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SIMULTANEOUS DETERMINATION OF AMPICILLIN SODIUM AND SULBACTAM SODIUM IN BINARY MIXITURES & COMMERCIAL DOSAGE FORMS USING CHEMOMETRICS-ASSISTED SPECTROPHOTOMETRIC TECHNIQUES

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Simultaneous determination of ampicillin sodium and sulbactam sodium in binary mixtures and commercial dosage forms using chemometrics assisted spectrophotometric techniques

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LIST OF ABBREVIATIONS

CLS: Classical Least Squares

CV: Coefficient of variation

D1 ratio: First derivative ratio

LOD: Limit of detection

LOQ: Limit of quantification

Ml: Milliliter

NBW: Normal band width

PCR: Principal component regression

SNR: Signal-to -noise ratio

Thiram: tetramethyldithiocarbamate

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ABSTRACT

The binary mixture of ampicillin sodium and sulbactam sodium is officially analyzed by HPLC but they can not be simultaneously determined by the normal spectrophotometric techniques since their absorption spectra have a strong degree of overlapping.

This study applied four Chemometrics-assisted spectrophotometric techniques for the simultaneous determination of the two drugs without undergoing any physical separation: derivative spectrophotometry, derivative ratio technique, classical least square techniques and principal component regression techniques.

The derivative spectrophotometric techniques could not be successful since the overlapping absorption spectra of the two drugs could not be resolved from each other by this technique. The derivative ratio technique showed a better result since it has separated the two spectra to a better extent. The technique determined ampicillin in the mixture by using sulbactam sodium as a divisor at 251nm, 252nm & 253nm. The amount of sulbactam sodium was also determined using ampicillin sodium as a divisor at 205nm, 222nm & 245nm. The CLS and PCR methods were found to be capable of separating and hence determining the two drugs simultaneously better than the other techniques as can be seen from the recovery results.

The recovery results by all techniques were acceptable and very close to the results obtained by the official method and therefore the technique can equivalently be used for the simultaneous determination of the studied drugs.

Key words: Chemometrics, Spectrophotometry, Ampicillin sodium, Sulbactam sodium.

1. INTRODUCTION

1.1 Antibiotics

In modern usage, an antibiotic is a chemotherapeutic agent with activity against microorganisms such as bacteria, fungi or protozoa. The term was coined by Selman Washman i n 1942 to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution. This original definition excluded naturally occurring substances such as gastric juice and hydrogen peroxide (they kill microorganisms but are not produced by microorganisms), and also excluded synthetic compounds such as the Sulphonamides (which are anti-microbial agents). With advances in medicinal chemistry, most antibiotics are now modified chemically from original compounds found in nature. Some semi-synthetic antibiotics are produced and isolated from living organisms such as amino glycosides; in addition, many more have been created through purely synthetic means [1].

To be effective antibiotic drugs should be toxic to invading organisms and innocuous for the host; such selective toxicity depends on there being exploitable biochemical differences between the parasites (e.g. a bacterium) and the host. The three general classes of biochemical reactions are potential targets for chemotherapy [1].

Penicillin is one of beta-lactam antibiotics, which also include cephalosporins, monobactams and carbapenems, which has bactericidal activity. The basic nucleus of penicillins is 6-aminopenicillanic acid, which consists of a thiazolidine ring (A) linked to a beta–lactam ring (B). This latter ring carries a secondary amino group. The side-chain substituents at R1 determine the main anti-bacterial and pharmacological characteristics of particular penicillin. Penicillins may be destroyed by ß-lactamases or penicillinases through destruction of the ß –lactam ring and amidase by destructing the acylamino side chain, but the latter one is not significant since the ß-lactam ring is more important than the side chain for the pharmacological action of penicillins[2].

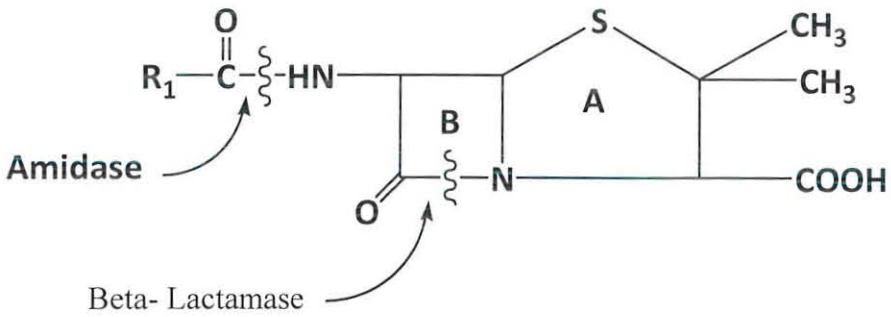

Figure1.1 Nucleus of penicillins

Table 1.1: Spectrum of activity of some antibiotics

Antibiotic	Gram-positive bacteria	Gram-negative bacteria
Penicillin	Streptococci, staphylococci	Anaerobes
Vancomycin	Clostridia, Staphylococci	Legionella, Campylobactor
Erythromycin	Corynebacteria,Streptococci	Legionella
Amino glycosides	*S .aureus*	Coliforms, pseudomonads

1.1.1 Mechanism of action of ß-lactam antibiotics

ß-lactam antibiotics inhibit the synthesis of peptidoglycon, which is the major polymer of the bacterial cell wall. In gram-positive organisms the peptidoglycan typically forms a thick layer external to the cytoplasmic membrane and accounts for 50 % of the dry weight of the bacterium, whereas in Gram-negative bacteria and mycobacteria, peptidoglycan is a thin layer, or hydrated gel, sandwiched between the outer &cytoplasmic membranes.

To manufacture peptidoglycan, bacteria first synthesize precursor molecules of uridine diphosphate (UDP) linked to N-acetylmuramic acid pentapeptide. The sequence of the pentapeptide varies among species ,but the two terminal residues are D-alanine, and the third amino acid, usually L-lysine or m-diaminopimelic acid, bears a free amino group, which may be substituted with bridge of additional amino acids.These antibiotics work by inhibiting the bacterial cell wall synthesis.This has a lethal effect on bacteria,

especially on Gram-positive once. Bacteria can become resistant to ß-lactam antibiotics by expressing ß-lactamase enzyme [3].

1.1.2 Resistance to antibiotics

Development of effective and safe drugs to deal with bacterial infections has revolutionized medical treatment. Morbidity & mortality from microbial disease have been dramatically reduced.

Unfortunately, the development of antibiotic defences against bacteria has been paralleled by the development of bacterial defences against antibiotic agents, resulting in the emergence of resistance.

Increasing resistance to antibiotics is a consequence of selective pressure, but the actual incidence of resistance varies between different bacterial species. For example, ampicillin resistance in *E. coli,* presumably under similar selective pressure as *S. aureus* with penicillin has remained at a level of 30-40% for many years with a slow rate of increase.

Antibiotic resistance is classified into two broad types: intrinsic and acquired.

1. ***Intrinsic resistance****:* This suggests that inherent properties of the bacterium are responsible for preventing antibiotic action.
2. ***Acquired resistance***: This occurs when bacteria which were previously Susceptible become resistant, usually, but not always, after exposure to the antibiotic concerned.

Intrinsic resistance is always chromosomally mediated, whereas acquired resistance may occur by mutations in the chromosome or by the acquisition of genes coding for resistance from an external source normally via a plasmid or transposon. Generally we can conclude that bacterial resistance to antibiotics is often achieved by the constitutive possession or inducibility of drug-inactivating or modifying enzymes [4].

1.2 Ampicillin sodium and Sulbactam combination

1.2.1 Ampicillin sodium

Ampicillin sodium is a semi synthetic penicillin antibiotic which contains ß-lactam ring. It kills or stops the growth of some bacteria by interfering with synthesis of the bacteria cell wall peptidoglycan.This drug is used to treat many kinds of infections of the blood, brain, stomach, intestines, heart, kidney, & Lung [5].

It is white powder, hygroscopic, freely soluble in water, sparingly soluble in acetone, practically insoluble in ether, fatty oils or liquid paraffin. Ampicillin sodium contains not less than 91.0 per cent and not more than the equivalent of 100.5 per cent of sodium (2*S*, 5R, 6R)-6-[[(2′ *R*)-2′-amino-2′-phenylacetyl] amino]-3, 3-dimethyl-7-oxo-4-thia-1-azabicycloheptane-2-carboxylate, calculated with reference to the anhydrous substance [6].

It is officially quantified by an HPLC method equipped with a 254 nm detector, a 4 mmx5 cm pre column containing 5 µm packing L1, and a 4mm x 30 cm analytical column containing 5 µm packing L1.The flow rate is about 2 ml per minute. The mobile phase is a filtered and degassed mixture of 0.005M tetrabutylammoniumhydroxide & acetoritrile in the ratio of 82.5 to 17.5, respectively [7].

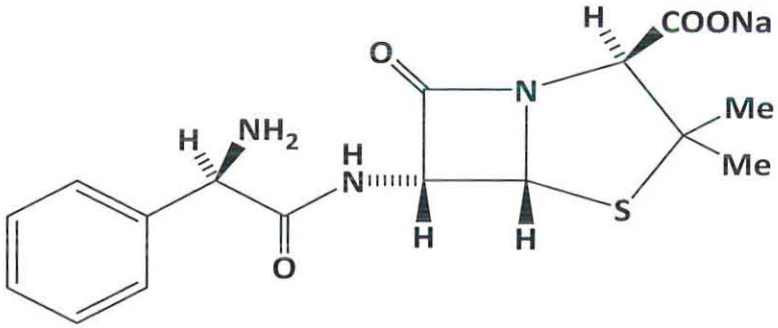

Figure 1.2 Chemical structure of ampicillin Sodium

1.2.2 Sulbactam Sodium

ß-Lactamase production is responsible for the development of resistance to ß-Lactam antibiotics, so its inhibitors are of great clinical importance. It is a chromosome and plasmid encoded enzyme. Several mechanism- based inhibitors of the enzyme have been synthesized or isolated; clavulanic acid, penicillanic sulfonate, & sulbactam, are among the best studied of these inhibitors. Sulbactam have been found to show synergism with standard penicillins such as ampicillin against penicillin resistant organisms; this observation is of clinical significance [8].

Sulbactam sodium is officially analysed by a liquid chromatography(HPLC) equipped with a 230 nm detector and a 4 mm by 30 cm column that contains L1 packing with a flow rate of 2 ml per minute and the mobile phase is prepared by mixing 0.005M tetrabutylammoniumhydroxide and acetonitrile in the ratio of 82.5 to 17.5 ,respectively [9].

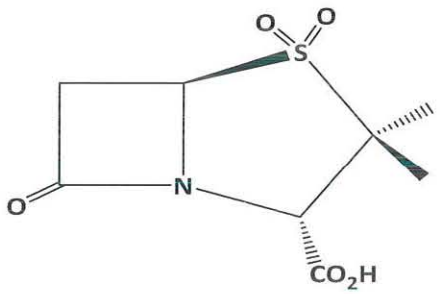

Figure 1.3 Chemical structure of Sulbactam

Ampicillin/Sulbactam sodium combined drug is an antibacterial combination consisting of the semisynthetic antibiotic, ampicillin sodium, and the beta-lactamase inhibitor, sulbactam sodium, for intravenous and intramuscular administration. To reduce the development of drug resistant bacteria and maintain the effectiveness of this drug it should be used only to treat or prevent infections that are proven to or strongly suspected to be caused by bacteria.

Ampicillin sodium/sulbactam sodium parenteral preparation is available as a white to off-white dry powder for reconstitution. The dry powder is freely soluble in aqueous

diluents to yield pale yellow to yellow solutions containing ampicillin sodium and sulbactam sodium equivalent to 250mg ampicillin per ml and 125mg sulbactam per ml. The pH of the solution is between 8.0 and 10.0.It is indicated for the treatment of infections due to susceptible strains of the designated micro organisms in the combinations listed below:

a. Skin and skin structure infections-caused by beta-lactamase producing strains of *Staphylococcus aureus, Escherichia coli, Klebsilella spp, Proteus mirabills, Bacteroides fragilis, Entrobacter spp, and Acinetobacter calcoaceticus.*

b. Intra-Abdominal Infections-caused by beta-lactamase producing strains of *Escherichia coli, Klebsiella spp.Bacteroides spp* [5].

1.3 Chemometrics-assisted spectrophotometric techniques in drug analysis

1.3.1 Chemometrics

Ampicillin sodium and sulbactam sodium combination is a combination used for the treatment of bacterial infections. The dosage form is normally analysed by an HPLC method used for quantification of both ampicillin sodium &sulbactam sodium [7].

The two drugs show a great sort of overlapping spectra when scanned in between 200nm and 300 nm and hence can not be quantified by the normal uv-vis spectrophotometry. Therefore a new chemometrics assisted spectrophotometric technique is required for simultaneous determination of the two drugs with out undergoing any sort of physical separation.

Chemometrics has been defined as "The chemical discipline that uses mathematical and statistical methods to design or select optimal procedures and experiments, and to provide maximum chemical information by analyzing chemical data". The most prominent part of chemometrics is data interpretation by multivariate methods. Chemometric methods are often applied in situations when no sufficient theory is available for describing or solving problems. Typical for problems of this type is the use of many

variables to describe a system; furthermore often only hidden relationships exist between the available data and the desired information and the aim of chemometrics is to find out some of these relationships. Examples of such widespread problems in chemistry are: recognition of the chemical structure from spectral data (spectral classification), quantitative analyses of substances in complex mixtures (multivariate calibration), and determination of the origin of samples (cluster analysis and classification), and prediction of properties or activities of chemical compounds or technological materials (quantitative structure-activity or structure-property relationships) [10].

Chemometrics provides powerful methods to reduce the large amount of data which is produced easily by automated instruments such as chromatographs coupled to a spectrometer. Another measure for the huge amount of chemical data available today is the number of registered chemical substances by the Chemical Abstract Service which reached 22.7 million at the begin of February 2000; the increase per day is more than 4000 new compounds [11].

The typical chemometric strategy (Figure 1.4) is data-driven and consists of the following steps. (a) Collection of data. (b) Generation of a mathematical model which is usually based on multivariate statistics or neural networks. (c) Interpretation of the model parameters in terms of the underlying chemistry. (d) Application of the model to new cases, or often the search for a better model or for more appropriate variables. During this process the possibility must always be carefully considered that a significant relationship does not really exist in the given data or cannot be extracted by the applied methods. The data-driven philosophy in chemometrics avoids prejudices to some extent but on the other hand it includes the danger of finding artifact correlations.

Consequently, results from chemometric methods must not be over-interpreted and it should always be tried to explain the resulting model parameters in terms of chemistry [11].

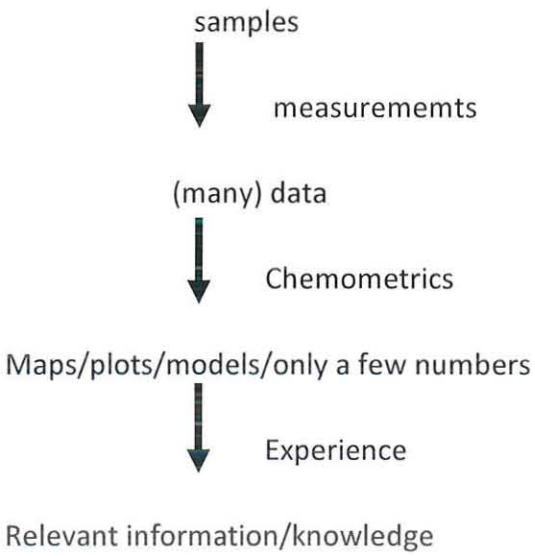

Figure 1.4 Typical strategy in chemometrics

Chemometric data analysis methods provide powerful tools for the analysis and interpretation of large, environmental, multivariate data sets generated within environmental monitoring programs. Chemometrics is complementary to laboratory automation. Just as automation is largely concerned with the tools with which to handle the mechanics and chemistry of laboratory manipulations and processes, so chemometrics seeks to apply mathematical and statistical operations to aid data handling and includes derivative spectrophotometry, CLS and PCR methods [11-12].

1.3.2. Derivative spectrophotometry

The range of application of derivative spectrophotometry is increasing regularly in the field of analysis. Derivative spectroscopy is a relatively modern technique, which was

introduced in 1953, that has proved to be very advantageous in solving particular analytical problems that normal spectroscopy is not able to solve. One of the most extensive fields of application of derivative spectroscopy is the quantitative analysis of two or more components. Derivative spectrophotometry has been applied to many chemical systems, as extensively reported giving rise to accurate and precise analytical results [12].

Derivative spectrophotometry is an analytical technique of great utility for extracting both qualitative and quantitative information from spectral curves composed of unresolved bands. In general, the derivative process discriminates against broad bands while emphasizing sharper features to an extent that increases with increasing derivative order. However, the use of higher-order spectra is not recommended as a general procedure because the signal to noise ratio becomes progressively larger. Thus, the information content of a spectrum is presented in a potentially more useful form, offering a convenient solution to a number of analytical problems, such as resolution of multi-component systems, removal of sample turbidity, matrix background and enhancement of spectral details. Derivative spectrophotometry is now a reasonably priced standard feature of modern micro-computerized UV/Vis spectrophotometry [13]. Derivative techniques have been used in pharmaceutical analysis, environmental analysis and in the fingerprint analysis of proteins, but few data have been published on the determination of mixtures of inorganic ions. Using fifth derivative spectrophotometry it is possible to determine cadmium-zinc & palladium – platinum mixtures [14-16].

Basic characteristics of derivative spectroscopy:

a. better resolution of spectra

The normal uv-vis spectrum of many compounds does not give a sharper band or peak. For example, the absorption spectrum of the complex of thiram with copper(II) sulphate recorded against a reagent blank shows absorption maximum at 423 nm(figure 1.5) and the

fourth derivative absorption spectra is shown in figure 1.6. Derivatization leads to sharper zero-order bands and gives higher signals in the resulting spectra. The characteristics of derivative spectra, such as peak height and noise level, depend on the choice of parameters such as order of derivative, scan speed and integration time during the recording of the spectra. These parameters should be optimized to give a well-resolved peak (better resolution) i.e., to good selectivity and higher sensitivity in a determination. Preliminary observations revealed that the best result were obtained from the fourth derivative with wavelength interval $\Delta\lambda$= 9 nm [15].

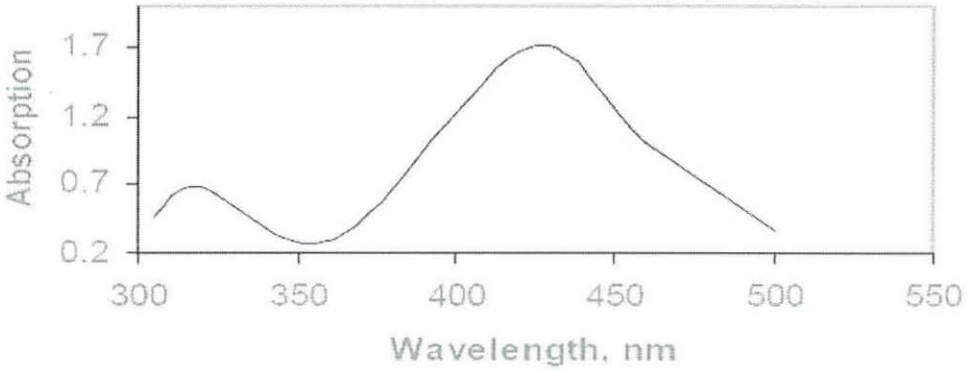

Figure 1.5 Normal absorption spectra of thiram as copper sulfate.

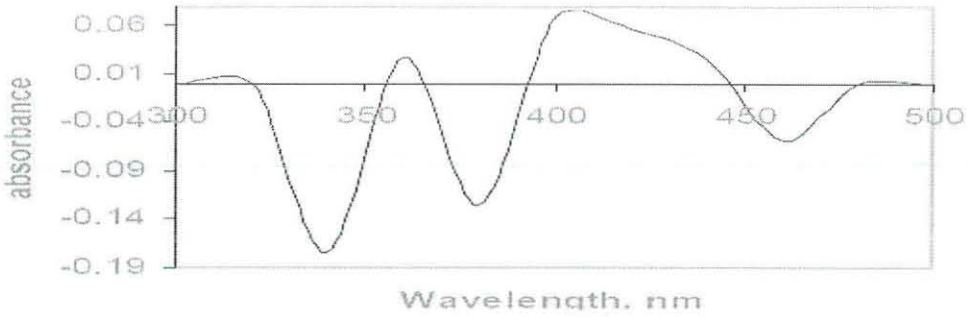

Figure 1.6 Fourth derivative absorption spectra of thiram as copper sulphate.

b. Elimination of the influence of baseline shift and matrix interferences

Qualitative and quantitative investigations of broad spectra are frequently difficult, especially where the measurement of small absorbances is concerned, because of uncontrollable baseline shift, great blank absorption and matrix interferences,

regardless of whether they are caused by irrelevant absorption of light scattering by turbid solutions and suspensions. All these influences can be overcome by derivatization (Figures. 1.7).

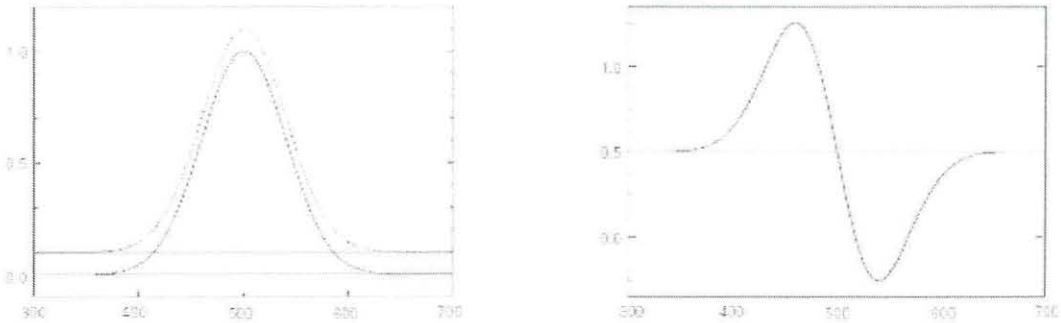

Figure 1.7 Left, normal spectra; right, first derivative of the same compound.

It is important to note that the order of derivatization depends on the order of the polynomial function used to describe interferences. In general, if n represents the highest degree of the polynomial equation used to define an interference, then the interference is reduced to a constant by using the n^{th} order derivative and is completely eliminated in the $(n+1)^{th}$ derivative as expressed in equations 1 to 3 :

$$P = a_o + a_1\lambda + a_2\lambda^2 + ... + a_n\lambda^n \qquad (1)$$

$$d^nP/\,d\lambda^n = n!a_n \qquad (2)$$

$$d^{(n+1)}P/\,d\lambda^{(n+1)} = 0 \qquad (3)$$

In many cases, matrix interference can be approximated by a linear or quadratic function. When interference can be described by a linear function ($P = a\lambda + b$), the first derivative yields a function where the interference is reduced to a constant $dP/d\lambda = a$ and in the second order derivative transformation the interference is completely eliminated ($d^2P/d\lambda^2 = 0$) [18].

c. Discrimination

Probably the most important effect of the derivative process is that broad bands are suppressed relative to sharp bands and this suppression increases with increasing

derivative order. This arises from the fact that the amplitude, *Dn*, of a Gaussian band in the *nth* derivative is inversely proportional to the original bandwidth, *W*, raised to the n^{th}degree as expressed by equation 4:

$$D^n=1/W^n \quad (4)$$

Thus for two coincident bands of equal intensity but different bandwidth in the zero order, the *nth* derivative amplitude of the sharper band, *X*, is greater than that of the broader band, *Y*, by a factor that is dependent on the relative bandwidth and the derivative order, equations 5 & 6 & figure 1.8 [19].

$$\frac{D^n_x}{} = \underline{W}^n{}_y \quad (5)$$

$$D^n{}_y \quad W^n{}_x \quad (6)$$

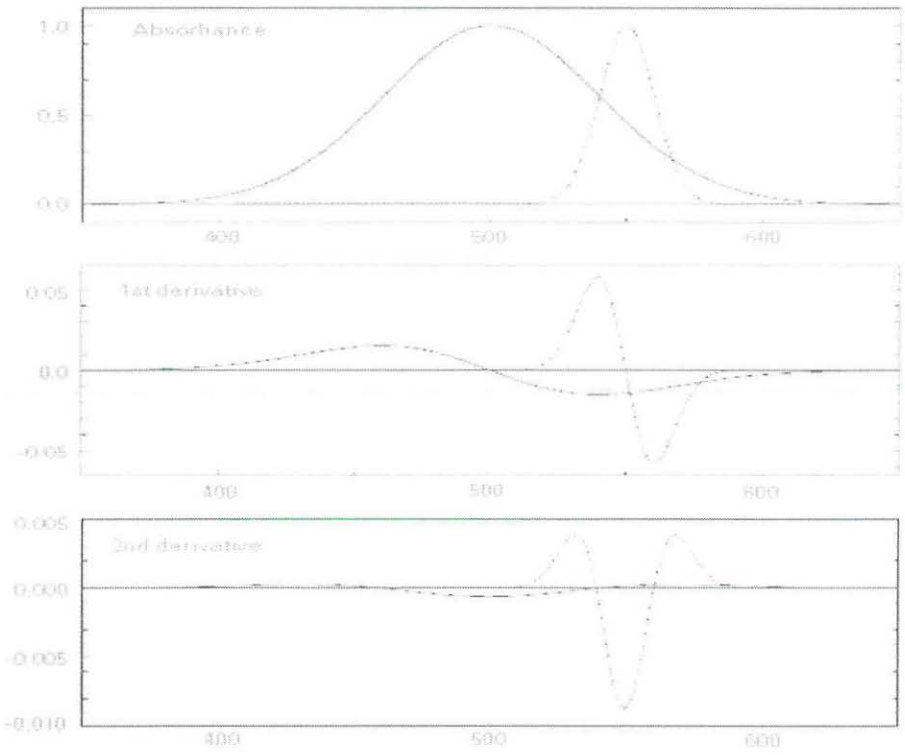

Figure 1.8 Derivatives of two bands, one with 160 nm NBW and one with 40 nm NBW.

d. Signal-to-noise ratio

An unwanted effect of the derivatization process is that the signal-to-noise ratio decreases as higher orders of derivatives are used. This follows from the discrimination effect and the fact that noise always contains the sharpest features in the spectrum.

Thus, if the spectral data used in the derivative calculation is at 2nm intervals, the noise has a 2 nm bandwidth. If the analyte band has a bandwidth of 20nm then the signal-to-noise ratio of the first derivative is ten times worse than the zero-order spectrum. Alternative techniques, such as using a reference wavelength or full spectrum multicomponent analysis with a scattering spectrum as standard, may often be used to achieve the same analytical goals but without the reduced signal-to-noise penalty [20].

e. Quantification

Of course, for quantitative analytical purposes, only the amplitudes versus concentrations are measured and here spectral distortion is an acceptable sacrifice, as long as the impact of signal-to noise ratio on the measurement precision is kept to a minimum. In general, the quantitative analytical problems can be divided in to two categories:

- ***Single or multy*** –component analysis of overlapping absorption peaks.

The quantitative determination of either one or several compounds depends on each compound obeying the Beer-Lambert Law in the zero order spectrum at a specified wavelength:

$$\text{Log } I_O/I = A = \varepsilon.b.c \qquad (7)$$

Where: I_o and I are incident and transmitted intensities, respectively and ε is the molar absorpitivity,c is molar concentration & b is the path thickness.

Similarly the analyte concentration is linearly related to the amplitude of the n^{th} derivative peak at the specified wavelength, equation 8 :

$$^{n}D = d^{n}A / d\lambda^{n} = d^{n}\varepsilon / d\lambda^{n} cb \qquad (8)$$

- ***Law of additive***

Furthermore, an important extension of the Beer-Lambert law is the law of additive, which states that the absorption of radiation by one species will be unaffected by the presence of other materials, whether they absorb or not. Thus the general form of the law may be written as equation 9:

$$A=\Sigma\varepsilon i.b.ci \qquad (9)$$

where the summation is over all substances, i, present [21].

1.3.3 Derivative ratio spectroscopy

Derivative ratio spectrophotometry is among the methods that utilize derivative spectrophotometry. It considers the ratio of the derivative (first derivative, second derivative, third derivative etc) rather than the derivatized value as it is. The method was developed for the simultaneous analysis of a ternary mixiture, without prior separation. It is based on the use of the derivative ratio spectrum obtained by dividing the absorption spectrum of the ternary mixture by a standard spectrum of a mixture of two of the three compounds in the mixture [22-24].

A spectrophotometric method was developed for resolving binary mixtures when the spectra of the components are overlapped. The method is based on the use of the first derivative of the ratios of the spectra. The absorption spectrum of the mixture is obtained and divided (amplitude by amplitude at appropriate wavelengths) by the absorption spectrum of a standard solution of one of the components (previously stored in a computer), and the first derivative (or another derivative order) of the ratio spectrum is obtained. The concentration of the other component is then determined from a calibration graph [25].

Derivative ratio spectrophotometric method has some reported advantages of being able to suppress matrix effects, easy of operation and obtaining results rapidly. Although results from the derivative ratio spectrophotometric method are sometimes not as accurate as those from the HPLC method, it is still regarded as a good analytical method without prior separation to determine coexisting similar components in a simple system [26].

Absorbance ratios have been used by the British pharmacopoeia for the identity and purity of certain pharmaceuticals. In the quantitative assay of mixtures, absorbance ratios were used at certain wavelengths for the analysis of binary mixtures. However, in the application of these methods, the presence of spectral interferences and/or spectral overlapping will certainly lead to erroneous results. [6]

Basic principle:

The method is based on the use of the derivative of the ratios of spectra. The absorption spectrum of the mixture is obtained and the amplitudes at appropriate wavelengths are divided by the corresponding amplitudes in the absorption spectrum of a standard solution of one of the components. The first derivative of the ratio spectrum is obtained. The concentration of the other component is then determined from a calibration graph [17, 27].

Ratio derivative methods permits the determination of components in mixtures at wavelengths corresponding to a maximum or minimum .The values at these points permit better sensitivity and accuracy. The main instrumental parameters that affect the shape of the derivative ratio spectra are the wavelength scanning speed, concentration of the divisor spectra, smoothing and scaling factor [28].

1.4 Multivariate calibration methods

Modern automatic analysis methods provide opportunities to collect large amounts of data very easily. For example, in clinical chemistry it is routine to determine many analytes for each specimen of blood, urine, etc. A number of chromatographic and spectroscopic methods can provide analytical data on many components of a single specimen. Situations like these, where several variables are measured for each specimen, yield multivariate data. For example dividing the stationary phases used in GC into groups with similar properties by studying the retention behaviour of a varity of solutes with different chemical properties. In this case it would be possible to compare specimens by considering each variable in turn, but modern computers allow more sophisticated processing methods where the variables are considered simultaneously. The traditional technique for this method is multiple linear regressions; it has been complemented by more robust and more powerful methods such as principal component regression and partial least squares regression [29].

1.4.1 Principal components regression (PCR)

The basis of principal component regression (PCR) is to reduce the number of predictor variables by using their first few principal components rather than the original variables. The method works well when there is a considerable degree of correlation between the predicted variables. This is usually the case in inverse calibration.PCR is also useful technique when the predictor variables are very highly correlated [29].

It is a two-step procedure. In the first step, one determines principal components that are linear combinations of the original variables. They can be considered as new variables that summarize in an optimal way the variation present in the spectra. In the second step, CLS is applied to the newly obtained latent variables. When co-linearity between original variables occurs, principal component plots often allow better interpretation of the variations observed in the data set than plots of original variables selected by CLS. As modelling method, it is less performant than CLS when performing

prediction within the calibration domain and when the model is indeed linear. It is more reliable if extrapolation may be required. It is a linear method, but it is able to perform quite well for moderately nonlinear data.

Advantages of PCR:

- Does not require wavelength selection. Any number can be used, usually the whole spectrum or large regions.
- Larger number of wavelengths gives averaging effect, making model less susceptible to spectral noise.
- Can be used for very complex mixtures since only knowledge of constituents of interest is required.
- Can sometimes be used to predict samples with constituents (contaminants) not present in the original calibration mixtures.

Disadvantages of PCR:

- Calculation are slower than most classical methods
- Generally, a large number of samples are required for accurate calibration.
- Collecting calibration samples can be difficult and must avoid collinear constituent concentrations [30].

1.4.2 Classical least squares analysis (CLS)

CLS technique assumes that responses (absorbance) at each frequency (wavelengths) are proportional to component concentration units. Model errors are assumed to derive from the measurement of spectral absorbance. So CLS requires that all interfering chemical components be known and included in the calibration data set. CLS has the advantage of improved precision when using many frequencies, due to signal averaging [31].

CLS technique assumes that responses (absorbance) at each frequency (wavelengths) are proportional to component concentration units. Model errors are assumed to derive

from the measurement of spectral absorbance. So CLS requires that all interfering chemical components be known and included in the calibration data set. CLS has the advantage of improved precision when using many frequencies, due to signal averaging. Calibration is realized by recording the spectra at n-wavelengths of m standard mixtures, of known composition of c components. The spectra (absorbance or emission) are arranged into the columns of matrix Y (dimensions n x m), with the composition of each mixture forming the columns of concentration matrix X (c x m), equation 10 :

$$Y = K.X \qquad (10)$$

With a prior knowledge of X and by recording data for Y, then the matrix of sensitivities, K, can be calculated, but after the rearrangement of equation 1 to the following equation by multiplying the equation components by X^t value as equation 11 & 12:

$$Y.X^t = K.X.X^t \qquad (11)$$

$$K = (X.X^t)^{-1}.Y.X^t \qquad (12)$$

To avoid being under-determined, there must be measurements at more wavelengths than there are components (i.e. n = c). If n > c then the component concentrations in an unknown mixture are obtained from its spectrum by equation 13:

$$X \text{ unknown} = (K^t.K)^{-1}.^{Kt} y \text{ unknown} \qquad (13)$$

This CLS method is intuitively appealing since it is based on some generally assumed relationship, e.g., Beer's law and it can be used for moderately complex composition of the calibration mixtures, i.e. the concentration of each absorbing species [32].

2. OBJECTIVES OF THE STUDY

2.1 General objective

The general objective of this study is to find a simple, cost effective and accurate chemometrics-assisted spectrophotometric method for the simultaneous determination of ampicillin sodium and sulbactam sodium in single combination dosage form.

2.2 Specific objectives

- To provide a simple and cost effective DS,CLS and PCR methods for determination of ampicillin sodium and sulbactam sodium in combined dosage forms.
- To compare the results obtained by the above methods with the results of the official or reported methods.

3. EXPERIMENTAL

3.1 Instruments and equipments

A Spectronic unicam Ultraviolet-visible double beam spectrophotometer (model number-UVA 083430 type Helison alpha) with a 1.00 cm quartz cells was used for the spectrophotometric measurements, Scaltec analytical balance (Model number SBC-31), Sonicator (Bandelin sonorex model number D2800), Perk inElmer's HPL C (model number series 200), glass wares of different sizes & suction filtration apparatus were used in the work. Statistical manipulation was performed by transferring the spectral data to Microsoft excel 2003 program and processing them with the standard curve fit package and matrix calculations using Harvard graphics 2.0 & VISTA 6 version 6.4.3435-EWU (may 10, 2001) soft wares.

3.2 Solvents and Chemicals

Distilled water, HPLC grade tetra butyl ammonium hydroxide & acetonitrile were used (BDH chemicals Ltd., England). Working reference standards of sulbactam sodium and ampicillin sodium were supplied by CSPC, China.

3.3 Formulation

Aurobennz® (Sterile powder for injection) with batch number JUXII-010 Aurobindo pharma Ltd,India, labelled to contain 2g of ampicillin sodium and 1g of sulbactam sodium per vial, as the only brand commercial pharmaceutical preparation in Ethiopia was purchased from the local market and subjected to analysis by the proposed and official methods.

3.4 Preparation of stock and working standard solutions

Stock solutions of ampicillin sodium and sulbactam sodium were prepared by dissolving an accurately weighed amount (50mg) of the studied drugs in about 80 ml of distilled water in 100 ml volumetric flask. The solutions are then made up to the volume with the same solvent. Suitable aliquots of the stock solutions (3ml to 10 ml) were diluted quantitatively

with the solvent to 50ml to obtain the suitable working standard solutions according to the linear calibration range for each drug (30 -100µg/ml).

3.5 Preparation of synthetic mixtures of ampicilln sodium and sulbactam sodium reference standards

Laboratory prepared synthetic mixtures of the two drugs were prepared by mixing known amounts of working solution of ampicillin sodium with known amounts of working solution of sulbactam sodium in different proportions (ratios) in order to verify the precision of the method for analysis of such mixtures and matching the commercial formulations with those having comparable concentrations. In all of the dilutions the measured volumes were diluted quantitatively to 50 ml to get completely different ratios. Ten different ratios of 1:10 to 10:1 of ampicillin sodium to sulbactam sodium were prepared.

3.6 Procedures for preparation of dosage form solution

3.6.1 Procedures for repeatability using standard addition technique

150mg of dosage form was quantitatively transferred to five 100ml volumetric flasks. Then different amounts (mg) of reference working standards of both drugs were added to each flask and the solution was completed to volume with distilled water.

I.e. 50:25, 60:30, 80:40, 100:50, & 120:60 ratio of ampicillin sodium to sulbactam sodium were added to 150mg of the dosage form in 100ml volumetric flask, dissolved & diluted to volume with distilled water.

2.5ml of each solution was then diluted to 50ml with the same solvent and absorbance of each solution was measured in the range between 200 and 300nm in triplicate.

3.6.2 Procedure for quantification of the dosage form.

150mg of the dosage form was dissolved and diluted to 100ml volumetric flask with distilled water; 5ml of the resulting solution was diluted to 100ml with the same solvent.

This was done three times and all of the solutions were scanned in between 200nm and 300nm, to evaluate repeatability of the absorbance reading.

3.7 Procedure for the official method (USP)

3.7.1 Standard preparation

60mg of ampicillin sodium and 30mg of sulbactam sodium reference standards were dissolved & diluted to 100ml with the mobile phase, which is a mixture of 0.005M tetra butyl ammonium hydroxide and Acetonitrile in the ratio of 82.5 to 17.5, to get 0.6mh/ml & 0.3mg/ml of each, respectively.

3.7.2 Sample preparation

100mg of the mixed contents of the dosage form was dissolved and diluted to 100ml with the mobile phase to get a solution of 1mg/ml of the sample.

3.7.3 Running the chromatography

10µl of the standard and sample preparations were injected one by one to an HPLC system equipped with a detector at 230nm, a reversed phase column, L1, at a flow rate of 2ml/min. The chromatograms were then utilized for the quantification of each drug in the dosage form.

4. RESULTS AND DISCUSSION

4.1. Preliminary studies

4.1.1 Overlay of absorption spectra

All of the experiments were done in triplicate in all cases for three different days employing the same analytical procedure, using the same laboratory equipment and by the same analyst (to confirm both repeatability and reproducibility). Average of the triplicate analysis for each day and all the three days was taken in all of the analysis.

UV-absorbance spectrum of pure, synthetic mixtures and dosage forms of ampicillin sodium and sulbactam sodium was recorded in a wavelength range of 200-300 nm in the linear calibration domain of ampicillin sodium (30-100µg/ml) and sulbactam sodium (30-100µg/ml), which was obtained by measuring absorbances of solutions of different concentrations. The 30µg/ml to 100µg/ml range gave us absorbance readings between 0.2 & 1.0 & hence this range is chosen to be the linear calibration range. First derivative zero crossing, first derivative of the ratio spectra, CLS, and PCR techniques are used for the determination of pure drugs (calibration step), laboratory prepared synthetic mixtures (testing step) and the dosage forms (prediction step). Form the results of this analysis various graphs are sketched using soft wares such as and Harvard graphics 2.0. and the absorption patterns of the two drugs are as given in Figures 4.1 and 4.2. In both figures the different colours express the different concentrations of each solution used in their linear calibration range.

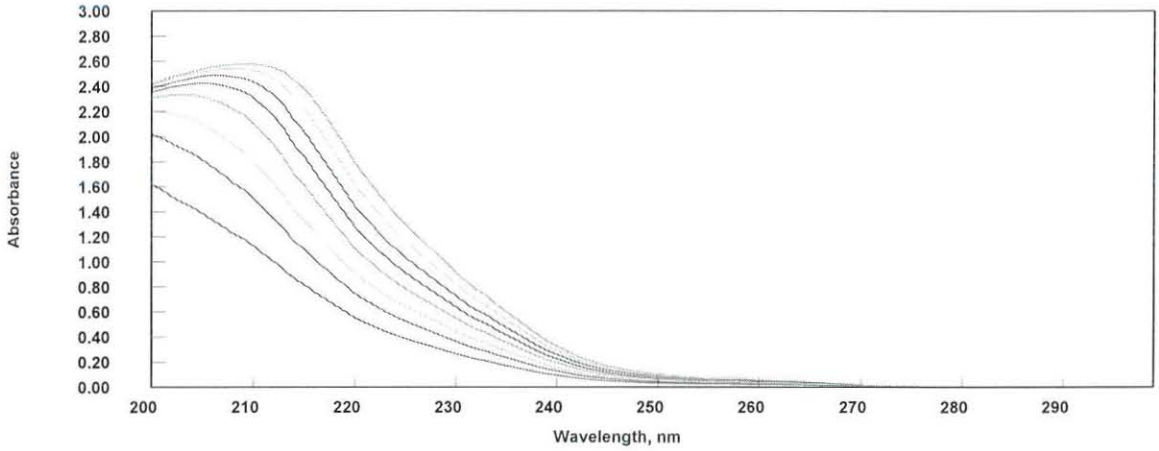

Figure4.1: Overlay absorption spectra for ampicillin sodium in the range from, 30-100µg/ml of the final dilution in aqueous solutions.

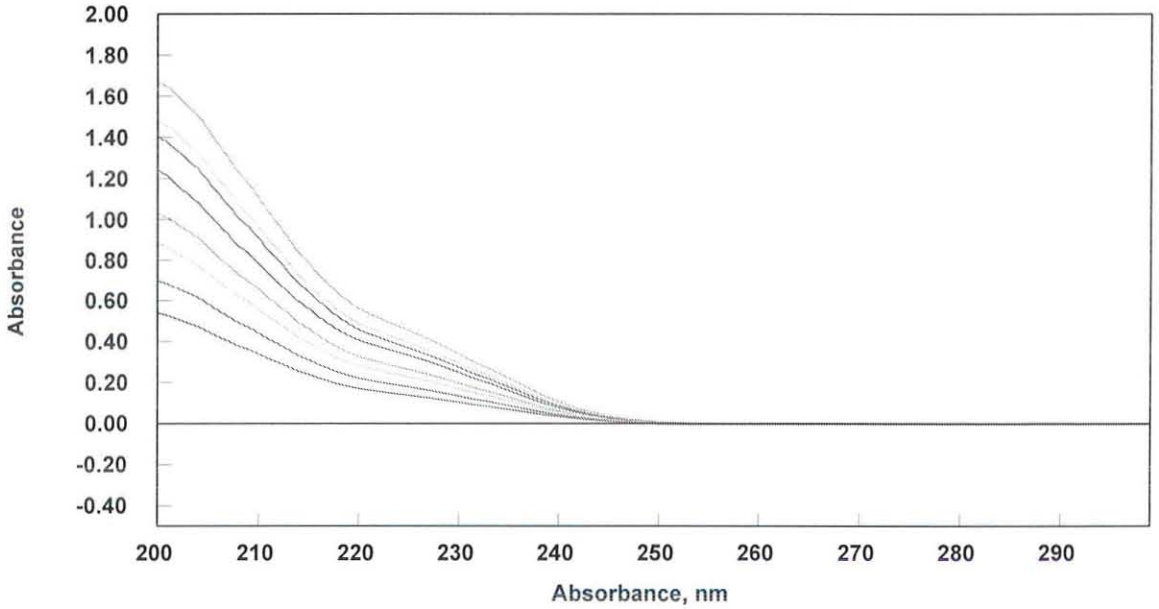

Figure 4.2: Overlay absorption spectra for sulbactam sodium in the range from, 30-100µg/ml of the final dilutions in aqueous solutions.

This preliminary study indicates that there is no cross linking of the spectrum of each solution with the other and linear response of absorbance against concentration is observed. These two premlinary studies are important to know before we proceed to applying the new techniques for the synthetic and commercial dosage form.

4.1.2 Histogram plots of the C-values

The histogram plots give sufficient information for the presence or absence of outliers and clusters in the concentration gradient (Figures 4.3 and 4.4). From the histogram plots of C-values one can conclude that there is a linear correlation between absorbance and concentration and also there is no outlier and clustering of data. These are important for PCR & CLS techniques; these techniques do no need clustering and outlying of data.

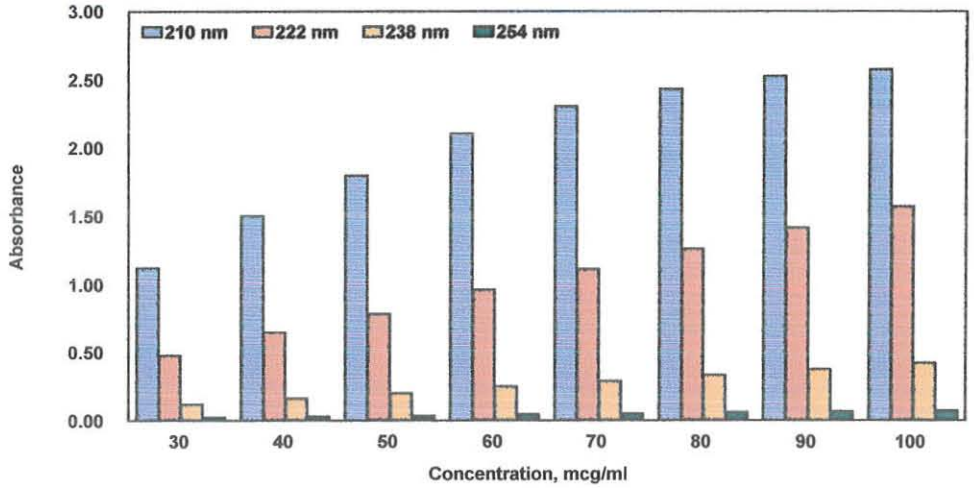

Figure 4.3: Histogram of C-values of ampicillin sodium against the corresponding absorbances at four randomly selected wavelengths

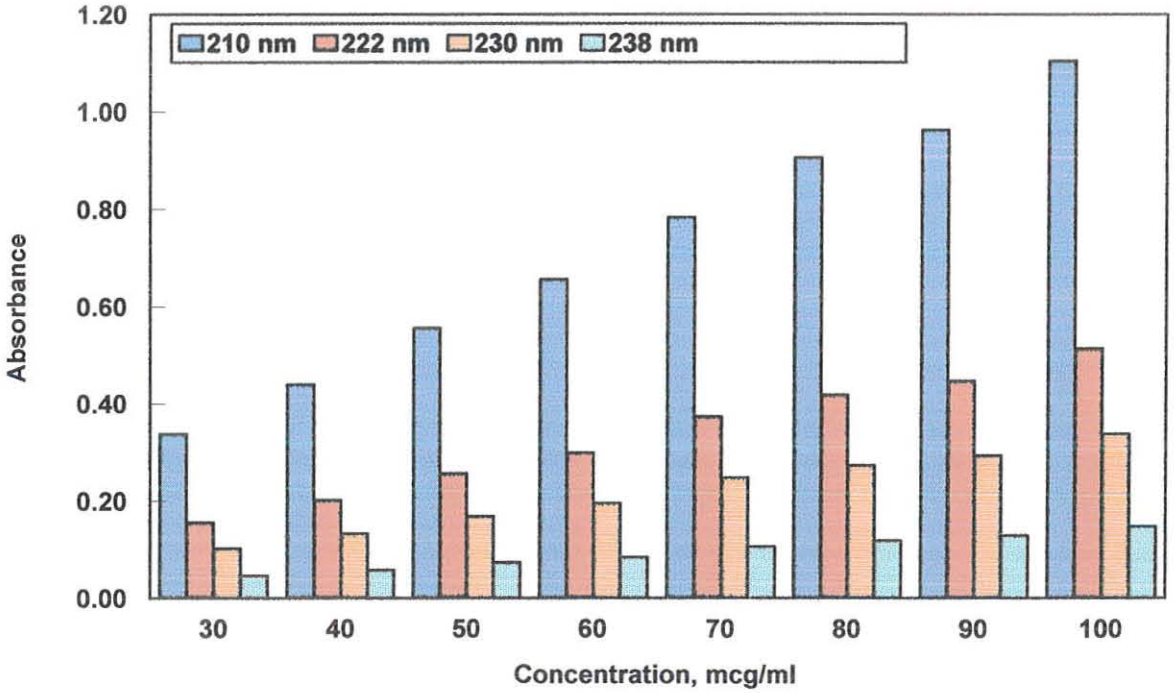

Figure 4.4: Histogram of C-values of sulbactam sodium against the corresponding absorbances at four randomly selected wavelengths

4.1.3. Relationship of the plot between the principal components

The figures that relate the relationship of the histogram plot with that of the principal components give additional information on the presence of extreme data, data which is not important and is related to errors in measuring absorbance, on reading (absorbance) which is very important in the case of PCR analysis since strong non-linear responses may be shown probably on one of the plots. That means when measurement of absorbance is done the absorbance reading is the amount of light that gets absorbed by the sample plus errors. Therefore the extreme data that is related to the errors is ignored by the plot. Figures 4.5 to 4.10 illustrate that most absorption data are found in PC1 and some in PC2 in the pure reference standards and the laboratory prepared binary mixtures of the two drugs.

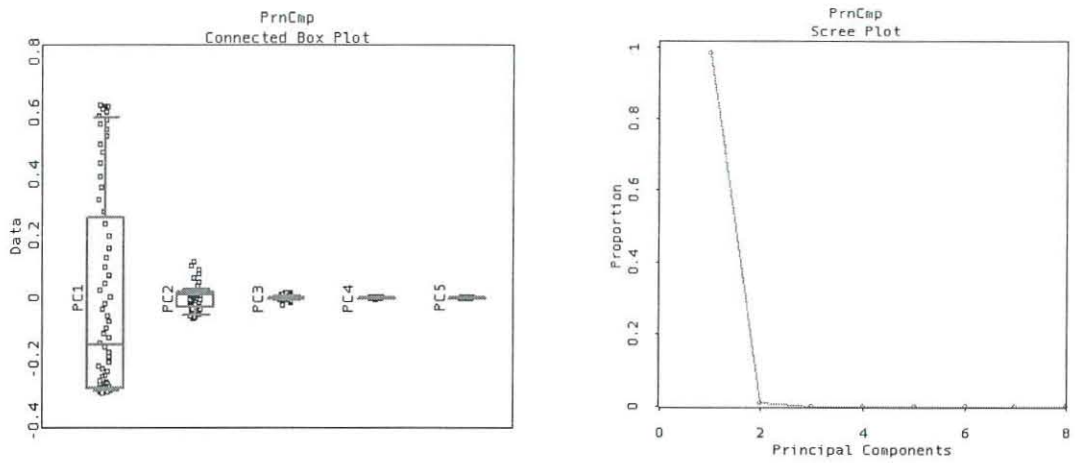

Figure 4.5: Scree (right) and connected box (left) plots for principal component model ampicillin sodium data

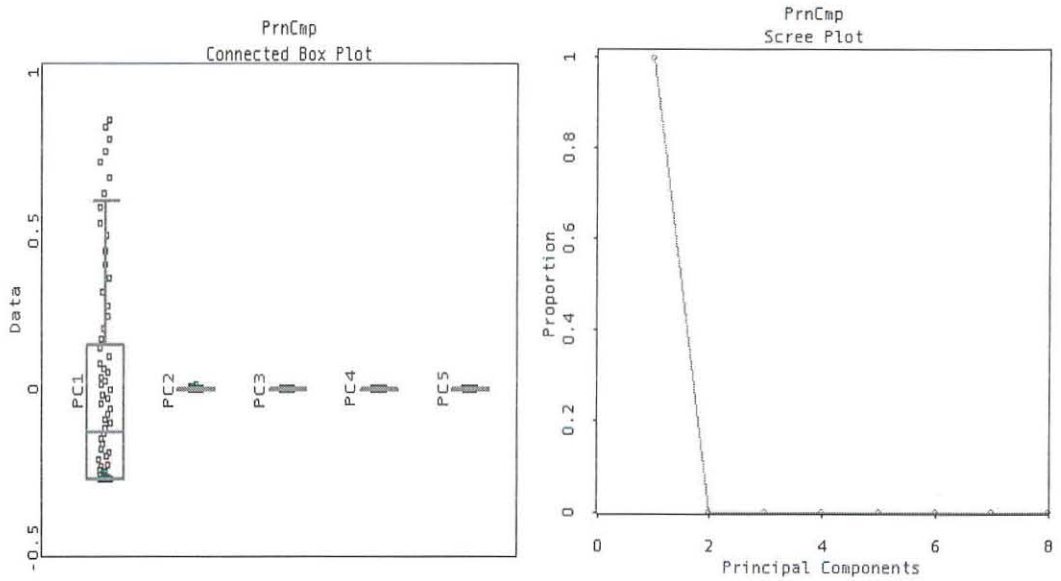

Figure 4.6: Scree (right) and connected box (left) plots for principal component model sulbactam sodium data

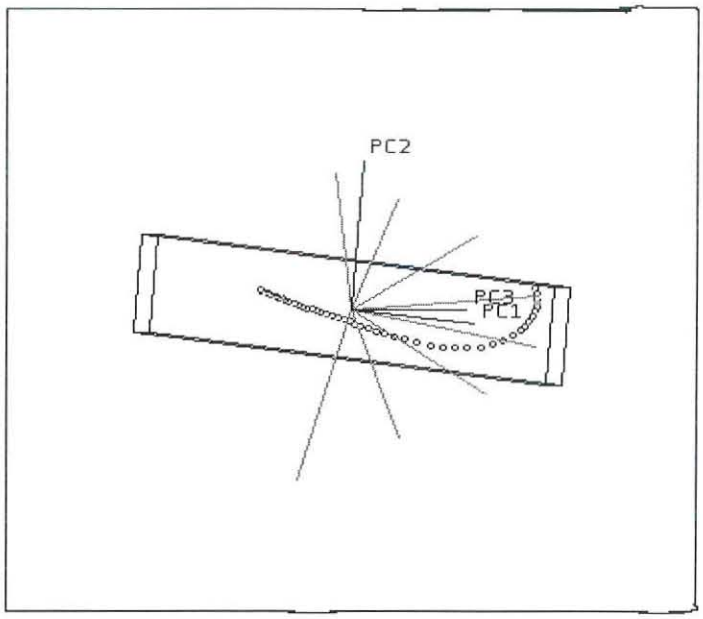

Figure 4.7: 3D Principal components analysis of variable correlation for ampicillin sodium

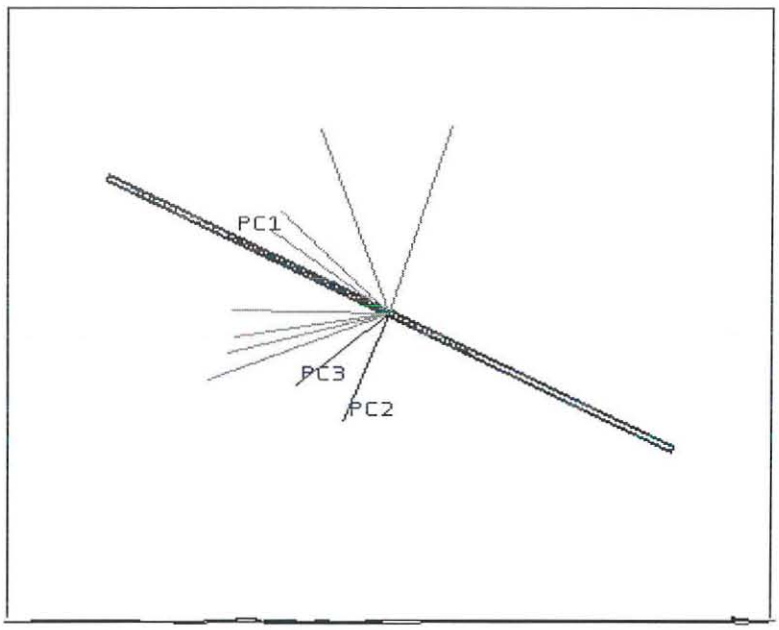

Figure 4.8: 3D Principal components analysis of variable correlation for sulbactam sodium

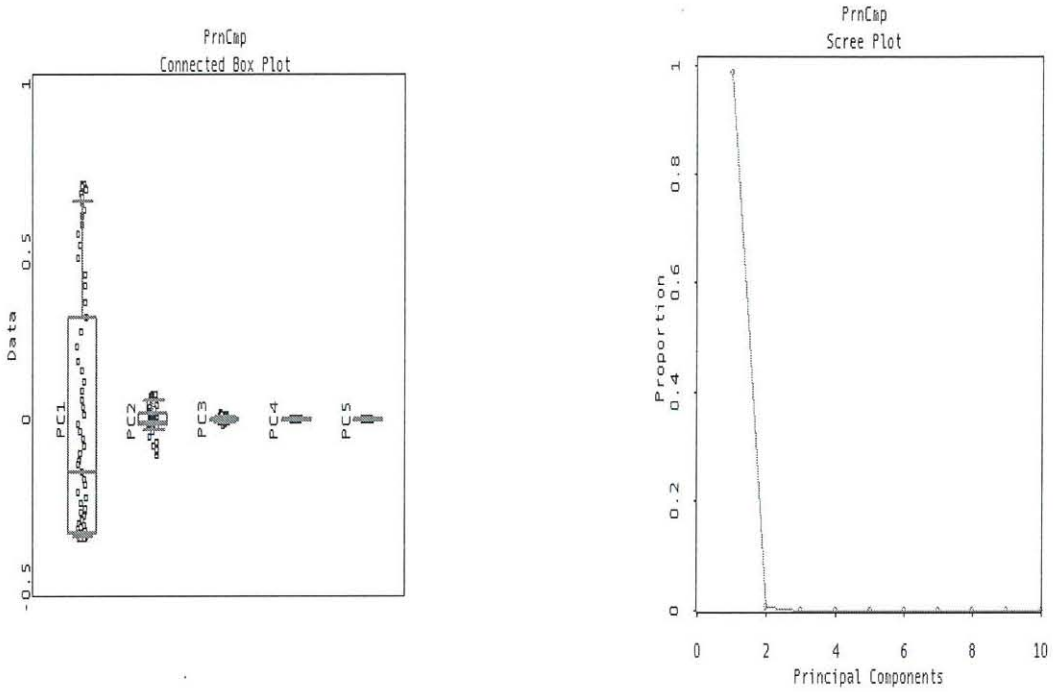

Figure 4.9: Scree (right) and connected box (left) plots for principal component model ampicillin sodium: sulbactam sodium mixture from 1:10 to 10:1.

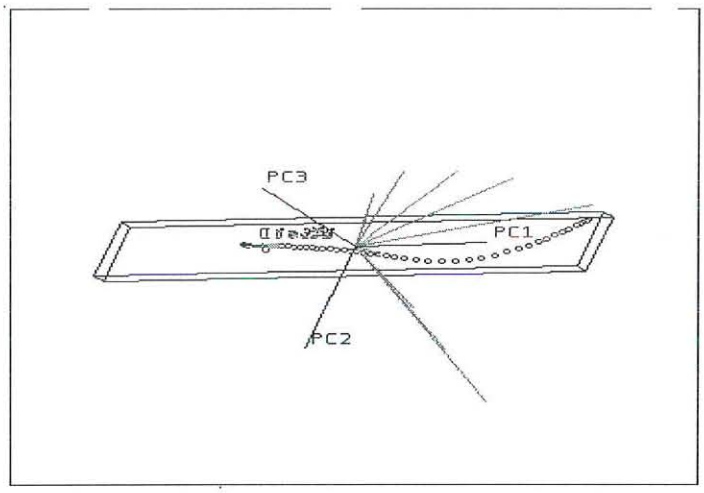

Figure 4.10: Scree (right) and connected box (left) plots for principal component model ampicillin sodium: sulbactam sodium mixture from 1:10 to 10:1. (3-D view).

4.2 Derivative spectrophotometric techniques:

4.2.1 Zero-crossing technique

Figures 4.1 shows ampicillin sodium absorption spectrum (the above) overlapped with the absorption spectrum of sulbactam sodium (the bottom) in the range between 200nm and 300nm. From the figure we can easily understand that the two drugs have a high degree of overlap.

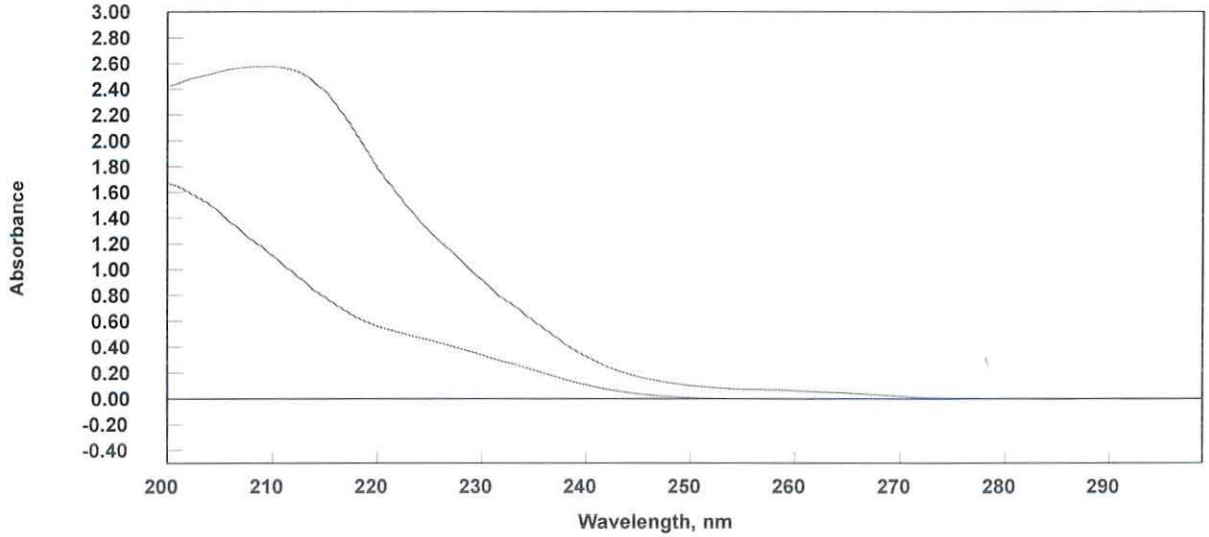

Figure4.11: Degree of overlapping for ampicillin sodium (the above) & 100µg/ml, sulbactam sodium (the bottom), 100µg/ml each, spectra in aqueous solutions in the range from 200-300nm.

Derivative spectrophotometry is one of the techniques used for simultaneous determination of two component systems with overlapping spectra. This technique of analysis was tried to analyze the binary mixture, ampicillin sodium/sulbactam sodium, which have an overlapping spectrum. But the method could not be effective in separating one of the components from the other since the degree of overlapping is high. Figures 4.12 and 4.13 show that it is not possible get any zero-crossing point for the sample analyzed and hence this technique cannot be used for simultaneous determination of this compound drug.

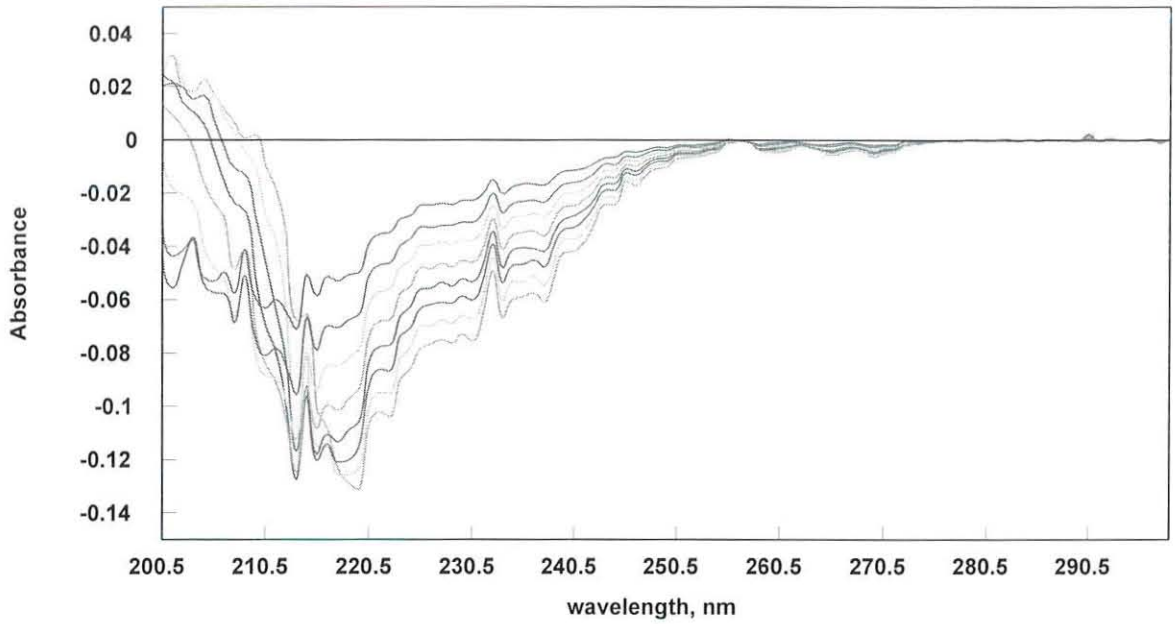

Figure 4.12: First derivative spectra of ampicillin sodium in aqueous solutions in the range from 30-100µg/ml of the final dilutions.

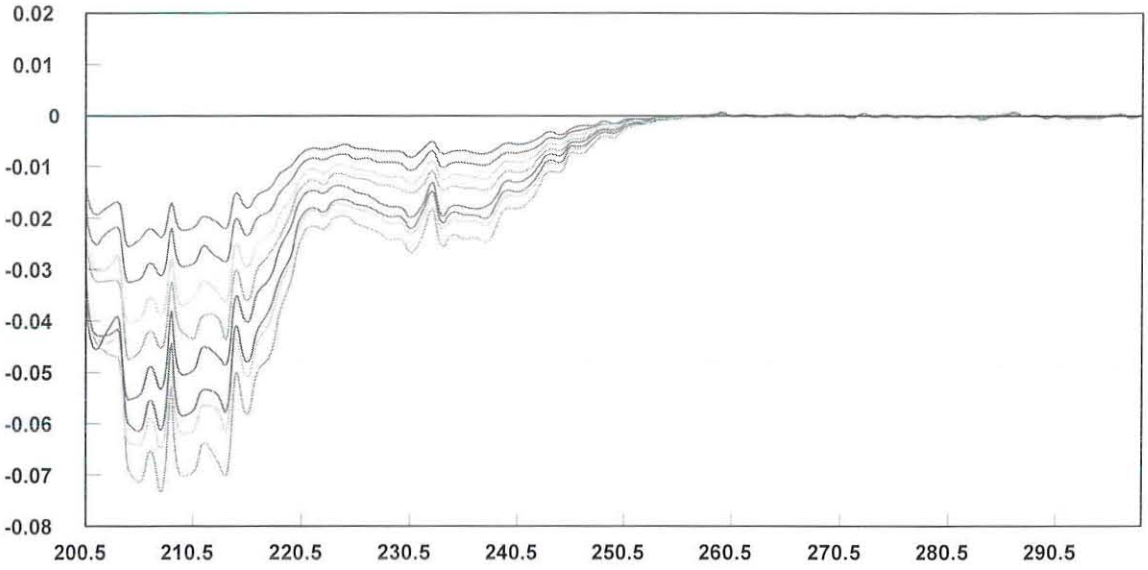

Figure 4.13: First derivative spectra of sulbactam sodium in aqueous solutions in the range from 30-100µg/ml of the final dilutions.

4.2.2 Derivative ratio technique

Ratio derivative methods were used for analysis of mixtures with overlapped spectra. This method permits the determination of components in mixtures at wavelengths corresponding to a maximum or minimum absorbances. The values at these points permit better sensitivity and accuracy. The main instrumental parameters that affect the shape of the derivative ratio spectra are the wavelength scanning speed, the concentration of divisor spectra, smoothing (Δλ) and scaling factor. The effects of these parameters were studied by trying different values and fast scanning speed, smoothing factor (Δλ = 2nm), scaling factor (= 10) was selected. Divisor concentration is the main instrumental parameter: the standard spectrum of 60µg/ml of ampicillin sodium and 80µg/ml of sulbactam sodium was considered as divisor for the determination of ampicillin sodium and sulbactam sodium in mixtures. Then from these figures ampicillin sodium can be determined in the mixture by measuring the absorbance or amplitude at 251nm, 252nm and 253nm where there is no contribution from sulbactam sodium (Figures 4.13 and 4.14)

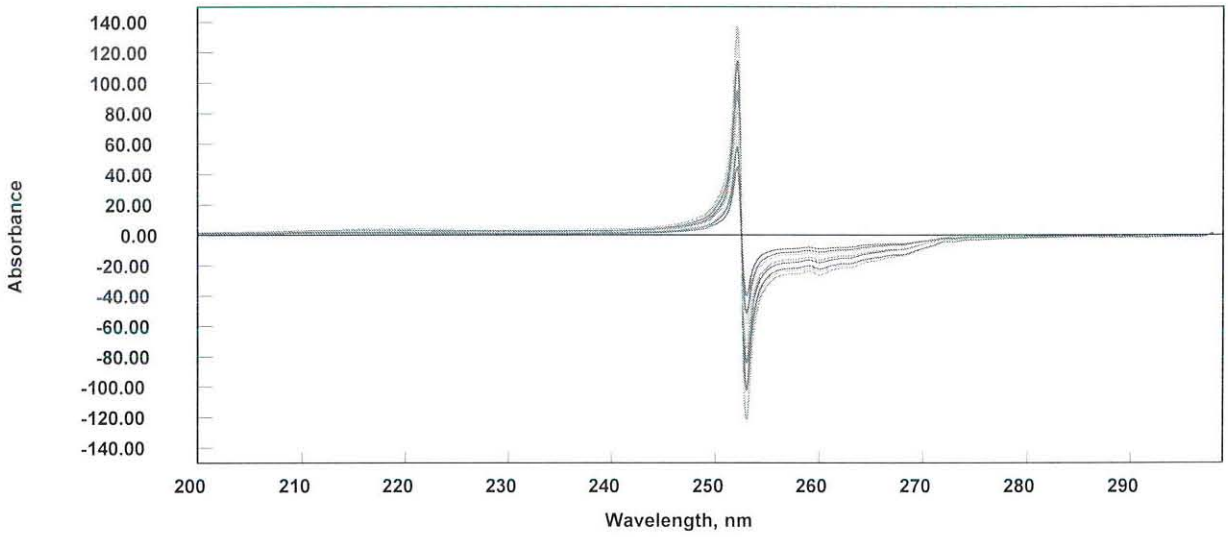

Figure 4.14: First derivative ratio spectra of ampicillin sodium (30 – 100µg/ml), divisor is 80µg/ml of sulbactam sodium (2nm intervals).

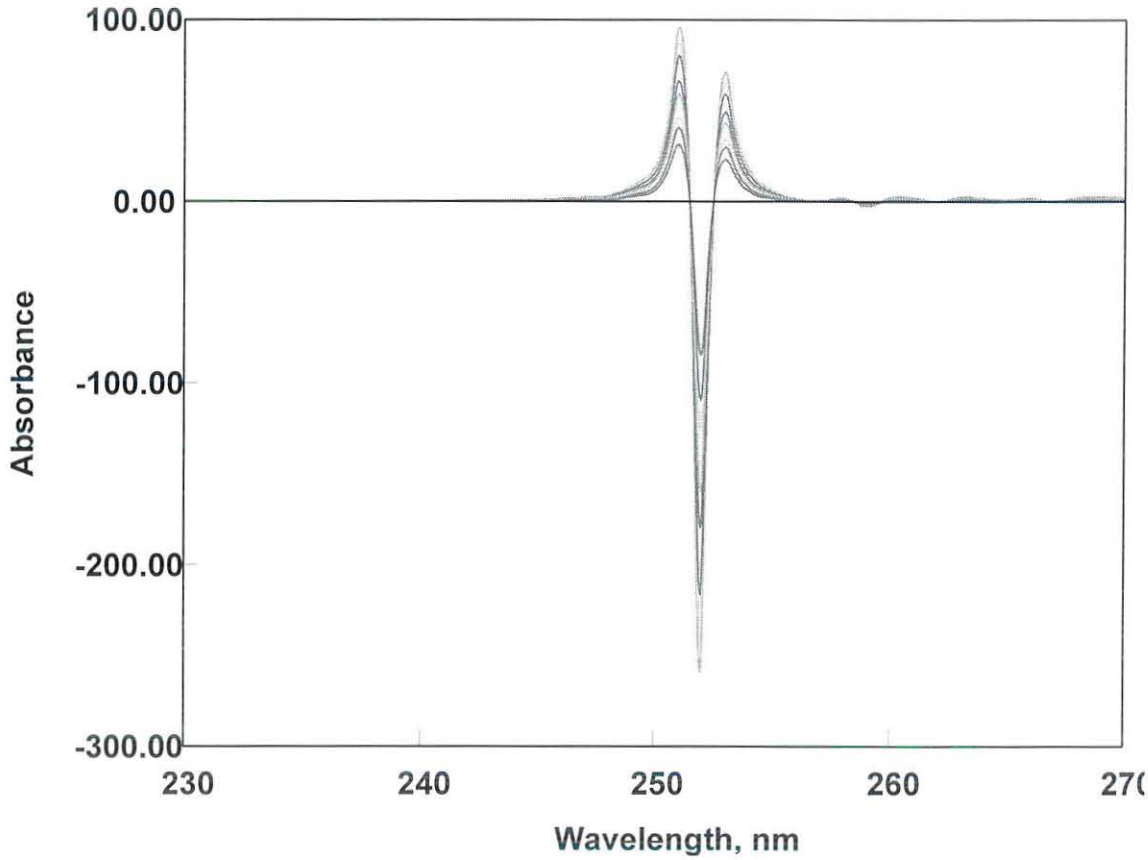

Figure 4.15: First derivative Ratio spectra of sulbactam sodium (30 – 100µg/ml), divisor is 80µg/ml of ampicillin sodium.

Similarly sulbactam sodium was also determined by dividing its spectra by that of a solution of ampicillin sodium, 80µm/ml, to get the first derivative of the ratio spectra by calculating at 2nm intervals. From the first derivative ration spectra it is seen that amplitude of ampicillin does not interfere with that of sulbactam sodium at 205nm, 222nm and 245 nm and hence the latter was easily determined at these wavelengths (Figures 4.6 and 4.7).

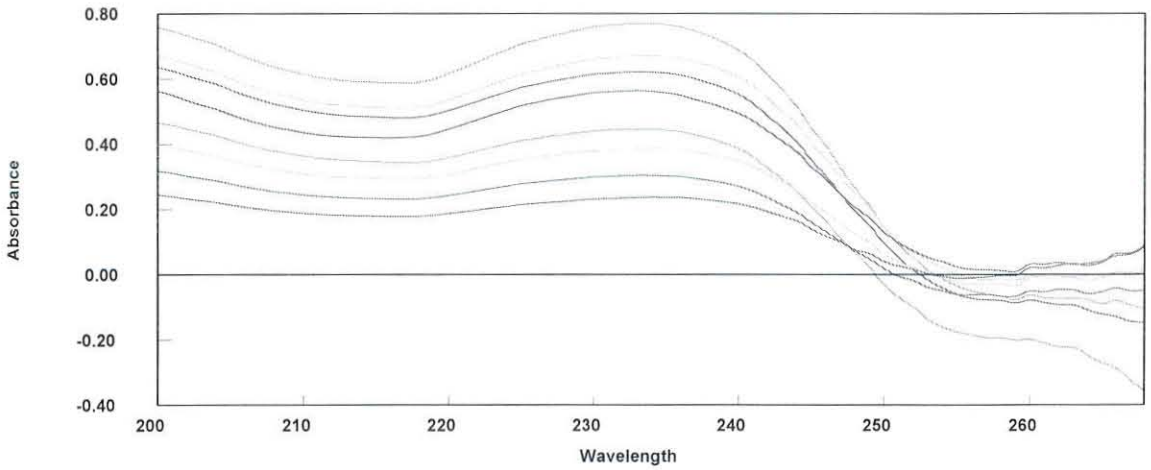

Figure 4.16: Ratio spectra of sulbactam sodium (30 – 100µg/ml), divisor is 60µg/ml of ampicillin sodium.

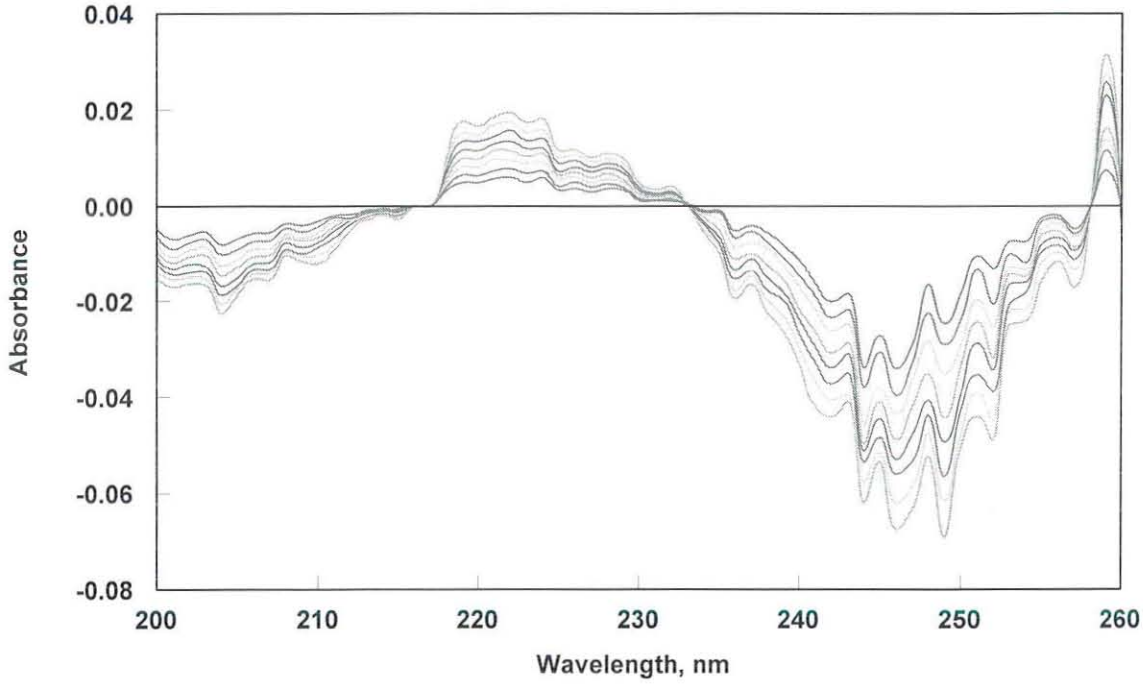

Figure 4.17: First derivative ratio spectra of sulbactam sodium (30 – 100µg/ml), divisor is 60µg/ml of ampicillin sodium (2nm intervals).

The calculated values obtained by the derivative ratio technique are illustrated in Tables 4.1 and 4.2. The proposed method was successfully applied for the determination of the two drugs in laboratory prepared mixtures and commercial dosage

form. Recoveries and relative standard deviations are given in Tables 4.3 and 4.4 and are found to excellent as can be seen from the results in the tables mentioned.

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to formula 14 & 15:

$$LOD = 3\,\sigma / S \qquad (14)$$

$$LOQ = 10\,\sigma / S \qquad (15)$$

Where σ is the standard deviation of the intercept and S is the sensitivity ,and the results are mentioned in Tables 4.1 and 4.2.

Table 4.1 Calculated values for determination of ampicillin sodium with the derivative ratio spectrophotometric technique.

Conc. range (µg/ml)	Wavelength of determination (nm)	Intercept (a)±SD	Slope (b)±SD	Correlation Coefficient (r)	Determination Coefficient (r^2)	LOD* (µg/ml)	LOQ** (µg/ml)
30 – 100	251	1.9457± 1.9261	0.9486± 0.0279	0.9974	0.9948	6.0915	20.3051
30 – 100	252	-5.2094± 4.9629	2.5517± 0.07201	0.9976	0.9952	5.8347	19.4491
30 – 100	253	1.1224 ± 1.2762	0.7034± 0.01852	0.9979	0.9959	5.4431	18.1435

* LOD Limit of detection

**LOQ Limit of quantification

Table 4.2: Calculated values for determination of sulbactam sodium with the derivative ratio spectrophotometric technique.

Conc.range (µg/ml)	Wavelength of determination (nm)	Intercept (a)±SD	Slope (b)±SD	Correlation Coefficient (r)	Determination Coefficient (r^2)	LOD* (µg/ml)	LOQ** (µg/ml)
30 – 100	205	-0.00107± .000029	-0.000197± .000005	0.9987	0.9975	4.3262	14.4207
30 - 100	222	0.00023± .000304	-0.000182± .000044	0.9982	0.9964	5.0547	16.8491
30 - 100	245	-0.00604± 0.00214	-0.00061± 0.000031	0.9923	0.9847	10.5086	35.0286

*LOD Limit of detection

**LOQ Limit of quantification

A critical evaluation of this method was performed by statistical analysis of the data, where slopes, intercepts and correlation coefficients are shown in Tables 4.1 and 4.2. Summary of the assay results for commercial preparation are also shown in Tables 4.3 and 4.4. The selectivity of the proposed method was also assayed by the analysis of synthetic mixtures, where satisfactory results were obtained over the stated calibration range.

Table 4.3: Actual and predicted amounts of ampicillin sodium given by applying first derivative ratio technique for Pure, laboratory-prepared mixtures with sulbactam sodium and commercial dosage form

		Measured at 251 nm			Measured at 252 nm			Measured at 253 nm		
	Real concentrations	Found			Found			Found		
Form	µg/ml	µg/ml	%	CV*	µg/ml	%	CV*	µg/ml	%	CV*
Pure	30	30.621	102.07	2.13	30.548	101.83	2.13	30.570	101.90	2.22
	40	40.738	101.85	1.95	40.777	101.94	2.11	40.701	101.75	2.04
	50	49.381	98.76	1.88	49.497	98.99	1.87	49.308	98.62	1.84
	60	60.420	100.70	1.03	60.350	100.58	1.85	60.053	100.08	1.23
	70	69.136	98.77	1.32	69.338	99.05	1.21	69.582	99.40	1.46
	80	80.703	100.88	1.22	80.678	100.85	1.34	80.478	100.60	1.74
	90	90.311	100.34	1.75	90.238	100.26	1.22	90.134	100.14	1.33
	100	99.386	99.38	0.96	99.371	99.37	1.45	99.569	99.57	0.98
ıthetic mixs. Amp./Sulb.										
	10/100	10.322	103.22	2.12	10.272	102.72	2.32	10.301	103.01	2.34
	20/90	20.351	101.76	1.82	20.253	101.27	1.76	20.344	101.72	2.01
	30/80	30.453	101.51	1.13	30.322	101.07	1.23	30.432	101.44	1.77
	40/70	39.866	99.67	1.22	39.911	99.78	1.45	39.807	99.52	1.39
	50/60	50.048	100.09	1.54	50.118	100.29	1.76	50.148	100.30	1.28
	60/50	59.986	99.98	1.65	60.032	100.05	1.11	59.886	99.81	1.22
	70/40	70.456	100.65	0.98	70.222	100.32	1.55	70.488	100.70	0.97
	80/30	80.855	101.07	1.11	80.462	100.60	0.92	80.612	100.76	0.98
	90/20	90.943	101.05	1.34	90.539	100.59	1.22	90.333	100.37	1.33
	100/10	100.83	100.83	0.94	100.713	100.71	1.01	100.774	100.77	0.93
sage form**	50/25	49.782	99.56	1.87	49.880	99.76	1.82	49.707	99.41	1.79

Table 4. 4: Actual and predicted amounts of sulbactam sodium given by applying first derivative ratio technique for Pure, laboratory-prepared mixtures with ampicillin sodium and commercial dosage form

	Real concentrations	Measured at 205 nm			Measured at 222 nm			Measured at 245 nm		
		Found			Found			Found		
Form	µg/ml	µg/ml	%	CV*	µg/ml	%	CV*	µg/ml	%	CV*
Pure	30	30.845	102.82	1.88	30.833	102.78	1.67	30.901	103.00	2.01
	40	40.922	102.31	1.73	40.945	102.36	1.65	40.976	102.44	2.02
	50	50.581	101.16	1.22	50.555	101.11	1.44	50.678	101.36	1.95
	60	60.887	101.48	1.54	60.763	101.27	1.59	60.977	101.63	1.78
	70	70.332	100.47	1.76	70.567	100.81	1.92	70.615	100.88	1.94
	80	81.173	101.47	1.92	81.178	101.47	1.87	81.182	101.48	1.84
	90	91/223	101.36	1.87	91.231	101.37	1.21	91.113	101.24	1.37
	100	1-1.547	101.55	1.55	101.571	101.57	1.43	102.07	102.07	1.59
Lab. prepared	10/100	10.341	103.41	1.94	10.317	103.17	1.95	10.411	104.11	2.01
Mixture	20/90	20.562	102.81	1.81	20.559	102.8	1.87	20.754	103.77	1.88
Sulb/Amp.	30/80	30.734	102.45	1.65	30.427	101.42	1.85	30.821	102.74	1.65
	40/70	40.766	101.92	1.22	40.615	101.54	1.32	40.815	102.04	1.78
	50/60	50.593	101.19	1.34	50.422	100.84	1.55	50.773	101.55	1.07
	60/50	60.765	101.28	1.98	60.472	100.79	1.81	60.821	101.37	1.93
	70/40	70.833	101.19	1.91	70.564	100.81	1.98	70.917	101.31	1.92
	80/30	80.748	100.94	1.87	80.557	100.70	1.65	80.933	101.17	1.55
	90/20	90.972	101.08	1.54	90.722	100.80	1.33	91.012	101.12	1.38
	100/10	101.476	101.48	1.39	101.211	101.21	1.28	101.733	101.73	1.46
Dosage form**	25/50	25.2195	100.88	1.83	25.161	100.64	1.75	24.3535	101.41	1.94

4.3 Multivariate calibration techniques

4.3.1 Classical Least Squares technique

This CLS method is intuitively appealing since it is based on some generally assumed relationship, e.g. Beer's law, and it can be used for moderately complex composition of the calibration mixtures, i.e. the concentration of each absorbing species.

The absorption spectra for the studied drugs showed a considerable degree of spectral overlapping (Figure 4.17). The degree of spectral overlapping can be conveniently given by $(D_i)^{0.5}$, where D_i is the magnitude of the dependency which can be calculated for a two component mixture from equation 16:

$$Di = \frac{\Sigma(k_1 k_2^t)^2}{\Sigma k_1 k_2 t \Sigma k_2 k_2^t} \qquad (16)$$

where k_1 and k_2 are the *l x n* matrices of regression coefficients for studied drugs and k^t is the transposed k matrix.

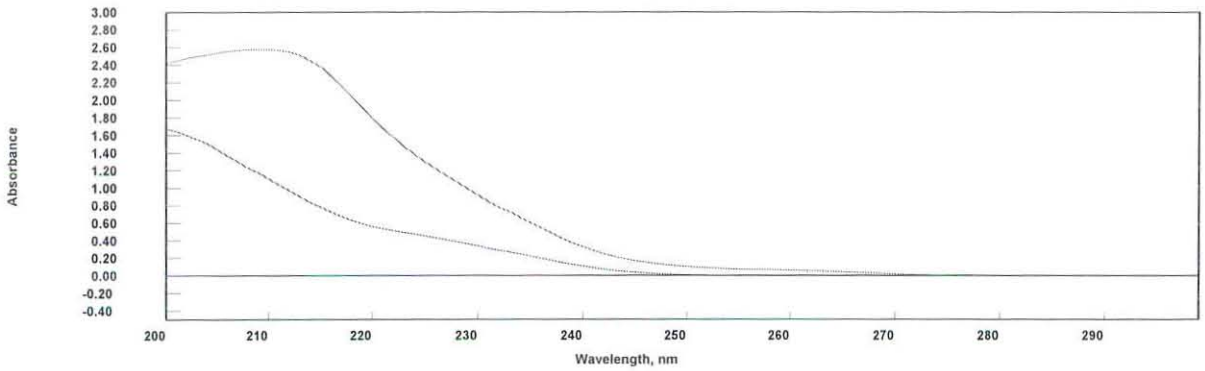

Figure 4. 18: Degree of overlapping for ampicillin sodium (the above) & sulbactam sodium, 100µg/ml, spectra in aqueous solutions in the range from 200-300nm.

The degree of overlapping for the studied drug was calculated using the calculated D_i value (0.9613) and the corresponding $D_i^{0.5}$ value is 98.05% showing more or less completely overlapping for ampicillin sodium and sulbactam sodium.

In this technique a linear relationship between sample concentration and absorbance is assumed which is given by the equation 17:

$$A=CK+E \qquad (17)$$

where A is the m x n matrix of calibration

Calibration is based on a set of *n* samples of known concentrations for which the spectra are measured. By means of the calibration sample set, estimation of coefficients is possible by solving for the matrix *K* according to the general least-squares solution, equation 18:

$$K = (C^T \cdot C)^{-1} \cdot C^T \cdot A \qquad (18)$$

Where *C* is calibration matrix, and *A* is absorbance matrix.

The analysis is then based on the spectrum *a*0 of the unknown sample by use of:

$$C_0 = a_0 K^T (KK^T)^{-1} \qquad (19)$$

where Co is the vector of sought for concentration of the unknown.

Tables 4.5 and 4.6 show the practically determined and claimed quantities with plus or minus the relative standard deviations of the drugs studied by the CLS technique. Results of the analysis ensures that there is an acceptable recovery, above 98%, conforming a good accuracy of the proposed technique and hence the technique can be used for the simultaneous determination of the two mixed drugs .Synthetic binary mixtures with different ratio were subjected to CLS analysis to confirm suitability of the technique for dosage form analysis (Tables 4.5 and 4.6)

From the results obtained we can easily understand that concentrations determined by the proposed technique are very close to the real value as the recovery values are acceptable

too and this reality is shown by results of the statistical treatment of the data obtained (Tables 4.5 and 4.6). The technique was also applied to the commercial dosage form and the result obtained was very satisfactory and therefore the technique is feasible for the determination.

Table 4.5: Actual and predicted amounts of ampicillin sodium given by applying CLS multivariate techniques for Pure, laboratory-prepared mixtures with sulbactam sodium and commercial dosage forms.

	Real concentrations	CLS Technique		
				Found
Form	µg/ml	µg/ml	%	RSE*
Pure	30	30.335	101.11	1.33
	40	39.754	99.39	1.78
	50	50.455	100.91	1.88
	60	60.176	100.29	1.21
	70	70.264	100.38	1.92
	80	80.245	100.31	1.43
	90	90.084	100.09	1.73
	100	100.306	100.31	1.87
Lab. prepared mixtures				
Amp. /Sulb.	10/100	9.885	98.85	1.28
	20/90	19.837	99.19	1.65
	30/80	30.348	101.16	1.91
	40/70	40.321	100.80	1.76
	50/60	49.444	98.89	1.33
	60/50	60.459	100.77	1.94
	70/40	70.472	100.67	1.82
	80/30	80.672	100.84	1.44
	90/20	90.768	100.85	1.54
	100/10	101.027	101.03	1.28
Dosage form(**)	50/25	49.569	99.14	1.66

* RSE is the relative standard errors calculated from the original matrix data for each concentration

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Table 4.6: Actual and predicted amounts of sulbactam sodium given by applying CLS multivariate techniques for Pure, laboratory-prepared mixtures with ampicillin sodium and commercial dosage forms.

	Real concentrations	CLS Technique		
		Found		
Form	(µg/ml)	µg/ml	%	RSE*
Pure	30	30.477	101.59	1.65
	40	39.466	98.67	1.51
	50	50.092	100.18	1.90
	60	59.655	99.42	1.92
	70	70.828	101.18	1.31
	80	81.592	101.99	1.57
	90	90.883	100.98	1.82
	100	100.514	100.51	1.73
Lab. Prepared mixture				
Sulb./Amp.	10/100	9.915	99.15	0.95
	20/90	19.755	98.78	1.12
	30/80	30.229	100.76	1.93
	40/70	40.428	101.07	1.76
	50/60	49.562	99.12	1.36
	60/50	60.327	100.55	1.91
	70/40	70.371	100.53	1.83
	80/30	80.559	100.70	1.82
	90/20	90.651	100.72	1.22
	100/10	100.911	100.91	0.88
Dosage form (**)	25/50	25.097	100.39	1.67

* RSE is the relative standard errors calculated from the original matrix data for each Concentration.

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4.3.2 Principal component regression (PCR) technique

PCR is a two step procedure, the first step is estimating the number of principal components by one or more of the following criteria, the percentage of explained variance, eigen value-one criterion, the scree-test and cross validation. The preliminary work on ampicillin sodium and sulbactam sodium indicates that a high sort of correlation is seen between principal component one (PC1) and principal component two (PC2); from about 99.95% to 100.97% of the total data variance fit by ampicillin sodium, sulbactam sodium and the mixture of ampicillin sodium and sulbactam sodium as seen in Figures 4.5 to 4.9 . They can be considered as new variables that summarize in an optimal way the variation present in the spectra.

In the second step, CLS is applied to the newly obtained latent variables. When co-linearity between original variables occurs, principal component plots often allow better interpretation of the variations observed in the data set than plots of original variables selected by CLS. As modelling method, it is less performant than CLS when performing prediction within the calibration domain and when the model is indeed linear. It is more reliable if extrapolation may be required. It is a linear method, but it is able to perform quite well for moderately nonlinear data.

The main equation for calculating the concentration can be written as:

$$C = F.\ Aproj. \quad (20)$$

Where C is the concentration components and F is the regression matrix of the new coordinates & A proj. Is the projected absorbance, thus equation 21 can be derived from equation 20:

$$\mathbf{C}.\ A_{Proj}.\ [AProj\ A^{t}_{Proj}]^{-1} = F \quad (21)$$

Then the calculated F values are used to predict the concentrations in an unknown sample from its measured spectrum using equation 22:

$$C_{un} = F.\ V^{t}_{c}\ A_{un} \text{ or } C_{un} = F_{cal}.A_{un} \quad (22)$$

Where $F_{cal} =$ the quantity $V^{t} c$ t.A pre-calculated at calibration time.

Tables 4.7 and 4.8 give the actual and expected amounts of ampicillin sodium and sulbactam sodium. From the tables it can be deduced that there is a strong correlation between concentration and response (absorbance) when the sample is analyzed by PCR in the absorption range between 200nm and 300nm. The same technique was also used to test the commercial dosage form and the results obtained are in acceptable range. Therefore this method of analysis can be effectively used for the drug mixture without undergoing any physical separation of one from the other.

Table 4.7: Actual and predicted amounts of ampicillin sodium given by applying PCR multivariate techniques for Pure, laboratory-prepared mixtures with sulbactam sodium and commercial dosage forms.

Form	Real concentrations (µg/ml)	PCR technique Found µg/ml	%	RSE*
Pure	30	30.324	101.08	1.27
	40	39.822	99.56	1.56
	50	50.539	101.08	1.94
	60	60.155	100.26	1.84
	70	70.266	100.38	1.11
	80	80.222	100.28	1.43
	90	90.019	100.02	0.96
	100	100.267	100.27	1.72
Lab. prepared mixtures amp./sulb.	10/100	9.903	99.03	1.65
	20/90	19.885	99.43	1.73
	30/80	30.265	100.88	1.11
	40/70	40.333	100.83	1.39
	50/60	49.658	99.32	1.26
	60/50	60.329	100.55	1.31
	70/40	70.411	100.59	1.32
	80/30	80.348	100.44	1.78
	90/20	90.554	100.62	1.55
	100/10	100.789	100.79	1.37
Dosage form(**)	50/25	49.672	99.34	1.22

* RSE is the relative standard errors calculated from the original matrix data for each concentration;

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Table 4.8: Actual and predicted amounts of sulbactam sodium given by applying PCR multivariate techniques for Pure, laboratory-prepared mixtures with ampicillin Sodium and commercial dosage forms.

	Real concentrations	PCR technique		
				Found
Form	µg/ml	µg/ml	%	RSE*
Pure	30	30.283	100.94	1.45
	40	39.877	99.69	1.87
	50	50.086	100.17	1.52
	60	60.034	100.06	1.94
	70	70.622	100.89	1.69
	80	80.941	101.18	1.42
	90	90.766	100.85	1.62
	100	100.359	100.36	1.85
Lab. prepared mixtures	10/100		99.37	
Sulb./Amp.		9.937		1.37
	20/90	19.826	99.13	1.56
	30/80	30.278	100.93	1.90
	40/70	40.389	100.97	1.82
	50/60	49.674	99.35	1.86
	60/50	60.412	100.69	1.11
	70/40	70.654	100.93	0.94
	80/30	80.387	100.48	0.95
	90/20	90.571	100.63	1.32
	100/10	100.762	100.76	1.84
Dosage form (**)	25/50	25.203	100.81	1.77

* RSE is the relative standard errors calculated from the original matrix data for each concentration

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4.4 Comparison of results of the proposed method with that of the official method

Tables 4.9 & 4.10 show the results of quantification of the compound drug by using D^1 ratio, PCR, CLS and the official method. From the table we can observe that the compound drug studied could be quantified by all of the three proposed techniques with acceptable limit of precision and accuracy.

A comparison of results of the proposed techniques with that of the official method revealed that there is no significant difference between them as tested by t-test and F-test for each pair since the t-theoretical (2.228) and F- theoretical (3.88) values are greater than the calculated values of each, which is a statistical requirement for a new method to be accepted.

Table 4.9: Statistical analysis of results of sulbactam ampicillins's assay in a pharmaceutical preparation that contains sulbactam sodium by the proposed methods and official method

Brand	Statistical parameter	D^1 ratio			CLS	PCR	Official method*
		251 nm	252 nm	253nm			
	X	99.56%	99.76%	99.41%	99.14%	99.34%	99.52%
	n	9	9	9	9	9	3
Aurobennz	C.V	1.87	1.82	1.79	---------	---------	-------
	RSD	------	-------	------	1.66	1.22	1.86
	t	0.03	0.19	0.09	0.18	0.13	-----
	f	1.01	1.04	1.07	1.25	2.32	-------

*United states Pharmacopoeia 2007

Theoretical values at 95% confidence limit are: t = 2.228 and F = 3.88 (n_1 = 3and n_2 = 9); X= mean; n = number of observations; RSD= relative standard deviation; C.V =coefficient of variation.

Table 4.10: Statistical analysis results of sulbactam sodium's assay obtained in a pharmaceutical preparation that contains ampicillin sodium by the proposed methods and official method

Brand	Statistical parameter	D^1 ratio					Official method*
		205 nm	222nm	245nm	CLS	PCR	
	X	100.88%	100.64%	100.41%	100.39%	100.81%	100.28%
	n	9	9	9	9	9	3
Aurobennz	C.V	1.83	1.75	1.94	---------	---------	-------
	RSD	------	-------	------	1.67	1.77	1.65
	t	0.68	0.42	0.22	0.19	0.91	-----
	f	1.23	1.13	1.07	1.38	1.15	-------

*United states Pharmacopoeia 2007

Theoretical values at 95% confidence limit are: t = 2.228 and F = 3.88 (n_1 = 3and n_2 = 9); X= mean; n = number of observations; RSD= relative standard deviation; C.V =coefficient of variation.

5. CONCLUSION

Simultaneous determination of combined drugs with overlapping uv-vis spectra has been a serious problem in pharmaceutical quality control laboratories and research institutes since the normal spectrophotometric technique does not resolute them. As components absorb light at the same fixed wavelength it is difficult to know the exact amount of each component in the compound in the presence of the other.

Therefore, for routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with due accuracy and precision with out undergoing any physical separation. Spectrophotometric techniques can generate large amounts of data within a short period of analysis time; however, when coupled with chemometrics tools, the quality of the spectral information can be markedly increased, converting this combined technique into a powerful and highly convenient analytical tool.

Simultaneous determination of ampicillin sodium-sulbactam sodium in a mixture parenteral preparation was attempted using chemometrics-assisted spectrophotometric techniques. The absorption spectra of both drugs showed high degree of overlapping in the range of working wavelengths (200nm to 300nm). The derivative method was unable to quantify the drugs since the UV spectra of the two drugs could not be resolved from each other by this method because the degree of overlapping is so high (about 98%). But The D^1 ratio and multivariate (CLS and PCR) methods enabled the simultaneous quantization of ampicillin sodium and s ulbactam sodium binary mixture. T hey were developed for the analysis of the laboratory prepared mixtures and pharmaceutical dosage forms. The acceptable recoveries obtained in all cases as well as the reliable agreement with the official procedures proved that, the proposed methods could be applied efficiently for determination of ampicillin sodium and sulbactam sodium binary mixture with quite excellent precision and could be easily used in a quality control laboratory for their analysis.

Comparing the results of the three proposed methods it can be concluded that results of the D^1 ratio method are very good in the determination and that of CLS & PCR are superior over the D^1 ratio technique, specially the PCR's. The PCR is the best method for the studied drugs because in this case there is no averaging effect making model less susceptible to spectral noise. The statistical treatments done to compare results of the new method with that of the official, HPLC, method also conformed that the newly developed method can be equivalently used for the simultaneous determination of ampicillin sodium and sulbactam sodium in a binary mixture.

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DECLARATION

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted for examination with approval as a University Advisor.

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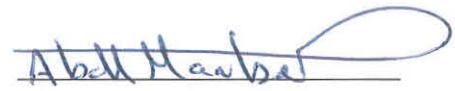
Prof. Dr. Abdel Maaboud I.M. Attaya	
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