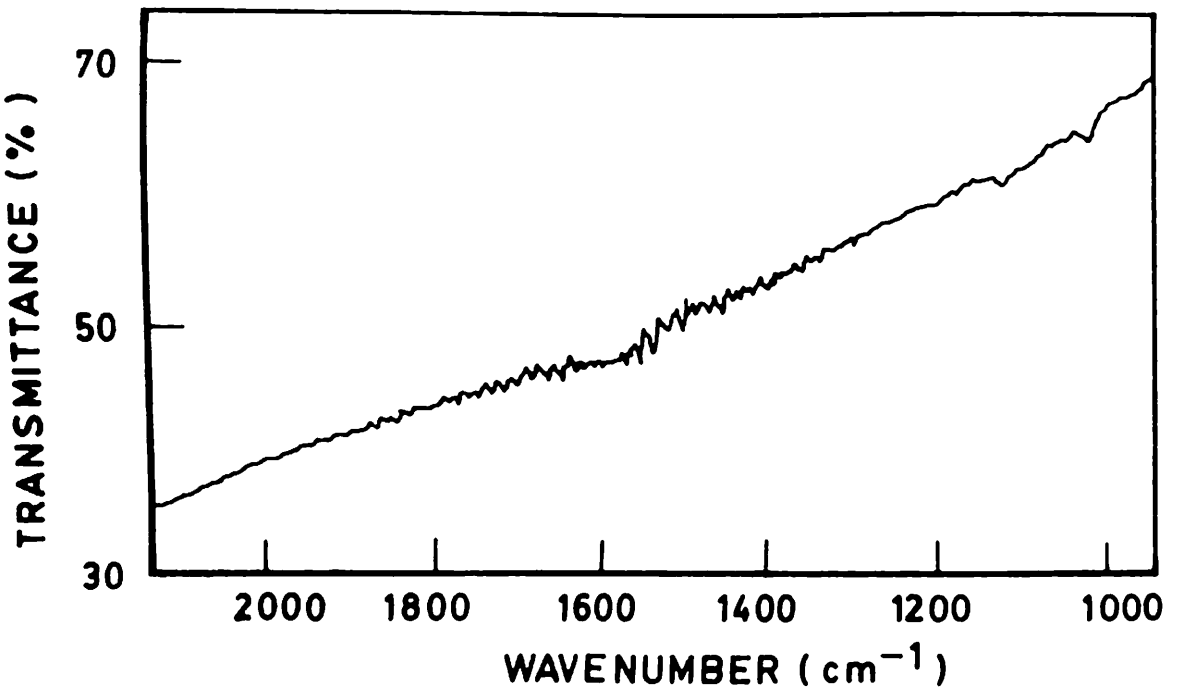


Fig 4. An interference pattern (infra-red) recorded by scanning from 2000 to 1000 cm^{-1} with an empty cell in the sample beam

$$L = \frac{10}{2\,(1700\text{-}1600)}\ \text{cm} = 0.05\ \text{cm}$$

Preparation of Standard and Sample Solutions

Four series (Series A,B,C and D) of standard solutions were prepared from pure drugs. Solutions of Series A solutions were prepared by transferring quantitatively accurately weighed quantities of aspirin (250,375,500,625,750, 875 and 1000 mg) to 25 ml volumetric flasks (low actinic Pyrex Glass A) and dissolving and diluting to volume with chloroform to obtain solutions containing 10-40 mg/ml of aspirin. In a similar manner, accurately weighed quantities of dipyridamole (250 to 1500 mg) were used to prepare Series C solutions (in 25ml volumetric flasks) which comprised of 10-60 mg/ml of dipyridamole in chloroform. The Series B solutions were mixtures of aspirin and dipyridamole in chloroform containing a varying concentration of aspirin (10-40 mg/ml) and a constant concentration of dipyridamole (35 mg/ml) and the Series D solutions were mixtures of the drugs containing a varying concentration of dipyridamole (10-60 mg/ml) and a constant concentration of aspirin (25 mg/ml).

Preparation of Sample solutions

Seventy tablets of Brand A and fifty each of Brands B and C were weighed, powdered and appropriate weights of the tablet powders containing approximately 250 mg of aspirin and 468 mg of dipyridamole (Brand A), 250 mg of aspirin and 312 mg of dipyridamole (Brand B) and 250 mg of aspirin and 187 mg (Brand C) were transfered quantitatively to 25 ml volumetric flasks for the assay of aspirin. For the assay of dipyridamole, fifty tablets of each of the Brand were weighed, powdered and appropriate weights of the tablet powders containing approximately 350 mg of dipyridamole and 186 mg of aspirin (Brand A), 350 mg of

dipyridamole and 280 mg of aspirin (Brand B) and 300 mg of dipyridamole and 400 mg of aspirin (Brand C) were quantitatively transfered to 25ml volumetric flasks. These were dissolved by thorough shaking and diluted to volume with the chloroform. The solution was filtered through Whatman No.1 filter paper. The first and last 5 ml of the filtrate were discarded and the remainder was collected in dry 25 ml volumetric flasks. All the above solutions were stored in low actinic volumetric flasks at a room temperature of 20-25°C till the recording of their absorbances.

RESULTS AND DISCUSSION

As described in the beginning of this section, the quantitative IR spectrophotometry has some distinct advantages over uv-visible spectrophotometry for pharmaceutical analysis in spite of its certain inherent disadvantages. It is especially useful for the quantitation of drug mixtures in formulations since it may often be possible to select a strong band for each component in a mixture so that there is little or non-interference of one component in the absorption of the other. The colouring agents are less likely to interfere in IR spectrophotometry when compared to uv-visible spectrophotometry due to the high concentrations required for infra-red spectrophotometry when compared to uv-visible spectrophotometry as well as the provision of more number of peaks to choose for estimation.

In the present investigation, the peak heights (absorbance values) determined by the base line technique [29-31] at the wavenumbers of 920 cm^{-1} and 2935 cm^{-1} (Figures 5 and 6) were found to fulfill many requirements including the linearity with the concentrations [32]. The peaks heights at the wavenumbers of 920 cm^{-1} and 2935 cm^{-1} were found to be linear with the concentration range of 10-40 mg/ml of aspirin and 10-60 mg/ml of dipyridamole respectively and have been used for the

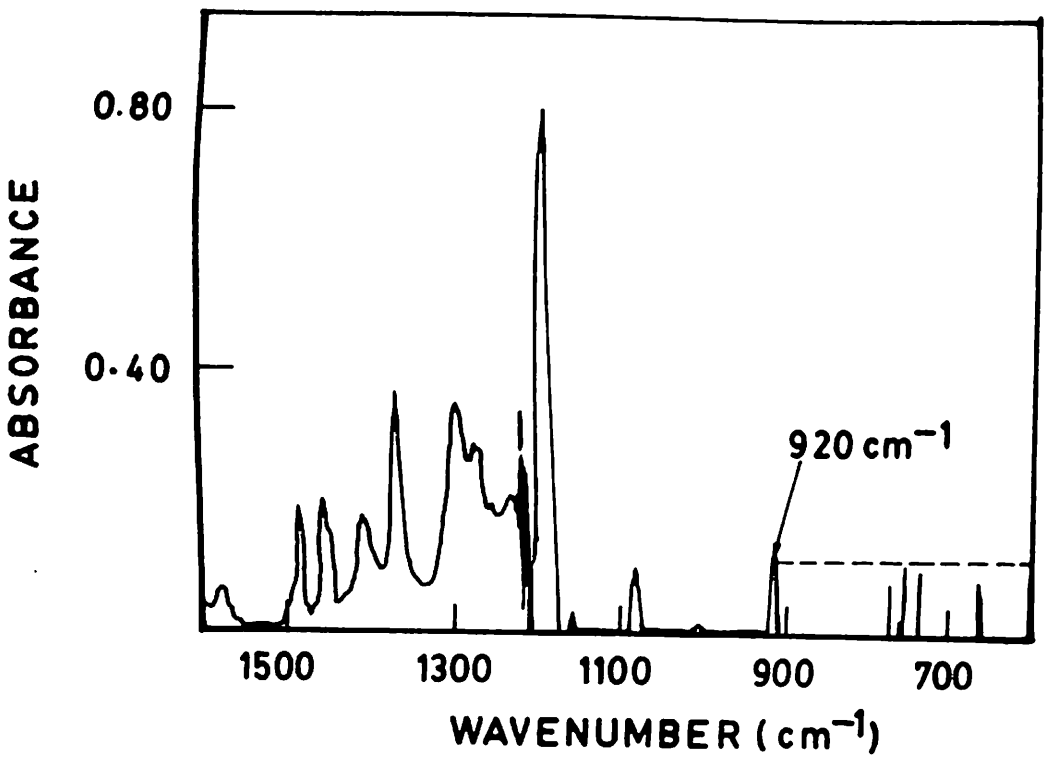


Fig 5. Infra-red spectrum of aspirin (20 mcg ml^{-1}); sample was solution of aspirin in spectal grade chloroform solvent

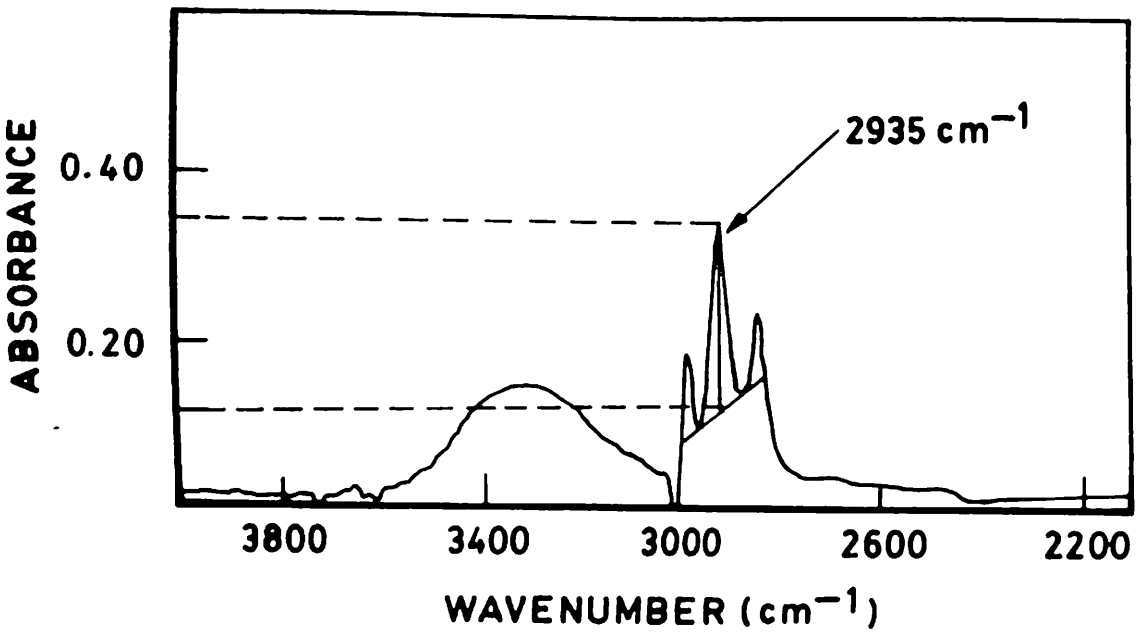


Fig 6. Infra-red spectrum of dipyridamole (30 mcg ml^{-1}); sample was a solution of dipyridamole

quantitation of the drugs in tablet formulations.The amounts of aspirin and dipyridamole in the tablets have been calculated from calibration curves obtained as best fits based on least square method using the data presented in Tables 3 and 4.

The statistical analysis had been done on the data of pure drug solutions, their admixtures as well as commercial samples. The small standard deviation values associated with the determination (Tables 3 and 4) indicated the high level of precision of the proposed method as well as the independence of one drug in the absorption measurement of the other. The negligible intercepts of the equations indicated regression through or close to the origin at the chosen wavelengths.

The coefficient of variation values (Table 3 and 4) were less and the standard error (which is the standard deviation of the mean) values are also quite small.

The percentage ratio of the residuals in tables 3 and 4 clearly indicated a random scatter in case of both pure drug solutions as well as their admixtures. In addition, the F test for non-linearity which is a quantitative test for non-linearity [4] was done and the results have been presented in tables 3 and 4. This test is based on the fact that the population standard deviation applicable to single measurements of y should be the population standard deviation for the residuals (Sy) also. In case of repeat determinations at a single concentration, the standard deviation of the measurement is used [5]. If a linear relationship holds, the standard deviation of the residuals (standard error of estimate, Sy, in tables 3 and 4) would represent an estimate (with (n-2) degrees of freedom) of the standard deviation of the sample. Therefore, we would have an evidence of non-linearity if we can show that Sy has too large a value to be compatible with the sample estimate (S).The values in tables 3 and 4 show that the calculated F values were less than that of critical values at 5% level of significance and evidence the linear relationship.

Table 3. Selectivity of the Method for the Determination of Aspirin in the presence Dipyridamole by Infra- red Spectrophotometry

Composition of the solution ($mg\ ml^{-1}$) ASP	DIP	Mean value of [a] absorbance ($920\ cm^{-1}$)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
10	0	0.0879 ± 0.0038	3.27	0.0009	93.72	3.48	3.20
15	0	0.1246 ± 0.0026	2.08	0.0008	104.21	3.48	3.60
20	0	0.1751 ± 0.0027	1.55	0.0009	101.81	3.48	3.37
25	0	0.2256 ± 0.0024	1.05	0.0009	100.47	3.48	3.37
30	0	0.2840 ± 0.0033	1.15	0.0010	96.86	3.48	2.50
35	0	0.3150 ± 0.0037	1.19	0.0012	102.69	3.48	3.37
40	0	0.3755 ± 0.0025	0.68	0.0008	99.04	3.48	3.04
10	35	0.0905 ± 0.0021	2.34	0.0007	94.82	3.48	2.42
15	35	0.1302 ± 0.0023	1.77	0.0007	102.23	3.48	2.60
20	35	0.1799 ± 0.0028	1.56	0.0009	100.27	3.48	2.25
25	35	0.2214 ± 0.0031	1.40	0.0010	102.84	3.48	1.84
30	35	0.2789 ± 0.0028	0.99	0.0009	98.59	3.48	2.25
35	35	0.3217 ± 0.0020	0.62	0.0006	100.17	3.48	2.09
40	35	0.3712 ± 0.0022	0.60	0.0007	99.55	3.48	2.42

DIP - Dipyridamole ASP - Aspirin

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(5,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 4. Selectivity of the Method for the Determination of Dipyridamole in the Presence of Aspirin by Infra- red Spectrophotometry

Composition of the solution (mg ml^{-1}) ASP	DIP	Mean value of [a] absorbance (920 cm^{-1})	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
0	10	0.1168 ± 0.0054	2.05	0.0007	99.44	3.18	1.32
0	15	0.1738 ± 0.0048	1.60	0.0009	99.88	3.18	1.67
0	20	0.2308 ± 0.0049	1.27	0.0009	100.11	3.18	1.00
0	25	0.2882 ± 0.0049	1.03	0.0009	100.11	3.18	1.00
0	30	0.3475 ± 0.0042	0.94	0.0010	99.56	3.18	1.05
0	35	0.4057 ± 0.0041	0.72	0.0009	99.44	3.18	2.29
0	40	0.4458 ± 0.0044	0.82	0.0012	103.38	3.18	1.60
0	45	0.5258 ± 0.0049	0.64	0.0010	98.58	3.18	1.60
0	50	0.5812 ± 0.0047	0.81	0.0015	99.07	3.18	1.74
0	55	0.6361 ± 0.0045	0.71	0.0014	99.55	3.18	1.89
0	60	0.6861 ± 0.0034	0.45	0.0009	100.67	3.18	3.32
25	10	0.1169 ± 0.0032	2.74	0.0010	100.66	3.18	3.28
25	15	0.1746 ± 0.0033	1.91	0.0011	100.22	3.18	3.19
25	20	0.2366 ± 0.0041	1.75	0.0013	98.18	3.18	2.07
25	25	0.2869 ± 0.0030	0.99	0.0009	100.95	3.18	3.86
25	30	0.3473 ± 0.0035	0.76	0.0008	99.85	3.18	2.84
25	35	0.4067 ± 0.0033	0.73	0.0009	99.39	3.18	3.19
25	40	0.4483 ± 0.0039	0.64	0.0009	102.96	3.18	2.28
25	45	0.5270 ± 0.0034	0.60	0.0010	98.45	3.18	3.01
25	50	0.5800 ± 0.0035	0.55	0.0010	99.39	3.18	2.84
25	55	0.6357 ± 0.0033	0.53	0.0010	99.65	3.18	3.19
25	60	0.6866 ± 0.0037	0.54	0.0012	100.61	3.18	2.58

DIP - Dipyridamole ASP - Aspirin

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical}$ = F (9,9) values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Similarly, the other F test results which was based on mean square due to regression and mean square about the regression clearly showed the non-linearity since the calculated F values were far larger than the critical values leading to rejection of null hypothesis (Table 5). The regression equations of the pure drug solutions and those of admixtures (Table 5) were similar. This similarity as well as the correlation coefficient values in the range of 0.9990 to 0.9995 indicated the non-interference of one drug in the estimation of the other. The co-efficient of determination (which is ratio of the sum of squares due to regression to the sum of squares about the mean) values ranged from 99.65 to 99.91 indicating that this much of variation in the absorbance is accounted for by the concentration of the drug in the solutions. A comparison of T test values at a significance level of 5% showed that the calculated values are far larger than the critical values obtained from the t table and confirmed the existence of strong positive correlation [5]. The standard error of slope and intercept are the standard deviation values of slope and intercept and the standard error of estimate (which is the standard deviation value of residuals of y on x line and which is an indicator of the precision of the fit by regression) values for the various solutions. This standard error of estimate was less relative to the typical change in absorbance value from point to point in the calibration curve based on the regression equations for pure admixtures [5].

The table 6 gives the actual values of the pure aspirin (25 mg/ml) and dipyridamole (35 mg/ml) as well as the value calculated from the regression line for admixtures. The standard error of prediction is also given in this table.The 95% confidence level concentration ranges presented in table 6 (calculated using the standard error of prediction values) appear to be narrow.

The assay results of commercial formulations have also been given in table 6. In the case of aspirin, the estimation was done using a solution of the tablet sample containing approximately 25 mg/ml of aspirin for all brands and the dipyridamole was estimated by using solutions containing

Table 5. Regression Analysis of Aspirin and Dipyridamole Standard Solutions

Sample	Composition of Solution (mg ml^{-1})		Regression Equation [a] (at 920 cm^{-1} for aspirin, at 2935 cm^{-1} for dipyridamole)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	ASP	DIP				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	10-40	0	y = 0.0097x - 0.0015	0.9990	99.65	6.61	1407	2.57	37	0.0003	0.0069	0.0043
Series B	10-40	35	y = 0.0095x - 0.0087	0.9992	99.86	6.61	3536	2.57	59	0.0002	0.0043	0.0042
Series C	0	10-60	y = 0.0114x + 0.0012	0.9995	99.90	5.12	9344	2.26	96	0.0001	0.0046	0.0062
Series D	25	10-60	y = 0.0115x + 0.0031	0.9996	99.91	5.12	10429	2.26	102	0.0001	0.0043	0.0059

ASP- Aspirin DIP - Dipyridamole [a] Based on *7 and 11 calibration values* for aspirin and dipyridamole respectively; concentration of drug in mg ml^{-1}

[b] Coefficient of determination which is the ratio of the *sum of squares due to regression* to the sum of squares *about the mean*

[c] F test based on F statistic (a one tail test); F value is the ratio of *mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1,n-2*) value at 5% significance level; F *crit* is the F (*1,n-2*) value from the F ratio table for 5% significance level; n is 7 for aspirin and 11 for dipyridamole

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T (n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for t distribution table at 5% significance level; n is 7 for aspirin and 11 for dipyridamole

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of y on x regression where y is the absorbance and x is the concentration

Table 6. Results of the Assay of Aspirin and Dipyridamole in Pure Drug Admixtures and Commercial Formulations by Infra-red Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	ASP	DIP	ASP	DIP	ASP	DIP	ASP	DIP
Pure Drug Admixture	25.00	35.00	—	—	99.20	99.08	24.13-25.46	34.06-35.30
Brand A	25.00	46.87	40	75	97.92	101.19	23.91-25.04	46.87-47.99
Brand B	25.00	31.25	60	75	98.12	99.96	23.95-25.08	30.73-31.74
Brand C	25.00	18.75	100	75	100.28	100.64	24.51-25.63	18.26-19.48
Brand A	18.66	35.00	40	75	100.10	100.08	18.05-19.31	34.53-35.52
Brand B	28.00	35.00	60	75	97.86	100.68	26.82-27.97	34.74-35.73
Brand C	40.00	30.00	100	75	99.43	99.10	38.91-40.62	29.21-30.23

ASP - Aspirin DIP - Dipyridamole

[a] Average of ten determinations; assay as percentage of actual concentration / label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 5 degrees of freedom for aspirin and 9 degrees of freedom for dipyridamole

approximately 35 mg/ml. This was done to calculate the 95% confidence level concentration ranges in the last column at concentrations where the standard error of prediction will be minimal. As can be seen from tables 3 and 4, the mean points of the calibration data for aspirin and dipyridamole lie at around 25 mg/ml and 35 mg /ml respectively and it is a known fact that a linear regression fit is most precisely determined at the mean point of the calibration data. For comparison of the 95% confidence level ranges, the dipyridamole has also been determined at the appropriate concentrations corresponding to a concentration of 25 mg/ml of aspirin in the various Brands (2-5 rows in table 6) and aspirin at the appropriate concentrations corresponding to a concentration of 35 mg/ml of dipyridamole. The 95% confidence level ranges of concentration predicted from a regression equation uses the standard error of prediction which is minimal at the mean point of calibration [5]. In case of Brand C, the concentration of the dipyridamole solution has been 30 mg/ml instead of 35mg/ml since the value of aspirin concentration goes above 40 mg/ml at this concentration of dipyridamole whereas 40 mg/ml is the highest concentration of aspirin used for the calibration curve of aspirin. The detection limits at 5% level of significance were found to be 1.45 and 1.70 mg /ml respectively.

The results of the assay by the proposed method in table 6 indicated that the tested commercial formulations were conforming to the limits for the drug content in individual tablets of aspirin and dipyridamole prescribed by I.P. 1985 and U.S.P. 23. In fact, the 95% confidence level concentration ranges in table 6 were itself within the prescribed limit for these drugs by U.S.P. 23. Thus the proposed method of determination of aspirin and dipyridamole was found to be reasonable accurate and precise and may be used for the estimation of the drugs in commercial formulations.

3.00 SPECTROFLUOROMETRY

Fluorescence analysis is an analytical method closely related to spectrophotometry and is by far the most widely used luminescence technique in practice, primarily because of its intrinsic sensitivity and selectivity. The sensitivity is generally greater than that achieved by absorption spectrophotometry in which, at low drug concentrations, accurate and precise measurement of the difference in intensity of the two similar beams of radiation, I_o and I, becomes progressively more difficult. By contrast, the intensity of fluorescence is essentially measured against a `dark' background, or at least a background with relatively low fluorescence emission.

The selectivity of spectrofluorometry arises from the requirement that two wavelengths are involved, the excitation wavelength and fluorescence emission wavelength. Moreover, the ability of a molecule to fluoresce is in itself a characteristic which discriminates it from many compounds which do not display significant fluorescence.

In order for a molecule to fluoresce it must first absorb radiation. If the concentration of the absorbing substance is very high, all the incident light may be absorbed by the first layers of solution, with very little light even reaching more distant portions of the sample. The fluorescence of such a sample will therefore be non-uniform and will not be proportional to the concentration of the substance. Because this is undesirable from the analytical point of view, solution concentration of fluorescing substances are always held to very low levels to avoid the absorption of an appreciable fraction of the incident beam. Thus a necessary condition for quantitative spectrofluorometric analysis is that the total absorbance of the system should not exceed 0.05 absorbance units. Otherwise progressively greater negative

deviations from linearity are usually observed. Hence it is essential to establish the range of linearity of the calibration curve of I_f versus concentration, using at least five standard solutions, for which the condition that absorbance at the excitation wavelength maximum is less than 0.05 absorbance unit holds.

Thus the expression for fluorescence intensity [74]

$$F = 2.31\, I_o\, \phi\, abc$$

(where F is the fluorescence intensity (the intensity of emitted radiation), Io is the intensity of the incident radiation, ϕ is the quantum yield of fluorescence, a is the molar absorptivity of the substance, b the pathlength and c the molar concentration) shows that F is proportional to I_o, and hence increased sensitivity can be achieved for a given concentration simply by increasing the intensity of the incident excitation radiation. This is a fundamental difference between fluorometry and spectrophotometry, for in the later technique the absorbance is independent of the incident intensity [74]. Since the above equation shows that the fluorescence intensity is proportional to molar absorptivity, it is usual to use excitation radiation corresponding to the absorption band maximum [74].

The outstanding advantage of fluorescence analysis, as mentioned earlier, is its sensitivity. Although the amounts of drugs present in dosage forms are seldom so small as to require sensitivity of this order, the concentrations of drugs and drug metabolites in blood, urine, and other biological samples may be extremely low, and fluorescence analysis finds wide application in quantitative studies of rates and mechanisms of drug absorption, metabolism and excretion [74]. In addition spectro fluorometry will be a useful analytical technique to estimate drugs in combined preparations whose uv absorption spectra interfere with each other requiring the application of some kind of simultaneous method. As described in the section on infra-red spectrophotometry, the combination of

aspirin and dipyridamole, if to be estimated by uv spectrophotometry, do require some kind of simultaneous estimation. Hence a successful attempt has been made in this thesis to design a spectrofluorometric method of estimation of these drugs. This section describes the details of the estimation of aspirin and dipyridamole by spectrofluorometric method.

Fluorimetric drug analyses may be classified into three chemical types. First are those assays of drugs that possess intrinsic fluorescence, in the sense that they require no chemical reactions to create a fluorescent compound. The second type of analysis involves the formation of a derivative of the drug by attaching a fluorescent tag to the drug and the third class of assays involve development of a fluorophore by more extensive molecular change than simple derivative formation [74]. The fluorimetric assay procedure developed for the assay of aspirin and dipyridamole in this thesis falls under the first category in which chemical reactions have not been used for the fluorescence of the drugs.

3.02 Estimation of Aspirin and Dipyridamole in Pure Admixtures and in Tablets by Spectrofluorometry

The combination of aspirin with dipyridamole is widely used as an anti-anginal preparation. Of the several methods mentioned for the estimation of acetylsalicylic acid in the infra-red spectrophotometric method (section 2.02) for estimation of aspirin and dipyridamole [33-68], six were on the spectrofluorometric estimation of aspirin [41, 47-52]. These reports deal with the estimation of acetylsalicylic acid in 1% v/v acetic acid in chloroform [50], estimation of salicylic acid in the presence of acetylsalicylic acid at pH 4.0 since acetylsalicylic acid does not fluoresce at this pH [52], simultaneous determination of acetylsalicylic acid and salicylic acid in solid phase [47] and estimation of salicylic acid in buffered acetylsalicylic acid products in formic acid chloroform solution [49]. Although both acetylsalicylic acid and salicylic acid may be simultaneously determined on the basis of the pH-dependent shift in their individual absorption spectra, such a shift results in a partial overlap of the absorptions of salicylic acid and acetylsalicylic acid and corrections may be required for these spectral interferences [75]. Chromatographic methods have also been used for the determination of acetylsalicylic acid but with these methods separation of the tablet additives (excipients and antacids) was reported to be essential [47]. In the GLC methods requiring chemical derivatisations of acetylsalicylic acid, separation is necessary to avoid interferences of additives with the chemical derivatisations. Estimation of acetylsalicylic acid as free acid by GLC is difficult because of the low vapour pressures of acetylsalicylic acid and the presence of polar functional groups, which cause absorption and tailing [47].

Masking of the functional groups by derivatisation makes these molecules much less polar, more volatile, and consequently, amenable to GLC analysis. However, acetylsalicylic acid samples, when analysed for salicylic acid as an impurity by GLC, show higher salicylic acid content than that obtained by spectrophotometric methods which is due to the generation of salicylic acid by the slight hydrolysis of acetylsalicylic acid during the methylation or other derivatisation processes. The most specific quantitative methods used to date are liquid chromatographic methods. With HPLC methods, insoluble additives should be removed completely to prevent the column from being blocked. The extraction procedures must be carried out with great care so as not to induce hydrolysis of acetylsalicylic acid to salicylic acid, especially when the tablets contain buffers or antacids [47]. HPLC methods using methanol and water may cause hydrolysis of acetylsalicylic acid [47]. In this section, the spectrofluorometric estimation of acetyl salicylic acid and dipyridamole in pure admixtures as well as tablet preparations has been described.

METHODS

Materials, Reagents and Equipment

1. *Chloroform - Spectroscopic grade (Spectrochem.India Ltd)*
2. *Glacial acetic acid - A.R. Grade (Glaxo Laboratories Ltd)*
3. *Pure drug samples of aspirin I.P. and dipyridamole U.S.P. were obtained as gifts.*

The fluorescence spectra were recorded with a Jasco FP-777 scanning spectrofluorometer using 1 cm cuvettes.

Standard and sample solutions

Four series of solutions (Series A-D) of acetylsalicylic acid and dipyridamole were prepared by using appropriate aliquots of stock

solutions of acetylsalicylic acid (1 mg/ml) and of dipyridamole (1 mg/ml) in chloroform. Series A comprised of solutions of acetylsalicylic acid of various concentrations (2-12 mcg/ml) in 1% v/v acetic acid in chloroform and series C comprised of solutions of dipyridamole of various concentrations (2-12 mcg /ml) in pure chloroform. Series B comprised of solutions of acetylsalicylic acid of varying concentration (2-12 mcg/ml) along with constant concentration of dipyridamole (6 mcg/ml) in 1% v/v acetic acid in chloroform and Series D was made up of solutions containing a varying concentration of dipyridamole (2-12 mcg/ml) and a constant concentration of acetylsalicylic acid (6mcg /ml) in pure chloroform (Table 7). In order to assess the non-interference of salicylic acid which may be present as an impurity is small quantities, another two series of solutions were prepared. The series E comprised of varying concentration of acetylsalicylic acid (2-12 mcg/ ml) and a constant concentration of salicylic acid (6 mcg/ml) in 1% v/v acetic acid in chloroform. The series F comprised of varying concentration of dipyridamole (2-12 mcg /ml) and a constant concentration of salicylic acid (6 mcg /ml) in pure chloroform.

Sample Preparation

Twenty tablets of each brand were finely ground and a weight of the powder equal to the average weight of a tablet was transfered to a 100/ml volumetric flask and dissolved in pure chloroform by thorough shaking, made up to volume and filtered (Whatman No. 1 filter paper). The first and last 5 ml of the filtrate were discarded. Appropriate volumes of aliquots of the filtrate were used to prepare sample solutions (using the solvents of 1% v/v acetic acid in spectroscopic grade chloroform for estimation of acetylsalicylic acid and pure spectroscopic grade chloroform for the estimation of dipyridamole) containing approximately 7 mcg/ml of dipyridamole and approximately 4.6 or 5.6 or 9 mcg/ml of acetylsalicylic acid. The solutions of pure drugs, their admixtures and the tablet sample

solutions were scanned in a Jasco FP-777 scanning spectrofluorometer. The results of the scan have been presented in tables 7-10 and figures 7 and 8.

Results and Discussion

Aspirin has been reported to possess both fluorescence and phosphorescence [51]. Its hydrolytic product salicylic acid is more fluorescent than acetylsalicylic acid although its wavelength of excitation and emission maxima (310 nm and 455 nm respectively) were different from that of acetylsalicylic acid . Thus far, acetylsalicylic acid in combined formulations has been quantified by fluorometry after its quantitative conversion to salicylic acid [48,51] as well as in the presence of other components as acetylsalicylic acid itself [47,50,75] and the estimation of acetylsalicylic acid by phosphorimetry requires liquid nitrogen temperatures [51]. The objective of the present work was to estimate acetylsalicylic acid as acetylsalicylic acid itself (in the presence of dipyridamole) and vice versa and not as salicylic acid so that the method may be used to estimate acetylsalicylic acid without conversion to salicylic acid in the presence of dipyridamole. Although acetylsalicylic acid fluoresces both in pure chloroform as well as in 1% v/v acetic acid in spectroquality chloroform, the later has been used as a solvent for the estimation since the hydrolysis of acetylsalicylic acid to salicylic acid is very less in this solvent [51]. In addition, acetylsalicylic acid has been reported to fluoresce weakly in chloroform alone but the presence of 1% acetic acid, chloroacetic acid or dichloroacetic acid greatly enhances the emission in proportion to the strength of these acids [50]. Since strong acids can hydrolyse acetylsalicylic acid to acetic and salicylic acids, aliphatic carboxylic acids were reported to be better for enhancing the emission of otherwise weakly fluorescent acetylsalicylic acid [50]. Dipyridamole also shows fluorescence in both pure as well as 1 % v/v acetic acid in chloroform solvent but the rectilinear response is found only in the former

Table 7. Selectivity of the Method for the Determination of Aspirin in the Presence of Dipyridamole by Spectrofluorometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] fluorescence λ_{ex} = 246nm λ_{em} = 346nm	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
ASP	DIP					Crit	Calc
2	0	261.60 ± 7.09	2.71	2.24	98.01	3.63	2.40
4	0	529.20 ± 7.31	1.38	2.31	98.62	3.63	2.21
6	0	769.90 ± 7.72	1.00	2.44	102.27	3.63	2.02
8	0	1047.60 ± 7.27	0.69	2.29	100.50	3.63	2.28
10	0	1326.30 ± 8.77	0.66	2.77	99.40	3.63	1.51
12	0	1586.20 ± 7.60	0.48	2.40	99.85	3.63	2.09
2	7	259.30 ± 6.73	2.59	2.13	98.23	3.63	2.19
4	7	528.60 ± 8.37	1.58	2.64	98.65	3.63	1.42
6	7	773.20 ± 6.16	0.79	1.95	101.95	3.63	2.63
8	7	1048.60 ± 8.14	0.78	2.58	100.61	3.63	1.50
10	7	1328.80 ± 8.09	0.61	2.56	99.48	3.63	1.52
12	7	1591.60 ± 7.39	0.46	2.33	99.81	3.63	1.82

DIP - Dipyridamole ASP - Aspirin

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical}$ = F (4,9) values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 8. Selectivity of the Method for the Determination of Dipyridamole in the Presence of Aspirin by Spectrofluorometry

Composition of the solution (mcg ml^{-1}) ASP	DIP	Mean value of [a] fluorescence λ_{ex} = 420nm λ_{em} = 475nm	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
0	2	940.20 ± 5.72	0.61	1.81	100.65	3.63	2.77
0	4	1887.70 ± 5.62	0.29	1.77	99.79	3.63	2.87
0	6	2817.90 ± 5.96	0.21	1.88	100.11	3.63	2.55
0	8	3773.90 ± 8.64	0.23	2.73	99.59	3.63	2.22
0	10	4888.90 ± 5.66	0.12	1.78	100.15	3.63	2.83
0	12	5629.80 ± 5.66	0.10	1.79	100.05	3.63	2.83
7	2	939.80 ± 6.13	0.65	1.93	100.72	3.63	2.24
7	4	1888.20 ± 6.11	0.32	1.93	99.77	3.63	2.26
7	6	2819.00 ± 6.73	0.24	2.13	100.07	3.63	1.86
7	8	3774.20 ± 8.00	0.22	2.52	99.59	3.63	1.82
7	10	4688.00 ± 5.66	0.12	1.79	100.17	3.63	2.64
7	12	5630.30 ± 6.25	0.11	1.98	100.05	3.63	2.16

DIP - Dipyridamole ASP - Aspirin

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

solvent. Hence acetylsalicylic acid was estimated in 1% v/v acetic acid in chloroform. The emission spectra of pure acetylsalicylic acid and pure dipyridamole are given in figures 7 and 8 and the excitation spectra of the drugs in their respective solvents have been shown in figure 9.

The excitation spectra in figure 9 indicated that the chosen excitation wavelengths were highly specific for the particular drug. Thus, in an admixture of acetylsalicylic acid and dipyridamole, when acetylsalicylic acid was estimated by excitation at 246 nm (and emission at 346 nm), the dipyridamole did not interfere. Similarly, during the estimation of dipyridamole by excitation at 420 nm and emission at 475 nm, acetylsalicylic acid did not interfere. The wavelength of 246 nm rather than 285 nm was chosen for the excitation of acetylsalicylic acid since salicylic acid does not get excited at 246 nm (figure 10) whereas a solution of pure salicylic acid in 1% v/v acetic acid in chloroform solvent, when excited at 285 nm, showed a weak fluorescence at 346 nm (where the fluorescence of acetylsalicylic acid was being measured). Hence traces of salicylic acid, if present as impurity in acetylsalicylic acid, would not interfere with the emission of acetylsalicylic acid, if acetylsalicylic acid is estimated by excitation at 246 nm. Similarly, salicylic acid does not get excited at 420 nm (figure 10) and hence would not interfere with the estimation of dipyridamole when it is estimated by excitation at 420 nm and emission at 475 nm.

The results of statistical analysis of the spectral data have been presented in tables 7-10. The small standard deviation values indicate the high level of precision of the proposed method as well as the independence of the fluorescence emission measurement of the drugs in the presence of each other. The negligible intercepts of the regression equations indicated the regression through or close to the origin. The small standard deviation values (Table 7 and 8) indicated a high level of precision of estimation. The similarity between the equations (Table 9) for pure drugs and their admixtures (pure and with salicylic acid) as well as the correlation coefficient

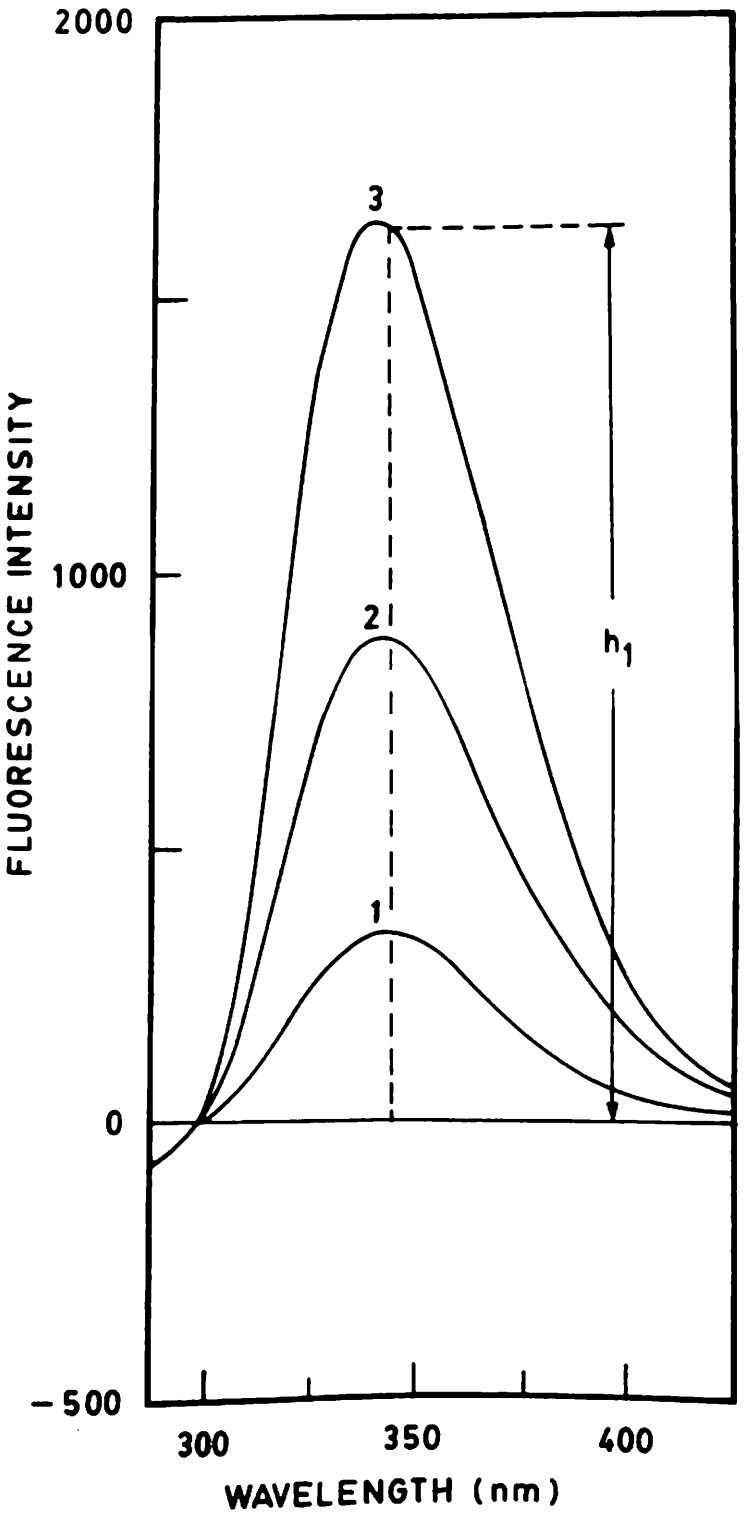


Fig 7. Fluorescence emission spectra of aspirin; excitation wavelength was 246 nm; emission spectra recorded for solutions of aspirin in 1% v/v acetic acid in spectral grade chloroform solvent; concentration of aspirin was 2, 6 and 12 mcg ml^{-1} in curves 1, 2 and 3 respectively

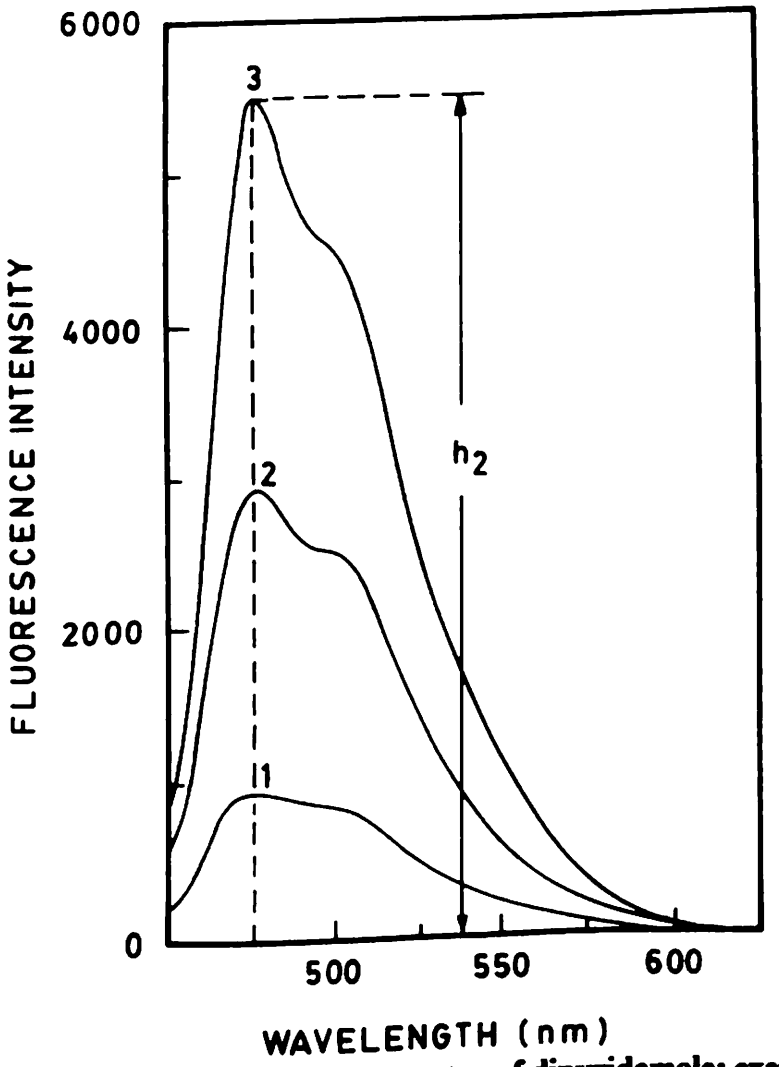


Fig 8. Fluorescence emission spectra of dipyridamole; excitation wavelength was 420 nm; emission spectra recorded for solutions dipyridamole in spectral grade chloroform solvent; concentration of dipyridamole was 2, 6 and 12 mcg ml^{-1} in curves 1, 2 and 3 respectively

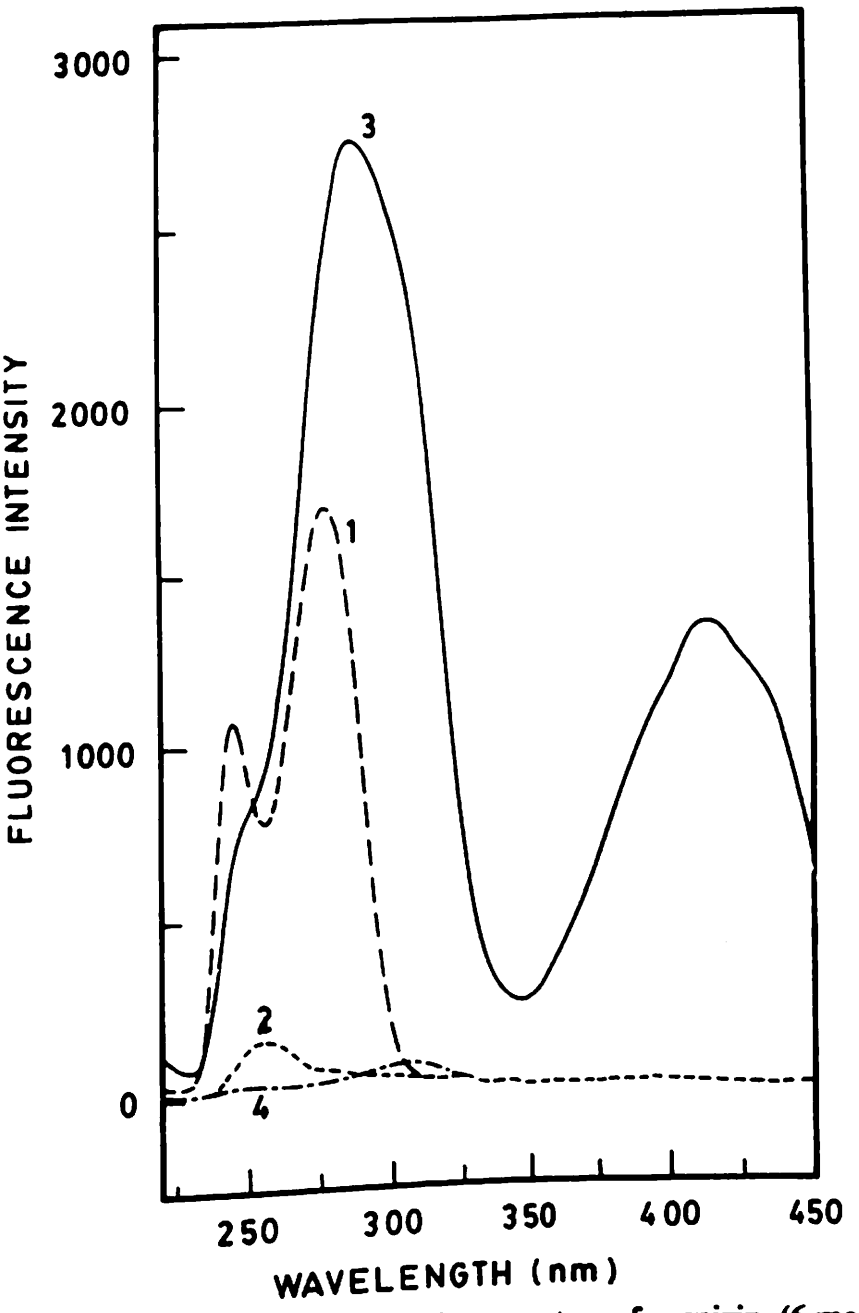


Fig 9. Fluorescence excitation spectra of aspirin (6 mcg ml^{-1}) in 1% v/v acetic acid in spectral grade chloroform with emission at 346 nm (curve 1) and 475 nm (curve 2); excitation spectra of dipyridamole (6 mcg/ml) in pure spectral grade chloroform with emission as 475 nm (curve 3) and 346 nm (curve 4)

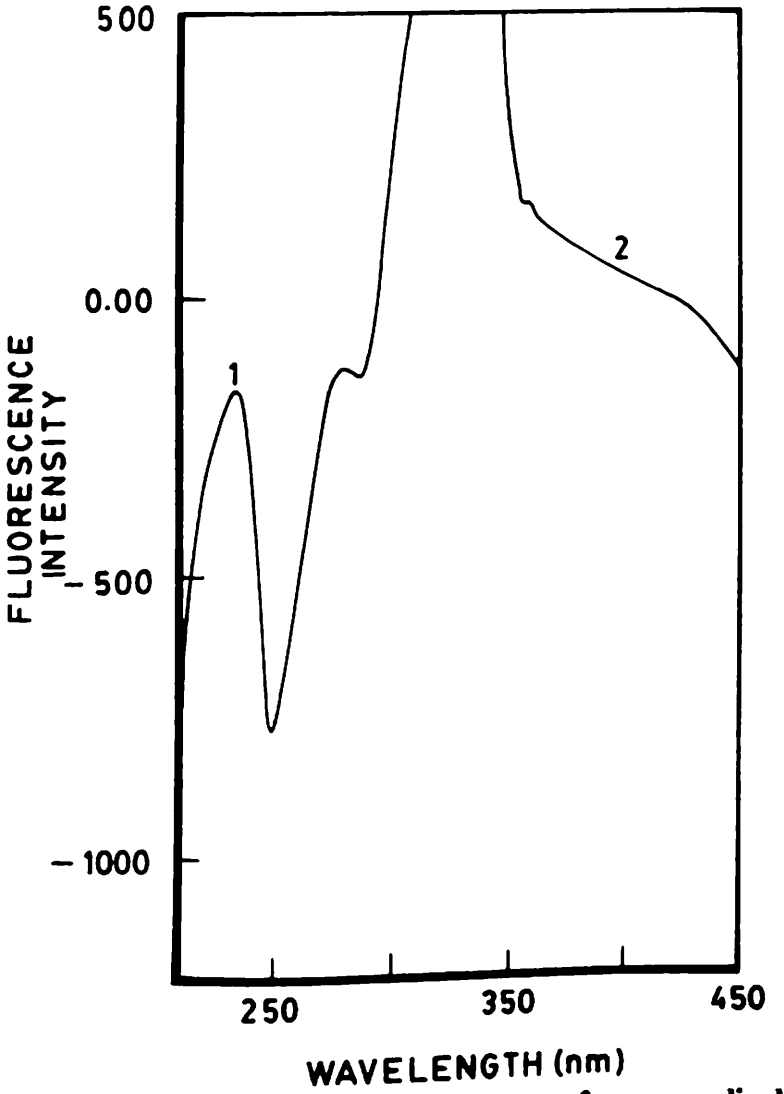


Fig 10. Fluorescence excitation spectra of pure salicylic acid (6 mcg ml^{-1}); Curve 1 is spectrum in 1% v/v acetic acid in spectral grade chloroform solvent with emission at 346 nm and curve 2 is spectrum in pure spectral grade chloroform solvent with emission at 475 nm

values in the range of 0.9998 - 0.9999 indicated the non-interference of one drug in the fluorescence emission measurement of the other at the selected wavelengths of emission for estimation.

The percentage ratio of the residuals in tables 7 and 8 clearly indicated a random scatter in case of both pure drug solutions as well as their admixtures. In addition, the F test for non-linearity which is a quantitative test for non-linearity [4] was done and the results have been presented in tables 7 and 8. With this test, we would have an evidence of non-linearity if we can show that Sy (standard error of estimate) has too large a value to be compatible with the sample estimate (Ss). The values in tables 7 and 8 show that the calculated F values were less than that of critical values at 5% level of significance and evidence the linear relationship. Similarly, the other F test results which was based on mean square due to regression and mean square about the regression clearly showed the non-linearity since the calculated F values were far larger than the critical values leading to rejection of null hypothesis (Table 9).

The co-efficient of determination (which is ratio of the sum of squares due to regression to the sum of squares about the mean) values ranged from 99.96 to 99.99 indicating that this much of variation in the absorbance was accounted for by the concentration of the drug in the solutions. A comparison of T test values at a significance level of 5% showed that the calculated values were far larger than the critical values obtained from the t table and confirmed the existence of strong positive correlation [5]. The standard error of slope and intercept are the standard deviation values of slope and intercept and the standard error of estimate (which is the standard deviation value of residuals of y on x line and which is an indicator of the precision of the fit by regression) values for the various solutions. This standard error of estimate was very less relative to the typical change in

Table 9. Regression Analysis of Aspirin and Dipyridamole Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})			Regression Equation [a] (λ_{em} = 346nm for aspirin and λ_{em} = 475 nm for dipyridamole)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	ASP	DIP	SAL				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	2-12	0	0	y = 132.74x - 9.07	0.9998	99.96	7.71	10217	2.77	101	1.31	10.22	10.98
Series B	2-12	7	0	y = 133.39x - 12.06	0.9998	99.96	7.71	12522	2.77	111	1.19	9.28	9.97
Series C	0	2-12	0	y = 468.68x + 8.97	0.9999	99.99	7.71	16482	2.77	406	1.15	8.99	9.51
Series D	7	2-12	0	y = 468.67x + 9.21	0.9999	99.99	7.71	15947	2.77	399	1.17	9.13	9.80
Series E	2-12	0	6	y= 132.66x - 8.24	0.9998	99.95	7.71	10534	2.77	96	1.26	9.45	9.24
Series F	0	2-12	6	y= 467.92x + 9.11	0.9999	99.97	7.71	14326	2.77	402	1.15	9.04	9.52

ASP- Aspirin DIP - Dipyridamole SAL- Salicylic acid [a] Based on six calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 6 for aspirin as well as dipyridamole

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is six for both aspirin as well as dipyridamole

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *fluorescence* and *x* is the *concentration*

fluorescence value from point to point in the calibration curve (Tables 7, 8 and 9)based on the regression equations for pure admixtures [5].

The table 10 gives the actual values of the pure aspirin (7 mcg/ml) and dipyridamole (7 mcg/ml) as well as the value calculated from the regression line for admixtures. The standard error of prediction is also given in this table.The 95% confidence level concentration ranges had been presented in table 10 (calculated using the standard error of prediction values).

The assay results of commercial formulations have also been given in table 6. In the case of aspirin, the estimation was done using a solution of the tablet sample containing approximately 7 mcg/ml of aspirin for all brands and the dipyridamole was also estimated by using solutions containing approximately 7 mcg/ml. This was done to calculate the 95% confidence level concentration ranges in the last column at concentrations where the standard error of prediction will be minimal. As can be seen from tables 3 and 4, the mean points of the calibration data for aspirin and dipyridamole lie around 7 mcg/ml and since the linear regression fit is most precisely determined at the mean point of the calibration data, the sample solutions were prepared so as to have the drugs in this concentration. For comparison of the 95% confidence level ranges, the dipyridamole has also been determined at the appropriate concentrations corresponding to a concentration of 7 mcg/ml of aspirin in the various Brands (Table 10) and aspirin at the appropriate concentrations corresponding to a concentration of 7 mcg/ml of dipyridamole.This was done because as can be seen from table 10, the proportion in which aspirin and dipyridamole are present in commercial formulations would not allow a single solution to be used for the estimation of both aspirin and dipyridamole since one of them cannot be estimated near its mean point of the calibration range. The 95% confidence level ranges of concentration predicted from a regression equation uses the standard error of prediction which is minimal at the

Table 10. Results of the Assay of Aspirin and Dipyridamole in Pure Drug Admixtures and Commercial Formulations by Spectrofluorometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	ASP	DIP	ASP	DIP	ASP	DIP	ASP	DIP
Pure Drug Admixture	7.00	7.00	—	—	98.71	99.71	6.80-7.02	6.95-7.01
Brand A	7.00	3.70	40	75	98.58	100.10	6.81-6.99	3.66-3.74
Brand B	7.00	5.60	60	75	98.74	99.98	6.80-7.02	5.55-5.62
Brand C	7.00	9.30	100	75	98.97	99.59	6.83-7.00	9.23-9.29
Brand A	11.35	6.00	40	75	98.85	99.66	11.07-11.37	5.95-6.01
Brand B	8.75	7.00	60	75	98.33	99.28	8.49-8.71	6.72-6.93
Brand C	5.20	7.00	100	75	98.85	99.57	5.00-5.23	6.94-6.99

ASP - Aspirin DIP - Dipyridamole

[a] Average of ten determinations; assay as percentage of actual concentration / label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 4 degrees of freedom for aspirin as well as dipyridamole

mean point of calibration [5]. In case of Brand A, the concentration of the dipyridamole solution has been 6 mcg/ml instead of 7 mcg/ml since the value of aspirin concentration goes above 12 mcg/ml at this concentration of dipyridamole whereas 12 mcg/ml is the highest concentration of aspirin used for the calibration curve of aspirin. The detection limits at 5% level of significance were found to be 0.25 and 0.07 mcg /ml respectively.

The assay results in table 10 show that the aspirin and dipyridamole contents in the different commercial formulations conform to the monographical requirements of I.P. 1985 (95 to 105 % of the stated amount of aspirin) as well as U.S.P. 1995 (90 to 110 % of stated amount for aspirin and dipyridamole) in single component tablets. Thus the proposed method of determination of aspirin and dipyridamole by spectrofluorometry was found to be accurate and would be very useful for the determination of aspirin and dipyridamole simultaneously without separation from each other. The method also does not require the conversion of aspirin to salicylic acid and hence can be used for the estimation of aspirin as acetylsalicylic acid itself in the presence of dipyridamole in commercial formulations.

4.00 Ultra-violet Spectrophotometry

4.01 INTRODUCTION - Difference Spectrophotometry

The electronic absorption spectrophotometry is perhaps the most widely used technique both officially and non-officially as the measurement step for the quantitative assay of drugs in dosage forms [76] The quantitation of single drugs in pure as well as dosage forms may be done rapidly if the Beer's Law is complied with and if the excipients do not absorb appreciably in the wavelenghts at which the absorbance is measured. Once the absorptivity of the drug at a specific wavelength has been determined, the quantitation of the unknown sample may be done. A simplified version of these methods uses a "one point" concomitant measurement of the sample solution and a solution prepared with a reference standard [74]. This standard is the same compound as the sample, but is a specimen whose purity is known. The standard and sample are carried through exactly the same procedure and Beer's Law is written for each solution. Thus,

$$A^s = a\,b\,c^s$$
$$A^r = a\,b\,c^r$$

where the superscript s refers to the sample and r to the reference standard.

Dividing these equations and solving for the unknown, C^s, we obtain

$$C^s = \frac{C^r\,A^s}{A^r}$$

Spectrophotometric assay procedure in the monographs [1-3] usually specify this method of analysis, and the final equation may include a numerical factor accounting for dilution of sample.

In the case of systems which contain two or more substances in a transparent solvent each of which absorbs light, the problem becomes complex. If the absorption spectra of the two components are so different that two wavelengths can be found at which each substance absorbs light without interference from each other, the problem reduces to that of single component analysis since neither componenet interfaces with the analysis of the other [6] In a more general case, both solutions will absorb light at the same wavelengths, but if their absorption spectra are markedly different, the mixture can still be analysed in various ways.

One of the advantages of absorption spectrophotometry is its specificity or freedom from interference which holds good even when absorption bands overlap if one of the multicomponent analytical method is used. The possibility of interfering absorbers must always be kept in mind while designing spectrophotometric analysis. This is especially true in cases of analysis of drugs in dosage forms which usually present the problems of 'background absorption' by excipients, that is, the dosage from components other than the active drug. This background often consists of relatively featureless absorption that increases in intensity toward shorter wavelength. Several techniques have been devised to compensate for background absorption which includes the simultaneous equation method [29], absorbance ratio method [77], graphical method or linear plot method [78], orthogonal function method [79],three point correction method [29], difference spectrophotometric method [76] and derivative spectrophotometric method [80].

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 solutions should be identical in all respects except for the presence of the analyte.

In practice, however, when analysing pharmaceutical samples, there is always uncertainity concerning interfering materials which may have accompanied the sample and which remain uncompensated for by the reference solution. Difference spectrophotometry is a method of compensating for the presence of extraneous materials in a sample which would otherwise interfere with the spectrum of the drug being determined. It involves the measurement of the absorbance difference, at a defined wavelength, between two samples in one of which a physical or chemical property of the drug has been changed [76]. It is assumed that the spectrum of the drug can be changed without affecting the spectrum of the interfering material. Alternatively, the absorbance difference may be measured between the sample and an equivalent solution without the drug. Thus, difference spectrophotometry, in case of systems containing drugs as well as excipients uses an approximation of the ideal reference solution by employing an aliquot of the sample solution itself as reference, adjusted by change in pH or other parameter but containing both the substance being analysed and all extraneous substances at exactly the same concentrations as the sample. If the pH or other variation, causes an alteration in the spectrum of the sample, the instrument records this as a characteristic difference spectrum. If other materials present are unaffected by the change in conditions, their contribution to the total absorbance of each solutions will be identical and their effect will be exactly cancelled [76].

The technique of difference spectrophotometry has proved particularly useful in the determination of medicinal substances by eliminating specific interference from degradation products and co-formulated drugs and involves the reproducible alteration of the spectral properties of the analyte in equimolar solutions and the measurement of the absorbance difference between the two solutions provided the absorbances of the other absorbing interferents are not affected by the reagents used for the

spectral property alteration [76] Many suitable methods for physical and chemical modification of the drug absorbance have been reported [76]. Although reactions such as hydrolysis or formation of Schiff bases have been described for their use in difference spectrophotometry it is usual to use simple aqueous acids, alkalis and buffers frequently for inducing spectral alterations since many drugs are weak acids or bases whose state of ionisation and absorptivity depend on the pH of the solution .

It should, however, be established that the absorbance difference (δA) is a linear function of concentration over the range required. It is convenient to select for the analytical wavelength a value corresponding to a maximum in the difference spectrum, obtained by scanning the sample and reference solution over an appropriate wavelenth range [80].

Difference spectrophotometry can be used for quality control in cases where the interfering material is well-defined, because an appropriate dilution of a suitable reference solution can be used in the reference cell. The difference absorbance is, however, susceptible to systematic error when there is uncertainity in the concentration of interfering materials in the samples to be assayed. This error increases in proportion to the ratio of the molar absorptivity of the interference to that of the drug [80]

Many workers have use difference spectrophotometry as an analytical technique for assay of single or binary mixtures in pure as well as dosage forms and have reported the technique to be satisfactory for the assay of the drugs. The effect of excipients on the measurement of absorption by difference spectrophotometry has been discussed by various workers. The various parameters which ought to be considered while designing spectrophotometric method had been discussed [76] using the spectral shifts of benzthiazide, hydrochlorothiazide, triamterene and phenobarbitone, The difference spectra of these drugs were recorded with the acidic and

alkaline equimolar solutions of the drugs. Other methods of producing spectral shifts such as reduction of ring of corticosteroids with sodium borohydride, formation of Schiff's base by primary aromatic amine such as procaine as well as hydrolysis of pyridostigmine bromide by sodium hydroxide had also been discussed [76].

Chlordiazepoxide and demoxepam in pure and dosage forms have been assayed by using different buffers for each of the drug and using the bathochromic shift of chlordiazepoxide [81].

A method for the rapid determination of phenothiazine drugs in a wide variety of pharmaceutical preparations by using a difference spectrophotometric technique based upon the absorbance of sulphoxide derivative of the phenothiazine drugs relative to the absorbance of a solution of the underivated drug had also been described [82]. Non-interference of oxidative and photochemical decomposition products, coloring and flavouring agents, excipients and co-formulated drugs has been reported.

The quantitation of 1,2-diphenolic drugs in pharmaceutical formulations by producing a bathochromic shift using germanium-dioxide reagent and estimation of steroids with conjugated keto chromophores via lithium borohydride reduction have also been reported [83,84]. Drugs such as acetaminophen and chlorzoxazone [85], tyrosine and tryptophan [86], santonin [87], p-hydroxybenzoic acid in the presence of its esters [88] have been estimated by the technique of difference spectrophotometry.

Isosbestic points are indication of the cancellation of the spectral interference [76] and the existence of such isosbestic points i.e. the wavelengths at which the absorption of the solutions are equal and hence zero may be used to analyse mixtures without interference from each other. Such 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 the other drug and vice versa. An experimental design which would involve the measurement of absorption of the pure drugs as well as their mixtures at the isosbestic points involving mixtures in which the concentration of the drug whose isosbestic point at which the measurement is being made is kept constant and the concentration of the other drug to be quantified is varied would give a clear idea about the applicability of the method for the determination of the drugs in binary mixtures. The same type of experimental design has been used for the estimation of various drug combinations by zero-order difference spectrophotometry as well as derivative difference spectrophotometry.

This section describes the estimation of four drug combinations as pure admixtures and dosage forms by zero-order difference spectrophotometry.

4.02 SIMULTANEOUS QUANTITATIVE DETERMINATION OF TINIDAZOLE AND DILOXANIDE FUROATE IN TABLET PREPARATIONS BY DIFFERENCE SPECTROPHOTOMETRY

The combination of tinidazole and diloxanide furoate as a tablet preparation is being widely used for acute and chronic intestinal amoebiasis and hepatic amoebiasis. The literature reports for the estimation of tinidazole spectrophotometry [89-91], dc polarography for estimation in tablets [92], thin layer chromatography [93] and HPLC [94,95] and for estimation of diloxanide furoate spectrophotometry [96-97]. But thus far there have been no reports on the simultaneous estimation of tinidazole and diloxanide furoate by spectrophotometry. This section of the thesis comprises of details of the successful design of a zero-order difference spectrophotometric method for the estimation of these drugs in pure admixtures and in tablet preparations.

METHODS

Materials, Reagents and Apparatus

The methanol used was of spectroscopic grade. The spectra were recorded using a Jasco-7800 uv-visible scanning double beam spectrophotometer using 1cm matched cuvettes. The scan rate was set at 240nm/ min.

Standard Solutions

Appropriate aliquots of stock solutions of pure tinidazole (1mg/ml) and diloxanide furoate (1mg/ml) in methanol were used to prepare two series of equimolar solutions of each drug in 0.1M NaOH and 0.1M HCl containing 20-60 mcg/ml of tinidazole (series A) and 20-60 mcg/ml of diloxanide furoate

(series C). Similarly, two more series of equimolar solutions of mixtures of tinidazole and diloxanide furoate, the first containing a constant concen tration of 40 mcg/ml of diloxanide furoate and a varying concentration of 20-60 mcg/ml of tinidazole (series B) and a second containing a constant concentration of 40 mcg/ml of tinidazole and a varying concentration of 20-60 mcg ml of diloxanide furoate (series D) were prepared with 0.1M HCl and 0.1M NaOH (Tables 11-13).

Preparation of Sample Solutions

Twenty tablets of each brand were accurately weighed, powdered and a weight of the powder equivalent to the average weight of the tablet was transferred to a 100ml volumetric flask, dissolved in methanol by thorough shaking, diluted to volume and filtered using Whatman No. 1 filter paper. The first and last 5 ml of the filtrate were rejected. Appropriate volumes of the aliquots of the filtrate were diluted with 0.1M HCl and 0.1M NaOH to obtain equimolar solutions containing approximately the concentrations given in tables 11 and 12.

The normal absorbance as well as the absorbance difference of the acidic and basic equimolar drug solutions were recorded with a Jasco 7800 uv-visible double beam spectrophotometer using 10 mm matched cuvettes by placing the basic solution in the reference beam and acidic solution in the sample beam. The scan rate was set at 240 nm / min. The results of the scan have been presented in Tables 11-14 and the spectra in figures 11-13.

RESULTS AND DISCUSSION

The combination of tinidazole and diloxanide furoate in tablets requires some kind of simultaneous estimation since their absorption spectra interfere with each other (figure 11). As can be seen from the

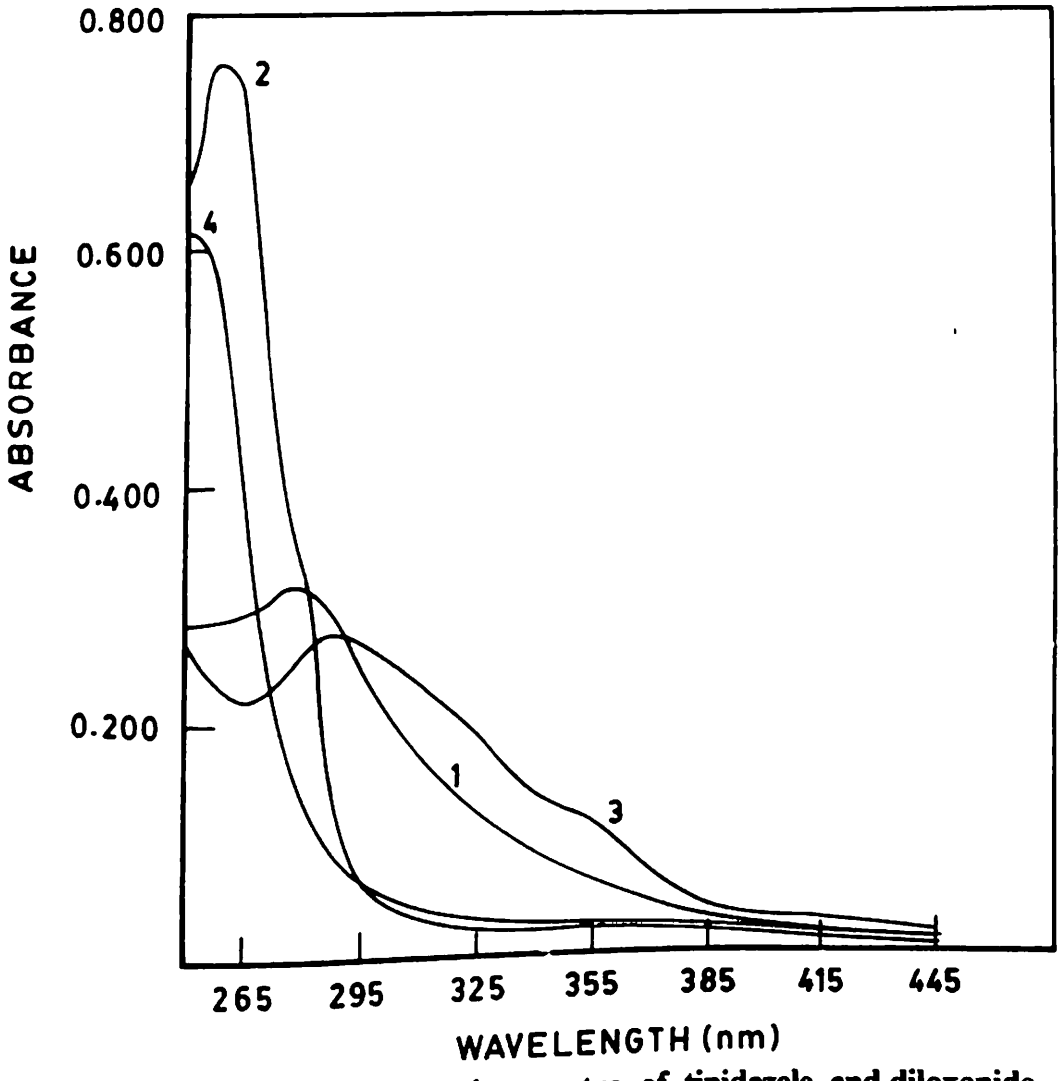


Fig 11. Normal absorption spectra of tinidazole and diloxanide furoate in 0.1M HCl and 0.1M NaOH; curves 1 & 2 are spectra of tinidazole (10 mcg ml^{-1}) and diloxanide furoate (10 mcg ml^{-1}) in 0.1M HCl respectively; curves 3 & 4 are spectra of tinidazole (10 mcg ml^{-1}) and diloxanide furoate (10 mcg ml^{-1}) respectively

figures, the diloxanide furoate absorbs strongly before 295nm in both acidic and basic solvents while the absorption of tinidazole is also appreciable in this range. Hence direct estimation of diloxanide furoate in the presence of tinidazole would not be possible although the absorption of tinidazole in the region between 355 to 305 nm may be useful in estimation of tinidazole in the presence of diloxanide furoate. Nevertheless, some amount of absorption was shown by diloxanide furoate in this range and this would lead to errors when direct estimation of tinidazole is done.

As mentioned in the introduction of this section, the difference absorbance of a compound can be readily related to concentration by prior calibration of the δA (absorbance difference) values so as to establish that δA is a linear function of concentration over the range required [80]. In this work, the δA values of standard solutions of tinidazole and diloxanide furoate were used to calculate regression equations (Tables 11-13). These equations were calculated using the data points obtained from drug solutions (Series A-D in Tables 11 and 12). The experimental design is such that the regression equations of pure individual drugs in solution (Series A and C) may be compared with that of their admixtures in which the concentration of the drug to be estimated is varied and that of the other is kept at a constant value (Series B and D in table 12). Such a comparison helps is easy determination of the interference of one drug in the estimation of the other at the chosen wavelength as well as the rectilinearity and precision of the method in the particular concentration range.

The figures 12 and 13 show the zero-order difference spectra of tinidazole and diloxanide fuorate respectively. The isosbestic point (i.e. the wavelength at which the absorption of the solutions are equal) of tinidazole occur at 285nm whereas diloxanide furoate did not exactly exhibit isosbestic point but the difference spectra touch the baseline above 312nm.

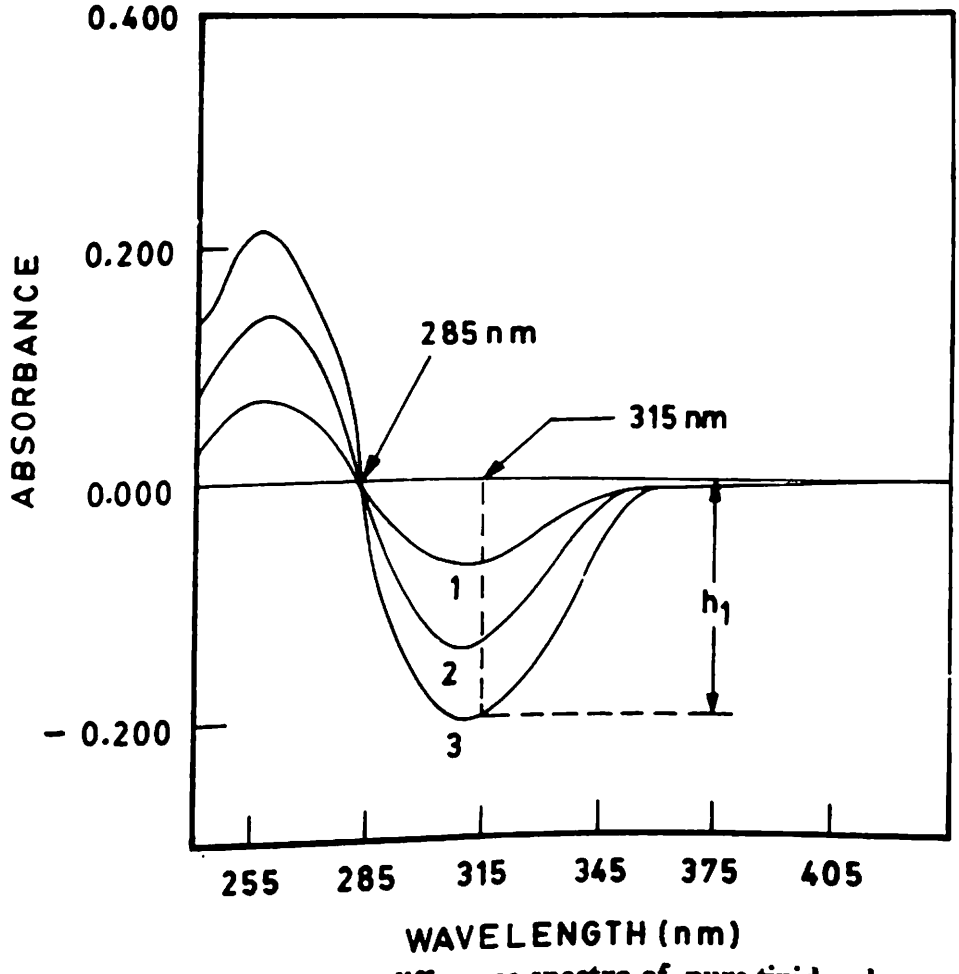


Fig 12. Zero-order difference spectra of pure tinidazole; spectra recorded by scanning equimolar solutions of tinidazole in 0.1M HCl and 0.1M NaOH;concentration of tinidazole tinidazole is 20, 40 and 60 mcg ml^{-1} in curves 1, 2 and 3 respectively

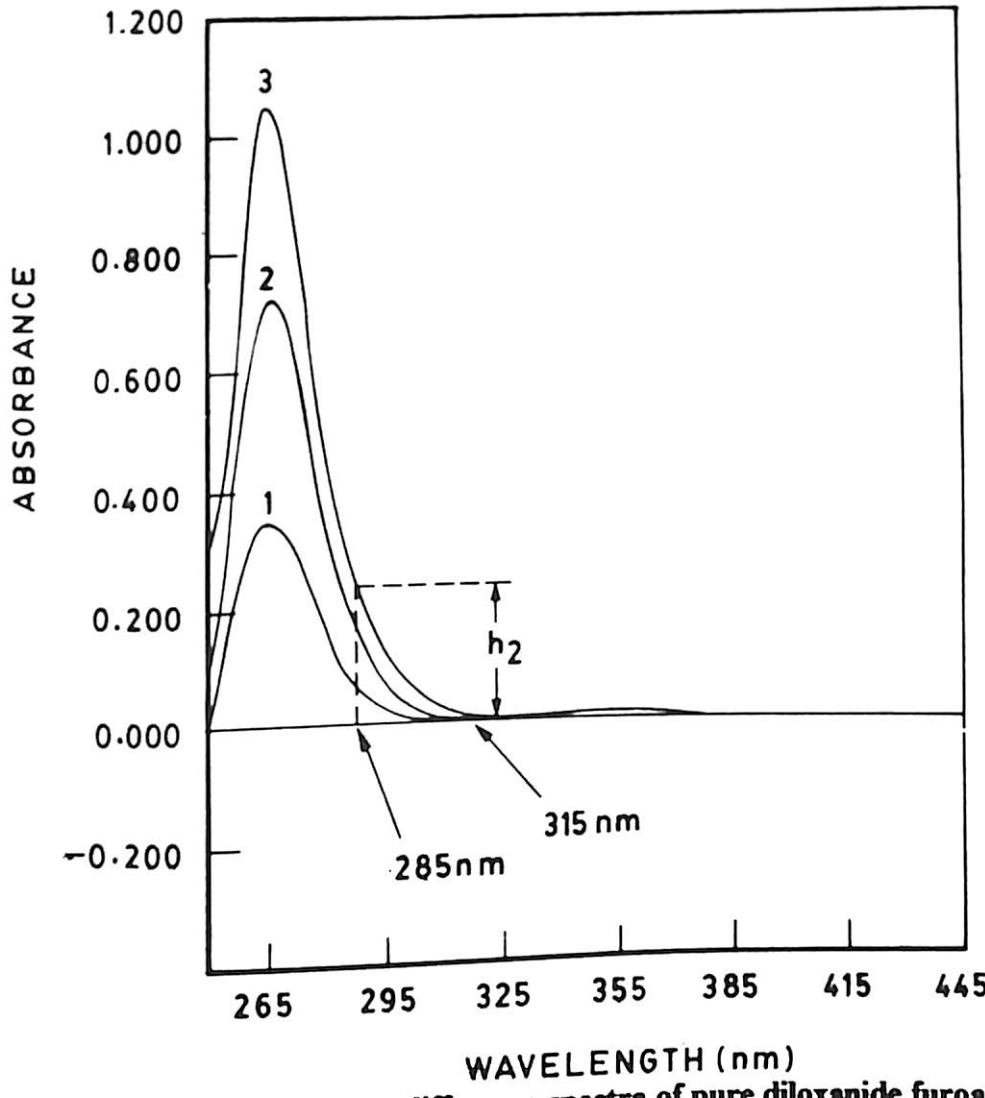


Fig 13. Zero-order difference spectra of pure diloxanide furoate; spectra recorded by scanning equimolar solutions of diloxanide furoate in 0.1M HCl and 0.1M NaOH; concentration of diloxanide furoate is 20, 40 and 60 mcg ml^{-1} in curves 1, 2 and 3 respectively

Using the amplitudes of the peak to baseline of the corresponding zero-order difference spectra, (h_1 and h_2 in figures 12 and 13) the drugs may be estimated in combinations without interference from each other. Thus, the amplitude of the spectra at 315 nm (figure 12) would be a function only of the concentration of tinidazole and the contribution of diloxanide furoate at this wavelength at this wavelength will be nil as can be seen from figure 13. Similarly, the amplitudes of the difference spectra of diloxanide furoate will be a function of only the concentrations of the drug in solutions at the wavelength of 285nm since the difference spectra of pure tinidazole shows isosbestic point at this wavelength indicating that the absorption contribution of tinidazole at this wavelength would be nil when the difference spectra of the admixture of tinidazole and diloxanide furoate is recorded. Thus the measurements made at these wavelengths would be a function only of concentration of one of the component.In case of tinidazole and diloxanide furoate combination, the heights of the zero-order difference spectra (δA) at the wavelengths of 315nm and 285nm were found to be proportional to the concentration of 20-60 mcg/ml.

The statistical analysis had been done on the data of pure drug solutions, their admixtures as well as commercial samples.

The small standard deviation values associated with the determination (Tables 11 and 12) indicated the high level of precision of the proposed method as well as the independence of one drug in the absorption measurement of the other. The negligible intercepts of the equations indicated regression through or close to the origin at the chosen wavelengths.

The coefficient of variation values (Table 11 and 12) were less and the standard error (which is the standard deviation of the mean) values were also less indicating the precision of the method.

Table 11. Selectivity of the Method for the Determination of Tinidazole in the Presence of Diloxanide fuorate by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) TIN	DIF	Mean value of [a] absorbance(δA) (315nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
20	0	0.0698 ± 0.0013	1.88	0.0004	99.26	3.86	0.59
30	0	0.1044 ± 0.0015	1.44	0.0005	100.37	3.86	0.44
40	0	0.1405 ± 0.0021	1.47	0.0007	99.86	3.86	0.25
50	0	0.1745 ± 0.0020	1.12	0.0006	100.75	3.86	0.26
60	0	0.2123 ± 0.0022	2.22	0.0007	99.54	3.86	0.21
20	40	0.0694 ± 0.0011	1.54	0.0003	99.14	3.86	1.44
30	40	0.1042 ± 0.0014	1.34	0.0004	100.39	3.86	0.75
40	40	0.1406 ± 0.0027	1.90	0.0008	99.87	3.86	0.20
50	40	0.1747 ± 0.0021	1.20	0.0007	100.88	3.86	0.33
60	40	0.2132 ± 0.0024	1.15	0.0008	99.46	3.86	0.25

TIN - Tinidazole DIF - Diloxanide furoate

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical}$ = F (3,9) values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 12. Selectivity of the Method for the Determination of Diloxanide fuorate in the Presence of Tinidazole by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) TIN	DIF	Mean value of [a] absorbance (δA) (285nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
20	0	0.0754 ± 0.0016	2.09	0.0005	98.46	3.86	1.13
30	0	0.1110 ± 0.0015	1.41	0.0005	99.99	3.86	1.19
40	0	0.1458 ± 0.0021	1.47	0.0007	101.74	3.86	0.66
50	0	0.1858 ± 0.0018	0.98	0.0006	99.78	3.86	0.89
60	0	0.2234 ± 0.0031	1.42	0.0010	99.57	3.86	0.29
20	40	0.0755 ± 0.0018	2.36	0.0006	98.12	3.86	1.23
30	40	0.1117 ± 0.0016	1.41	0.0005	99.96	3.86	1.64
40	40	0.1455 ± 0.0021	1.42	0.0007	102.02	3.86	0.91
50	40	0.1848 ± 0.0021	1.11	0.0007	99.96	3.86	0.91
60	40	0.2242 ± 0.0029	1.29	0.0009	99.38	3.86	0.51

TIN - Tinidazole DIF - Diloxanide furoate

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(3,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 13. Regression Analysis of Tinidazole and Diloxanide furoate Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (315 nm for TIN and 285 nm for DIF)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significanc [d] of Evidence of Correlation		Standard Error [e]		
	TIN	DIF				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	20-60	0	y = 0.0035x - 0.0016	0.9998	99.98	10.13	13950	3.18	118	0.0001	0.0013	0.0010
Series B	20-60	40	y= 0.0036x - 0.0022	0.9998	99.96	10.13	8825	3.18	93	0.0001	0.0016	0.0012
Series C	0	20-60	y = 0.0037x - 0.0002	0.9997	99.95	10.13	6192	3.18	78	0.0001	0.0020	0.0017
Series D	40	20-60	y = 0.0037x - 0.0002	0.9995	99.91	10.13	3720	3.18	61	0.0001	0.0026	0.0020

TIN - Tinidazole DIF - Diloxanide furoate [a] Based on five calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 5 for tinidazole as well as diloxanide furoate

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 5 for both tinidazole as well as diloxanide furoate

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance(δA)* and *x* is the *concentration*

The percentage ratio of the residuals in tables 10 and 11 clearly indicated a random scatter in case of both pure drug solutions as well as their admixtures. In addition, the F test for non-linearity which is a quantitative test for non-linearity [4] was done and the results have been presented in tables 10 and 11. If a linear relationship holds, the standard deviation of the residuals (standard error of estimate, Sy, in tables 10 and 11) would represent an estimate (with (n-2) degrees of freedom) of the standard deviation of the sample. Therefore, we would have an evidence of non-linearity if we can show that Sy has too large a value to be compatible with the sample estimate (Ss).The values in tables 10 and 11 show that the calculated F values were less than that of critical values at 5% level of significance and evidence the linear relationship. Similarly, the other F test results which was based on mean square due to regression and mean square about the regression clearly showed the non-linearity since the calculated F values were far larger than the critical values leading to rejection of null hypothesis (Table 13).

The regression equations of the pure drug solutions and those of admixtures (Table 13) were similar. This similarity as well as the correlation coefficient values in the range of 0.9995 to 0.9998 indicated the non-interference of one drug in the estimation of the other. The co-efficient of determination (which is ratio of the sum of squares due to regression to the sum of squares about the mean) values ranged from 99.91 to 99.98 indicating that this much of variation in the absorbance is accounted for by the concentration of the particular drug in the solutions. A comparison of T test values at a significance level of 5% showed that the calculated values are far larger than the critical values obtained from the t table and confirmed the existence of strong positive correlation [5]. The standard error of slope and intercept are the standard deviation values of slope and intercept and the standard error of estimate (which is the standard deviation value of residuals of y on x line

and which is an indicator of the precision of the fit by regression. This standard error of estimate was less relative to the typical change in δA value from point to point in the calibration curve based on the regression equations for pure admixtures [5].

The table 14 gives the actual values of the pure tinidazole and diloxanide furoate as well as the value calculated from the regression line for admixtures. The standard error of prediction is also given in this table.The 95% confidence level concentration ranges presented in table 14 (calculated using the standard error of prediction values) show a narrow range. The assay results of commercial formulations have also been given in table 14. In the case of tinidazole as well as diloxanide furoate the estimation will be best at the mean point of the calibration which is 40 mcg/ml. The estimation of tinidazole was done using a solution of the tablet sample containing approximately 42-45 mcg/ml of tinidazole for all brands and the diloxanide furoate was estimated using solutions containing approximately 37.5 mcg/ml. This was done to calculate the 95% confidence level concentration ranges in the last column at concentrations where the standard error of prediction will be minimal. For comparison of the 95% confidence level ranges, the drugs were also determined at concentrations other than those mentioned above depending on the ratio in which they are present in the commercial formulations. The 95% confidence level ranges of concentration predicted from a regression equation uses the standard error of prediction which is minimal at the mean point of calibration [5]. The detection limits at 5% level of significance were found to be 1.10 and 1.60 mcg/ml for tinidazole and diloxanide furoate respectively.

Table 14. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Tinidazole and Diloxanide furoate by Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	TIN	DIF	TIN	DIF	TIN	DIF	TIN	DIF
Pure Drug Admixture	40.00	37.50	—	—	99.47	99.23	98.89-100.05	98.26-100.19
Brand A	42.00	52.50	600	750	98.33	99.06	97.74 - 98.91	97.88-100.23
Brand B	45.00	37.50	300	250	98.97	99.38	98.37 - 99.56	99.02-99.74
Brand C	45.00	37.50	300	250	98.73	99.12	98.13 - 99.32	98.76-99.48
Brand D	45.00	37.50	300	250	99.62	99.81	99.02 -100.21	99.45-100.17
Brand A	30.00	37.50	600	750	98.56	98.50	97.88 - 99.24	98.13-98.86

TIN- Tinidazole DIF - Diloxanide furoate

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 3 degrees of freedom for tinidazole as well as diloxanide furoate

The stability of the standard solutions of tinidazole (40 mcg/ml) and diloxanide furoate (40 mcg/ml) in 0.1M HCl and 0.1M NaOH (stored prior to scanning in low actinic flasks at 28-32°C) were monitored spectrophotometrically (at the wavelengths of 315nm for tinidazole and 285nm for diloxanide furoate) for a period of two hours and were found to vary by the following absorbance units (AU): 0.1N HCl solution of tinidazole and diloxanide furoate by± 0.003 and ± 0.006 and 0.1M NaOH solutions of tinidazole by ±0.005 and ±0.004 respectively.

The assay results show a range of 98.23 - 99.81 percent of stated amount of diloxanide furoate which conforms to the I.P. 1985 requirements for diloxanide furoate tablets. Similarly, the results for tinidazole tablets (98.33 to 98.62% of stated amount) also conform to the I.P. 1985 requirement.

Thus the proposed method of determination of tinidazole and diloxanide furoate was found to be accurate and precise and may be used for the estimation of the drugs in commercial formulations.

4,03 SIMULTANEOUS DETERMINATION OF METRONIDAZOLE AND DI-IODOHYDROXYQUINOLINE IN TABLET PREPARATIONS BY DIFFERENCE SPECTROPHOTOMETRY

The combination of metronidazole and di-iodohydroxyquinoline as a tablet preparation is being widely used for amoebiasis and giardiasis. The literature reports for the estimation of metronidazole include [98-103], dc polarography and voltametry [104,105] high performance thin layer chromatography [106], gas chromatography [107] and HPLC [108-110] and for di-iodohydroxyquinoline non-aqueous titrimetry [13] and spectrophotometry [14-18]. The official procedures for the estimation of metronidazole are HPLC [1] and non-aqueous titrimetry [3] and for di-iodohydroxyquinoline oxygen flask method [1,3].This section of the thesis comprises of details of the successful design of a zero-order difference spectrophotometric method for the estimation of metronidazole and di-iodohydroxyquinoline in pure admixtures and in tablet preparations.

METHODS

Materials, Reagents and Apparatus

The dimethylformamide used was of spectroscopic grade. The spectra were recorded using a Jasco-7800 uv-visible scanning double beam spectrophotometer using 1cm matched cuvettes. The scan rate was set at 240nm/ min.

Standard Solutions

Appropriate aliquots of stock solutions of pure metronidazole (1mg/ml) and di-iodohydroxyquinoline (1mg/ml) in dimethylformamide were used to prepare two series of equimolar solutions of each drug in 0.1M NaOH and 0.1M HCl containing 10-50 mcg/ml of metronidazole (series A) and 5-15 mcg/ml of di-iodohydroxyquinoline (series C). Similarly, two more series of equimolar solutions of mixtures of metronidazole and di-iodohydroxyquinoline, the first containing a constant concentration of 10mcg/ml of di-iodohydroxyquinoline and a varying concentration of 10-50 mcg/ml of metronidazole (series B) and a second containing a constant concentration of 30 mcg/ml of metronidazole and a varying concentration of 5-15mcg/ml of di-iodohydroxyquinoline (series D) were prepared with 0.1M HCl and 0.1M NaOH (Tables 15,16).

Preparation of Sample Solutions

Twenty tablets of each brand were accurately weighed, powdered and a weight of the powder equivalent to the 100mg of di-iodohydroxyquinoline was transferred to a 100ml volumetric flask, dissolved in dimethylformamide by thorough shaking, diluted to volume and filtered using Whatman No. 1 filter paper. The first and last 5 ml of the filtrate were rejected. Appropriate volumes of the aliquots of the filtrate were diluted with 0.1M HCl and 0.1M NaOH to obtain equimolar solutions containing approximately the concentrations given in table 17.

The normal absorbance as well as the absorbance difference of the acidic and basic equimolar drug solutions were recorded with a Jasco 7800 uv-visible double beam spectrophotometer using 10 mm matched cuvettes by placing the basic solution in the reference beam and acidic solution in the sample

beam. The scan rate was set at 240 nm / min. The results of the scan have been presented in Tables 15 -17 and the spectra in figures 14 and 15.

RESULTS AND DISCUSSION

The combination of metronidazole and di-iodohydroxyquinoline in tablets had been estimated simultaneously as well as individually. Di-iodohydroxyquinoline in tablets had been estimated by colorimetry by using sodium nitrite with ammonia to produce yellow color [18] and by forming colored complex with vandal sulphate, extracting the complex with cyclohexane and measuring the absorbance at 415nm [15]. Metronidazole had been estimated in the presence of di-iodohydroxyquinoline by forming a colour complex with sodium nitroprusside and the absorbance was measured at 660nm [100].

As mentioned in the introduction of this section, the difference absorbance of a compound can be readily related to concentration by prior calibration of the δA (absorbance difference) values so as to establish that δA is a linear function of concentration over the range required [80] and is likely to minimise the interference due to excipients by cancellation of their absorbance during the recording of the difference spectrum. The δA values of standard solutions of metronidazole and diloxanide furoate were used to calculate regression equations (Tables 15 and 16). These equations were calculated using the data points obtained from drug solutions (Series A-D in Table 17). The experimental design is such that the regression equations of pure individual drugs in solution (Series A and C) may be compared with that of their admixtures in which the concentration of the drug to be estimated is varied and that of the other is kept at a constant value (Series B and D in table 17). This helps in easy determination of the interference of one drug in the estimation of the other at the chosen wavelength as well as in establishing the rectilinearity and precision of the method in the particular concentration range.

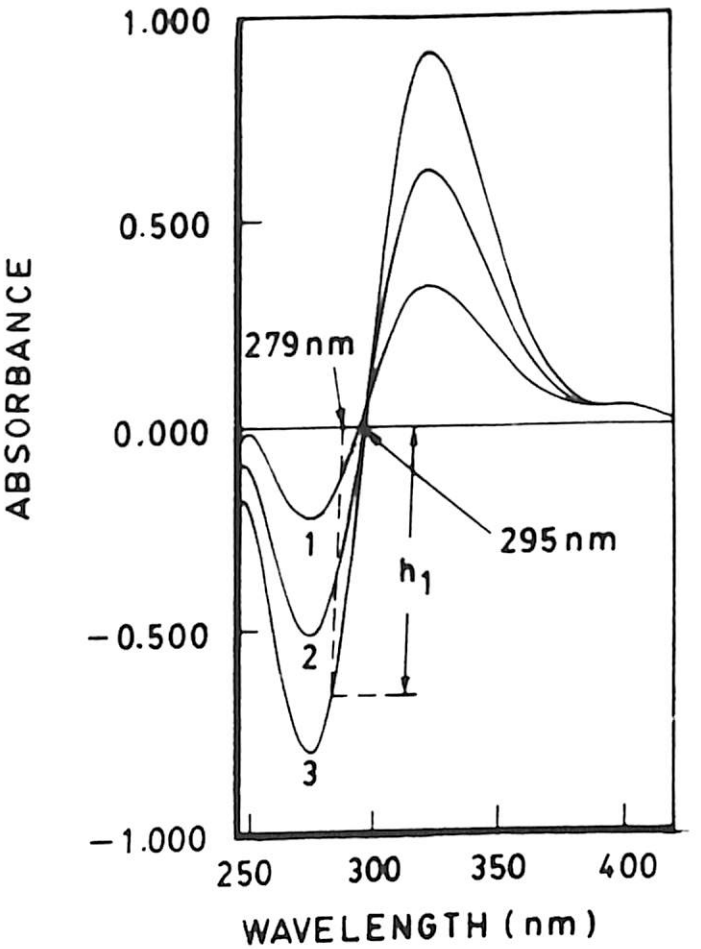


Fig 14. Zero-order difference spectra of pure metronidazole; spectra recorded by scanning equimolar solutions of metronidazole in 0.1M HCl and 0.1M NaOH; concentration of metronidazole is 10,30 and 50 mcg ml^{-1} in curves 1, 2 and 3 respectively

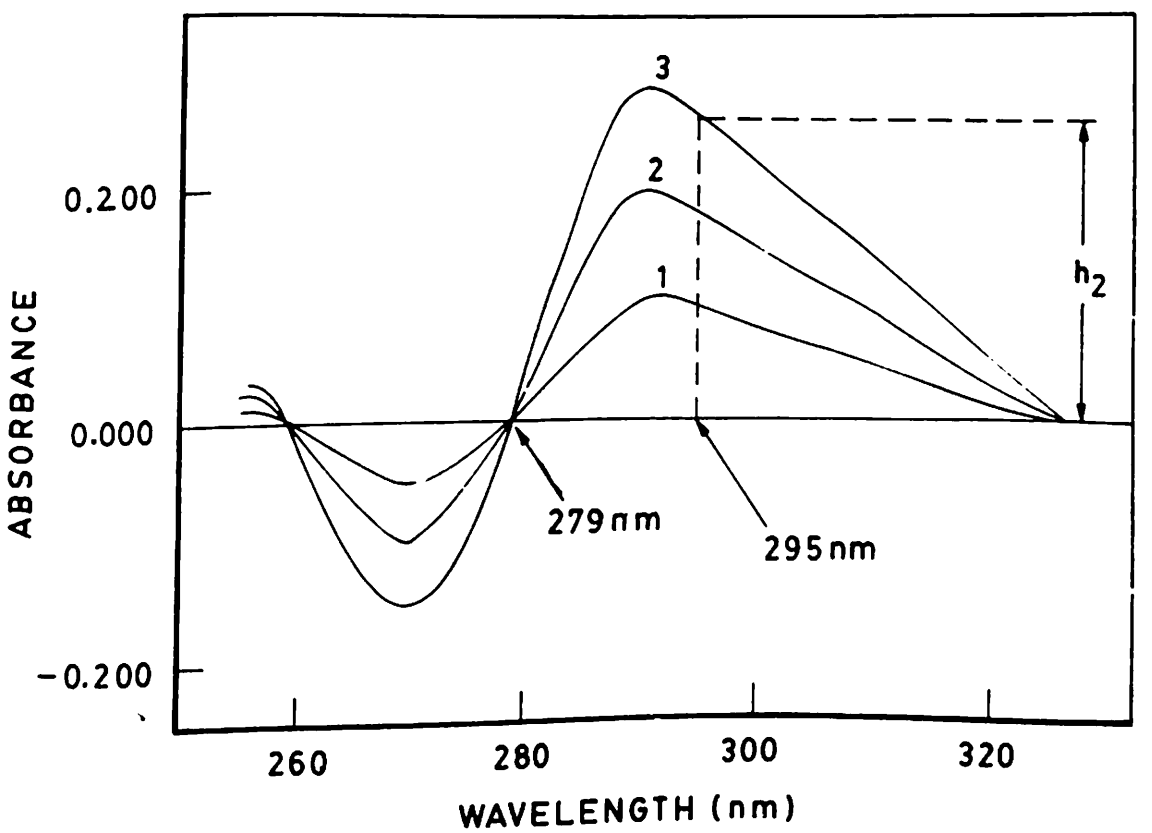


Fig 15. Zero-order difference spectra of pure di-iodohydroxy-quinoline; spectra recorded by scanning equimolar solutions of di-iodohydroxyquinoline in 0.1M HCl and 0.1M NaOH; concentration of di-iodohydroxy quinoline is 5,10 and 15 mcg ml^{-1} in curves 1, 2 and 3 respectively

The figures 14 and 15 show the zero-order difference spectra of metronidazole and di-iodohydroxyquinoline respectively. The isosbestic point (i.e. the wavelength at which the absorption of the solutions are equal) of metronidazole occured at 295nm while that of di-iodohydroxyquinoline occured at 279nm.

Using the amplitudes of the peak to baseline of the corresponding zero-order difference spectra, (h1 and h2 in figures 14 and 15 respectively) the drugs may be estimated in combinations without interference from each other. Thus, the amplitude of the spectra at 279nm (figure 14) would be a function only of the concentration of metronidazole since the contribution of di-iodohydroxyquinoline at this wavelength will be nil as can be seen from figure 15. The wavelength of 279nm is the isosbestic point of di-iodohydroxyquinoline at which the absorption of the drug molecule in the acidic and basic solvents are exactly equal and hence cancel each other when the difference spectrum is recorded by simultaneous scanning of the acidic and basic solutions of di-iodohydroxyquinoline. Similarly, the amplitudes of the difference spectra (δA) of di-iodohydroxyquinoline will be a function of only the concentrations of the di-iodohydroxyquinoline in solutions at the wavelength of 295nm since the difference spectra of pure metronidazole shows isosbestic point at this wavelength indicating that the absorption contribution of metronidazole at this wavelength would be nil when the difference spectra of the admixture of metronidazole and di-iodohydroxyquinoline is recorded. Thus the measurements made at these wavelengths would be a function only of concentration of one of the component.

The heights of the zero-order difference spectra (δA) at the wavelengths of 279nm and 295nm were found to be proportional to the concentration of 10-50 mcg/ml of metronidazole and 5-15 mcg/ml of di-iodohydroxyquinoline respectively.

The statistical analysis had been done on the data of pure drug solutions, their admixtures as well as commercial samples.

The small standard deviation values associated with the determination (Tables 15 and 16) indicated the high level of precision of the proposed method as well as the independence of one drug in the absorption measurement of the other since the deviation occurred only in the third decimal place when compared to the change in the absorbance from point to point in the calibration range. The negligible intercepts of the equations indicated regression through or close to the origin at the chosen wavelengths.

The coefficient of variation values (Table 15 and 16) were less and the standard error (which is the standard deviation of the mean) values were also less indicating the precision of the method.

The percentage ratio of the residuals in tables 15 and 16 clearly indicated a random scatter in case of both pure drug solutions as well as their admixtures. In addition, the F test for non-linearity which is a quantitative test for non-linearity [4] was done and the results have been presented in tables 15 and 16. If a linear relationship holds, the standard deviation of the residuals (standard error of estimate, Sy) would represent an estimate (with (n-2) degrees of freedom) of the standard deviation of the sample. Therefore, we would have an evidence of non-linearity if we can show that Sy has too large a value to be compatible with the sample estimate (Ss).The values in tables 15 and 16 show that the calculated F values were less than that of critical values at 5% level of significance and evidence the linear relationship. Similarly, the other F test results which was based on mean square due to regression and mean square about the regression clearly showed the non-linearity since the calculated F values were far larger than the critical values leading to rejection of null hypothesis (Table 17).

Table 15. Selectivity of the Method for the Determination of Metronidazole in the Presence of Di-iodohydroxyquinoline by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) MND	DIQ	Mean value of [a] absorbance (δA) (279nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
10	0	0.1349 ± 0.0032	2.36	0.0010	99.41	3.29	0.56
15	0	0.1965 ± 0.0035	1.79	0.0011	101.26	3.29	0.47
20	0	0.2671 ± 0.0042	1.57	0.0013	98.78	3.29	0.33
25	0	0.3279 ± 0.0032	0.99	0.0010	100.25	3.29	0.56
30	0	0.3902 ± 0.0030	0.76	0.0009	100.87	3.29	0.64
35	0	0.4615 ± 0.0039	0.84	0.0012	99.35	3.29	0.38
40	0	0.5240 ± 0.0043	0.82	0.0014	99.88	3.29	0.31
45	0	0.5872 ± 0.0036	0.61	0.0011	100.18	3.29	0.47
50	0	0.6532 ± 0.0043	0.67	0.0014	99.99	3.29	0.31
10	10	0.1338 ± 0.0031	2.30	0.0010	98.82	3.29	0.34
15	10	0.1964 ± 0.0036	1.85	0.0011	100.58	3.29	0.25
20	10	0.2606 ± 0.0033	1.27	0.0010	100.86	3.29	0.30
25	10	0.3302 ± 0.0033	0.98	0.0010	99.38	3.29	0.22
30	10	0.3917 ± 0.0039	0.99	0.0012	100.46	3.29	0.15
35	10	0.4598 ± 0.0047	1.03	0.0015	99.78	3.29	0.27
40	10	0.5248 ± 0.0037	0.71	0.0011	99.87	3.29	0.25
45	10	0.5909 ± 0.0038	0.64	0.0012	99.75	3.29	0.25
50	10	0.6532 ± 0.0047	0.71	0.0015	100.24	3.29	0.15

MND - Metronidazole DHQ - Di-iodohydroxyquinoline

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(7,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 16. Selectivity of the Method for the Determination of Di-iodohydroxyquinoline in the Presence of Metronidazole by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) DHQ	MND	Mean value of [a] absorbance (δA) (295nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
5	0	0.1029 ± 0.0019	1.86	0.0006	99.89	3.63	0.34
7	0	0.1422 ± 0.0020	1.44	0.0064	101.04	3.63	0.30
9	0	0.1861 ± 0.0032	1.73	0.0010	99.18	3.63	0.12
11	0	0.2260 ± 0.0040	1.78	0.0013	99.76	3.63	0.07
13	0	0.2661 ± 0.0035	1.31	0.0011	100.09	3.63	0.10
15	0	0.3068 ± 0.0031	1.03	0.0009	100.14	3.63	0.12
5	30	0.1022 ± 0.0018	1.71	0.0006	100.18	3.63	0.25
7	30	0.1425 ± 0.0016	1.56	0.0005	100.67	3.63	0.32
9	30	0.1856 ± 0.0032	1.75	0.0010	99.42	3.63	0.08
11	30	0.2264 ± 0.0040	1.74	0.0012	99.65	3.63	0.05
13	30	0.2666 ± 0.0033	1.24	0.0010	100.03	3.63	0.08
15	30	0.3071 ± 0.0025	0.82	0.0008	100.21	3.63	0.13

MND - Metronidazole DHQ - Di-iodohydroxyquinoline

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

The regression equations of the pure drug solutions and those of admixtures (Table 17) were similar. This similarity as well as the correlation coefficient values 0.9999 for all four series of solutions indicated the non-interference of one drug in the estimation of the other. The co-efficient of determination (which is ratio of the sum of squares due to regression to the sum of squares about the mean) values ranged from 99.98 to 99.99 indicating that this much of variation in the absorbance was accounted for by the concentration of the particular drug in the solutions. A comparison of T test values at a significance level of 5% showed that the calculated values are far larger than the critical values obtained from the t table and confirmed the existence of strong positive correlation [5]. The standard error of slope and intercept are the standard deviation values of slope and intercept and the standard error of estimate is the standard deviation value of residuals of y on x line indicating the precision of the fit by regression. This standard error of estimate was less, when compared to the typical change in δA value from point to point in the calibration curve based on the regression equations for pure admixtures [5].

The table 18 gives the actual values of the pure metronidazole and di-iodohydroxyquinoline as well as the value calculated from the regression line for admixtures. The standard error of prediction is also given in this table.The 95% confidence level concentration ranges presented in table 18 (calculated using the standard error of prediction values) show a narrow range. The assay results of commercial formulations have also been given in table 18. In the case of metronidazole as well as di-iodohydroxyquinoline the estimation will be best at the mean point of the calibration which is 20 and 10 mcg/ml respectively (Tables 15 and 16). The estimation of metronidazole was done using a solution of the tablet sample containing approximately 30 mcg/ml of metronidazole for both the brands. Similarly, di-iodohydroxyquinoline was estimated using solutions containing

Table 17. Regression Analysis of Metronidazole and Di-iodohydroxyquinoline Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (279 nm for MND and 295 nm for DIQ)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significanc[e] of Evidence of Correlation		Standard Error[e]		
	MND	DIQ				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	10-50	0	y = 0.0130x + 0.0043	0.9999	99.98	5.59	43746	2.37	209	0.0001	0.0020	0.0024
Series B	10-50	10	y = 0.0131x + 0.0016	0.9999	99.99	5.59	31916	2.37	286	0.0001	0.0015	0.0018
Series C	0	5-15	y = 0.0204x + 0.0006	0.9999	99.98	7.71	23219	2.78	152	0.0001	0.0014	0.0011
Series D	30	5-15	y = 0.0205x - 0.0003	0.9999	99.98	7.71	37186	2.78	192	0.0001	0.0011	0.0009

MND - Metronidazole DIQ - Di-iodohydroxyquinoline [a] Based on 9 and 6 calibration values of MND and DIQ; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 7 for metronidazole and 4 for di-iodohydroxyquinoline

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 7 for metronidazole and 4 for di-iodohydroxyquinoline

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance* (δA) and *x* is the *concentration*

Table 18. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Metronidazole and Di-iodohydroxyquinoline by Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	MND	DIQ	MND	DIQ	MND	DIQ	MND	DIQ
Pure Drug Admixture	30.00	9.75	---	---	99.40	99.17	99.25-99.54	99.10-99.23
Brand A (Batch 1)	30.00	48.75	250	325	98.43	----	98.28-98.57	-------
Brand A (Batch 2)	30.00	48.75	250	325	98.36	----	98.21-98.50	-------
Brand B (Batch 1)	31.25	40.62	200	325	99.58	----	99.43-99.72	-------
Brand B (Batch 2)	31.25	40.62	200	325	99.93	----	99.78-100.07	-------
Brand A (Batch 1)	7.50	9.75	250	325	----	97.47	-------	97.40-97.54
Brand A (Batch 2)	7.50	9.75	250	325	----	101.43	-------	101.36-101.50
Brand B (Batch 1)	6.50	9.75	200	325	----	99.16	-------	99.10-99.22
Brand B (Batch 2)	6.50	9.75	200	325	----	101.72	-------	101.65-101.78

MND - Metronidazole DIQ - Di-iodohydroxyquinoline

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 7 degrees of freedom for metronidazole and 4 degrees of freedom for di-iodohydroxyquinoline

approximately 9.75 mcg/ml of the drug. This was done to calculate the 95% confidence level concentration ranges in the last column at concentrations where the standard error of prediction will be minimal. Because of the proportion in which metronidazole and di-iodohydroxyquinoline are present in commercial formulations when compared to their linear calibration ranges of 10-30 mcg/ml for metronidazole and 5-15mcg/ml for di-iodohydroxyquinoline, it was not possible to estimate both the drugs at the same time. Such an attempt would make the estimation of metronidazole at a concentration value of around 10 mcg/ml since the concentration of di-iodohydroxyquinoline would be approximately 13 mcg/ml at this concentration of metronidazole because of the ratio of metronidazole to di-iodohydroxyquinoline of 1:1.3 in chosen commercial formulations. This will be highly undesirable since the calibration range of metronidazole starts at 10 mcg/ml and estimating the drug at approximately the same value will lead to a high error of prediction [5]. The 95% confidence level ranges of concentration predicted from a regression equation uses the standard error of prediction which is minimal at the mean point of calibration [5]. Always the estimation using a linear calibration plot is best at the mean point of the calibration. However it should be noted that the maximum solubility of di-iodohydroxyquinoline in aqueous 0.1M HCl as well as 0.1M NaOH is around 25 mcg/ml and hence when the commercial samples were dissolved to produce a concentration of 60mcg/ml of metronidazole, the di-iodohydroxyquinoline precipitated out and has to be filtered using a Whatman No. 1 filter paper prior to estimation of metronidazole in the tablets. Because of these facts, the estimation of the drugs had been done using separate working solutions prepared from the same stock solution of the tablet samples whereas the pure drug admixture estimation (Table 18) had been done to find out the concentration as predicted from the regression equations obtained earlier since these equations were fit by the least square method. The results in the table 18 indicated a mean recovery of 99.40 and 99.17 percent for metronidazole and di-iodohydroxyquinoline respectively. The assay results

show a mean recovery value of 98.36 to 99.93 for metronidazole in the commercial formulations. These values were conforming to the I.P.1985 limits of not less than 95% and not more than 105% of the stated amount of metronidazole as well as the 90-110% range of U.S.P. 1995 although these ranges were for pure metronidazole tablets. The limits for the combination are not available in both I.P. 1985 and U.S.P. 1995. Similarly, the assay results show a mean recovery range of 97.47 to 101.72% for di-iodohydroxyquinoline in commercial samples which is within the limits of I.P.1985 (92.5-107.5%) and U.S.P. 1995 (95-105%) for di-iodohydroxyquinoline alone in tablets. In fact, the 95% confidence level ranges in table 18 for the drugs falls within the prescribed limits. The detection limits at 5% level of significance were found to be 0.50 and 0.10 mcg/ml for metronidazole and di-iodohydroxyquinoline respectively.

The stability of the standard solutions of metronidazole (20 mcg/ml) and di-iodohydroxyquinoline (10 mcg/ml) in 0.1M HCl and 0.1M NaOH (stored prior to scanning in low actinic flasks at 28-32°C) were monitored spectrophotometrically (at the wavelengths of 279nm for metronidazole and 295nm for di-iodohydroxyquinoline) for a period of two hours and were found to vary by the following absorbance units (AU): 0.1N HCl solution of metronidazole and di-iodohydroxyquinoline by± 0.005 and ± 0.004 and 0.1M NaOH solutions of metronidazole by ±0.007 and ±0.006 respectively. The pKa values of metronidazole and di-iodohydroxyquinoline were 2.5 [111] and 10.5 [14] respectively and hence the pH of 0.1M HCl (approximate pH 1.0) and that of 0.1 M NaOH (approximate pH 13.0) were at least 1.5 pH units away from the pK_a values of the drugs. Therefore, small variations in the pH of the solvents did not lead to appreciable changes in the absorbance values since these pH values produce about 90% of the individual species of the drugs in the respective solvents [76].

Thus the proposed method of determination of metronidazole and diiodohydroxyquinoline was found to be accurate and precise and may be used for the estimation of the drugs in commercial formulations without prior separation of each other. The results in table 18 show that the interference from the formulation matrix is not likely in the assay of the drugs.

4.04 SIMULTANEOUS DETERMINATION OF SALBUTAMOL SULPHATE AND BROMHEXINE HYDROCHLORIDE IN PURE ADMIXTURES AND TABLET PREPARATIONS BY DIFFERENCE SPECTROPHOTOMETRY

The combination of salbutamol sulphate and bromhexine hydrochloride as a tablet preparation is being widely used for bronchodilation in asthma, chronic bronchitis and emphysema. The literature reports for the estimation of salbutamol sulphate include normal and derivative spectrophotometric estimation as a single constituent in tablets [19-22], low frequency dielectric spectroscopy [23] as well as zero-order estimation of salbutamol sulphate in the presence of bromhexine hydrochloride in tablets [24] and spectrofluorometric estimation [25]. There are also reports on the HPLC method for the estimation of salbutamol sulphate alone as well as in the presence of bromhexine hydrochloride in dosage forms [27]. The methods for estimation of bromhexine hydrochloride include spectrophotometry in the presence of salbutamol sulphate using Vierordt's method [24], colorimetric estimation [112], atomic absorption spectrophotometry [113], HPLC methods for bromhexine hydrochloride alone [114-116] as well as in the presence of salbutamol sulphate [27]. The official methods for the estimation of salbutamol sulphate in tablets are HPLC [1] This section of the thesis describes a zero-order difference spectrophotometric method for the estimation of salbutamol sulphate and bromhexine hydrochloride in pure admixtures and in tablet preparations without prior separation from each other as well as formulation additives.

METHODS

Materials, Reagents and Apparatus

The methanol used was of spectroscopic grade. The spectra were recorded using a Jasco-7800 uv-visible scanning double beam spectrophotometer using 1cm matched cuvettes. The scan rate was set at 240nm/ min.

Standard Solutions

Appropriate aliquots of stock solutions of pure salbutamol sulphate (1mg/ml) and bromhexine hydrochloride (1mg/ml) in methanol were used to prepare two series of equimolar solutions of each drug in 0.1M NaOH and 0.1M HCl containing 25-100 mcg/ml of salbutamol sulphate (series A) and 50-200 mcg/ml of bromhexine hydrochloride (series C). Similarly, two more series of equimolar solutions of mixtures of salbutamol sulphate and bromhexine hydrochloride, the first containing a constant concentration of 120mcg/ml of bromhexine hydrochloride and a varying concentration of 25-100 mcg/ml of salbutamol sulphate (series B) and a second containing a constant concentration of 60 mcg/ml of salbutamol sulphate and a varying concentration of 50-200mcg/ml of bromhexine hydrochloride (series D) were prepared with 0.1M HCl and 0.1M NaOH (Tables 19 and 20).

Preparation of Sample Solutions

One hundred tablets of each brand were accurately weighed, powdered and a weight of the powder equivalent to approximately 50mg of salbutamol sulphate was transferred to a 50ml volumetric flask, dissolved in methanol by thorough shaking, diluted to volume and filtered using Whatman No. 1 filter paper. The first and last 5 ml of the filtrate were rejected. Appropriate volumes of the aliquots of the filtrate were diluted with 0.1M HCl and

methanolic 0.1M NaOH to obtain equimolar solutions containing approximately the concentrations given in table 21.

The normal absorbance as well as the absorbance difference of the acidic and basic (methanolic 0.1M NaOH) equimolar drug solutions were recorded with a Jasco 7800 uv-visible double beam spectrophotometer using 10 mm matched cuvettes by placing the basic solution in the reference beam and acidic solution in the sample beam. The scan rate was set at 240 nm / min. The results of the scan have been presented in Tables 19-21 and the spectra in figures 16 and 17.

RESULTS AND DISCUSSION

The combination of salbutamol sulphate and bromhexine hydrochloride in tablets had been estimated simultaneously as well as individually. The simultaneous estimation reported involves the estimation of bromhexine hydrochloride directly at 319nm since salbutamol sulphate does not interfere at this wavelength whereas salbutamol sulphate had been estimated in the presence of bromhexine hydrochloride by using Vierordt's method [24]. Although the method had been reported to be satisfactory, it would involve the use of equation for the estimation of salbutamol sulphate since bromhexine hydrochloride interferes. The equation method may not be able to eliminate the contribution of additives which would get eliminated in the zero-order difference spectrophotometry provided the spectral properties of such additives does not get altered by the pH change which has been used in this work to produce the spectral shift so as to enable the recording of the difference spectra [76].

The difference absorbance of a compound can be readily related to concentration by prior calibration of the δA (absorbance difference) values so as to establish that δA is a linear function of concentration over the range required [80] and is likely to minimise the interference due to excipients

by cancellation of their absorbance during the recording of the difference spectrum. The δA values of standard solutions of salbutamol sulphate and bromhexine hydrochloride were used to calculate regression equations (Tables 19 and 20). These equations were calculated using the data points obtained from drug solutions (Series A-D in Table 21). The experimental design is such that the regression equations of pure individual drugs in solution (Series A and C) may be compared with that of their admixtures in which the concentration of the drug to be estimated is varied and that of the other is kept at a constant value (Series B and D in table 21). This helps in easy determination of the interference of one drug in the estimation of the other at the chosen wavelength as well as in establishing the rectilinearity and precision of the method in the particular concentration range.

The figures 16 and 17 show the zero-order difference spectra of salbutamol sulphate and bromhexine hydrochloride respectively. The isosbestic point (i.e. the wavelength at which the absorption of the solutions are equal) of salbutamol sulphate occurred at 280nm while that of bromhexine hydrochloride occurred at 310nm.

Using the amplitudes of the peak to baseline of the corresponding zero-order difference spectra, (h1 and h2 in figures 16 and 17 respectively) the drugs may be estimated in combinations without interference from each other. Thus, the amplitude of the spectra at 310nm (figure 16) would be a function only of the concentration of salbutamol sulphate since the contribution of bromhexine hydrochloride at this wavelength will be nil as can be seen from figure 17. The wavelength of 310nm was the isosbestic point of bromhexine hydrochloride at which the absorption of the drug molecule in the acidic and basic (methanolic 0.1M NaOH) solvents are exactly equal and hence cancel each other when the difference spectrum is recorded by simultaneous scanning of the acidic and basic solutions of bromhexine hydrochloride. Similarly, the amplitudes of the difference spectra (δA) of bromhexine hydrochloride will be a function of only the concentrations of the

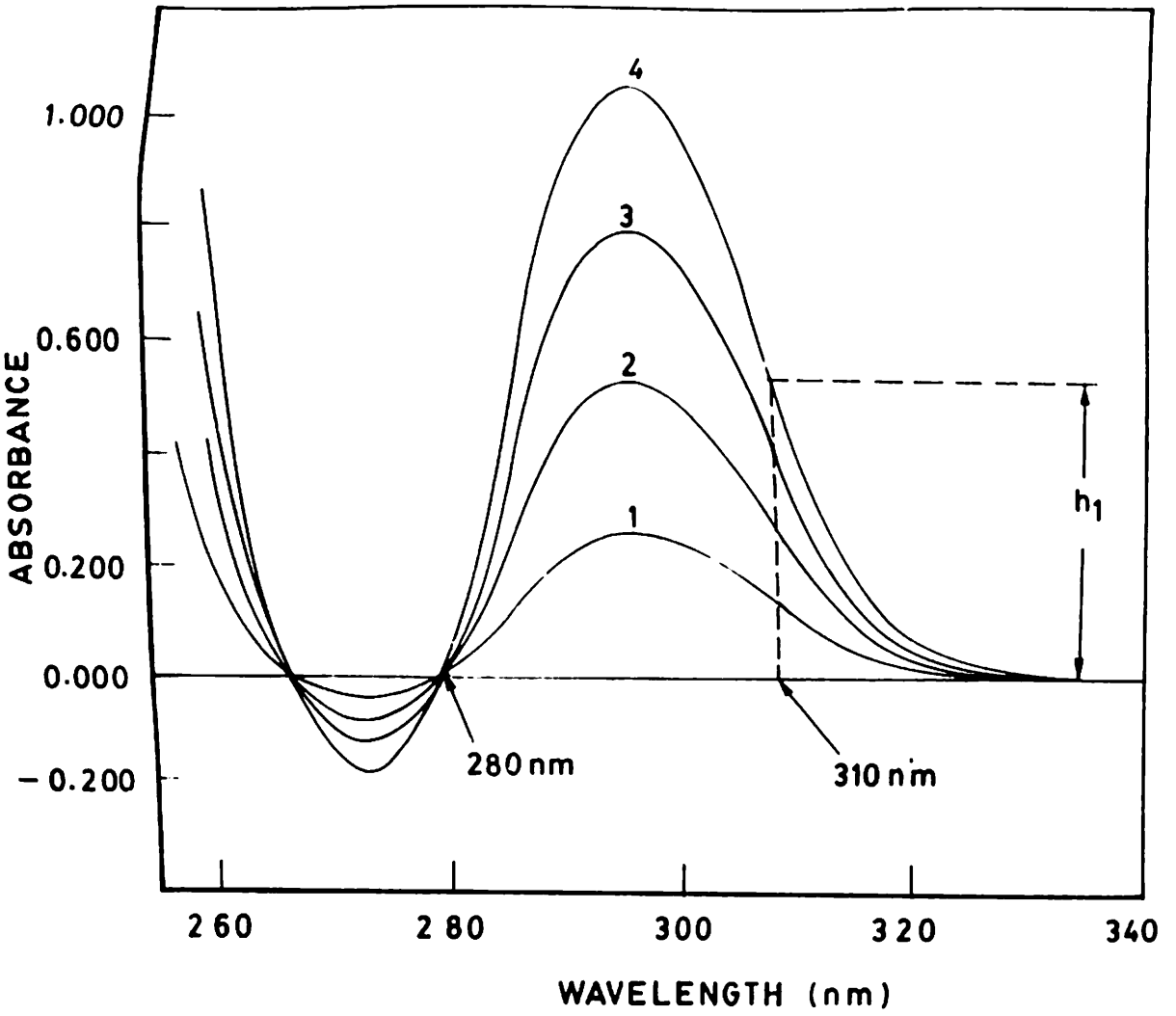


Fig 16. Zero-order difference spectra of pure salbutamol sulphate; spectra recorded by scanning equimolar solutions of salbutamol sulphate in 0.1M HCl and 0.1M NaOH; concentration of salbutamol sulphate is 25, 50, 75 and 100 mcg ml^{-1} in curves 1,2, 3 and 4 respectively

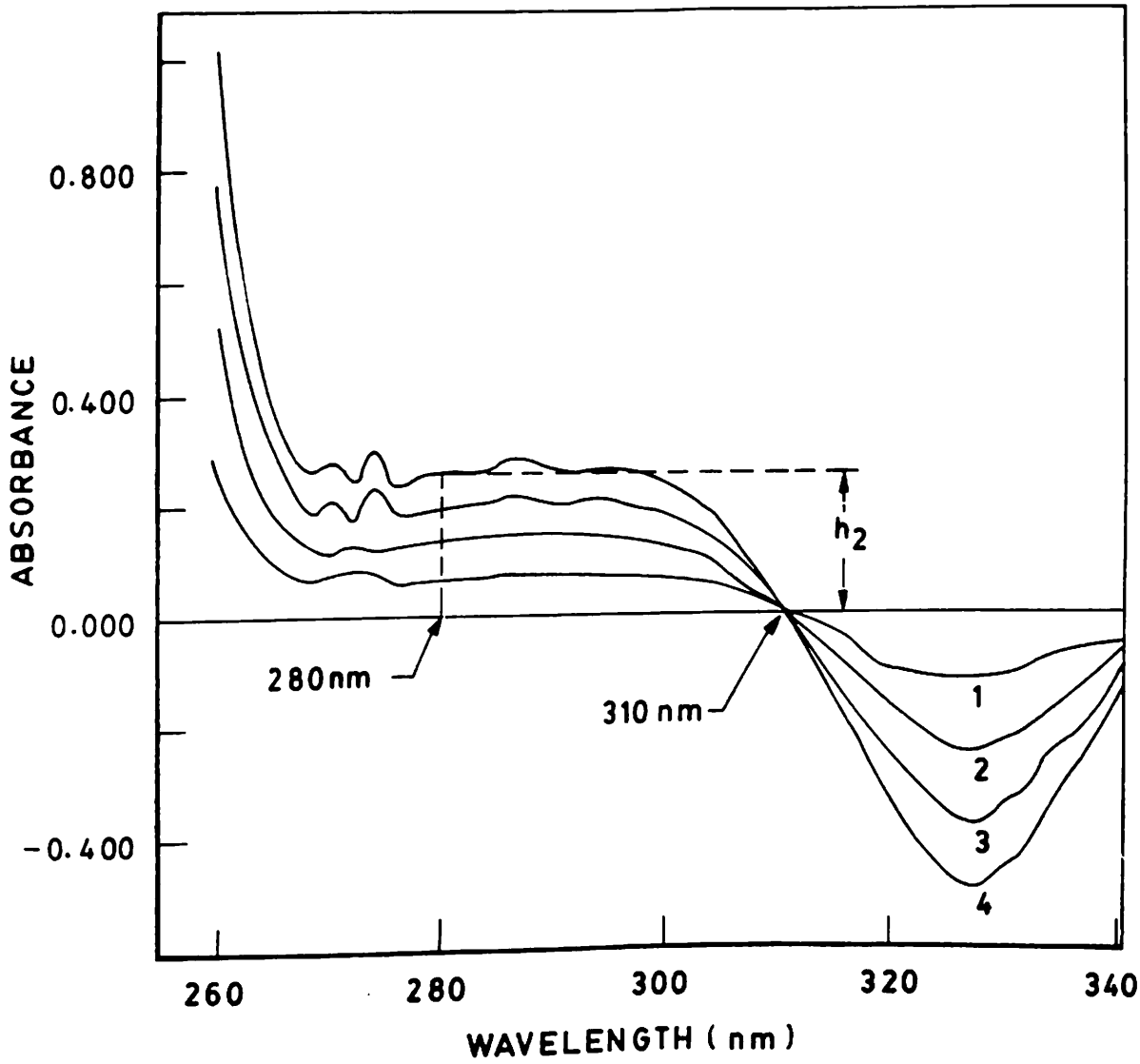


Fig 17. Zero-order difference spectra of pure bromhexine HCl; spectra recorded by scanning equimolar solutions of bromhexine HCl in 0.1M HCl and 0.1M NaOH; concentration of bromhexine HCl is 50,100,150 and 200 mcg ml^{-1} in curves 1, 2, 3 and 4 respectively

bromhexine hydrochloride in solutions at the wavelength of 280nm since the difference spectra of pure salbutamol sulphate shows isosbestic point at this wavelength indicating that the absorption contribution of salbutamol sulphate at this wavelength would be nil when the difference spectra of the admixture of salbutamol sulphate and bromhexine hydrochloride is recorded. Thus the measurements made at these wavelengths would be a function only of concentration of one of the component. The solvent of methanolic 0.1M NaOH was used to prepare the basic solutions of the drugs because of the insolubility of bromhexine hydrochloride in aqueous 0.1M NaOH.

The heights of the zero-order difference spectra (δA) at the wavelengths of 310nm and 280nm were found to be proportional to the concentration of 25-100 mcg/ml of salbutamol sulphate and 50-200 mcg/ml of bromhexine hydrochloride respectively.

The statistical analysis had been done on the data of pure drug solutions, their admixtures as well as commercial samples.

The small standard deviation values associated with the determination (Tables 19 and 20) indicated the high level of precision of the proposed method as well as the independence of one drug in the absorption measurement of the other since the deviation occurred only in the third decimal place when compared to the change in the absorbance (δA) from point to point in the calibration range. The negligible intercepts of the equations indicated regression through or close to the origin at the chosen wavelengths.

The coefficient of variation values (Tables 19 and 20) were less and the standard error (which is the standard deviation of the mean) values were also less indicating the precision of the method.

Table 19. Selectivity of the Method for the Determination of Salbutamol sulphate in the Presence of Bromhexine Hydrochloride by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] absorbance (δA) (310nm))	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
SAL	BRH					Crit	Calc
25	0	0.1360 ± 0.0032	2.33	0.0010	102.85	3.29	0.43
35	0	0.1952 ± 0.0023	1.18	0.0007	98.69	3.29	0.83
45	0	0.2477 ± 0.0034	1.39	0.0011	99.49	3.29	0.38
55	0	0.3010 ± 0.0026	0.87	0.0008	99.74	3.29	0.65
65	0	0.3557 ± 0.0026	0.74	0.0008	99.53	3.29	0.65
75	0	0.4071 ± 0.0029	0.73	0.0009	100.18	3.29	0.52
85	0	0.4599 ± 0.0039	0.86	0.0013	100.37	3.29	0.29
95	0	0.5161 ± 0.0032	0.62	0.0010	99.86	3.29	0.32
100	0	0.5416 ± 0.0037	0.69	0.0012	100.13	3.29	0.43
25	120	0.1369 ± 0.0031	2.30	0.0010	101.04	3.29	0.18
35	120	0.1940 ± 0.0035	1.82	0.0011	99.12	3.29	0.14
45	120	0.2469 ± 0.0033	1.33	0.0010	99.74	3.29	0.16
55	120	0.2998 ± 0.0025	0.72	0.0007	100.15	3.29	0.27
65	120	0.3546 ± 0.0030	0.84	0.0009	99.89	3.29	0.19
75	120	0.4077 ± 0.0030	0.73	0.0009	100.12	3.29	0.19
85	120	0.4611 ± 0.0029	0.63	0.0009	100.23	3.29	0.20
95	120	0.5150 ± 0.0036	0.70	0.0011	100.22	3.29	0.13
100	120	0.5449 ± 0.0033	0.62	0.0011	99.67	3.29	0.16

SAL - Salbutamol sulphate BRH - Bromhexine hydrochloride

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(7,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 20. Selectivity of the Method for the Determination of Bromhexine Hydrochloride in the Presence of Salbutamol sulphate by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) SAL	BRH	Mean value of [a] absorbance (δA) (280nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
0	50	0.0660 ± 0.0035	5.35	0.0011	100.22	3.63	0.06
0	80	0.1072 ± 0.0027	2.52	0.0009	99.54	3.63	0.11
0	110	0.1461 ± 0.0040	2.77	0.0013	100.79	3.63	0.05
0	140	0.1885 ± 0.0029	1.53	0.0010	99.64	3.63	0.10
0	170	0.2292 ± 0.0033	1.42	0.0010	99.64	3.63	0.07
0	200	0.2682 ± 0.0038	1.43	0.0012	100.27	3.63	0.06
60	50	0.0673 ± 0.0029	4.38	0.0009	99.51	3.63	0.12
60	80	0.1066 ± 0.0029	2.73	0.0009	100.64	3.63	0.12
60	110	0.1481 ± 0.0034	2.33	0.0011	99.67	3.63	0.09
60	140	0.1887 ± 0.0039	2.09	0.0012	100.70	3.63	0.07
60	170	0.2267 ± 0.0029	1.30	0.0009	100.68	3.63	0.12
60	200	0.2694 ± 0.0029	1.07	0.0009	99.69	3.63	0.12

Sal - Salbutamol sulphate BRH - Bromhexine hydrochloride

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

The percentage ratio of the residuals in tables 19 and 20 indicated a random scatter in case of both pure drug solutions as well as their admixtures . In addition, the F test for non-linearity which is a quantitative test for non-linearity [4] was done and the results have been presented in tables 19 and 20. If a linear relationship holds, the standard deviation of the residuals (standard error of estimate, Sy) would represent an estimate (with (n-2) degrees of freedom) of the standard deviation of the sample. Therefore, we would have an evidence of non-linearity if we can show that Sy has too large a value to be compatible with the sample estimate (Ss).The values in tables 19 and 20 show that the calculated F values were less than that of critical values at 5% level of significance and evidence the linear relationship. Similarly, the other F test results which was based on mean square due to regression and mean square about the regression clearly showed the non-linearity since the calculated F values were far larger than the critical values leading to rejection of null hypothesis (Table 21).

The regression equations of the pure drug solutions and those of admixtures (Table 21) were similar. This similarity as well as the correlation coefficient values 0.9999 for all four series of solutions indicated the non-interference of one drug in the estimation of the other. The co-efficient of determination (which is ratio of the sum of squares due to regression to the sum of squares about the mean) values ranged from 99.98 to 99.99 indicating that this much of variation in the absorbance was accounted for by the concentration of the particular drug in the solutions. A comparison of T test values at a significance level of 5% showed that the calculated values are far larger than the critical values obtained from the t table and confirmed the existence of strong positive correlation [5]. The standard error of slope and intercept are the standard deviation values of slope and intercept and the standard error of estimate is the standard deviation value of residuals of y on x line indicating the precision of the fit by regression. This standard error of

Table 21. Regression Analysis of Salbutamol sulphate and Bromhexine HCl Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (310 nm for SAL and 280 nm for BRH)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	SAL	BRH				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	25-100	0	y = 0.0054x + 0.0044	0.9999	99.98	5.59	37134	2.37	192	0.0001	0.0019	0.0020
Series B	25-100	120	y = 0.0054x + 0.0034	0.9999	99.99	5.59	99017	2.37	314	0.0001	0.0012	0.0013
Series C	0	50-200	y = 0.0014x - 0.0014	0.9999	99.98	7.71	34801	2.78	186	0.0001	0.0010	0.0009
Series D	60	50-200	y = 0.0013x - 0.0002	0.9999	99.98	7.71	26784	2.78	163	0.0001	0.0011	0.0010

SAL - Salbutamol sulphate BRH - Bromhexine HCl [a] Based on 9 and 6 calibration values for Salbutamol sulphate and bromhexine HCl respectively; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 9 for salbutamol sulphate and 6 for bromhexine HCl

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 9 for salbutamol sulphate and 6 for bromhexine HCl

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance (δA)* and *x* is the *concentration*

estimate was less, when compared to the typical change in δA value from point to point in the calibration curve based on the regression equations for pure admixtures [5].

The table 22 gives the actual values of the pure salbutamol sulphate and bromhexine hydrochloride as well as the value calculated from the regression line for admixtures. The standard error of prediction is also given in this table. The 95% confidence level concentration ranges presented in table 22 (calculated using the standard error of prediction values) show a narrow range. The assay results of commercial formulations have also been given in table 22. In the case of salbutamol sulphate as well as bromhexine hydrochloride the estimation will be best at the mean point of the calibration which is 60 and 120 mcg/ml respectively (Tables 19 and 20). The estimation of salbutamol sulphate was done using a solution of the tablet sample containing approximately 60 mcg/ml of salbutamol sulphate for both the brands. During this estimation, the proportion of bromhexine hydrochloride in Brand A was such that it produced a solution of approximate concentration 120 mcg/ml as such. Therefore, both the drugs in Brand A were estimated using a single solution whereas in the cases of the other Brands (Brand B,C and D) the proportion was such that it produced a bromhexine hydrochloride concentration of approximately 240 mcg/ml (which was outside the linear calibration range of 50-200 mcg/ml of bromhexine hydrochloride) when the salbutamol concentration was approximately 60mcg/ml. Hence, bromhexine hydrochloride in Brands B, C and D have been estimated by using separate tablet sample solutions which were prepared so as to give an approximate concentration of 120 mcg/ml of bromhexine hydrochloride. When such solutions were prepared, the proportion of the drugs in these Brands led to an approximate concentration of 30 mcg/ml of salbutamol sulphate which was almost near the lower limit of the calibration range of salbutamol sulphate (25-100 mcg/ml). Nevertheless, since the concentration was within the calibration range, salbutamol sulphate had been estimated at these concentrations as well in the Brands B, C and D in addition

Table 22. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Salbutamol sulphate and Bromhexine Hydrochloride by Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	SAL	BRH	SAL	BRH	SAL	BRH	SAL	BRH
Pure Drug Admixture	60	120	—	—	100.05	99.65	99.75-100.31	98.54-100.75
Brand A	60	120	4	8	99.33	100.27	99.07-99.59	99.16-101.37
Brand B	60	240	2	8	99.65	—	99.38-99.92	———
Brand C	60	240	2	8	100.23	—	99.96-100.50	———
Brand D	60	240	2	8	99.86	—	99.60-100.12	———
Brand B	30	120	2	8	—	99.28	———	98.17-100.38
Brand C	30	120	2	8	—	98.85	———	97.74-99.95
Brand D	30	120	2	8	—	99.03	———	97.92-100.13

SAL - Salbutamol sulphate BRH - Bromhexine hydrochloride

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 7 degrees of freedom for salbutamol sulphate and 4 degrees of freedom for bromhexine hydrochloride

to its estimation in the same Brands using a concentration of 60mcg/ml which is near the mean point of its calibration range.

A comparison of the assay results in terms of the mean recovery as well as the 95% confidence level concentration range shows that the values of mean recovery were not quite different although the confidence level limits seem to be bit higher in case of the estimation of salbutamol sulphate using 30mcg/ml solutions since the calculation of this range uses the value of standard error of prediction which increases as we move away from the mean point of the calibration [5]. Thus, although the proportion of the drugs in commercial formulations permit the estimation of both salbutamol sulphate and bromhexine hydrochloride using the same solution by difference spectrophotometry in the case of all the four Brands, it would be advisable to estimate salbutamol sulphate using a concentration of 60mcg/ml if the errors are to be minimal. The 95% confidence level ranges of concentration predicted from a regression equation uses the standard error of prediction which is minimal at the mean point of calibration [5]. The results of the estimation of the drugs in pure admixture (Table 22) had been done to find out the concentration as predicted from the regression equations obtained earlier since these equations were fit by the least square method. The results indicated a mean recovery of 100.05 and 99.65% for salbutamol sulphate and bromhexine hydrochloride respectively which is quite impressive. The assay results show a mean recovery value of 99.33 to 100.23 for salbutamol sulphate in the commercial formulations. These values were conforming to the I.P.1985 as well as U.S.P. 1995 limits of not less than 90% and not more than 110% of the stated amount of salbutamol sulphate for pure salbutamol sulphate tablets. Similarly, the assay results show a mean recovery range of 99.33 to 100.23% for bromhexine hydrochloride in commercial samples but cannot be compared with the official limits since they are not official in I.P.1985 or U.S.P. 1995. In the case of salbutamol sulphate, the 95% confidence level ranges themselves fall within the prescribed limits (Table 22). The detection limits at 5% level of

significance were found to be 0.38 and 2.45 mcg/ml for salbutamol sulphate and bromhexine hydrochloride respectively.

The stability of the standard solutions of salbutamol sulphate (60 mcg/ml) and bromhexine hydrochloride (120 mcg/ml) in 0.1M HCl and methanolic 0.1M NaOH (stored prior to scanning in low actinic flasks at 24-27°C) were monitored spectrophotometrically (at the wavelengths of 310nm for salbutamol sulphate and 280nm for bromhexine hydrochloride) for a period of two hours and were found to vary by the following absorbance units (AU): 0.1N HCl solution of salbutamol sulphate and bromhexine hydrochloride by ±0.006 and ± 0.007 and 0.1M NaOH solutions of salbutamol sulphate by ±0.009 and ±0.010 respectively. The pKa values of salbutamol sulphate were 9.3, 10.3 [111] and that of bromhexine hydrochloride is 8.5 [117] and hence the pH of 0.1M HCl (approximate pH 1.0) and that of 0.1 M NaOH (approximate pH 12.0) were at least 1.5 pH units away from the pK_a values of the drugs. Therefore, small variations in the pH of the solvents did not lead to appreciable changes in the absorbance values since these pH values produce about 90% of the individual species of the drugs in the respective solvents [76].

Thus the proposed method of determination of salbutamol sulphate and bromhexine hydrochloride was found to be accurate and precise and may be used for the estimation of the drugs in commercial formulations without prior separation of each other. The results in table 22 show that the interference from the formulation matrix is not likely in the assay of the drugs.

4.05 SIMULTANEOUS DETERMINATION OF THEOPHYLLINE AND PHENOBARBITONE IN PURE ADMIXTURES AND TABLET PREPARATIONS BY DIFFERENCE SPECTROPHOTOMETRY

The combination of theophylline and phenobarbitone as a tablet preparation is being widely used for bronchial asthma and bronchitis. The literature reports for the estimation of theophylline include difference spectrophotometry in pharmaceutical dosage forms as a single constituent [118-120] and in blood [121]. There are also reports on the estimation by differential scanning calorimetry in suppositories [122] and spectrophotometric estimation in syrup preparations [123,124]. Spectrofluorometric estimation in pharmaceutical preparations by solid surface room temperature photochemiluminescence [125], estimation using NMR [126], gas chromatography [127,128] including capillary GC-MS [128] and by HPLC [129,130] have also been reported. The reported methods for the estimation of phenobarbitone thus far include titrimetry [131], polarography [132], spectrophotometry [133-138] including difference spectrophotometry, spectrophosphorimetry [139,140], spectrofluorometry [141], thin layer chromatography [142,143], gas chromatography [144,145] and HPLC [146-148]. The official methods for the estimation of theophylline are HPLC [1], and those for phenobarbitone include HPLC [1], argentometry [2].This section of the thesis describes a zero-order difference spectrophotometric method for the estimation of theophylline and phenobarbitone in pure admixtures and in tablet preparations without prior separation from each other as well as formulation additives.

METHODS

Materials, Reagents and Apparatus

The spectra were recorded using a Jasco-7800 uv-visible scanning double beam spectrophotometer using 1cm matched cuvettes. The scan rate was set at 240nm/ min.

Standard Solutions

Appropriate aliquots of stock solutions of pure theophylline (1mg/ml) and phenobarbitone (1mg/ml) in distilled water were used to prepare two series of equimolar solutions of each drug in 0.1M NaOH and 0.1M HCl containing 10-40 mcg/ml of theophylline (series A) and 5-30 mcg/ml of phenobarbitone (series C). Similarly, two more series of equimolar solutions of mixtures of theophylline and phenobarbitone, the first containing a constant concentration of 17mcg/ml of phenobarbitone and a varying concentration of 10-40 mcg/ml of theophylline (series B) and a second containing a constant concentration of 25 mcg/ml of theophylline and a varying concentration of 5-30mcg/ml of phenobarbitone (series D) were prepared with 0.1M HCl and 0.1M NaOH (Tables 23 and 24).

Preparation of Sample Solutions

Twenty tablets of each brand were accurately weighed, powdered and a weight of the powder equivalent to the average weight of the tablet was transferred to a 100ml volumetric flask, dissolved in water by thorough

shaking, diluted to volume and filtered using Whatman No. 1 filter paper. The first and last 5 ml of the filtrate were rejected. Appropriate volumes of the aliquots of the filtrate were diluted with 0.1M HCl and 0.1M NaOH to obtain equimolar solutions containing approximately the concentrations given in table 26.

The normal absorbance as well as the absorbance difference of the acidic and basic equimolar drug solutions were recorded with a Jasco 7800 uv-visible double beam spectrophotometer using 10 mm matched cuvettes by placing the basic solution in the reference beam and acidic solution in the sample beam. The scan rate was set at 240 nm / min. The results of the scan have been presented in Tables 24,25 and the spectra figures 18 and 19.

RESULTS AND DISCUSSION

The combination of theophylline and phenobarbitone in tablets had been estimated individually by difference spectrophotometry in pharmaceutical dosage forms as well as in blood [118-120]. Thus far, the simultaneous estimation of the combination has not been done mainly because of the presence of ephedrine as hydrochloride salt in the combination. The presence of ephedrine as hydrochloride salt would invariably lead to interference in the estimation of theophylline and phenobarbitone and hence separation of constituents will be required prior to estimation. But in the case of the commercial formulation estimated in this report, ephedrine was not present as its hydrochloride salt but as resinate along with theophylline and phenobarbitone. This when dissolved in water, lead to elimination of ephedrine since ephedrine resinate was not soluble in water. Of course, phenobarbitone was also not highly soluble in water and required a thorough shaking for atleast twenty minutes for the drug to go into solution completely. Thus, the tablet sample dissolved in water, when filtered, contained only

phenobarbitone and theophylline in solution and the ephedrine as resinate was filtered off along with the other excipients prior preparation of working solutions.

The difference absorbance of the drugs can be readily related to concentration by prior calibration of the δA (absorbance difference) values so as to establish that δA is a linear function of concentration over the range required [80] and is likely to minimise the interference due to excipients by cancellation of their absorbance during the recording of the difference spectrum. The δA values of standard solutions of theophylline and phenobarbitone were used to calculate regression equations (Tables 23 and 24). These equations were calculated using the data points obtained from drug solutions (Series A-D in Table 25). The experimental design is such that the regression equations of pure individual drugs in solution (Series A and C) may be compared with that of their admixtures in which the concentration of the drug to be estimated is varied and that of the other is kept at a constant value (Series B and D in table 25). This helps in easy determination of the interference of one drug in the estimation of the other at the chosen wavelength as well as in establishing the rectilinearity and precision of the method in the particular concentration range.

The figures 18 and 19 show the zero-order difference spectra of theophylline and phenobarbitone respectively. The isosbestic points (i.e. the wavelength at which the absorption of the solutions are equal) of theophylline occurred at 251 and 269nm while phenobarbitone does not show any exact isosbestic point but the absorption of the difference spectra became negligible after 290nm.

Using the amplitudes of the peak to baseline of the corresponding zero-order difference spectra, (h1 and h2 in figures 18 and 19 respectively) the drugs may be estimated in combinations without interference from each other. Thus, the amplitude of the spectra at 291 (figure 18) would be a

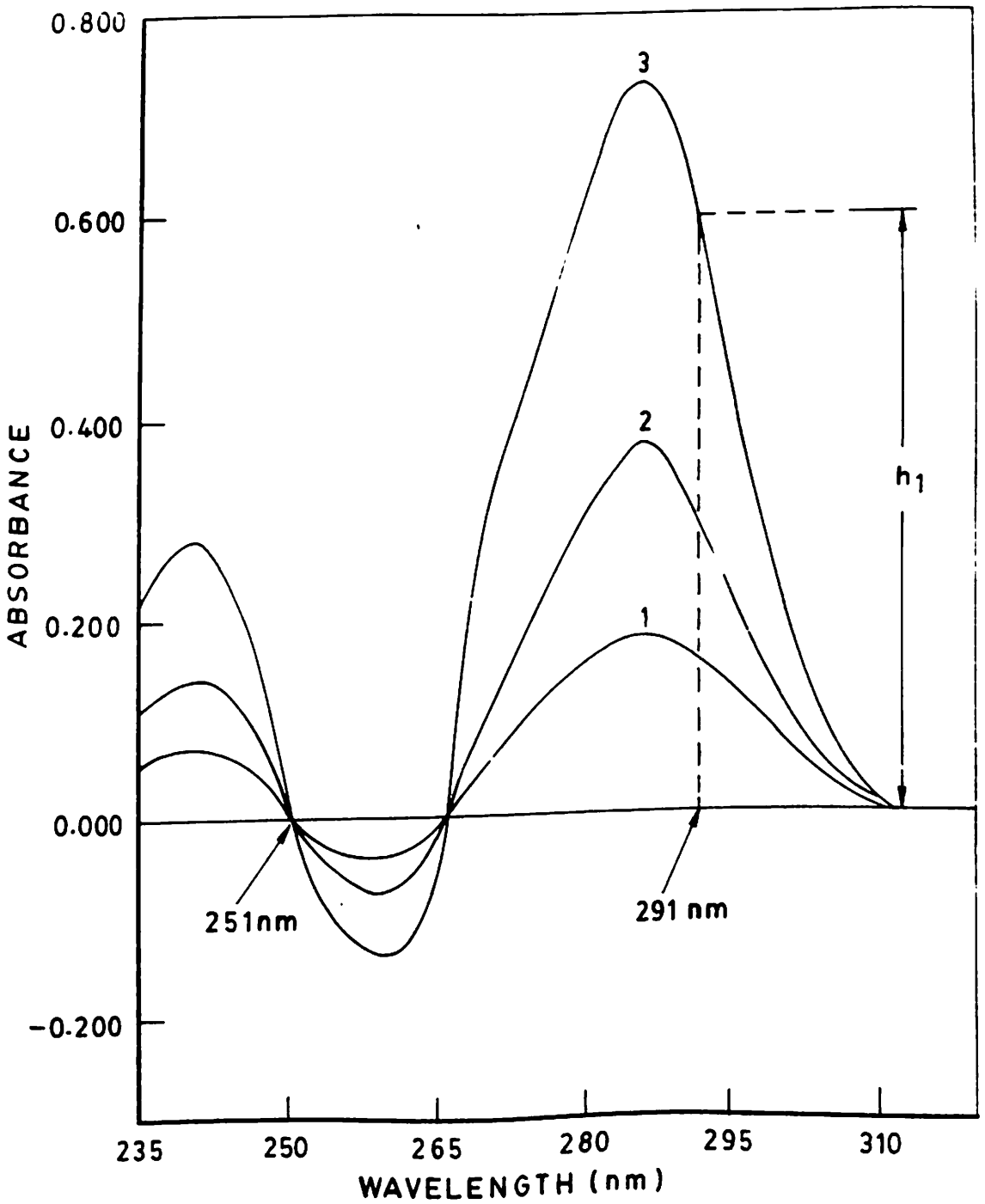


Fig 18. Zero-order difference spectra of pure theophylline; spectra recorded by scanning equimolar solutions of theophylline in 0.1M HCl and 0.1M NaOH; concentration of pure theophylline is 10, 20 and 40 mcg ml^{-1} in curves 1, 2 and 3 respectively

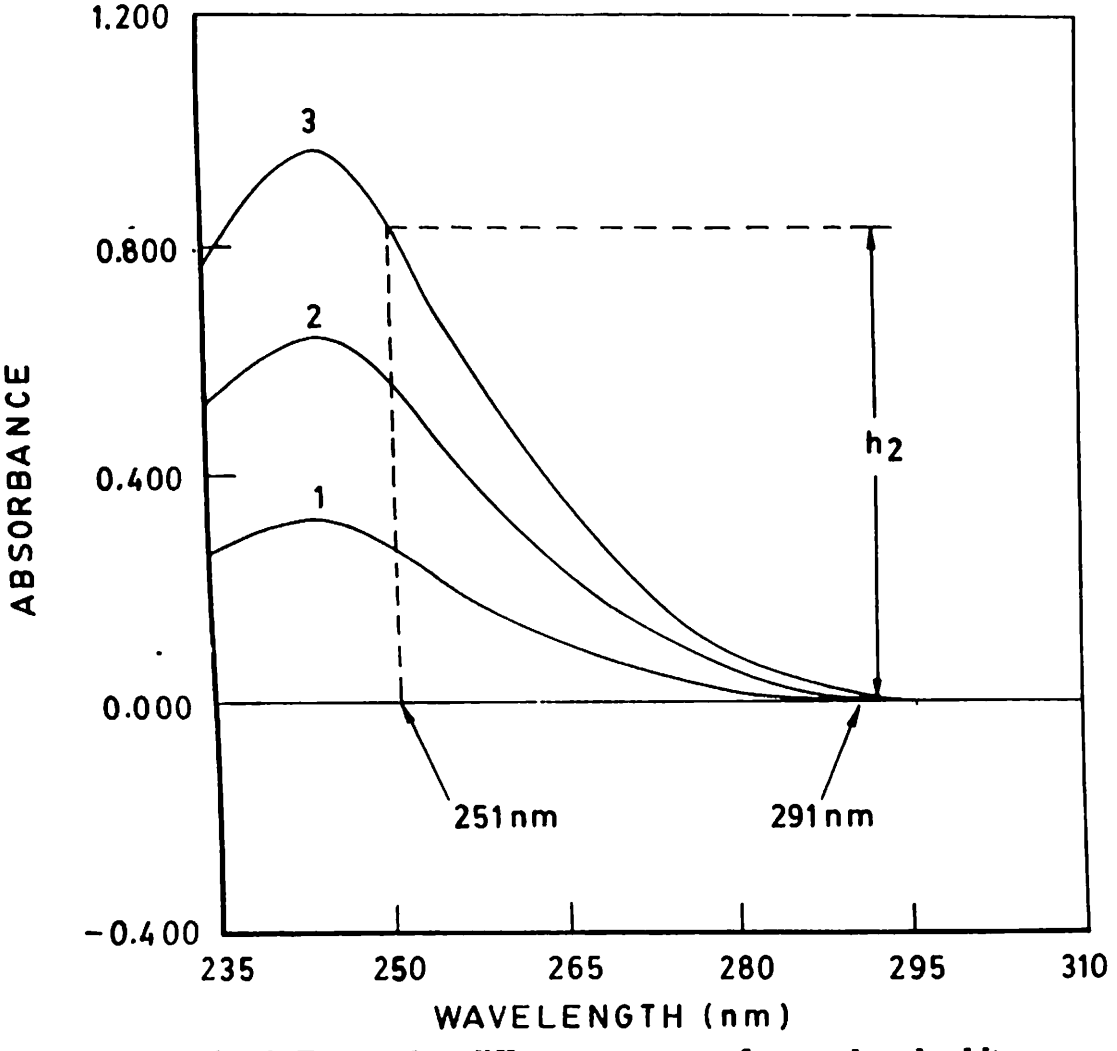


Fig 19. Zero-order difference spectra of pure phenobarbitone; spectra recorded by scanning equimolar solutions of phenobarbitone in 0.1M HCl and 0.1M NaOH; concentration of phenobarbitone is 10, 20 and 30 mcg/ml in curves 1,2 and 3 respectively

function only of the concentration of theophylline since the contribution of phenobarbitone at this wavelength will be nil as can be seen from figure 19. Similarly, the absorbance (δA) values of phenobarbitone at 251nm will be a function of only phenobarbitone since the difference spectra of pure theophylline shows isosbestic point at this wavelength indicating that the absorption contribution of theophylline at this wavelength would be nil when the difference spectra of the admixture of theophylline and phenobarbitone is recorded. Thus the measurements made at these wavelengths would be a function only of concentration of one of the component. Although the solubility was not very high, distilled water instead of a solvent like methanol was used for the preparation of the stock solutions because of the slight solubility of ephedrine resinate in methanol and its subsequent interference in the estimation.

The heights of the zero-order difference spectra (δA) at the wavelengths of 291 and 251nm were found to be proportional to the concentration of 10-40 mcg/ml of theophylline and 5-30 mcg/ml of phenobarbitone respectively.

The statistical analysis had been done on the data of pure drug solutions, their admixtures as well as commercial samples.

The small standard deviation values associated with the determination (Tables 23 and 24) indicated the high level of precision of the proposed method as well as the independence of one drug in the absorption measurement of the other since the deviation occurred only in the third decimal place when compared to the change in the absorbance (δA) from point to point in the calibration range. The negligible intercepts of the equations indicated regression through or close to the origin at the chosen wavelengths.

Table 23. Selectivity of the Method for the Determination of Theophylline in the Presence of Phenobarbitone by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) THE	PHN	Mean value of [a] absorbance (δA) (291nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
10	0	0.1505 ± 0.0032	2.11	0.0010	101.94	3.48	0.47
15	0	0.2290 ± 0.0037	1.61	0.0012	99.64	3.48	0.35
20	0	0.3040 ± 0.0031	1.00	0.0009	99.65	3.48	0.50
25	0	0.3805 ± 0.0037	0.98	0.0012	99.26	3.48	0.35
30	0	0.4526 ± 0.0028	0.63	0.0009	99.97	3.48	0.62
35	0	0.5279 ± 0.0033	0.62	0.0010	99.88	3.48	0.44
40	0	0.5995 ± 0.0030	0.51	0.0009	100.42	3.48	0.54
10	17	0.1503 ± 0.0031	2.08	0.0010	100.94	3.48	0.50
15	17	0.2259 ± 0.0024	1.05	0.0008	100.39	3.48	0.84
20	17	0.3039 ± 0.0035	1.14	0.0011	99.32	3.48	0.39
25	17	0.3769 ± 0.0032	0.86	0.0010	99.99	3.48	0.47
30	17	0.4531 ± 0.0031	0.68	0.0010	99.74	3.48	0.50
35	17	0.5292 ± 0.0032	0.61	0.0010	99.58	3.48	0.47
40	17	0.5989 ± 0.0042	0.69	0.0013	100.53	3.48	0.27

THE - Theophylline PHN - Phenobarbitone

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(5,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 24. Selectivity of the Method for the Determination of Phenobarbitone in the Presence of Theophylline by Difference Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] absorbance (δA) (251 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
THE	PHN					Crit	Calc
0	5	0.1325 ± 0.0034	2.59	0.0011	102.06	3.63	1.75
0	10	0.2713 ± 0.0032	1.18	0.0010	99.54	3.63	1.97
0	15	0.4029 ± 0.0041	1.02	0.0013	100.49	3.63	1.20
0	20	0.5469 ± 0.0026	0.47	0.0008	98.68	3.63	1.97
0	25	0.6747 ± 0.0032	0.47	0.0010	99.97	3.63	1.97
0	30	0.8054 ± 0.0032	0.39	0.0010	100.49	3.63	1.97
25	5	0.1301 ± 0.0033	2.49	0.0010	102.27	3.63	0.83
25	10	0.2691 ± 0.0036	1.33	0.0011	99.55	3.63	0.69
25	15	0.4037 ± 0.0031	0.77	0.0010	99.76	3.63	0.94
25	20	0.5399 ± 0.0026	0.48	0.0008	99.56	3.63	1.33
25	25	0.6746 ± 0.0026	0.38	0.0008	99.67	3.63	1.33
25	30	0.8034 ± 0.0026	0.32	0.0008	100.48	3.63	1.33

THE - Theophylline PHN - Phenobarbitone

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

The coefficient of variation values (Tables 23 and 24) were less and the standard error (which is the standard deviation of the mean) values were also low.

The percentage ratio of the residuals in tables 23 and 24 showed a random scatter in case of both pure drug solutions as well as their admixtures . In addition, the F test for non-linearity which is a quantitative test for non-linearity [4] was done and the results have been presented in tables 23 and 24. If a linear relationship holds, the standard deviation of the residuals (standard error of estimate, Sy) would represent an estimate (with (n-2) degrees of freedom) of the standard deviation of the sample. Therefore, we would have an evidence of non-linearity if we can show that Sy has too large a value to be compatible with the sample estimate (Ss).The values in tables 23 and 24 show that the calculated F values were less than that of critical values at 5% level of significance and evidence the linear relationship. Similarly, the other F test results which was based on mean square due to regression and mean square about the regression clearly showed the non-linearity since the calculated F values were far larger than the critical values leading to rejection of null hypothesis (Table 25).

The regression equations of the pure drug solutions and those of admixtures (Table 25) were similar. This similarity as well as the correlation coefficient values 0.9998-0.9999 for all four series of solutions indicated the non-interference of one drug in the estimation of the other. The co-efficient of determination (which is ratio of the sum of squares due to regression to the sum of squares about the mean) values ranged from 99.97 to 99.99 indicating that this much of variation in the absorbance was accounted for by the concentration of the particular drug in the solutions. A comparison of T test values at a significance level of 5% showed that the calculated values are far larger than the critical values obtained from the t

Table 25. Regression Analysis of Theophylline and Phenobarbitone Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (291 nm for THP and 251 nm for PHN)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	THP	PHN				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	10-40	0	y = 0.0149x + 0.0039	0.9999	99.98	6.61	31809	2.57	178	0.0001	0.0023	0.0022
Series B	10-40	17	y = 0.0150x + 0.0016	0.9999	99.99	6.61	33691	2.57	183	0.0001	0.0022	0.0022
Series C	0	5-30	y = 0.0269x + 0.0004	0.9998	99.97	7.71	15812	2.48	125	0.0002	0.0042	0.0045
Series D	25	5-30	y = 0.0270x - 0.0018	0.9999	99.98	7.71	34773	2.48	186	0.0001	0.0028	0.0030

THN - Theophylline PHN - Phenobarbitone [a] Based on 7 and 6 calibration values of theophylline and phenobarbitone respectively; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 7 for theophylline and 6 for phenobarbitone

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 7 for theophylline and 6 for phenobarbitone

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance* and *x* is the *concentration*

table and confirmed the existence of strong positive correlation [5]. The standard error of slope and intercept are the standard deviation values of slope and intercept and the standard error of estimate is the standard deviation value of residuals of y on x line indicating the precision of the fit by regression. This standard error of estimate was less, when compared to the typical change in δA value from point to point in the calibration curve based on the regression equations for pure admixtures [5].

The table 26 gives the actual values of the pure theophylline and phenobarbitone as well as the value calculated from the regression line for admixtures. The standard error of prediction is also given in this table. The 95% confidence level concentration ranges presented in table 26 (calculated using the standard error of prediction values) show a narrow range. The assay results of commercial formulations have also been given in table 26. In the case of theophylline as well as phenobarbitone the estimation will be best at the mean point of the calibration which is 25 and 17 mcg/ml respectively (Tables 23-25). The estimation of theophylline was done using a solution of the tablet sample containing approximately 25 mcg/ml of theophylline for all the batches of the commercial formulation. The concentration of phenobarbitone was approximately 11.50 mcg/ml in these solutions. Although this was not the mean point of the calibration range of phenobarbitone (5-25 mcg/ml), the phenobarbitone was also estimated in these solutions since the concentration of 11.50 mcg/ml was within the calibration range. In order minimise the error of prediction while estimating phenobarbitone in the formulations, the phenobarbitone was also estimated by using solutions containing approximately 17 mcg/ml of phenobarbitone. These solutions contained theophylline at a concentration of approximately 36.90 mcg/ml and the theophylline also was estimated in these second set of solutions. The mean recovery values within the first set of solutions and second set of solutions did not show much variation in the estimation of theophylline as well as phenobarbitone. The confidence limit ranges in the table 26 were also not much different between the solutions which were

Table 26 Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Theophylline and Phenobarbitione by Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})			Label Claim (mg/tablet)			Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	THP	PHN	EPH	THP	PHN	EPH	THP	PHN	THP	PHN
Pure Drug Admixture	25	17	19	----	-----	------	99.76	99.35	99.57-99.94	99.18-99.51
Brand A (Batch 1)	25	11.50	---	65	30	50	99.04	99.56	98.85-99.22	99.37-99.74
Brand A (Batch 2)	25	11.50	---	65	30	50	100.12	98.95	99.93-100.30	98.76-99.13
Brand A (Batch 3)	25	11.50	---	65	30	50	99.20	98.60	99.01-99.38	98.41-98.78
Brand A (Batch 1)	36.90	17	---	65	30	50	99.91	99.35	99.63-100.15	99.18-99.51
Brand A (batch 2)	36.90	17	---	65	30	50	100.62	98.82	100.37-100.86	98.65-98.98
Brand A (Batch 3)	36.90	17	---	65	30	50	100.08	98.23	99.83-100.32	98.06-98.39

THP - Theophylline PHN - Phenobarbitone EPH - Ephedrine (resinate)

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 5 degrees of freedom for theophylline and 4 degrees of freedom for phenobarbitone

estimated at the mean point of their calibration as well as at a concentration corresponding to the ratio in which they are present in commercial formulations. Since ephedrine as resinate was not soluble in water it was not indicated under the composition of solution in table 26 although it had been mentioned under the label claim.

Thus, although the proportion of the drugs in commercial formulations permit the estimation of both theophylline and phenobarbitone using the same solution by difference spectrophotometry in the case of all the four Brands, it would be advisable to estimate theophylline using a concentration of 60mcg/ml if the errors are to be minimal.

The 95% confidence level ranges of concentration predicted from a regression equation uses the standard error of prediction which is minimal at the mean point of calibration [5]. The results of the estimation of the drugs in pure admixture (Table 26) had been done to find out the concentration as predicted from the regression equations obtained earlier since these equations were fit by the least square method. The results indicated a mean recovery of 99.76 and 99.35% for theophylline and phenobarbitone respectively which was impressive. The assay results show a mean recovery value of 99.04 to 100.62 for theophylline in the commercial formulations. These values were conforming to the U.S.P. 1995 limits of 90 - 110% for the combination as well as the limits of 94 to 106% for single component tablets. Similarly, the assay results show a mean recovery range of 98.23 to 99.56% for phenobarbitone which was conforming to the limits for individual tablets in I.P 1985 (94-106%) as well as in U.S.P. 1995 (90-110%). In case of both the drugs, the 95% confidence level ranges themselves fall within the prescribed limits (Table 26). The detection limits at 5% level of significance were found to be 0.50 and 0.40 mcg/ml for theophylline and phenobarbitone respectively.

The stability of the standard solutions of theophylline (25 mcg/ml) and phenobarbitone (17 mcg/ml) in 0.1M HCl and 0.1M NaOH (stored prior to

scanning in low actinic flasks at 24-27°C) were monitored spectrophotometrically (at the wavelengths of 291 for theophylline and 251nm for phenobarbitone) for a period of two hours and were found to vary by the following absorbance units (AU): 0.1N HCl solution of theophylline and phenobarbitone by ±0.007 and ± 0.009 and 0.1M NaOH solutions of theophylline by ±0.008 and ±0.011 respectively. The pKa values of theophylline was 8.6 whereas that of phenobarbitone is 7.5 [111] and hence the pH of 0.1M HCl (approximate pH 1.0) and that of 0.1 M NaOH (approximate pH 13.0) were at least 1.5 pH units away from the pK_a values of the drugs. Therefore, small variations in the pH of the solvents did not lead to appreciable changes in the absorbance values since these pH values produce about 90% of the individual species of the drugs in the respective solvents [76].

Thus the proposed method of determination of theophylline and phenobarbitone was found to be accurate and precise and may be used for the estimation of the drugs in commercial formulations without prior separation of each other. The results in table 26 show that the interference from the formulation matrix is not likely in the assay of the drugs.

5.00 ULTRAVIOLET SPECTROPHOTOMETRY

DERIVATIVE SPECTROPHOTOMETRY

5.01 INTRODUCTION

As mentioned in the introduction to difference spectrophotometry, the practical limitation derived from variable and non-specific spectral interferences has been treated by mathematical and graphical methods, ranging from very simple to tedious and lengthy procedures according to the shape of irrelevant absorption spectrum. The Mortan's three point correction method [29] has been used extensively when there is an unknown or variable contribution to the measured absorption.

However, this method assumes linearity of irrelevant absorption over the wavelength range.

The simultaneous equation method as applied by Vierordt [29] involves the use of extinction measurements at a pair of suitable wavelengths but cannot be applied with high precision to binary mixtures of components which do not exhibit well separated peaks.

In general, for majority of mixtures, accuracy of the order of ±2% were obtained provided (i) the absorption curves of the two components are sufficiently different (ii) a wavelength is available at which the component in question contributes a reasonable proportion of the mixture's total absorption and (iii) the amount of irrelevant absorption is small. In practice, the last requirement places a considerable restriction upon the application of the method, since the term, "irrelevant absorption," must also include variations of the absorbing impurity content of the components, which occur between batches. Thus, if the mixture has been prepared from batches of material that differ from

the "reference" samples used to establish the assay coefficients, the overall effect is equivalent to the introduction of irrelevant absorption and the results suffer accordingly [79]. In cases, where the general shape of the impurity absorption curve is known, even a cursory comparison usually shows a marked difference in shape between the impurity absorption curve and the curve of the component whose concentration is sought [79]. Hence Glenn proposed the use of orthogonal functions to extract some information from an absorption curve which was fundamentally related to its overall shape, which would, in most instances, help in coping with irrelevant absorption. In adapting a traditional method, i.e. the use of simultaneous equations, to the use of orthogonal functions, the essential modification occurs at the final stage of calculation, when it is necessary to substitute suitable analogues for the entities, "extinction" and "wavelength". Thus, extinction is replaced by coefficient of an orthogonal function and wavelength by orthogonal function over a specified range (or set) of wavelengths. Once these substitutions have been made (in terms of Legendre polynomials) the orthogonal functions may be readily incorporated into the usual methods and equations of spectrophotometric analysis [152-156].

Abdine et al. [153] who investigated the applicability of Glenn's method [79] to spectrophotometric analysis of drugs in tablets acknowledge that the choice of a proper polynomial, number of points, wave length range and intervals ought to be based on specific absorption patterns.

The graphic correction or linear plot method [78] permits data to be taken at multiple wavelengths to generate linear plots from which the concentrations of the drugs in two or three component mixtures can be determined. This method is based on the assumptions of adherence to Beer's Law and additivity of absorbances and can be applied only if

these conditions are met. Another method for simultaneous deter mination of compounds by spectrophotometry is the absorbance ratio method [77].

The background absorption due to various excipients may also be eliminated by difference spectrophotometry [76] which had been discussed and applied in the section 4.00 of this report for the estimation of drug combinations.

LINEAR PLOT OR GRAPHICAL METHOD

For a two component (A and B) mixture, the linear plot method [83] uses data at multiple wavelengths to generate linear plots according to the equation:

$$\frac{A_t}{E_A} = C_A + \frac{E_B}{E_A} C_B$$

where A_t is the absorbance of the mixture, E_i and C_i are the molar absorptivity and molar concentration of species 'i' with the absorption and absorptivity referring to a common wavelength.

Thus when a plot of A_t / E_A vs E_B/E_A with all quantities evaluated at the same wavelength is made, the concentration C_B may be obtained from the slope of the plot and C_A can be evaluated by extrapolation of the plot to $E_B/E_A = 0$; alternatively the sum $C_A + C_B$ may be determined by extrapolation at the point where $E_B/E_A = 1$.

This method has been used for the estimation of drugs in 0.1N HCl and 0.1N NaOH solutions in the formulations.

Absorbance Ratio method

The absorbance ratio method uses the ratio of absorbance of the substances at the wavelength of maximum absorption to that of the absorbance at the isoabsorptive point (Q value) for the estimation [84].

In this method for analysis of a binary mixture, the spectral characteristics of the individual components (usually in 0.1M HCl or 0.1M NaOH) was initially determined.

The next step was to determine the existence of isoabsorptive point for the mixture. An isoabsorptive point may be defined as the wavelength at which two dissimilar substances have identical absorptivity values, the solvent being the same for both substances [77]. This may be easily located by finding out the wavelength at which the absorbance of the components are equal, i.e. where the spectra of the two components cross each other provided the solvent is same and the initial concentrations of the two substances are equal [77]. Once the isoabsorptive point has been located, the two wavelengths to be used in the analysis have to be chosen. If we consider a binary mixture of A and B, to estimate A, two wavelengths have to be chosen. These will usually be the wavelength at which A exhibits maximum absorption (λ_{Am}) and the isoabsorptive point (λ_{Is}).

Next the absorbancy ratio values (Q values) have to be calculated. This is calculated by dividing the absorbancy of solution A at λ_{Am} by the absorbancy at the isoabsorptive wavelength, namely, λ_{Is} ($Q(\lambda_{Am:Is})$). The next step is to plot the Q curve which refers to the curve resulting from a plot of Q values vs. the relative concentration of one of the two components in the binary mixture. Such a curve is constructed from data

accumulated on mixtures containing known amounts of A and B and the equation of the plot is determined by subjecting the data to the method of least squares [84].

The equation for component A will be of the form

$$Q(\lambda_{Am:Is}) = (\text{Slope of the curve})\, F_A + (\text{Intercept of the curve})$$

Once the equation for Q curve is known, the component A in an unknown mixture may be determined by substituting the $Q(\lambda_{Am:Is})$ value for the unknown mixture in the equation and solving the equation for F_A. The amount of component B in the mixture may be determined by using an equation similar to that above but expressed in terms of the absorbance values of component B at its wavelength of maximum absorption (λ_{Bm}) and the isoabsorptive wavelength.

The final accuracy of this method will depend on a number of factors such as accuracy of absorbancy measurement, relative concentrations of the active ingredients in and nature of the pharmaceutical being analysed and spectral characteristics of the components in the mixture [77]. More important among these is the proportion of the active ingredient in the pharmaceutical preparation being analysed. If one of the ingredient is present at a very low proportion, then the precision with which this ingredient could be determined would be very low. This may not be true only in cases where the molar absorptivity of the minor component is very high when compared to the major component. Under these circumstances, the absorption shown by the minor ingredient would be adequate enough to give accuracy and precision.

Although difference spectrophotometry is suppose to take care of the interference from the matrix, it may not be useful when the interferents change their spectral pattern under the conditions used for the recording of the difference spectra (such as change of pH) since the absorption due to the interferents will not get exactly cancelled if they have different spectral shapes in the acidic and basic solutions. The presence of excipients in large quantities in the final dilution for determination may sometimes lead to interference even in difference spectrophotometry. Examples of such excipients include lactose, dicalcium phosphate and polyvinylpyrrolidone which give different type of absorption spectra in acidic and alkaline solvents [153] leading to non-cancellation of the absorbance which would occur automatically in the difference spectrophotometry if the absorption pattern remains the same in b the solvents.

Under these circumstances, the technique of difference spectrophotometry cannot be used with accuracy and precision because of the interference of the excipients. The above mentioned excipients do interfere even at concentration ranges of 10-50mcg/ml which is the likely range of concentration to be present in the final dilutions of commercial formulations such as tablets. Hence cases of formulations which use these excipients in such proportions as to yield an excipient concentration of above 10mcg/ml in the final dilutions should be carefully evaluated for likely interference. The absorption values of the excipient solutions may vary from batch to batch as well as depending on grades [153].

The requirements of a satisfactory analytical method are many, but certainly selectivity must be counted being the most important. Often the

measurement techniques that are being used lack the inherent selectivity to allow straightforward application to the kind of highly complex materials for which the analytical chemist is often called for to develop analytical methods. The separation procedures involving techniques such as chromatography etc. are useful and indeed essential for such cases. But there are applications for which, for reasons of speed and simplicity a more direct approach will be desirable. Thus, there has always been interest in techniques that can improve the selectivity of measurement methods themselves. Among the conceptually simple of these is the derivative spectrophotometry.

Essentially, in the derivative spectra the ability to detect and to measure minor spectral features is considerably enhanced. This enhancement of characteristic spectral detail can distinguish very similar spectra and follow subtle changes in a spectrum. Moreover, it can be of use in quantitative analysis when it is desired to measure the concentration of an analyte whose peak is obscured by a larger overlapping peak due to something else in the sample [157, 158]

In derivative spectrophotometry the absorbance (A) of a sample is differentiated with respect to wavelength (λ) to generate the first, second or higher order derivatives

$A = f(\lambda)$ (Zero order)

$dA/d\lambda = f'(\lambda)$ (First Derivative)

$d^2A/d\lambda^2 = f''(\lambda)$, etc. (Second derivative)

Derivative spectra often yield a characteristic profile, where subtle changes of gradient and curvature in the normal (zero order) spectrum are observed as distinctive bipolar features (figure 20).

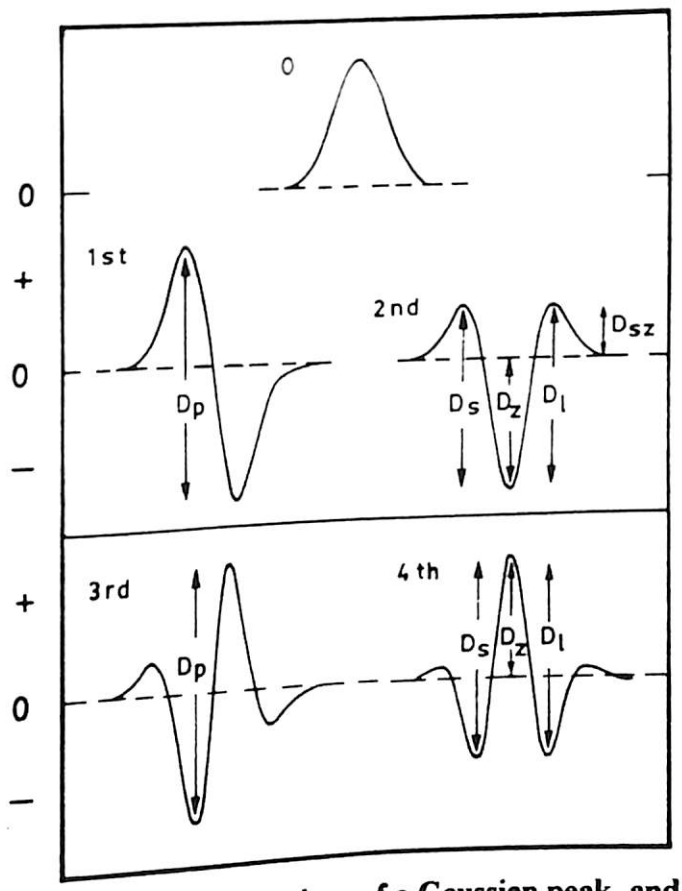


Fig 20. First to fourth derivatives of a Gaussian peak, and some graphical measures of derivative amplitude (D). D_p, peak-to-peak; D_s, peak-to-satellite at short wavelength; D_z, peak- to -derivative zero; D_l peak- to -satellite at long wavelength; D_{sz}, satellite peak-to-derivative zero

The first derivative of an absorption spectrum, represents the gradient at all points of the spectrum and can be used to locate `hidden peaks', since $dA/d\lambda = 0$ at peak maxima. However, the second and higher even-order derivative are potentially more useful in analysis [16]

The even-order derivatives are bipolar functions of alternating sign at the centroid (i.e. negative for 2^{nd}, positive for 4^{th},etc.), whose position coincides with that of the original peak maximum (figure 20) To this extent, even-derivative spectra bear a similarity to the original spectrum, although the presence of satellite peaks flanking the centroid adds a degree of complexity to the derivative profile. A key feature is that the derivative centroid peak width of a Guassian peak decreases to 53%, 41% and 34% of the original peak width, in the second, fourth, and sixth orders respectively. This feature can increase the resolution of overlapping peaks. However, the increasingly complex satellite patterns detract from resolution enhancement in higher derivative spectra. An important property of the derivative process is that broad bands are suppressed relative to sharp bands [157, 158, 80]. This effect increases with increasing order of the derivative since the amplitude (D_n) of a Guassian peak in the n^{th} derivative is inversely related to the original peak width (W), raised to the n^{th} degree

$$D_n \alpha (W)^{-n}$$

Thus, for two coincident peaks of equal intensity, the nth derivative amplitude of the sharper peak (x) is greater that that of the broader peak (y) by a factor which increases with derivative order

$$\frac{D_{n,\,x}}{D_{n,\,y}} = \frac{(W_y)^n}{(W_x)^n}$$

This property leads to selective rejection of broad, additive, spectral interferences such as Rayleigh scattering.

If the Beer-Lambert Law is obeyed, i.e.

$$A = \varepsilon bc$$

then

$$\frac{dA}{d\lambda} = \frac{d\varepsilon}{d\lambda} . b . c$$

$$\frac{d^2A}{d\lambda^2} = \frac{d^2\varepsilon}{d\lambda^2} . b. c$$

and similarly for higher derivatives, where ε = molar absorptivity (litre/ mol/cm), b = cell path-length (cm), and c = concentration (mol/litre) [16] .

Thus, for second-order derivative spectrophotometry, if the absorbance follows the Beer-Lambert relationship, the second derivative at any wavelength λ, is related to concentration by the following equation, as per the B.P. 1993.

$$\frac{d^2A}{d\lambda^2} = \frac{d^2A_{(1\%,\,1\,cm)}}{d\lambda^2} \times c\,d$$

where A = the absorbance at wavelength λ, $A_{(1\%,\,1\,cm)}$ = the specific absorbance at wavelength λ ,c = the concentration of the absorbing solute expressed as a percentage w/v and d = the thickness of the absorbing layer

For quantitative work, the amplitude of a derivative peak can be measured in various ways (figure 20). Although the true derivative amplitude is that measured with respect to the derivative zero, it may also be possible to record the amplitude with respect to a satellite in the spectrum which affords an extra degree of suppression of interference from extraneous substances. These peak-to-baseline and peak-to-peak measurements are called as graphical measurements and prior to their use it should be established that the graphical derivative adopted fulfills the analytical criteria of linear response with concentration, regression through or close to the origin, independence from interfering substances, and optimum precision [80].

The work by O'Haver and Green [159] on the numerical analysis of derivative spectrophotometry for the quantitative analysis of mixtures was based on assumption of analyte band overlap by a single interfering band. The authors have analysed analytical curves of zero, first and second order. They have reported the effect of systematic and random errors on the analyte band height of band pairs varying in terms of ratio of interfering band height to analyte band height, ratio of interfering band-half width to analyte half width and ratio of band separation to half width of analyte band . The authors reported that the derivative measures represent a significant improvement in total error compared to the normal measure (zero order) by at least a factor of three and usually by much more. The authors have concluded that derivative measures treated in the work [159] on guassian peaks would be useful for the reduction of band overlap errors in quantitative analysis if the systematic error caused by the overlap is large compared to random noise errors and if the interfering band is either known or constant (in which case zero-crossing measure are useful) or is broader than the analyte band (in which case the graphical measures are useful). This work is based on the

assumptions of overlapping of analyte band by a single interfering band and the total spectrum which is measured is the linear sum of the analyte and interfering band. But in the case of binary mixtures in pure or dosage forms on which the work has been done in this report, the matrix usually contains, in addition to two analytes, more than one excipient. In addition, when the quantitation of two analytes ought to be done, it would be very convenient to use the zero crossing point of derivative of one drug for the assay of the other and vice versa since measurements made at the zero-crossing point of the derivative spectrum of one of the two components would be a function only of concentration of the other component [160-166]. The pharmaceutical excipients such as lactose, PVP and dicalcium phosphate exhibit a broad band zero order spectra in aqueous pH 1.0 and pH 13.0 buffers in the wavelength region of 400-250nm when compared to the zero order spectra of the drugs which have been investigated in this report. Although Haver and Green [159] have recommended the use of graphical measurement (figure 20) for such band pairs where the band width of interfering substance is very large when compared to analyte band width due to the absence of systematic errors in graphical measurements, zero crossing method has been used for the various assays developed in the present work for the following reasons.

Firstly, as mentioned earlier, the recommendations of Haver and Green is based on certain assumptions which do not hold good for binary mixtures containing excipients except under certain special circumstances [31,33] such as a fortuitous juxtaposition of the spectra in such a fashion that at the wavelengths where the graphical measurement (peak-to-baseline and peak-to-peak) are made for one analyte, the contribution of derivative spectra of the other analyte should be zero either due to complete absence or negligible absorption [162]. Thus the work by Morelli [162] reports graphical measurement

for the assay of aztreonam in the presence of L-arginine which had been made possible by the fact that the value of the second derivative of L-arginine had been near zero at the wavelengths at which these graphical measurements have been made for the assay of aztreonam.

Otherwise, if the derivative spectrum of L-arginine have exhibited large $d^2A/d\lambda^2$ values, these values would have contributed to the graphical measurement of the derivative spectra of aztreonam leading to large errors. Similarly, the work by Morelli [164] reports graphical measurements which had been rendered possible by fortuitous juxtaposition of the derivative spectra of the vitamins in the combination investigated. Secondly, the pharmaceutical formulations may contain more than one uv absorbing excipient in which case the recommendations of O'Haver and Green [159] do not hold good.

The authors have suggested that the zero crossing measurements may be affected by errors due to variations in the exact abscissa (wavelength) value when an electronic or mechanical tachometer derivative attachment being used for the production of the derivative values. In general, methods for generating derivative spectra fall into two classes. These are optical methods, which operate on the radiation beam itself, and electronic or digital methods operating on the photometric detector output. The electronic device generates the required derivative as a function of time as the spectrum is scanned at constant speed and therefore the derivative amplitude varies with the scan speed, slit-width, and gain.

Alternatively, a microcomputer can be used, employing one of a number of digital algorithms to produce smoothed derivative spectra. This is done in real time or by post-run processing of the digitised spectrum. The digital approach is increasingly employed due to the widespread adoption of microprocessors for instrument control, data

handling and processing [80]. Thus the errors due to variation in the exact abscissa (wavelength) have been eliminated by use of digital algorithms to produce derivative spectra from zero order spectra as well as almost perfect scan-to-scan wavelength reproducibility as a result of use of microprocessors in spectrophotometers. In practice, during an assay by derivative spectrophotometry, that measurement which exhibits the best known response to analyte concentration which gives a zero intercept on the ordinate axis of the calibration graph and which is least affected by the concentration of any other componenet is selected [80]. Thus in the various mixtures which have been assayed by derivative method in this report, the method of measurement (graphical or zero crossing) have been decided upon on the basis of the zero crossing points of the analytes as well as rectilinearity. One problem associated with the zero-crossing measurements sometimes is that of the measurement of the derivative spectra of one analyte at wavelengths (zero crossing point of the other analyte) where the slope of derivative spectra may be steep leading to large errors with small changes in wavelength [164] making it a little hazardous . However, this kind of measurement is not uncommon [160-164, 166]. Moreover, the method may be used if the high degree of accuracy and reproducibility of the method are evidenced by statistical evaluations.

Another problem associated with zero crossing measurements is that if the width of the interfering band changes, then the zero crossing point of the interfering band would change resulting in errors which would depend on the amount of derivative value contributed at the wavelength of previous zero crossing . Thus, the bandwidth of the interfering band should be constant ideally. In the case of pharmaceutical excipients, the change in the bandwidth is not likely to affect the zero crossing point as can be seen from figures 21-30 which shows the second-order derivative spectra of lactose, pvp and indigocaramine. These

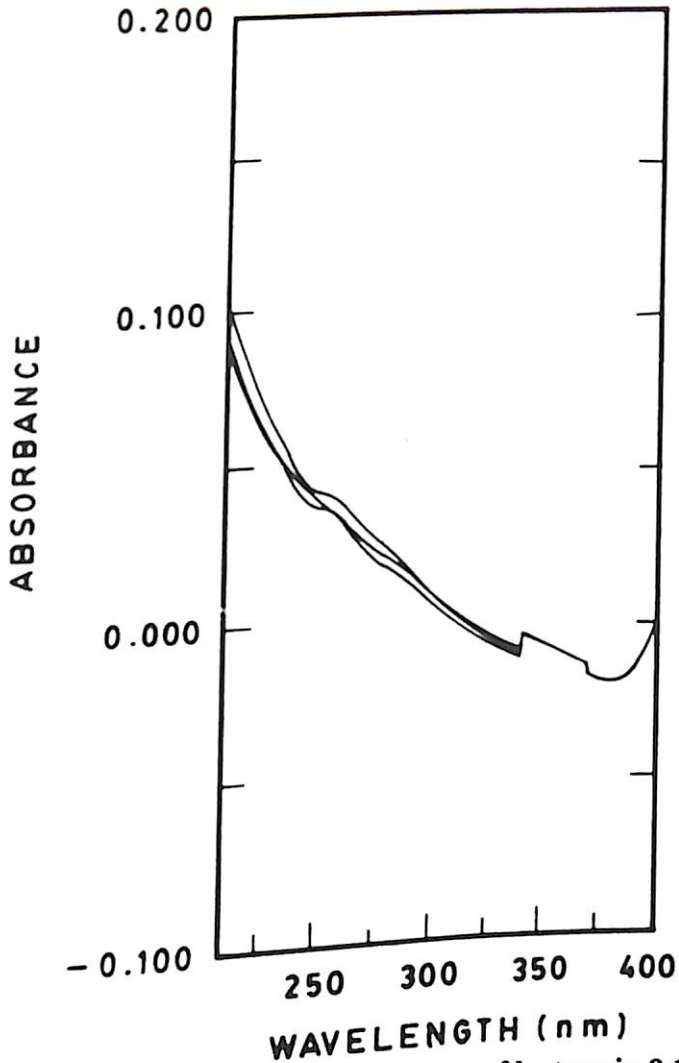


Fig 21. Normal absorption spectra of lactose in 0.1M HCl; concentration of lactose is 2, 4 and 8 mg ml^{-1}

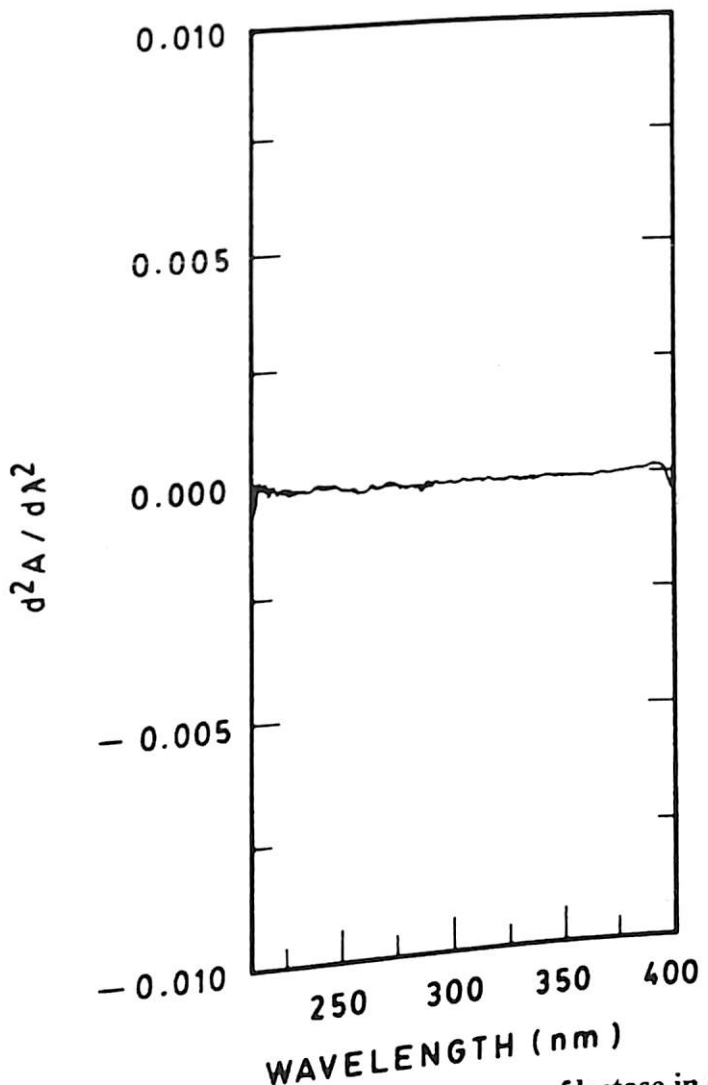


Fig 22. Second-order derivative spectra of lactose in 0.1M HCl; concentration of lactose is 2, 4 and 8 mg ml^{-1}

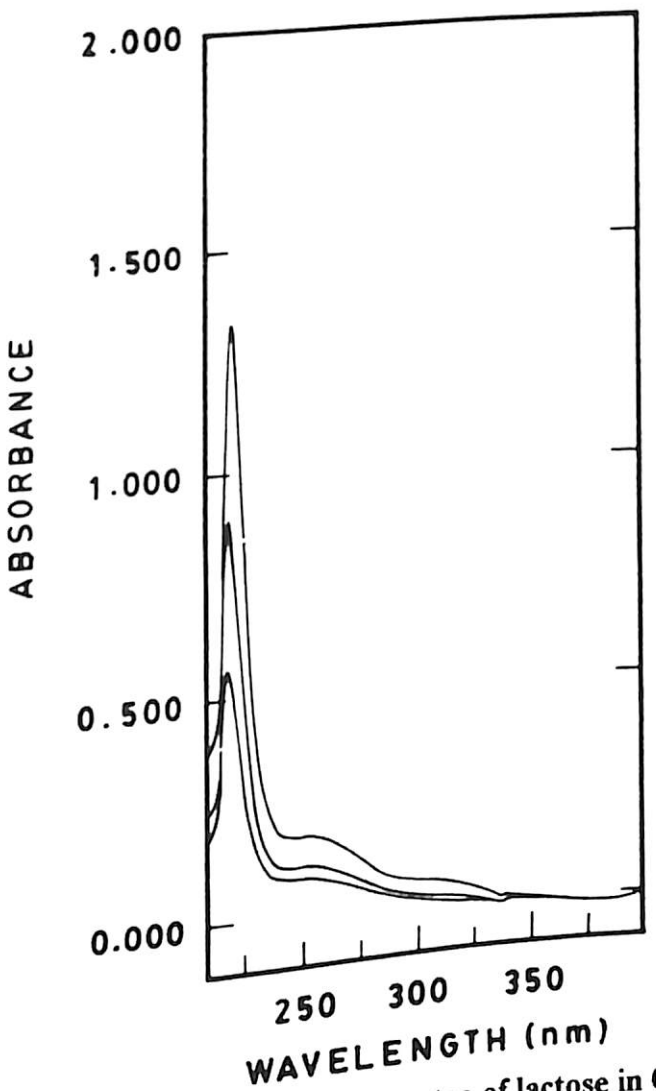


Fig 23. Normal absorption spectra of lactose in 0.1M NaOH; concentration of lactose is 2, 4 and 8 mg ml^{-1}

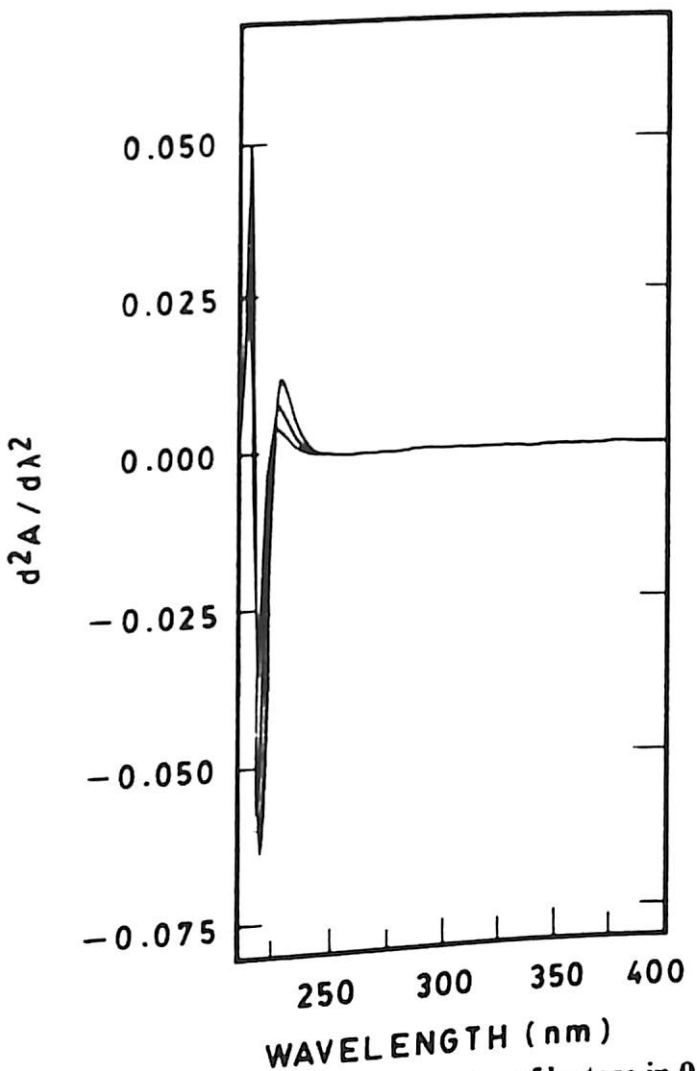


Fig 24. Second-order derivative spectra of lactose in 0.1M NaOH; concentration of lactose is 2, 4 and 8 mg ml^{-1}

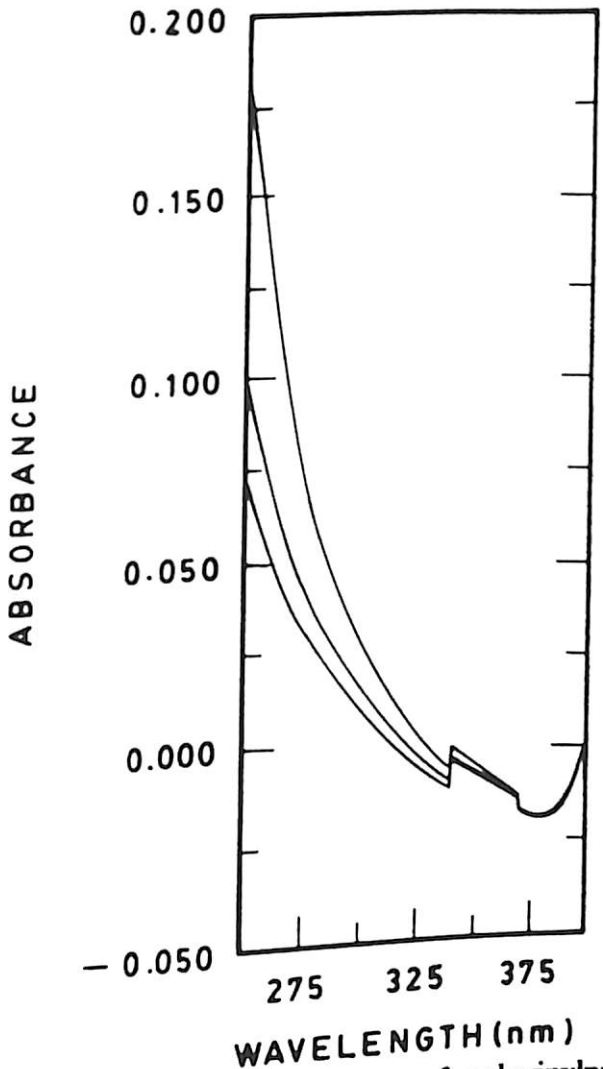


Fig 25. Normal absorption spectra of polyvinylpyrrolidone in 0.1M HCl; concentration of polyvinylpyrrolidone is 1, 2 and 4 mg ml^{-1}

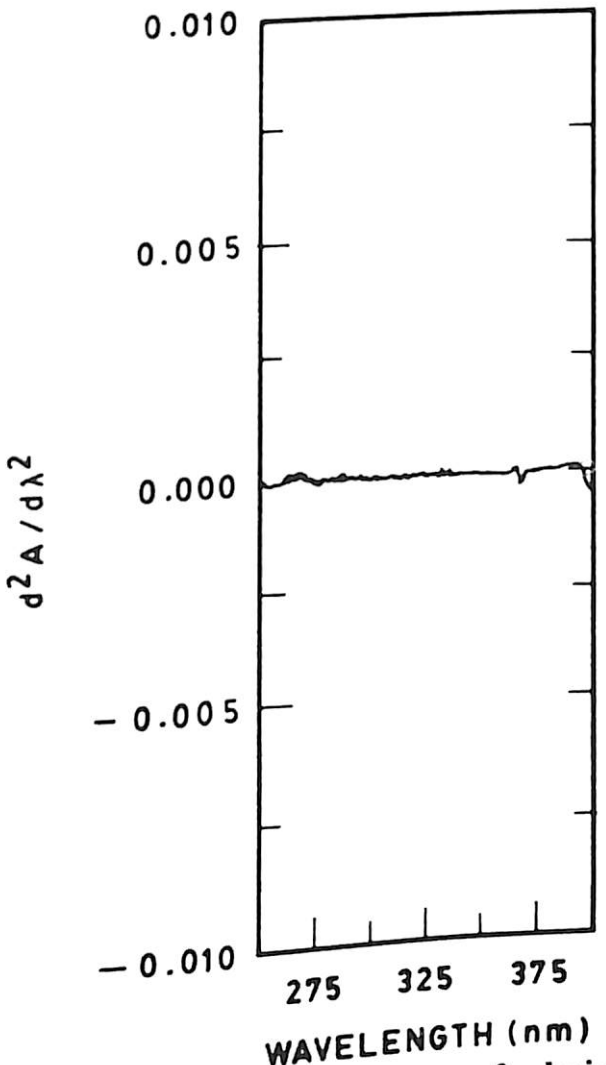


Fig 26. Second-order derivative spectra of polyvinylpyrrolidone in 0.1M HCl; concentration of polyvinylpyrrolidone is 1, 2 and 4 mg ml^{-1}

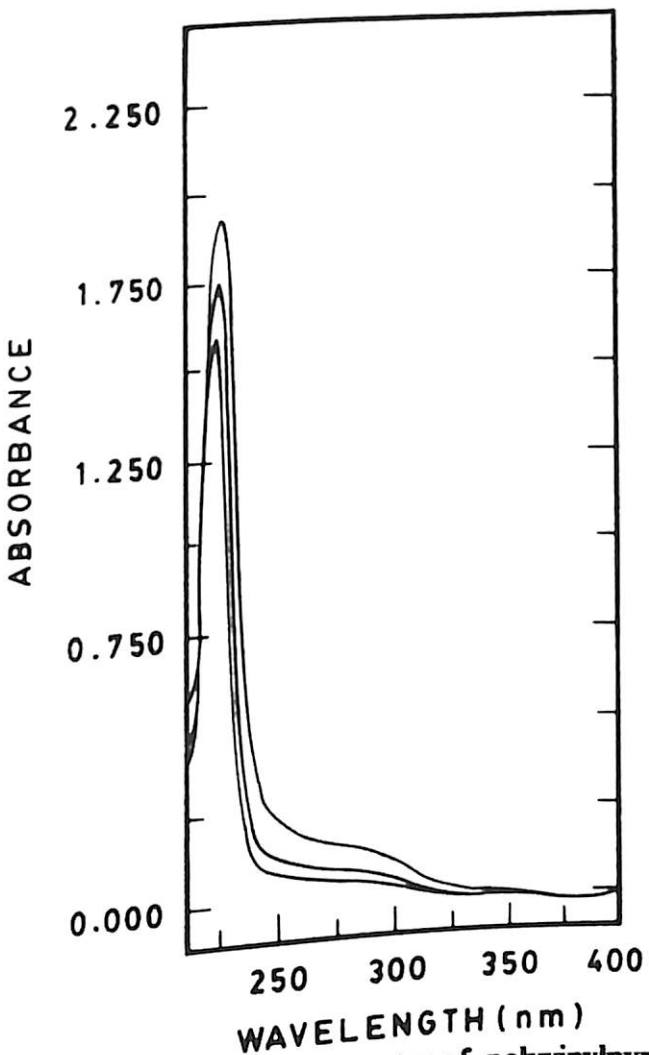


Fig 27. Normal absorption spectra of polyvinylpyrrolidone in 0.1M NaOH; concentration of polyvinylpyrrolidone is 1, 2 and 4 mg ml^{-1}

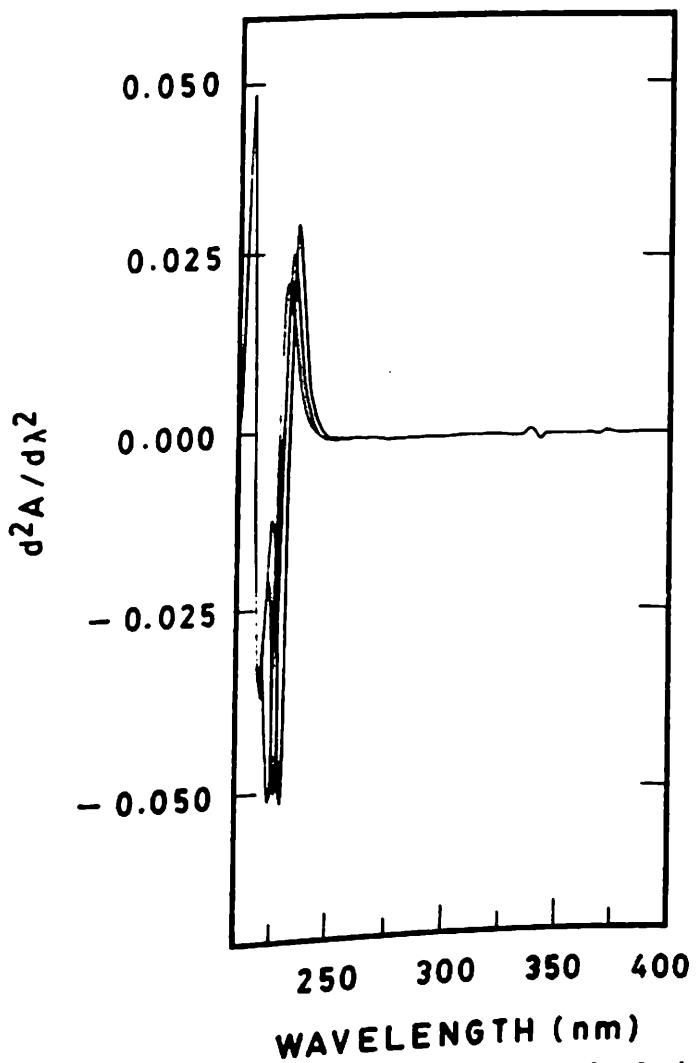


Fig 28. Second-order derivative spectra of polyvinylpyrrolidone in 0.1M NaOH; concentration of polyvinylpyrrolidone is 1, 2 and 4 mg ml^{-1}

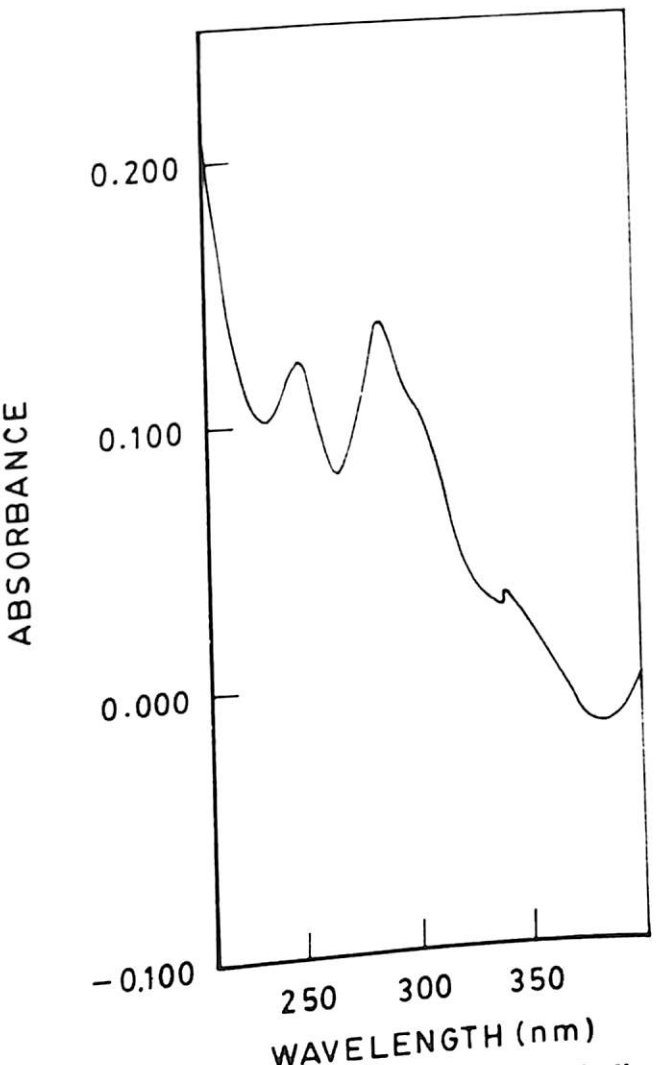


Fig 29. Normal absorption spectra of indigocarmine in 0.1M HCl; concentration : 4mcg ml^{-1}

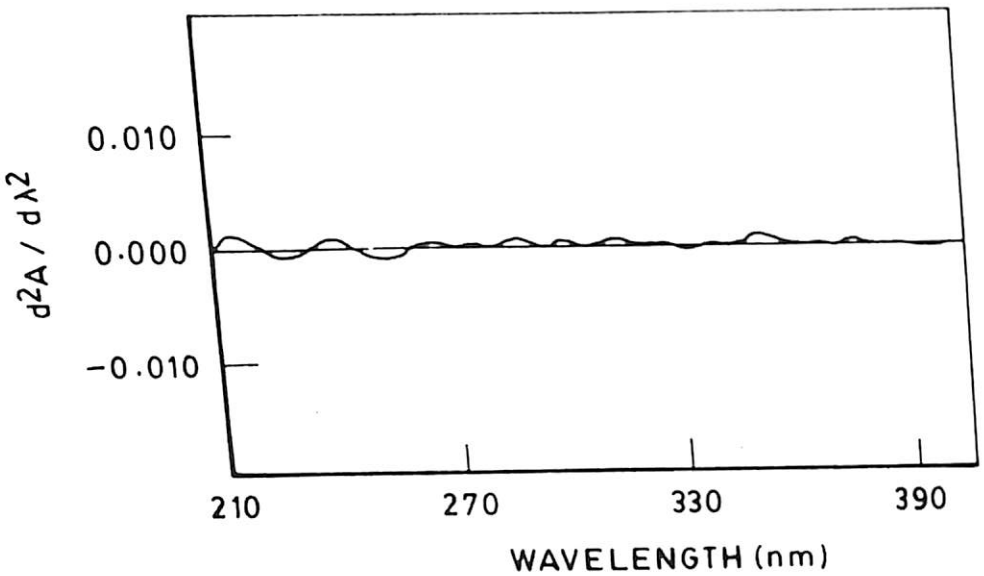


Fig 30. Second-order derivative spectra of indigocarmine in 0.1M HCl; concentration : 4 mcg ml^{-1}

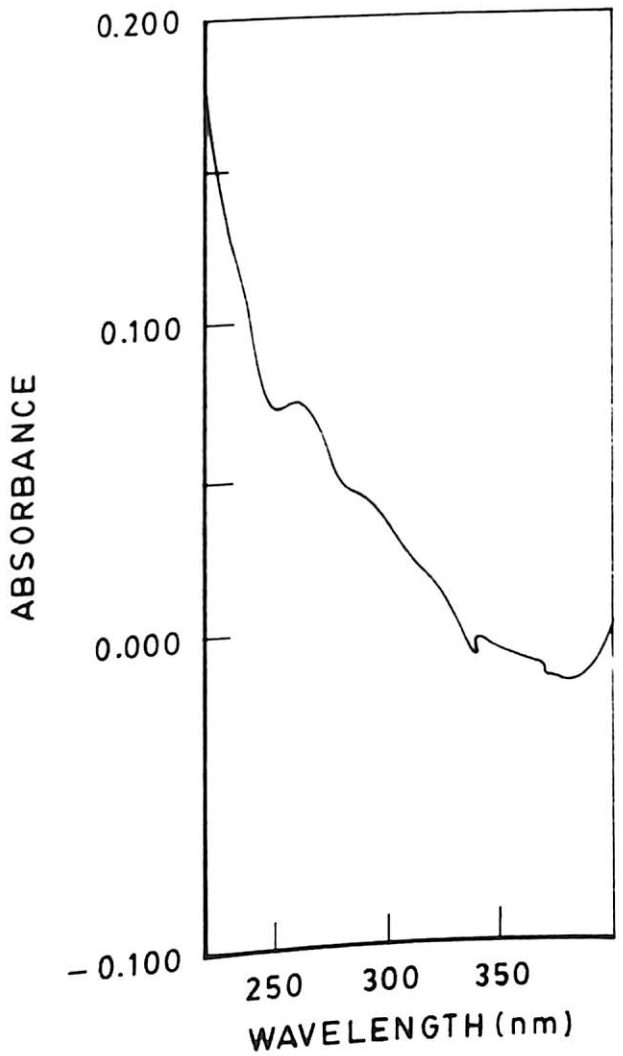


Fig 31. Normal absorption spectra of indigocarmine in 0.1M NaOH; concentration : 4 mcg ml^{-1}

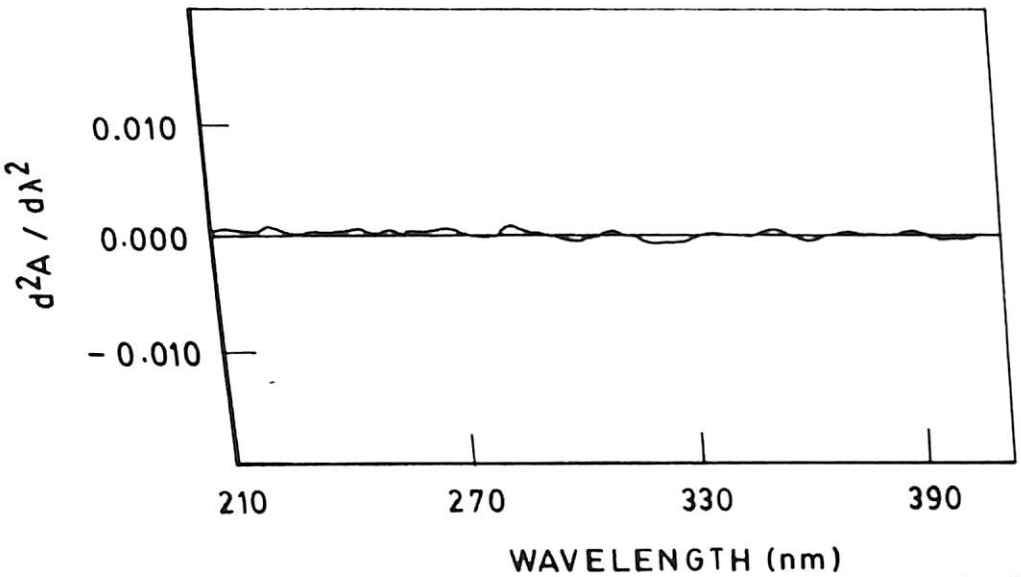


Fig 32. Second-order derivative spectra of indigocarmine in 0.1M NaOH; concentration : 4 mcg ml^{-1}

excipients were chosen on the basis of their wide use in tablet formulations. In most of the work in this section, these additives have been intentionally added to pure admixtures and the effect of them on the measurement of the derivative values had been studied. As can be seen from the figures, the second-order transformation of these common additives leads to a spectrum whose amplitude in negligible when compared to the derivative spectrum of many drugs as can been seen in the following pages. Although the second-order transformation is not effective in eliminating the interference at wavelengths below 250nm, the transformation is likely to be very effective in the estimation of drugs which show appropriate spectral patterns above 250nm. Even below 250nm, the amplitude of the interfering derivative spectrum may not be always as high as shown in the figures since it would also depend on the concentration in which these additives are finally present in the working solutions. This in turn would depend on the solubility of the interfering additive in the solvent used for the preparation of the stock solution and as well as in the solvent used for obtaining the derivative spectra. It would also depend on the proportion in which the additives have been used in the formulation.

Although transformation of a spectrum to its second or higher order derivative often yields a more highly characteristic profile than the zero order spectrum, the intrinsic information content of the data is not increased [80]. However, the derivative method tends to emphasise subtle spectral features by presenting them in a new and visually more accessible way. The method is generally applicable in analytical chemistry and can be used equally for resolution enhancement of electrochemical, chromatographic, or thermal analysis data. Usually, normal spectra recorded with a particular solvent is used for the production of the derivative spectra. This would involve digital conversion

of the data points of the normal spectra and is called as n-order derivative spectra where n refers to the order of transformation.

Derivative method can be successfully combined with difference spectrophotometry, to give second derivative- difference spectra, when enhanced discrimination against interfering substances and sharpened fine structural features are observed [80]. This would involve the use of difference spectra for second-order transformation and may be done by initially recording the zero-order difference spectrum by scanning the equimolar drug solutions in acidic and basic solvents and using the data points of these difference spectra to produce the second-order derivative spectrum using digital algorithms. This technique had been applied for the estimation of metronidazole with furazolidone and nalidixic acid, estimation of aspirin and dipyridamole and estimation of orciprenaline sulphate and bromhexine hydrochloride in this thesis work.

Derivative spectrophotometry has found significant application in clinical, forensic, and biomedical analysis. In forensic toxicology the suppression of the absorbance due to interfering substances by second derivative spectrophotometry is well demonstrated in studies on amphetamine in an homogenised liver extract [80].

A number of workers have reported the assay of drug by first, second and fourth order derivative spectrophotometry. These include assay of aromatic amino acids [166], progesterone [161], dicloxacillin sodium and ampicillin [160], ephedrine and pseudoephedrine by second and fourth derivative [167], vitamins [164], procyclidine [168], tryptophan and tyrosine [164], captopril and hydrochlorothiazide [169], ascorbic acid and acetylsalicylic acid [170], nortryptylline and perphenazine [171] and methylene blue and resorcinol [172]. The current B.P. 1993 also has

recognised the second-order derivative spectrophotometry as a method of assay of drugs.

DESIGN OF THE EXPERIMENT

Essentially, all the tablet formulations for which the second-order derivative or second-order derivative difference spectrophotometry has been applied were two component formulations.

The method of estimation (whether second-order derivative or second-order derivative difference) has been determined on the basis of the spectral shape as well as the occurrence of the shift of the peak of the normal spectra with change in pH. Thus in cases where the zero-order difference spectra may be recorded (metronidazole and nalidixic acid, metronidazole and furazolidone and aspirin and dipyridamole and orciprenaline sulphate and bromhexine hydrochloride),the second-order derivative difference has been tried and the results have been compared with the linear plot method and absorbance ratio method.

In the cases of atenolol and nifedipine, imipramine HCl and diazepam and diphenhydramine HCl and diazepam the spectral shifts did not favour the recording of the zero-order difference spectra and hence the second-order derivative spectrophotometry has been used.

The general experimental approach has been as follows:

a. To prepare standard solutions of the pure drugs at an appropriate concentration range

b. To check the rectilinearity of the concentration range in terms of the $d^2A / d\lambda^2$ values

c. To choose an appropriate concentration range so as to fulfill both the requirements of compliance of Beer-Lambert's Law as well as to suit the proportion of the drugs in commercial formulations which ought to be estimated by the method.

To prepare different series of the solutions (from stock solutions of pure drugs) for the construction of the calibration curve. The series of solutions were always prepared in such a fashion so as to be representative of both pure drugs as well as their admixtures to enable the comparison of the regression equations of pure drug solutions with that of the admixtures which would establish the selectivity of the chosen wavelength for the determination of the particular drug. Thus, the pure drug standard solutions are prepared in the concentration range showing linearity of derivative values and the drug admixture solutions (for comparison of the equations) were prepared by using the same concentration range of the drug in the presence of a constant concentration of the second drug to be estimated. This gives an opportunity to observe whether one drug is likely to interfere in the absorption measurement of the other and vice versa.

As mentioned in the introduction for derivative spectrophoto metry, the zero-crossing point of one drug has always been chosen as the wavelength for the estimation of the other and vice versa for the aforementioned reasons.

In order to assess the applicability of the derivative method to the estimation of drugs in formulations without interference from the additives which are normally present in the tablets, three such additives, namely lactose, polyvinylpyrrolidone and indigocarmine were selected. The concentration of lactose and pvp were maintained at 600 mcg/ml whereas the concentration of indigocarmine was kept at 4 mcg/ml. These concentrations were approximately chosen on the basis of the amount of

additives which may be present in the tablets and these additives were added to pure drug admixtures to study whether the second-order transformation is able to eliminate the interference due to the additives. This had been done by comparing the regression equations of the pure drug admixtures with those of the admixtures containing the added additives. In addition, wherever applicable, the results obtained by the second-order derivative or derivative difference spectrophotometry had been compared with the results obtained by the linear plot method and absorbance ratio method.

5.02 DETERMINATION OF ATENOLOL AND NIFEDIPINE, IN PURE ADMIXTURES AND TABLETS BY SECOND-ORDER DERIVATIVE SPECTROPHOTOMETRY

INTRODUCTION

The combination of atenolol and nifedipine as a tablet preparation has been introduced in the market for essential hypertension. The methods reported thus far for the estimation of atenolol include spectrophotometry [176-178], calorimetry and thermogravimetry [179], TLC [180], GLC [181] and HPLC [182-185]. The methods for nifedipine include titrimetry [186], polarography [187], spectrophotometry [188-190], spectro fluorometry [191], thin layer chromatography [186,118,192], GLC [186,188,193,194], HPLC [186, 183,195-197] and GC-MS [198,199]. The official methods for the estimation of atenolol include spectrophotometric estimation [2] and HPLC [1] and the official methods for nifedipine are titrimetry [2] and HPLC [3]. The present work investigates the simultaneous estimation of atenolol and nifedipine in combined preparations without prior separation from each other as well as formulation excipients by uv derivative spectrophotometry.

Materials, Reagents and Apparatus

1. Hydrochloric Acid - A.R.Grade (E.Merck India Ltd.)
2. Methanol - Spectroscopic Grade (Spectrochem. India)
3. Atenolol and Nifedipine were as obtained as gift samples.

The second-order derivative spectra were recorded at a scan rate of 240nm/min with a Jasco 7800 uv-visible double beam scanning spectro photometer using 1cm matched quartz cuvettes. The resolution of the spectrophotometer for recording the second-order derivative spectra was checked as per the procedure in B.P. 1993 by recording the second-order

derivative spectra of 0.02% v/v solution of toluene in methanol and was found to be satisfactory (figure 33). The data thus recorded with the various solutions have been given in tables.

Standard and Sample Solutions

The stock solutions of the drugs containing 1mg/ml of atenolol and nifedipine were prepared in methanol by dissolving the pure drugs in methanol by thorough shaking.

The solutions for linear plot method were prepared (Table 27) by using appropriate volumes of the aliquots of the stock solutions so as to obtain the concentrations of the drugs given in table 27. Similarly, appropriate volumes of aliquots from the stock solutions were used to prepare four series of solutions for second-order derivative method.

The first series (Series A) comprised of solutions of atenolol of varying concentrations (10-30mcg/ml) prepared by pipetting out appropriate volumes of aliquots (1.0, 1.4, 1.8, 2.2, 2.6, 3.0 ml) from the stock solution of atenolol (1mg/ml) into 100ml volumetric flasks and making up the volume with 0.1N HCl.The second series (Series C) consisted of solutions of nifedipine of varying concentration (5-20mcg/ml) prepared in a similar fashion in 0.1N HCl. The third series (Series B) comprised of mixtures of the drugs having a constant concentration of nifedipine (10mcg /ml) and a varying concentration of atenolol (10-30 mcg/ml) prepared by pipetting out the same volumes of aliquots from atenolol stock solution as in the preparation of Series A solutions into 100ml volumetric flasks , adding 1ml of nifedipine stock solution (1mg/ml) to each flask and making up the volume with 0.1N HCl. Similarly, the fourth series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the atenolol and nifidepine stock solutions

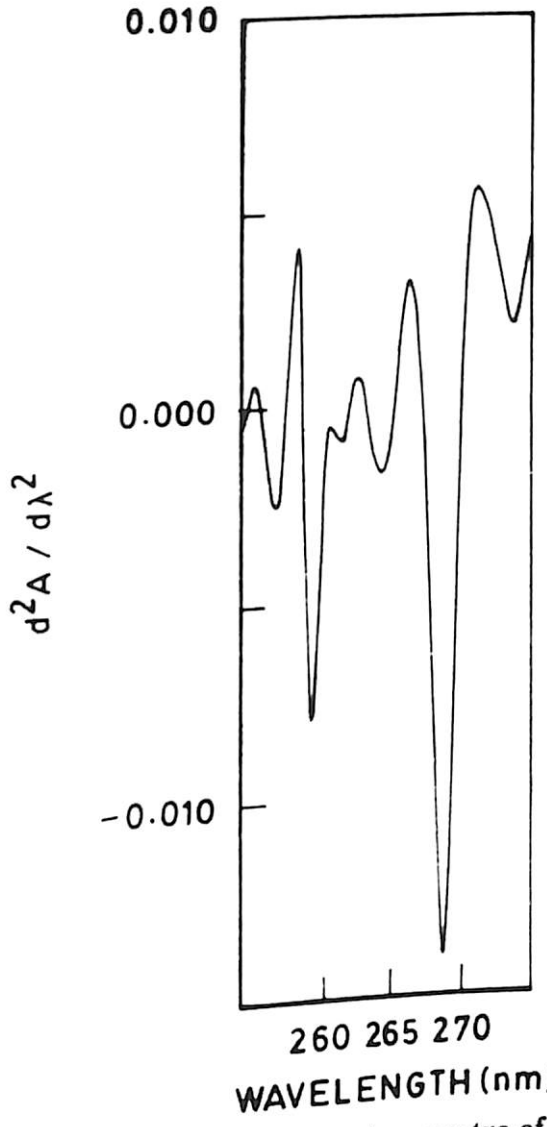


Fig 33. Second-order derivative spectra of 0.020% v/v solution of toluene in methanol

so as to give solutions containing various concentra tions of nifedipine (5-20mcg/ml) along with a constant concentration of atenolol (20 mcg/ml) in 0.1M HCl.

Twenty tablets of atenolol and nifedipine (of each brand) were finely ground and a weight of the powder equal to the average weight of the tablet was dissolved in methanol, filtered (Whatman No 1. filter paper) and appropriate volumes of aliquots of the filtrate were used to prepare sample solutions containing approximately the concentrations of atenolol and nifedipine as given in table 32.

The solutions containing nifedipine (pure, mixtures with atenolol as well as tablet samples) were prepared under red light and stored in low actinic Pyrex volumetric flasks at room temperature till their analysis to avoid photodegradation.The stability of the solutions (0.1M HCl) were monitored spectrophotometrically for a period of three hours and were found to vary by the following absorbance units (AU) : atenolol in 0.1M HCl by ± 0.002AU and nifedipine in 0.1M HCl by ± 0.005AU. All the measurements for replicate determinations were recorded within a time interval of 60-90 minutes after preparation of the solutions in 0.1M HCl to minimise the variations in absorbance with time.

Recording of the Spectra

The normal spectra in 0.1M HCl were recorded by using 0.1M HCl as the blank in the Jasco 7800 spectrophotometer. The second-order derivative spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by initially recording their normal absorption spectra at the appropriate concentrations (tables 29 and 30) and converting the normal spectra to second-order derivative spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the

recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. The conversion follows Savitzky-Golay method [200] and a data interval of one was found to be satisfactory. No smoothing of the derivative spectra was found necessary.

RESULTS AND DISCUSSION

As mentioned in the introduction to derivative spectrophotometry, the technique of derivative spectrophotometry may be used for the quantitation of one analyte whose peak is obscured by a larger overlapping peak of some other analyte with minimum error. The advantage of second-order derivative spectrophotometry in eliminating the back ground absorption due to formulation excipients had been studied [161].The first derivative of an absorption spectra represents the gradient at all points of the spectrum and may be used to locate hidden peaks since $dA / d\lambda = 0$ at peak maxima, but the higher even order derivatives are potentially more useful for analysis. The absorption of two or more compounds in the same wavelength region which would create inseparable interference in direct absorption spectrophotometry can often be resolved in the derivative mode by choosing a wavelength at which one analyte's derivative signal goes through a value of zero.

The spectra of the drugs atenolol and nifedipine in 0.1M HCl (figure 34) indicated that the drugs cannot be estimated without interference from each other since both the drugs absorbed strongly only in the lower wavelength region of uv range. The nifedipine absorbs more strongly than atenolol between 275 and 250 nm and hence may be estimated in this region with less interference from atenolol but it should be noted that the concentration of the drugs in this figure is 10mcg/ml whereas the concentration of atenolol will be more than twice that of nifedipine in the commercial formulations (Table 32). Hence, when the actual analysis of

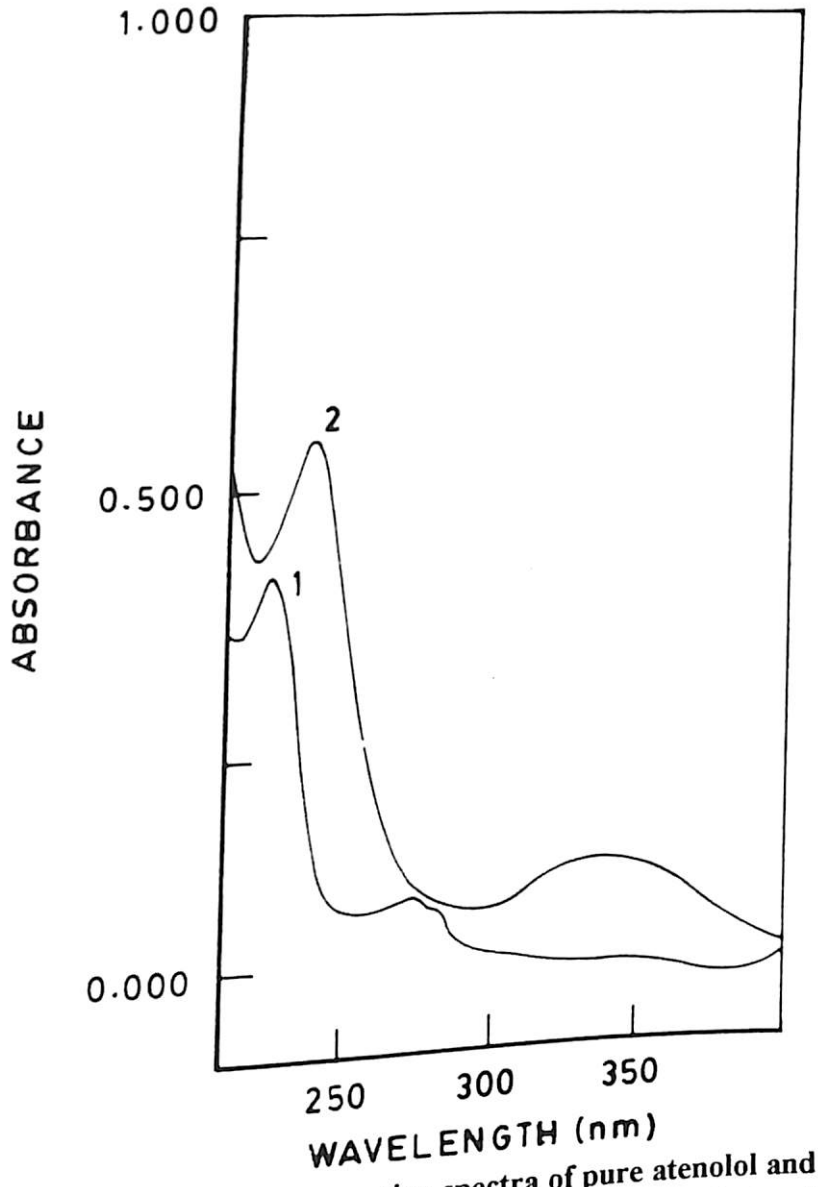


Fig 34. Normal absorption spectra of pure atenolol and nifedipine in 0.1M HCl; concentration of atenolol, 10 mcg ml^{-1} (curve 1); concentration of nifedipine, 10 mcg ml^{-1} (curve 2)

the drugs ought to be done, the interference of atenolol will certainly be there. As can be seen from figure 34, atenolol cannot be estimated without interference from nifedipine because of the strong absorption of the later even when the concentration is same for both the drugs. Of course, in the commercial formulations, the concentration may be less than half the concentration of atenolol in the working solution, but still the interference will be there and hence some kind of simultaneous estimation is required for the determination of these drugs in tablets.

In this section, the methods of linear plot as well as second-order derivative difference have been designed for the estimation of these drugs in combined formulations. The absorbance ratio method using isoabsorptive point had not been used because of the absence of the isoabsorptive point [77] between atenolol and nifedipine (figure 34). The second-order derivative difference method was not applied because of the absence of appropriate shift with change of pH in the case of atenolol.

Linear Plot Method

This method [78], as mentioned in the introduction, uses data at multiple wavelengths to generate linear plots. For the admixture of atenolol and nifedipine, the data points between the wavelengths of 238-245nm were used and the results of the analysis have been presented in table 27 and 28. Although the percentage relative error was a bit high for atenolol (table 27) in pure admixture, the results of the assay of the drug in commercial formulations gave a mean recovery which ranged from 97.36 to 100.34% for atenolol and 98.45 to 99.43% for nifedipine.

Table 27. Results of Determination of Atenolol and Nifedipine in Pure Admixtures by Linear Plot Method

Solution	Solv.			Concentration of [a, b] Drugs (mcg ml^{-1})				% Relative Error	
				ATN		NIF		ATN	NIF
				ACT	FND	ACT	FND		
Pure Drugs	A	----	----	22.00	20.54	8.00	8.04	-6.63	+2.12

ATN - Atenolol NIF - Nifedipine A - 0.1M HCl
ACT - Actual value FND - Found value
[a] Wavelength range used for collection of data points was 238-245nm; concentration obtained from the slope and intercept of the linear curve plot
[b] Based on five replicate determinations

Table 28. Results of Assay of Atenolol and Nifedipine in Commercial Samples by Linear Plot Method

Sample	Label Claim (mg/tablet)		Mean Recovery, % [a]	
	ATN	NIF	ATN	NIF
Brand A	50	20	97.36 ± 1.22	98.45 ± 0.76
Brand B	50	20	98.74 ± 1.53	99.43 ± 0.94
Brand C	50	20	100.34 ± 1.77	98.69 ± 0.56

[a] Average of ten determinations; assay as percentage of label claim

Second-order derivative method

For this method, the spectra of the drug solutions were recorded in 0.1M HCl as normal spectra and converted to second-order derivative spectra using the digital algorithms. The zero-crossing points of the second-order derivative spectra of atenolol occured at 209, 219, 234, 270 and 285nm whereas those of nifedipine occured at 210, 227, 252 and 297nm (figures 35 and 36). Of these wavelengths, the wavelength of 227nm was chosen for the estimation of atenolol and 219nm for nifedipine since the amplitude of the derivative spectra (h_1 and h_2 in figures 35 and 36) were found to be proportional to the concentration. The amplitude of the spectra of the drugs at these wavelengths were also found to be independent of each other at these wavelengths (figures 37 and 38). Thus these zero crossing points were used for the estimation of the drugs without interference from each other.

The small standard deviation values showed the precision of the derivative method (Tables 29 and 30) and the negligible intercepts of the equations indicated regression through or close to the origin. The correlation coefficient values in the range of 0.9996-0.9998 indicated the non-interference of one drug in the estimation of the other. The calculated F test values for each concentration of the drugs (Tables 29 and 30) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.92 - 99.97% variation in the derivative values were accounted for by the concentration of the drugs in solution. The results of F test using ratio of mean square due to regression to the mean square about the regression (Table 31) showed the calculated values to be much higher than the critical values at 5% level of significance proving

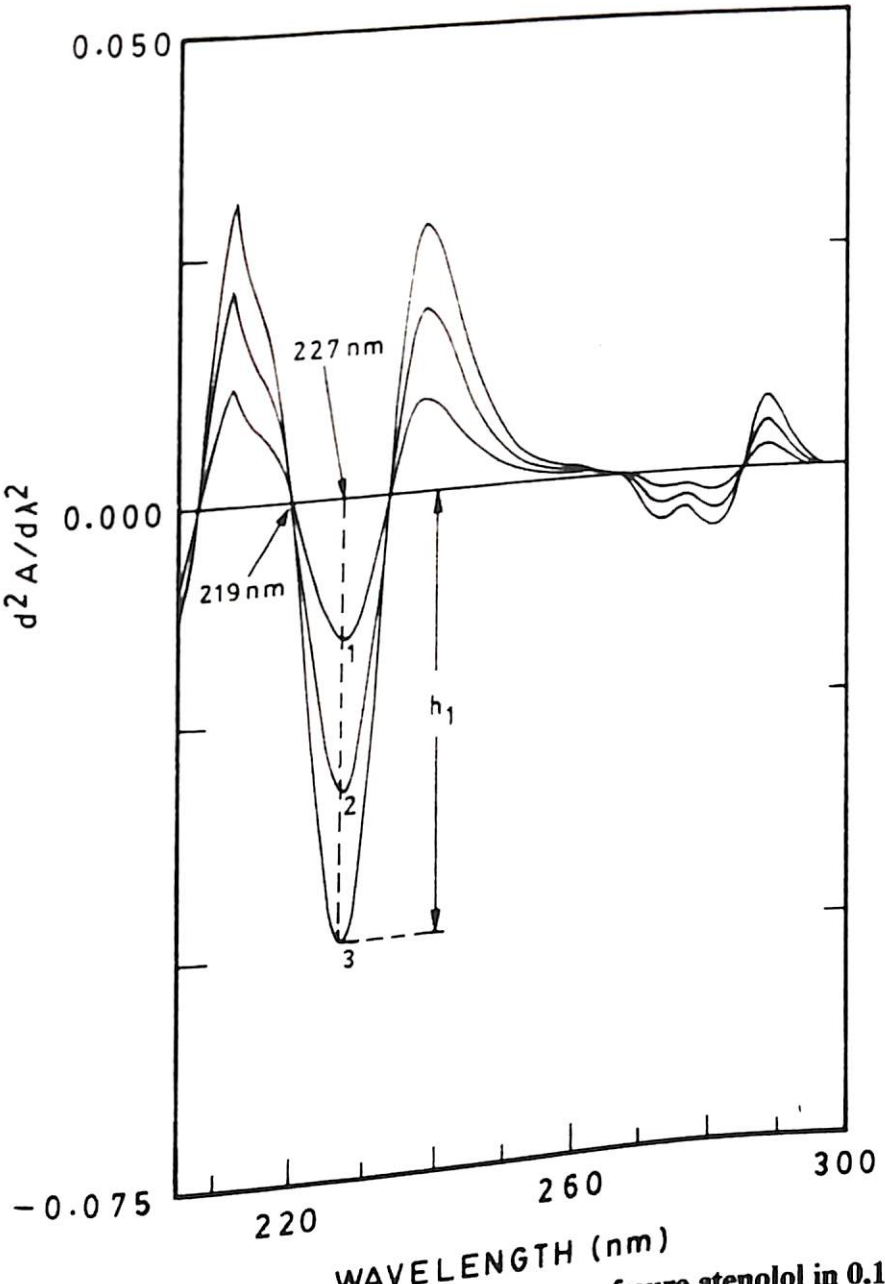


Fig 35. Second-order derivative spectra of pure atenolol in 0.1M HCl (atenolol concentration: 10, 20 and 30 mcg ml^{-1} in curves 1,2 and 3 respectively)

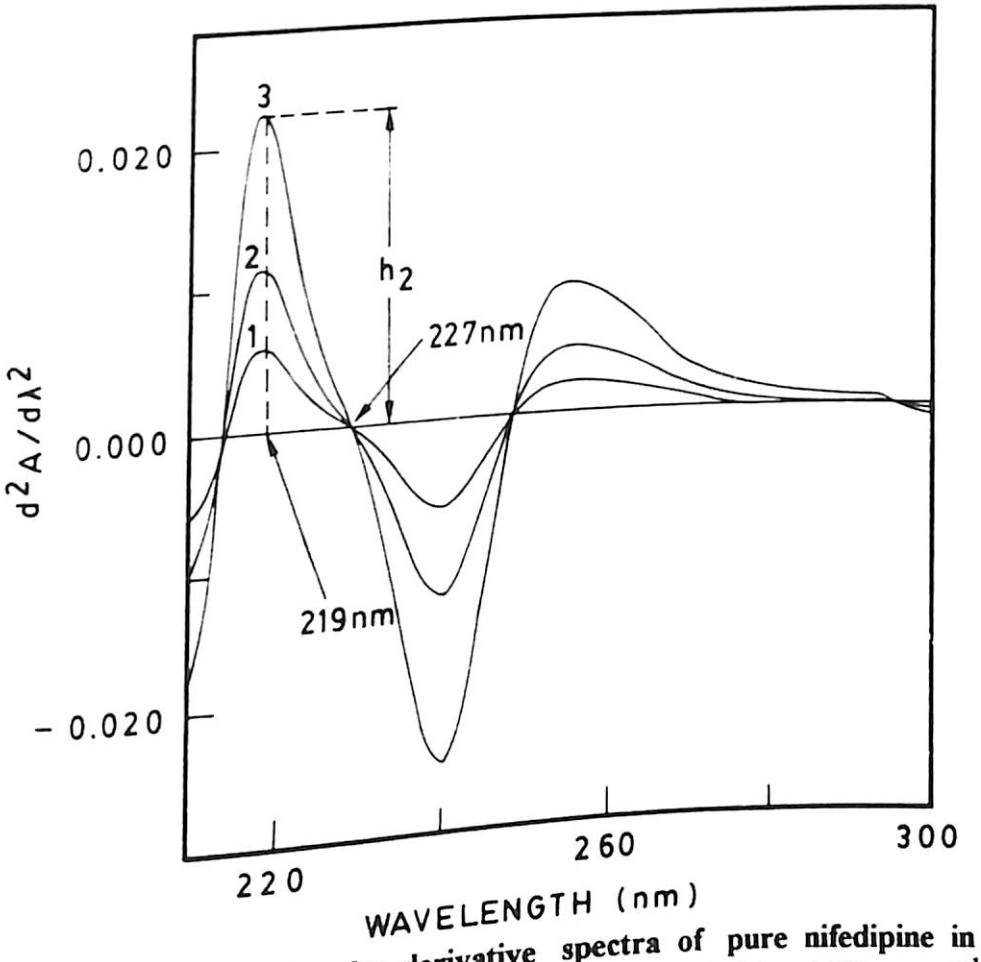


Fig 36. Second-order derivative spectra of pure nifedipine in 0.1M HCl (nifedipine concentration: 5, 10 and 20 mcg ml^{-1} in curves 1,2 and 3 respectively)

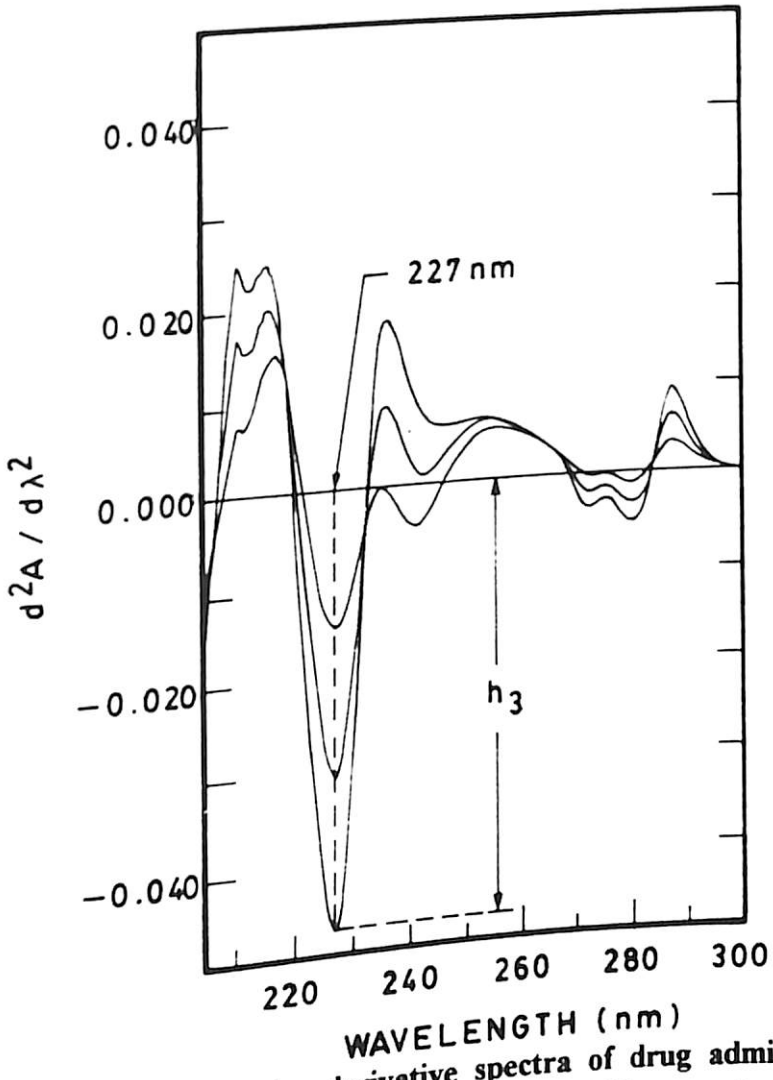


Fig 37. Second-order derivative spectra of drug admixture of atenolol and nifedipine in 0.1M HCl; concentration of nifedipine 10 mcg ml^{-1} ; concentration of atenolol 10, 20 and 30 mcg ml^{-1} in curves 1,2 and 3 respectively

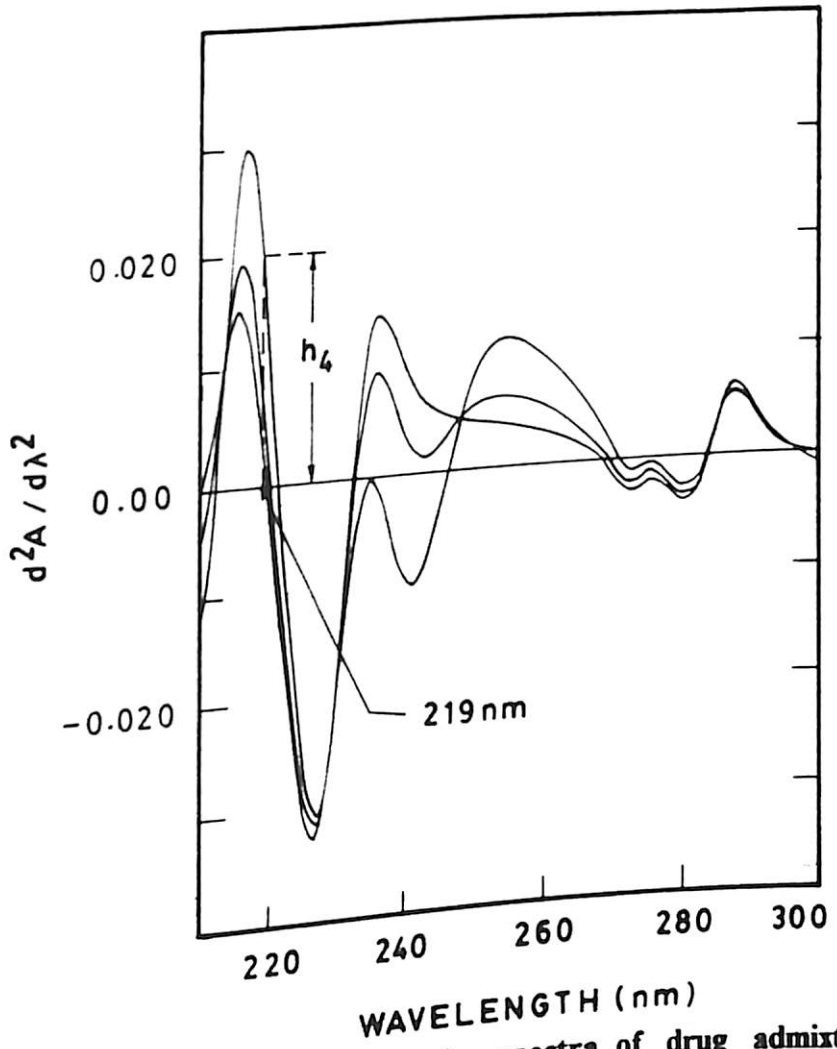


Fig 38. Second-order derivative spectra of drug admixture of nifedipine and atenolol in 0.1M HCl; concentration of atenolol 20 mcg ml^{-1} ; concentration of nifedipine 5,10 and 20 mcg ml^{-1} in curves 1,2 and 3 respectively

Table 29. Selectivity of the Method for the Determination of Atenolol in the Presence of Nifedipine by Second-order Derivative Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (227nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
ATN	NIF					Crit	Calc
10	0	0.0163 ± 0.0003	1.57	0.0001	98.53	3.63	1.77
14	0	0.0220 ± 0.0003	1.60	0.0001	102.83	3.63	1.31
18	0	0.0296 ± 0.0003	0.89	0.0001	98.87	3.63	2.36
22	0	0.0359 ± 0.0003	0.95	0.0001	99.64	3.63	1.38
26	0	0.0425 ± 0.0003	0.63	0.0001	99.91	3.63	2.36
30	0	0.0489 ± 0.0003	0.65	0.0001	100.23	3.63	1.56
10	12	0.0164 ± 0.0003	1.80	0.0001	98.01	3.63	1.90
14	12	0.0220 ± 0.0004	1.71	0.0001	102.79	3.63	1.17
18	12	0.0295 ± 0.0003	0.90	0.0001	99.04	3.63	2.37
22	12	0.0358 ± 0.0004	1.09	0.0001	100.14	3.63	1.17
26	12	0.0425 ± 0.0003	0.63	0.0001	99.79	3.63	2.36
30	12	0.0490 ± 0.0003	0.60	0.0001	100.07	3.63	1.90

ATN - Atenolol NIF - Nifedipine

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 30. Selectivity of the Method for the Determination of Nifedipine in the Presence of Atenolol by Second-order Derivative Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (219nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
NIF	ATN					Crit	Calc
5	0	0.0005 ± 0.0003	4.69	0.0001	100.12	3.63	0.44
8	0	0.0088 ± 0.0003	3.54	0.0001	100.51	3.63	0.42
11	0	0.0123 ± 0.0003	1.67	0.0001	99.69	3.63	0.44
14	0	0.0158 ± 0.0003	1.67	0.0001	99.05	3.63	0.47
17	0	0.0188 ± 0.0002	1.26	0.0001	101.06	3.63	0.47
20	0	0.0224 ± 0.0003	1.49	0.0001	99.72	3.63	0.27
5	20	0.0056 ± 0.0003	5.78	0.0001	97.15	3.63	0.16
8	20	0.0085 ± 0.0003	3.90	0.0001	102.94	3.63	0.10
11	20	0.0122 ± 0.0003	2.58	0.0001	99.26	3.63	0.10
14	20	0.0156 ± 0.0003	1.92	0.0001	99.54	3.63	0.15
17	20	0.0187 ± 0.0003	1.54	0.0001	101.10	3.63	0.16
20	20	0.0223 ± 0.0004	1.73	0.0001	99.40	3.63	0.11

ATN - Atenolol NIF - Nifedipine

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 31. Regression Analysis of Atenolol and Nifedipine Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (227 nm for ATN and 219 nm for NIF)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	ATN	NIF				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	10-30	0	y = 0.0016x - 0.0004	0.9996	99.92	7.71	5173	2.78	71	0.0016	0.0005	0.0004
Series B	10-30	12	y = 0.0016x - 0.0005	0.9996	99.92	7.71	5294	2.78	72	0.0017	0.0005	0.0004
Series C	0	5-20	y = 0.0011x - 0.0001	0.9998	99.97	7.71	11480	2.78	107	0.0001	0.0001	0.0001
Series D	20	5-20	y = 0.0011x - 0.0002	0.9996	99.92	7.71	4897	2.78	69	0.0001	0.0002	0.0002

ATN - Atenolol NIF - Nifedipine [a] Based on six calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 6 for atenolol as well as nifedipine

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 6 for both atenolol as well as nifedipine

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

the linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve (Table 31). The ratio of residuals expressed as percentage showed a scatter which was random.

The results of the estimation of atenolol and nifedipine in synthetic admixtures and commercial formulations by second-order derivative spectrophotometry have been given in table 32. The estimation of synthetic admixtures were done using a concentration of 20 mcg/ml of atenolol and 12 mcg/ml of nifedipine which resulted in a mean recovery of 99.80 and 99.83% for atenolol and nifedipine respectively. These concentrations of the drugs were mean points of the calibration ranges of the drugs. Similarly, the estimation of the drugs in commercial formulations was also done in such a fashion that the concentrations of the drugs in the final solution of the commercial sample remained approximately 20 mcg/ml for atenolol and 8 mcg/ml for nifedipine. The proportion in which these drugs are present in commercial samples precludes the use of the same solution for the determination of both the drugs at their corresponding mean points of calibration (Table 32). However, the calibration ranges of the drugs did allow the estimation of the drugs using a single solution at concentrations different from that of their mean points of calibration. Therefore, atenolol and

Table 32. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Atenolol and Nifedipine by Second-order Derivative Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	ATN	NIF	ATN	NIF	ATN	NIF	ATN	NIF
Pure Drug Admixture	20	12	—	—	99.80	99.83	99.47-100.12	99.57-100.08
Brand A	20	8	50	20	99.61	99.75	99.28-99.93	99.43-100.06
Brand B	20	8	50	20	100.20	98.12	99.87-100.53	97.80-98.43
Brand C	20	8	50	20	99.27	98.50	98.94-99.59	98.18-98.81
Brand A	25	12	50	20	99.64	100.25	99.26-100.01	99.99-100.50
Brand B	25	12	50	20	100.16	98.65	99.78-100.53	98.39-98.90
Brand C	25	12	50	20	99.32	98.57	98.94-99.69	98.31-98.82

ATN - Atenolol NIF - Nifedipine

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 4 degrees of freedom for both atenolol as well as nifedipine

nifedipine have been estimated twice in each commercial sample. One determination was done by diluting the sample solution to obtain approximately the mean point of calibration concentration of the drug and the other at the actual concentration in which one drug would be present when the second drug was estimated at its mean point of calibration.

Atenolol was estimated both at 20 mcg/ml and 25 mcg/ml and nifedipine at both 8 mcg/ml and 12 mcg/ml (Table 32). The 95% confidence level range in table 32 showed a slightly wider range in the case of determination at concentrations other than mean point of calibration which was normal since this range calculation was dependent on the standard error of prediction which will be minimal at the mean point of the calibration.

The assay results in tables 28 and 32 showed difference in the mean recovery by the two methods for the same commercial formulation. But it may be concluded that the second-order derivative method was more accurate since it uses the calibration data at a particular wavelength which had been fit by least square method whereas the linear plot method uses data at multiple wavelengths. But it should be noted that the basic advantage of the second-order transformation i.e., the elimination of the spectral contribution of additives such as lactose, pvp and coloring agents will be lost in the determination of atenolol and nifedipine since the absorption of these additives does not get completely eliminated at wavelengths approximately below 250nm (figures 21-30). This is mainly due to the steep slope exhibited by the additives at the lower wavelengths which cannot be approximated by a linear function and hence will not get eliminated by second-order transformation.

5.03 DETERMINATION OF IMIPRAMINE HYDRO CHLORIDE AND DIAZEPAM IN PURE ADMIXTURES AND TABLETS BY SECOND-ORDER DERIVATIVE SPECTROPHOTOMETRY

INTRODUCTION

The combination of imipramine hydrochloride with diazepam as a tablet preparation has been introduced in the market for treatment of depression associated with anxiety and agitation. The methods reported thus far for the estimation of imipramine hydrochloride include spectrophotometry [201-205], titrimetry [206], polarography[207], TLC [208-211] GLC [209, 212] and HPLC [210] and spectrofluorometry [213]. The methods for estimation of diazepam include potentiometry [214], differential pulse polarography [215], spectrophotometry [216-219], spectrofluorometry [220], TLC [221], GLC [222], HPLC [223-226]. The official methods for the estimation of imipramine hydrochloride include spectrophotometric estimation [1,3] , non-aqueous titrimetry [2] and those for diazepam include non-aqueous titrimetry [2] and spectrophotometry [3].

Materials, Reagents and Apparatus

1. Hydrochloric Acid - A.R.Grade (E.Merck India Ltd.)
2. Methanol - Spectroscopic Grade (Spectrochem. India)
3. Imipramine hydrochloride and diazepam were obtained as gift samples.

The second-order derivative spectra were recorded at a scan rate of 240nm/min with a Jasco 7800 uv-visible double beam scanning spectro photometer using 1cm matched quartz cuvettes. The resolution of the spectrophotometer for recording the second-order derivative spectra was checked as per the procedure in B.P. 1993 by recording the second-order

derivative spectra of 0.02% v/v solution of toluene in methanol and was found to be satisfactory. The data thus recorded with the various solutions have been given in tables 33-39.

Standard and Sample Solutions

The stock solutions of the drugs containing 1mg/ml of imipramine hydrochloride and diazepam were prepared in methanol by dissolving the pure drugs in methanol by thorough shaking. Appropriate volumes of aliquots from the stock solutions were used to prepare different series of solutions in 0.1M HCl.

The solutions for linear plot method were prepared with the composition as shown in table 33. The solutions for the estimation by absorbance ratio method were of the composition as given in table 34. In addition, the series A-J solutions were prepared for estimation by second-order derivative spectrophotometry.

The first series (Series A) comprised of solutions of imipramine HCl of varying concentrations (10-70mcg/ml) prepared by pipetting out appropriate volumes of aliquots from the stock solution of imipramine HCl (1mg/ml) into 100ml volumetric flasks and making up the volume with 0.1N HCl.The second series (Series C) consisted of solutions of diazepam of varying concentration (2-8 mcg/ml) prepared in a similar fashion in 0.1N HCl. The third series (Series B) comprised of mixtures of the drugs having a constant concentration of diazepam (5 mcg /ml) and a varying concentration of imipramine HCl (10-70 mcg/ml) prepared by pipetting out the same volumes of aliquots from imipramine HCl stock solution as in the preparation of Series A solutions into 100ml volumetric flasks , adding 0.5ml of diazepam stock solution (1mg/ml) to each flask and making up the volume with 0.1N HCl. Similarly, the fourth

series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the diazepam and imipramine HCl stock solutions so as to give solutions containing various concentrations of diazepam (2-8 mcg/ml) along with a constant concentration of imipramine HCl (40 mcg/ml) in 0.1N HCl. The series E,F and G were exactly similar to that of series C except that they had lactose (600 mcg/ml), pvp (600mcg/ml) and indigocarmine (4 mcg/ml) in them. These additives were added by using appropriate volumes of aliquots of dissolution in water to produce stock solutions of 10mg/ml since lactose, pvp as well as indigocarmine are freely soluble in water.

Twenty tablets (of each brand) were finely ground and a weight of the powder equal to the average weight of the tablet was dissolved in methanol, filtered (Whatman No 1. filter paper) and appropriate volumes of aliquots of the filtrate were used to prepare sample solutions containing approximately the concentrations of imipramine HCl and diazepam as given in table 39.

The solutions were stored in low actinic Pyrex volumetric flasks at room temperature till their analysis. The stability of the solutions (0.1M HCl) were monitored spectrophotometrically for a period of three hours and were found to vary by the following absorbance units (AU) : imipramine HCl in 0.1N HCl by $\pm$ 0.005AU and diazepam in 0.1N HCl by $\pm$ 0.009AU. All the measurements for replicate determinations were recorded within a time interval of 45-60 minutes after preparation of the solutions in 0.1N HCl to minimise the variations in absorbance with time.

Recording of the Spectra

The normal spectra in 0.1M HCl were recorded by using 0.1M HCl as the blank in the Jasco 7800 spectrophotometer. The second-order derivative spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by initially recording their normal absorption spectra at the appropriate concentrations and converting the normal spectra to second-order derivative spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. A data interval of one was found to be satisfactory. No smoothing of the derivative spectra was found necessary.

RESULTS AND DISCUSSION

The combination of imipramine HCl and diazepam in 0.1M HCl will require a simultaneous estimation since both the drugs absorb strongly between 250-310nm although imipramine HCl did not show any prominent peak in this region. Nevertheless, the interference will be present and it becomes more pronounced as the absorbance values reach the range of 0.300-0.800 AU (absorbance units) which would give the minimum error. Although during actual estimation the concentration of diazepam is likely to get reduced approximately to one fifth or one tenth of imipramine HCl concentration (due to the ratio in which the drugs are present in commercial formulations), the interference by imipramine HCl in the estimation of diazepam will remain almost the same as indicated in figure 39 whereas the interference of diazepam will get reduced by one fifth but will not get completely eliminated. Hence the methods of linear plot, absorbance ratio and second-order derivative spectrophotometry have been designed for the estimation in this section.

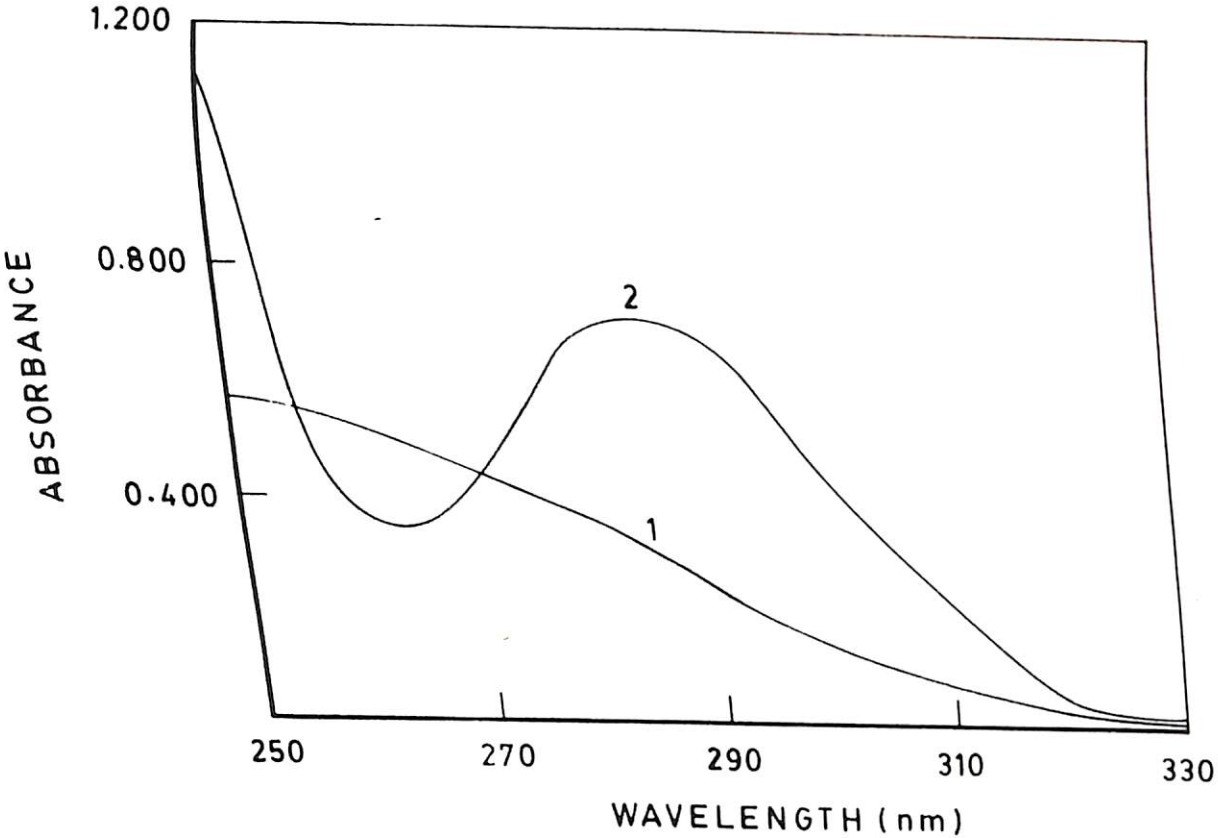


Fig 39. Normal absorption spectra of pure imipramine HCl and diazepam in 0.1M HCl; concentration of imipramine HCl is 20 mcg ml^{-1} (curve 1); concentration of diazepam is 20 mcg ml^{-1} (curve 2)

The linear plot method using the data points in the region of 264-300 nm was done by using the 0.1M HCl solutions of composition as given in table 33. The amount of drugs were estimated from the slope and intercept of the equations of the plots. It can be seen from the results in table 33 that the linear plot method will not be able to completely eliminate the interference by additives, if any, since the percentage relative error associated with the estimation of the drugs increased in the presence of lactose, pvp and indigocarmine due to their contribution to the absorbance at the chosen wavelengths.

The isoabsorptive point of the drugs in 0.1M HCl occurs at 255nm and 270nm as can be seen from figure 39. The Q curve for estimation was plotted using 270nm as the isoabsorptive point. The values of the imipramine HCl at 262nm (10-30 mcg/ml) and those of diazepam at 290nm (10-20 mcg/ml) were used for the Q curve plot. The concentration range of the drugs were chosen on the basis of the proportion in which the drugs were present in commercial formulations. The Q curves were plotted using the quotient obtained by dividing absorbance values at the wavelengths of 262nm (for imipramine HCl) and 290nm (for diazepam) by the isoabsorptive wavelengths *vs* concentration of the drug (Table 34). The amounts of the drugs were estimated by solving the equations of the Q curves for the corresponding unknown Q values of the admixtures. Since the Q values were fit by least square method, the F test for non-linearity and T test for correlation were also done and the results indicated the rejection of null hypothesis for both the tests since the calculated values were larger than the critical values although the correlation coefficient for imipramine HCl curve was not very high (0.9985). The results of the assay of the commercial formulations by the absorbance ratio method have been given in table 35. Although the accuracy of the absorbance ratio method will be maximum when the proportion of the drugs in the solution is 50:50,

Table 33.Results of Determination of Imipramine Hydrochloride and Diazepam in Pure Admixtures by Linear Plot Method

Solution	Solv.	Concentration of Additives (mcg ml^{-1})			Concentration of Drugs[a, b] (mcg ml^{-1})				% Relative Error	
		LAC	PVP	CAR	IMP ACT	IMP FND	DIZ ACT	DIZ FND	IMP	DIZ
Pure Drugs	A	----	------	------	25.00	24.97	5.00	4.81	-0.12	-3.80
Pure Drugs	A	600	------	------	25.00	26.20	5.00	4.68	4.80	-6.40
Pure Drugs	A	----	600	------	25.00	26.60	5.00	4.70	6.40	8.87
Pure Drugs	A	----	----	4	25.00	26.06	5.00	7.85	4.24	30.83

IMP - Imipramine hydrochloride DIZ - Diazepam A - 0.1M HCl
ACT - Actual value FND - Found value LAC - Lactose CAR- Indigocarmine
PVP - Polyvinylpyrrolidone

[a] Wavelength range used for collection of data points was 264-300nm; concentration obtained from the slope and intercept of the linear curve plot

[b] Based on five replicate determinations

Table 34. Regression Analysis of Absorbance Ratio Values of Imipramine hydrochloride and Diazepam

Solvent	Composition of Solution (mcg ml^{-1}) IMP	DIZ	Regression Equation [a] (262 nm for IMP and 290 nm for DIZ)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept
0.1M HCl	10-30	0-20	$Q = 0.0164X_{imp} + 1.1910$	0.9985	99.70	10.31	1011	3.18	31	0.0005	0.0109
0.1M HCl	10-30	0-20	$Q = 0.0499X_{diz} + 0.8194$	0.9999	99.99	10.31	35108	3.18	187	0.0003	0.0033

IMP- Imipramine hydrochloride DIZ - Diazepam [a] Based on values used for Q curve plot; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 5 for both the drugs

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 5 for both the drugs

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance ratio* value of the admixture and *x* is the *concentration*

the results of the assay of imipramine HCl and diazepam showed that the method was suitable for the estimation of the drugs in spite of the 5:1 ratio in which the drugs were present in the commercial samples. One of the reasons for the applicability of the method for the estimation of the drugs was that the absorption intensity of diazepam was almost twice as that of imipramine HCl in 0.1M HCl solvent at the wavelength of 290nm.

As a result, from the point of view of absorbance measurement, the ratio became 5:2. The concentration range chosen for the method did not permit the estimation of the drugs in Brand D in which the ratio of imipramine HCl : diazepam was 25:2 although the estimation in this Brand had been possible by second-order derivative spectrophotometry. The disadvantage of this method when compared to second-order derivative method was that the absorption of the excipients, if any, at the chosen wavelengths of 262 and 290nm will not be eliminated and would lead to errors. But during the assay of the formulations, it was found that the assayed formulations did not contribute additives in such concentrations in the final working solutions as to interfere in the absorption measurements. This was evidenced by the assay results in table 35.

Second-order derivative method

For this method, the spectra of the drug solutions were recorded in 0.1M HCl as normal spectra and converted to second-order derivative spectra using the digital algorithms. The zero-crossing point of the second-order derivative spectra of imipramine HCl at 258nm and that of diazepam at 275 (figures 40 and 41) were chosen for the estimation of the drugs on the basis of the linear response as well as optimum derivative values. Thus the derivative values of imipramine HCl at 275nm (h_1 and h_3 in figures 40 and 42) and the derivative values of diazepam at 258nm (h_2 and h_4 in figures 41 and 43) were found to be proportional to the corresponding concentrations and independent of each other and hence used for the estimation of the

Table 35. Results of Assay of Imipramine Hydrochloride and Diazepam in Commercial Samples by Linear Plot and Absorbance Ratio Method

Sample	Method	Label Claim (mg/tablet)		Mean Recovery, % [a]	
		IMP	DIZ	IMP	DIZ
Brand A	LPM	25	5	98.26 ± 0.22	100.35± 0.38
Brand B	LPM	25	5	99.89 ± 0.27	99.23 ± 0.94
Brand C	LPM	25	2	99.26 ± 0.57	99.71 ± 0.56
Brand A	ARM	25	5	99.32 ± 0.47	98.83 ± 0.88
Brand B	ARM	25	5	100.33 ± 0.96	98.83 ± 0.63
Brand C	ARM	25	2	99.55 ± 0.33	99.93 ± 0.12

[a] Average of ten determinations; assay as percentage of label claim

LPM - Linear Plot Method ARM - Absorbance Ratio Method

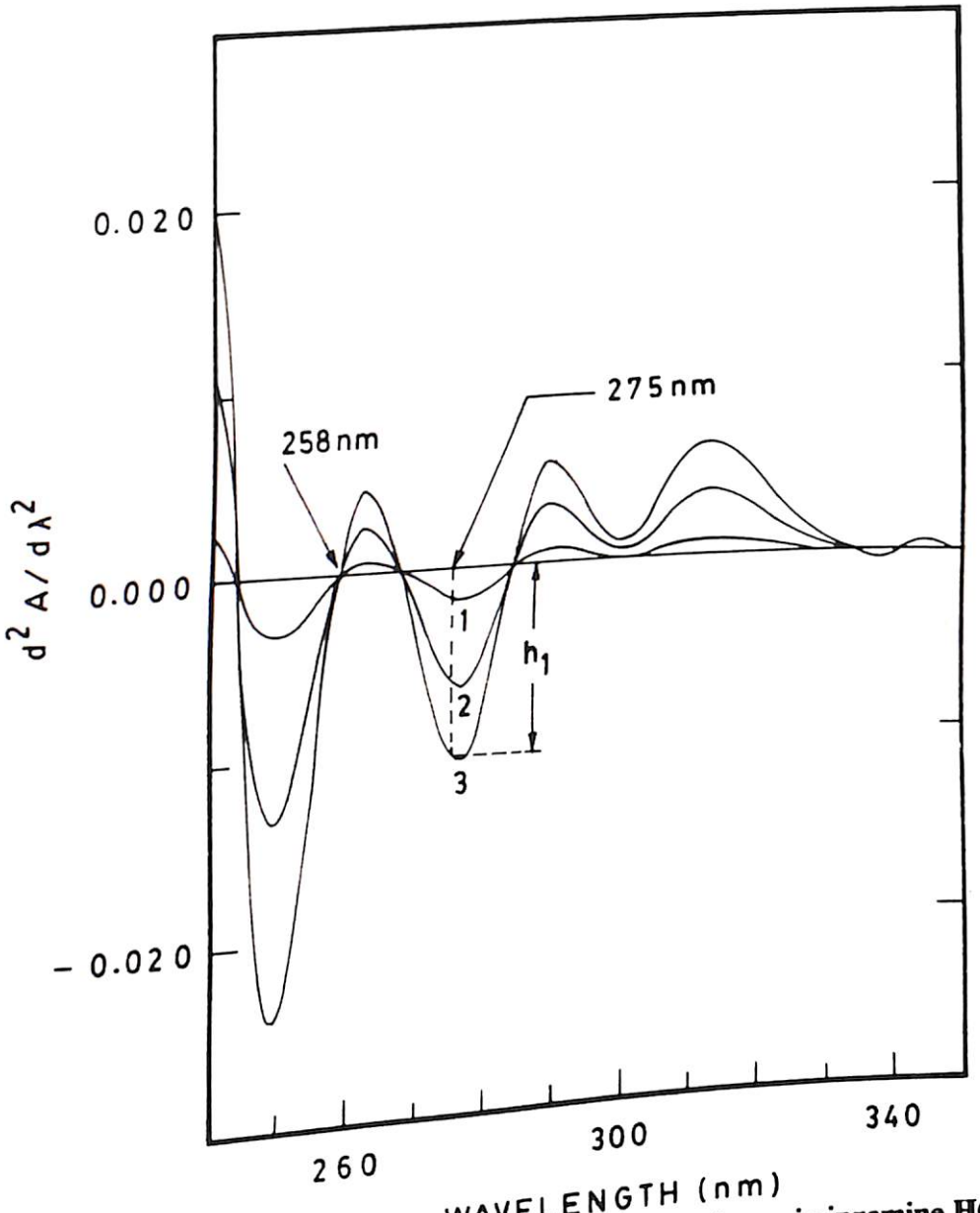


Fig 40. Second-order derivative spectra of pure imipramine HCl in 0.1M HCl (imipramine HCl concentration: 10, 40 and 70 mcg ml^{-1} in curves 1,2 and 3 respectively)

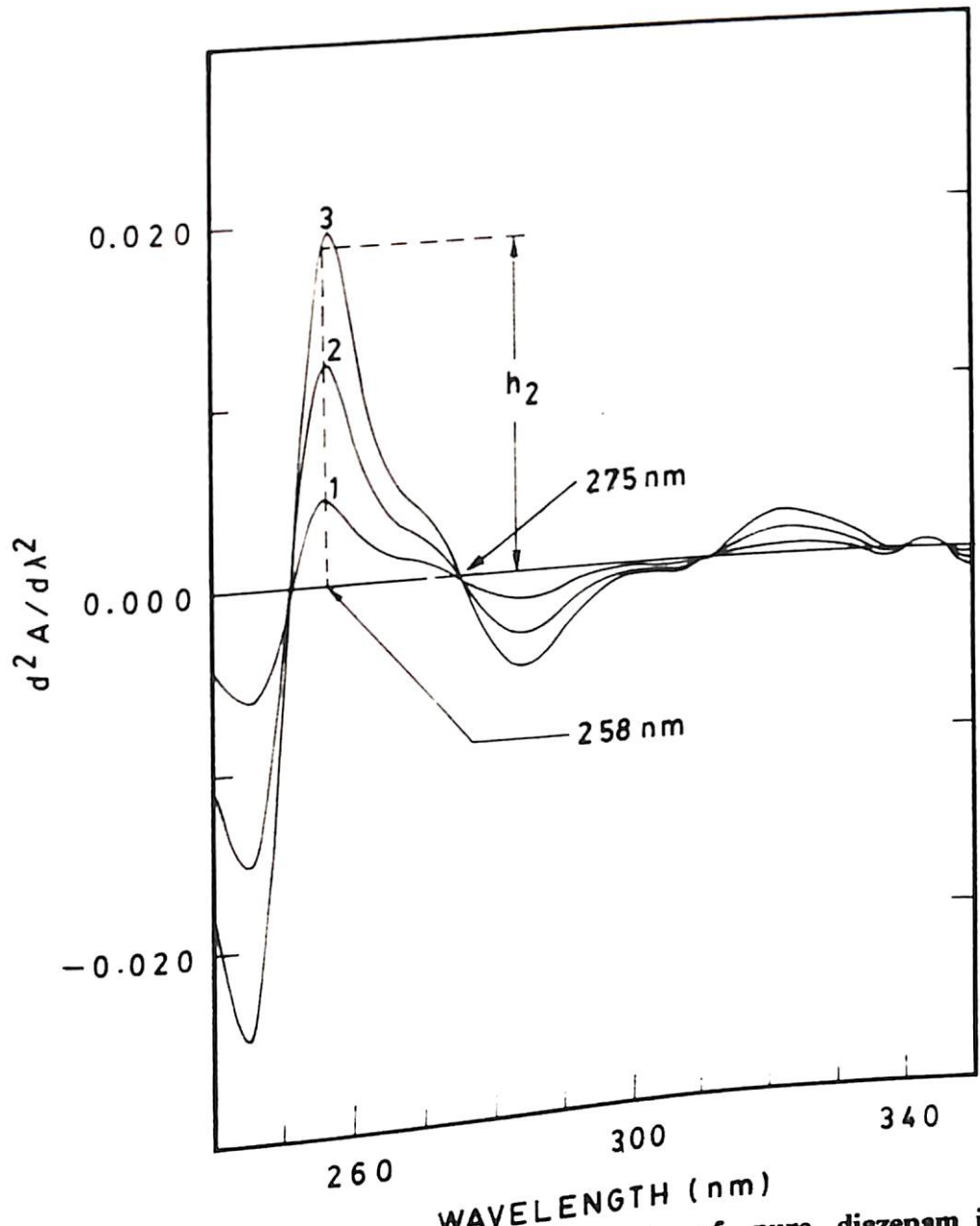


Fig 41. Second-order derivative spectra of pure diazepam in 0.1M HCl (diazepam concentration: 2, 5 and 8 mcg ml^{-1} in curves 1,2 and 3 respectively)

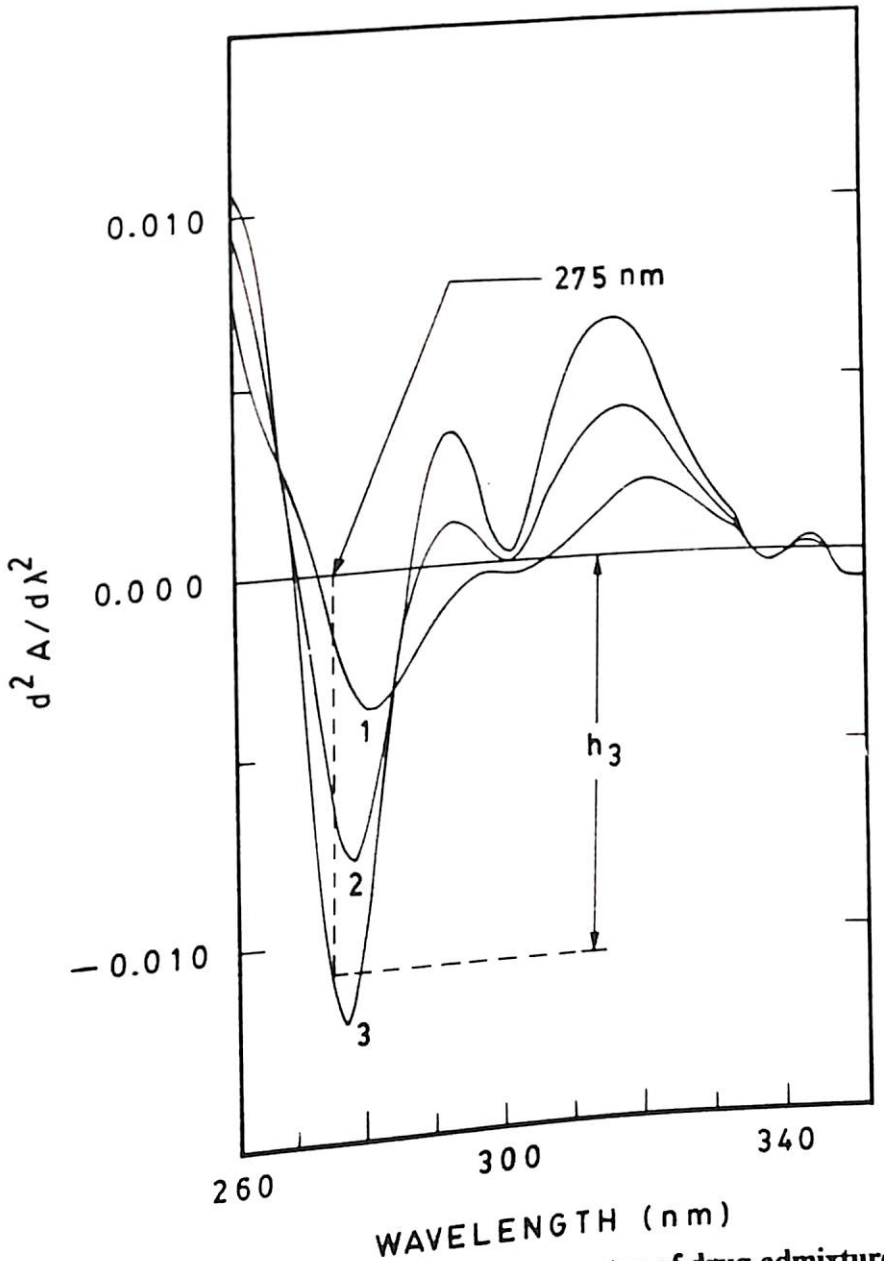


Fig 42. Second-order derivative spectra of drug admixture of imipramine HCl and diazepam in 0.1M HCl; concentration of diazepam 5 mcg ml^{-1}; concentration of imipramine HCl 10, 40 and 70 mcg ml^{-1} in curves 1, 2 and 3 respectively

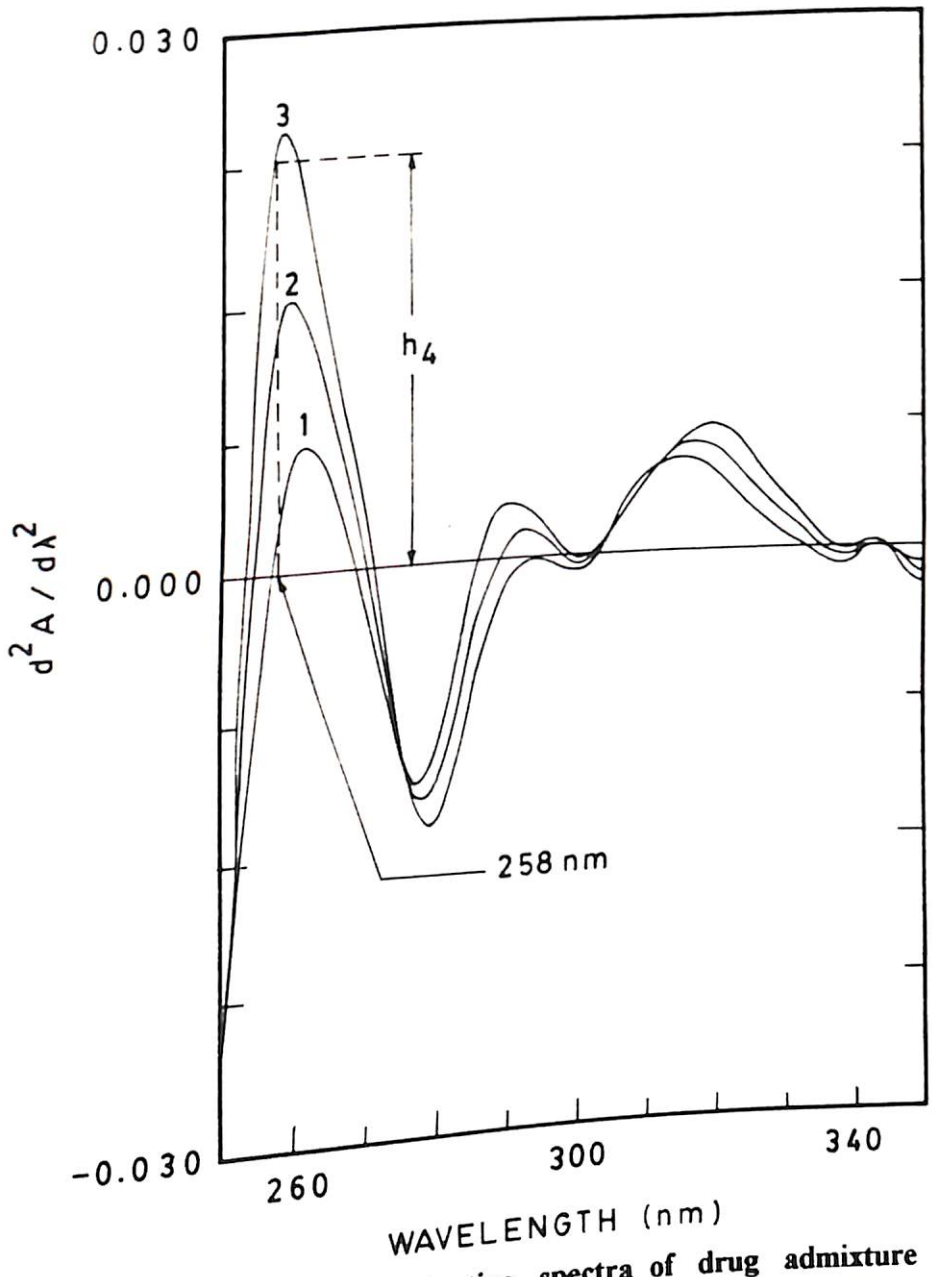


Fig 43. Second-order derivative spectra of drug admixture of diazepam and imipramine HCl in 0.1M HCl; concentration of imipramine HCl 40 mcg ml^{-1}; concentration of diazepam is 2, 5 and 8 mcg ml^{-1} in curves 1,2 and 3 respectively

drugs. Thus these zero crossing points were used for the estimation of the drugs without interference from each other.

The small standard deviation values showed the precision of the derivative method (Tables 36 and 37) and the negligible intercepts of the equations indicated regression through or close to the origin. The co-efficient of variation as well as the correlation co-efficient values were found to be quite satisfactory. The calculated F test values for each concentration of the drugs (Tables 36 and 37) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.83 - 99.95% variation in the derivative values were accounted for by the concentration of the- drugs in solution. The results of F test using ratio of mean square due to regression to the mean square about the regression (Table 38) showed the calculated values to be much higher than the critical values at 5% level of significance proving the linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve (Table 36-38). The ratio of residuals expressed as percentage showed a scatter which was random. The similarity of the regression equations of pure admixture of the drugs to those of admixtures with excipients evidenced the elimination of interference of the excipients on second-order transformation. Such a transformation, as mentioned earlier, will be able to eliminate the interference by producing a spectra which is almost a straight line. The order of the transformation will depend on the degree of the function

Table 36. Selectivity of the Method for the Determination of Imipramine Hydrochloride in the Presence of Diazepam by Second-order Derivative Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (275nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
IMP	DIZ					Crit	Calc
10	0	0.0015 ± 0.0001	6.67	0.0001	94.46	3.48	1.47
20	0	0.0030 ± 0.0001	3.26	0.0001	97.27	3.48	1.56
30	0	0.0042 ± 0.0001	3.53	0.0001	102.86	3.48	0.64
40	0	0.0057 ± 0.0002	4.89	0.0001	102.15	3.48	0.19
50	0	0.0073 ± 0.0002	2.79	0.0001	99.65	3.48	0.36
60	0	0.0087 ± 0.0002	2.37	0.0001	101.11	3.48	0.36
70	0	0.0104 ± 0.0002	2.58	0.0001	98.53	3.48	0.20
10	5	0.0015 ± 0.0001	5.77	0.0001	94.25	3.48	2.97
20	5	0.0029 ± 0.0001	3.65	0.0001	97.54	3.48	1.83
30	5	0.0042 ± 0.0002	5.67	0.0001	103.30	3.48	0.36
40	5	0.0057 ± 0.0003	5.39	0.0001	102.11	3.48	0.23
50	5	0.0075 ± 0.0002	2.78	0.0001	98.56	3.48	0.52
60	5	0.0087 ± 0.0004	4.23	0.0001	101.78	3.48	0.16
70	5	0.0105 ± 0.0003	2.65	0.0001	98.53	3.48	0.29

IMP - Imipramine Hydrochloride DIZ - Diazepam

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(5,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 37.Selectivity of the Method for the Determination of Diazepam in the Presence of Imipramine Hydrochloride by Second-order Derivative Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (258nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
DIZ	IMP					Crit	Calc
2	0	0.0487 ± 0.0002	0.59	0.0001	97.94	3.48	0.37
3	0	0.0725 ± 0.0005	0.69	0.0001	100.13	3.48	0.07
4	0	0.0970 ± 0.0002	0.28	0.0001	100.41	3.48	0.25
5	0	0.1216 ± 0.0003	0.29	0.0001	100.52	3.48	0.15
6	0	0.1469 ± 0.0003	0.23	0.0001	100.13	3.48	0.17
7	0	0.1703 ± 0.0004	0.26	0.0001	100.93	3.48	0.11
8	0	0.1987 ± 0.0004	0.19	0.0001	99.03	3.48	0.13
2	40	0.0486 ± 0.0003	0.59	0.0001	97.95	3.48	0.19
3	40	0.0722 ± 0.0003	0.48	0.0001	100.33	3.48	0.14
4	40	0.0097 ± 0.0003	0.26	0.0001	100.25	3.48	0.25
5	40	0.1218 ± 0.0004	0.29	0.0001	100.37	3.48	0.13
6	40	0.1469 ± 0.0004	0.21	0.0001	100.16	3.48	0.17
7	40	0.1703 ± 0.0003	0.24	0.0001	101.11	3.48	0.10
8	40	0.1991 ± 0.0004	0.22	0.0001	98.96	3.48	0.10

IMP - Imipramine Hydrochloride DIZ - Diazepam

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(5,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 38. Regression Analysis of Imipramine HCl and Diazepam Standard Solutions

Sample	Composition of Solution (mcg ml^{-1}) IMP	DIZ	Regression Equation [a] (275 nm for IMP and 258 nm for DIZ)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept	Estimate
Series A	10-70	0	y = 0.0001x - 0.0001	0.9993	99.87	6.61	3987	2.57	63	0.0001	0.0001	0.0001
Series B	10-70	5	y = 0.0001x - 0.0001	0.9992	99.83	6.61	2956	2.57	54	0.0001	0.0001	0.0001
Series C	0	2-7	y = 0.0248x - 0.0019	0.9997	99.95	6.61	1079	2.57	104	0.0002	0.0012	0.0001
Series D	40	2-7	y = 0.0249x - 0.0022	0.9997	99.95	6.61	9406	2.57	96	0.0003	0.0014	0.0001
Series E	10-70	5	y = 0.0001x - 0.0002	0.9990	99.76	6.61	2157	2.57	46	0.0001	0.0001	0.0001
Series F	10-70	5	y = 0.0001x - 0.0002	0.9991	99.79	6.61	2459	2.57	49	0.0001	0.0001	0.0001
Series G	10-70	5	y = 0.0001x - 0.0001	0.9994	99.76	6.61	2157	2.57	46	0.0001	0.0001	0.0001
Series H	40	2-7	y = 0.0249x - 0.0025	0.9996	99.93	6.61	7462	2.57	86	0.0003	0.0015	0.0001
Series I	40	2-7	y = 0.0249x - 0.0025	0.9996	99.92	6.61	6803	2.57	82	0.0003	0.0016	0.0001
Series J	40	2-7	y = 0.0248x - 0.0020	0.9998	99.90	6.61	8455	2.57	84	0.0003	0.0015	0.0001

IMP -Imipramine HCl DIZ - Diazepam [a] Based on seven calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 7 for imipramine HCl as well as diazepam.

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 7 for both imipramine HCl as well as diazepam.

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

which would approximate the spectral pattern. For example, the second-order transformation will be able to eliminate spectra which approximate a linear function whereas interferences which may be approximated by a quadratic function would require a third-order transformation. As can be seen from figures 21-32, the second-order transformation will be able to eliminate the interference of typical additives such as lactose, pvp and indigocarmine above 250nm. This was clearly seen in the case of the estimation of imipramine HCl and diazepam which were estimated at 258 and 275nm. The regression equations were very similar.

The results of the estimation of imipramine HCl and diazepam in synthetic admixtures and commercial formulations by second-order derivative spectrophotometry have been given in table 39. The estimation of synthetic admixtures were done using a concentration of 25 mcg/ml of imipramine HCl and 5 mcg/ml of diazepam which resulted in a mean recovery of 100.68 and 99.40% for imipramine HCl and diazepam respectively. Actually, the concentration of diazepam was the mean point of the calibration range whereas that of imipramine HCl was not the mean point of calibration which was 40 mcg/ml. But the assay results as well as the 95% confidence level range were found to be satisfactory since the assay results were well within the official limits (for single component tablets) of 92.5-107.5 [3] and 93-107 [1] for imipramine HCl and 92.5-107.5 [3] and 90-110 [3] for diazepam.

The assay results in tables 35 and 39 showed difference in the mean recovery by the three proposed methods for the same commercial formulation. But it may be concluded that the second-order derivative method was more accurate since it uses the calibration data at a particular wavelength which had been fit by least square method whereas the linear plot method uses data at multiple wavelengths. The absorbance ratio method also uses the least square method, but will not be able to eliminate

Table 39. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Imipramine Hydrochloride and Diazepam by Second-order Derivative Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	IMP	DIZ	IMP	DIZ	IMP	DIZ	IMP	DIZ
Pure Drug Admixture	25	5	—	—	100.68	99.40	99.05-102.30	99.31-99.48
Brand A	25	5	25	5	97.48	99.40	95.85-99.10	99.31-99.48
Brand B	25	5	25	5	100.70	99.42	99.07-102.30	99.33-99.50
Brand C	25	5	25	5	96.92	99.50	95.29-98.54	99.42-99.58
Brand D	50	4	25	2	103.16	99.87	101.59-104.72	99.78-99.95

IMP - Imipramine hydrochloride DIZ - Diazepam

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 5 degrees of freedom for both imipramine hydrochloride as well as diazepam

the contribution of the additives, if any, at the chosen wavelengths for estimation. During the estimation of imipramine HCl and diazepam by second-order derivative method, the basic advantage of the second-order transformation i.e., the elimination of the spectral contribution of additives such as lactose, pvp and coloring agents was not lost since the additives show a spectral pattern which can be approximated by a linear function at the wavelengths of 275 and 258nm making their elimination possible by second-order transformation.

5.04 DETERMINATION OF DIPHENHYDRAMINE HYDROCHLORIDE AND DIAZEPAM IN PURE ADMIXTURES AND TABLETS BY LINEAR PLOT AND SECOND-ORDER DERIVATIVE SPECTROPHOTOMETRIC METHODS

INTRODUCTION

The combination of diphenhydramine hydrochloride with diazepam as a tablet preparation has been introduced in the market for treatment of insomnia, allergy and status epilepticus. Chemically, diphenhydramine hydrochloride is (RS) -2-benzhydryloxyethyldimethyl amine hydrochloride and diazepam is 7-chloro-1,3-dihydro-1-methyl-5-phenly-1,4-benzodiazepin-2-one. The methods reported thus far for the estimation of diphenhydramine hydrochloride include non-aqueous titrimetry [227], atomic absorption spectrophotometry [228], spectrofluorometry [229], spectrophotometry [230-233] including application of orthogonal function method [230] and second-order derivative method for estimation in nasal drops [233] and HPLC [234,235]. The methods for estimation of diazepam include potentiometry [214], differential pulse polarography [215], spectrophotometry [216-219], spectrofluorometry [220], TLC [221], GLC [222], HPLC [223-226]. The official methods for the estimation of diphenhydramine hydrochloride in capsules include titrimetry [2,3] and HPLC [1] and those for diazepam include non-aqueous titrimetry [2] and spectrophotometry [3].

Materials, Reagents and Apparatus

1. Hydrochloric Acid - A.R.Grade (E.Merck India Ltd.)
2. Methanol - Spectroscopic Grade (Spectrochem. India)
3. Diphenhydramine hydrochloride and diazepam were obtained as gift samples.

The second-order derivative spectra were recorded at a scan rate of 240nm/min with a Jasco 7800 uv-visible double beam scanning spectro

photometer using 1cm matched quartz cuvettes. The resolution of the spectrophotometer for recording the second-order derivative spectra was checked as per the procedure in B.P. 1993 by recording the second-order derivative spectra of 0.02% v/v solution of toluene in methanol and was found to be satisfactory. The data thus recorded with the various solutions have been given in tables 40-45.

Standard and Sample Solutions

The stock solutions of the drugs containing 10mg/ml of diphenhydramine hydrochloride and 1mg/ml of diazepam were prepared in methanol by dissolving the pure drugs in methanol by thorough shaking. Appropriate volumes of aliquots from the stock solutions were used to prepare different series of solutions in 0.1M HCl.

The solutions for linear plot method were prepared with the composition as shown in table 40. In addition, the series A-J solutions were prepared for estimation by second-order derivative spectrophotometry.

The first series (Series A) comprised of solutions of diphenhydramine HCl of varying concentrations (100-300mcg/ml) prepared by pipetting out appropriate volumes of aliquots from the stock solution of diphenhydramine HCl (10mg/ml) into 100ml volumetric flasks and making up the volume with 0.1N HCl.The second series (Series C) consisted of solutions of diazepam of varying concentration (10-30 mcg/ml) prepared in a similar fashion in 0.1N HCl. The third series (Series B) comprised of mixtures of the drugs having a constant concentration of diazepam (20 mcg /ml) and a varying concentration of diphenhydramine HCl (100-300 mcg/ml) prepared by pipetting out the same volumes of aliquots from diphenhydramine HCl stock solution as in the preparation of Series A solutions into 100ml volumetric flasks , adding 2 ml of diazepam stock solution (1mg/ml) to each

flask and making up the volume with 0.1N HCl. Similarly, the fourth series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the diazepam and diphenhydramine HCl stock solutions so as to give solutions containing various concentrations of diazepam (10-30 mcg/ml) along with a constant concentration of diphenhydramine HCl (200 mcg/ml) in 0.1N HCl. The series E,F and G were exactly similar to that of series C except that they had lactose (600 mcg/ml), pvp (600mcg/ml) and indigocarmine (4 mcg/ml) in them. These additives were added by using appropriate volumes of aliquots of stock solutions of lactose (10mg/ml), pvp (10mg/ml) and indigocarmine (1mg/ml) in water since lactose, pvp as well as indigocarmine were freely soluble in water.

Thirty tablets (of each batch) were finely ground and a weight of the powder containing approximately 500mg of diphenhydramine HCl and 50 mg of diazepam was dissolved in methanol, filtered (Whatman No 1. filter paper) and appropriate volumes of aliquots of the filtrate were used to prepare sample solutions containing approximately the concentrations of diphenhydramine HCl and diazepam as given in table 45.

The solutions were stored in low actinic Pyrex volumetric flasks at room temperature till their analysis. The stability of the solutions (0.1M HCl) were monitored spectrophotometrically for a period of three hours and were found to vary by the following absorbance units (AU) : diphenhydramine HCl in 0.1N HCl by ± 0.008AU and diazepam in 0.1N HCl by ± 0.009AU. All the measurements for replicate determinations were recorded within a time interval of 45-60 minutes after preparation of the solutions in 0.1N HCl to minimise the variations in absorbance with time.

Recording of the Spectra

The normal spectra in 0.1M HCl were recorded by using 0.1M HCl as the blank in the Jasco 7800 spectrophotometer. The second-order derivative spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by initially recording their normal absorption spectra at the appropriate concentrations and converting the normal spectra to second-order derivative spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. A data interval of one was found to be satisfactory. No smoothing of the derivative spectra was found necessary.

RESULTS AND DISCUSSION

The normal absorption spectra of diphenhydramine HCl (20 mcg/ml) and diazepam (20 mcg/ml) in figure 44 clearly indicated the necessity of a simultaneous estimation method. The absorption of diphenhydramine HCl would increase approximately ten fold when the concentration of diazepam was around 20 mcg/ml since the ratio of diphenhydramine HCl :diazepam in commercial formulation was around 10:1. Thus it may be possible to estimate diazepam in the presence of diphenhydramine HCl but the estimation of the later without interference from the former will not be possible.

The diphenhydramine HCl and diazepam in 0.1M HCl showed no isoabsorptive point and hence the absorbance ratio method could not be applied for the estimation of this combination (figure 44). The linear plot method was used for the estimation of these drugs using the data points between 253-295nm and the percentage relative error increases in pure admixtures due to the presence of additives. But the results of the estimation of the drugs in commercial formulations in table 41 showed that the

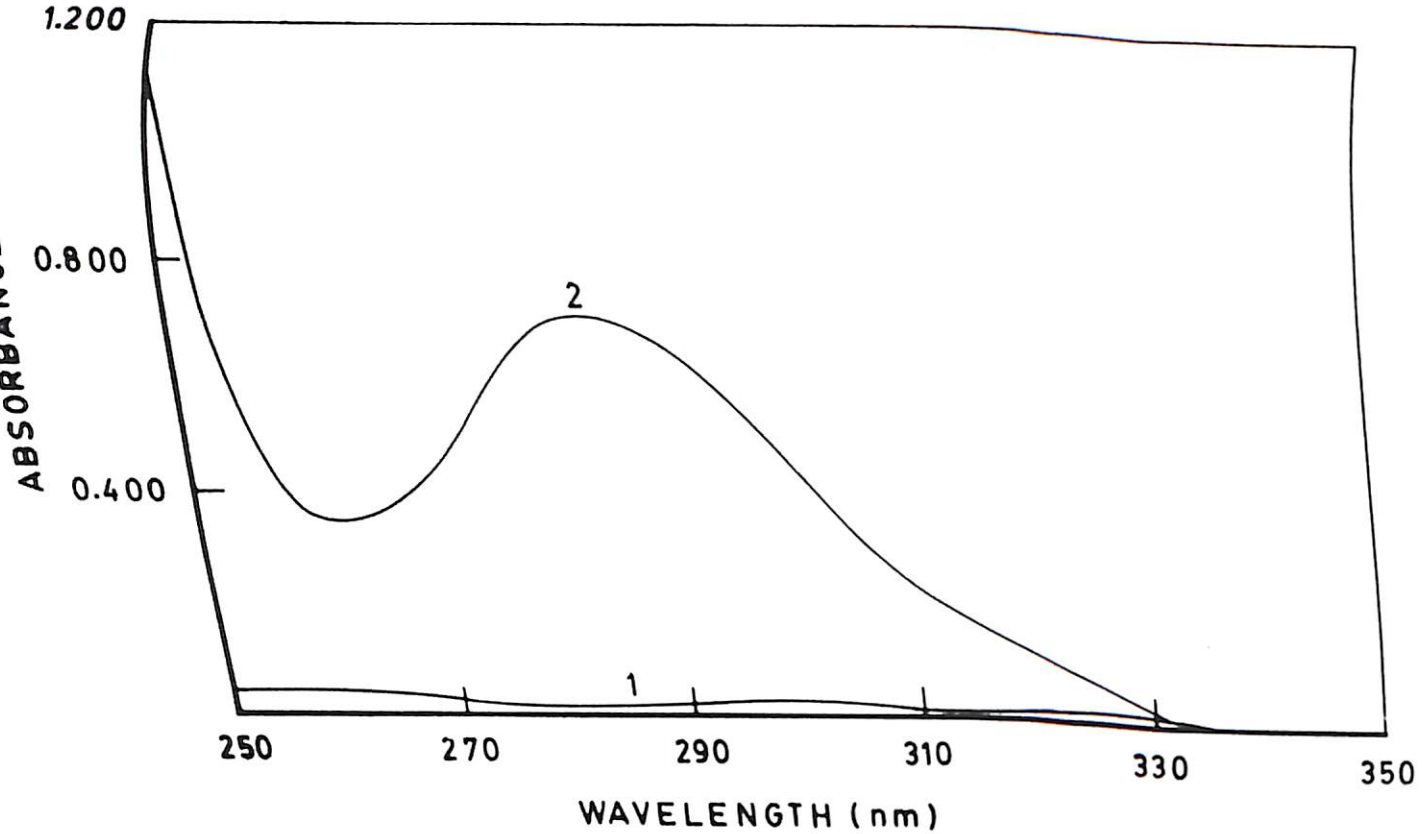


Fig 44. Normal absorption spectra of pure diphenhydramine HCl and diazepam in 0.1M HCl ; concentration of pure diphenhydramine HCl is 20 mcg ml^{-1} (curve 1); concentration of diazepam is 20 mcg ml^{-1} (curve 2)

Table 40.Results of Determination of Diphenhydramine Hydrochloride and Diazepam in Pure Admixtures by Linear Plot Method

Solution	Solv.	Concentration of Additives (mcg ml^{-1})			Concentration of Drugs[a, b] (mcg ml^{-1})				% Relative Error	
		LAC	PVP	CAR	DPN ACT	DPN FND	DIZ ACT	DIZ FND	DPN	DIZ
Pure Drugs	A	----	------	------	200	200.89	20.00	19.66	0.44	-1.70
Pure Drugs	A	600	------	------	200	268.87	20.00	20.20	34.43	1.00
Pure Drugs	A	----	600	------	200	222.60	20.00	20.40	11.30	2.00
Pure Drugs	A	-----	- ----	4	200	217.35	20.00	22.91	8.67	14.50

DPN - Diphenhydramine hydrochloride DIZ - Diazepam A - 0.1M HCl
ACT - Actual value FND - Found value LAC - Lactose CAR- Indigocarmine
PVP - Polyvinylpyrrolidone

[a] **Wavelength range used for collection of data points was 253-295nm;**
concentration obtained from the slope and intercept of the linear curve plot

[b] **Based on five replicate determinations**

Table 41. Results of Assay of Diphenhydramine Hydrochloride and Diazepam in Commercial Samples by Linear Plot Method

Sample	Method	Label Claim (mg/tablet)		Mean Recovery, % [a]	
		DPH	DIZ	DPH	DIZ
Brand A (Batch 1)	LPM	50	5	99.35 ± 0.39	99.35 ± 0.68
Brand A (Batch 2)	LPM	50	5	99.25 ± 0.78	99.43 ± 0.54

[a] Average of ten determinations; assay as percentage of label claim

LPM - Linear Plot Method ARM - Absorbance Ratio Method

formulation did not contain additives which would solubilise in the final dilution and interfere in the estimation of the drugs. The mean recovery of the drugs in commercial formulations by linear plot method was almost the same as that of pure drug admixtures (Tables 40 and 41).

Second-order derivative method

For this method, the spectra of the drug solutions were recorded in 0.1M HCl as normal spectra and converted to second-order derivative spectra using the digital algorithms. The zero crossing point of 275nm of diazepam was used for the estimation of diphenhydramine HCl and the wavelength of 285nm at which the $d^2A/d\lambda^2$ value of diphenhydramine HCl became zero was chosen for the estimation of diazepam. Thus the derivative values of diphenhydramine HCl at 275nm (h_1 and h_3 in figures 45 and 47) and the derivative values of diazepam at 285nm (h_2 and h_4 in figures 46 and 48) were found to be proportional to the corresponding concentrations and independent of each other and hence used for the estimation of the drugs.

The small standard deviation values showed the precision of the derivative method (Tables 42 and 43) and the negligible intercepts of the equations indicated regression through or close to the origin. The co-efficient of variation, the correlation co-efficient values, the calculated F test and T test values have been given in tables 42-44. The calculated F test values for each concentration of the drugs (Tables 42 and 43) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.46 - 99.96% variation in the derivative values were accounted for by the concentration of the drugs in solution. The results of F test using ratio of mean square due to regression to the mean square about the regression (Table 44) showed the calculated values to be much higher than the critical values at 5% level of significance proving the

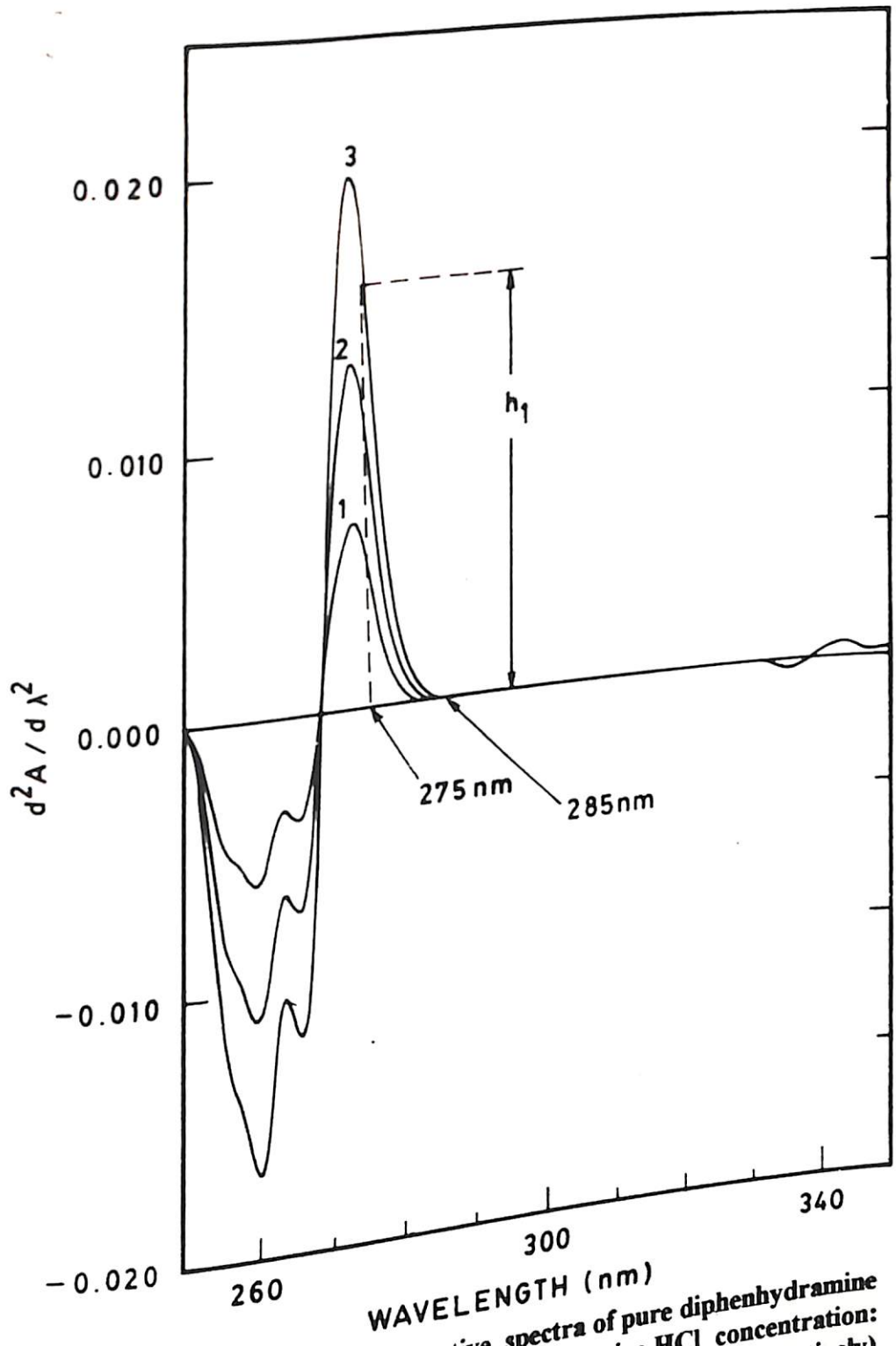


Fig 45. Second-order derivative spectra of pure diphenhydramine HCl in 0.1M HCl (diphenhydramine HCl concentration: 100, 200 and 300 mcg ml^{-1} in curves 1,2 and 3 respectively)

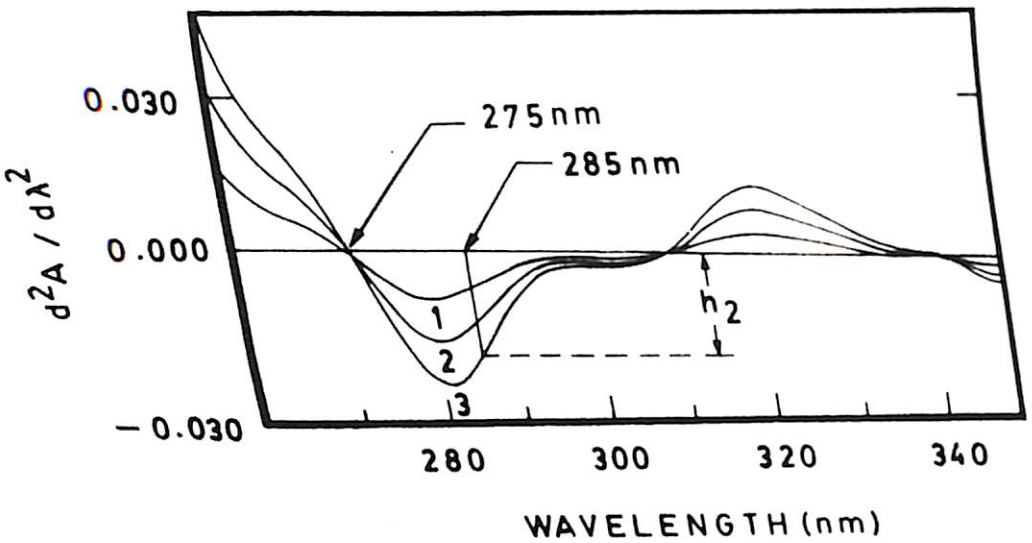


Fig 46. Second-order derivative spectra of pure diazepam in 0.1M HCl (diazepam concentration:10,20 and 30 mcg ml^{-1} in curves 1,2 and 3 respectively)

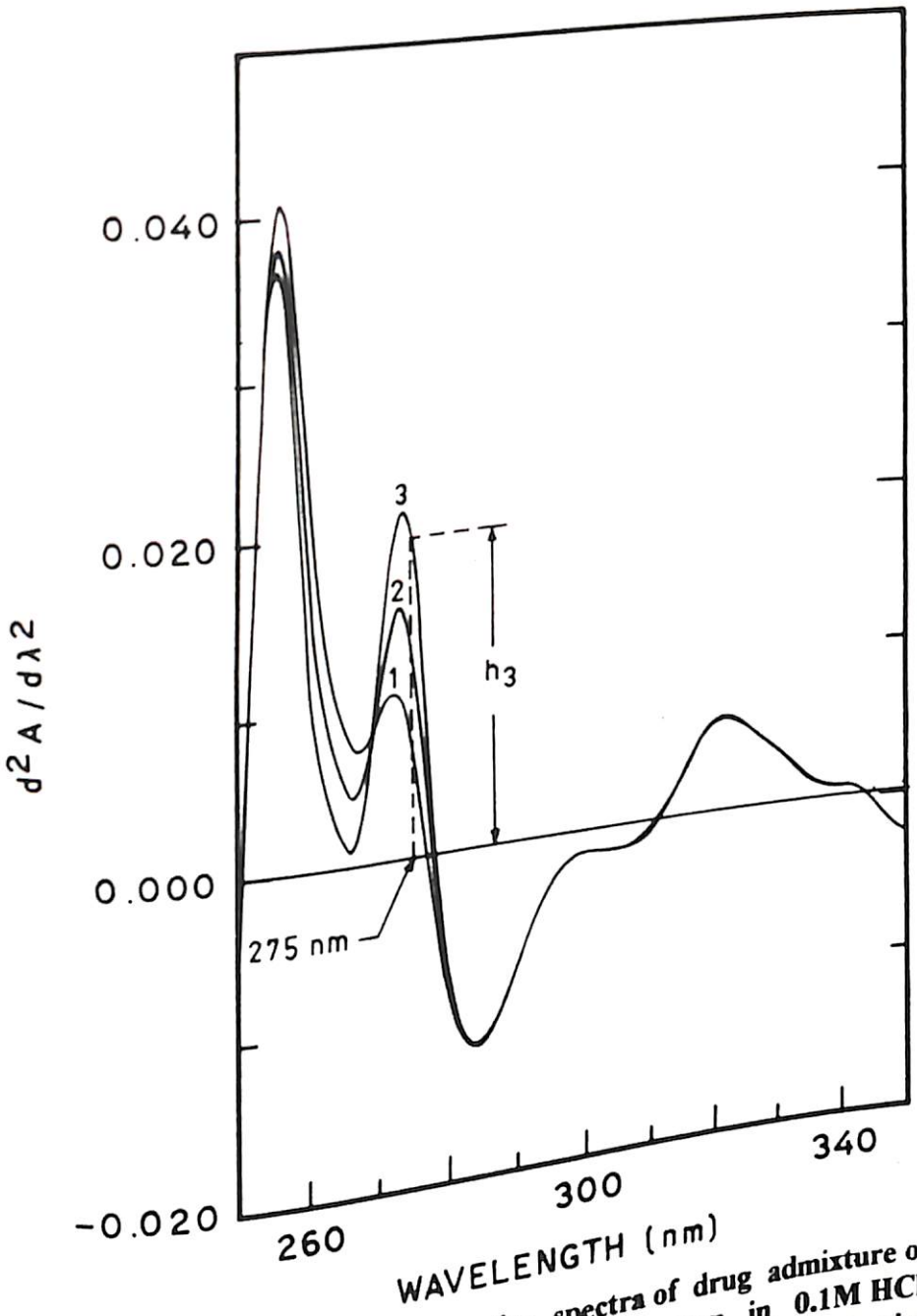


Fig 47. Second-order derivative spectra of drug admixture of diphenhydramine HCl and diazepam in 0.1M HCl; concentration of diazepam, 20 mcg ml^{-1}; concentration of diphenhydramine HCl: 100, 200 and 300 mcg ml^{-1} in curves 1,2 and 3 respectively

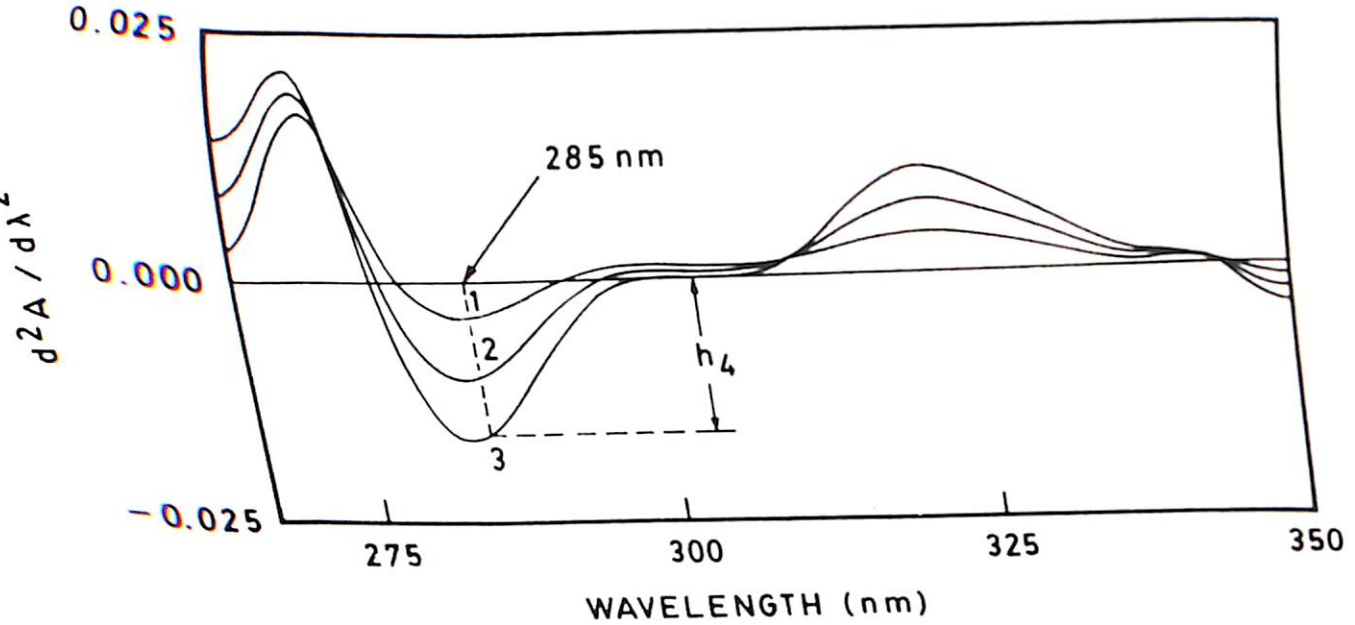


Fig 48. Second-order derivative spectra of drug admixture of diazepam and diphenhydramine HCl in 0.1M HCl; concentration of diphenhydramine HCl, 200 mcg ml^{-1} ; concentration of diazepam : 10, 20 and 30 mcg ml^{-1} in curves 1,2 and 3 respectively

Table 42. Selectivity of the Method for the Determination of Diphenhydramine Hydrochloride in the Presence of Diazepam by Second-order Derivative Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (275nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
DPH	DIZ					Crit	Calc
100	0	0.0061 ± 0.0002	3.86	0.0001	100.11	3.63	0.32
140	0	0.0086 ± 0.0002	2.69	0.0001	99.73	3.63	0.32
180	0	0.0109 ± 0.0003	1.91	0.0001	99.88	3.63	0.43
220	0	0.1346 ± 0.0002	1.69	0.0001	99.52	3.63	0.32
260	0	0.0156 ± 0.0003	2.09	0.0001	101.45	3.63	0.17
300	0	0.0184 ± 0.0003	1.75	0.0001	99.27	3.63	0.17
100	20	0.0061 ± 0.0002	3.85	0.0001	100.02	3.63	0.43
140	20	0.0086 ± 0.0003	3.82	0.0001	99.73	3.63	0.22
180	20	0.0109 ± 0.0002	2.72	0.0001	100.29	3.63	0.24
220	20	0.1357 ± 0.0003	2.31	0.0001	99.03	3.63	0.25
260	20	0.0156 ± 0.0004	2.41	0.0001	101.62	3.63	0.16
300	20	0.0184 ± 0.0004	2.17	0.0001	99.29	3.63	0.14

DPH - Diphenhydramine HCl DIZ - Diazepam

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 43. Selectivity of the Method for the Determination of Diazepam in the Presence of Diphenhydramine Hydrochloride by Second-order Derivative Spectrophotometry

Composition of the solution (mcg ml^{-1}) DIZ	DPH	Mean value of [a] absorbance (δA) (285nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
10	0	0.0061 ± 0.0002	3.34	0.0001	98.52	3.63	0.36
14	0	0.0085 ± 0.0002	2.83	0.0001	99.83	3.63	0.36
18	0	0.0107 ± 0.0003	3.32	0.0001	101.89	3.63	0.12
22	0	0.0135 ± 0.0004	3.16	0.0001	99.41	3.63	0.08
26	0	0.0158 ± 0.0005	3.14	0.0001	100.36	3.63	0.06
30	0	0.0184 ± 0.0003	1.69	0.0001	99.59	3.63	0.34
10	200	0.0062 ± 0.0002	3.64	0.0001	98.53	3.63	0.21
14	200	0.0085 ± 0.0003	3.62	0.0001	100.62	3.63	0.11
18	200	0.0109 ± 0.0004	3.43	0.0001	100.88	3.63	0.09
22	200	0.0135 ± 0.0004	3.24	0.0001	99.56	3.63	0.05
26	200	0.0158 ± 0.0005	2.99	0.0001	100.61	3.63	0.04
30	200	0.0184 ± 0.0004	1.91	0.0001	99.61	3.63	0.09

DPH - Diphenhydramine HCl DIZ - Diazepam

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non- linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 44. Regression Analysis of Diphenhydramine Hydrochloride and Diazepam Standard Solutions

Sample	Composition of Solution (mcg ml^{-1}) DPH	DIZ	Regression Equation [a] (275 nm for DPH and 285 nm for DIZ)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept	Estimate
Series A	100-300	0	y = 0.0001x + 0.0001	0.9996	99.92	3.63	5553	2.77	74	0.0001	0.0001	0.0001
Series B	100-300	20	y = 0.0001x + 0.0001	0.9995	99.90	3.63	4134	2.77	64	0.0001	0.0002	0.0001
Series C	0	10-30	y = 0.0006x - 0.0001	0.9996	99.94	3.63	6477	2.77	80	0.0001	0.0002	0.0001
Series D	200	10-30	y = 0.0006x - 0.0001	0.9998	99.96	3.63	12186	2.77	110	0.0001	0.0001	0.0001
Series E	100-300	20	y = 0.0001x + 0.0001	0.9992	99.92	3.63	2588	2.77	50	0.0001	0.0002	0.0001
Series F	100-300	20	y = 0.0001x - 0.0001	0.9994	99.89	3.63	3671	2.77	60	0.0001	0.0002	0.0001
Series G	100-300	20	y = 0.0001x - 0.0002	0.9997	99.90	3.63	3512	2.77	51	0.0001	0.0001	0.0001
Series H	200	10-30	y= 0.0006x + 0.0001	0.9990	99.89	3.63	1314	2.77	36	0.0001	0.0003	0.0001
Series I	200	10-30	y = 0.0006x - 0.0004	0.9995	99.50	3.63	804	2.77	28	0.0001	0.0004	0.0001
Series J	200	10-30	y = 0.0006x - 0.0003	0.9998	99.45	3.63	809	2.77	109	0.0001	0.0004	0.0001

DPH - Diphenhydramine Hydrochloride DIZ - Diazepam

[a] Based on six calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n*-2) value at 5% significance level; F *crit* is the F (*1*, *n*-2) value from the *F ratio table* for 5% significance level; n is 6 for both diphenhydramine hydrochloride as well as diazepam

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 6 for both diphenhydramine hydrochloride and diazepam

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

Table 45. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Diphenhydramine hydrochloride and Diazepam by Second-order Derivative Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	DPH	DIZ	DPH	DIZ	DPH	DIZ	DPH	DIZ
Pure Drug Admixture	200	20	----	----	99.67	99.15	95.31-104.02	98.90-99.39
Brand A (Batch 1)	200	20	----	----	98.68	100.60	94.32 -102.97	100.35-100.84
Brand A (Batch 2)	200	20	----	----	99.34	99.95	94.98-103.69	99.70-100.19
Brand A (Batch 3)	200	20	----	----	98.20	101.30	93.84-102.50	101.05-101.54

DPH - Diphenhydramine hydrochloride DIZ - Diazepam

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 4 degrees of freedom for both diphenhydramine hydrochloride as well as diazepam

linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve (Table 42-44). The ratio of residuals expressed as percentage showed a scatter which was random. The similarity of the regression equations of pure admixture of the drugs to those of admixtures with excipients evidenced the elimination of interference of the excipients on second-order transformation.

The results of the estimation of diphenhydramine HCl and diazepam in synthetic admixtures and commercial formulations by second-order derivative spectrophotometry have been given in table 45. The estimation of synthetic admixtures was done using a concentration of 200 mcg/ml of diphenhydramine HCl and 20 mcg/ml of diazepam which resulted in a mean recovery of 99.67 and 99.15% for diphenhydramine HCl and diazepam respectively. Both the concentrations were the mean points of the calibration range of the corresponding drugs and hence the error of prediction was minimum at this concentration. The assay results by the linear plot method as well as by second-order derivative method showed that the drug contents in the commercial samples were within the official limits (in capsules as single ingredients) of 90-110% [1] and 93-107 [3] for diphenhydramine HCl and 92.5-107.5 [3] and 90-110 [1] for diazepam. Among the two methods, the second-order derivative method is more advantageous since it can eliminate the interference of the excipients, if any, in the formulation provided the interfering spectral pattern could be approximated by a linear function.

5.05 DETERMINATION OF ASPIRIN AND DIPYRIDAMOLE IN PURE ADMIXTURES AND TABLETS BY LINEAR PLOT AND SECOND-ORDER DERIVATIVE DIFFERENCE SPECTROPHOTOMETRIC METHODS

INTRODUCTION

The combination of aspirin and dipyridamole is being widely used as an anti-anginal preparation. The various methods of estimation of aspirin [33-68] and dipyridamole [68-73] had already been discussed in the sections 2.02 and 3.02 of this report. The official methods for the estimation of aspirin are HPLC [1] and titrimetry [3] and for dipyridamole HPLC [1]. Aspirin is chemically O-acetylsalicylic acid and dipyridamole is 2,2',2'', 2''' -[(4,8-dipiperidino pyrimido [5,4-d] pyrimidine-2,6-diyl) dinitrilo] tetraethanol. This section of the thesis report describes the estimation of these drugs by linear plot method and second-order derivative difference spectrophotometric method.

Materials, Reagents and Apparatus

1. Hydrochloric Acid - A.R.Grade (E.Merck India Ltd.)
2. Sodium hydroxide - A. R. Grade (Qualigens India Ltd)
3. Methanol - Spectroscopic Grade (Spectrochem. India)
4. Aspirin and dipyridamole were obtained as gift samples.

The second-order derivative spectra were recorded at a scan rate of 240nm/min with a Jasco 7800 uv-visible double beam scanning spectro photometer using 1cm matched quartz cuvettes. The resolution of the spectrophotometer for recording the second-order derivative spectra was checked as per the procedure in B.P. 1993 by recording the second-order derivative spectra of 0.02% v/v solution of toluene in methanol and was

nd to be satisfactory. The data thus recorded with the various solutions ve been given in tables 46-51.

tandard and Sample Solutions

he stock solutions of the drugs containing 1mg/ml of aspirin and 1mg/ml f dipyridamole were prepared in methanol by dissolving the pure drugs n methanol by thorough shaking. Appropriate volumes of aliquots from the stock solutions were used to prepare different series of solutions in 0.1M HCl and 0.1M NaOH.

The solutions for linear plot method were prepared with the composition as shown in table 46. In addition, the series A-J solutions were prepared for estimation by second-order derivative difference spectrophotometry. Each of the solution in the series were prepared in duplicate as equimolar solutions in 0.1M HCl and 0.1M NaOH so as to record the zero-order difference spectrum of each of the solution by scanning acid solution vs basic solution.

The first series (Series A) comprised of solutions of aspirin of varying concentrations (20-40mcg/ml) prepared by pipetting out appropriate volumes of aliquots from the stock solution into 100ml volumetric flasks and making up the volume with 0.1N HCl and 0.1M NaOH. The second series (Series C) consisted of solutions of dipyridamole of varying concentration (20-40 mcg/ml) prepared in a similar fashion in 0.1M HCl and 0.1M NaOH. The third series (Series B) comprised of mixtures of the drugs having a constant concentration of dipyridamole (30 mcg /ml) and a varying concentration of aspirin (20-40 mcg/ml) and the fourth series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the stock solutions so as to give solutions containing various concentrations of dipyridamole (20-40 mcg/ml) along

with a constant concentration of aspirin (30 mcg/ml) in the acidic and basic solvents. The series E,F and G were exactly similar to that of series C except that they had lactose (600 mcg/ml), pvp (600mcg/ml) and indigocarmine (4 mcg/ml) in them. These additives were added by using appropriate volumes of aliquots of stock solutions of the additives in water since lactose, pvp as well as indigocarmine were freely soluble in water. The stock solution of the lactose and pvp were prepared at a concentration of 20mg/ml and that of indigocarmine at 1mg/ml.

Thirty tablets (of each brand) were finely ground and a weight of the powder equal to the average weight of the tablet was dissolved in methanol by thorough shaking and filtered (Whatman No 1. filter paper). The first and last 5 ml of the filtrate were discarded and appropriate volumes of aliquots of the filtrate, after dilution in methanol, were used to prepare sample solutions containing approximately the concentrations of aspirin and dipyridamole given in table 51.

The solutions were stored in low actinic Pyrex volumetric flasks at room temperature till their analysis. The stability of the solutions were monitored spectrophotometrically for a period of three hours at the chosen wavelengths for estimation and were found to vary by the following absorbance units (AU) : aspirin and dipyridamole in 0.1N HCl by ± 0.020AU and ± 0.007 AU respectively and in 0.1M NaOH by ± 0.016 and ± 0.007 respectively. All the measurements for replicate determinations were recorded within a time interval of 30-45 minutes after preparation of the solutions to minimise the variations in absorbance with time.

cording of the Spectra

he spectra for linear plot method were recorded as normal spectra using he appropriate blanks of 0.1M HCl and 0.1M NaOH.

The zero-order difference spectra of the various solutions were initially recorded by scanning equimolar solutions in 0.1M HCl and 0.1M NaOH solvents in the Jasco 7800 spectrophotometer. The second-order derivative difference spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by converting the zero-order difference spectra to second-order derivative difference spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. A data interval of one was found to be satisfactory. Both the aspirin and dipyridamole spectra were smoothed once using a data interval of 10.

RESULTS AND DISCUSSION

The necessity of a simultaneous method for the estimation of aspirin and dipyridamole has been discussed in section 2.02.

The linear plot method was used for the estimation of these drugs using the data points between 240-275nm for acidic solutions and 270-325nm for basic solutions. The results of the estimation of the drugs in admixtures in the presence and absence of additives have been given in table 46. They show that the method has a very high relative error for the estimation of the drugs in 0.1M NaOH. Hence the estimation of the drugs had been done in the solvent of 0.1M HCl for commercial formulations the results of which have been presented in table 47.

Table 46.Results of Determination of Aspirin and Dipyridamole in Pure Admixtures by Linear Plot Method

Solution	Solv.	Concentration of Additives (mcg ml^{-1})			Concentration of Drugs[a, b] (mcg ml^{-1})				% Relative Error	
		LAC	PVP	CAR	ASP		DIP		ASP	DIP
					ACT	FND	ACT	FND		
Pure Drugs	A	—	—	—	10.00	9.86	14.00	13.80	-1.40	-1.42
Pure Drugs	A	600	—	—	10.00	10.65	14.00	14.30	6.50	2.14
Pure Drugs	A	—	600	—	10.00	12.12	14.00	14.50	21.20	3.57
Pure Drugs	A	—	—	4	10.00	12.96	14.00	15.43	29.50	10.21
Pure Drugs	B	—	—	—	10.00	15.31	14.00	15.48	53.10	10.57
Pure Drugs	B	600	—	—	10.00	14.44	14.00	15.99	44.40	11.21
Pure Drugs	B	—	600	—	10.00	13.43	14.00	16.62	34.30	18.71
Pure Drugs	B	—	—	4	10.00	16.44	14.00	16.19	64.40	15.42

ASP - Aspirin DIP - Dipyridamole A - 0.1M HCl B - 0.1 M NaOH
ACT - Actual value FND - Found value LAC - Lactose CAR- Indigocarmine
PVP - Polyvinylpyrrolidone

[a] Wavelength range used for collection of data points was 240-275nm for acidic solution and 270-325 for basic solution ; concentration obtained from the slope and intercept of the linear curve plot

[b] Based on five replicate determinations

Table 47. Results of Assay of Aspirin and Dipyridamole in Commercial Samples by Linear Plot Method

Sample	Method	Label Claim (mg/tablet)		Mean Recovery, % [a]	
		ASP	DIP	ASP	DIP
Brand A	LPM	40	75	99.86 ± 0.42	98.35 ± 0.97
Brand B	LPM	60	75	98.25 ± 0.57	99.66 ± 0.49
Brand C	LPM	100	75	99.77 ± 0.84	99.22 ± 0.46

[a] Average of ten determinations; assay as percentage of label claim

LPM - Linear Plot Method using 0.1M Hydrochloric acid

Second-order derivative difference method

For this method, the spectra of the drug solutions were recorded as zero-order difference spectra and converted to second-order derivative difference spectra using the digital algorithms. The zero crossing point of 310nm of aspirin and 272nm of dipyridamole were used for the estimation. Thus the derivative values of aspirin at 272nm (h_1 and h_3 in figures 49 and 51) and the derivative values of dipyridamole at 310nm (h_2 and h_4 in figures 50 and 52) were found to be proportional to the corresponding concentrations and independent of each other and hence used for the estimation of the drugs.

The small standard deviation values showed the precision and the negligible intercepts of the equations indicated regression through or close to the origin. The co-efficient of variation, the correlation co-efficient values, the calculated F test and T test values have been given in tables 48-50. The calculated F test values for each concentration of the drugs (Tables 48 and 49) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.76 - 99.98% variation in the derivative values were accounted for by the concentration of the drugs in solution. The results of F test using ratio of mean square due to regression to the mean square about the regression (Table 50) showed the calculated values to be much higher than the critical values at 5% level of significance proving the linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard

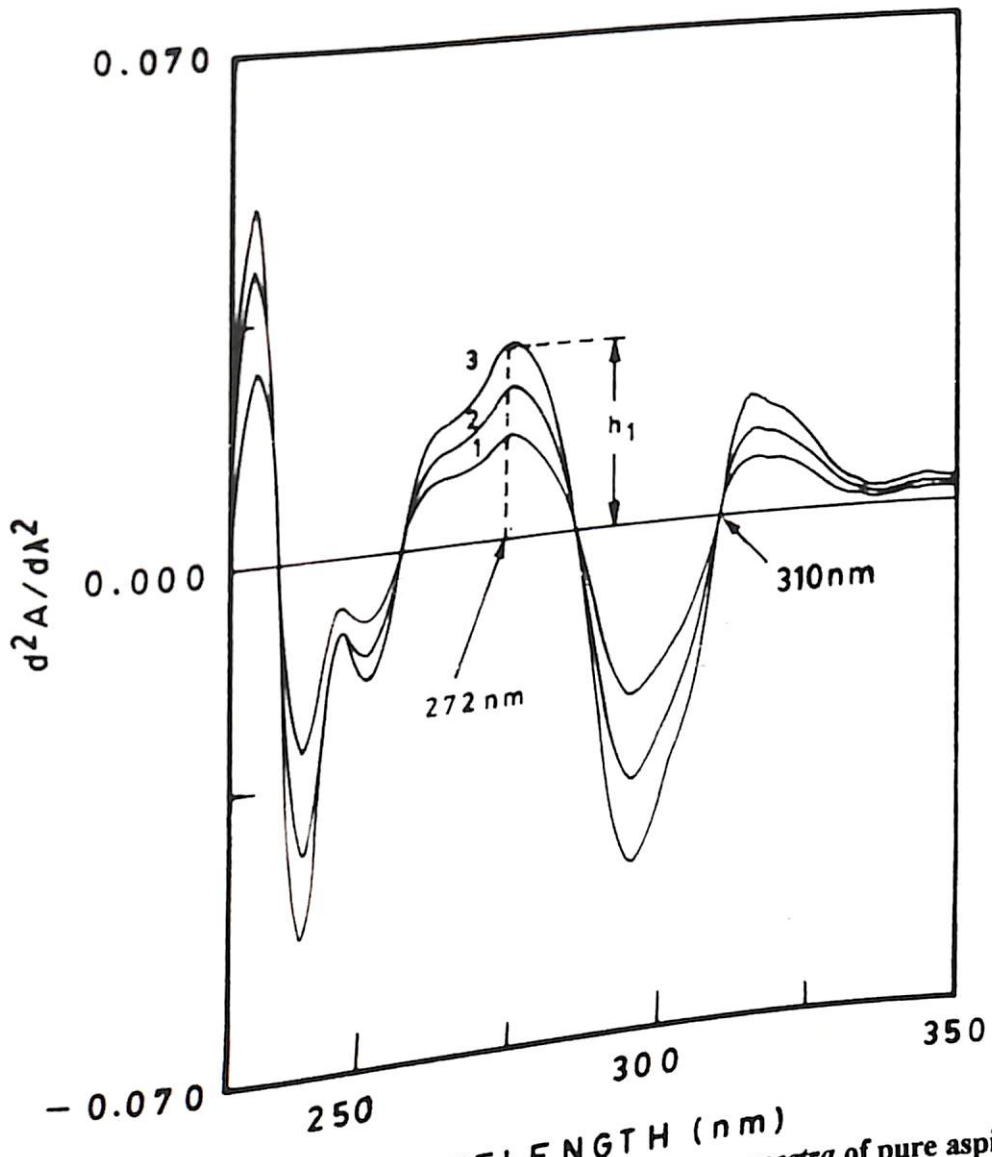


Fig 49. Second-order *derivative difference spectra* of pure aspirin obtained by scanning 0.1M HCl *vs* corresponding equimolar 0.1M NaOH solutions; (aspirin concentration: 20, 30 and 40 mcg ml^{-1} in curves 1,2 and 3 respectively)

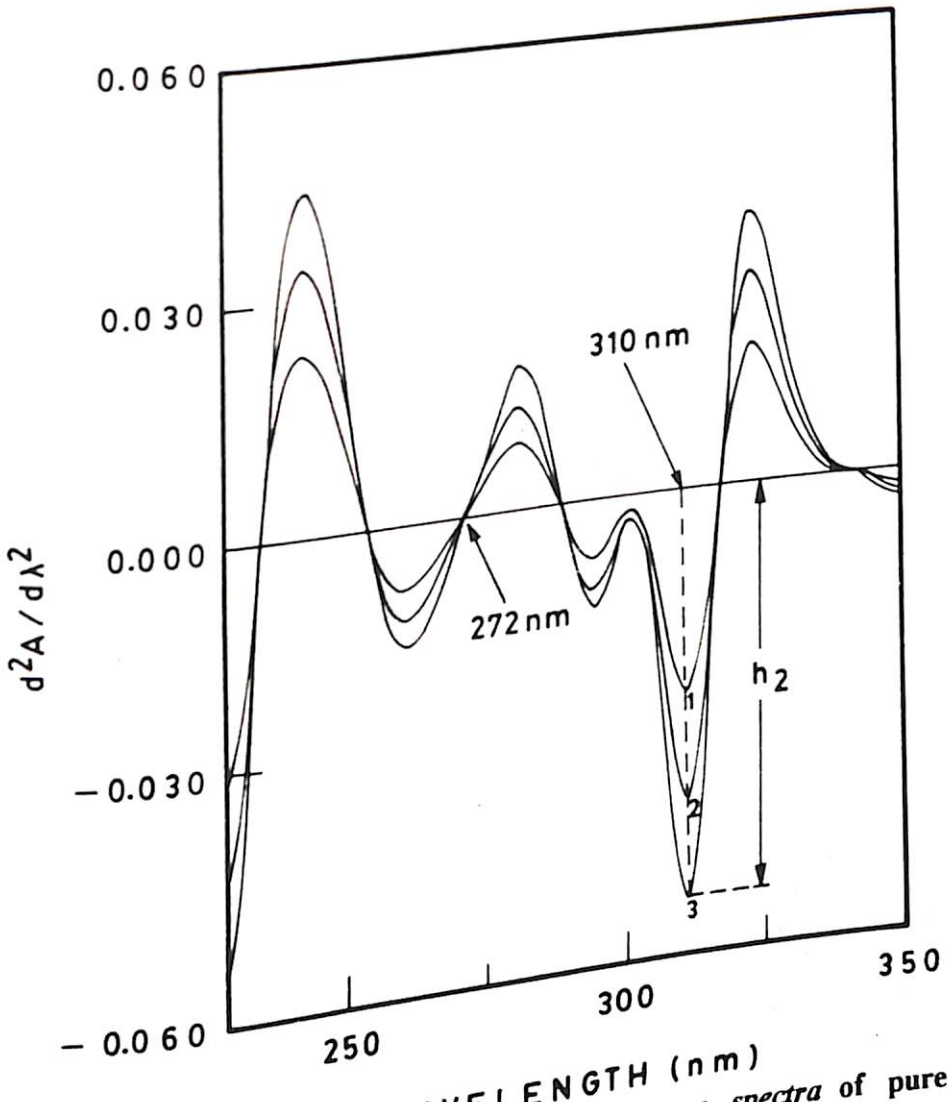


Fig 50. Second-order *derivative difference spectra* of pure dipyridamole obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (dipyridamole concentration: 20, 30 and 40 mcg ml^{-1} in curves 1,2 and 3 respectively)

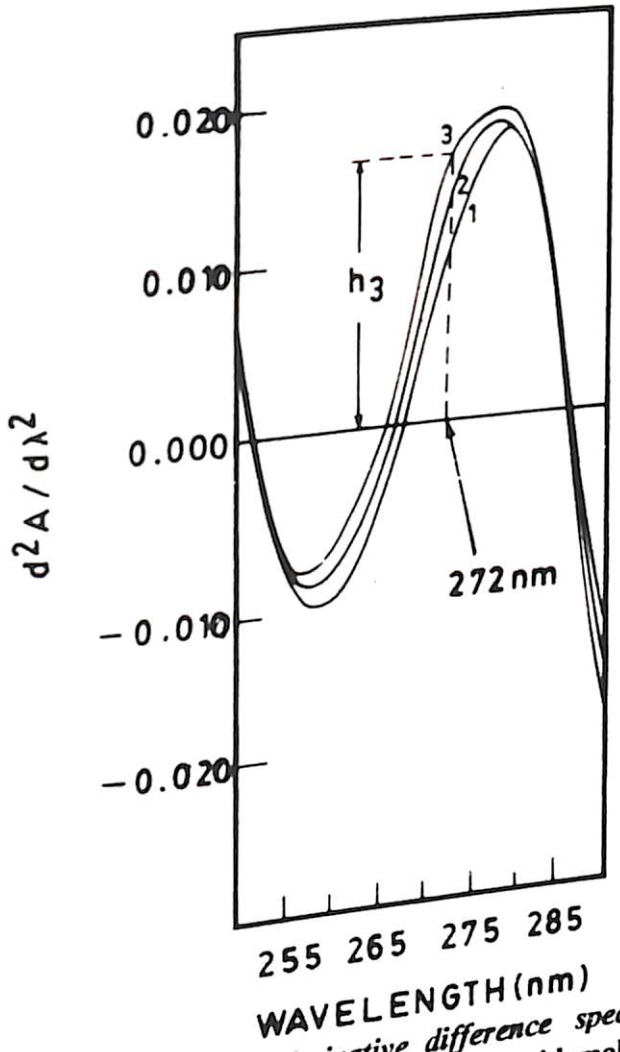


Fig 51. Second-order *derivative difference spectra* of drug admixture of aspirin and dipyridamole obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions;(concentration of dipyridamole, 30 mcg ml^{-1} ; concentration of aspirin 20, 30 and 40 mcg ml^{-1} in curves 1, 2 and 3 respectively)

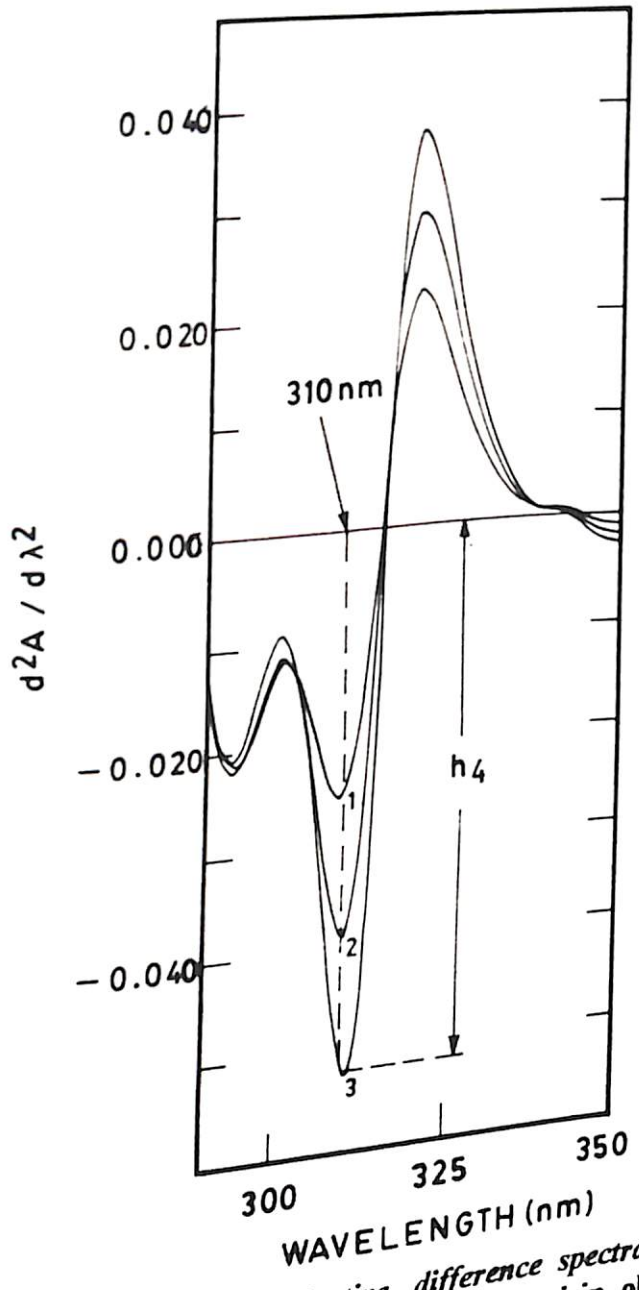


Fig 52. Second-order *derivative difference spectra* of drug admixture of dipyridamole and aspirin obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions;(concentration of aspirin, 30 mcg ml^{-1} ;concentration of dipyridamole 20, 30 and 40 mcg ml^{-1} in curves 1,2 and 3 respectively)

Table 48. Selectivity of the Method for the Determination of Aspirin in the Presence of Dipyridamole by Second-order Derivative Difference Spectrophotometry

nposition of e solution ncg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (272nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
SP	DIP					Crit	Calc
	0	0.0144 ± 0.0003	2.09	0.0001	100.92	3.63	0.27
	0	0.0176 ± 0.0003	1.64	0.0001	99.87	3.63	0.31
	0	0.0207 ± 0.0003	1.50	0.0001	99.03	3.63	0.22
	0	0.0234 ± 0.0003	1.44	0.0001	100.31	3.63	0.22
	0	0.0265 ± 0.0004	1.27	0.0001	99.53	3.63	0.22
	0	0.0292 ± 0.0004	1.39	0.0001	100.47	3.63	0.15
	0	0.0146 ± 0.0004	2.53	0.0001	100.25	3.63	0.16
)	30	0.0175 ± 0.0003	1.84	0.0001	100.43	3.63	0.22
4	30	0.0206 ± 0.0004	1.73	0.0001	99.40	3.63	0.18
8	30	0.0234 ± 0.0003	1.46	0.0001	100.15	3.63	0.19
2	30	0.0266 ± 0.0004	1.47	0.0001	99.27	3.63	0.15
6	30	0.0291 ± 0.0003	1.33	0.0001	100.57	3.63	0.15
0	30						

DIP - Dipyridamole ASP - Aspirin

[a] verage of ten replicate determinations; [b] Standard deviation of the mean

[c] atio of the *calculated* y value to *actual* y value expressed as %

[d] ased on *F test for non-linearity*; $F_{critical}$ = F (4,9) values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 49. Selectivity of the Method for the Determination of Dipyridamole in the Presence of Aspirin by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (310nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
DIP	ASP					Crit	Calc
20	0	0.0258 ± 0.0004	1.69	0.0001	100.02	3.63	0.09
24	0	0.0308 ± 0.0004	1.19	0.0001	100.30	3.63	0.13
28	0	0.0363 ± 0.0004	1.11	0.0001	99.49	3.63	0.11
32	0	0.0412 ± 0.0003	0.67	0.0001	100.29	3.63	0.22
36	0	0.0466 ± 0.0003	0.77	0.0001	99.79	3.63	0.14
40	0	0.0516 ± 0.0004	0.71	0.0001	100.11	3.63	0.13
20	30	0.0256 ± 0.0004	1.63	0.0001	100.26	3.63	0.11
24	30	0.0309 ± 0.0004	1.44	0.0001	100.04	3.63	0.09
28	30	0.0363 ± 0.0004	1.15	0.0001	99.48	3.63	0.16
32	30	0.0411 ± 0.0003	0.82	0.0001	100.33	3.63	0.10
36	30	0.0466 ± 0.0004	0.93	0.0001	99.79	3.63	0.13
40	30	0.0516 ± 0.0004	0.73	0.0001	100.13	3.63	0.06

DIP - Dipyridamole ASP - Aspirin

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(4,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve (Table 48-50). The ratio of residuals expressed as percentage showed a scatter which was random. The similarity of the regression equations of pure admixture of the drugs to those of admixtures with excipients evidenced the elimination of interference of the excipients on second-order transformation coupled with difference spectrophotometry.

The results of the estimation in synthetic admixtures and commercial formulations by second-order derivative difference spectrophotometry have been given in table 51. The estimation of synthetic admixtures was done using a concentration of 30 mcg/ml of the drugs which resulted in a mean recovery of 99.12 and 99.81% for aspirin and dipyridamole respectively. The concentration of 30mcg/ml was the mean point of the calibration range of both the drugs and hence the error of prediction was minimum at this concentration. But the assay of the tablets was done by using different concentrations of aspirin and dipyridamole since the mean recovery was good even at this concentration. The assay results by the linear plot method as well as by second-order derivative method showed that the drug contents in the commercial samples were within the official limits (in tablets as single ingredients) of 95-105% [3] and 90-110 [1] for aspirin and 90-110 [1] for dipyridamole. The pk_a of aspirin was 3.5 and that of dipyridamole was 6.4. Hence the drug solutions in 0.1M HCl (pH ≈ 1.0) and in 0.1M NaOH (pH ≈ 13.0) which were at least 1.5 pH units away from their respective pk_a values did not show appreciable changes in absorbance with small changes in the pH of the solvents [76]. Among the two methods, the second-order derivative difference method is more advantageous since it can eliminate the interference of the excipients, if any, in the formulation. This elimination, in the case of derivative

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (272 nm for ASP and 310 nm for DIP)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	ASP	DIP				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	20-40	0	y = 0.0007x - 0.0002	0.9996	99.94	7.71	6258	2.78	79	0.0001	0.0002	0.0002
Series B	20-40	30	y = 0.0007x - 0.0001	0.9997	99.94	7.71	6793	2.78	82	0.0001	0.0002	0.0001
Series C	0	20-40	y = 0.0013x - 0.0001	0.9999	99.98	7.71	26155	2.78	161	0.0001	0.0002	0.0001
Series D	30	20-40	y = 0.0013x - 0.0002	0.9999	99.98	7.71	25689	2.78	160	0.0001	0.0002	0.0001
Series E	20-40	30	y = 0.0007x - 0.0001	0.9995	99.90	7.71	8769	2.73	85	0.0001	0.0002	0.0001
Series F	20-40	30	y = 0.0007x - 0.0002	0.9994	99.90	7.71	7655	2.73	102	0.0001	0.0002	0.0001
Series G	20-40	30	y = 0.0007x - 0.0001	0.9998	99.84	7.71	7768	2.73	99	0.0001	0.0002	0.0001
Series H	30	20-40	y = 0.0013x - 0.0002	0.9997	99.92	7.71	22341	2.73	180	0.0001	0.0002	0.0001
Series I	30	20-40	y = 0.0013x - 0.0001	0.9996	99.87	7.71	21234	2.73	220	0.0001	0.0002	0.0001
Series J	30	20-40	y = 0.0013x - 0.0002	0.9995	99.76	7.71	22675	2.73	176	0.0001	0.0002	0.0001

ASP - Aspirin DIP - Dipyridamole [a] Based on six calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level; n is 6 for both aspirin and dipyridamole

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 6 for both aspirin and dipyridamole

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

difference method, will not only be a function of the transformation using digital algorithms but also the simultaneous scanning of the equimolar solutions in acidic and basic solvents. Hence there is always a possibility of enhanced discrimination against interfering substances with second-order derivative difference method in comparison to that of second-order derivative method, especially when the interfering substances do not undergo spectral alteration due to change of pH resulting in cancellation of interfering absorbance when the equimolar acidic and basic solutions are scanned for recording of the zero-order difference spectra.

Table 51. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Aspirin and Dipyridamole by Second-order Derivative Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	ASP	DIP	ASP	DIP	ASP	DIP	ASP	DIP
Pure Drug Admixture	30	30	—	—	99.12	99.81	98.73-99.50	99.62-99.99
Brand A	27.60	34.50	60	75	101.19	99.42	100.84-101.53	99.23-99.60
Brand B	35	26.25	100	75	98.85	100.14	98.47-99.22	99.95-100.32

ASP - Aspirin DIP - Dipyridamole

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 4 degrees of freedom for both aspirin and dipyridamole

furazolidone (20 mcg /ml) and a varying concentration of metronidazole (10-50 mcg/ml) and the fourth series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the stock solutions so as to give solutions containing various concentrations of furazolidone (10-30 mcg/ml) along with a constant concentration of metronidazole (30 mcg/ml) in the acidic and basic solvents. The series E,F and G were exactly similar to that of series C except that they had lactose (600 mcg/ml), pvp (600mcg/ml) and indigocarmine (4 mcg/ml) in them. These additives were added by using appropriate volumes of aliquots of stock solutions of the additives in water since lactose, pvp as well as indigocarmine were freely soluble in water. The stock solution of the lactose and pvp were prepared at a concentration of 20mg/ml and that of indigocarmine at 1mg/ml.

Twenty tablets (of each brand) were finely ground and a weight of the powder equal to the average weight of the tablet was dissolved in dimethylformamide by thorough shaking and filtered (Whatman No 1. filter paper). The first and last 5 ml of the filtrate were discarded and appropriate volumes of aliquots of the filtrate, after dilution in dimethylformamide, were used to prepare sample solutions containing approximately the concentrations of metronidazole and furazolidone given in table 58.

The solutions were stored in low actinic Pyrex volumetric flasks at room temperature till their analysis. The stability of the solutions were monitored spectrophotometrically for a period of three hours at the chosen wavelengths for estimation and were found to vary by the following absorbance units (AU) : metronidazole and furazolidone in 0.1N HCl by ± 0.007AU and ± 0.004 AU respectively and in 0.1M NaOH by ± 0.022 and ± 0.007 respectively. All the measurements for replicate

determinations were recorded within a time interval of 30-45 minutes after preparation of the solutions to minimise the variations in absorbance with time.

Recording of the Spectra

The spectra for linear plot and absorbance ratio methods were recorded as normal spectra using the appropriate blanks of 0.1M HCl and 0.1M NaOH.

The zero-order difference spectra of the various solutions were initially recorded by scanning equimolar solutions in 0.1M HCl and 0.1M NaOH solvents in the Jasco 7800 spectrophotometer. The second-order derivative difference spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by converting the zero-order difference spectra to second-order derivative difference spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. A data interval of one was found to be satisfactory. No smoothing of the spectra was found necessary.

RESULTS AND DISCUSSION

The estimation of metronidazole and furazolidone in combined formulations will require some kind of simultaneous estimation in the solvents of 0.1M HCl and 0.1M NaOH as can be seen from figure 53. The estimation of furazolidone in the presence of metronidazole may be possible directly since the absorbance of metronidazole above the wavelength of 350nm in 0.1M HCl and above 375nm in 0.1M NaOH is practically nil whereas furazolidone absorbs appreciably at these wavelengths in these solvents but furazolidone will always interfere in the

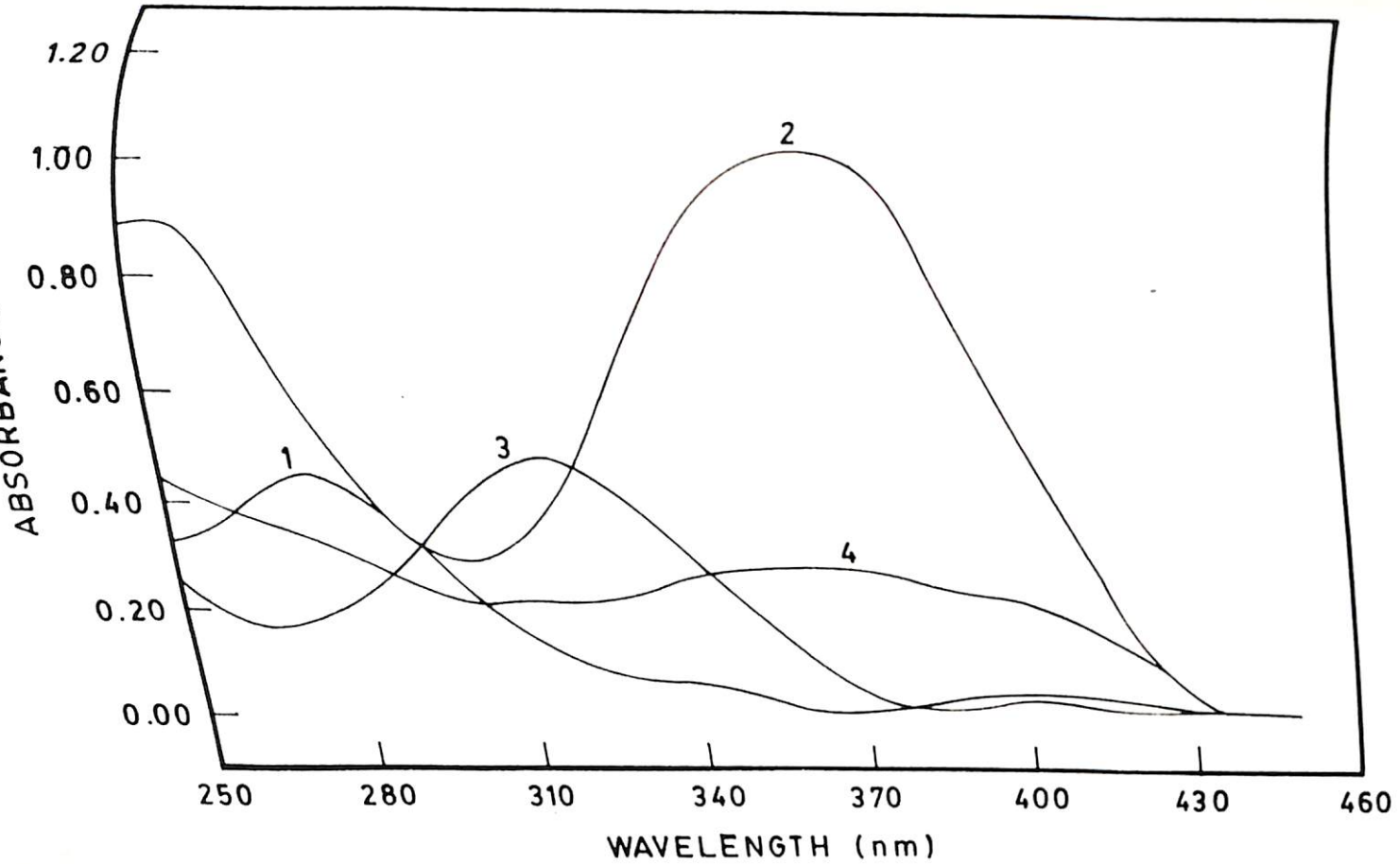


Fig 53. Normal absorption spectra of pure metronidazole and furazolidone in 0.1M HCl and 0.1M NaOH; concentration of metronidazole in 0.1M HCl and 0.1M NaOH is 10 mcg ml^{-1} (curves 1 and 3 respectively);concentration of furazolidone in 0.1M HCl and 0.1M NaOH is 10 mcg ml^{-1} (curves 2 and 4 respectively)

estimation of metronidazole although the interference will be less in 0.1M NaOH since the commercial formulations contain metronidazole and furazolidone in the ratio of 3:1 or 4:1.

The linear plot method was used for the estimation of these drugs using the data points between 310-325nm for acidic solutions and 355-380nm for basic solutions. The results of the estimation of the drugs in admixtures in the presence and absence of additives have been given in table 52. They show that the method has a very high relative error for the estimation of the drugs in 0.1M NaOH. Hence the estimation of the drugs had been done in the solvent of 0.1M HCl for commercial formulations the results of which have been presented in table 54.

The absorbance ratio method was based on the ratio of absorbance of the drugs at the wavelength maximum absorption to that of the absorbance at the isoabsorptive point (Q value) for the estimation [77]. In 0.1M HCl, the maximum absorption of metronidazole occurred at 277nm and that of furazolidone at 365nm and the isoabsorptive point at 295nm. Hence the wavelengths chosen for the analysis of metronidazole were 277nm and 295nm and those for furazolidone were 365nm and 295nm (figure 53). For estimation of metronidazole in 0.1M HCl, first the $Q_{(277:295)}$ values were plotted against the fraction of metronidazole in the mixture (X_{mnd}) to get a straight line (Q curve) by the least square method. The data for plotting this curve was obtained from various synthetic mixtures of metronidazole and furazolidone. Once the equation of the curve was known, the metronidazole in the unknown mixture (synthetic admixture or tablet sample) may be determined by substituting the $Q_{(277:295)}$ value for the unknown mixture in the equation and solving the equation for X_{mnd}. The amount of furazolidone in the mixture may be determined by using an equation similar to the above equation but expressed in terms of

Table 52.Results of Determination of Metronidazole and Furazolidone in Pure Admixtures by Linear Plot Method

Solution	Solv.	Concentration of Additives (mcg ml^{-1})			Concentration of Drugs (mcg ml^{-1})				% Relative Error	
		LAC	PVP	CAR	MND ACT	MND FND	FZD ACT	FZD FND	MND	FZD
Pure Drugs	A	----	----	----	45.00	44.52	15.00	14.87	-1.07	-0.87
Pure Drugs	A	600	----	----	45.00	44.62	15.00	14.87	-0.84	0.86
Pure Drugs	A	----	600	----	45.00	44.84	15.00	14.91	-0.36	-0.60
Pure Drugs	A	----	----	4	45.00	50.63	15.00	14.74	12.44	-1.73
Pure Drugs	B	----	----	----	45.00	44.34	15.00	15.23	-1.46	1.53
Pure Drugs	B	600	----	----	45.00	44.44	15.00	15.21	-1.24	1.40
Pure Drugs	B	----	600	----	45.00	44.16	15.00	15.34	-1.87	2.27
Pure Drugs	B	----	----	4	45.00	44.14	15.00	15.36	1.91	2.40

MND - Metronidazole FZD - Furazolidone A - 0.1M HCl B - 0.1 M NaOH
ACT - Actual value FND - Found value LAC - Lactose CAR- Indigocarmine
PVP - Polyvinylpyrrolidone

[a] Wavelength range used for collection of data points was 310 - 325 and 355-380 nm for acidic and basic solutions;concentration obtained from the slope and intercept of the linear curve plot

[b] Based on five replicate determinations

Table 53. Regression Analysis of Absorbance Ratio Values of Metronidazole and Furazolidone

Solvent	Composition of Solution (mcg ml^{-1}) MND	FZD	Regression Equation [a] (277 and 318 nm for MND 365 and 260 nm for FZD)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept
0.1MHCl	0-10	0-10	$Q = -0.0401X_{mnd} + 1.7689$	0.9988	99.76	7.71	1718	2.77	41	0.0009	0.0058
0.1M HCl	0-10	0-10	$Q = 0.3240X_{fzd} + 0.1114$	0.9999	99.99	7.71	42409	2.77	205	0.0015	0.0095
0.1M NaOH	0-10	0-10	$Q = 0.0894X_{mnd} + 0.8651$	0.9997	99.95	7.71	9538	2.77	97	0.0009	0.0055
0.1M NaOH	0-16	0-16	$Q = 0.0689X_{fzd} + 1.1351$	0.9996	99.93	7.71	4428	2.77	66	0.0010	0.0101

MND- Metronidazole FZD - Furazolidone [a] Based on values used for Q curve plot; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n-2*) value at 5% significance level; F *crit* is the F (*1*, *n-2*) value from the *F ratio table* for 5% significance level;

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level;

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance ratio* value of the admixture and *x* is the *concentration*

furazolidone (Table 53). A similar curve was used for the estimation of the drugs in 0.1M NaOH with the wavelengths of maximum absorption for metronidazole and furazolidone to be 318nm and 260nm respectively and the isoabsorptive point at 344nm (figure 53). The results of the F and T tests on these values have been given in table 53 and the assay results in table 54.

Second-order derivative difference method

For this method, the spectra of the drug solutions were recorded as zero-order difference spectra and converted to second-order derivative difference spectra using the digital algorithms. The zero crossing point of 410nm of metronidazole and 323nm of furazolidone were used for the estimation. Thus the derivative values of metronidazole at 323nm (h_1 and h_3 in figures 54 and 56) and the derivative values of furazolidone at 410nm (h_2 and h_4 in figures 55 and 57) were found to be proportional to the corresponding concentrations and independent of each other and hence used for the estimation of the drugs.

The equations showed a regression close to the origin and the standard deviation values associated with the determinations were small. The co-efficient of variation, the correlation co-efficient values, the calculated F test and T test values have been given in tables 55-57. The calculated F test values for each concentration of the drugs (Tables 55 and 56) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.91 - 99.99% variation in the derivative values were accounted for by the concentration of the drugs in solution. The results of F test using ratio of mean square due to regression to the mean square about the regression (Table 57)

Table 54. Results of Assay of Metronidazole and Furazolidone in Commercial Samples by Linear Plot and Absorbance Ratio Method

Sample	Method	Label Claim (mg/tablet)		Mean Recovery, % [a]	
		MND	FZD	MND	FZD
Brand A	LPM	300	100	99.16 ± 0.34	100.22 ± 0.45
Brand B	LPM	400	100	98.89 ± 0.41	98.97 ± 0.83
Brand C	LPM	200	50	99.96 ± 0.78	98.91 ± 0.67
Brand A	ARM	300	100	99.56 ± 0.76	99.83 ± 0.72
Brand B	ARM	400	100	99.21 ± 0.82	98.76 ± 0.57
Brand C	ARM	200	50	99.34 ± 0.73	99.33 ± 0.42

[a] Average of ten determinations; assay as percentage of label claim

LPM - Linear Plot Method using 0.1M HCl

ARM - Absorbance Ratio Method using 0.1M HCl

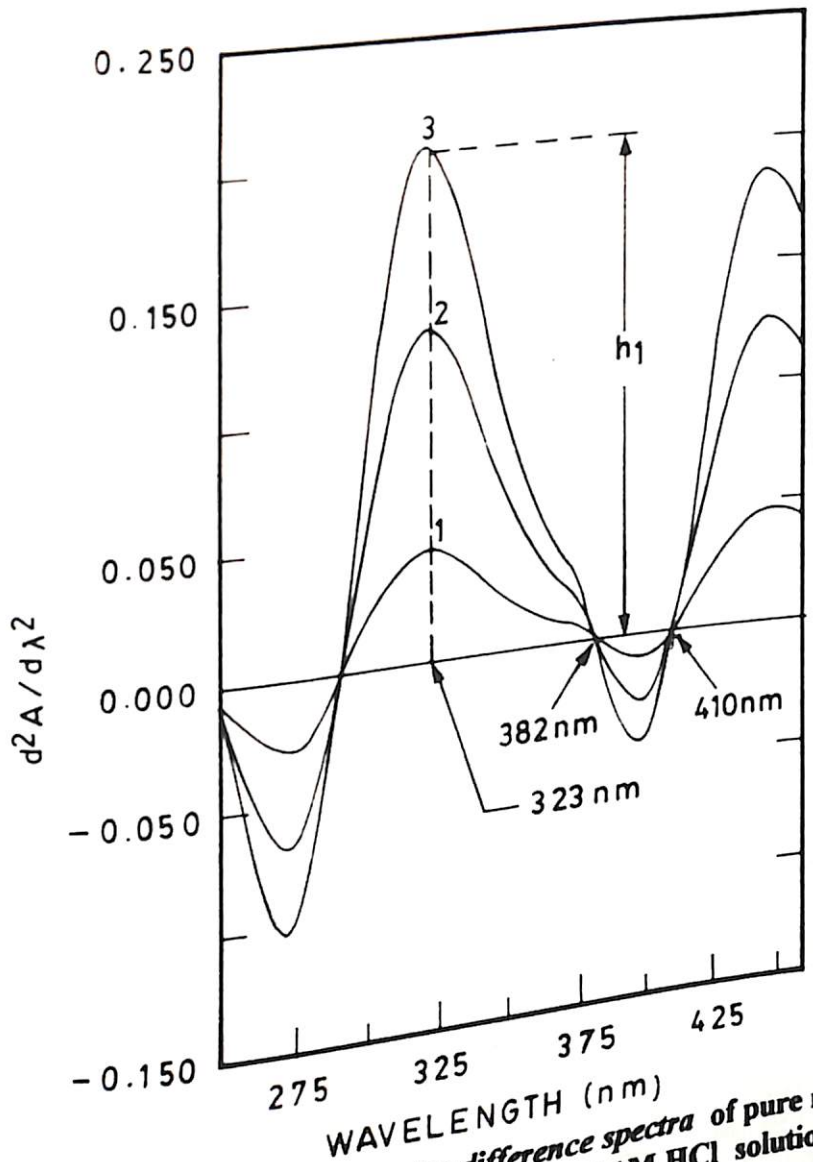


Fig 54. Second-order *derivative difference spectra* of pure metronidazole obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; metronidazole concentration:10, 30 and 50 mcg ml^{-1} in curves 1, 2 and 3 respectively)

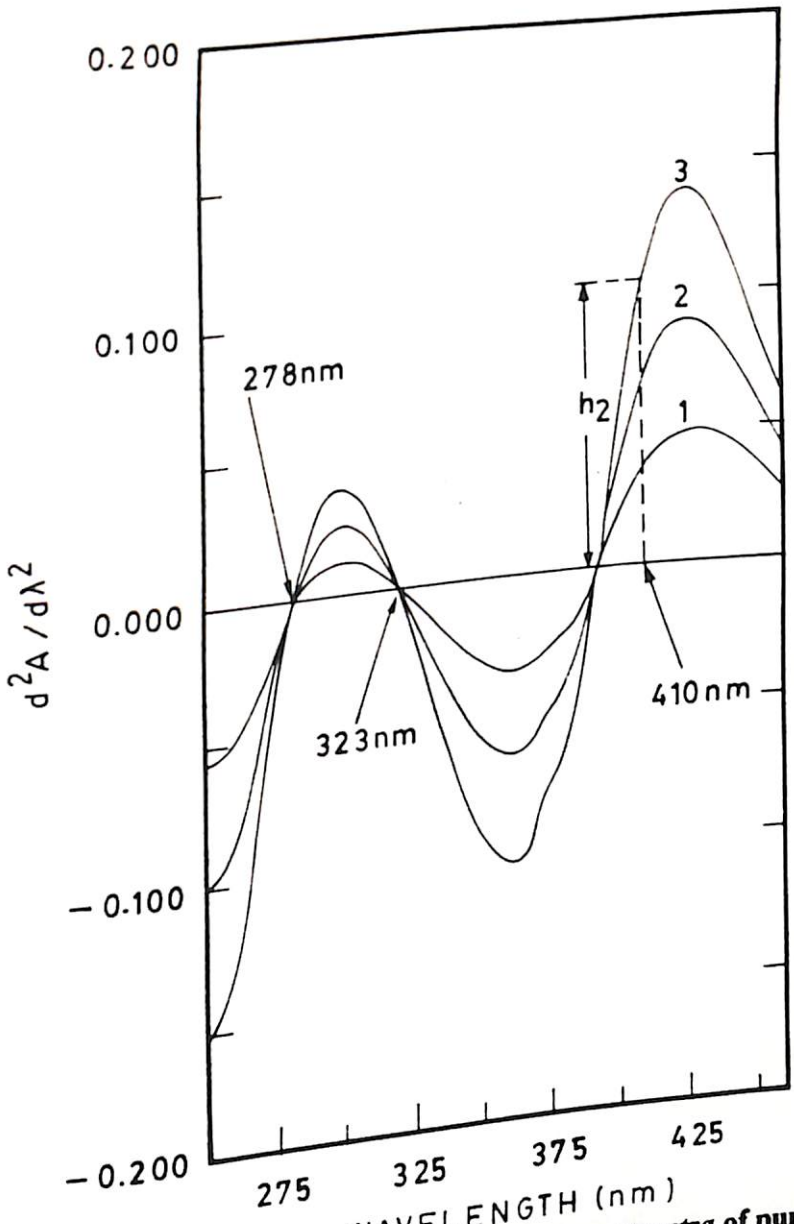


Fig 55. Second-order *derivative difference spectra* of pure furazolidone obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (furazolidone concentration:10,20 and 30 mcg ml^{-1} in curves 1, 2 and 3 respectively)

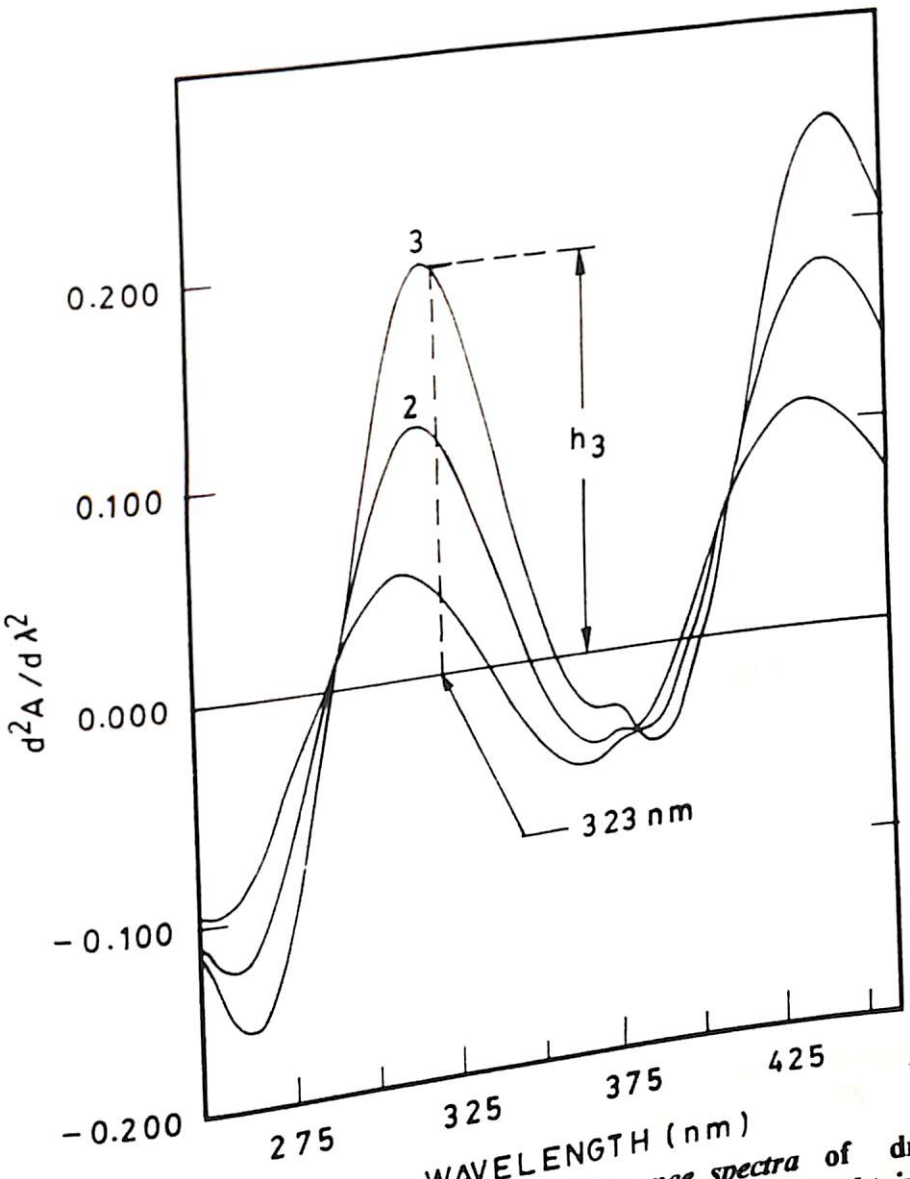


Fig 56. Second-order *derivative difference spectra* of drug admixture of metronidazole and furazolidone obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (concentration of furazolidone, 20 mcg ml^{-1}; concentration of metronidazole: 10, 30 and 50 mcg ml^{-1} in curves 1,2 and 3 respectively)

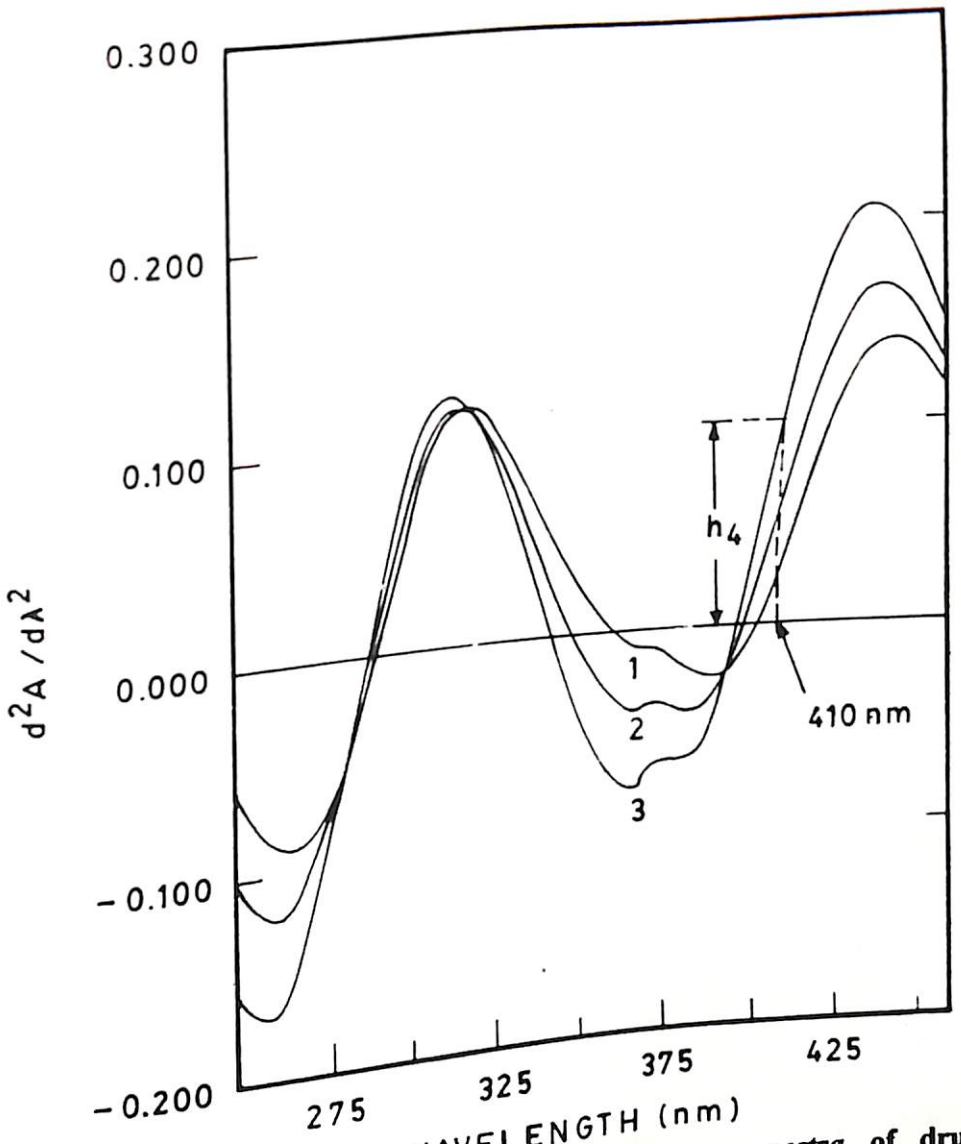


Fig 57. Second-order *derivative difference spectra* of drug admixture of furazolidone and metronidazole obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (concentration of metronidazole, 30 mcg ml^{-1}; concentration of furazolidone: 10, 20 and 30 mcg ml^{-1} in curves 1,2 and 3 respectively)

Table 55. Selectivity of the Method for the Determination of Metronidazole in the Presence of Furazolidone by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (323 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
MND	FZD					Crit	Calc
10	0	0.0405 ± 0.0003	0.91	0.0001	99.02	3.29	1.18
15	0	0.0604 ± 0.0004	0.59	0.0001	100.04	3.29	1.26
20	0	0.0804 ± 0.0004	0.48	0.0001	100.37	3.29	1.09
25	0	0.1006 ± 0.0004	0.36	0.0001	100.50	3.29	1.25
30	0	0.1214 ± 0.0003	0.24	0.0001	99.92	3.29	1.86
35	0	0.1415 ± 0.0004	0.25	0.0001	100.13	3.29	1.25
40	0	0.1626 ± 0.0003	0.21	0.0001	99.64	3.29	1.43
45	0	0.1826 ± 0.0003	0.16	0.0001	99.82	3.29	1.86
50	0	0.2022 ± 0.0004	0.20	0.0001	100.18	3.29	1.01
10	20	0.0405 ± 0.0003	0.85	0.0001	99.41	3.29	0.85
15	20	0.0604 ± 0.0004	0.59	0.0001	100.03	3.29	0.85
20	20	0.0805 ± 0.0004	0.51	0.0001	100.33	3.29	0.61
25	20	0.1006 ± 0.0004	0.37	0.0001	100.44	3.29	0.74
30	20	0.1218 ± 0.0005	0.43	0.0001	99.69	3.29	0.36
35	20	0.1416 ± 0.0004	0.27	0.0001	100.07	3.29	0.71
40	20	0.1624 ± 0.0004	0.23	0.0001	99.74	3.29	0.73
45	20	0.1823 ± 0.0004	0.19	0.0001	99.97	3.29	0.81
50	20	0.2024 ± 0.0004	0.17	0.0001	100.11	3.29	0.81

MND - Metronidazole FZD - Furazolidone

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(7,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 56. Selectivity of the Method for the Determination of Furazolidone in the Presence of Metronidazole by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) FZD	MND	Mean value of [a] $d^2A/d\lambda^2$ (410 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
10	0	0.0344 ± 0.0004	0.98	0.0001	100.08	3.86	0.17
15	0	0.0515 ± 0.0003	0.62	0.0001	100.09	3.86	0.18
20	0	0.0687 ± 0.0004	0.62	0.0001	99.93	3.86	0.10
25	0	0.0859 ± 0.0004	0.41	0.0001	99.79	3.86	0.15
30	0	0.1028 ± 0.0004	0.43	0.0001	100.14	3.86	0.09
10	30	0.0343 ± 0.0003	0.83	0.0001	100.16	3.86	0.12
15	30	0.0515 ± 0.0004	0.73	0.0001	99.15	3.86	0.07
20	30	0.0687 ± 0.0004	0.57	0.0001	99.93	3.86	0.06
25	30	0.0859 ± 0.0004	0.44	0.0001	99.93	3.86	0.07
30	30	0.1029 ± 0.0004	0.41	0.0001	100.06	3.86	0.06

MND - Metronidazole FZD - Furazolidone

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(3,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 57. Regression Analysis of Metronidazole and Furazolidone Standard Solutions

Sample	Composition of Solution (mcg ml^{-1}) MND	FZD	Regression Equation [a] (323 nm for MND and 410 nm for FZD)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept	Estimate
Series A	10-50	0	y = 0.0041x - 0.0004	0.9999	99.99	5.59	15248	2.37	390	0.0001	0.0003	0.0004
Series B	10-50	20	y = 0.0041x - 0.0003	0.9999	99.99	5.59	24258	2.37	492	0.0001	0.0003	0.0003
Series C	0	10-30	y = 0.0034x - 0.0001	0.9999	99.99	10.13	16369	3.18	404	0.0001	0.0002	0.0001
Series D	30	10-30	y = 0.0034x - 0.0001	0.9999	99.99	10.13	51326	3.18	716	0.0001	0.0001	0.0001
Series E	10-50	20	y = 0.0041x - 0.000	0.9998	99.99	5.59	23234	2.37	385	0.0001	0.0002	0.0001
Series F	10-50	20	y = 0.0041x - 0.0003	0.9999	99.92	5.59	14254	2.37	404	0.0001	0.0003	0.0001
Series G	10-50	20	y = 0.0041x - 0.0002	0.9999	99.96	5.59	13245	2.37	509	0.0001	0.0003	0.0001
Series H	30	10-30	y = 0.0034x - 0.0001	0.9997	99.92	10.13	47123	3.18	654	0.0001	0.0001	0.0001
Series I	30	10-30	y = 0.0034x - 0.0001	0.9998	99.95	10.13	34567	3.18	608	0.0001	0.0002	0.0001
Series J	30	10-30	y = 0.0034x - 0.0001	0.9999	99.91	10.13	42316	3.18	556	0.0001	0.0002	0.0001

MND - Metronidazole FZD - Furazolidone [a] Based on 9 and 5 calibration values of MND and FZD respectively; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n*-2) value at 5% significance level; F *crit* is the F (*1*, *n*-2) value from the *F ratio table* for 5% significance level; n is 9 and 5 for MND and FZD respectively

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 9 and 5 for MND and FZD respectively

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

showed the calculated values to be much higher than the critical values at 5% level of significance proving the linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve (Table 55-57). The ratio of residuals expressed as percentage showed a scatter which was random. The similarity of the regression equations of pure admixture of the drugs to those of admixtures with excipients evidenced the elimination of interference of the excipients on second-order transformation coupled with difference spectrophotometry.

The results of the estimation in synthetic admixtures and commercial formulations by second-order derivative difference spectrophotometry have been given in table 58. The estimation of synthetic admixtures was done using a concentration of 30 mcg/ml of metronidazole and 20mcg/ml of furazolidone which resulted in a mean recovery of 99.80 and 100.05% for metronidazole and furazolidone respectively. The concentration of 30mcg/ml was the mean point of the calibration range of metronidazole. But when the concentration was kept at 30mcg/ml, the furazolidone concentration was below the lower limit of its calibration range in brand B and C and hence was estimated in these brands separately by using the sample solutions containing approximately 20 mcg/ml of furazolidone. Hence the error of prediction was minimum during the estimation of both the drugs.. The assay results by the linear plot method as well as by second-order derivative method showed that the drug contents in the commercial samples were within the official limits (in tablets as single ingredients) of 95-105% [3] and 90-110 [1] for metronidazole and 90-110 [1,3] for furazolidone.

Table 58. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Metronidazole and Furazolidone by Second-order Derivative Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	MND	FZD	MND	FZD	MND	FZD	MND	FZD
Pure Drug Admixture	30	20	----	----	99.80	100.05	99.71-99.81	100.01-100.09
Brand A	30	10	300	100	100.30	99.80	100.21-100.39	99.74-99.86
Brand B	30	7.5	400	100	99.79	----	99.71-99.89	-------
Brand C	30	7.5	200	50	100.30	-----	100.21-100.39	-------
Brand A	60	20	300	100	----	99.25	-------	99.21-99.28
Brand B	80	20	400	100	----	99.55	-------	99.51-99.59
Brand C	80	20	200	50	----	100.30	-------	100.26-100.34

MND - Metronidazole FZD - Furazolidone

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 7 degrees of freedom for metronidazole and 3 degrees of freedom for furazolidone

Among the two methods, the second-order derivative difference method may more advantageous since it can eliminate the interference of the excipients, if any, in the formulation although the recovery by the linear plot and absorbance ratio methods were also comparable.

5.07 DETERMINATION OF METRONIDAZOLE AND NALIDIXIC ACID IN PURE ADMIXTURES AND TABLETS BY LINEAR PLOT, ABSORBANCE RATIO AND SECOND-ORDER DERIVATIVE DIFFERENCE SPECTROPHOTOMETRIC METHODS

INTRODUCTION

The combination of metronidazole and nalidixic acid as a tablet preparation is being widely used for the treatment of diarrhoea or dysentery of amoebic, bacterial or mixed origin. The reported methods for the estimation of nalidixic acid include titrimetry [240-242], polarography [240], NMR [243], spectrophotometry [242, 244-245], spectrofluorometry [246-248], TLC [240, 247, 249] and HPLC [250]. The official methods of analysis of metronidazole in tablets are HPLC [1] and titrimetry [3] and for nalidixic acid spectrophotometry [1,3]. Metronidazole is chemically 2-(2-methyl-5-nitroimidazol-1-yl)ethanol and nalidixic acid is 1-Ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid. This section of the thesis report describes the estimation of these drugs by linear plot, absorbance ratio and second-order derivative difference spectrophotometric methods.

Materials, Reagents and Apparatus

1. Hydrochloric Acid - A.R.Grade (E.Merck India Ltd.)
2. Sodium hydroxide - A. R. Grade (Qualigens India Ltd)
3. Dimethylformamide - Spectroscopic Grade (Spectrochem. India)
4. Metronidazole and nalidixic acid were obtained as gift samples.

The second-order derivative spectra were recorded at a scan rate of 240nm/min with a Jasco 7800 uv-visible double beam scanning spectro photometer using 1cm matched quartz cuvettes. The resolution of the

spectrophotometer for recording the second-order derivative spectra was checked as per the procedure in B.P. 1993 by recording the second-order derivative spectra of 0.02% v/v solution of toluene in methanol and was found to be satisfactory. The data thus recorded with the various solutions have been given in tables 59-65.

Standard and Sample Solutions

The stock solutions of the drugs containing 1mg/ml of metronidazole and 1mg/ml of nalidixic acid were prepared in dimethylformamide by dissolving the pure drugs in the solvent by thorough shaking. Appropriate volumes of aliquots from the stock solutions were used to prepare different series of solutions in 0.1M HCl and 0.1M NaOH.

The solutions for linear plot method were prepared with the composition as shown in table 59 and the solutions for absorbance ratio were of the composition as given in table 60. In addition, the series A-J solutions were prepared for estimation by second-order derivative difference spectrophotometry. Each of the solution in the series were prepared in duplicate as equimolar solutions in 0.1M HCl and 0.1M NaOH so as to record the zero-order difference spectrum of each of the solution by scanning the acidic solution vs basic solution.

The first series (Series A) comprised of solutions of metronidazole of varying concentrations (10-50 mcg/ml) prepared by pipetting out appropriate volumes of aliquots from the stock solution into 100ml volumetric flasks and making up the volume with 0.1N HCl and 0.1M NaOH. The second series (Series C) consisted of solutions of nalidixic acid of varying concentration (10-50mcg/ml) prepared in a similar fashion in 0.1M HCl and 0.1M NaOH. The third series (Series B) comprised of mixtures of the drugs having a constant concentration of

Table 59. Results of Determination of Metronidazole and Nalidixic Acid in Pure Admixtures by Linear Plot Method

Solution	Solv.	Concentration of Additives (mcg ml^{-1})			Concentration of Drugs[a, b] (mcg ml^{-1})				% Relative Error	
		LAC	PVP	CAR	MND ACT	MND FND	NAL ACT	NAL FND	MND	NAL
Pure Drugs	B	----	----	----	8.00	8.13	12.00	12.32	1.62	2.66
Pure Drugs	B	600	----	----	8.00	8.31	12.00	12.51	3.87	4.25
Pure Drugs	B	----	600	----	8.00	8.59	12.00	12.76	7.37	6.33
Pure Drugs	B	----	----	4	8.00	8.87	12.00	12.74	10.87	6.16
Pure Drugs	A	----	----	----	10.00	9.93	15.00	14.93	0.70	0.46
Pure Drugs	A	600	----	----	10.00	10.49	15.00	14.85	4.90	1.90
Pure Drugs	A	----	600	----	10.00	10.78	15.00	14.79	7.80	1.40
Pure Drugs	A	----	----	4	10.00	12.22	15.00	16.28	22.22	8.53

MND - Metronidazole NAL - Nalidixic Acid A - 0.1M HCl B - 0.1 M NaOH
ACT - Actual value FND - Found value LAC - Lactose CAR- Indigocarmine
PVP - Polyvinylpyrrolidone

[a] Wavelength range used for collection of data points was 270-330 nm for acidic solution and 270-350 nm for basic solution; concentration obtained from the slope and intercept of the linear curve plot

[b] Based on five replicate determinations

Table 60. Regression Analysis of Absorbance Ratio Values of Metronidazole and Nalidixic Acid

Solvent	Composition of Solution (mcg ml^{-1}) MND	NAL	Regression Equation [a] (275 and 320 nm for MND 315 and 234 nm for NAL)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept
0.1MHCl	0-20	0-20	$Q = 0.0418X_{mnd} + 0.6345$	0.9984	99.68	7.71	1276	2.77	35	0.0011	0.0141
0.1M HCl	0-20	0-20	$Q = 0.0694X_{nal} + 0.3006$	0.9999	99.99	7.71	40805	2.77	202	0.0003	0.0041
0.1M NaOH	0-20	0-20	$Q = 0.0440X_{mnd} + 1.8701$	0.9997	99.94	7.71	6740	2.77	82	0.0005	0.0065
0.1M NaOH	0-20	0-20	$Q = 0.0126X_{nal} + 2.2590$	0.9904	98.10	7.71	206	2.77	14	0.0008	0.0106

MND- Metronidazole NAL - Furazolidone [a] Based on values used for Q curve plot; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n-2*) value at 5% significance level; F *crit* is the F (*1*, *n-2*) value from the *F ratio table* for 5% significance level;

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level;

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance ratio* value of the admixture and *x* is the *concentration*

nalidixic acid (30 mcg /ml) and a varying concentration of metronidazole (10-50 mcg/ml) and the fourth series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the stock solutions so as to give solutions containing various concentrations of nalidixic acid (10-50 mcg/ml) along with a constant concentration of metronidazole (30 mcg/ml) in the acidic and basic solvents. The series E,F and G were exactly similar to that of series C except that they had lactose (600 mcg/ml), pvp (600mcg/ml) and indigocarmine (4 mcg/ml) in them. These additives were added by using appropriate volumes of aliquots of stock solutions of the additives in water. The stock solution of the lactose and pvp were prepared at a concentration of 20mg/ml and that of indigocarmine at 1mg/ml.

Twenty tablets (of each brand) were finely ground and a weight of the powder equal to the average weight of the tablet was dissolved in dimethylformamide by thorough shaking and filtered (Whatman No 1. filter paper). The first and last 5 ml of the filtrate were discarded and appropriate volumes of aliquots of the filtrate, after dilution in dimethylformamide, were used to prepare sample solutions containing approximately the concentrations of metronidazole and nalidixic acid given in table 65.

The solutions were stored in low actinic Pyrex volumetric flasks at room temperature till their analysis. The stability of the solutions were monitored spectrophotometrically for a period of three hours at the chosen wavelengths for estimation and were found to vary by the following absorbance units (AU) : metronidazole and nalidixic acid in 0.1N HCl by ± 0.005AU and ± 0.003 AU respectively and in 0.1M NaOH by ± 0.027 and ± 0.005 respectively. All the measurements for replicate determinations were recorded within a time interval of 30-45 minutes

after preparation of the solutions to minimise the variations in absorbance with time.

Recording of the Spectra

The spectra for linear plot and absorbance ratio methods were recorded as normal spectra using the appropriate blanks of 0.1M HCl and 0.1M NaOH.

The zero-order difference spectra of the various solutions were initially recorded by scanning equimolar solutions in 0.1M HCl and 0.1M NaOH solvents in the Jasco 7800 spectrophotometer. The second-order derivative difference spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by converting the zero-order difference spectra to second-order derivative difference spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. A data interval of one was found to be satisfactory. No smoothing of the derivative spectra was found necessary.

RESULTS AND DISCUSSION

The estimation of metronidazole and nalidixic acid in combined formulations will require some kind of simultaneous estimation in the solvents of 0.1M HCl and 0.1M NaOH as can be seen from figure 58. The estimation of one drug in the presence of the other will invariably lead to interference since both the drugs absorb strongly between 250-360nm. The ratio in which the drugs are present in the formulations may not be helpful in eliminating the interference since the ratio of metronidazole: nalidixic acid is either 2:5 or 2:3. Hence, three methods of estimation of these drugs, namely linear plot, absorbance ratio and second-order derivative

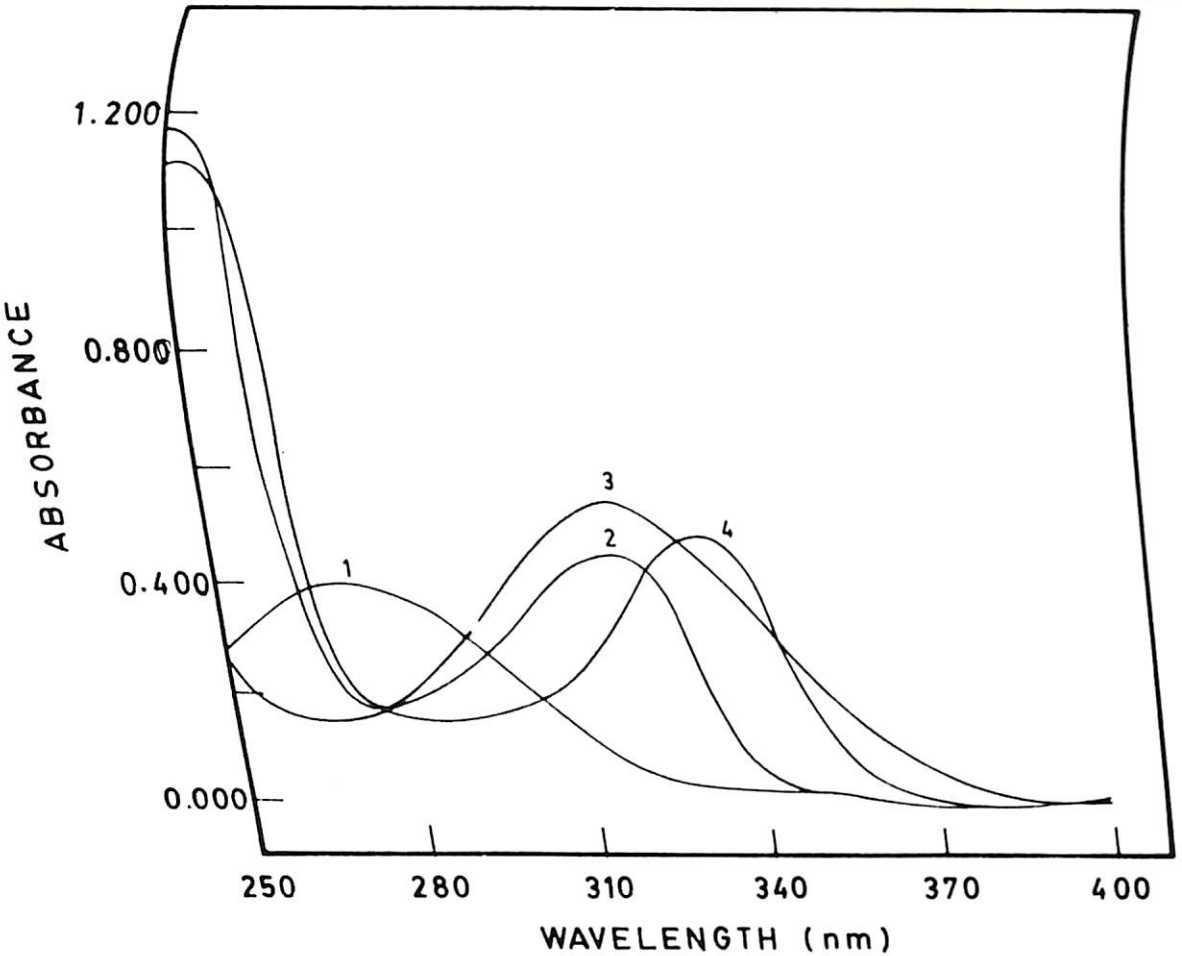


Fig 58. Normal absorption spectra of pure metronidazole and nalidixic acid in 0.1M HCl and 0.1M NaOH; concentration of metronidazole in 0.1M HCl and 0.1M NaOH is 10 mcg ml^{-1} (curves 1 and 3 respectively);concentration of nalidixic acid in 0.1M HCl and 0.1M NaOH is 10 mcg ml^{-1} (curves 2 and 4 respectively)

difference methods have been successfully designed and validated in this section.

The linear plot method was used for the estimation of these drugs using the data points between 270-330nm for acidic solutions and 270-350 for basic solutions (figure 58). The results of the estimation of the drugs in admixtures in the presence and absence of additives have been given in table 59. The relative error for the estimation of the drugs in 0.1M NaOH and 0.1M HCl by the linear plot method appeared to be comparable to any other method in the case of pure admixtures but increased in the presence of added additives due to their contribution at the chosen wavelengths. The results of the estimation of the drugs in commercial formulations have been given in table 61.

The absorbance ratio method was based on the ratio of absorbance of the drugs at the wavelength maximum absorption to that of the absorbance at the isoabsorptive point (Q value) for the estimation [77]. In 0.1M HCl, the maximum absorption of metronidazole occurred at 275nm and that of nalidixic acid at 315nm and the isoabsorptive point at 294nm. Hence the wavelengths chosen for the analysis of metronidazole were 275nm and 294nm and those for nalidixic acid were 315nm and 294nm (figure 58). For estimation of metronidazole in 0.1M HCl, first the $Q_{(275:294)}$ values were plotted against the fraction of metronidazole in the mixture (X_{mnd}) to get a straight line (Q curve) by the least square method. The data for plotting this curve was obtained from various synthetic mixtures of metronidazole and nalidixic acid. Once the equation of the curve was known, the metronidazole in the unknown mixture (synthetic admixture or tablet sample) may be determined by substituting the $Q_{(275:294)}$ value for the unknown mixture in the equation and solving the equation for X_{mnd}. The amount of nalidixic acid in the mixture may be determined by using an

Table 61. Results of Assay of Metronidazole and Nalidixic Acid Commercial Samples by Linear Plot and Absorbance Ratio Method

Sample	Method	Label Claim (mg/tablet)		Mean Recovery, % [a]	
		MND	NAL	MND	NAL
Brand A	LPM	200	500	99.37 ± 0.42	98.45 ± 0.68
Brand B	LPM	200	300	100.79 ± 0.47	99.53 ± 0.74
Brand C	LPM	200	300	99.56 ± 0.87	99.21 ± 0.76
Brand A	ARM	200	500	99.77 ± 0.41	98.12 ± 0.66
Brand B	ARM	200	300	98.97 ± 0.56	99.23 ± 0.73
Brand C	ARM	200	300	99.63 ± 0.52	99.23 ± 0.52

[a] Average of ten determinations; assay as percentage of label claim

LPM - Linear Plot Method (0.1M Hydrochloric acid)

ARM - Absorbance Ratio Method (0.1M Hydrochloric acid)

equation similar to the above equation but expressed in terms of nalidixic acid (Table 60). A similar curve was used for the estimation of the drugs in 0.1M NaOH with the wavelengths of maximum absorption for metronidazole and nalidixic acid to be 320nm and 334nm respectively and the isoabsorptive point at 274nm (figure 58). The results of the F and T tests on these values have been given in table 60 and the assay results in table 61.

Second-order derivative difference method

For this method, the spectra of the drug solutions were recorded as zero-order difference spectra and converted to second-order derivative difference spectra using the digital algorithms. The zero crossing point of 346nm of metronidazole and 281nm of nalidixic acid were used for the estimation. Thus the derivative values of metronidazole at 281nm (h_1 and h_3 in figures 59 and 61) and the derivative values of nalidixic acid at 346nm (h_2 and h_4 in figures 60 and 62) were found to be proportional to the corresponding concentrations and independent of each other and hence used for the estimation of the drugs.

The equations showed a regression close to the origin and the standard deviation values associated with the determinations were small. The co-efficient of variation, the correlation co-efficient values, the calculated F test and T test values have been given in tables 62-64. The calculated F test values for each concentration of the drugs (Tables 62 and 63) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.86 - 99.99% variation in the derivative values were accounted for by the concentration of the drugs in solution. The results of F test using ratio of mean square

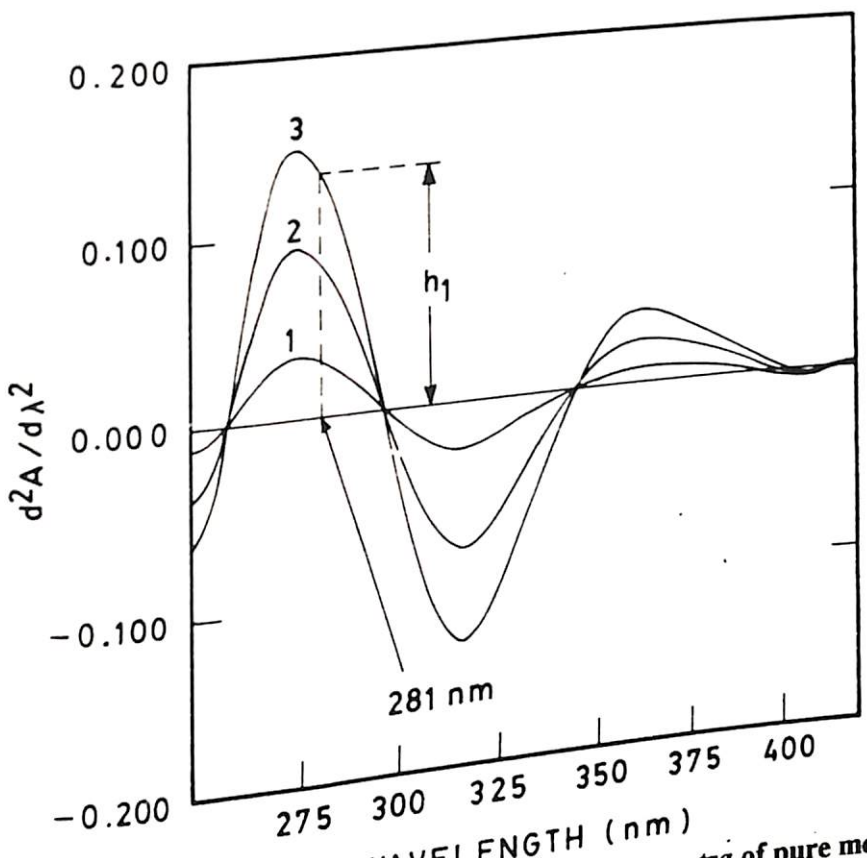


Fig 59. Second-order *derivative difference spectra* of pure metronidazole obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; metronidazole concentration:10,30 and 50 mcg ml^{-1} in curves 1, 2 and 3 respectively)

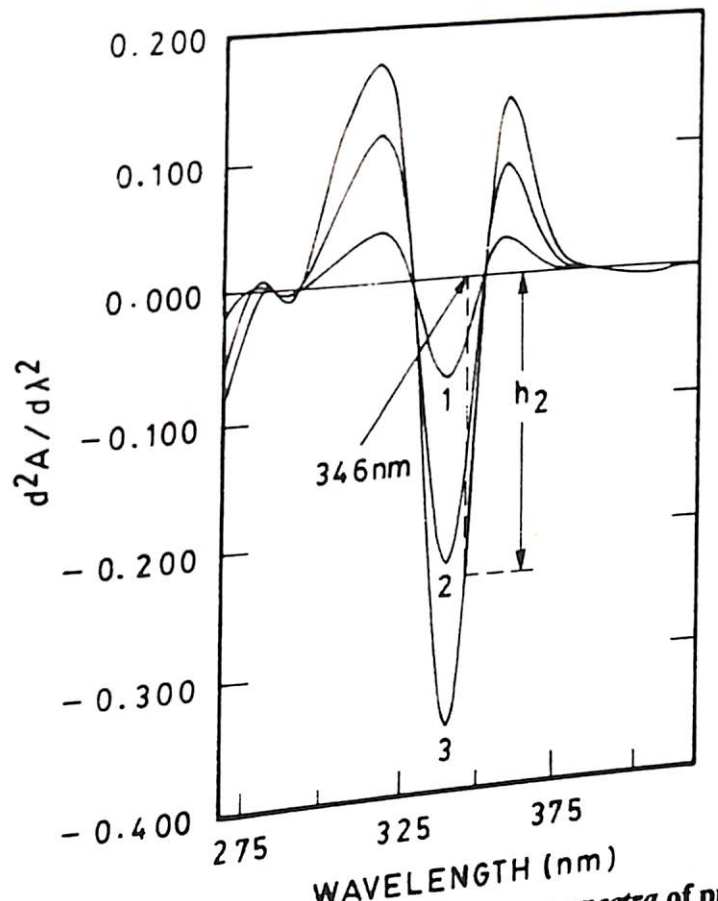


Fig 60. Second-order *derivative difference spectra* of pure nalidixic acid obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (nalidixic acid concentration:10, 30 and 50 mcg ml^{-1} in curves 1, 2 and 3 respectively)

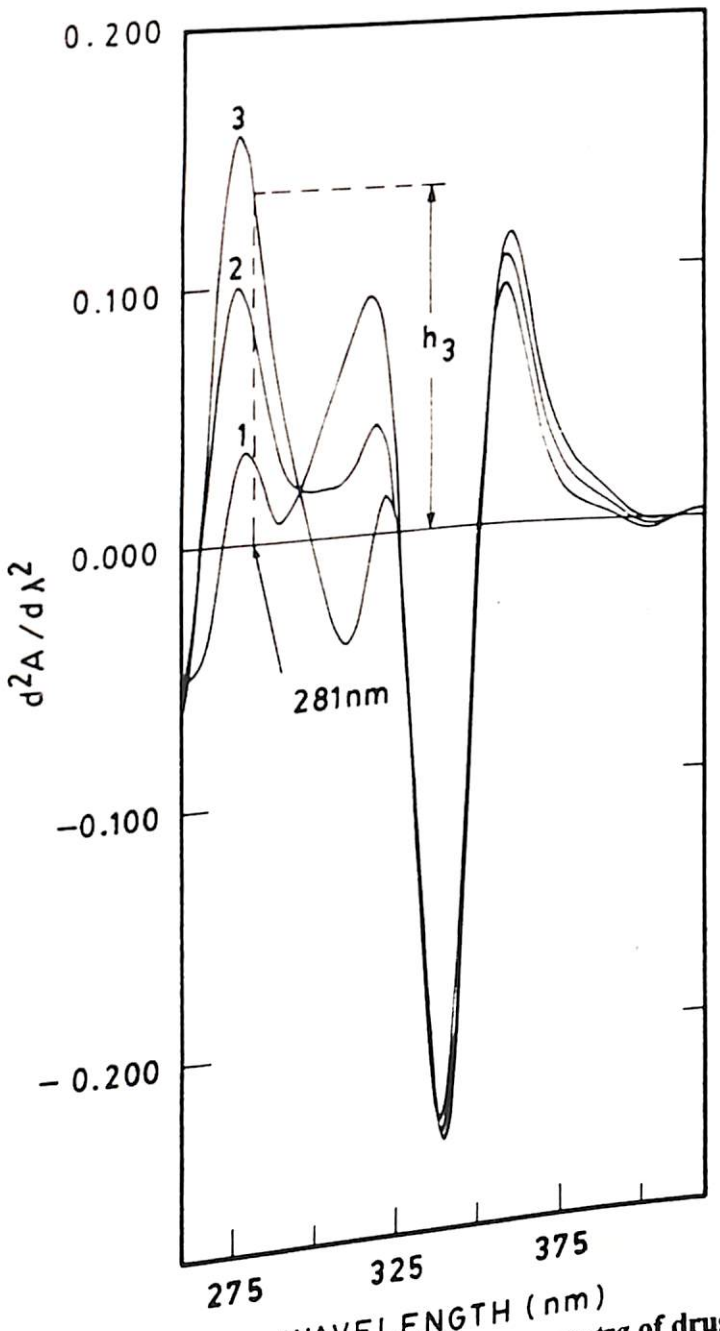


Fig 61. Second-order *derivative difference spectra* of drug admixture of metronidazole and nalidixic acid obtained by by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; concentration of nalidixic acid, 30 mcg ml^{-1} ;concentration of metronidazole:10, 30 and 50 mcg ml^{-1} in curves 1,2 and 3 respectively)

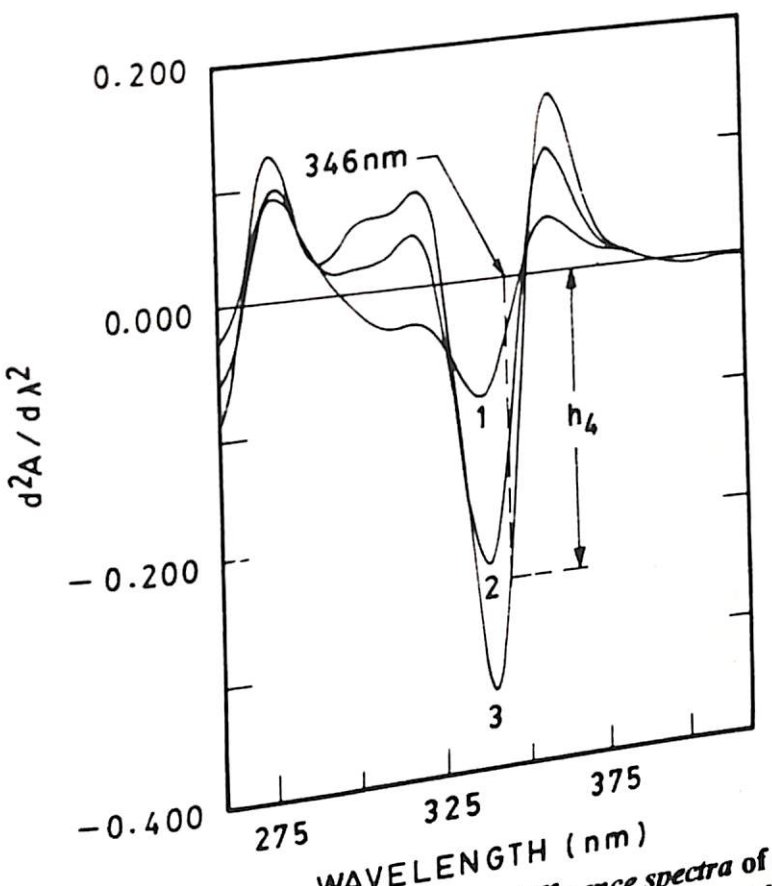


Fig 62. Second-order *derivative difference spectra* of drug admixture of nalidixic acid and metronidazole obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (concentration of metronidazole is 30 mcg ml^{-1}; concentration of nalidixic acid: 10, 30 and 50 mcg ml^{-1} in curves 1,2 and 3 respectively)

Table 62. Selectivity of the Method for the Determination of Metronidazole in the Presence of Nalidixic Acid by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (281 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
MND	NAL					Crit	Calc
10	0	0.0294 ± 0.0003	1.05	0.0001	99.80	3.29	0.29
15	0	0.4419 ± 0.0003	0.67	0.0001	100.03	3.29	0.33
20	0	0.0589 ± 0.0003	0.49	0.0001	100.07	3.29	0.35
25	0	0.0736 ± 0.0003	0.41	0.0001	100.20	3.29	0.30
30	0	0.0886 ± 0.0004	0.44	0.0001	99.97	3.29	0.18
35	0	0.1036 ± 0.0004	0.39	0.0001	99.73	3.29	0.17
40	0	0.1181 ± 0.0004	0.27	0.0001	99.98	3.29	0.27
45	0	0.1326 ± 0.0003	0.23	0.0001	100.21	3.29	0.29
50	0	0.1478 ± 0.0003	0.17	0.0001	99.99	3.29	0.42
10	30	0.0296 ± 0.0002	0.76	0.0001	99.43	3.29	0.94
15	30	0.0439 ± 0.0003	0.73	0.0001	100.51	3.29	0.47
20	30	0.0589 ± 0.0003	0.46	0.0001	100.03	3.29	0.66
25	30	0.0736 ± 0.0003	0.46	0.0001	100.23	3.29	0.42
30	30	0.0886 ± 0.0004	0.46	0.0001	99.89	3.29	0.29
35	30	0.1037 ± 0.0004	0.39	0.0001	99.64	3.29	0.29
40	30	0.1181 ± 0.0004	0.30	0.0001	99.95	3.29	0.37
45	30	0.1326 ± 0.0003	0.26	0.0001	100.21	3.29	0.41
50	30	0.1476 ± 0.0004	0.24	0.0001	99.97	3.29	0.37

MND - Metronidazole NAL - Nalidixic acid

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(7,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 63. Selectivity of the Method for the Determination of Nalidixic Acid in the Presence of Metronidazole by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) NAL	MND	Mean value of [a] $d^2A/d\lambda^2$ (346 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
10	0	0.0467 ± 0.0004	0.80	0.0001	99.78	3.29	0.25
15	0	0.0697 ± 0.0004	0.55	0.0001	100.24	3.29	0.24
20	0	0.0934 ± 0.0004	0.40	0.0001	99.78	3.29	0.25
25	0	0.1166 ± 0.0004	0.31	0.0001	99.99	3.29	0.27
30	0	0.1399 ± 0.0004	0.27	0.0001	100.04	3.29	0.25
35	0	0.1632 ± 0.0004	0.22	0.0001	99.99	3.29	0.27
40	0	0.1864 ± 0.0004	0.20	0.0001	100.11	3.29	0.25
45	0	0.2098 ± 0.0004	0.17	0.0001	100.07	3.29	0.27
50	0	0.2336 ± 0.0005	0.22	0.0002	99.87	3.29	0.14
10	30	0.0466 ± 0.0004	0.76	0.0001	99.75	3.29	0.37
15	30	0.0697 ± 0.0005	0.72	0.0001	100.25	3.29	0.18
20	30	0.0935 ± 0.0004	0.39	0.0001	99.68	3.29	0.34
25	30	0.1165 ± 0.0004	0.34	0.0001	100.05	3.29	0.28
30	30	0.1397 ± 0.0003	0.23	0.0001	100.04	3.29	0.43
35	30	0.1631 ± 0.0003	0.24	0.0001	100.08	3.29	0.28
40	30	0.1865 ± 0.0003	0.16	0.0001	100.09	3.29	0.49
45	30	0.2098 ± 0.0004	0.21	0.0001	100.06	3.29	0.24
50	30	0.2336 ± 0.0005	0.23	0.0001	99.85	3.29	0.16

MND - Metronidazole NAL - Nalidixic acid

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(7,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 64. Regression Analysis of Metronidazole and Nalidixic Acid Standard Solutions

Sample	Composition of Solution (mcg ml^{-1}) MND	NAL	Regression Equation [a] (281 nm for MND and 346 nm for NAL)	Corr. coeff.	R^2, % [b]	F test Values [c] Crit	Calc	Test for Significance [d] of Evidence of Correlation Crit	Calc	Standard Error [e] Slope	Intercept	Estimate
Series A	10-50	0	y = 0.0029x - 0.0001	0.9999	99.99	5.59	44789	2.37	669	0.0001	0.0001	0.0001
Series B	10-50	30	y = 0.0030x - 0.0001	0.9999	99.99	5.59	27421	2.37	523	0.0001	0.0002	0.0002
Series C	0	10-50	y = 0.0046x - 0.0001	0.9999	99.99	5.59	99058	2.37	995	0.0001	0.0001	0.0002
Series D	30	10-50	y = 0.0047x - 0.0002	0.9999	99.99	5.59	66826	2.37	817	0.0001	0.0002	0.0002
Series E	10-50	30	y = 0.0030x - 0.0001	0.9999	99.97	5.59	26541	2.37	556	0.0001	0.0001	0.0002
Series F	10-50	30	y = 0.0030x - 0.0002	0.9999	99.99	5.59	25431	2.37	425	0.0001	0.0001	0.0002
Series G	10-50	30	y = 0.0030x - 0.0001	0.9996	99.92	5.59	23167	2.37	549	0.0001	0.0001	0.0003
Series H	30	10-50	y = 0.0047x - 0.0001	0.9995	99.90	5.59	55672	2.37	789	0.0001	0.0002	0.0002
Series I	30	10-50	y = 0.0047x - 0.0002	0.9998	99.86	5.59	43567	2.37	654	0.0001	0.0002	0.0002
Series J	30	10-50	y = 0.0047x - 0.0001	0.9996	99.94	5.59	44321	2.37	542	0.0001	0.0002	0.0002

MND- Metronidazole NAL - Nalidixic acid [a] Based on 9 calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n-2*) value at 5% significance level; F *crit* is the F (*1*, *n-2*) value from the *F ratio table* for 5% significance level; n is 9 for both metronidazole as well as nalidixic acid

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 9 for both aspirin as well as dipyridamole

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

due to regression to the mean square about the regression (Table 64) showed the calculated values to be much higher than the critical values at 5% level of significance proving the linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve (Table 62-64). The ratio of residuals expressed as percentage showed a scatter which was random. The similarity of the regression equations of pure admixture of the drugs to those of admixtures with excipients evidenced the elimination of interference of the excipients on second-order transformation coupled with difference spectrophotometry.

The pK_a of metronidazole is 2.5 and that of nalidixic acid is 6.0 [111] and hence any small change in the pH values of the solvents did not produce any appreciable change in the spectrum since the pK_a values were at least 1.5 units away from the pH values of 0.1M HCl (pH ≈ 1.0) and 0.1M NaOH (pH ≈ 13.0).

The results of the estimation in synthetic admixtures and commercial formulations by second-order derivative difference spectrophotometry have been given in table 65 The estimation of synthetic admixtures was done using a concentration of 30 mcg/ml of metronidazole and nalidixic acid which resulted in a mean recovery of 99.16 and 99.93% for metronidazole and nalidixic acid respectively. The concentration of 30mcg/ml was the mean point of the calibration range of both the drugs. But when the concentration of one drug was kept at 30mcg/ml, the other drug concentration was either above the calibration range (Brand A) or

Table 65. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Metronidazole and Nalidixic Acid by Second-order Derivative Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/tablet)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	MND	NAL	MND	NAL	MND	NAL	MND	NAL
Pure Drug Admixture	30	30	-----	-----	99.16	99.93	99.06-99.26	99.87-99.99
Brand A	30	75	200	500	98.83	----	98.74-98.91	-------
Brand B	30	45	200	300	99.40	99.64	99.30-99.51	99.64-99.77
Brand C	30	45	200	300	100.30	99.57	100.20-100.40	99.50-99.63
Brand A	12	30	200	500	99.00	99.33	98.88-99.11	99.27-99.38
Brand B	20	30	200	300	98.80	99.60	98.70-98.90	99.54-99.66
Brand C	20	30	200	300	98.15	99.40	98.05-98.25	99.34-99.46

MND - Metronidazole NAL - Nalidixic Acid

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures (Equations of Series B and Series D)

[b] Concentration range at 95% confidence level using t test (a two tail test) with 7 degrees of freedom for both metronidazole as well as nalidixic acid

was not at its mean point of calibration (Table 65). Hence the drugs were estimated in these brands separately by using the sample solutions diluted in such a fashion as to have 30 mcg/ml of one of the drug. The other drug was estimated at the concentration in which it was present in the formulation as well as by a separate solution which had 30 mcg/ml of the second drug. Thus metronidazole in the Brands had been estimated twice and nalidixic acid in Brands B and C had been estimated twice.The assay results by the linear plot method as well as by second-order derivative method showed that the drug contents in the commercial samples were within the official limits (in tablets as single ingredients) of 95-105% [3] and 90-110 [1] for metronidazole and 93-107 [1,3] for nalidixic acid.

Among the two methods, the second-order derivative difference method may more advantageous since it can eliminate the interference of the excipients, if any, in the formulation although the recovery by the linear plot and absorbance ratio methods were also comparable. Although the standard error of prediction will be more in solutions containing drugs at concentrations other than the mean point of calibration range, the difference in the recovery was not much in the estimation of metronidazole and nalidixic acid as could be seen from the results in table 65.

5.08 DETERMINATION OF ORCIPRENALINE SULPHATE AND BROMHEXINE HYDROCHLORIDE IN PURE ADMIXTURES AND TABLETS BY LINEAR PLOT, ABSORBANCE RATIO AND SECOND-ORDER DERIVATIVE DIFFERENCE SPECTROPHOTOMETRIC METHODS

INTRODUCTION

The combination of orciprenaline sulphate and bromhexine HCl as a syrup is being used for the treatment of asthma and chronic obstructive bronchitis. The reported methods for the estimation of bromhexine HCl has already been mentioned in section 4.04 and those for the estimation of orciprenaline sulphate were coulometric estimation in formulations [251] and spectrophotometric methods [252-254] including diazo coupling with o-nitroaniline and p-aminobenzoic acid [253]. The official method for estimation of orciprenaline sulphate in syrup preparations is HPLC [1]. Orciprenaline sulphate is chemically (RS)-1-(3,5-dihroxypheny)-2-isopropylaminoethanol sulphate and bromhexine HCl is 2-amino-3,5-dibromobenzyl(cyclohexyl)methylamine hydrochloride. This section of the thesis report describes the estimation of these drugs by linear plot, absorbance ratio and second-order derivative difference spectrophotometric methods.

Materials, Reagents and Apparatus

1. Hydrochloric Acid - A.R.Grade (E.Merck India Ltd.)
2. Sodium hydroxide - A. R. Grade (Qualigens India Ltd)
3. Methanol- Spectroscopic Grade (Spectrochem. India)
4. Orciprenaline sulphate and bromhexine HCl were obtained as gift samples.

The second-order derivative spectra were recorded at a scan rate of 240nm/min with a Jasco 7800 uv-visible double beam scanning spectro

photometer using 1cm matched quartz cuvettes. The resolution of the spectrophotometer for recording the second-order derivative spectra was checked as per the procedure in B.P. 1993 by recording the second-order derivative spectra of 0.02% v/v solution of toluene in methanol and was found to be satisfactory. The data thus recorded with the various solutions have been given in tables 66-72.

Standard and Sample Solutions

The stock solutions of the drugs containing 1mg/ml of orciprenaline sulphate and 1mg/ml of bromhexine HCl were prepared in methanol by dissolving the pure drugs in the solvent by thorough shaking. Appropriate volumes of aliquots from the stock solutions were used to prepare different series of solutions in 0.1M HCl and methanolic 0.1M NaOH.

For the preparation of all the subsequent dilutions, 0.1M HCl (aqueous) and methanolic 0.1M NaOH were used. The methanolic 0.1M NaOH was actually a mixture of aqueous 0.1M NaOH and methanolic 0.1M NaOH in the ratio of 2:3. Such a mixture was required to act as a common solvent for both the drugs since orciprenaline sulphate does not dissolve in methanolic 0.1M NaOH prepared with methanol alone and will dissolve only in aqueous 0.1M NaOH. But when aqueous 0.1M NaOH was used as the solvent, bromhexine HCl did not dissolve in it. Hence a mixture of the aqueous and methanolic 0.1M NaOH had been used in this work and all subsequent reference to methanolic 0.1M NaOH in this section refer to the mixture prepared by mixing aqueous 0.1M NaoH and methanolic 0.1M NaOH in the ratio of 2:3.

The solutions for linear plot method were prepared with the composition as shown in table 66 and the solutions for absorbance ratio were of the composition as given in table 67. In addition, the series A-D solutions were

prepared for estimation by second-order derivative difference spectrophotometry. Each of the solution in the series were prepared in duplicate as equimolar solutions in 0.1M HCl and methanolic 0.1M NaOH so as to record the zero-order difference spectrum of each of the solution by scanning the acidic solution vs basic solution.

The first series (Series A) comprised of solutions of orciprenaline sulphate of varying concentrations (20-80 mcg/ml) prepared by pipetting out appropriate volumes of aliquots from the stock solution into 100ml volumetric flasks and making up the volume with 0.1M HCl and methanolic 0.1M NaOH. The second series (Series C) consisted of solutions of bromhexine HCl of varying concentration (20-80mcg/ml) prepared in a similar fashion in 0.1M HCl and methanolic 0.1M NaOH. The third series (Series B) comprised of mixtures of the drugs having a constant concentration of bromhexine HCl (50 mcg /ml) and a varying concentration of orciprenaline sulphate (20-80 mcg/ml) and the fourth series (Series D) of solutions were prepared by using appropriate volumes of aliquots from the stock solutions so as to give solutions containing various concentrations of bromhexine HCl (20-80 mcg/ml) along with a constant concentration of orciprenaline sulphate (50 mcg/ml) in the acidic and basic solvents.

Accurately measured volume (2ml) of the syrup was dissolved in methanol by shaking in a 100ml volumetric flask and making up the volume. Appropriate volumes of the stock solution were used to prepare sample solutions containing approximately the concentrations of orciprenaline sulphate and bromhexine HCl given in table 72.

The solutions were stored in low actinic Pyrex volumetric flasks at room temperature till their analysis. The stability of the solutions were monitored spectrophotometrically for a period of two hours at the

chosen wavelengths for estimation and were found to vary by the following absorbance units (AU) : orciprenaline sulphate and bromhexine HCl in 0.1N HCl by ± 0.003AU and ± 0.009 AU respectively and in methanolic 0.1M NaOH by ± 0.006 and ± 0.004 respectively. All the measurements for replicate determinations were recorded within a time interval of 30-45 minutes after preparation of the solutions to minimise the variations in absorbance with time.

Recording of the Spectra

The spectra for linear plot and absorbance ratio methods were recorded as normal spectra using the appropriate blanks of 0.1M HCl and methanolic 0.1M NaOH.

The zero-order difference spectra of the various solutions were initially recorded by scanning equimolar solutions in 0.1M HCl and methanolic 0.1M NaOH solvents in the Jasco 7800 spectrophotometer. The second-order derivative difference spectra of the pure drugs, their admixtures and the tablet sample solutions were produced by converting the zero-order difference spectra to second-order derivative difference spectra by using digital algorithms (programmed in Jasco 7800). The scan rate used for the recording of the normal spectra was 240nm/min and spectral bandwidth 3nm. A data interval of one was found to be satisfactory. The derivative spectra were smoothed once using a data interval of 10.

RESULTS AND DISCUSSION

The estimation of orciprenaline sulphate and bromhexine HCl in combined formulations will require some kind of simultaneous estimation in the solvents of 0.1M HCl and methanolic 0.1M NaOH as can be seen from

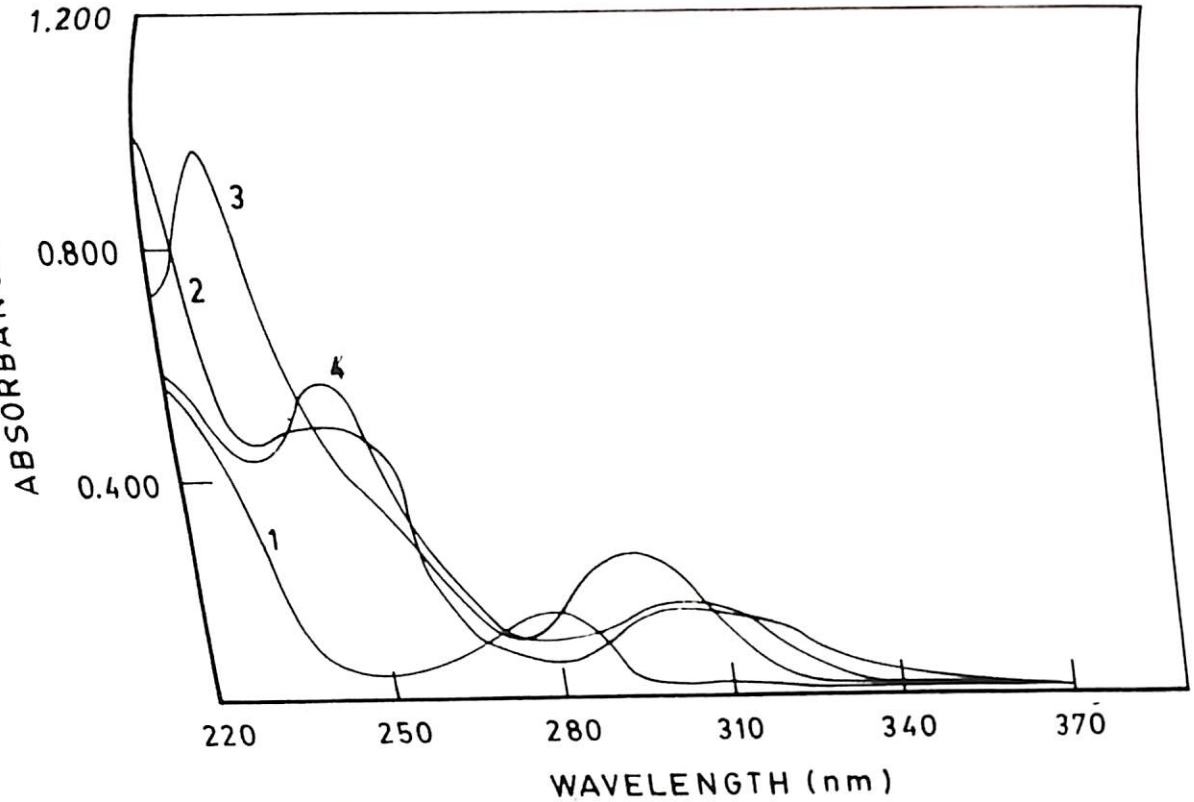


Fig 63. Normal absorption spectra of pure orciprenaline sulphate and bromhexine HCl: orciprenaline sulphate (20 mcg ml^{-1}) and bromhexine HCl (20 mcg ml^{-1}) in 0.1M HCl (curves 1 and 2);orciprenaline sulphate (20 mcg ml^{-1}) and bromhexine HCl (20 mcg ml^{-1}) in 0.1M NaOH (curves 3 and 4)

Table 66.Results of Determination of Orciprenaline sulphate and Bromhexine in Pure Admixtures Hydrochloride by Linear Plot Method

Solution	Solvent	Concentration of [a, b] Drugs (mcg ml^{-1})				% Relative Error	
		ORP		BRH		ORP	BRH
		ACT	FND	ACT	FND		
Pure Drugs	A	30.00	30.53	24.00	24.72	1.76	3.00
Pure Drugs	B	30.00	28.92	20.00	19.88	-3.60	- 0.60

ORP - Orciprenaline sulphate BRH - Bromhexine hydrochloride A - 0.1M HCl
B - 0.1 M NaOH ACT - Actual value FND - Found value

[a] Wavelength range used for collection of data points was 270-325nm;
concentration obtained from the slope and intercept of the linear curve plot

[b] Based on five replicate determinations

Table 67. Regression Analysis of Absorbance Ratio Values of Orciprenaline sulphate and Bromhexine hydrochloride

Solvent	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (225 and 230 nm for ORH 250 and 248 nm for BRH)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]	
	ORH	BRH				Crit	Calc	Crit	Calc	Slope	Intercept
0.1MHCl	5-25	2-22	$Q = -0.0141X_{orh} + 1.7576$	0.9988	99.77	10.13	1337	3.18	36	0.0004	0.0064
0.1M HCl	5-25	2-22	$Q = 0.0426X_{brh} + 0.1369$	0.9961	99.23	10.13	388	3.18	19	0.0021	0.0301
0.1M NaOH	6-14	4-12	$Q = 0.2309X_{orh} + 2.6732$	0.9998	99.96	10.13	7481	3.18	86	0.0023	0.0245
0.1M NaOH	6-14	4-12	$Q = 0.0326X_{brh} + 2.6372$	0.9998	99.96	10.13	10172	3.18	100	0.0003	0.0027

MND- Metronidazole FZD - Furazolidone [a] Based on values used for Q curve plot; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1, n-2*) value at 5% significance level; F *crit* is the F (*1, n-2*) value from the *F ratio table* for 5% significance level;

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level;

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the *absorbance ratio* value of the admixture and *x* is the *concentration*

Table 68. Results of Assay of Orciprenaline sulphate and Bromhexine Hydrochloride in Commercial Samples by Linear Plot and Absorbance Ratio Method

Sample	Method	Label Claim (mg/5ml)		Mean Recovery, % [a]	
		ORP	BRH	ORP	BRH
Brand A (Batch 1)	LPM	5	4	99.28 ± 0.53	98.92 ± 0.77
Brand A (Batch 2)	LPM	5	4	99.72 ± 0.44	99.24 ± 0.63
Brand A (Batch 1)	ARM	5	4	99.31 ± 0.27	99.62 ±0.42
Brand A (Batch 2)	ARM	5	4	99.31 ± 0.32	98.94 ±0.71

[a] Average of ten determinations; assay as percentage of label claim

LPM - Linear Plot Method using 0.1M HCl

ARM - Absorbance Ratio Method using 0.1M HCl

figure 63. Hence, three methods of estimation of these drugs, namely linear plot, absorbance ratio and second-order derivative difference methods have been successfully designed and validated in this section.

The linear plot method was used for the estimation of these drugs using the data points between 270-325nm for acidic as well as methanolic 0.1M NaOH solutions. The results of the estimation of the drugs in admixtures have been given in table 66. The relative error for the estimation of the drugs in methanolic 0.1M NaOH and 0.1M HCl by the linear plot method appeared to be comparable to second-order derivative difference method. The results of the estimation of the drugs in commercial formulations have been given in table 68.

The absorbance ratio method was based on the ratio of absorbance of the drugs at the wavelength maximum absorption to that of the absorbance at the isoabsorptive point (Q value) for the estimation [77]. In 0.1M HCl, the wavelengths chosen for the analysis of orciprenaline sulphate and bromhexine HCl were 225nm and 250nm with the isoabsorptive point at 265nm and in methanolic 0.1M NaOH the wavelengths of 230nm and 248nm were used for orciprenaline sulphate and bromhexine HCl respectively with the isoabsorptive point at 275nm. The results of the estimation have been given in tables 67 and 68.

Second-order derivative difference method

For this method, the spectra of the drug solutions were recorded as zero-order difference spectra and converted to second-order derivative difference spectra using the digital algorithms. The zero crossing point of 345nm of orciprenaline sulphate and 270nm of bromhexine HCl were used for the estimation. Thus the derivative values of orciprenaline sulphate at 270nm (h_1 and h_3 in figures 64 and 66) and the derivative values of

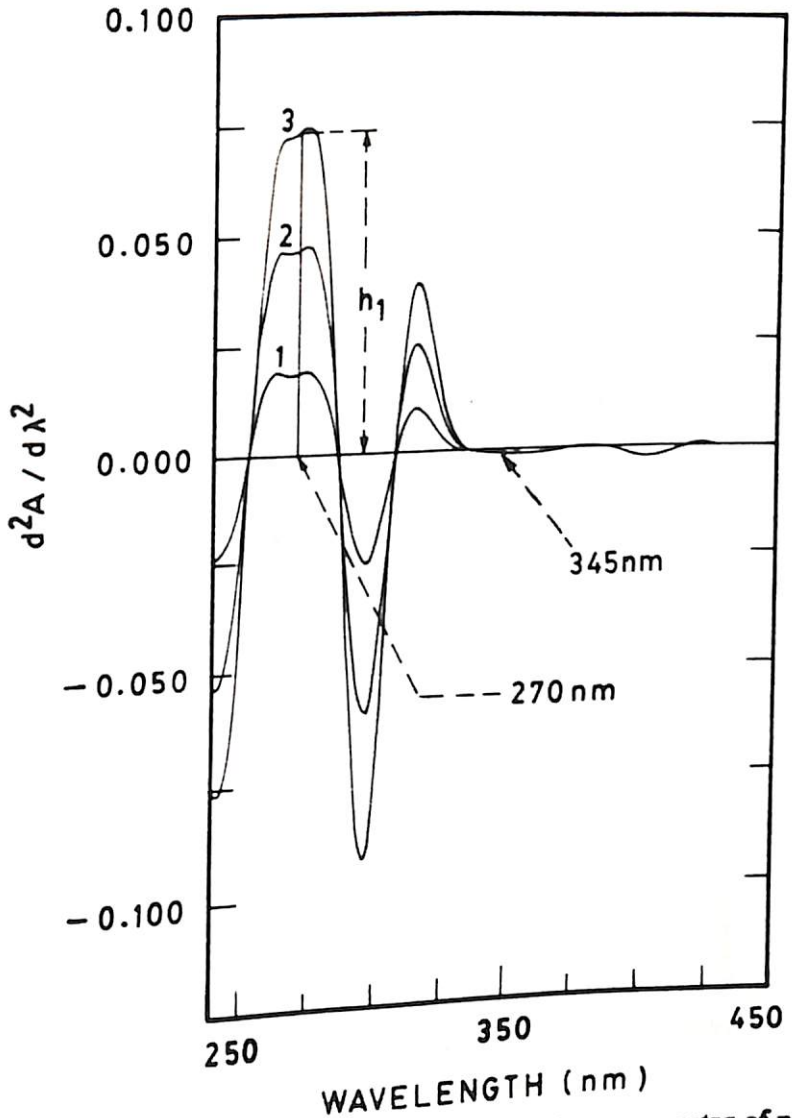


Fig 64. Second-order *derivative difference spectra* of pure orciprenaline sulphate obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; concentration of orciprenaline sulphate: 20, 50 and 80 mcg ml^{-1}

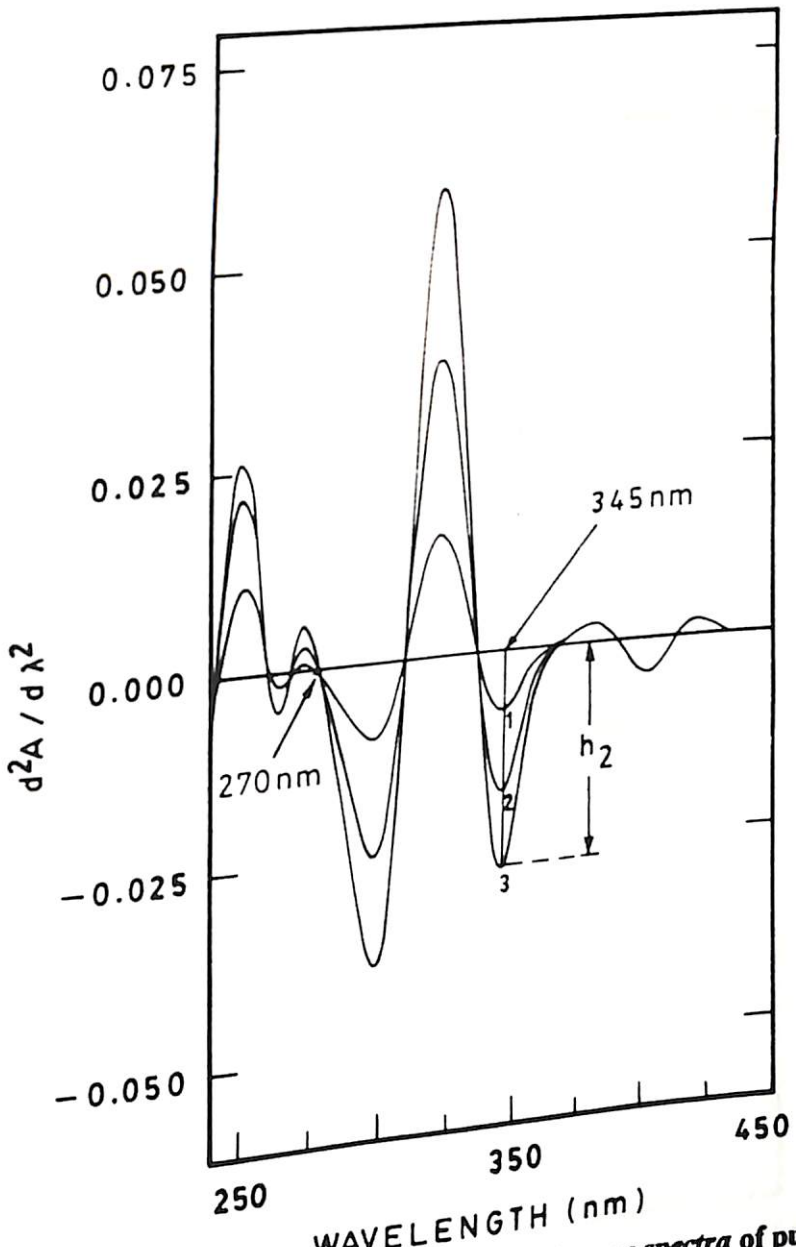


Fig 65. Second-order *derivative difference spectra* of pure bromhexine HCl obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (concentration of bromhexine HCl: 20, 50 and 80 mcg ml^{-1})

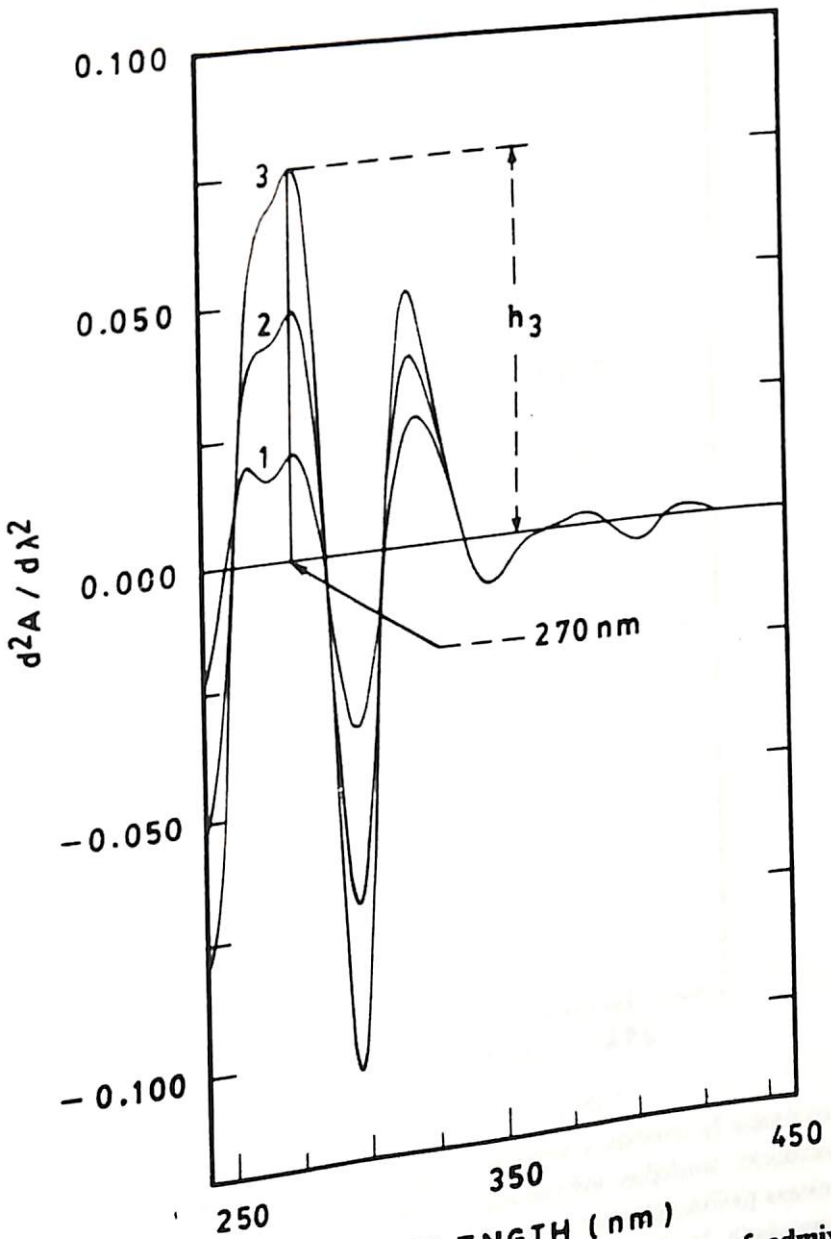


Fig 66. Second-order *derivative difference spectra* of admixture of orciprenaline sulphate and bromhexine HCl obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (concentration of bromhexine HCl is 50 mcg ml^{-1} ; concentration of orciprenaline sulphate : 20, 50 and 80 mcg ml^{-1})

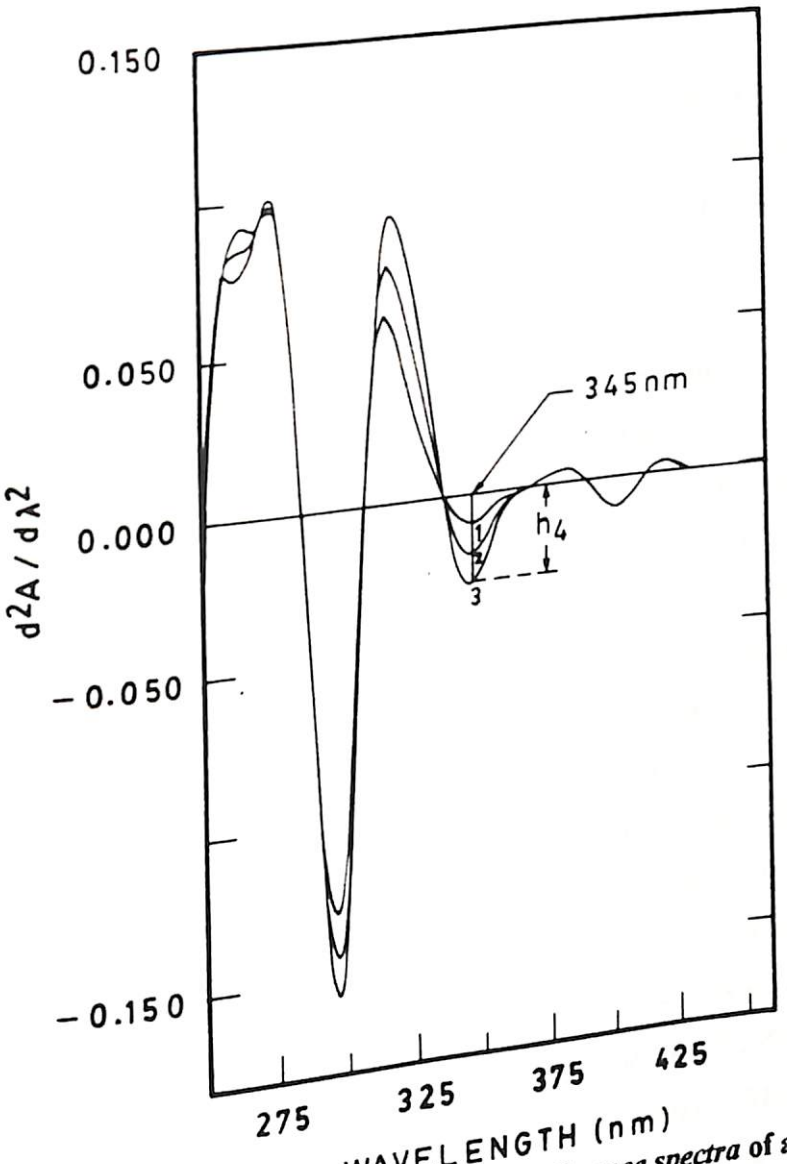


Fig 67. Second-order *derivative difference spectra* of admixture of bromhexine HCl and orciprenaline sulphate obtained by scanning 0.1M HCl solutions *vs* corresponding equimolar 0.1M NaOH solutions; (concentration of orciprenaline sulphate is 50 mcg ml^{-1}; concentration of bromhexine HCl: 20, 50 and 80 mcg ml^{-1})

bromhexine HCl at 345nm (h_2 and h_4 in figures 65 and 67) were found to be proportional to the corresponding concentrations and independent of each other and hence used for the estimation of the drugs.

The equations showed a regression close to the origin and the standard deviation values associated with the determinations were small. The co-efficient of variation, the correlation co-efficient values, the calculated F test and T test values have been given in tables 69-71. The calculated F test values for each concentration of the drugs (Tables 69 and 70) were less than that of the critical values at 5% significance level and proved the linearity of the $d^2A/d\lambda^2$ values with that of the concentration of the drugs. The co-efficient of determination values showed that 99.98 - 99.99% variation in the derivative values were accounted for by the concentration of the drugs in solution. The results of F test using ratio of mean square due to regression to the mean square about the regression showed the calculated values to be much higher than the critical values at 5% level of significance proving the linear relationship between concentration and the derivative values. The calculated T test values were also greater than the critical values confirming the existence of correlation at 5% level of significance. The standard error of slope and intercept were quite small and the standard error of estimate for the various series of solutions was less when compared to the typical change in the $d^2A/d\lambda^2$ values from point to point in the corresponding calibration curve. The ratio of residuals expressed as percentage showed a scatter which was random. The similarity of the regression equations of pure drugs to those of admixtures evidenced the non-interference of one drug in the absorption measurement of the other.

Table 69. Selectivity of the Method for the Determination of Orciprenaline Sulphate in the Presence of Bromhexine Hydrochloride by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1}) ORP	BRH	Mean value of [a] $d^2A/d\lambda^2$ (270 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d] Crit	Calc
20	0	0.0179 ± 0.0003	1.70	0.0001	98.95	3.48	0.72
30	0	0.0263 ± 0.0003	1.20	0.0001	101.88	3.48	0.68
40	0	0.0359 ± 0.0003	0.76	0.0001	99.53	3.48	0.88
50	0	0.0450 ± 0.0003	0.61	0.0001	99.61	3.48	0.88
60	0	0.0539 ± 0.0003	0.49	0.0001	99.83	3.48	0.97
70	0	0.0628 ± 0.0003	0.46	0.0001	100.03	3.48	0.82
80	0	0.0718 ± 0.0003	0.33	0.0001	100.13	3.48	1.18
20	50	0.0179 ± 0.0003	1.67	0.0001	98.94	3.48	0.47
30	50	0.0264 ± 0.0003	1.04	0.0001	101.46	3.48	0.56
40	50	0.0359 ± 0.0004	1.04	0.0001	99.67	3.48	0.29
50	50	0.0449 ± 0.0003	0.55	0.0001	99.78	3.48	0.68
60	50	0.0539 ± 0.0003	0.41	0.0001	99.92	3.48	0.85
70	50	0.0628 ± 0.0003	0.51	0.0001	100.05	3.45	0.28
80	50	0.0719 ± 0.0002	0.39	0.0001	100.03	3,45	0.54

ORP - Orciprenaline sulphate BRH - Bromhexine hydrochloride

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(5,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 70. Selectivity of the Method for the Determination of Bromhexine hydrochloride in the Presence of Orciprenaline sulphate by Second-order Derivative Difference Spectrophotometry

Composition of the solution (mcg ml^{-1})		Mean value of [a] $d^2A/d\lambda^2$ (345 nm)	Coeff. of variation (%)	Standard error [b]	Ratio of residual [c] (%)	F test for non-linearity [d]	
BRH	ORP					Crit	Calc
20	0	0.0070 ± 0.0001	2.43	0.0001	99.24	3.48	0.34
30	0	0.0105 ± 0.0002	1.67	0.0001	100.47	3.48	0.34
40	0	0.0140 ± 0.0002	1.22	0.0001	100.44	3.48	0.26
50	0	0.0176 ± 0.0003	1.50	0.0001	99.97	3.48	0.20
60	0	0.0212 ± 0.0002	0.98	0.0001	99.47	3.48	0.17
70	0	0.0246 ± 0.0002	0.99	0.0001	100.20	3.48	0.17
80	0	0.0282 ± 0.0003	1.05	0.0001	100.01	3.48	0.11
20	50	0.0070 ± 0.0002	2.91	0.0001	99.97	3.48	0.24
30	50	0.0106 ± 0.0002	1.46	0.0001	99.75	3.48	0.42
40	50	0.0141 ± 0.0002	1.22	0.0001	100.42	3.48	0.34
50	50	0.0175 ± 0.0002	1.50	0.0001	100.60	3.48	0.14
60	50	0.0213 ± 0.0002	0.98	0.0001	99.07	3.48	0.24
70	50	0.0247 ± 0.0003	1.33	0.0001	99.91	3.48	0.10
80	50	0.0281 ± 0.0003	1.05	0.0001	100.29	3.48	0.11

ORP - Orciprenaline sulphate BRH - Bromhexine Hydrochloride

[a] Average of ten replicate determinations; [b] Standard deviation of the mean

[c] Ratio of the *calculated* y value to *actual* y value expressed as %

[d] Based on *F test for non-linearity*; $F_{critical} = F(5,9)$ values from F table for 5% level of significance; $F_{calculated} = Sy^2 / Ss^2$ where Sy is the *standard error of estimate* and Ss is the *standard deviation* of ten replicate determinations for a single concentration of the drug (measurement of y)

Table 71. Regression Analysis of Orciprenaline Sulphate and Bromhexine Hydrochloride Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (270 nm for ORP and 345 nm for BRH)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	ORP	BRH				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	20-80	0	y = 0.0009x - 0.0002	0.9999	99.98	6.61	31800	2.57	178	0.0001	0.0002	0.0003
Series B	20-80	50	y = 0.0009x - 0.0002	0.9999	99.98	6.61	53197	2.57	230	0.0001	0.0002	0.0002
Series C	0	20-80	y = 0.0004x - 0.0001	0.9999	99.99	6.61	72051	2.57	268	0.0001	0.0002	0.0001
Series D	50	20-80	y = 0.0004x - 0.0001	0.9999	99.98	6.61	28060	2.57	167	0.0001	0.0001	0.0001

ORP - Orciprenaline sulphate BRH - Bromhexine hydrochloride [a] Based on 7 calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n*-2) value at 5% significance level; F *crit* is the F (*1*, *n*-2) value from the *F ratio table* for 5% significance level; n is 7 for both ORP as well as BRH

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 7 for both orciprenaline sulphate as well as bromhexine hydrochloride

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

Table 71. Regression Analysis of Orciprenaline Sulphate and Bromhexine Hydrochloride Standard Solutions

Sample	Composition of Solution (mcg ml^{-1})		Regression Equation [a] (270 nm for ORP and 345 nm for BRH)	Corr. coeff.	R^2, % [b]	F test Values [c]		Test for Significance [d] of Evidence of Correlation		Standard Error [e]		
	ORP	BRH				Crit	Calc	Crit	Calc	Slope	Intercept	Estimate
Series A	20-80	0	y = 0.0009x - 0.0002	0.9999	99.98	6.61	31800	2.57	178	0.0001	0.0002	0.0003
Series B	20-80	50	y = 0.0009x - 0.0002	0.9999	99.98	6.61	53197	2.57	230	0.0001	0.0002	0.0002
Series C	0	20-80	y = 0.0004x - 0.0001	0.9999	99.99	6.61	72051	2.57	268	0.0001	0.0002	0.0001
Series D	50	20-80	y = 0.0004x - 0.0001	0.9999	99.98	6.61	28060	2.57	167	0.0001	0.0001	0.0001

ORP - Orciprenaline sulphate BRH - Bromhexine hydrochloride [a] Based on 7 calibration values; concentration of drug in mcg ml^{-1}

[b] *Coefficient of determination* which is the *ratio of the sum of squares due to regression* to the *sum of squares about the mean*

[c] F test based on F statistic (a one tail test); F value is the *ratio of mean square due to regression* to the *mean square about regression*; F *calc* is the F (*1*, *n-2*) value at 5% significance level; F *crit* is the F (*1*, *n-2*) value from the *F ratio table* for 5% significance level; n is 7 for both ORP as well as BRH

[d] *Student's t test* for correlation (a two tail test): T *calc* is the T(n-2) value at 5 % level of significance and T *crit* is the T(n-2) value for *t* distribution table at 5% significance level; n is 7 for both orciprenaline sulphate as well as bromhexine hydrochloride

[e] Standard error of slope and intercept are the standard deviations of slope and intercept; standard error of estimate is the standard deviation of residuals of *y on x regression* where *y* is the $d^2A/d\lambda^2$ value and *x* is the *concentration*

Table 72. Results of the Assay of Pure Drug Admixtures and Commercial Formulations of Orciprenaline sulphate and Bromhexine Hydrochloride by Second-order Derivative Difference Spectrophotometry

Sample	Composition of Solution (mcg ml^{-1})		Label Claim (mg/5ml)		Mean[a] Recovery		95% Confidence[b] Level Concen. Range	
	ORP	BRH	ORP	BRH	ORP	BRH	ORP	BRH
Brand A (Batch 1)	50	40	5	4	99.82	98.86	99.70-99.94	98.76-98.96
Brand A (Batch 2)	50	40	5	4	99.34	99.65	99.20-99.48	99.52-99.78

ORP - Orciprenaline sulphate BRH - Bromhexine hydrochloride

[a] Average of ten determinations; assay as percentage of label claim calculated from the regression equations of pure drug admixtures

[b] Concentration range at 95% confidence level using t test (a two tail test) with 5 degrees of freedom for both oricprenaline sulphate as well as bromhexine hydrochloride

The pK_a values of orciprenaline sulphate are 9.0, 10.1 and 11.4 and that of bromhexine HCl is 8.5 [117] and hence any small change in the pH values of the solvents did not produce any appreciable change in the spectrum since the pK_a values were at least 1.5 units away from the pH values of 0.1M HCl ($pH \approx 1.0$) and 0.1M NaOH ($pH \approx 13.0$).

The results of the estimation in synthetic admixtures and commercial formulations by second-order derivative difference spectrophotometry have been given in table 72. The results in tables 68 and 72 showed that the drug contents in the commercial samples were within the official limits of 90-110 [1] for orciprenaline sulphate (in syrup preparation) whereas the limits for bromhexine HCl in syrups was not available.

6.00 Summary and Conclusion

The various methods of estimation proposed and validated in this thesis report indicate that these new methods of analysis may be used for the simultaneous estimation of drugs in combined formulations with ease. At present, the separation techniques, especially HPLC is the most popular method for the analysis of the drugs but the methods proposed in this report show that it is always not necessary to go for the chromatographic methods for the routine estimation of drugs in pharmaceutical formulations.

One of the main problem in the analysis of drugs in tablet formulations is usually the interference by the matrix constituents. These matrix constituents are, by and large, the additives such as binders, disintegrating agents, diluents and coloring agents. A properly designed analytical method will be able to eliminate this interference. In this report, most of the proposed methods were designed with this objective in mind. The common additives exhibit a spectral shape which may be eliminated either by difference spectrophotometry or by derivative and /or derivative difference spectrophotometry. The common additives of lactose, pvp and the coloring agent of indigocarmine were used to demonstrate the use of second-order transformation in eliminating the interference by additives. These additives are commonly present in most of the tablet preparations and are soluble in many solvents including water and hence are likely to be present in the final dilutions used for estimating the drugs.

The HPLC method is nowadays used for the estimation of drugs in tablets to a large extent. The results of the methods proposed in this report are of high precision and accuracy as evidenced by the statistical tests and may be preferable from the point of view of cost and ease of operation.

The main advantage of the proposed methods is that all of them (except non-aqueous titrimetry for salbutamol sulphate) involve simultaneous estimation of the drugs. This is prefarable to the official methods since none of the monographs (I.P., B.P. 1993 and U.S.P. 23) give the method for the simultaneous estimation of the drug combinations for which the methods have been developed.

However, there are certain facts about the proposed methods ought to be kept in mind while using them for analysis. In the case of estimation of aspirin and dipyridamole by infra-red spectrophotometric method, care must be taken not to allow the chloroform solution to evaporate while the spectra is being recorded since the chloroform evaporates easily. Similarly, during the estimation of aspirin and dipyridamole by spectrofluorometry, the chloroform used must be of spectroscopic grade since the presence of large amounts of moisture leads to hydrolysis of aspirin.

During the application of difference spectrophotometry for estimation of any combination, the fact that difference spectrophotometry will be able to eliminate only the interference of substances which do not undergo spectral alteration due to the condition used (change of pH) for spectral shift should be remembered. Similarly, the fact that the spectral shape as well as amplitude of the spectra in the derivative spectrophotometry depend on the instrument conditions such as scan speed, spectral bandwidth and data interval value should also be carefully noted. For example, the $d^2A/d\lambda^2$ values mentioned in this report were obtained under certain instrument conditions as mentioned in the text. These values should not be used as such while applying the method for estimation of the drugs. The method should be validated in the particular instrument which is to be used for the

estimation. In addition, the zero-crossing point may also shift depending on the scan speed and the wavelengths mentioned in this report should be taken as `working wavelengths'. On changing the instrumental parameters and /or type of the instrument, it would be advisable to verify the working wavelengths.

The results by the linear plot method, absorbance ratio method and derivative method showed that these methods yield comparable results in many cases. However, among the various proposed methods, the derivative method may be most advantageous one since it can eliminate the matrix interference by most of the common additives. Hence this method seems to be more versatile in terms of simultaneous estimation of the drugs without interference from each other as well as formulation additives. However, the degree of interference by the absorbing additives will depend on the drug-to-additive ratio in the formulation as well as the solubility of the additive in the solvents used for preparing the stock solution and final dilutions. Similarly, the presence of certain additives (such as dicalcium hydrogen phosphate) may alter the pH of the solution, resulting in a pH value of sample solution which will be different from that of the pure drug admixtures used for calibration. In cases where the direct use of derivative technique is not possible, a suitable extraction procedure to reduce the effect of interfering substances may be used prior to recording of the derivative spectra although such a requirement did not arise in the formulations assayed in this report.

13. K.L.K. Paranjothy and Banerjee, J.Pharm.Sci., 62 1697 (1973)

14. R. Abu-Eittah, J.Pharm.Sci., 63 1866 (1974)

15. D.M. Shingbal and G.B. Natekar, Ind.J.Pharm.Sci., 42 181 (1980).

16. F. Belal, Analyst, 109 615 (1984).

17. S.K. Bhowal and T.K. Das., Ind.J.Pharm.Sci., 52 202 (1990).

18. D.J. Vadidaria, B.R. Desai and S.P. Mukherji, Ind.J. Pharm.Sci., 27 257 (1965).

19. M.N. Reddy, D.G. Sankar, G.D. Rao and K. Sreedhar, East. Pharm., 34 127 (1991).

20. G. Mukherji and N. Agarwal, Int.J.Pharm.,71 187 (1991).

21. E. Hassan, I. Hewala, A.A. Wahbi and Y. Hassan, Farmaco, 48 1137 (1993).

22. E.R. Hackman, S.A. Benetton and M.I. Santoro, J.Pharm. Pharmacol., 43 285 (1991).

23. D.Q.Craig and K.M.Taylor, Int.J.Pharm., 114 129 (1995).

24. Y.K. Rathore, N. Murugesan, S.C. Mathur, Y.Kumar and P.D. Sethi, Ind.J.Pharm.Sci., 54 206 (1992).

25. R. Aroor and P. Gundu Rao, Ind.J.Pharm.Sci., 54 79 (1992).

26. R.E. Blend, R.J. Tannel and J.R. Lang, J.Pharm.Biomed. Anal., 8 591 (1990).

27. R.T. Sane, D.P. Gangal, R.V. Tendolkar and L. Joshi, Ind.J.Pharm.Sci., 52 197 (1990).

28. B.S.Furniss, A.J.Hannaford, P.W.Smith and A.R.Tatchell, Vogel's Textbook of Practical Organic Chemistry, 5th edn., ELBS and Longman, London, 1989, p.398.

29. W.D. Williams, Infra-red Spectrophotometry; Analytical Applications of Absorption Spectra. In A.H.Beckett and J.B. Stenlake (Eds), Practical Pharmaceutical Chemistry, Vol 2, 3rd edn., CBS Publishers and Distributors, New Delhi, 1986, pp. 340-342, 246-252.

30. J.R.Kincaid, Infra-red and Raman Spectroscopy; K.L. Cheng and V.Y. Young, Ultraviolet and Visible Absorption Spectroscopy. In G.D. Christian and J.E.O'Reilly (Eds), Instrumental Analysis, 2nd edn., Allyn and Bacon, Inc., Boston, 1986, pp. 240-242, 184-197.

31. H.H. Willard, L.L.Merritt,JR., J.A.Dean and F.A. Settle, Instrumental Methods of Analysis, 6th edn., CBS Publishers and Distributors, New Delhi, 1986, pp. 209-210.

32. W.O. George and P.S.Mcintyre, Quantitative Analysis. In D.J. Mowthorpe (Ed), Infrared Spectroscopy, John Wiley & Sons, New York, 1987, pp. 227-268, 149-151.

33. S.L. Lin and M.I. Blake, J.Pharm. Sci., 56 43 (1967)

34. H.J. Rhodes, J.J. DeNardo, D.W. Bode and M.I.Blake, J. Pharm. Sci., 64 1386 (1975)

35. S.L. Lin, J. Pharm. Sci.,, 56 1130 (1967)

36. R.A. Glenn and J.T.Peake, Anal.Chem., 27 205 (1955)

37. N. Shane and M. Knowbalnsky, J. Pharm. Sci., 57 1218 (1968)

38. M.I. Walash, S.P.Agarwal and Blake, Can.J.Pharm.Sci., 7 123 (1972).

39. M. Angberg, C. Nystrom, S. Cartensson, Int.J.Pharm., 61 67 (1990)

40. I.A. Muni,J.L. Leching, R. J. Helmo and N. Johnson, J. Pharm. Sci., 67 289 (1978)

41. S. Torrado and R. Cadorniga, J. Pharm. Biomed.Anal., 12 383 (1994)

42. F. Onur and N. Acar, S.T.P.Pharm.Sci., 5 152 (1995).

43. G.Jovicic, L.Nikolic, K.K.Rajic, Farmaco, 50 285 (1995)

44. M. Ustun and S. Sungur, Pharmazie, 47 459 (1992).

45. P.Mazseo, M.G. Quaghia and F. Segnabieu, J.Pharm. Pharmacol., 34 470 (1982).

46. M.I. Walash, A.M. El-Brashy and M.A. Sultan, Pharmazie, 49 698 (1994).

47. A. Villari, N. Micali, M. Fresta and G. Puglisi, J. Pharm. Sci., 81 895 (1992)

48. Z.G. Shao, Z. Pam and Z.O. Gan, Chinese J.Pharm., 26 222 (1991).

49. N.Shane and R. Stilmore, J.Pharm.Sci., 60 114 (1971).

50. G.H. Schenk, F.H. Boyer and C.I.Miles, Anal.Chem., 44 1593 (1972).

51. C.I. Miles and G.H. Schenk, Anal.Chem., 42 656 (1970).

52. N. Shane and C.I. Miles, J.Pharm.Sci., 59 397 (1970).

53. J.D. Winefordner and H.W. Latz, Anal. Chem., 3 1517 (1963).

54. L. Khemani and J.W. French, J. Chromatogr., 41 274 (1969).

55. H. Hsiu, T. Shih, K. Wang, J. Chromatogr., 41 489 (1969).

56. H.Chiang, T.Chiang, J.Chromatogr., 47 128 (1970).

57. W. Serfontein, D. Botha and L.S. DeVilliers, J. Chromatogr., 115 507 (1975).

58. A.N. Masoud. J. Pharm. Sci., 65 1585 (1976).

59. A.J. Hoffman and H.I. Mitchell, J. Pharm. Sci., 52 305 (1963).

75. A.W.clayton and R.E.Thiers, J.Pharm.Sci., 55, 404 (1966).

76. T.D. Doyle and F.R. Fezzari, J.Pharm.Sci., 12, 1921 (1974).

77. M.Pernarowski, A.M. Knevel and J.E.Christian, J.Pharm. Sci., 50, 946 (1961).

78. K.A. Connors and C.J. Eboka, Anal.Chem., 51, 1262 (1979).

79. A.L. Glenn, J.Pharm.Pharmacol., 15 Suppl., 123T (1963).

80. A.F.Fell, Ultraviolet, Visible and Fluorescence Spectrophotometry. In A.C.Moffat (Ed), Clarke's Isolation and Identification of Drugs, 2nd edn., The Pharmaceutical Press, London, 1986 pp. 221-236.

81. A.G. Davidson, J.Pharm.Sci.,73, 55 (1984).

82. A.G. Davidson, J.Pharm.Pharmacol., 28, 795 (1976).

83. A.G. Davidson, J.Pharm.Sci.,73, 1582 (1984).

84. L. Chafetz, D.C. Tsilifonis and J.M. Riedl, J.Pharm.Sci., 61, 148 (1972).

85. P.K.Chatterjee, C.L. Jain and P.D. Sethi, Pharm.Biomed.Anal., 7, 693 (1989).

86. J.Demeester, M.Bracke, R.Vochten and A. Lauwers, J.Pharm.Sci., 67, 729 (1978).

87. A.A.M. Habib, A.A.Omar and T.M. Sarg, J.Ass.Off.Anal. Chem., 67, 939 (1984).

88. D.W. Fink, H.C. Fink, J.W. Tolan and J. Blodinger, J.Pharm.Sci., 67, 837 (1978).

89. R.G. Bhatkar and C.V.Nagrankar, East.Pharm., 25 11 (1982).

90. R.B.Patel,A.A.Patel,B.K.Patel,T.N.Bhatt and S.C. Manakivala, East.Pharm 28, 137 (1985).

91. M. Aruna and D.Vijaya, Ind.J.Pharm.Sci., 49 110 (1987)

92. S.Manjan, J.Pharm.Sci., 65 736 (1976).

93. J.Nachbaur and J.Joly, J.Chromatogr.Biomed.Appl., 145 325 (1978).

94. K.Laubeck and B. Lindstorm, J.Chromatogr.Biomed.Appl., 162, 117 (1979).

95. P.P.Shah and R.C.Mehta, Ind.J.Pharm.Sci., 43 147 (1981).

96. S.K.Talwar, S.G. Sharma and S.Das, J.Pharm.Biomed.Anal., 4 511 (1986).

97. N.M.Sanghan and S.P.Kulkarni, Ind.J.Pharm.Sci., 40 101 (1978).

98. R. Godfrey and R.E.Duardo, J.Pharm.Sci., 80 212 (1991)

99. S.K.Talwar, S.C.Sharma and S.Das, J.Pharm.Biomed.Anal., 4 511 (1986)

100. C.S.Sastry, M.Aruna and D.Vijaya, Ind.J.Pharm.Sci., 49 190 ((1987)

101. B.A.Moussa, Int.J.Pharm., 10 199 (1982)

102. N.M.Sanghavi and H.S.Chandramohan, Ind.J.Pharm.Sci., 36 151 (1974)

103. P.K.Chaterjee, C.L.Jain and P.D.Sethi, Ind.J.Pharm.Sci., 48 195 (1986)

104. W.F.Smith and C.Yamitzsky, Int.J.Pharm., 75 161 (1991)

105. M.A.Brooks, L D'arconte and J.A.F. de Silva, J.Pharm.Sci., 65 (1976)

106. P.Shirke, M.K.Patel, V.B. Tinodkar, V.A.Tamhane and P.D.Sethi, Ind.J.Pharm.Sci., 56 108 (1994)

107. S.C.Bhatia, J.Chromatogr.Biomed.Appl., 305 325 (1984)

108. K.Rana and B.Gachalji, J.Chromatogr.Biomed.appl., 420 228 (1987)

109. R.A.Marques, B.Stafford, N.Flynn and W.Sadee, J.Chromatogr. Biomed.Appl., 146 163 (1978)

110. S.Ray, East.Pharm., 33 139 (1990)

111. Martindale, The Extra Pharmacopoeia, 29th edn., The Pharmaceutical Press, London, 1989, pp. XXIV-XXV.

112. J.Emmanuel and A.J.Viera, East.Pharm., 29 207 (1986)

113. M.Eisman, M.Gallego and M.Valcarcel, J.Pharm.Biomed.Anal., 11 301 (1993)

114. G.J.Bhomsule, V.S. Gomle, Ind.Drugs., 30 258 (1993)

115. E.Bechagaard and A.Nielson, J.Chromatogr.Biomed.Appl., 228 392 (1982)

116. T. Schmid and F.W.Kass, J.Chromatogr.Biomed.Appl., 227 71 (1982)

117. N.C.Paul, In J.D.Colin (Ed), Comprehensive Medicinal Chemistry, 1st edn., Pergamon, NewYork, 1990, Vol 6, pp. 234, 236.

118. H.W.Schultz and B.C. Paveen, J.Pharm.Sci., 62 1995 (1973)

119. Y.S.Chae and W.H. Shelver, J.Pharm.Sci., 65 1178 (1976)

120. T.F. Woodman, A.T.Conada and J.Scidman, Am.J.Hosp.Pharm., 34 984 (1977)

121. R.C.Gupta and G.D.Lendberg, Anal.Chem., 45 2403 (1973)

122. R.H.Pryee-Jones, G.M.Ecelesten and B.B.Abu-Bhakar, Ind.J. Pharm.Sci., 86 2317 (1992)

123. F. de Fabrizio, J.Pharm.Sci., 67 572 (1978)

124. G.Graham, Aus.J.Pharm.Sci., NS3 29 (1974)

125. J.Fidamza and J.J.Aaron, J.Pharm.Biomed.Anal., 5 619 (1987)

126. A.L.Thakkar and L.G.Tensmeyer, J.Pharm.Sci., 63 1319 (1974)

127. V.P.Shah and S.Riegalman, J.Pharm.Sci., 68 1283 (1974)

128. E.Bailey, P.B.Farmer, J.A.Peal, J.Caldwell and S.A.Hotchkiss, J.Chromatogr.Biomed.Appl., 416 81 (1987)

129. D.M.Mordelet, J.Y.Baglin, A.Roux and D.Dusser, Ther.Drug. Monitor., 8 106 (1986)

130. E.R.Venkata Rao, S.Raguveer and P.Khadgapathi, Ind.J. Pharm.Sci., 49 180 (1987)

131. M.E.Krahl, J.Phy.Chem., 44 446 (1940)

132. T.C.Butler, J.M.Ruth and G.F.Tucker, J.Am.Chem.Soc., 77 1486 (1955)

133. S.P.Agarwal and M.I.Blake, Anal.Chem., 41 1104 (1969)

134. M.A.Brooks, J.A.F. deSilva and M.R.Hackman, Anal.Chim. Acta, 64 165 (1973)

135. E.L.Cohen, AmJ.Pharm., 118 40 (1946)

136. V.Chromy and J.S.Babjuk, Clin.Chim.Acta, 37 547 (1972)

137. P.Jatlow, Am.J.Clin.Pathol., 59 167 (1973)

138. D.J.Blackmore, A.S.Curry, T.S.Hayes and E.R.Rutter, Clin. Chem., 17 896 (1971)

139. N.A.Zhakani, M.I.Walash and S.M.Ahmed, Farmaco, 46 601 (1991)

140. F.Onur and N.Acar, S.T.P.Pharm.Sci., 5 152 (1995)

141. J.D.Winefordner and M.Tin, Anal.Clin.Acta., 31 239 (1964)

142. K.F.Harbaugh, C.M.O'Dannell and J.D.Wineforder, Anal. Chem., 46 1206 (1974)

143. S.Udenfriend,D.Duggan, B.Vasta and B.Brodie, J.Pharmacol. Expt.Therap., 120 26 (1957)

144. S.H.Atwell, V.A.Green and W.G.Hanfy, J.Pharm.Sci., 64 806 (1975)

145. E.Kammerel and W.Schlemmer, J.Chromatogr., 82 143 (1973)

146. H.L.Davis, K.J.Falk and D.Bailey, J.Chromatogr.,107 61 (1975)

147. R.Osiewicz, V.Agarwal, R.M.Young and I.Sunshine, J.Chromatogr., 88 157 (1976)

148. H.Ehresson, Anal.Chem., 46 922 (1974)

149. A.M.Wahbi, M.A.Korany, M.M.Bedair and E.Elgindy, Drug.Dev.Ind.Pharm., 17 2215 (1991)

150. R.D.Sophia, L.A.Ramanyshyn and J.K.Wickmann, Ther. Drug.Monitor., 16 90 (1994)

151. H.Liu, M.Delgado,L.J.Forman and C.M.Eggers, J.Chromatogr.Biomed.Appl., 127 105 (1993)

152. A.M.Wahbi and H.Abdine, J.Pharm.Pharmacol., 25 69 (1973)

153. H.Abdine, A.M.Wahbi and M.A.Korany, J.Pharm.Pharmacol., 23 444 (1971)

154. M.M.Amar, A.K.S.Ahmed and S.M.Hassan, J.Pharm.Pharmacol., 29 355 (1977)

155. M.A.Korany, M.Bedair and F.A.Elyah, Analyst, 111 1986 (1986).

156. A.M.Wahbi, S.Beleal ,M.Bedair and H.Abdine, J.Assoc.Off.Anal. Chem., 64 1179 (1981)

157. T.C.O'Haver, Anal.Chem., 51 91A (1979)

158. R.N.Hager, Anal.Chem., 45 1131A (1973)

159. T.C.O'Haver and G.L.Green, Anal.Chem., 48 312 (1976)

160. B.Morelli, J.Pharm.Sci., 77 1042 (1988)

161. J.Traveset, V.Such, R.Gonzalo and E.Gelpi, J.Pharm.Sci., 69 629 (1980)

162. B.Morelli, J.Pharm.Sci., 79 261 (1990)

163. B.Morelli, J.Pharm.Sci., 77 615 (1988)

164. B. Morelli, J.Pharm.Sci., 94 34 (1995)

165. J.Bertinni, C.Mannucci, A.Pevico and P.Rovero, J.Pharm.Sci., 82 179 (1993)

166. A.F.Fell, J.Pharm.Pharmacol., 31 23 (1979)

167. A.G.Davidson and H.Elsheikh, Analyst, 107 879 (1982)

168. R.Jones and G.Marnham, J.Pharm.Pharmacol., 33 458 (1981)

169. I.Panderi and M.P.Parlou, Int.J.Pharm., 86 99 (1992)

170. M.Ustum and S.Sungur, Pharmazie, 47 459 (1992)
171. S.Atamaca, Z.Bilgic and M.Acikkol, Pharmazie, 46 532 (1991)
172. F. Onur and N.Acar, Int.J.Pharm., 78 89 (1992)
173. A.Parra, V.J.Garcia, V.Rodenas and M.d.Gomez, J.Pharm.Biomed. Anal., 12 653 (1994)
174. E.M. Hassan,M.A.El Sayed and E.F.Khamis, S.T.P.Pharm.Sci., 4 305 (1994)
175. F.Onur and N.Acar, S.T.P.Pharm.Sci., 5 152 (1995)
176. L.Hansson, J.Aberg, S.Jameson, B.Karlberg and R.Malmcrona, Acta.Med.Scand., 194 549 (1973)
177. C.Vetuschi and G.Ragno, Int.J.Phar.., 65 177 (1990)
178. A.D.Dale and S.E.Turner, J.Pharm.Biomed.Anal., 8 1055 (1990)
179. G.Pyramiede, J.W.Robinson and S.W.Zito, J.Pharm.Biomed.Anal., 13 103 (1995)
180. L. Zhou, C.F.Poole, J.Triska and A.Zlatkis, J.High.Resolut. Chromatogr.Commun., 3 440 (1980)
181. G.R.Rao, A.B.Avadhanulu, R.Giridhar, A.R.Pantulu and C.K.Kokate, East.Pharm., 33 113 (1990)
182. B.R.Patel and R.B.Poet, J.Pharm.Sci., 70 336 (1981)
183. S.V.Erram and H.P.Tipnis, Ind.Drugs, 29 436 (1992)
184. M.T.Rossel, A.M.Veriem and F.M.Pelpaire, J.Chromatogr. Biomed. Appl., 568 239 (1991)
185. R.T.Sane, L.Joshi, R.v.Tendolkar and D.P.Gangal, Ind.J.Pharm.Sci., 52 204 (1990)
186. F.Eiden, DAZ, 123 2003 (1983)
187. K.Thoma and R.Klimek, DAZ , 120 1967 (1980)
188. S.Ebel, H.Schutz and A.Hornitschek, Drug.Res., 28 2188 (1978)
189. K.R.Mahadik, G.B.Byale, H.N.More and S.S.Kadam, East.Pharm., 34 121 (1991)
190. S.P.Vyas and S.K.Goswami, Ind.Drugs, 30 342 (1993)
191. K.Schlobmann, Drug Res., 22 60 (1972)

192. K.Thoma and R.Klimek, Pharm.Ind., 47 319 (1985)

193. L.J.Lesko, A.K.Miller, R.L.Yeager and D.C.Chatterjee, J.Chromatogr. Sci., 21 415 (1983)

194. K.D.Ramsch and J.Sommer, Hypertension, 11 18 (1983)

195. K.Miyazaki, N.Kohri and T.Arita, J.Chromatogr., 310 219 (1984)

196. J.S.Grundy, R.Kherani, R.T.Foster, J.Pharm.Biomed.Anal., 12 1529 (1994)

197. T.Okhubo, H.Noro and K.Sugawara, J.Pharm.Biomed.Anal., 10 67 (1992)

198. P.Jakobsen, J.Chromatogr., 162 81 (1979)

199. S.Higuchi and J.Shiobara, Biomed.Mass.Spectrom., 5 220 (1978)

200. A.Savitzky and M.Golay, Anal.Chem., 36 1627 (1964)

201. W.French, F.Matsui and J.Truelove, Can.J.Pharm.Sci., 3 33 (1968)

202. T. Fitzgerald and E.Walaszek, Clin.Toxicol., 6 599 (1973)

203. M.Yamamoto and T.Uno, Chem.Pharm.Bull., 24 2237 (1976)

204. R.A.Kennedy, Drug.Dev.Ind.Pharm., 17 2127 (1991)

205. S.Ahuja, C.Spitzer and F.Brofazi, J.Pharm.Sci., 57 1979 (1968)

206. A.Olech, Acta.Ploon.Pharm., 32 73 (1975)

207. K.Brunt and J.Franke, Pharm.Weekblad., 112 481 (1977)

208. H.Heck, R.simon and M.Anbar, J.Chromatogr., 133 281 (1977)

209. K.McErlane, N.Curran and E.Lovering, J.Pharm.Sci., 66 1015 (1977)

210. A.Villet, J.alary and A.Coeur, Talanta, 27 659 (1980)

211. D.Thompson, J.Pharm.Sci., 71 536 (1982)

212. F.Fricke, J.Assoc.Off.Anal.Chem., 55 1162 (1972)

213. H.L.Rao, A.R.Aroor, P.G.Rao, Ind.J.Pharm.Sci., 53 31 (1991)

214. L.H.Nie, D.Z.Liu and S.Z.Yao, J.Pharm.Biomed.Anal., 8 379 (1990)

215. N.Y.Sreedhar and S.J.reddy, Ind.Drugs., 54 22 (1992)

216. T.R.Baggi, S.N.Mahajan and G.R.Rao, J.Assoc.Off.Anal.Chem., 58 875 (1975)

217. J.Berett, W.F.Smith and I.E.Davidson, J.Pharm.Pharmacol., 25 387 (1973)

218. J.M.Cacheza and L.J.Rodriguez, J.Pharm.Biomed.Anal., 5 395 (1987)
219. K.M.Emara and N.M.Mahfarz, Egypt.J.Pharm.Sci., 34 267 (1993)
220. G.R.Saggara, B.C.Gamez and C.J.Martinez, J.Pharm.Biomed.Anal., 11 1357 (1993)
221. N.T.Wad and E.J.Hanifal, J.Chromatogr.Biomed.Appl., 148 214 (1977)
222. G.Ramana Rao, A.B.Avadhanalu and A.R.Pantulu, East.Pharm., 34 123 (1991)
223. G.Santani and L.Fabbri, Int.J.Pharm., 71 1 (1991)
224. C.Lacroix and P.Danger, J.Chromatogr.Biomed.Appl., 128 285 (1993)
225. F.Kamali, J.Pharm.Biomed.Anal., 11 625 (1993)
226. C.Mannucci, J.Bertinni, A.Cochini and A. Perico, J.Pharm.Sci., 82 367 (1993)
227. S.A.Soliman, H.Abdine and M.g.Marcos, Can.J.Pharm.Sci., 11 63 (1976)
228. C.Nerin, J.Cacho and A.Barnica, J.Pharm.Biomed.Anal., 11 411 (1993)
229. D.R.Wirz, D.L.Wilson and D.H.Schenk, Anal.Chem., 46 896 (1974)
230. M.A.Korany, M.Bedair and F.A.El-Yazbi, Analyst, 111 41 (1986)
231. F.A.Shamsha and R.H. Magsouddi, J.Pharm.Sci., 65 761 (1976)
232. M.A.Korany, M.M.Bedair and A. Elgindy, Drug.Dev.Ind.Pharm., 16 1555 (1990)
233. G.Santani, P.Mura and S.Pinjauti, Int.J.Pharm., 50 75 (1989)
234. C.C.Webb, Pharm.Res., 8 1448 (1991)
235. H.P.Yuan and D.C.Locke, Drug.Dev.Ind.Pharm., 17 2319 (1991)
236. A.Morales, P.Richter and M.I.Toral, Analyst, 112 971 (1987)
237. M.Tuncel and G.Altiokka, Pharmazie, 47 304 (1992)
238. T.Das, C.Das and S.Ray, Ind.J.Pharm.Sci., 54 111 (1992)
239. G.S.Sadana and A.B.Ghogare, Ind.J.Pharm.Sci., 52 240 (1990)
240. R. Staroscik, Pharmazie, 29 387 (1974)
241. M. Bachrata, Pharmazie, 32 398 (1977)
242. E. Salim and I. Shupe, J.Pharm.Sci., 55 1289 91966)

243. Aboul-Ensin, K.A. Al-Rashood and H.M. El-Fatory, Pharma.Acta. Helv., 56 262 (1981)

244. N.Murgu, Pharmazie, 19 724 (1964)

245. H. Nangia, A.Munjal and M.Ahuja, Ind.Drugs, 29 457 (1992)

246. E. McChesney, Toxicol. and Appl.Pharmacol., 6 292 (1964)

247. G.Portmann, J.Pharm.Sci., 55 72 (1966)

248. G.Portmann, J.Pharm.Sci., 55 59 (1966)

249. E. McChesney, Toxicol.and Appl. Pharmacol., 14 138 (1969)

250. T.B.Vree, B.M.Van-Den, V.E.B. Kolmer and Y.A.Hekster, Pharm.World.Sci., 15 98 (1993)

251. K.Nikolic and M.Bogarae, J.Pharm.Biomed.Anal., 11 207 (1993)

252. E.Pawlak and S.Admski, Acta.Pol.Pharm., 29 409 (1972)

253. M.El Sadek, H.A.Gatef and A.A.Khier, J.Pharm.Biomed.Anal., 9 83 (1991)

254. F.A.El-Yazbi, M.H.Abdul and M.A.Korany, Farmaco Ed.Prat., 40 50 (1985)

A. Appendix I

Analytical Profile of Drugs

A. Di-iodohydroxyquinoline

5,7-Di-iodoquinolin-8-ol

Empirical formula: $C_9H_5I_2NO$

Molecular weight: 397.0

Melting point: 200-215° C with decomposition

pK_a : 10.5 [14]

Solubility: [111]

Practically insoluble in water and sparingly soluble in alcohol and ether

Official Methods of Analysis:

Oxygen flask combustion method [1]

Oxygen flask method [3]

B. Salbutamol sulphate

(RS)-1-(4-hydroxy-3-hydroxymethylphenyl)-2-(*tert*-butylamino)ethanol sulphate

Characteristics: A white or almost white, crystalline powder.

Empirical formula: $(C_{13}H_{21}NO_3)_2, H_2SO_4$

Molecular weight: 576.7

pK_a : 9.3, 10.3 [111]

Solubility: [2]

Freely soluble in water; slightly soluble in ethanol (96%) and in ether, very slightly soluble in dichloromethane

Official Methods of Analysis:

Spectrophotometry [2,3]

HPLC [1]

C. Aspirin

O-acetylsalicylic acid

Charecteristics: Colourless crystals or a white, crystalline powder; odourless or almost odourless.

Empirical formula: $C_9H_8O_4$

Molecular weight: 180.2

Melting point: 143° C [2]

pK_a : 3.5 at 25° C [111]

Solubility: [2]

Slightly solube in water; freely soluble in ethanol (96%); soluble in chloroform and in ether.

Official Methods of Analysis:

Titrimetry [2,3]

HPLC [1]

D. Dipyridamole

2,2',2'' , 2''' - [(4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine-2,6-diyl)dinitrilo]tetraethanol

Characteristics: A bright yellow, crystalline powder; odourless or almost odourless

Empirical formula: $C_{24}H_{40}N_8O_4$

Molecular weight: 504.6

Melting point: 164-167° C [2]

pK_a : 6.4 [111]

Solubility: [2]

Practically insoluble in water; freely soluble in chloroform; soluble in ethanol (96%).

Official Methods of Analysis:

Spectrophotometry [2]

HPLC [1]

K. Atenolol

(RS)-4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide

Characteristics: A white or almost white powder

Empirical formula: $C_{14}H_{22}N_2O_3$

Molecular weight: 266.3

Melting point: 146-148° C

pK_a : 9.6 at 24 [111]

Solubility: [2]

Sparingly soluble in water; soluble in absolute ethanol; slightly soluble in dichloromethane; practically insoluble in ether

Official Methods of Analysis:

Spectrophotometry [2]

HPLC [1]

E. Tinidazole

1-[2-(Ethylsulphonyl)ethyl]-2-methyl-5-nitroimidazole

Characteristics: Pale yellow crystals or crystalline powder with a slight characteristic odour

Empirical formula: $C_8H_{13}N_3O_4S$

Molecular weight: 247.3

Melting point: 125-128° C [3]

Solubility [1]:

Sparingly soluble in water; slightly soluble in alcohol, in chloroform and in solvent ether.

Official Methods of Analysis:

Spectrophotometry [3]

F. Diloxanide furoate

4-(N-methyl-2,2-dichloroacetamido)phenyl 2-furoate

Characteristics: A white or almost white, crystalline powder; odourless or almost odourless.

Empirical formula: $C_{14}H_{11}Cl_2NO_4$

Molecular weight: 328.2

Melting point: 114-116° C [2]

Solubility: [2]

Very slightly soluble in water; freely soluble in chloroform; slightly soluble in ethanol (96%) and in ether

Official Methods of Analysis:

Spectrophotometry [2,3]

G. Metronidazole

2-(2-methyl-5-nitroimidazol-1-yl)ethanol

Characteristics: A white or yellowish, crystalline powder.

Empirical formula: $C_6H_9N_3O_3$

Molecular weight: 171.2

Melting point: 159-163° C

pK_a : 2.5 [111]

Solubility: [2]

Slightly soluble in water, in acetone, in dichloromethane and in ethanol (96%); very slightly soluble in ether

Official Methods of Analysis:

HPLC [1]

Non-aqueous titrimetry [3]

H. Bromhexine Hydrochloride

2-amino-3,5-dibromobenzyl(cyclohexyl)methylamine hydrochloride

Characteristics: A white or almost white, crystalline powder

Empirical formula: $C_{14}H_{20}Br_2N_2$, HCl

Molecular weight: 412.6

Melting point: 235° C

pK_a : 8.5 [117]

Solubility: [2]

Very slightly soluble in water; slightly soluble in dichloromethane and in ethanol (96%)

Official Methods of Analysis:

Spectrophotometry [2]

I. Theophylline

1,3-dimethylpurine-2,6(3H,1H)-dione

Characteristics: A white, crystalline powder; odourless.

Empirical formula: $C_7H8N_4O_2$

Molecular weight: 180.2

Melting point: 270-274° C

pK_a : 8.6 at 25° C [111]

Solubility: [2]

Slightly soluble in water; sparingly soluble in absolute ethanol; slightly soluble in chloroform; very slightly soluble in ether.

Official Methods of Analysis:

HPLC [1]

Titrimetry [2]

J. Phenobarbitone

5-ethyl-5-phenylbarbituric acid

Characteristics: Colourless crystals or a white, crystalline powder; odourless

Empirical formula: $C_{12}H_{12}N_2O_3$

Molecular weight: 232.2

pK_a : 7.4 at 25 [111]

Solubility: [2]

Very slightly soluble in water; freely soluble in ethanol (96%); soluble in ether; sparingly soluble in chloroform.

Official Methods of Analysis:

HPLC [1]

Argentometry [2]

Spectrophotometry [3]

L. Nifedipine

Dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate

Characteristics: A yellow, crystalline powder.

Empirical formula: $C_{17}H_{18}N_2O_6$

Molecular weight: 346.3

Melting point: 171-175° C

Solubility: [2]

Practically insoluble in water, freely soluble in acetone; sparingly soluble in absolute ethanol.

Official Methods of Analysis:

HPLC [1]

Titrimetry [2]

Spectrophotometry [3]

M. Imipramine Hydrochloride

3-(10,11-dihydo-5H-dibenz[b,f]azepin-5-yl)propyldimethylamine hydrochloride

Characteristics: A white or slightly yellow crystalline powder; almost odourless

Empirical formula: $C_{19}H_{24}N_2$, HCl

Molecular weight: 316.9

pK_a : 9.5 at 24° C [111]

Solubility: [2]

Freely soluble in water, in chloroform and in ethanol (96%); practically insoluble in ether

Official Methods of Analysis:

Spectrophotometry [1,3]

Titrimetry [2]

N. Diazepam

7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2-one

Characteristics: A white or almost white crystalline powder; odourless.

Empirical formula: $C_{16}H_{13}ClN_2O$

Molecular weight: 284.7

pK_a : 3.3 at 20° C [111]

Solubility: [2]

Very slightly soluble in water; freely soluble in chloroform; soluble in ethanol (96%).

Official Methods of Analysis:

HPLC [1]

Titrimetry [2]

Spectrophotometry [3]

O. Diphenhydramine Hydrochloride

(RS)- 2-benzhydryloxyethyldimethylamine hydrochloride

Characteristics: A white crystalline powder; odourless or almost odourless

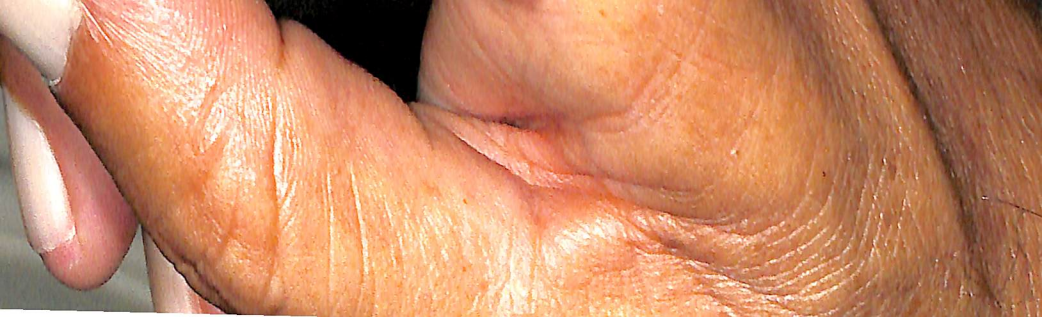
Empirical formula: $C_{17}H_{21}NO$, HCl

Molecular weight: 291.8

pK_a : 9.0 at 25° C [111]

Solubility: [2]

Very soluble in water, freely soluble in chloroform and in ethanol (96%); practically insoluble in ether



Solubility: [3]

Practically insoluble in water; slightly soluble in chloroform and in alcohol; very slightly soluble in solvent ether

Official Methods of Analysis:

Spectrophotometry [1, 3]

R. Orciprenaline sulphate

(RS)- 1-(3,5-dihyroxyphenyl)-2-isopropylaminoethanol sulphate

Characteristics: A white crystalline powder; odourless or almost odourless

Empirical formula: $(C_{11}H_{17}NO_3)_2$, H_2SO_4

Molecular weight: 520.6

pK_a: 9.0, 10.1 and 11.4 [111]

Solubility: [2]

Freely soluble in water and in ethanol (96%); practically insoluble in chloroform and in ether

Official Methods of Analysis:

HPLC [1]

Titrimetry [2]

Appendix II

List of Research Publications

1. Determination of Metronidazole and Furazolidone in Tablet Preparations and in Admixtures by Second-order Derivative Difference Spectrophotometry *(Communicated)*

2. Estimation of Metronidazole and Nalidixic Acid in Pharmaceutical Preparations by Second-order Derivative Difference Spectrophotometry *(Communicated)*

3. Spectrofluorometric Estimation of Aspirin and Dipyridamole in Pure Admixtures and Tablet Preparations, *J.Pharm.Biomed.Anal. (In press)*

4. Second-order Derivative Spectrophotometric Assay for Imipramine Hydrochloride and Diazepam in Pure Admixtures and in Dosage Forms, *J.Pharm.Biomed.Anal.*, 13 1003 (1995)

5. Simultaneous Quantitative Estimation of Tinidazole and Diloxanide furoate in Tablet Preparations by Difference Spectroscopy, *Drug Dev. and Ind.Pharm.*, 20 2143 (1994)

11. Quantitative Estimation of Paracetamol and Ibuprofen in Tablet Preparations by Non-aqueous Titrimetry, *Indian Drugs*, 30 596 (1993)

Appendix II

List of Research Publications

1. Determination of Metronidazole and Furazolidone in Tablet Preparations and in Admixtures by Second-order Derivative Difference Spectrophotometry *(Communicated)*

2. Estimation of Metronidazole and Nalidixic Acid in Pharmaceutical Preparations by Second-order Derivative Difference Spectrophotometry *(Communicated)*

3. Spectrofluorometric Estimation of Aspirin and Dipyridamole in Pure Admixtures and Tablet Preparations, *J.Pharm.Biomed.Anal. (In press)*

4. Second-order Derivative Spectrophotometric Assay for Imipramine Hydrochloride and Diazepam in Pure Admixtures and in Dosage Forms, *J.Pharm.Biomed.Anal.*, 13 1003 (1995)

5. Simultaneous Quantitative Estimation of Tinidazole and Diloxanide furoate in Tablet Preparations by Difference Spectroscopy, *Drug Dev. and Ind.Pharm.*, 20 2143 (1994)

6. Determination of Atenolol, Nifedipine, Aspirin and Dipyridamole in Tablet Preparations by Second-order Derivative Spectrophotometry, *Int.J.Pharm.*, 108 11 (1994)

7. Simultaneous Quantitative Determination of Salbutamol Sulphate and Bromhexine Hydrochloride in Drug Preparations by Difference Spectrophotometry, *Int.J.Pharm.*, 100 227 (1993)

8. Simultaneous Quantitative Determination of Metronidazole and Di-iodohydroxyquinoline in Tablet Preparations by Difference Spectrophotometry, *J.Ind.Chem.Soc.*, 72 111 (1995)

9. Simultaneous Determination of Aspirin and Dipyridamole in Pure and Dosage Forms by IR Spectrophotometry, *Indian Drugs*, 31 489 (1994)

10. Simultaneous Quantitative Determination of Phenobarbitone and Theophylline in Drug Preparations by Difference Spectroscopy, *Indian Drugs*, 29 442 (1992)

11. **Quantitative Estimation of Paracetamol and Ibuprofen in Tablet Preparations by Non-aqueous Titrimetry, *Indian Drugs*, 30 596 (1993)**