Flow Injection Spectrophotometric Assay of Tetracyclines in Drug Formulations

by

Sulieman Fakhreldin Osman

A Thesis Presented to the

FACULTY OF THE COLLEGE OF GRADUATE STUDIES

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In Partial Fulfillment of the
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This thesis, written by SULIEMAN FAKHRELDIN OSMAN under the direction of his Thesis Advisor and approved by his Thesis Committee, has been presented to and accepted by the Dean of the College of Graduate studies, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in CHEMISTRY.

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to my parents

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THESIS ABSTRACT

NAME OF STUDENT: SULIEMAN FAKHRELDIN OSMAN

TITLE OF STUDY : FLOW INJECTION SPECTROPHOTOMETRIC ASSAY OF TETRACYCLINES IN DRUG FORMULATIONS

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A flow injection spectrophotometric method was successfully applied to the determination of tetracyclineHCl, oxytetracycline, chlorotetracycline, and demeclocycline. Both, the univarient and the simplex method of optimization were adopted for the selection of the proper experimental conditions, that is, the chemical and flow injection parameters.

In the method 157 μl sample was injected, for all compounds, into the carrier stream of iron(III) solution of concentration 554, 626, 701, and 447 ppm flowing at rates of 3.72, 3.72, 4.37, and 3.72 ml/min, thus passing through reaction coils of a lengths of 55, 45, 85, and 45 cm respectively. 0.001M Sulphuric acid was used as an overall reaction medium. These experimental conditions were attained by the modified simplex method and were considered for the determination of the above compounds.

Very high sampling frequency of the order of at least 170 samples per hour was attained for all compounds. High precision with a relative standard deviation of less than 0.9 was obtained. The accuracy was found to be high when the results were compared to those obtained by the conventional spectrophotometric method. The present method was applied to the determination of tetracycline or its derivatives in propietry drugs in both capsules and injection formulation, and interferences from exciepients was found to be insignificant.

MASTER OF SCIENCE DEGREE

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خلاصة الرسالة

اسم الطالب الكامل : فخر الدين عثمان سليمان

عنوان الدراسة : التحديد الكمى المطياقى للتتراسايكلين فى المستحضرات الدوائية بواسطة الحقن الانسيابى .

التخصص : كيمياء تحليلية

تاريخ الشهادة : يناير ١٩٩٢

طبقت طريقة الحقن الانسيابى المطياقى بنجاح تام لتحديد تركيز التراسايكلين هايدروكلوريد ، الأكسى تتراسايكلين ، التلوروتتراسايكلين والديوسايكلين . وقد استخدمت كل من الطريقتين الاحصائيتين وهما طريقة الإفراد المعدلة وطريقة الإبدال الموحدة للوصول لانسب المتغيرات وأحسن الظروف العملية الكيميائية منها والنظامية .

وفى هذه الطريقة أمكن حقن ١٥٧ مايكروليتر من الدواء فى جميع الحالات ولكل المركبات الدوائية فى تيار الحمل والسابق تحضيره من مركبات الحديد الثلاثى الذى تركيزه ٥٥٤ جزء من المليون للتراسايكلين ، ٦٢٦ للاكس تتراسايكلين و ٧٠١ للكلور تتراسايكلين و ٤٤٧ للديوسايكلين وذلك بمعدل إنسيابى مقداره ٣ر٧٢ ، ٣ر٧٢ ، ٤ر٣٧ و ٣ر٧٢ مل/دقيقة للمركبات المذكورة على التوالى حيث يمر تيار الحمل مخلوطاً بالمركب على أنبوب للتفاعل بطول ٥٥ ، ٤٥ ، ٨٥ ، ٤٥ سم للمركبات نفسها على التوالى . كان تركيز حمض الكبريتيك عبارة عن ٠ر٠١ مولار لكل المركبات . لقد تم التوصل لكل المتغيرات أعلاه بواسطة طريقة الافراد المعدلة حيث استخدمت فى طريقة التحديد الكمى .

لقد تم التوصل أيضاً الى نسبة عالية جداً من معدل عدد العينات التى يمكن تحليلها بواسطة هذه الطريقة حيث لايقل المعدل عن ١٧٠ عينة فى الساعة الواحدة وذلك لكل المركبات التى درست . كما ان الدقة كانت متناهية ونسبة معدل الانحراف وصلت الى أقل من ٩ر٠ . أما مدى صحة القياسات فكان كبيراً جداً عند مقارنة الطريقة بالطريقة المطياقية السابقة .

تم تطبيق الطريقة المذكورة للتحديد الكمى للتتراسايكلين ومشتقاته فى بعض المركبات الدوائية فى هيئتها الحقنية والكبسولات ولم تسجل تداخلات تذكر من المكونات الدوائية المساعدة .

درجة الماجستير في العلوم

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CHAPTER ONE

CHAPTER ONE

TETRACYCLINES

1.1. Introduction

Tetracyclines and derivatives form an important class of antibiotics, and are extensively used in current therapy due to their broad spectrum of antibacterial activity. Tetracyclines comprise a family of biosynthetic compounds and semisynthetic derivatives, all characterized by the broad antimicrobial spectrum. This spectrum includes gram-positive and gram negative bacteria, some protozoa, actinomycetes and some other microbial classes.

Tetracycline hydrochloride, oxytetracycline, chlorotetracycline, and demeclocycline have been recently[1] assayed spectrophotometrically through complexation with iron(III) in sulphuric acid media and subsequent measurement of the soluble brown complex formed, at the corresponding wavelength. The introduction of flow injection analysis (FIA) and the present direction to utilize this technique for continuous analysis, led the investigators to adopt it for effective and full advantages, towards highest sensitivity and maximization of response along with the maximum sample frequency and economy of the sample and reagents.

Two FIA methods for the assay of tetracyclines have been reported, one[2] with a special and complicated amperometric detection and the other[3] with a chemiluminescence detector suitable for the determination of tetracycline only in low detection limits and high concentrations. Both methods require the use of a difficult buffering procedure and suffer low throughput. In the British pharmacopiea (BP) tetracyclines were assayed biologically, this method is tedious and time consuming, requiring long incubation periods[4]

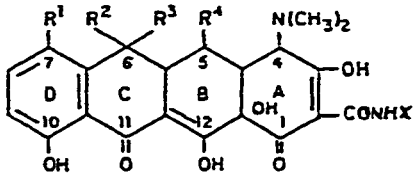
1.2 Tetracyclines

The term tetracyclines is commonly accepted as including eight antibiotics, namely, chlorotetracycline, oxytetracycline, tetracycline, demeclocycline, rolitetracycline, methacycline, doxycycline and minocycline. Tetracyclines and some derivatives are presented in Table 1.1. The first four compounds are all produced by appropriate microorganisms when there is a suitable substrate. The last four compounds are strictly semisynthetic and are not known in nature. Indeed, several hundred semisynthetic tetracyclines have been prepared by selective removal of various substituents with or without replacement by other functional groups.

Chlorotetracycline, more specifically, 7-chlorotetracycline, was the first discovered tetracycline in 1948 by Duggar[5] and Co-workers, and

TABLE 1.1: STRUCTURES AND TRADE NAMES OF TETRACYCLINE ANTIBIOTICS

General Structure:



Drug	R1	R2	R3	R4	Year First Reported	Representative Trade Names
Chlortetracycline (7-chlor-tetracycline)	Cl	CH_3	OH	H	1948	Aureomycin (Lederle)
Oxytetracycline (5-hydroxy-tetracycline)	H	CH_3	OH	OH	1950	Terramycin (Pfizer)
Tetracycline	H	CH_3	OH	H	1953	Achromycin (Lederle) Panmycin (Upjohn) Steclin (Squibb) Sumycin (Squibb) Tetracyn (Roerig) Tetrex (Bristol)*
Demeclocycline (6-demethyl-7-chlortetracycline)	Cl	H	OH	H	1957	Declomycin (Lederle)

was separated from the metabolic products of the actinomycet *Streptomycesaureofaciens*. The second member of the family, oxytetracycline, which is produced by *Streptomyces rimosus*, was first reported in 1950 by Finlay et al[6] The third member of this group, tetracycline, was prepared by Boothe et al[7] and Conver et al[8] by replacing the halogen by hydrogen with a simple catalytic hydrogenation process. Also tetracyclines isolation from the culture filtrates of a streptomyces species has been reported[9]

1.3 Chemistry of Tetracyclines

All of the tetracyclines have a common skeleton which can be considered an octahydro analogue of napthacene, but they differ in the specific substituents, as indicated in Table 1.1[10] and these have significant effects on the solubility of the compounds and on their biological properties.

All tetracyclines are amphoteric, capable of forming salts with both acids and bases. Salts with acids are generally more stable than those formed with bases. Salts of tetracycline with acids are preferred for most medical and pharmaceutical uses because they are more soluble in water than the tetracyclines.

All tetracyclines form stable chelates with many metals. Albert and

Reese[11] suggested that the antimicrobial action of tetracyclines is primarily due to the ability of these drugs to remove metal ions essential for microorganism activity by chelation. Also it has been reported[12] that tetracyclines act principally by interferences with protein synthesis or by disturbance of the metabolic pathways in the cells.

Tetracyclines inhibit the formation of adenosine triphosphate (A.T.P.) which is a primary source of energy for cellular function by chelation with magnesium ion which is essential to form A.T.P. and this was prevented by the addition of excess magnesium ion[13]. Hamner[14] reported that zinc ions pontentiate the antimicrobial activity of dimethlchlorotetracycline hydrochloride whereas the reverse effect was noticed in the presence of nickel, magnesium, copper and aluminium ions.

The stability of metal-tetracycline complexes has been extensively studied by many authors, and was recently reviewed[15]. The most stable metal-tetracycline complexes listed in the order of decreasing stability are: Fe(III), Al(III), Cu(II), Ni(III), Fe(II), Co(II), Zn(II), and Mn(II). The affinity of various tetracycline derivatives to cations is dependent on the nature of both the tetracycline derivative and the cation.

1.4 Sites of Complexation in Tetracyclines

The tetracycline structure contains numerous sites at which chelation with metallic cations might occur[16 19]. The most important of these being in the lower tier of the molecule which contains two, 1,3-diketones with two of the ketones in the enol form[20 22]. Such monoenols in 1,3- diketones chelate with metallic ions very readily to form six-membered rings. In each ring, the two atoms that bind the metallic ion are oxygen atoms[22,23]. Hochestein et al[24] assumed that the formation of a hydrogen bond structure in tetracycline from the keto-enol system of tricarbonyl-methane group which they believed to be involved in chelation. However this assumption is not possible for the high acidity of tetracyclines. Sakaguch et al[17]. studied the process of chelate formation of tetracyclines with metal ions by following the shift of the maximum absorbance of the chelates with the pH-drop. They concluded that chelation must involve the 10-12 enol system, in the case of thorium and other metal ions. However, zirconium ion involves the two positions, (in 11-12 and 10-11 positions) assuming that ring (A) and (B) (table 1.1) to be almost in the same plane. Further experiments by the same authors on anhydrochlorotetracycline chelates substantiated these conclusions.

1.5. Alkaline and Acid Degradation of Tetracyclines

Alkaline hydrolysis results in rupture of ring (C), (general structure in Table 1.1) between Carbons 11 and 11a[25,26], acid hydrolysis effects cleavage through rings (A) and (B). All tetracyclines are susceptible to these process, at different extents. Demeclocycline, which is the most stable of the natural tetracyclines, is 200-times more stable than chlorotetracycline which is the least stable one. The chlorine on ring (D) in chlorotetracycline is assumed to have an important role in determining instability of the compound[13] since tetracycline which is 20 times more stable differs from the former in lacking the chlorine atom. However it is clear that the chlorine atom alone is not responsible for the instability because demeclocycline, which has chlorine atom in the same location but lacks the CH_3 group at carbon (6), is much more resistant to degradation by both acid and alkali. Oxytetracycline hydrochloride is stable in dilute acidic solution. Regna and Solomons[27] reported that dilute solutions are stable for a similar period through the pH range 1.0-9.0 when stored at 5^0 C. At higher temperatures, however, stability of oxytetracycline solutions is pH dependent.

1.6.Quantitative Determination of Tetracyclines :

Spectrophotometric methods based on complexation of tetracycline with transition metal ions have been reviewed[15] recently. Spectrophotometric methods using different oxidative reagents have been reported[29-36] Reagents used for this purpose include sodium hydroxide, ferric chloride, thorium nitrate, ammonium vanadate or molybdate, aminophrine, sodium tungestate, alkaline cu(II), cerium acetate, sodium cobaltinitrite, and sodium vanadate.

Many of these methods suffer from many drawbacks such as narrow Beer's Law range, instability of colour, critical reaction and heating times, low sensitivity, multiple reaction steps and multiple pH adjustments. Some of them are suitable for the assay of some derivatives.

Titrimetric[37,38] and chromatographic methods[39-42] have been reported for the determination of tetracyclines. Liquid chromatographic methods for identification of tetracyclines were reviewed[43]. Reversed-phase chromatography was explored for the determination of chlorotetracycline in capsules, ointments, and aerosols[44] A collaborative study was conducted to evaluate the applicability of a recent method using polystirine-divinyl benzene stationary phases for the determination of

tetracyclines[45] also another method which has utilized a polymeric support for the determination of tetracyclines[46] has been reported. Many HPLC methods were reported, for determination of tetracyclines in honey, serum, tissue, and pharmaceutical preparations[47 52]

Tetracycline hydrochloride was determined by a polarographic method[53] in the presence of anhydrotetracycline. A potentiometric method[54] using ammonia electrode, and another method[55] utilizing a tetracycline plastic membrane ion selective electrode, were used for the determination of tetracyclines.

Two FIA methods have been reported, for the determination of tetracyclines, in one of them amperometric detection[2] was used whereas in the other one[3] chemiluminescence detection was used. Oxytetracycline was determined by FIA with a spectrophotometric method[56]

1.7 Spectroscopy

Spectroscopy comprises separation, detection, and recording of energy changes due to emission, or absorption of electromagnetic radiation. The experimental applications of spectroscopic methods in chemical problems are diverse, but all have in common the interaction of electro-

magnetic radiation with the quantized energy states of matter. Electromagnetic radiation consists of discrete particles called photons which have definite energies and travel through space with the velocity of light.

As a requirement for absorption to occur, the energy of the radiation must match the energy difference between the quantized energy levels that correspond to different states of the molecule. If the energy difference between two of these states is represented by E, the wavelength of the radiation, λ, necessary for matching is given by the equation

$$E = \frac{hc}{\lambda} \qquad (1.1)$$

where h is Plank's constant, 6.623×10^{-27} erg sec/molecule and c is the speed of light in cm sec^{-1}, giving E in units of erg/molecule. In order for matter to absorb the electric field components of radiation, other requirements in addition to energy matching must be met[57]. The energy transition in the molecule must be accompanied by a change in the electrical center of the molecule in order that electrical work can be done on the molecule by the electromagnetic radiation field. Only if this condition is satisfied can absorption occur.

In atomic spectroscopy, electrons are excited to higher energy states by thermal or electrical energy, and the energy absorbed or emitted is measured. Atomic spectra were often examined as emission spectra in the UV and visible region. In molecular spectroscopy, absorption of energy is usually measured. Molecular spectra in the UV and visible region appears as bands, the width of which in liquid samples is due to their unresolved vibrational structure. The appearance of these vibrational structure of the bands can be explained in terms of Frank-Condon principle[58]

1.7.1.Quantitative Analysis by molecular absorption in UV-visible Region

Quantitative analysis depends on the relationship between the amount of light absorbed by certain system and the concentration of the absorbing species, which is expressed by the Beer-Lambert law[59-60]

$$A = \log \frac{I_o}{I} = \varepsilon bc \qquad (1.2)$$

where A is the absorbance, Io is the intensity of the incident radiation. I is the intensity of the transmitted radiation, ε is the molar absorptivity at a given wavelength and temperature, C is the concentration in mole l^{-1}

The Beer-Lambert's Law is usually valid for dilute analyte solutions, for strongly monochromatic, parallel and coherent radiation and in optically homogeneous media. Its validity is demonstrated by strictly linear plots of A vs cell path length 'b' or concentration of absorbing species in solution[61]. On the other hand, deviations from the direct proportionality between the measured absorbance, and concentration where b is constant are frequently encountered. Some of these deviations are fundamental and represent real limitations of the Law for example highly concentrated solutions and dependence of ε upon the refractive index[62]. Other deviations occur as a consequence of the way in which the absorbance measurements are made or as a result of chemical changes associated with concentration changes; these are called, respectively, instrumental deviations[63,64] and chemical deviations[65]

Determination of an unknown analyte concentration is carried out with the aid of a particularly spectrophotometric procedure under optimized conditions, and a suitable treatment of the absorbance data at selected wavelengths. It is assumed that Beer's Law is obeyed and that the measurements are of sufficient precision and accuracy. Appropriate calibration plots are prepared by using standard solutions. The optimum concentration interval of the analyte corresponds to the strictly

linear part of the plot of absorbance vs analyte concentration for which Beer's Law is strictly obeyed and measurement precision is acceptable. The calibration plots are prepared from a sufficient number of standard solutions or aliquots of different concentrations under optimum conditions.

1.7.2. Interferences

Accompanying ions, interfering species, masking agents, or various matrix components may introduce bias into absorbance measurements of analyte solution or into calibration graphs. Constant or proportional bias may be observed in the analyte calibration plot if various levels of interfering species are present. The constant bias shifts the plot along the Y- coordinates; the multiplicative influence of the interfering species, which is proportional to the analyte concentration, changes the slope of the calibration plot simultaneously. Parallel shifts in the analyte calibration plot with increasing concentration of interfering species are observed if the reagent binds both the analyte and interferent quantitatively. On the contrary, the intercept and the slope are dependent on the concentrations of the interfering species and analyte if the reagent is displaced from the complex by the interferent[66]. If complexation is the basis of formation of absorbing species of the ana-

lyte and interferent, which is often the case, the interference effect at a particular wavelength depends on the values of molar absorption coefficient of both complexes, their stability constants and the concentrations of the analyte and interfering species[67]

A well considered and tested masking of the interfering species may decrease or remove interferences, but there are many problems in practice. Analytical masking is often a compromise because even the calibration plot of the analyte is influenced to some degree by the presence of the masking agent and the concentration of the latter must be constant at a tested level. On the other hand influence of the sample matrix can be eliminated to a large extent by using the standard addition method[66].

1.7.3 Complexation and Spectrophotometry

As far as the spectrophotometric measurement of complexes are concerned, the first spectrophotometric requirement is the fast and quantitative formation of a single, stable, soluble, and highly absorbing complex under the experimental conditions. The second requirement is a sufficient colour contrast between the complex formed and the reagent form under the selected conditions.

Various approaches may be used to establish the optimum conditions for a spectrophotometric procedure with respect to the maximum sensitivity, precision, and accuracy. The true reaction equilibria and analyte-reagent speciation in solution are first evaluated under a wide range of experimental conditions. The number, stoichiometry and distribution of analyte species together with their thermodynamic, kinetic and spectral parameters are determined and simultaneous competition equilibria are tested. The optimum interval of the complex may be selected by means of experimental uni-or multivariant optimization procedures based on factorial design or simplex procedure[68,69] In such a case, the equilibria stoichiometry and other parameters of analyte species may be unknown.

1.7.4. Spectrophotometers

A spectrophotometer is an instrument that will resolve polychromatic light into different wavelengths[7,8]

The main components of a spectrophotometer are:

(1) A source of continuous radiation over the wavelength of interest. For the visible region a tungsten filament incandescent lamp is used, whereas for the UV region a low pressure hydrogen or deuterium dischargee tube is genera tube is generally used.

(2) A monochromator for selecting a narrow band of wavelength from the source spectrum. The components of a monochromator include; (a) an entrance slit which admits polychromatic radiation from the source; b) a collimating dev (b) a collimating device, either lense or a mirror; (c) a dispersion device, either a prism or grating

(3) A sample container; quartz or fused silica cells or cuvetts are used in ultra violet region; ordinary glass is used in the visible region. Solution cells have typical path lengths of 1-10 cm, whereas microcells used in flow techniques could have path lengths of 1 mm.

(4) A detection device; photoelectric detectors which include phototubes and phtovoltaic cells are usually employed.

1.7.4.1. Single and double beam spectrophotometers

Two basic instrument designs are employed in commercial spectrophotometers. One design uses only a single beam whereas the other provides a double beam.

In single beam spectrophotometers[70,71] a beam of radiation from the source enters a prism or a grating. As the dispersing element is rotated, the various resolved bands of radiation are focused at the entrance slit. The radiation then passes through the cells and on to a detector. The instrument is then calibrated (0% and 100%T), using a reagent blank.

A double-beam instrument employs some type of beam splitter prior to the sample cells. One beam is directed through the "blank" cell (or reference cell) and the other beam through the sample cell. The two beams are then compared either continuously or alternately many times a second. Thus, in the double-beam design, fluctuations in the source intensity, the detector response and amplifier gain are compensated for by observing the ratio signal between "blank" and sample. Therefore,

double beam instruments are more sophisticated electronically and mechanically than the single-beam designs and consequently are more expensive.

CHAPTER TWO

CHAPTER TWO

FLOW INJECTION ANALYSIS (FIA)

2.1. Introduction :

Flow injection analysis (FIA) was introduced in 1975 by Ruzicka and Hansen[72] FIA is based on the injection of a liquid sample into a moving non-segmented continuous stream of a suitable liquid. The injected sample forms a zone, which is then transported towards a detector that continuously records the absorbance, electrode potential, or other physical parameter as it continuously changes due to the passage of the sample material through the flow cell[72,73].

Since its introduction, the versatility of FIA has allowed the method to be adapted to different detectors and techniques using numerous configurations. Therefore, optimization and design of the flow channels to achieve maximum sampling frequency, best sample and reagent economies and proper exploitation of chemistries involved are possible only through a better understanding of the complex, physical and chemical process taking place during the movement of the fluids through the FIA channels.

In addition to the simple assay described previously, FIA systems may be designed to dilute or to preconcentrate the the analyte; to perform separations based on solvent extraction, ion exchange ,gas diffusion, or dialysis; to prepare unstable reagents in situ; and to dilute the reagents to the concentrations suitable for a given assay. Moreover, since FIA is being used increasingly for such diverse tasks as process monitoring and as a new information gathering tool in an industrial, agricultural, pharmaceutical, research, or clinical laboratories, there is a need to design parameters that would allow scaling the FIA system.

2.2. Principles of FIA

The simplest flow injection analyzer consists of a pump, which is used to propel the carrier stream through a narrow tube; an injection port by means of which a well defined sample solution is injected in the carrier stream in a reproducible manner; and a micro reactor in which the sample zone disperses and reacts with the components of the carrier stream, forming a species that is sensed by a flow-through detector, and recorded. A typical recorder output has the form of a peak, the height (H), width (W), or area of which is related to the concentration

of the analyte. The time span between the sample injection (S) and the peak maximum, which yields the analytical readout as the peak height, is the residence time (T) during which the chemical reaction takes place. A well designed FIA system has an extremely rapid response, because(T) is in the range of 5-20 seconds. Therefore, a sampling cycle is less than 30 seconds, and thus, typically two samples can be analyzed per minute. The injected sample volumes may be between 1 and 200 μl, which in turn requires no more than 0.5 ml of reagent per sampling cycle. This makes FIA a simple, automated, microchemical technique capable of having a high sampling rate and a minimum sample and reagent consumption.

It follows from the previous discussion that FIA is based on a combination of three principles : Sample injection, controlled dispersion of the injected sample zone, and reproducible timing of its movements from the injection point towards and into the detector. Thus the concept of dispersion, controlled within space and time, is a central issue of FIA.

Peak height and peak area are directly proportional to the concentration of the analyte, that is,

$$H = kC \tag{2.1}$$

or

$$A = \kappa C \tag{2.2}$$

Peak height(H) is the most frequently measured peak dimension, since it is easily identified and directly related to the detector response. Peak area readout suffers from two drawbacks, which are due to its integral character. Peak area A, cannot be related to spectra or concentration gradients, and it grossly distorts the readout of log(c) detectors, since that portion of response which is close to the baseline disproportionally weighs much more than portions of readout close to the peak apex.

Peak width, being proportional to the logarithm of the concentration, has a wide dynamic range but it is less precise than the peak height or area measurement. Being horizontal, it cannot be related directly to spectra, but it yields the readout as a time difference (W or Δt) between the rising and falling edges of the peak.

2.3. dispersion and flow injection design

The flow injection analysis response curve is a result of two processes, both kinetic in nature; the physical process of dispersion of the sample zone within the carrier stream and the chemical process of formation of a chemical species. These two processes occur simultaneously, and yield, together with the dynamic properties of the detector the FIA response.

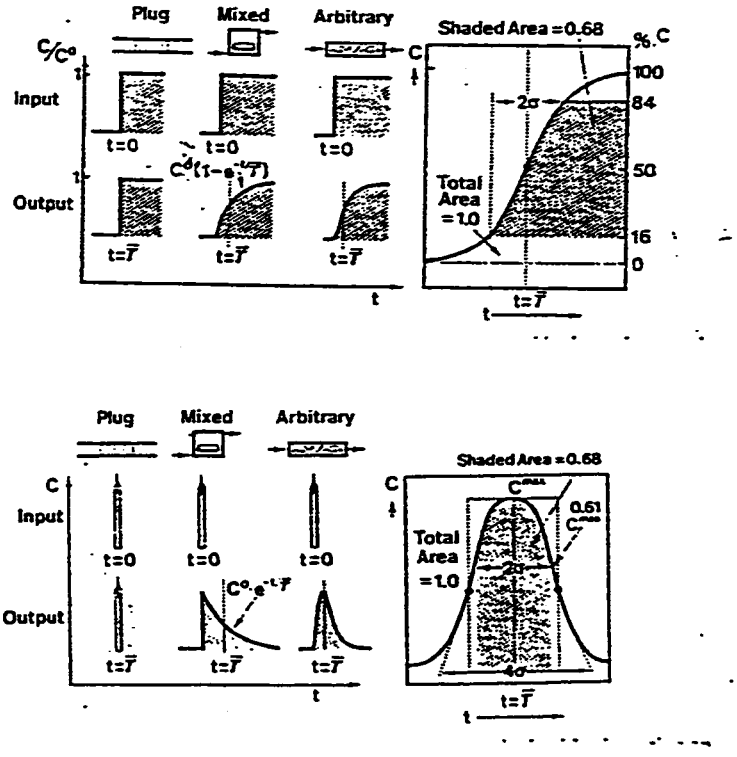


Fig. 2.1 Formalized plot of the observed concentration C versus time (a) F-curves for plug, mixed, and arbitrary flow. (b) C-curves for plug, mixed and arbitrary flow[73]

The physical process of material dispersion is due to the hydrodynamic process taking place in the flow through system, and is therefore conveniently investigated by the stimulus technique which is based on introduction of a tracer into a flowing stream and measurement of the dispersion of the tracer as caused by the transport process throughout the system. The tracer can be added as a step or as a pulse signal, which can be quantitatively measured at the exit of the vessel. In a vessel with stabilized flow two idealized patterns can be visualized (Fig.2.1) as plug flow and mixed flow, representing two extremes . In reality, neither of these extreme types exists, and the resulting arbitrary flow contains constitutions of both of these extreme types, in different proportions.

The so called F-curves[73] (Fig.2.1a) is measured by imposing a step impulse of a dye of concentration C_0 on the fluid stream entering the vessel, with the result that, with a certain delay, an increase of colour intensity is observed at the vessel output. For plug type of flow the output curve is the same as the input curve, since no mixing occur. The mixed flow representing the other extreme, where instantaneous, homogeneous mixing is taking place within a single stage, will yield a purely exponential concentration gradient, showing a gradual increase of colour intensity. In reality, an arbitrary flow will be observed,

resulting in an S-shaped curve, which will become progressively more symmetrical with an increase in the number of mixing stages through which the sample plug has passed.

Alternatively the signal can be injected as a pulse (width =0,while its area =1), the broadening of which is observed when, with a certain delay, the dyed zone reach the vessel output. This is represented by the C-curve (Fig.2.1b).

Also the tank-in-series model[73] has been used for the theoretical description of fluid behaviour in different types of FIA reactors . The concept highlights the influence of radial mixing on the physical and chemical processes and the importance of the uniform flow geometry . This model is based on the view that the liquid flows through a series of ideally stirred tanks of equal size or through a network of parallel ideally stirred tanks. The parameters of this model are (N), the number of mixin stages (tanks) through which an element of fluid has passed, and (T_i) the mean residence time of the element of fluid in one mixing stage. For one tank the curve has the form :

$$C = \frac{1}{T_i} e^{(-t/T_i)} \qquad (2.3)$$

while the system with any number of serial stages is described by the statistical (G) function written as a C-curve :

$$C = \frac{1}{T_i} (t/T_i)^{N-1} \frac{1}{(N-1)!} e^{-t/T_i} \qquad (2.4)$$

for large (N) the curves approach a Gaussian shape. An important feature of this model is that it formally covers the transition from mixed flow to plug flow, and therefore, the C-curves (Fig.2.1a) resemble the FIA curves. The extreme cases of this model, that is, one mixing stage, curve(N=1) and the multistage system (N>10)are very useful for describing and designing FIA manifolds[74-76]

It has been shown[72,77] that a larger number of uniform mixing stages connected in series yields symmetrical residence time distribution (RTD) curves, with standard deviations that decrease with increasing (N) relative to the path traveled. This is described by relating the height of an imaginary mixing stage (Hv) to the length (or volume Vr) of the path traveled.

$$H_v = \frac{V_r}{N} \quad or \quad H_v = \frac{T}{N} \qquad (2.5)$$

The important feature of this approach is that it allows estimation of the intensity of radial mixing from the mixing length (H), or more suitably from the radial mass transfer constant a which is the reciprocal value of the mean residence time of the individual tank, T_i.

$$a = \frac{1}{T_i} = \frac{N}{T} \qquad (2.6)$$

and the higher the a value is the more efficient the radial mixing is in a given component of the system.

Therefore the FIA component (e.g. reactor with the highest (a) and (N) will yield the highest σ / T ratio, and maximum sampling frequency for a given residence time . This concept is in harmony with the concept of dispersion factor ($\beta_{1/2}$), which has been defined[78] as:

$$\beta_{1/2} = \frac{t_{1/2}}{T} = \sigma\{\frac{\pi}{2}\}^{1/2} \qquad (2.7)$$

and which when lowerd, yields an optimized FIA channel in terms of sample, reagent, and time economies.

In a straight tube the dispersion of a sample plug is the result of the redistribution of material through countless repositioning of the element of fluid in axial and radial directions caused by the twin process

of convection and diffusion . The resulting concentration of the solute within the carrier stream is the result of the relative contribution of these two effects on the original homogeneous cylindrical sample plug. The extent of radial mixing primarily depends on the type of flow, that is, turbulent or laminar. The laminar flow prevails at Reynolds number (Re) lower than 2100.

$$Re = 4\rho \frac{Q}{\pi} 2 R \nu \qquad (2.8)$$

where (ρ) is the density (gm/ml), (R) is the channel radius, (Q) is the volumetric flow rate , (v) is the viscosity in poise .

In FIA the desired features is to increase the radial mixing while reducing the extent of axial dispersion, when the sample is injected into a laminar flow of carrier stream. The radial dispersion can be promoted by introducing turbulent flow or secondary flow, by diffusion alone or by a combination of these effects.

In turbulent flow, the stream line cross, the movement is chaotic, and mixing in all directions is intense and rapid, resulting in fast averaging of the individual extent of radial mixing, and plug flow with minimum dispersion. This needs to occur at very high (Re) values, which is not practical for FIA, since equation (2.3) requires either very high

flow rates, which are uneconomical ,or use of very narrow tubes and high linear velocities, which can cause high backpressure[79]

Secondary flow, combined with molecular diffusion promotes the the radial transport in a powerful way. Its intensity increases with geometrical disorientation of the flow path. Thus coiling of the tube induces a gentle secondary flow while knitting[88] of the tubes, or meandering of the channels, promotes an intense streaming in radial direction. Packing of tubes with single beads[81] is the most effective way to promote an intensive radial mixing .

Since the selection of the injected sample volume (S_v) is the main tool for optimizing the FIA readout, and since the FIA curves cover the whole range between the C-curves and the F-curves a useful theory must cover the whole range of response curves between the ideal pulse input and ideal step input. For this purpose an injection parameter ($\alpha=S_v/V_r$) must be introduced to the dispersion equation. Therefore equation (2.1) can be rewritten as :

$$\frac{C^{max}}{C^0} = \alpha\, e^{(-t/Tbar)} = \frac{\alpha\, e^{\cdot 1}}{D^{max}} \qquad (2.9)$$

from which the dispersion coefficient D^{max} can be computed from a given (α) value from a known system constant (χ). The system constant for N = 1 is simply the ratio of volume of liquid that passed at a given time (t) to the volume of the mixing stage (V_m) (χ =Qt/ V_m). Equation (2.7) can be written for peak maximum as follows :

$$\frac{C^{max}}{C^0} = 1\text{-}e^{(-0.693S_v/S_{1/2})} \qquad (2.10)$$

For N >>10 a more complicated mathematical models were derived[82] but were further simplified to read :

$$\frac{C^{max}}{C^0} = \alpha\left(\frac{N}{2\pi}\right)^{1/2} \qquad (2.11)$$

This equation is in agreement with the experimental results, that for S_v < $S_{1/2}$ the peak height increases linearly with the injected sample volume .

2.4. Dispersion coefficient

In order to design an FIA system rationally , it is important to know how much the original sample solution is diluted on its way

towards the detector and how much time elapsed between the sample injection and the readout. For this purpose the dispersion coefficient D_s has been defined[83,84] as the ratio of concentration of sample material before and after the dispersion process has taken place:

$$D_s = \frac{C_s^0}{C_s} \qquad (2.12)$$

at maximum peak height measurement this equation can be written as

$$D_s^{max} = C_s^{max} \qquad (2.13)$$

The dispersion coefficient can be measured by injecting a sample of a dyed solution into a colourless stream and monitoring the absorbance of the dispersed zone continuously by a spectrophotometer. To obtain the value of (D), at any time, the height of the recorded peak is measured and compared with the height of the signal obtained when the cell of the colourimeter has been filled with the undiluted dye.

For convenience, sample dispersion has been[84] as limited (D=1-3), medium (D=3-10), and large (D>10), and FIA systems are designed accordingly, have been used for a variety of analytical tasks

2.5.Dispersion and residence time (T)

It has been shown[74] that dispersion in a FIA system caused by the flow in the open narrow tube increases with the square root of the mean residence time (T),or the distance travelled (L):

$$T = \pi \frac{(d/2)^2 . L}{Q} = \frac{V_r}{Q} \qquad (2.14)$$

where (d) is the tube diameter, (L) is the tube length, and (Q) is the pumping rate. When the sample has to be mixed and made to react with the components of the carrier stream, in the first place one would tend to increase the tube length in order to increase T. However dispersion of the sample zone will increase with the distance travelled, and thus band broadening will eventually results in loss of sensitivity . Therefore to increase (T) , it is better to decrease the flow rate while keeping (L) as short as possible, however a compromise must be made .

2.6 Dispersion of sample zone in a reagent stream

When a chemical reaction is to take place between the sample solution and a reagent contained in the carrier stream, a mixing must take place. Similar to the value of the sample dispersion coefficient D_s the reagent dispersion coefficient is defined as:

$$D_R = \frac{C_R^o}{C_R} \qquad (2.15)$$

where C_R^o is the original reagent concentration C_R is the reagent concentration in the element of fluid that yields the analytical readout.

Let us consider a single line system (Fig.2.2a),where the carrier stream contains a reagent (C_R^o) into which sample (C_S^o) is injected yielding concentrations C_s and C_R which are obtained by mutual dispersion of sample zone and carrier stream (Fig 2.2a). Obviously when the sample concentration is highest (C_s^{max}), the reagent concentration is lowest C_R^{min} and therefore whenever D_s approaches 1, the reagent concentration approaches zero. Consequently , a species to be measured cannot be formed in the center of the sample zone whenever D_s = 1. It has been shown[72] that :

$$\frac{1}{D_s} + \frac{1}{D_R} = 1 \qquad (2.16)$$

and therefore

$$D_R = \frac{D_s}{(D_s - 1)} \qquad (2.17)$$

From which it follows that in one line system the sample and reagent concentrations cross at D=2 (where $D_R = D_s$). Furthermore, if at any point within a single channel the reagent concentration should be equal to the sample concentration then the following conditions must be fulfilled .

$$C_s = \frac{C_s^o}{D_s} \qquad (2.18)$$

or

$$C_s^o = C_R^o(D_s - 1) \qquad (2.19)$$

If sensitivity of measurement is to be increased by decreasing the sample zone dispersion then the original reagent concentration in the carrier stream must be increased correspondingly.

In the two line system, the dispersion of the sample and reagent solutions differ from a single line system in two ways. Firstly, the dispersion coefficient of the sample D_s cannot reach a value of 1 and, secondly the dispersion of the reagent becomes independent of that of the sample (Fig 2.2b). If the original concentration of the sample and reagent are equal, $C_R^o = C_s^o$ then

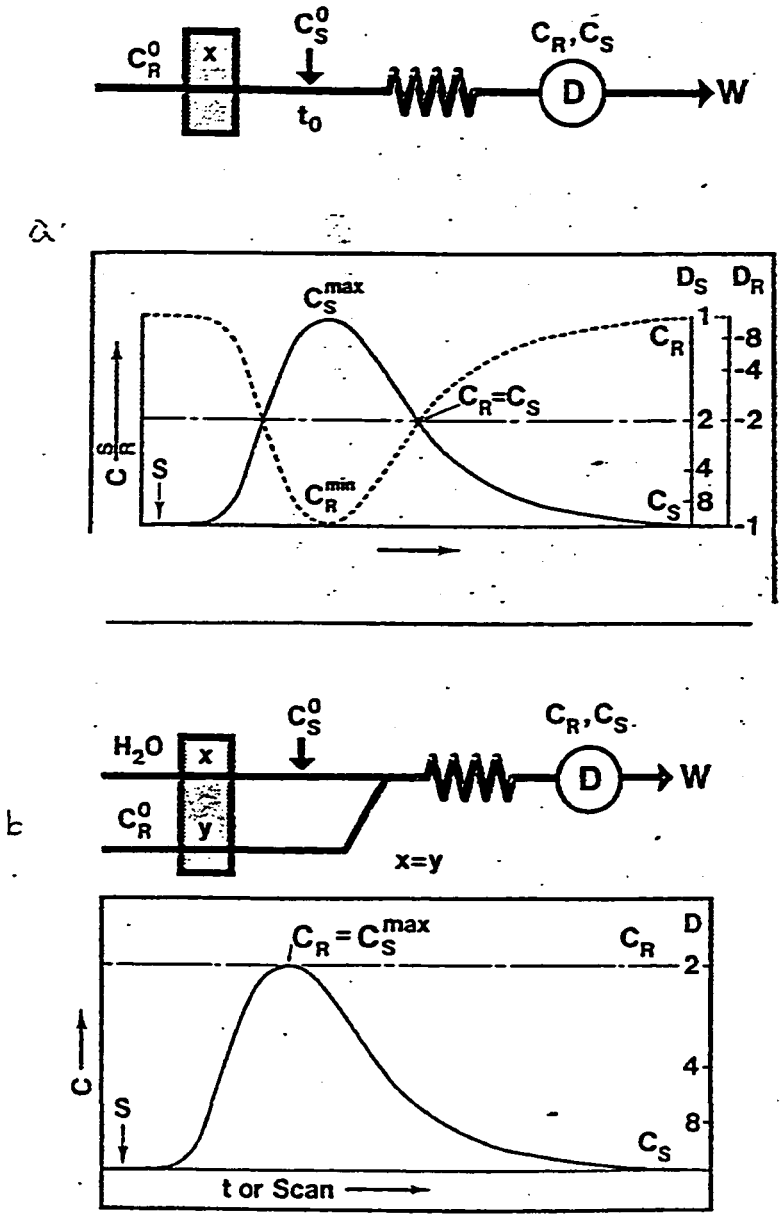


Fig. 2.2 Mutual dispersion profiles of sample (S) and reagent (R) in (a) single line manifold, (b) Double line manifold . Where sample of original concnetration C^o_s is injected into a carrier stream of original concentration C^o_R at time t_o.

$$C_R = \frac{C_R^o}{D_R} = C_R^{o\{y/(x+y)\}} = C_s^{max} \qquad (2.20)$$

where C_s^{max} is sample concentration at the steady state

$$C_s^{max} = C_s^{o\{x/(x+y)\}} \qquad (2.21)$$

When $C_R = C_s$, and $y=x$

$$C_s^o D_R = C_R^o D_s \qquad (2.22)$$

therefore, it is easy to maintain an excess of reagent through the entire sample zone, even when D_s is close to the steady state. Therefore, the original reagent concentration must be slightly higher than the that stoichiometrically corresponding to the concentration of the injected sample solution as compared to a single line system where the original reagent concentration must be increased dramatically whenever D_s approaches the steady state .

2.7.FIA Techniques:

2.7.1.Single FIA Manifold

The simplest FIA system consists of one tube through which the carrier stream moves towards the flow through detector. Depending on the injected volume (S_v) , tube length, flow geometry, and element of fluid along the gradient selected for the analytical readout, limited, medium, and large values of dispersion coefficient (D) might be achieved.

The limited dispersion (D = 1-3) is used in the original composition of a sample solution to be analyzed. In fact, in this case the FIA system serves merely as a means of rigorous and precise transport of the sample material to the flow cell in an undiluted form or as a means of reproducible sample introduction.

The systems with medium dispersion are the most interesting from an analytical point of view. They encompass a large number of procedures in which one or several reagents are mixed with the sample solution in order to form colored, fluorescent, electroactive or otherwise marked product, which can be sensed by a flow-through detector. In this type of determination not only sufficient mixing must take place,

but also sufficient time should elapse before the sample zone reaches the detector to ensure that the chemical reactions involved are allowed enough time to produce an adequate amount of detectable product. Again, because of the high reproducibility of the mixing and timing in the unsegmented stream, there is no need to reach chemical equilibrium in order to obtain valid analytical results.

2.7.2. Two and Multiline FIA Manifold

More often, the analytical scheme requires the application of two or more reagent solutions, which are either premixed shortly before use or added sequentially because the reagent or reaction products are incompatible. Initially, it was doubted[85] that FIA would be capable of accommodating procedures that require addition of further reagent downstream, because it was thought that the mixing at the confluence points would not be reproducible. Fortunately, it soon became apparent that the addition of several reagents in sequence was feasible[86], and the theory of dispersion confirmed that to perform analysis successfully, the sample and reagent solutions do not have to, and indeed should not be mixed homogeneously.

Premixing of reagents immediately prior to sample injection is a very useful technique in cases where a reagent mixture might deteriorate during storage. Sequential use of only two reagents is a simple matter because a manifold for this purpose involves only one confluence point at which a second reagent is added to the sample zone when carried past by the stream of the first reagent.

Two-line and Multi-line manifolds are now commonly used in FIA methods. Besides being based on one phase equilibria, multiline manifolds may also involve gas diffusion, solvent extraction, and liquid-liquid phase reactions in packed reactors. It should be emphasized, however, that a FIA system should always be kept as simple as possible and that a well designed chemical analysis will often require only the use of two line manifold.

2.7.3. High Sensitivity FIA:-

If FIA procedure is required to reach the same level of sensitivity as a batch procedure, two obstacles have to be overcome: the short reaction time which is due to the short residence time and may result in a relatively low yield of reaction product, and an excessive dispersion of the sample zone, which results in unwanted dilution of the species to

be measured. Leaving aside at this stage the problem of too short residence time, it might be helpful to consider an approach by means of which the dispersion can be minimized. Yet still remain sufficient for supplying the middle of the sample zone with an adequate amount of reagent. Obviously, lack of reagent in the centre of the sample zone will result in the absence of product to be sensed. Thus, instead of a sharp smooth peak, one will obtain either a double peak, which may be well pronounced or merely appear a noisy peak signal, or a nonlinear calibration curve at the upper range where the concentration of analyte in the standard solutions is high. Although an increase of the reagent concentration in the carrier stream may forsake the formation of humped peaks and widen the linear calibration range; this approach is not economical in terms of reagent consumption.

Injecting a sample into a carrier stream of pure solvent and adding the reagent by means of a confluence downstream is, however, an effective way to ensure the rapid and adequate mixing of sample with the reagent. Using such a flow arrangement, a high sensitivity of measurement can be obtained by injecting a large sample volumes.

The stopped flow approach is used to increase the sensitivity of measurement by increasing the residence time (T). The stopped-flow

technique is based on one of the most important observations in FIA, postulating that to increase the residence time, one should keep the reaction coil short and to decrease the velocity of the carrier stream. To prolong the time of reaction stopped flow technique is applied. The simplest way to investigate this parameter, which is different for each chemical reaction is to stop the flow when the sample is either in the reaction coil or the flow cell.

2.8.Components of FIA system:

2.8.1.Pumps:

It is common practice in FIA to use multiroller peristaltic pumps . The single flow fluctuations that may occur are damped to some extend by the use of flexible and somewhat elastic tubing and , if necessary, an additional pulse-damping devise can be used . The advantage of the peristaltic pumps is that they are capable , in , principle ,of maintaining a constant volumetric flow rate and correspondingly a constant residence time independent of minor changes in viscosity or variations in back pressure because of restriction changes in the remainder of the system . This does not apply to the use of gas- pressurized reservoirs for reagents and carrier or to the use of constant head vessels. How-

ever, both are cheap alternatives to pumping, and a part from the benefit of an almost completely pulse-free fluid flow, their main advantages are simplicity and lack of any moving parts .

2.8.2. Injection devices

FIA is based on creation of a well defined zone, which is the starting point of each measuring cycle in terms of concentration. The injection means designed for this purpose can be divided into two parts : (1)Volume based injection; and (2) Time-based injection. In the first category, the injection is based on the physical entrapment of sample solution into a geometrically well defined volumetric cavity and subsequent transfer of the formed sample zone into the non-segmented carrier stream. The oldest volumetric-based FIA injection technique utilized a syringe furnished with a hypodermic needle, by which piercing the wall of the carrier stream tube allowed the introduction of the sample material[72] This type of injection was later replaced by slider valves[87,88] and especially by rotary valves (with four , six , or eight ports). The rotary valves has two positions, the sampling and injection position. During the first stage sample is filled into the volumetric cavity while the carrier stream is shunted via a by-pass in order to prevent build up of back pressure. When the cavity is filled with

sample; either affected manually by a syringe or automatically by aspiration of a pump the valve is rapidly turned to the inject position and left there until the sample is completely swept by the carrier stream out of the volumetric cavity. In the time based injection procedures the sample volume is metered as a function of time. Thus the injected sample volume can be readily computer controlled, while in the volumetric methods the sample volume is changed by physically changing the volume of the dimensions of the sample loop. Admittedly, the latter methods have the advantage of being independent of volumetric flow rates, and therefore inherently more precise, because the precision of the time-based injection relies on maintenance in constancy of flow rates.

2.8.3.Reactor and Connectors

The most frequently used reactors are made of plastic tubing which can be coiled, knitted or knotted . The most frequently used internal tube diameter in FIA applications is 0.5 mm, but 0.8 mm is useful either to increase dispersion or to increase the hold up volume and decrease the flow resistance of extremely long reaction coil. The most suitable tube material is Teflon, which besides being chemically resistant, adsorbs the least solute on its surface . Packed reactors are readily

made from pieces of suitable tubing into which the solid material is placed and where if necessary, at both a small tuft of glass wool, fine plastic grid, or frit is placed to keep the packing material within the column. Typical column dimensions are 1-3 cm in length and 5 mm i.d.

2.8.4.Detectors:

Similar to chromatographic detectors, the linearity, noise level, and peak broadening effects are important criteria valid to FIA detectors. The peak broadening could be as a result of the flow rate in, the holdup of the detector, the speed of detector response, and of the time constant of the associated electronics. It is desirable that the peak broadening caused by detector, expressed as its variance, is no more than 5%.

the main difference between chromatographic and FIA detectors, is that the latter technique preferably uses selective detectors, whereas chromatography relies on nonselective detectors that should yield a readout for as many species as possible, preferably with the same sensitivity. Due to the formation of a concentration gradient of the dispersed sample zone in FIA, all detectors fall into two categories depending on the way of probing the dispersed sample zone, that is,

by bulk sensing or by surface sensing, the typical representatives of these two categories being optical detectors and electrochemical detectors. In most optical sensors the signal approximates the mean composition of the flowing stream present in the detector cell, and thus reflects the composition of the bulk of the solution. This is because in spectrometry the beam penetrates the sample zone either radially, or more often axially. Electrochemical methods on the other hand, rely on transport of an electroactive species towards an electrochemically active surface. If the species is not effectively transferred from the bulk solution to the diffusion layer and across the diffusion layer to the sensing surface, it cannot be sensed.

2.9.FIA Optimization:

Optimization can be defined as a collective process of finding the set of conditions required to achieve the best result from a given situation. Optimization techniques require at the start a definition of the response of the system that is to be maximized (or minimized) and of the experimental variables upon which it is dependent. Consider a two -variable system. The variables could be represented as the axis of a graph, with each point in the space described corresponding to one set of

experimental conditions. Each experiment will have a response and these could be shown as a third dimension. A response surface is thus created, the highest point of which corresponds to the optimum conditions for the system. A simple surface would look like a hill. Optimization procedures are a means of getting from a given set of starting conditions to those corresponding to a maximum . An ideal optimization procedure should be capable of finding the highest maximum reliably and rapidly, irrespective of the nature of the surface. This may be difficult to achieve as some surfaces are very complex, with an appearance more similar to mountain ranges than to simple hill.

When adapting a manual procedure to a FIA system, it is commonly necessary to investigate whether optimum experimental conditions have been chosen. Although one would, as a rule, always start from a well known equilibrium data or from a well known conditions as established for a manual method, these might serve merely as a guide, because at the dynamic conditions of FIA system, the kinetic factors may play an important role. Whether trying to eliminate their influence or possibly exploiting these kinetic factors as the basis for the analytical assay via kinetic discrimination procedures, it is nevertheless important to be able to investigate the execution of the actual assay under well-controlled conditions

2.9.1. Univarient Method of Optimization:

The univarient method of optimization involves keeping all but one variable constant, finding the best conditions ,and then repeating the procedure for each variable in turn, starting from the best point response achieved so far. The conventional univarient approach considers each variable once only. An iterative univarient optimization considers each variable several times, the procedure continuing until no further improvement is obtained.

In systems where the variables do not interact a univarient approach is quite successful. However the presence of interaction between the variables may cause the method to fail[69]

2.9.2. Simplex Method

The need for an efficient effective optimization procedure in analytical chemistry has been realized by many researchers[69,89] The main objective is to maximize response or yield in a minimum number of experiments. The simplex method, first presented by Spendley et al[90] and further modified by Nelder and Mead[91] to give not only a clear indication of when sufficiently precise optimum has been attained but also has the advantages of acceleration and adaptation to fit the partic-

ular response surface studied. This method was first introduced to analytical chemistry by Morgan and Dming[69] since then various modified versions have been suggested[92-98], each aiming to improve the speed and reliability of optimization.

The steps of the modified simplex method is given below :

Let

$$X_{k,i} = \{x_{k,i1}, \ldots, x_{k,ij}, \ldots, x_{k,in}\} \qquad (2.23)$$

i = 1,2,....,n+1

be the ith vertex of the simplex (point) in D^n (n-dimension space) on the kth stage of the search k =0,1,..., and let the value of the objective function which we want to maximize at $X_{k,i}$ be $f(X_{k,i})$.

Let us define the maximum and minimum value of f(x) on the simplex

$$f(X_{k,h}) = max\{f(X_{k,1}), \ldots, f(X_{k,n+1})\} \qquad (2.24)$$

$$f(X_{k,l}) = min\{f(X_{k,1}, \ldots, f(X_{k,n+1})\} \qquad (2.25)$$

Let $X_{k,n+2}$ be the centroid of the simplex. Then the procedure for finding a new vertex in n-dimension space, D^n, at which the yield of the experiment is expected to be better, is as follows:

Step 1:Reflection: Reflect $X_{k,l}$ through the centroid by computing

$$X_{k,n+3} = X_{k,n+2} + \alpha(X_{k,n+2}) - X_{k,l}) \tag{2.26}$$

where $\alpha > 0$. A recommended value for α is 1. Here we are moving away from the minimum point.

Step 2:Expansion: If $f(x_{k,n+3}) > f(X_{k,h})$ expand the vector $(X_{k,n+3} - X_{k,n+2})$ by computing

$$X_{k,n+4} = X_{k,n+2} + \gamma(X_{k,n+3} - X_{k,n+2} \tag{2.27}$$

where $\gamma > 1$. A recommended value for γ is 2. If $f(X_{k,n+4}) > f(X_{k,h})$ then replace $(X_{k,l})$ by $(X_{k,n+1})$ and continue from step 1. Otherwise replace $(X_{k,l})$ by $(X_{k,n+3})$ and continue from step 1 with k = k+1.

Step 3:Contraction: If $f(X_{k,n+3}) < f(X_{k,i})$ for all i contract the vector $(X_{k,l} - X_{k,n+2})$ by computing

$$X_{k,n+5} = X_{k,n+2} + \beta(X_{k,h} - X_{k,n+2}) \tag{2.28}$$

where $0 < \beta < 1$ is the contraction coefficient. A recommended value for β is 5. Replace (X_{l}) by (X_{n+5}) and return to step 1 to continue the search on the (k+1) stage.

Step 4: Reduction: If f($(X_{k,n+3})$ < f($X_{k,l)}$, replace $(X_{l)}$ by a point half way between $(X_{l)}$ and $(X_{h)}$

$$X_{k,l} = 0.5(X_{k,l} + X_{k,h}) \tag{2.29}$$

Sort for the minimum and the maximum again. Go to step 1.

if in all of the above steps the coordinate of the new obtained points is checked wether they lie between the upper and lower bound of the corresponding variable. If it is less, replace it by the the lower bound, if it is higher than the upper bound replace it by the upper bound .

There are two major differences between the classical method[90] and the one presented above. The first difference is that at each step in the modified simplex we check for the obtained point wether its components lie between the specified upper and lower bound for each variable, i.e. the method is modified to handle constrained situation. The second difference is the reduction step. In this step of the classical simplex, all points of the simplex are replaced by

$$X_{k,i} = X_{k,l} + 0.5(X_{k,i)} - X_{k,h}) \tag{2.30}$$

$i = 1,\ldots,n+1$

The required (n+1) new experiments to be performed after each reduction step. In the reduction step of the modified simplex the minimum point is replaced by a point half distance between the minimum and maximum (see step 4 of the algorithm). Therefore one experiment has to be conducted after each reduction step in order to proceed with the algorithm. This amount to a considerable reduction in number of experiments to be conducted.

The modified simplex method has been implemented using pascal language on IBM compatible computers in a friendly interactive environment. This allows the user to easily interact with the optimization procedure.

CHAPTER THREE

CHAPTER THREE

EXPERIMENTAL

3.1 Apparatus

(1) The Alitea USA/FIA lab apparatus was used for all FIA experimental work. The apparatus consists of the following units :

(a) Pump: A high quality peristaltic pump with cassette drive .It features eight stainless steel rollers on individual bearings with individual cassettes for as many as four pump tubing and a gearbox with a double bearing shaft . Pump tubings (PVC) of various internal diameters were used to give proper flow rates .

(b) Injector: A Rheodyne model 5041 4-way PTFE rotary valve mounted on an angle bracket in the injector position of the FIA lab apparutus was used

(c) Reactor module: Consisting of 0.5 mm i.d. PTFE tubing of different lengths was used. The length of this reaction coil tubing is chosen as appropriate and coiled in a way to ensure completion of reaction by mixing .

(d) Detector : A spectronic mini 20 spectrophotometer (Milton Roy) with a grating monochromator detector, a Unovic ultra-micro flow-through cell of 20 μl with a pathlength of 1.0 mm was used.

(e) Recorder : A model 0555 single channel-strip-chart recorder was used for peak absorbance-time recording .

(2) IBM compatible computer was used for all simplex experiments .

(3) Lambda-5 UV-Visible spectrophotometer (Perkin-Elmer) was used for all conventional spectrophotometric experiments .

3.2. Reagents:

Deionized water was used in all the work .Stock solutions were prepared from analytical reagent grade or pharmaceutical grade chemicals, from which working solutions were prepared by appropriate dilutions .

3.2.1 Sulphuric acid :

The required concentration of sulphuric acid was prepared by appropriate dilutions from concentrated anlytical reagent grade sulphuric acid.

3.2.2 Tetracycline and derivatives :

3.2.2.1 Pure tetracycline samples :

These were freshly prepared by using the powder form which was directly weighed and dissolved in 0.001M sulphuric acid stirred and made up to volume in a calibrated flask, except for oxytetracycline,

where warming at $60^{0}C$ for 20 minutes is necessary for complete dissolution .

3.2.2.2 Drug samples

For capsules the contents of 10 capsules were mixed, weighed and accurately a quantity of powder equivalent to the mass of the tetracycline in one capsule as claimed by the manufacturer was accurately weighed out and dissolved in 200 ml of 0.001M sulphuric acid.This was warmed for 20 minutes, and then diluted to 250 ml with 0.001M sulphuric acid in a calibrated flask after cooling to room temperature .

For syrups, the volume of syrup containing a certain mass of the tetracycline was pippetted and delivered into the standard flask and diluted to the mark with the required sulphuric acid to give a final solution of 0.001M acid .

3.2.3 Ferric ammonium sulphate :

Iron(III) stock solution 48.50 mg ml^{-1} was prepared by dissolving accurately about 24.20000 g of ferric ammonium sulphate

($NH_4(SO_4)_2 12H_2O$) which was previously dried in an oven, in 400 ml 0.001M sulphuric acid then diluted to 500 ml calibrated flask with 0.001M sulphuric acid to the mark . This solution was used as a carrier stream after being diluted with sulphuric acid of appropriate concentration.

3.3 FIA design and optimization :

To design a manifold suitable for the determination of tetracyclines, FIA and chemical variables are considered for optimization, and these include coil length, flow rate, reagent concentration, and sample loop size. The FIA system was then configured to give the required coil length, flow rate, and sample loop size. In all experiments a single FIA configuration was found to be suitable as shown in fig. 3.1

The boundaries for each variable were shown in table 3.1. These were chosen on ground of previous experimental experience. The iterative univarient method looked at the effect of each variable individually using an average seven values of variable per cycle. This method involves keeping all but one variable constant, finding the best of the variable under those conditions, and then repeating the procedure for each variable in turn, starting from the best response achieved so far.

Table 3.1: Boundary conditions for paremeters varied in the optimization procedures.

Variable	Upper bound	lower bound
Coil length	45 cm	400 cm
Flow rate	3.70 ml/min	5.40 ml/min
iron(III) concentration	50 ppm	2000 ppm
Sulphuric acid concentration	0.0001M	0.5M
Sample volume	110 ul	210 ul

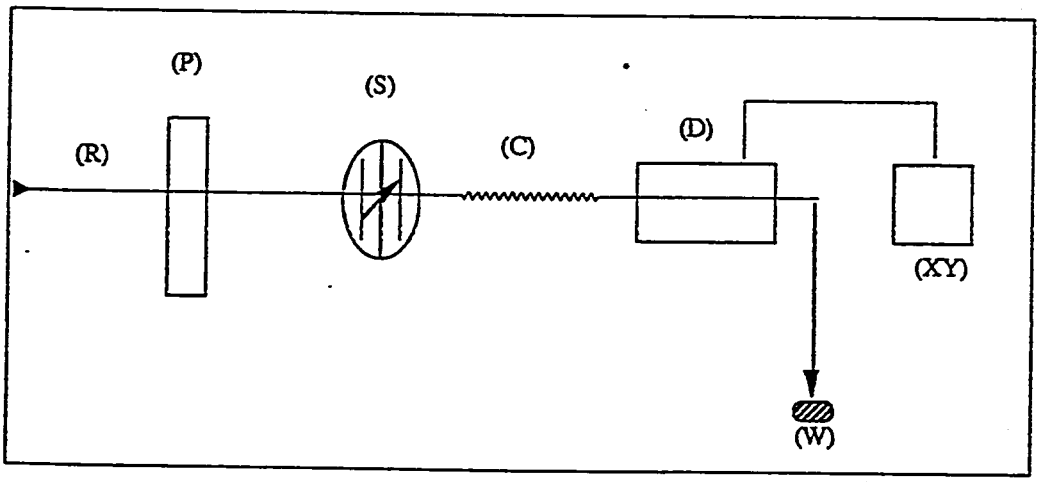


Fig.3.1: FIA schematic diagram manifold used: R, reagent carrier of iron(III) in sulphuric acid; P, Peristaltic pump; S, sample injector of 157 µl loop size; C, reaction coil; D, spectronic mini 20 spectrophotometer, XY, recorder and W, Waste.

The modified simplex method optimizations were carried out as follows ; the parameters of the programme were fed into the computer as well as the upper and lower boundaries for each variable. Since the simplex is a geometric figure whose vertices are n+1, where n is the number of variables, five experiments were needed to generate the initial simplex, the values of the variables and the response (peak absorbance) of each experiment were fed into the computer. The programme calculated the next set of experimental conditions to be tried and printed worksheet. Then by using these conditions an experiment is performed, and the peak absorbance value was collected and entered into the computer, and another experiment was generated. This process was continued, the programme giving a single set of conditions(one experiment) each time.

3.4 Procedure :

The carrier stream solution was allowed to flow for few minutes at a certain flow rate, absorbance was adjusted to zero in the spectrophotometer and the baseline of the chart recorder was brought to be stable and constant. The sample was then injected into the flowing stream and the reaction was allowed to take place in the reaction coil. The developed coloured compound was allowed to flow at the same flow rate of the

carrier stream already adjusted and passed through the micro-flow-through cell in the spectrophotometer where the absorbance was recorded at a fixed wavelength of the corresponding iron(III)-tetracycline complex.

CHAPTER FOUR

CHAPTER FOUR

Determination of TetracyclineHCl

4.1 Introduction

TetracyclineHCl and derivatives, (chlorotetracycline, democlocycline and oxytetracycline) have found extensive use in current therapy because of their broad spectrum antimicrobial activities. Chelation of tetracycline with some cations such as iron(III), aluminium(III), copper(II), nickel(II), cobalt(II), zinc(II), vanadium(III), thorium, lanthanum, magnesium, and calcium was recently reviewed[1]

The aim of this work is to establish a simple, fast and reliable method of quantitative determination of tetracycline by utilizing flow injection analysis technique. Thus, the quantitative spectrophotometric method[1] which involves complexation of iron(III) in sulphuric acid media, has been selected for this study. The system has been optimized on the basis of sensitivity by a modified simplex, and an iterative univarient procedures. All chemical and FIA variables have been taken into consideration.

Tetracycline hydrochloride is the hydrochloride salt of an antibiotic substance which was discovered in 1953[6]. It has the empirical formula $C_{22}\ H_{24}\ N_2\ O_8$. HCl and a molecular weight of 480.9. The structural formula of tetracycline and the reaction scheme of tetracycline with iron(III) are presented in Fig. 4.1.1. method[1] Aqueous solution of tetracyclineHCl absorbs at 355 nm whereas tetracycline-iron(III) complex, which is a brown soluble complex absorbs at 423 nm. The manifold used for the assay of tetracycline HCl has been presented earlier in Fig. 3.1

4.1.2 Optimization Procedure

In any optimization procedure, a response of the system that is to be maximized or minimized should be defined, as well as the experimental variables upon which this response is dependent. The variables could be represented as the axes of a graph, with each point in the space described corresponding to one set of experimental conditions. Each experiment will have a response which could be considered as an additional dimension. A response surface is thus created, the highest point of which corresponds to the optimum conditions for the system.

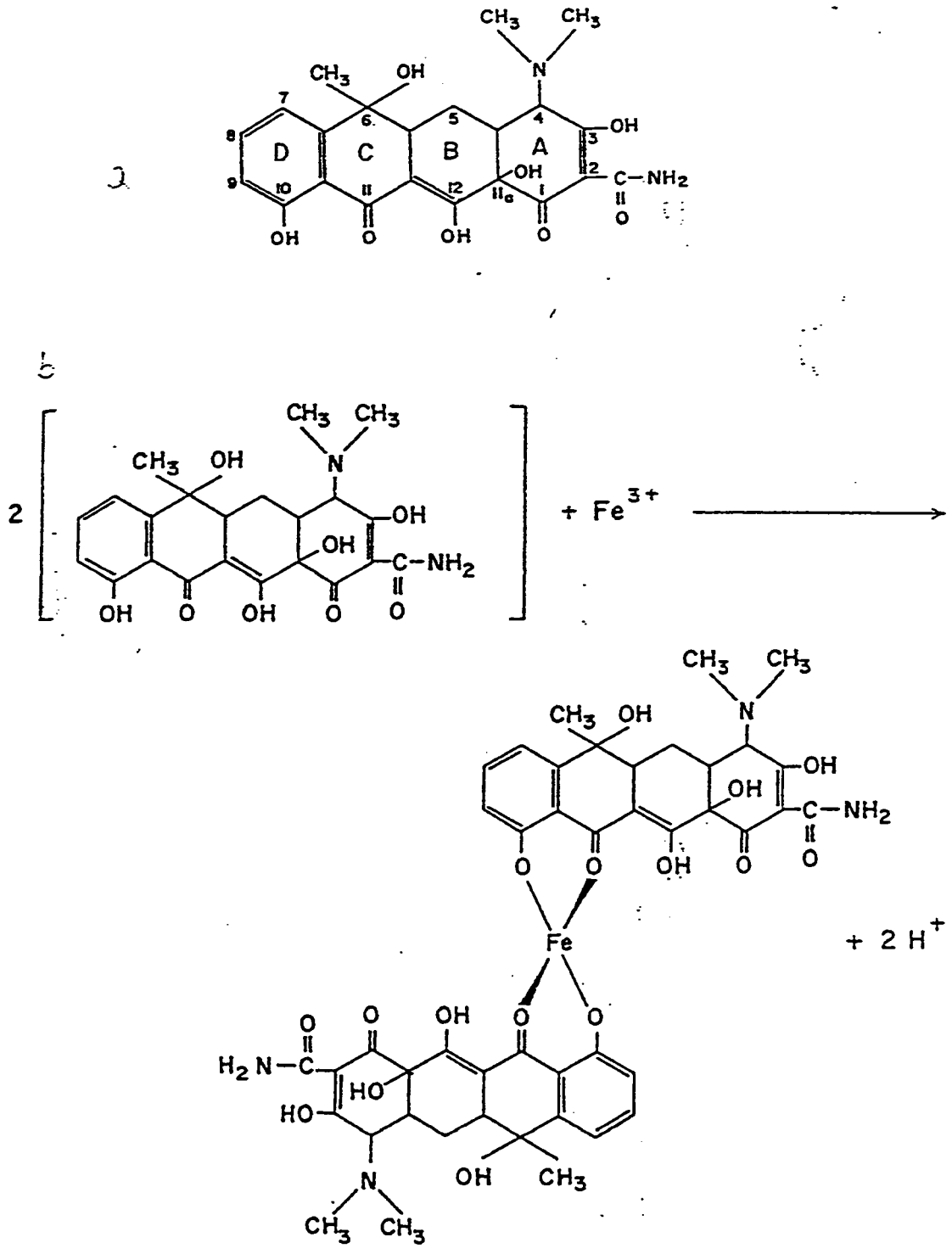


Fig. 4.1.1 (a) Structural formula of tetracycline (b) Reaction scheme of tetracycline with iron(III). 65

In the development of new analytical methods and in improvement or adaptation of established ones, factorial experiments and the analysis of variance are often used to investigate the effects of many continuous variables upon the results of given method. Those variables that exhibit a significant effect can then be adjusted to improve the results of the method. All of the above activities are concerned with optimization, the collective process of finding the set of conditions required to achieve the best result from a given situation. If the variables do not interact with each other, each variable can be optimized independently. In general, however, variables do interact with each other, and the one factor at a time approach will not always result in an optimum set of conditions.

4.1.2.1 Univarient method of Optimization

This method was implemented by varying one variable, while keeping the others constant, finding the best value of this variable under those conditions, and then repeating the procedure for each variable in turn, starting from the best point response achieved so far. An iterative univarient procedure, with two cycles was applied, and the results are presented in the following subsections.

4.1.2.1.1 Variation of Sulphuric Acid Concentration

The effect of sulphuric acid concentration on the peak absorbance has been investigated in the range of (0.001 - 0.50M), for the two cycles. Acid concentrations below 0.001M could result in hydrolysis of Fe(III), whereas acid concentrations greater than 0.5M could result in disproportionation of the complex formed. The other variables were kept at constant values, as follows; coil length 45 cm flow rate 3.9 ml/ min; sample loop size 110 μl and iron(111) concentration 50 ppm, in the first cycle, whereas in the second cycle they were as follows; coil length 120 cm, flow rate 3.81 ml/min, sample volume 157 μl, and Fe(III) concentration 600 ppm. The results are shown in Fig. 4.1.2. It is obvious that the acid concentration has an important effect on the peak absorbance. As the acid concentration increases the peak absorbance decreases very rapidly. At acid concentrations below 0.1M, the peak absorbance remains almost constant, this could be due to disproportionation of the complex. The maximum absorbance was obtained at 0.001M, which is the lowest acid concentration used. Acid concentrations below 0.001 M lead to hydrolysis of the iron(111), therefore, the optimum acid concentration was chosen as 0.001 M. It is observed that in Fig. 4.1.2, both the first and second cycle show the same behaviour, and the same optimum acid concentration was obtained, with a considerable

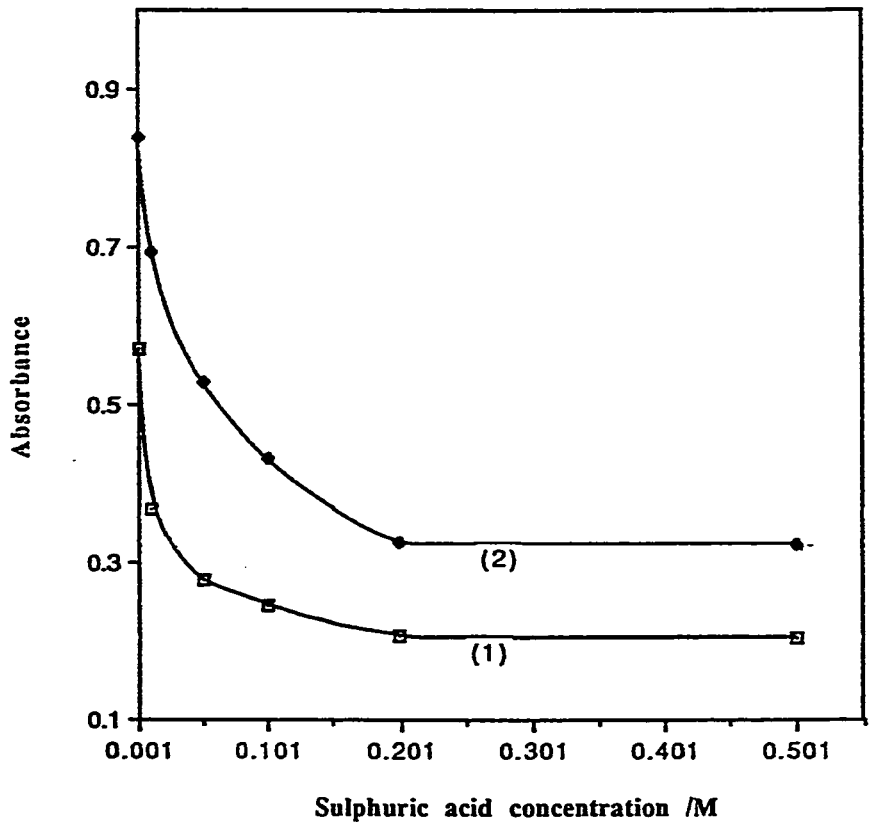


Fig 4.1.2. Variation of sulphuric acid concentration for tetracyclineHCl (1) first cycle where coil length is 45 cm, flow rate is 3.9 ml/min, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where coil length is 120 cm, flow rate is 3.81 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

improvement in the peak absorbance in the second cycle. In the first cycle, the initial conditions were selected arbitrarily, and each variable was then optimized separately. Therefore, in the second cycle the absorbance was measured under initially optimized conditions, this is manifested by the improvement of the peak absorbance.

4.1.2.1.2 Variation of Reaction Coil Length

The reaction coil length, which is defined as the total length from the point of sample introduction to the detector, is interchangeable with longer or shorter tubing of the same type and diameter. In this work the effect of coil length on the peak absorbance has been studied in the range 45 - 400 cm and 0.5 mm i.d.. Coil length was varied by keeping other variables at the following values in the first cycle; sulphuric acid concentration, 0.001M, flow rate 3.92 ml/min, sample volume 110 μl and iron(III) concentration, 50 ppm. However in the second cycle, sulphuric acid concentration, flow rate, sample loop size and iron(III) concentration were 0.001M, 3.81 ml/min, 157 μl and 600 ppm respectively. The results were plotted in Fig. 4.1.3. it was found that in a single line system, the concentration of the sample is highest when the concentration of the reagent is. lowest[84] Therefore, when

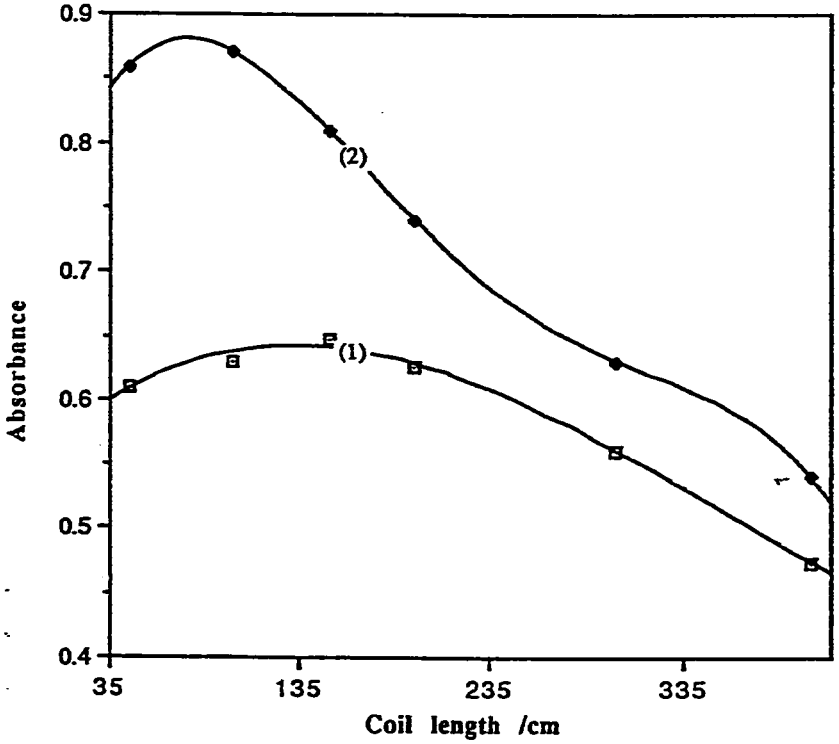


Fig 4.1.3. Variation of coil length for tetracyclineHCl (1) first cycle where sulphuric acid concentrationis 0.001M, flow rate is 3.9 ml/min, sample volum 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, flow rate is 3.81 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

the dispersion tends to lower values the mutual mixing of the reagent and the sample could be incomplete. Therefore, increasing the coil length results in increasing residence time (t) to complete mixing, but very long reaction coil lengths could result in an excessive dispersion and thus band broadening will eventually result in loss of sensitivity. This is clearly shown in Fig. 4.1.3, that is, as the coil length is increased, the peak absorbance is also increased until 150 cm tubing length in the first cycle and 85 cm in the second cycle, then the dilution of the complex formed became the dominant factor, and rapid decrease in the peak absorbance is observed.

4.1.2.1.3 Variation of Flow Rate

The flow rate is conveniently controlled by the peristaltic pump with a speed of up to 999 ramps. The relationship of pump speed in ramps to flow rate in ml/min could be obtained by applying the following equation, provided that the pump tubing i.d. is 1.3 mm.

$$\text{Flow rate (ml/min)} = 3.70 + 1.77 \times 10^{-3}\ rmps \qquad (4.1)$$

The flow rate was varied by changing the speed of the pump. Other variables were kept constant, that is in the first cycle the values were 0.001M, 120 cm, 110 μl, and 50 ppm, whereas in the second cycle, the values were 0.001M, 85 cm, 157 μl, and 600 ppm, for sulphuric acid concentration, coil length, sample loop size and iron(111) concentration respectively.

The results obtained were plotted in Fig. 4.1.4. Slight increase in the peak absorbance was observed which as a result of a slight decrease in the dispersion, then a rapid decrease in the peak absorbance was obtained, which is caused by a corresponding decrease in the residence time (t) of the sample, hence the mixing is not allowed to reach completion. This is clearly the opposite picture of the coil length, therefore these two factors should be varied simultaneously, to reach to a good and correct conclusion. The optimum flow rates were found to be 3.81 and 4.10 ml/min for the first and second cycle respectively, with an improvement in the peak absorbance in the second cycle.

4.1.2.1.4 Variation of Sample Volume

The sample volume was varied between 110 and 204 μl by changing the sample loop size. The conditions for the sample volume variation for

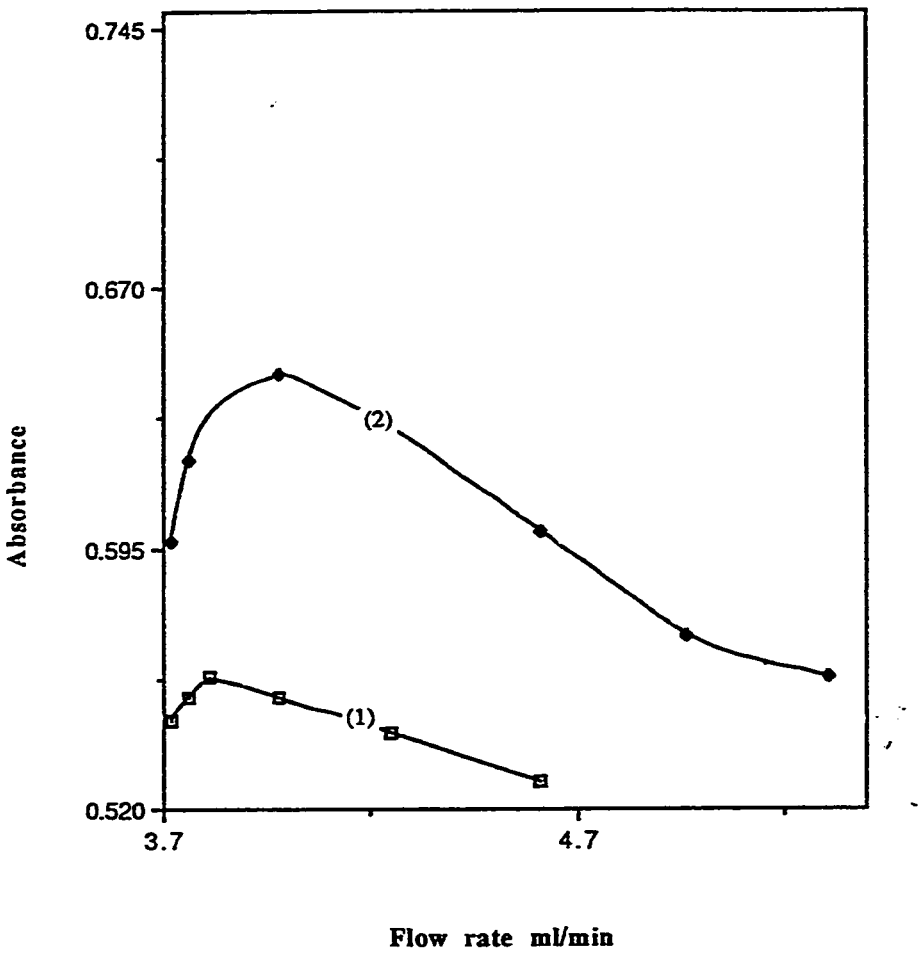


Fig 4.1.4. Variation of flow rate for tetracyclineHCl(1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 120 cm, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 85 cm, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

the first were, 0.001M, 120 cm, 3.81 ml/min and 50 ppm, whereas in second cycle the conditions were 0.001M, 85 cm, 4.10 ml/min and 600 ppm for sulphuric acid concentration, coil length, flow rate and iron(III) concentration respectively. The results obtained are shown in Fig. 4.1.5. The results obtained show a slight increase in the peak absorbance, which is insignificant, also it was found that the results were not reproducible at high sample volumes. Therefore, for sample economy considerations a sample loop size of 157 μl was chosen for further investigations. The first and second cycle have the same behaviour, with a considerable improvement of peak height in the second cycle.

4.1.2.1.5 Variation of Iron(III) Concentration

The concentration of iron(III) dissolved in 0.001M sulphuric acid concentration was varied between 50 - 2000 ppm. The results obtained were shown in Fig. 4.1.6. It was found that as the iron(III) concentration increases the peak absorbance increases, until it reaches a maximum at 600 ppm, then it starts to decrease, this could be attributed to overlapping of peak absorbance of iron(III) and the complex. Fe(III) was varied for first and second cycle. In the first cycle the values of

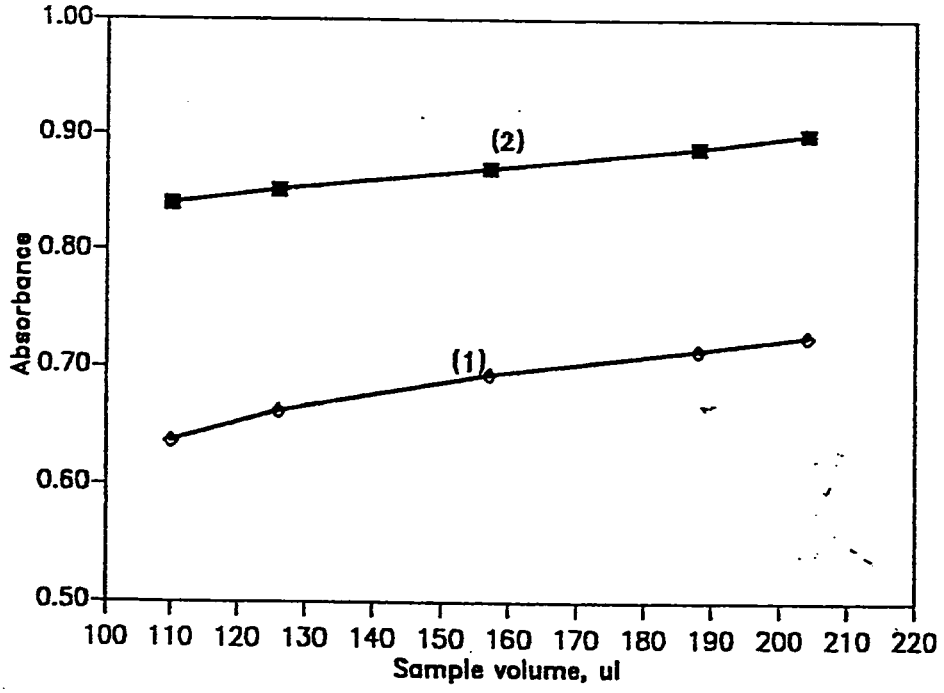


Fig 4.1.5. Variation of sample loop size for tetracyclineHCl (1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 120 cm, flow rate is 3.81 ml/min, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 85 cm, flow rate is 4.10 ml/min, and iron(III) concentration is 600 ppm.

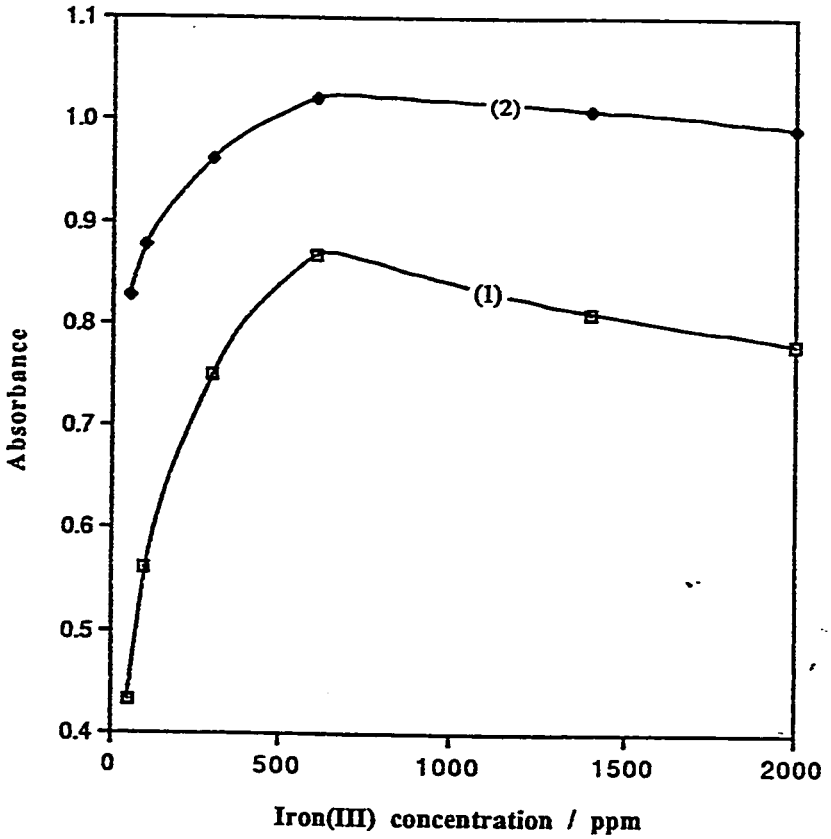


Fig 4.1.6. Variation of iron(III) concentration for tetracyclineHCl (1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 120 cm, flow rate is 3.81 ml/min and sample volume is 110 μl, (2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 85 cm, flow rate is 3.81 and sample volume is 157 μl.

other variables were, 0.001M, 120 cm, 3.81 ml/min and 157 μl for sulphuric acid concentration, coil length, flow rate and sample loop size respectively. In the second cycle, sulphuric acid concentration was the same as in the first cycle, whereas the other variables were as follows, 85 cm, 3.81 ml/min and 157 μl for coil length, flow rate, and sample volume respectively. As observed from the result shown above, the iron(III) concentration obtained at the optimum conditions, is very high compared to that obtained by the conventional spectrophotometric method.[1] This could be attributed to the fact that in single line systems the concentration gradient of sample and reagent are mirror images of each other, that the concentration of reagent is minimum when the concentration of the sample is maximum. Therefore, to maintain a sufficient excess of reagent throughout the whole sample zone, the original reagent concentration in the carrier stream must be increased correspondingly. In conclusion, the best experimental setting obtained by the univarient method, was found to be 0.001M for sulphuric acid concentration, 85 cm for coil length, 4.10 ml/min for flow rate, 157 μl for the sample size, and 600 ppm for iron(III) concentration.

4.1.2.2 Simplex Optimization

Procedures of determining optimum operating conditions often consisted of a trial and error approach, the results of which, although sometimes were successful, depend on the individual experiments and the skill of the operator. Systematic variation of a single factor at a time while holding all remaining factors constant sequentially produces individual optima, and can be successful only when the variables are independent of each other. Moreover, to reach real optimum conditions is usually difficult and can be attained slowly, by performing a huge number of experiments.

The sequential simplex method, does not use traditional testing of significance and therefore faster and simpler than the univarient method. Also it attains the experimental optimum rapidly, guided by calculations and decisions that are rigidly specified, this makes it particularly attractive to automated optimization.

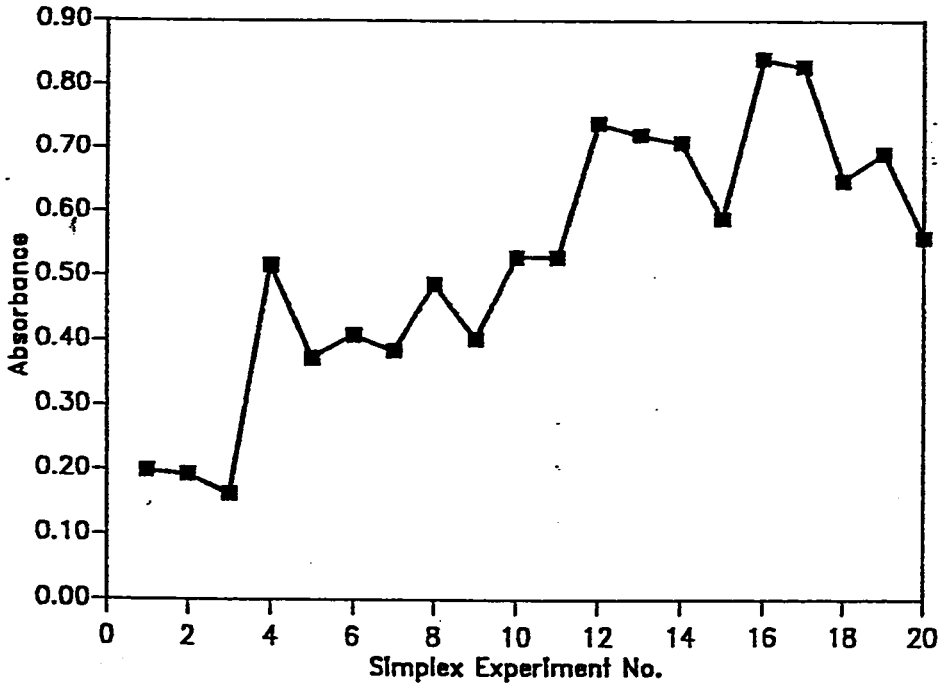
The modified simplex procedure was performed according to the procedure described previously (Chapter 3). The results are shown in Table 4.1.1 and plotted in Fig. 4.1.7. For a four variable system, five initial points were needed to form the first simplex. Then a new simplex is generated by the computer, by replacing one of the five points in the current simplex by a new point.

Table 4.1.1 Simplex optimization of chemical aand FIA variables for tetracyclineHCl

Exp #	Coil length cm	Flow rate ml/min	Acid conc. M	Reagent conc. ppm	peak absorbance
(1)	100	4.78	0.050	100	0.198
(2)	300	4.78	0.050	100	0.192
(3)	45	5.31	0.50	100	0.162
(4)	45	3.90	0.010	300	0.516
(5)	45	3.90	0.100	600	0.372
(6) R	200	3.72	0.055	450	0.408
(7) C	84	4.12	0.030	290	0.381
(8) R	136	3.72	0.046	544	0.462
(9) C	81	4.04	0.029	311	0.402
(10) R	45	3.72	0.035	649	0.528
11) E	45	3.72	0.028	924	0.528
(12) R	82	3.99	0.001	311	0.738
(13) E	101	4.03	0.001	167	0.720
(14) R	56	3.84	0.006	528	0.708
15) C	85	3.99	0.009	293	0.588
(16) R	55	3.72	0.001	554	0.840
(17) E	45	3.72	0.001	685	0.828
(18) R	93	3.83	0.009	736	0.648
(19) C	57	3.79	0.005	602	0.690
(20) C	99	4.05	0.001	50	0.558

C, contraction; R, reflection; E,expansion

Fig.4.1.7. Simplex optimization for tetracyclineHCl.



In Table 4.1.1, the reflections are marked R, expansions E, and contractions C. Points 1-5 which represent the first cycle covers an optimization area of 20 - 60%. The best point attained was point 4 with a peak absorbance of 0.516 and the worst point was point 3 with a peak absorbance of 0.162. Therefore, point 3 was reflected through the centroid of other points to obtain point 6. An experiment was then performed, utilizing the variables setting at the reflected point, and an absorbance of 0.408 was obtained. Since this value was better than that at point 3, point 3 was rejected and replaced by point 6. A contraction was then performed since this point is not better than the best point, point 4. Then by using of the experimental setting of variables generated by contraction, a peak absorbance of 0.384 was obtained, which is not better than the reflection point, then this completes the cycle. The reflection at point 10, gives a response better than the best one leading to an expansion point. The response of the expansion point was the same as that at the reflection point, therefore the reflection point replaced the worst point of the previous simplex. Point 16 represent the highest response obtained. However, attempts were made to reach further improvement towards maximization but this was found to cause more experiments, and does not worth the effort involved.

Also one of the reasons which led to termination of the procedure at this stage is that the results obtained at point 16 were very satisfactory. The maximum sample throughput at the maximum sensitivity was found to be 160 s/h.

The results obtained by the modified simplex procedure were found to resemble those obtained by the univarient method, except the flow rate, and reaction coil length. Lower flow rates were obtained by the simplex method namely 3.72 ml/min, compared to appreciably higher flow rates in the univarient method, where the optimum flow rate obtained was 4.10 ml/min. Also a lower reaction coil length was obtained by the simplex method 55 cm compared to 120 cm by the univarient method. This could be entirely in agreement with the FIA rules[84], where to increase the residence time (t) one either increases coil length or decreases flow rate.

The modified simplex method investigates the variation of sensitivity over a shorter range than do the univarient methods. This is shown clearly by the scattered diagrams 4.1.8 - 4.1.11. Fig. 4.1.8 showed that the simplex program investigated the sulphuric acid concentration in the range 0.001 - 0.050M, whereas in the univarient method the acid was varied in a wider range up to 0.5M. The reaction coil investiga-

tion, as shown in Fig. 4.1.9, was concentrated on the range 45 - 100 cm, compared to the range up to 400 cm in the univarient method. The same behaviour was observed for the flow rate, Fig. 4.1.10, and the iron(III) concentration, Fig. 4.1.11, where investigations were limited to the ranges 3.7 - 4.2 ml/min and 50 - 1000 ppm, respectively.

The number of experiments needed to reach optimum operating conditions by the simplex method was 16 experiments, compared to 50 experiments conducted by the univarient method.

The best operating conditions chosen for the determination of tetracyclineHCl were those obtained by the modified simplex method. This is because shorter coil length and lower flow rates give better sample and reagent economy, besides the sensitivity obtained by this experimental setting was comparable to that obtained by the univarient method. The peak absorbances obtained by the experimental settings of the simplex and the univarient method for the same concentration of drug were 0.840 and 0.814 respectively. Therefore, a coil length of 55 cm, a flow rate of 3.72 ml/min, a sulphuric acid concentration of 0.001 M, and an iron(111) concentration of 554 ppm were used for the determination of tetracyclineHCl in pharmaceutical preparations.

Fig.4.1.8 Simplex variation of acid concentration for tetracyclineHCl.

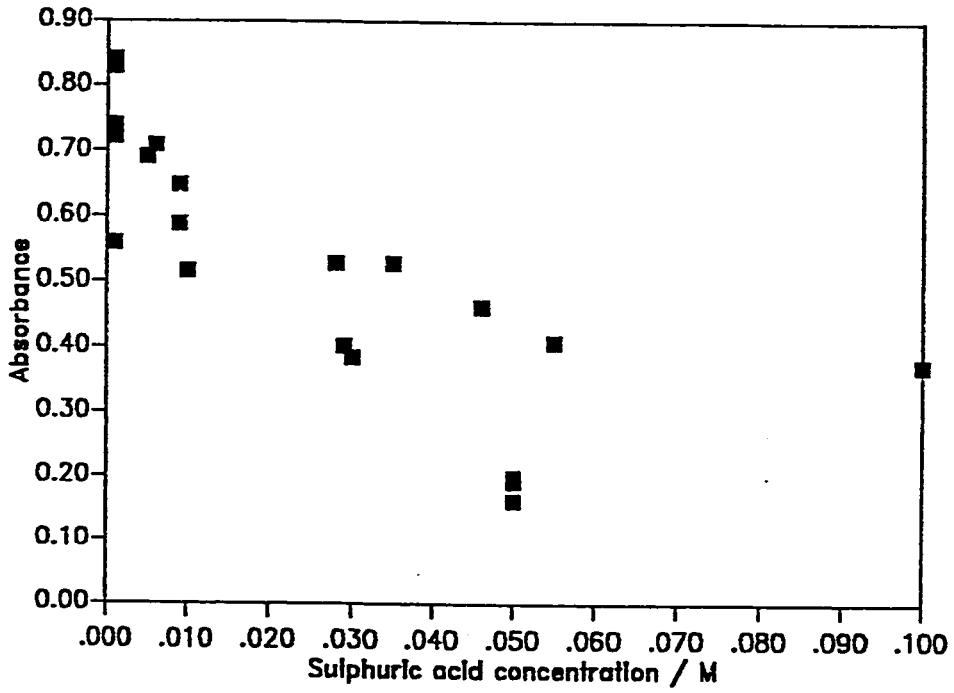


Fig.4.2.9 Simplex variation of coil length for tetracycline HCl

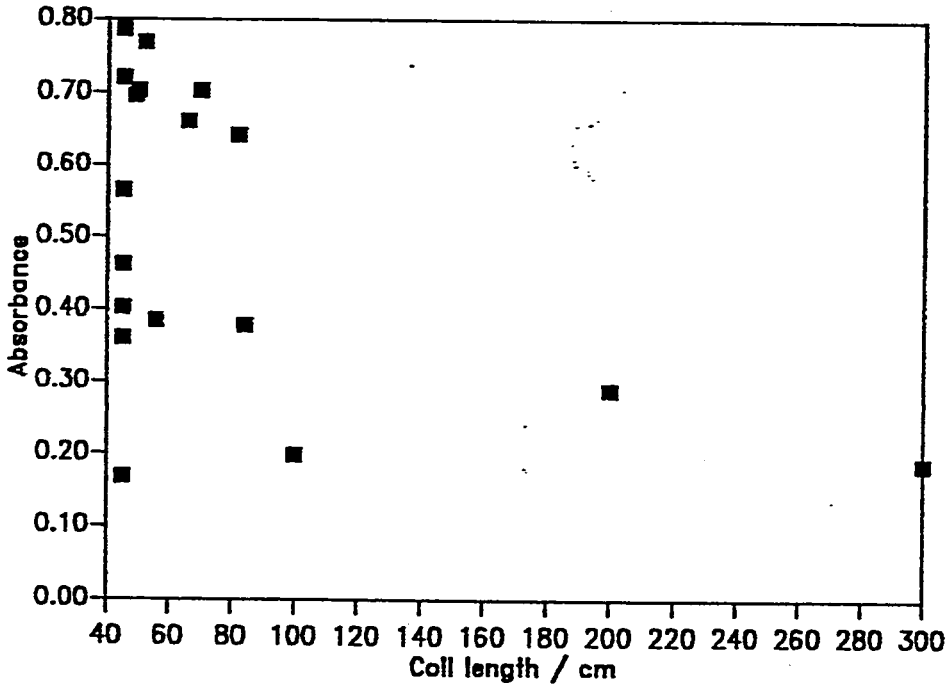


Fig.4.1.10 Simplex variation of flow rate for tetracyclineHCl

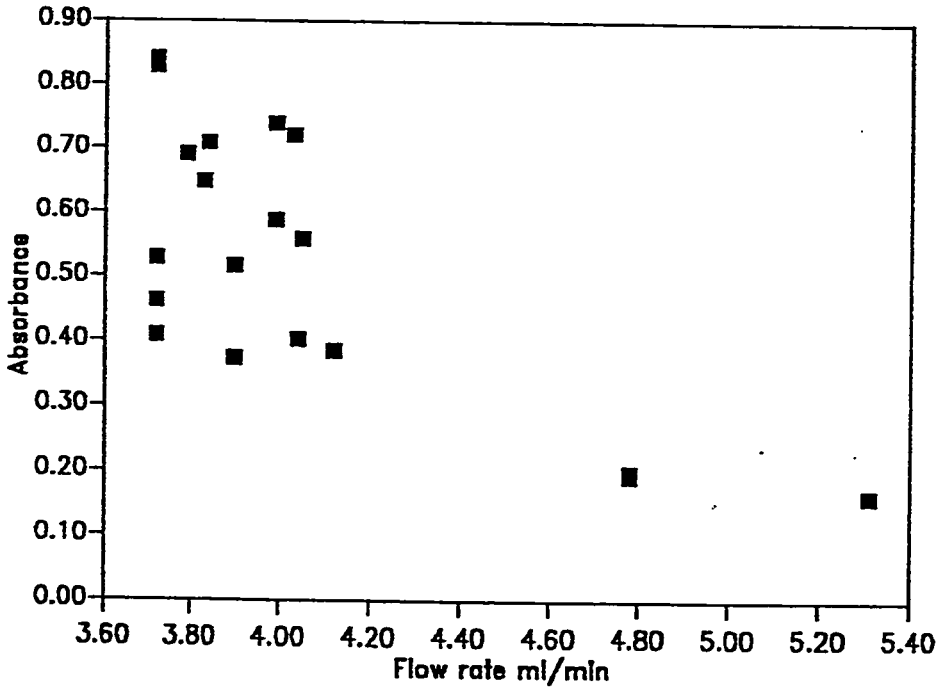
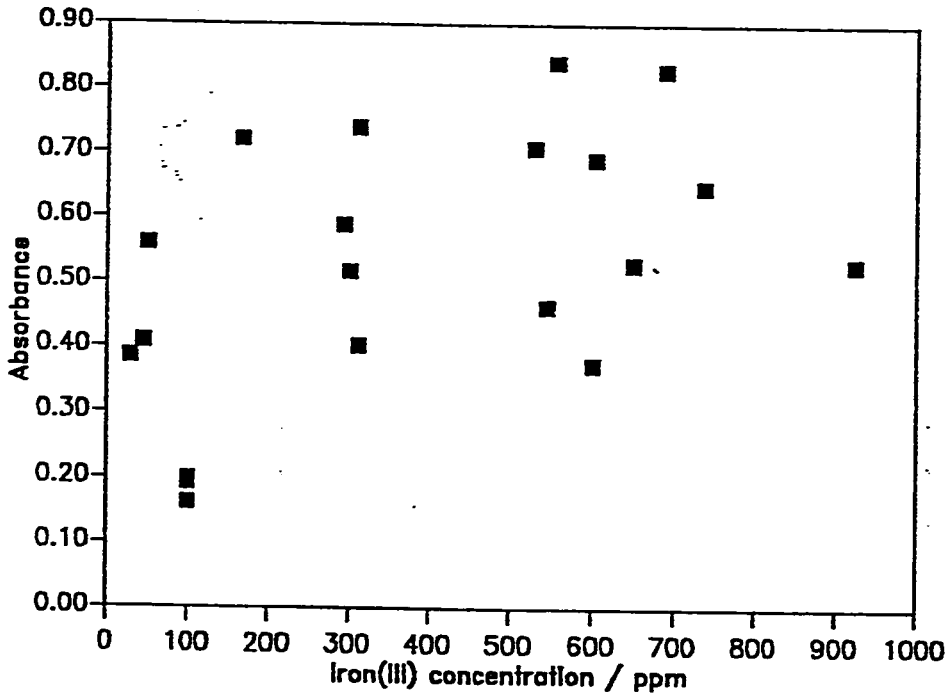


Fig.4.1.11 Simplex variation of iron(III) concentration for tetracyclineHCl.



4.1.3 Analytical Appraisal

Beer's law was found to be valid in the range of 20 - 200 $\mu g\ ml^{-1}$ tetracyclineHCl, the lower detection limit was slightly higher than that for the conventional spectrophotometric method (10 $\mu g\ ml^{-1}$). rules[1]

Accurately about 0.25000 gm of pure tetracycline-HCl was weighed. Different standard tetracyclineHCl solutions were prepared by adding an appropriate volume of stock tetracyclineHCl solution to a 50-ml volumetric flask, and the volume was then completed to the mark with 0.001M sulphuric acid.

Triplicate volumes of 157 μl of the standard solutions were injected into the carrier stream. The carrier stream consists of Iron(III) (554 ppm) dissolved in 0.001M sulphuric acid, flowing at the rate of 3.72 ml/min. The absorbance of the flowing stream is monitored at 423 nm, after crossing a reaction coil length of 55 cm. The results of the proposed method are presented in Table 4.1.2 and Fig. 4.1.12. The measured peak absorbancies are plotted versus different tetracyclineHCl concentrations as shown in Fig. 4.1.13 and Fig. 4.1.14.

The straight line was obtained in the range of 20 - 200 $\mu g\ ml^{-1}$, with a correlation coefficient (r) of 0.9994 and an intercept of 0.0249.

Table 4.1.2: Beers's law validation for tetracyclineHCl with the FIA proposed method.

concentration ppm	Absorbance
30	0.048
40	0.109
60	0.151
80	0.192
100	0.228
120	0.264
140	0.300
160	0.336
180	0.366
200	0.408
220	0.450
260	0.486
280	0.489

* Intercept = -0.0249
**Correlation coefficient for 13 different concentrations in the range 30 - 280 is 0.9994.

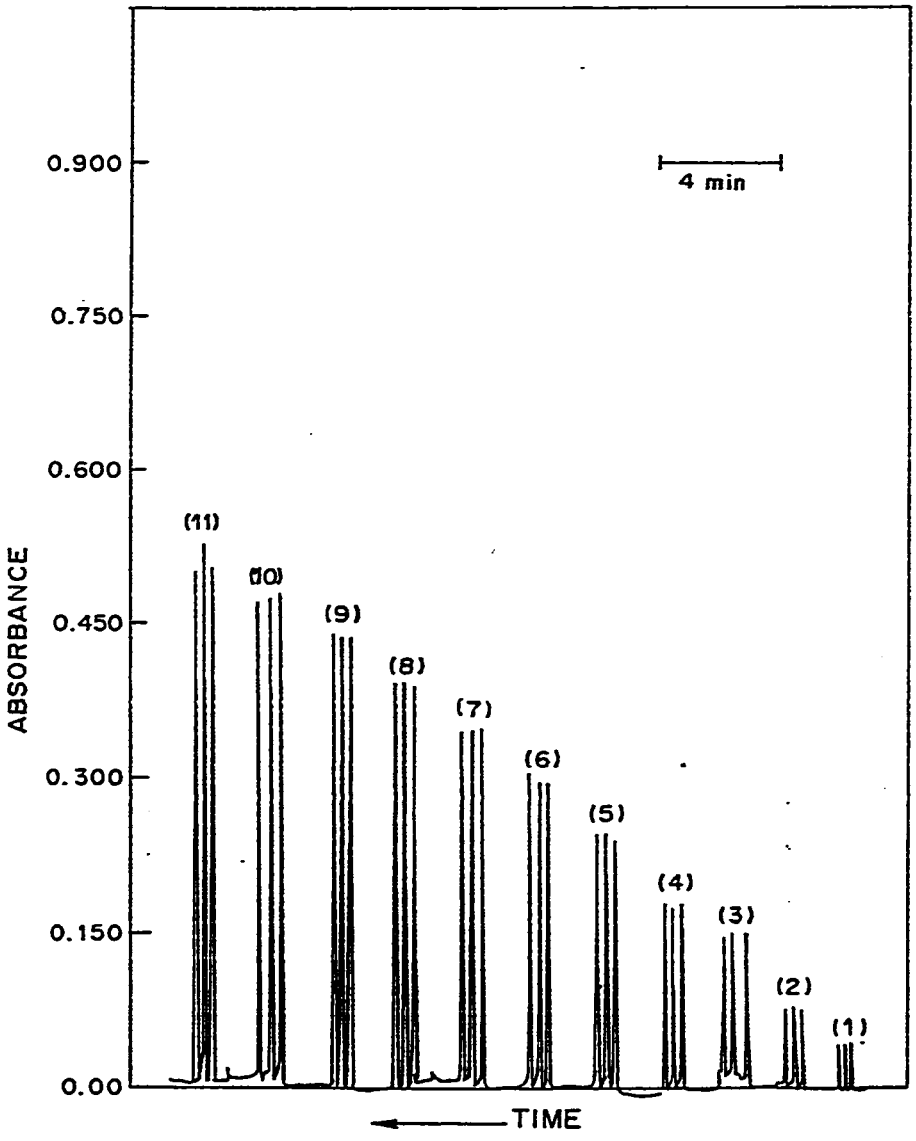


Fig.4.1.12. Typical FIA results (n = 3) for tetracyclineHCl standard solutions of : (1)30 (2)40 (3)60 (4)80 (5)100 (6)120 (7)140 (8)160 (9)180 (10)200 (11)220 ppm.

520

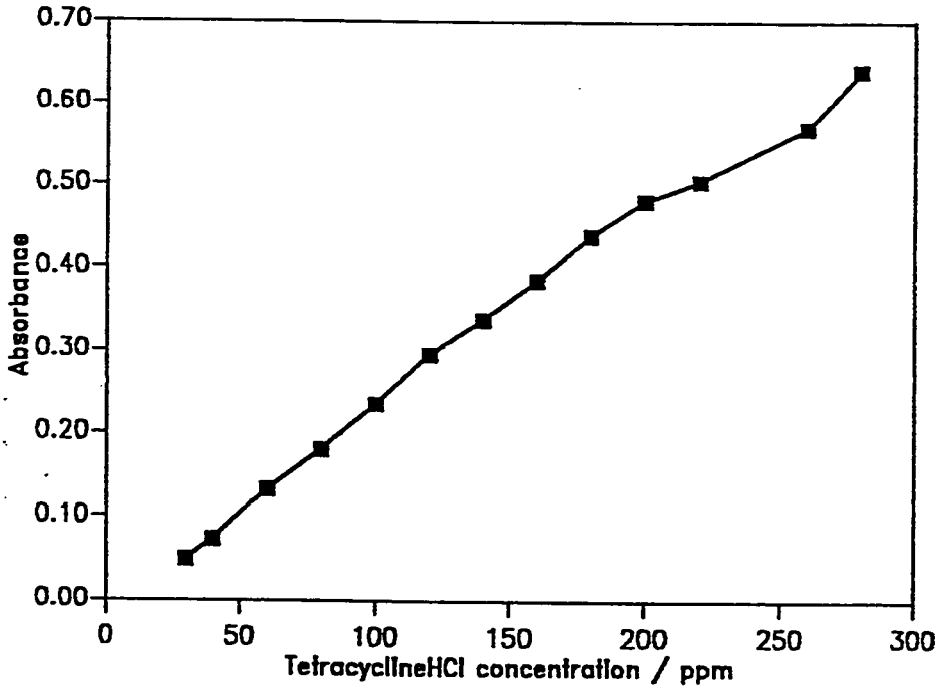


Fig.4.1.13 A typical computer plot of absorbance vs tetracyclineHCl concentration with the FIA proposed method.

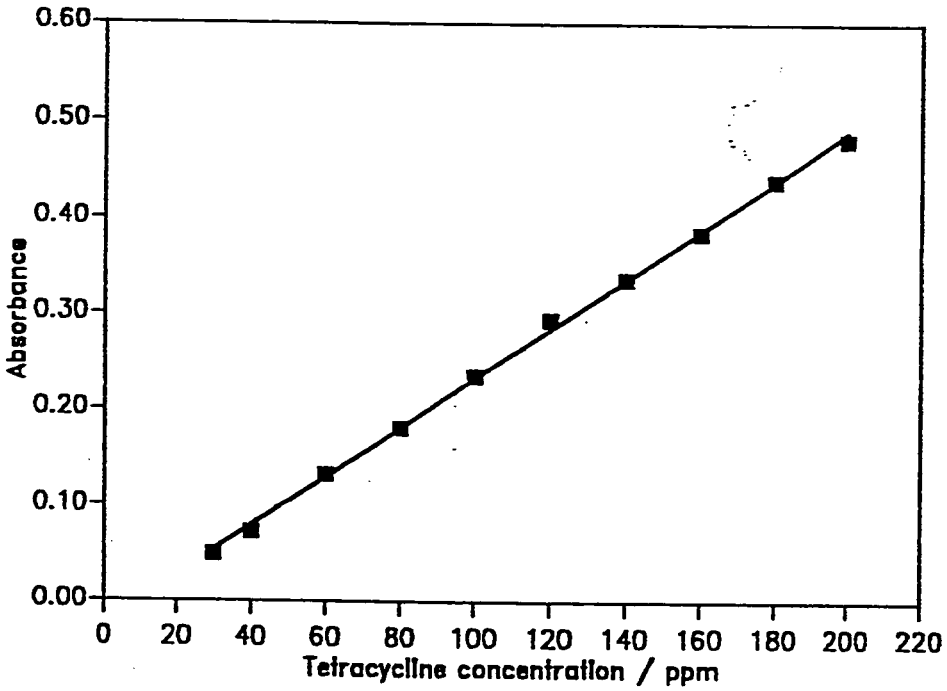


Fig.4.1.14 A typical calibration curve for tetracycline HCl in the range 20 - 200 ppm.

Above 200 $\mu g\ ml^{-1}$, there is a deviation towards the concentration axis, and the readings were not reproducible. Then, a calibration equation of the following type is obtained in the linear region:

$$A = - 0.0249 + 0.0026\ C \qquad (4.2)$$

where

A = absorbance at 423, C = concentration of tetracycline-HCl in $\mu g\ ml^{-1}$, r = 0.9994.

This equation was then used for determination of unknown concentrations of tetracyclineHCl directly by taking exact peak absorbance at the conditions specified.

The peak width (w) at 60% of the peak height was calculated as a measure of the total sample dispersion in the system, and it was found to be 3 sec. The peak width at the baseline was found to be 22 sec, from which the maximum sample throughout was found to be 160 sample/h. Repeatability for the system was confirmed by injecting one of the sample solutions seven times and estimating the relative standard deviation (RSD), this was found to be 0.57.

DETERMINATION OF TETRACYCLINE DERIVATIVES

4.2.1. Introduction

Tetracyclines have a common skeleton[10] which can be considered an octahydro analogue of naphthacene but they differ in the specific substituents, and these have significant effects on the solubility and stability of the compounds, and their complexes with cations. As mentioned previously, iron(III), has been used for the spectrophotometric determination of these compounds in a sulphuric acid media[1]. Also, for these compounds, as for tetracyclineHCl, a FIA method for quantitative determination had to be developed, utilizing the spectrophotometric method of determination of tetracyclines by iron(III)[1]. Optimization of chemical and FIA variables would rather be worked out for this compound as follows:

4.2.2. Determination of Oxytetracycline

Oxytetracycline is a yellow, optically active and amphoteric compound with an empirical formula $C_{22}H_{24}N_2O_9.2H_2O$ and a molecular weight of 496.891. It is the first biosynthetic antibiotic of the tetracycline group whose structural formula has been elucidated. It was elab-

orated by *Streptomyces rimosus* and it was first reported in 1950 by Finlay et al[6]. The structural formula is shown in Fig. 4.2.1.

In this part of this section, a quantitative FIA method for the analysis of oxytetracycline will be investigated, on the basis of the complexation of oxytetracycline with iron (III) in sulphuric acid media. The brown complex of iron(III)-oxytetracycline absorbs at λ_{max} 423 nm as reported earlier[1]. Pure oxytetracycline dissolved in water has a characteristic λ_{max} at 354 nm.

4.2.2.1. Optimization

As in tetracycline-HCl, the chemical and FIA variable were varied, by a modified simplex and an iterative univarient methods. The procedure was carried out in the same way as in tetracycline-HCl, so as to reach the best conditions for the assay of oxytetracycline, the details of the optimization procedure is as follows:

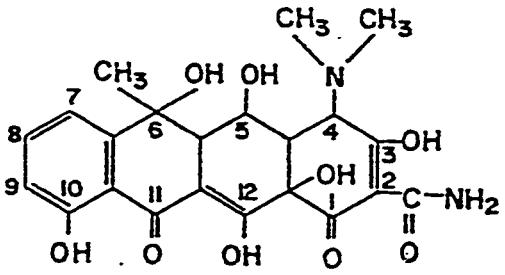


Fig. 4.2.1 Structural formula of oxytetracycline

4.2.2.1.1 Variation of Sulphuric Acid Concentration

Sulphuric acid concentration was also studied in the range of 0.001 - 0.50M, concentrations below 0.001M could result in hydrolysis of iron(III); whereas at concentrations higher than 0.50M disproportionation of the complex was expected as reported earlier. The other variables were kept constant at the following values: coil length was 45 cm, flow rate 3.9 ml/min sample volume 110 μl, and iron(III) concentration at 50 ppm as for the first cycle. In the second cycle the conditions were 45 cm, 4.2 ml/min 157 μl, and 600 ppm for coil length, flow rate, sample loop size and iron(III) concentration respectively. The effect of sulphuric acid concentration on the peak absorbance is similar to that for tetracycline-HCl. The results obtained were plotted in Fig. 4.2.1. The maximum peak absorbance 0.380 and 0.900 was obtained in the first and the second cycle respectively, at 0.001 M sulphuric acid concentration. In both cycles, the peak absorbance became almost constant beyond 0.1 M sulphuric acid concentration, which previously mentioned to be due to disproportionation of the complex. Also the increase in acid concentration resulted in a sharp decrease in peak absorbance, indicating that the acid concentration is a very important factor. Also improvement of conditions after the first cycle led to a great improvement in the peak absorbance in the second cycle. There-

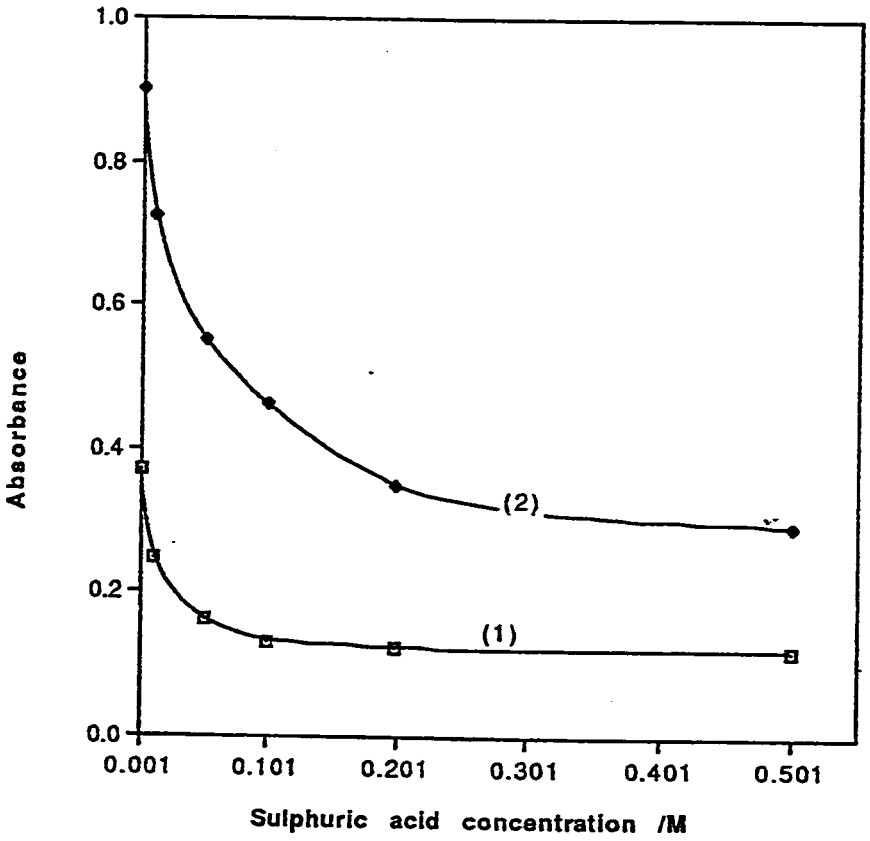


Fig 4.2.2. Variation of sulphuric acid concentration for oxytetracycline (1) first cycle where coil length is 45 cm, flow rate is 3.90 ml/min and sample volume is 110 μl, and iron(III) concentration is 50 ppm (2) second cycle, where coil length is 45 cm, flow rate is 4.20 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

fore, the optimum sulphuric acid concentration was chosen to be 0.001 M.

4.2.2.1.2. Variation of Reaction Coil Length

The reaction coil length represents the residence time "t", the reaction mixture can spend before reaching the detector. Therefore, to increase the residence time one of the choices is to increase the reaction coil length. But, by increasing the reaction coil length, the dispersion of the sample zone increases dramatically and eventually the peak absorbance will be greatly reduced. Putting these two factors into consideration, we have varied the reaction coil length between 45 - 400 cm, using 0.5 mm id. tubing. Coil lengths below 45 cm is limited by the physical distance between the injection port and the detector. The variation of the coil length was studied while the other factors were fixed at constant values. In the first cycle, the values of these variables were as follows: 0.001M, 3.9 ml/min 110 μl, and 50 ppm for sulphuric acid concentration, flow rate, sample loop size and iron(III) concentration respectively, whereas the values in the second cycle were 0.001 M, 4.20 ml/min 157 μl, and 600 ppm respectively. The results obtained were plotted in Fig. 4.2.3. In the first cycle, it is clear that the dispersion factor prevails, that is the increase in coil length

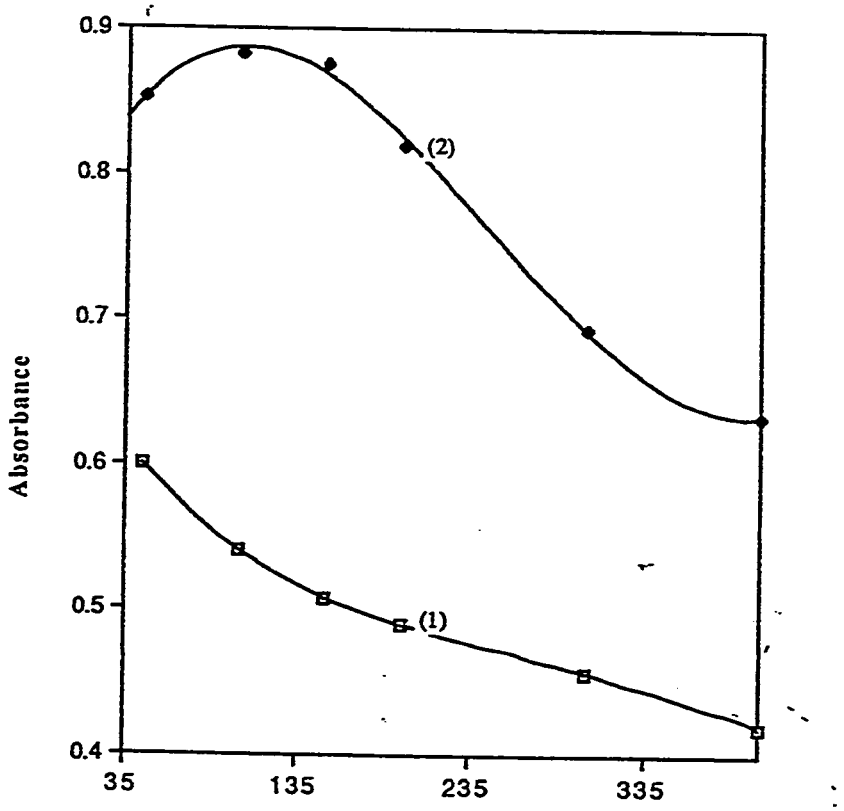


Fig 4.2.3. Variation of coil length for oxytetracycline (1) first cycle where sulphuric acid concentrationis 0.001M, flow rate is 3.9 ml/min, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, flow rate is 4.20 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

resulted in a decrease of the peak absorbance. In th second cycle, the peak absorbance increases with the coil length, reaches a maximum at 100 cm, then it starts to decrease to reach its minimum value at 400 cm. The residence time needed for complete mixing is also affected by the flow rate. Increasing the flow rate resulted in decreasing the residence time in the reaction coil. From these facts, one can say that the flow rate and the coil length are closely related and interacting variables. Therefore, to compensate for the shorter residence time resulted from the increase in flow rate, from 3.9 to 4.2 ml/min an increase in the coil length is observed. The decrease in the peak absorbance after that could be explained on the basis of the dilution of the resulted complex by dispersion. From this discussion, the optimum reaction coil length was found to be 100 cm.

4.2.2.1.3. Variation of Flow Rate

The flow rate measured in ml/min is easily regulated by the peristaltic pump as mentioned before. The relation between the pump speed in ramps and the flow rate in ml/min is stated in Eq. (4.1). The pump tubing used is 1.3 mm i.d. The conditions under which the flow rate was studied were as follows: in the first cycle, 0.001M, 45 cm, 110 μl,

and 50 ppm for sulphuric acid concentration, coil length, sample volume and Fe(III) concentration respectively, whereas in the second cycle, the conditions were 0.001M, 100 cm, 157 μl, and 600 ppm respectively. The results obtained were plotted in Fig. 4.2.4. The peak absorbance in the first and the second cycle increases with the flow rate, until it reaches a maximum value then it starts to decrease rapidly with the increasing flow rate. As mentioned before, a compromise should be made between the flow rate and the coil length to reach to the optimum residence time needed to complete mixing. The optimum flow rate obtained for the first cycle is 4.2 ml/min and that for the second cycle is 3.80 ml/min. Comparing these two values to the values of the corresponding coil lengths, 45 cm and 100 cm for the first and second cycle respectively, one can see these values are contraindicating to the previous discussion. Actually, this is not the case because the conditions for the variation of the flow rates were different, and this indicates that, there is a considerable interaction with other factors. Therefore, the optimum flow rate found by the univarient method is 3.80 ml/min

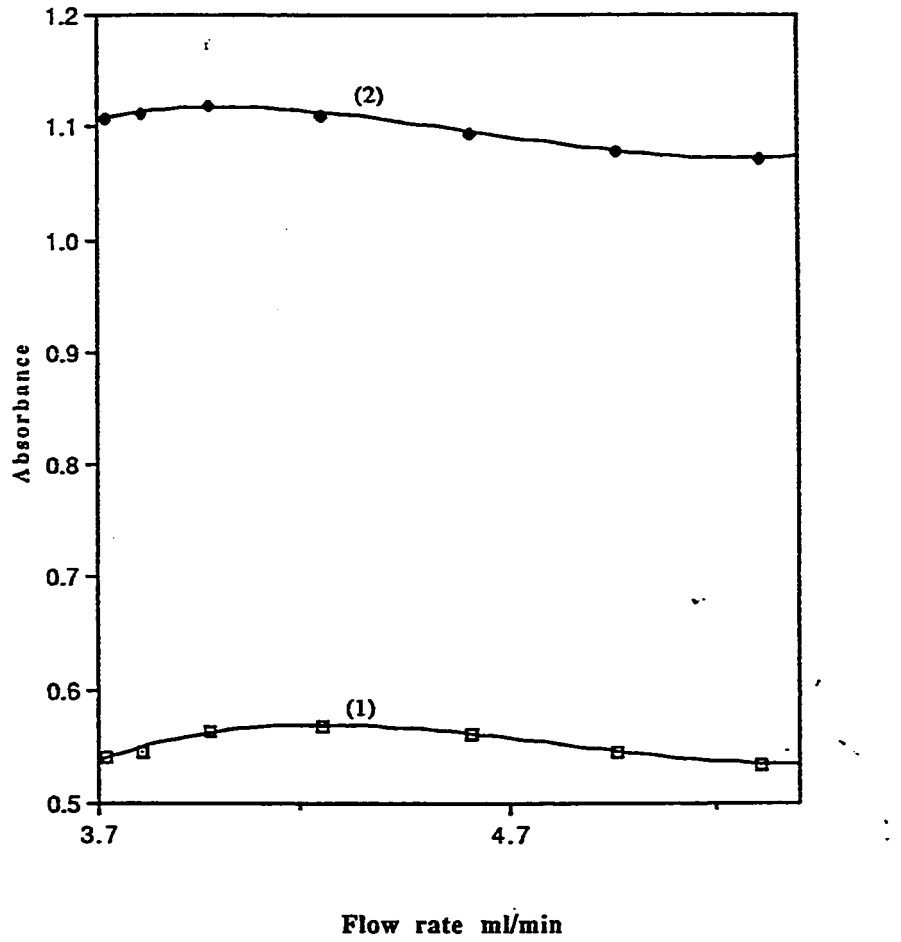


Fig 4.2.4. Variation of flow rate for oxytetracycline (1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 45 cm, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 100 cm, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

4.2.2.1.4. Variation of Sample Volume

The sample volume was varied between 110 - 204 μl, by changing the sample loop size. The values of the other factors were kept constant. In the first cycle, the conditions were as follows, 0.001M, 45 cm, 4.2 ml/min and 50 ppm for sulphuric acid concentration, coil length, flow rate and iron(III) concentration respectively. In the second cycle, the conditions were 0.001M, 100 cm, 3.81 ml/min and 600 ppm for sulphuric acid concentration, coil length flow rate and iron(III) concentration respectively. The results obtained were shown in Fig. 4.2.5. In the first cycle, a considerable increase in peak absorbance with the sample loop size is observed, but this is not the case in the second cycle where the increase in peak absorbance is insignificant as with flat upper curve. At high sample loop sizes, the peak produced were irreproducible, therefore for both cycles 157 μl was found to be the optimum sample loop size.

4.2.2.1.5. Variation of Iron(III) Concentration

The concentration of iron(III) was varied between 50 - 2000 ppm In the first cycle, the conditions in which the iron(III) concentration was varied, are 0.001M sulphuric acid, 45 cm coil length, 4.2 ml/min flow

rate, and 157 μl loop size. In the second cycle, the sulphuric acid concentration was 0.001M, the coil length was 100 cm, the flow rate was 3.82 ml/min and the sample loop size was 157 μl. The results obtained were presented in Fig. 4.2.6. The peak absorbance increases with increasing the iron(III) concentration, over the concentration of 600 ppm the increase in the peak absorbance was not sharp, and a lot of problems were necessary to practice eliminating the blank absorbance by adjusting the detector needle to zero. Also very irreproducible results were observed at high Fe(III) concentrations. Since one of the aims of using FIA techniques is to improve the sample and reagent economy, the iron(III) concentration chosen for the optimum conditions was 600 ppm, for both first and second cycle.

In all variations discussed above, it is observed that improvement of peak absorbance in the second cycle is considerably high, and this, as has been mentioned above, is due to improvement of reaction conditions by optimization of the variables in the first cycle. Interaction of variables was also observed, specially in the case of coil length and flow rate. The optimum conditions selected were, 0.001M, 100 cm, 3.82 ml/min 157 μl and 600 ppm for sulphuric acid concentration, coil length, flow rate, sample loop size and iron(III) concentration concentration.

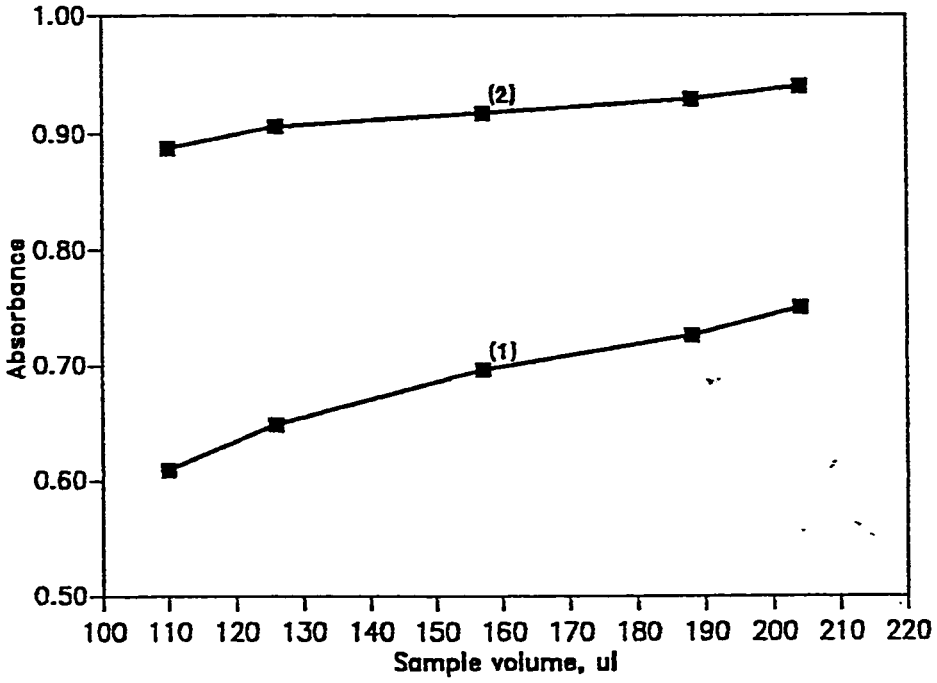


Fig 4.2.5. Variation of sample loop size for oxytetracycline (1) first cycle where sulphuric acid concentration is 0.001M, coil length is 45 cm, flow rate is 4.20 ml/min, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 100 cm, flow rate is 3.81 ml/min, and iron(III) concentration is 600 ppm.

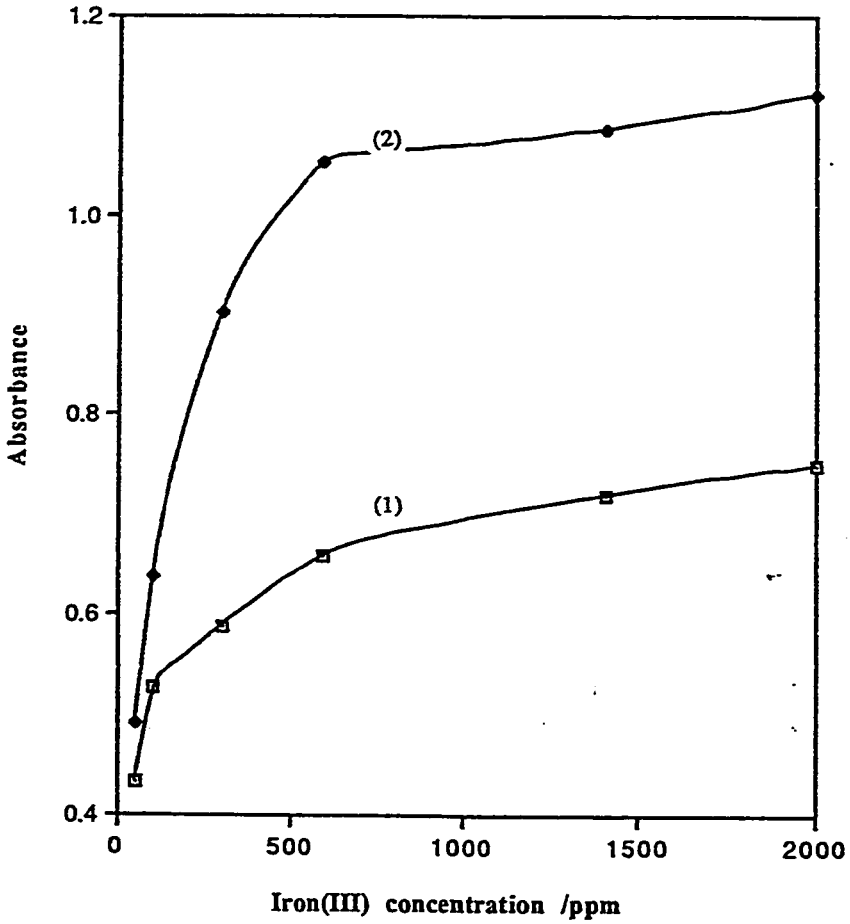


Fig 4.2.6. Variation of iron(III) concentration for oxytetracycline (1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 45 cm, flow rate is 4.20 ml/min and sample volume is 110 μl, (2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 100 cm, flow rate is 3.82 and sample volume is 157 μl.

4.2.2.2. Simplex Optimization

As could be seen from the univarient optimization for oxytetracycline, interaction between the variables is clearly manifested. The modified simplex procedure was then carried out. Five experiments were needed to form the initial simplex. Points 1 to 5 represent the initial simplex and covers 20 - 60% of the optimization area. The results obtained by the simplex method were presented in Table 4.2.1. and plotted in Fig. 4.2.7

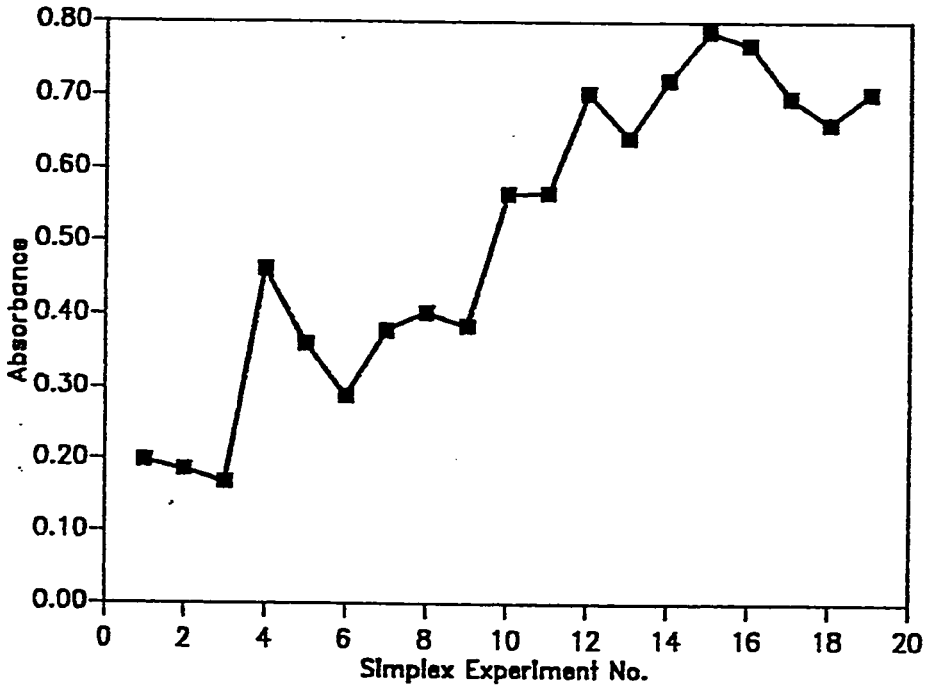
In Table 4.2.1. the reflections are marked R, expansions E, and contractions C. Point 3 which is the point with the worst response, was reflected through the centroid to give point 6. The reflected point gave a combination of experimental conditions, an experiment was carried out to get the response at this point. The response at this point was found to be better than that at all points except that at point 4, which is the best point, and point 5. Then the value of R was entered into the computer, a contraction was obtained, point 7, the peak absorbance of point 7 was measured, and it was found to be 0.378, which is better than that at the reflected point, 0.288, therefore, point 3 was rejected and replaced by point 7. The first expansion was obtained at point 11, the reflection which led to this expansion was having a peak

Table 4.2.1: Simplex optimization of chemical and FIA variables for oxytetracycline

EXP #	Coil Length /cm	Flow Rate/ ml min^{-1}	$[H_2SO_4]$ /M	[iron(III)] / ppm	Peak absorbance
(1)	100	4.78	0.050	100	0.198
(2)	300	4.78	0.050	100	0.186
(3)	45	5.31	0.050	100	0.168
(4)	45	3.90	0.010	300	0.462
(5)	45	3.90	0.100	600	0.36
(6) R	200	3.72	0.055	450	0.288
(7) C	84	4.12	0.031	288	0.378
(8) R	45	3.72	0.046	544	0.402
(9) C	56	4.04	0.029	311	0.384
(10) R	45	3.72	0.035	649	0.564
(11) E	45	3.72	0.028	924	0.564
(12) R	70	4.00	0.001	311	0.702
(13) E	82	4.03	0.001	167	0.642
(14) R	45	3.73	0.002	563	0.72
(15) E	45	3.72	0.001	701	0.786
(16) R	52	3.72	0.001	734	0.768
(17) C	49	3.78	0.005	612	0.696
(18) R	66	3.73	0.008	902	0.660
(19) C	50	3.77	0.005	651	0.702

R = Reflection, C = Contraction, E = Expansion

Fig.4.2.7 Simplex optimization for oxytetracycline.



absorbance of 0.564, the peak absorbance of the expansion at point 11, was also 0.564. Therefore, the reflection point replaced the worst point of the previous simplex and the cycle is completed. The best value for the peak absorbance was obtained at point 15. Additional experiments were made to get more improvement, but as shown in Table 4.2.1., this could result in more experiments, and was found not to be worth to the effort involved. Also, the results obtained at point 15 were very satisfactory. The maximum throughput at this point was found to be 170 s/h.

The results obtained by the simplex method were comparable to those obtained by the univarient method. Shorter coil length, 45 cm and lower flow rate of 3.72 ml/min were obtained by simplex method. Compared to 100 cm, and 3.82 ml/min obtained by the univarient method, for reaction coil length and flow rate respectively. The acid concentration obtained by the simplex method is the same as that obtained by the univarient method. The iron(III) obtained by the simplex method was 701 ppm, which is compatible to that obtained by the univarient method, but a little higher.

The modified simplex method shows the variation of variables to obtain the best sensitivity over a narrower range than do the univa-

rient method. In Figs. 4.2.8 - 4.2.11. the simplex data are represented as a series of scattered diagrams, this representation enables the same qualitative judgements to be made as with the iterative univarient procedure.

The number of experiments needed to reach optimum operating conditions by the simplex method was 15 experiments, compared to 52 experiments in case of the univarient method.

The optimum condition for the determination of oxytetracycline were proposed to be those obtained by the simplex procedure, where shorter coil length, and lower flow rate was obtained. Thus a better sample and reagent economy is obtained. The sensitivity obtained by the simplex method and the univarient method using the same drug concentration were comparable, namely 0.742 in the case of univarient method and 0.786 in case of simplex procedure. Therefore, a coil length of 45 cm, a sulphuric acid concentration of 0.001M, a flow rate of 3.72 ml/min and an iron(III) concentration of 701 ppm were used for the determination of oxytetracycline.

Fig.4.2.8 Simplex variation of acid concentration for oxytetracycline.

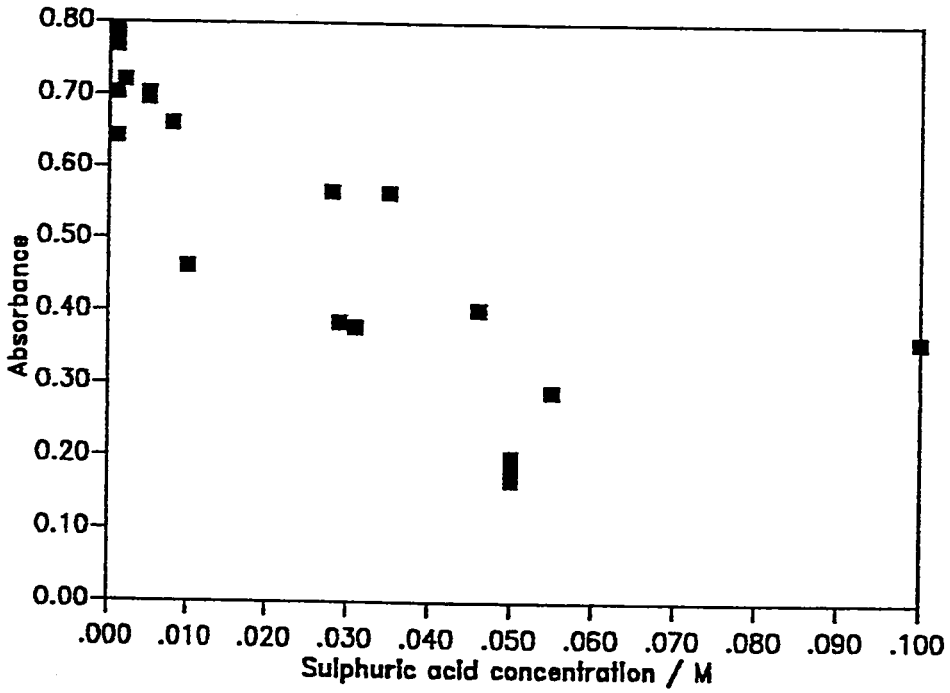


Fig.4.2.9 Simplex variation of coil length for oxytetracycline .

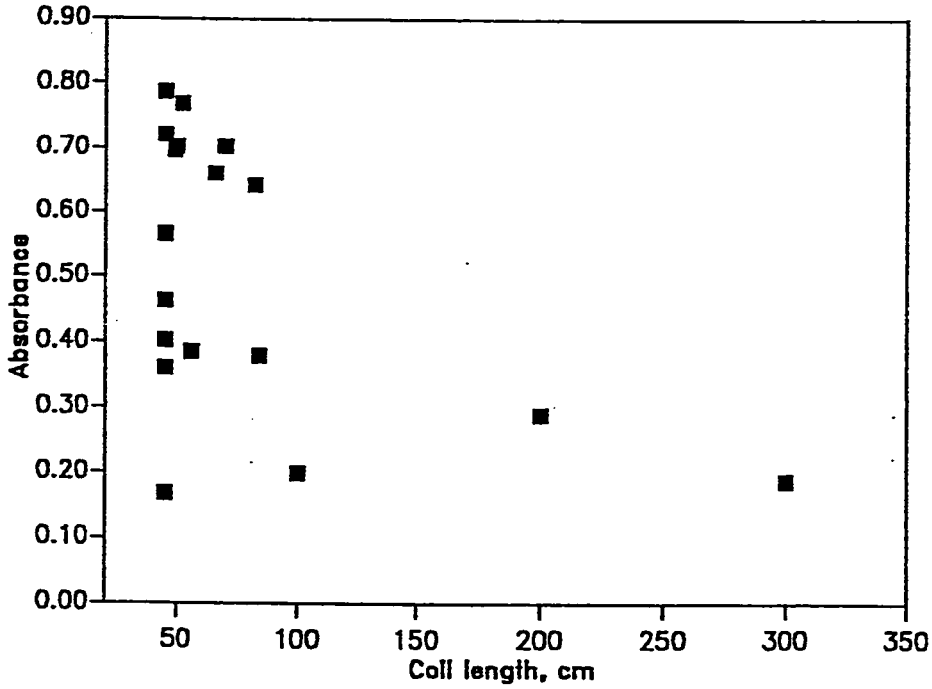


Fig.4.2.10 Simplex variation of flow rate for oxytetracyclineHCL.

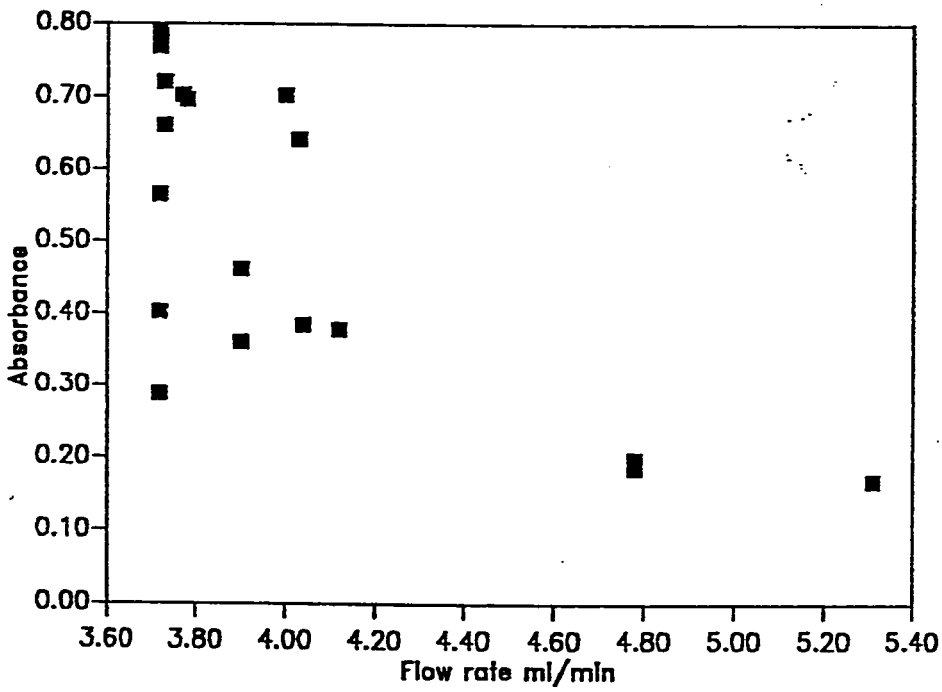
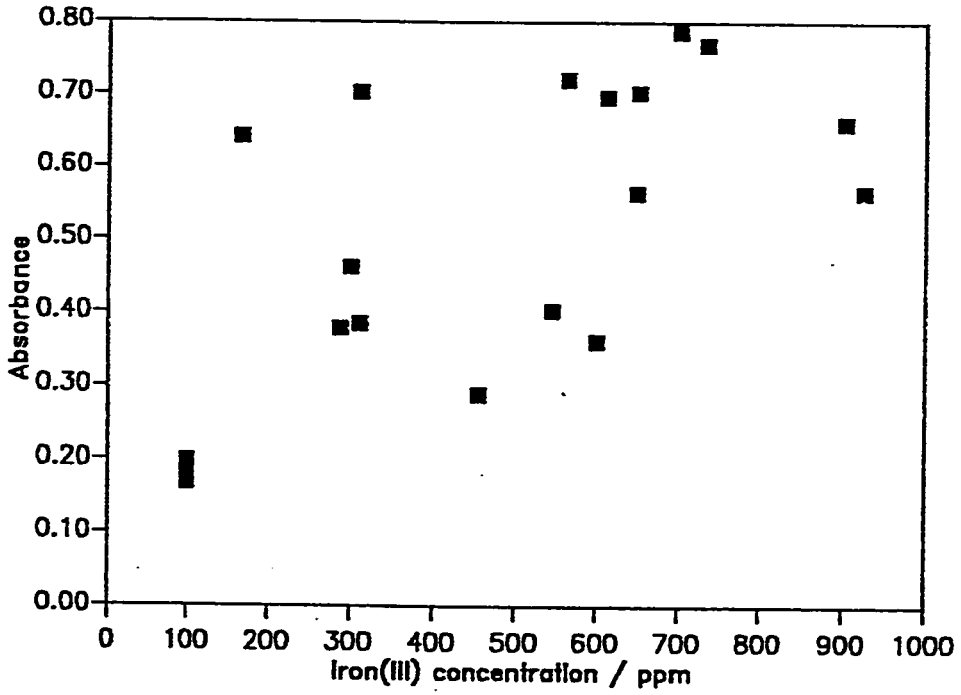


Fig.4.2.11 Simplex variation of Fe(III)concentration for oxytetracycline.



4.2.2.3. Analytical Appraisal

Beer's law was found to be valid in the range of 40 - 260 $\mu g\ ml^{-1}$. Accurately about 0.25000 g of pure oxytetracycline is weighed, dissolved in 0.001M sulphuric acid, and completed to volume to 250 ml with 0.001M sulpuric acid. Different standard oxytetracycline solutions were prepared by adding an appropriate volume of stock solution to 50 ml volumetric flasks, and the flasks were completed to volume with 0.001 M sulphuric acid.

The standard solutions were run in triplicate, by injecting a volume of 157 μl into the carrier stream. The carrier stream consists of 701 ppm iron(III) and 0.001 M sulphuric acid. The absorbance of the resulting iron(III)-oxytetracycline complex was monitored at 435 nm. The results of the proposed method are presented in Table 4.2.2. A typical recorder tracing for these results are presented in Fig. 4.2.12. The measured peak absorbancies are plotted versus different oxytetracycline concentrations as shown in Fig. 4.2.13 and Fig. 4.2.14.

The straight line was obtained in the range of 40 - 260 $\mu g\ ml^{-1}$ with a correlation coefficient (r) of 0.9996 and intercept of 0.000245. Above 260 $\mu g\ ml^{-1}$ there was a deviation towards the concentration axis. Therefore, a calibration equation of the following type was obtained:

Table 4.2.2: Beer's law validation for oxytetracycline with the FIA proposed method.

concentration ppm	Absorbance
40	0.072
60	0.210
80	0.152
100	0.191
120	0.227
140	0.263
160	0.299
180	0.335
200	0.365
220	0.407
240	0.451
260	0.485
280	0.490
300	0.515

* Intercept = 0.0019
** Correlation coefficient for 12 different determinations in the range 40 - 260 ppm;is equal to 0.9996

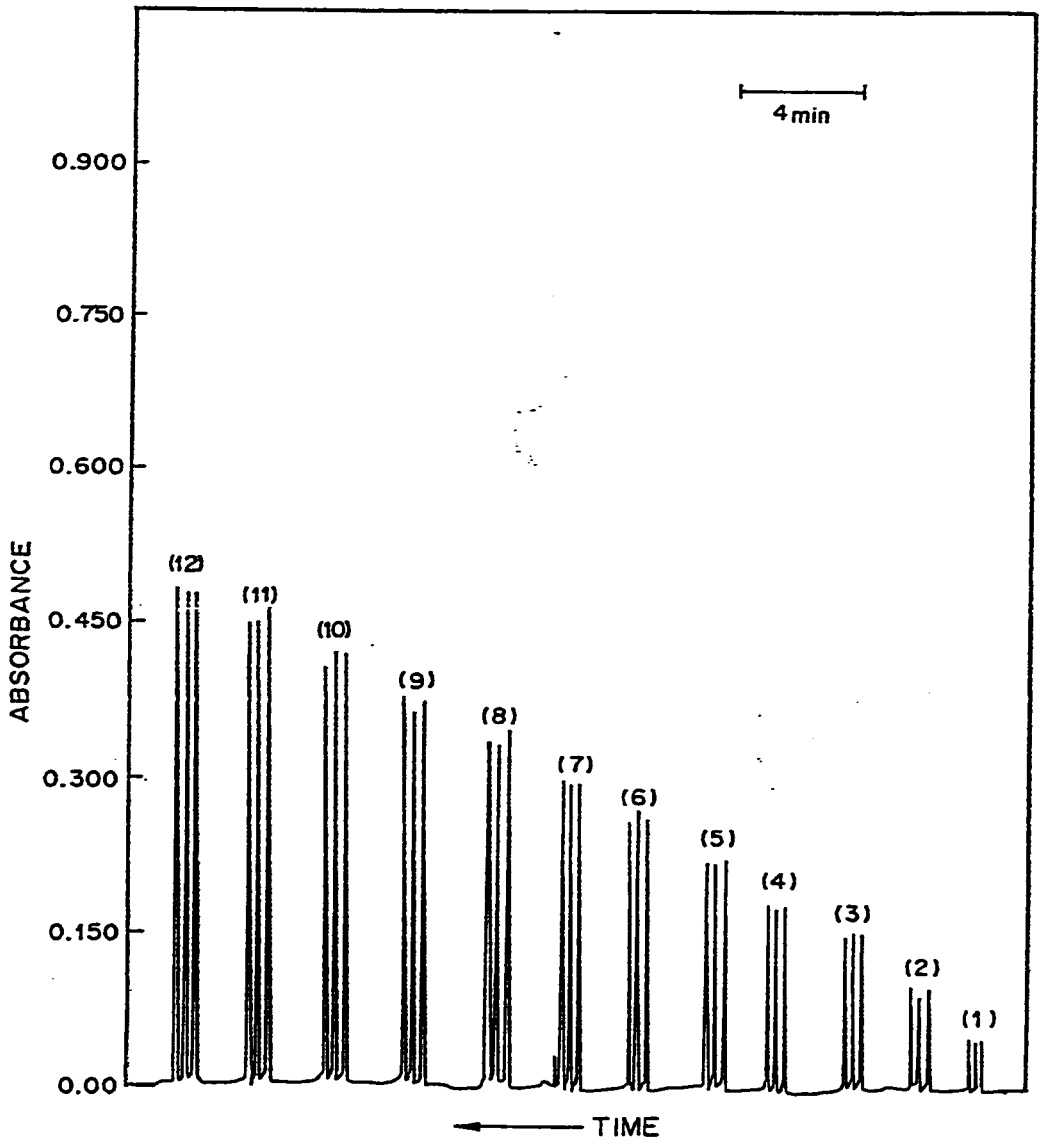


Fig.4.2.12. Typical FIA results(n = 3) graph for oxytetracycline standard solutions of : (1)40 (2)60 (3)80 (4)100 (5)120 (6)140 (7)160 (8)180 (9)200 (10)220 (11)240 (12)260 ppm.

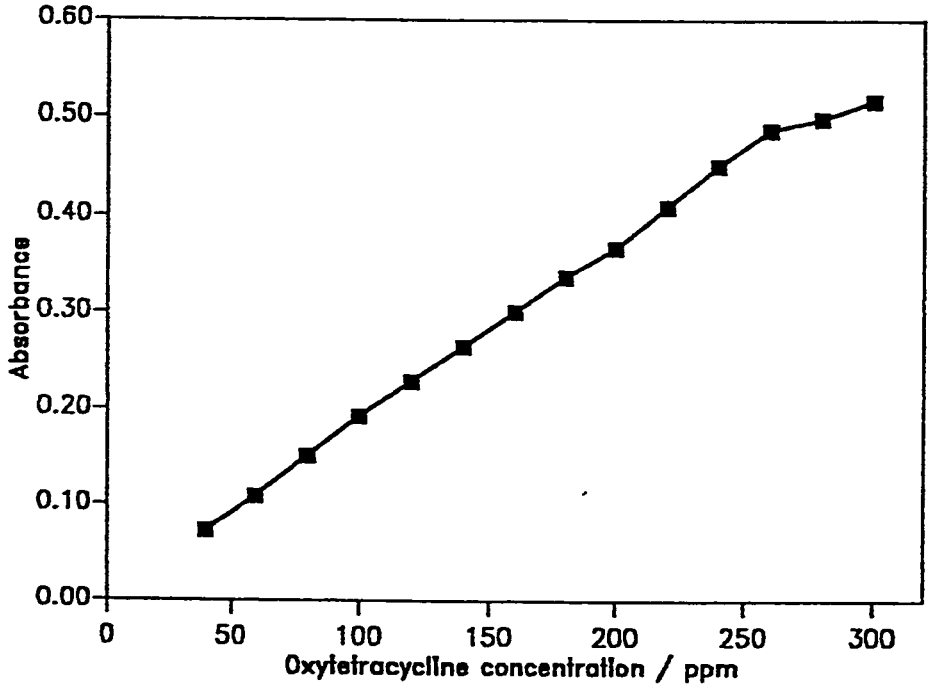


Fig.4.2.13 A typical computer plot of absorbance vs oxytetracycline concentration with the FIA proposed method.

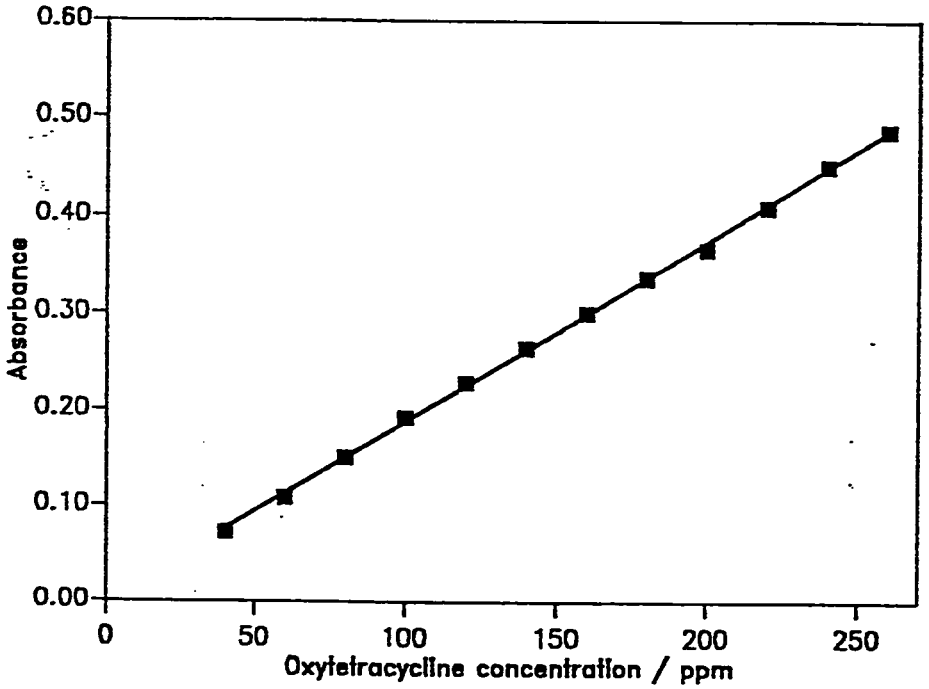


Fig.4.2.14 A typical calibration curve for oxytetracycline in the range 40 - 260 ppm.

$$A = 0.000245 + 0.0019\ C \qquad (4.3)$$

where

A = peak absorbance at 435,

C = concentration of oxytetracycline in $\mu g\ ml^{-1}$

This equation was then used for determination of unknown concentrations of oxytetracycline, by the proposed procedure.

The peak width (w) at 60% of the peak height was calculated as a measure of the total sample dispersion in the system, and was found to be 2.4 sec. The peak width at the base line was found to be 21 sec, from which the maximum sample frequency was found to be 170 s/h. Repeatability of the system was confirmed by injecting one of the sample solutions seven times, and estimating the relative standard deviation (RSD), which was found to be 0.53.

4.3.DETERMINATION OF CHLOROTETRACYCLINE

4.3.1. Introduction

Chlorotetracycline hydrochloride is the hydrochloride salt of an antibiotic substance produced by the growth of *Streptomyces aureofaciens*. It was discovered in 1948 by Duggar[5] and co-workers

ChlorotetracyclineHCl or 7-chlorotetracyclineHCl, as a dry powder, is a stable yellow crystalline material having the empirical formula $C_{22}H_{23}Cl\ N_2O_8.HCl$, and the molecular weight 515.341. The structural formula is shown in Fig. 4.3.1. ChlorotetracyclineHCl is the HCl salt of chlorotetracycline and consequently it is soluble in aqueous acid and base. The tertiary amine is responsible for the basic character, whereas the phenolic group is acidic.

The spectrum of pure sample of chlorotetracyclineHCl dissolved in water, showed a characteristic λ_{max} at 365.8 nm. Chlorotetracycline forms a brown soluble complex with iron(111) in the presence of 0.001M sulphuric acid, with a λ_{max} at 435 nm. In this work, the spectrophotometric method[1] which involves iron(III)-chlorotetracycline complex for-

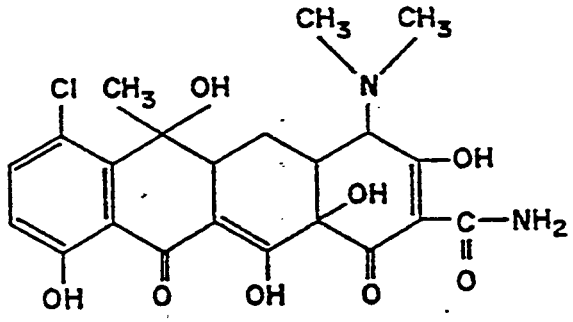


Fig. 4.3.1 Structural formula of chlorotetracycline

mation, was adapted to a FIA method, for quantitative determination of this compound. Optimum conditions for this complexation were studied for both chemical and FIA variables, by a modified simplex and an iterative univarient procedure.

4.3.2. Univarient Optimization

4.3.2.1. Variation of Sulphuric Acid Concentration

The sulphuric acid concentration was varied between 0.001M and 0.5M. Same behaviour was observed as in the case of tetracycline HCl and oxytetracycline. The results obtained were presented in Fig. 4.3.2. The acid concentration was studied by keeping other factors constant. In the first cycle the conditions were 45 cm, 3.9 ml/min 110 μl and 50 ppm for coil length, flow rate, sample loop size and iron(III) concentration respectively. In the second cycle, the conditions were, coil length, 45 cm, flow rate 3.79 ml/min, sample volume 157, μl and iron (III) concentration at 600 ppm As in the case of tetracycline HCl and oxytetracycline, the maximum peak absorbance was obtained at 0.001 M sulphuric acid. It was found that the peak absorbance decreases with increasing acid concentration. Disproportionation could occur at concentrations of sulphuric acid greater than 0.1 M.

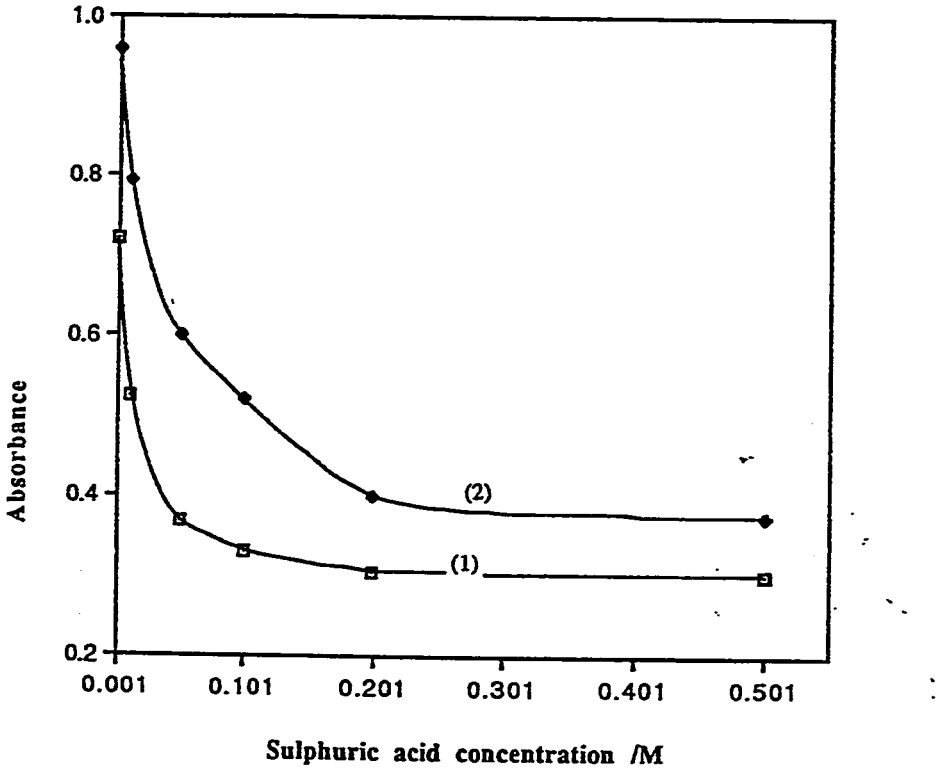


Fig 4.3.2. Variation of sulphuric acid concentration for chlorotetracycline (1) first cycle where coil length is 45 cm, flow rate is 3.90 ml/min and sample volume is 110 μl, and iron(III) concentration is 50 ppm (2) second cycle, where coil length is 45 cm, flow rate is 3.79 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

Therefore, 0.001 M sulphuric acid at which maximum peak absorbance was obtained, was found to be optimum. Improvement in peak absorbance is observed in the second cycle, this has been explained previously on the basis of improvement of other variable by the first cycle.

4.3.2.2. Variation of Reaction Coil Length

The reaction coil length was varied between 45 - 400 cm. The study of the effect of the coil length on the peak absorbance was carried out, while other variables were kept constant. In first cycle, the sulphuric acid concentration at 0.001M, flow rate of 3.9 ml/min sample column of 110 μl, and iron(III) concentration at 50 ppm In the second cycle, the sulphuric acid concentration at 0.001M, flow rate of 3.79 ml/min, sample size of 157 μl, and iron(III) concentration at 600 ppm. The results obtained were presented in Fig. 4.3.3. In the first cycle, the peak absorbance decreases with the increase in coil length indicating dispersion, therefore 45 cm was found to be the optimum coil length. In the second cycle, an increase in peak absorbance was first observed, until a maximum at 140 cm was obtained then it starts to decrease due to dispersion. This behaviour manifests the interaction between the coil length and other factors, specially the flow rate. Therefore, 140 cm was found to be the optimum coil length.

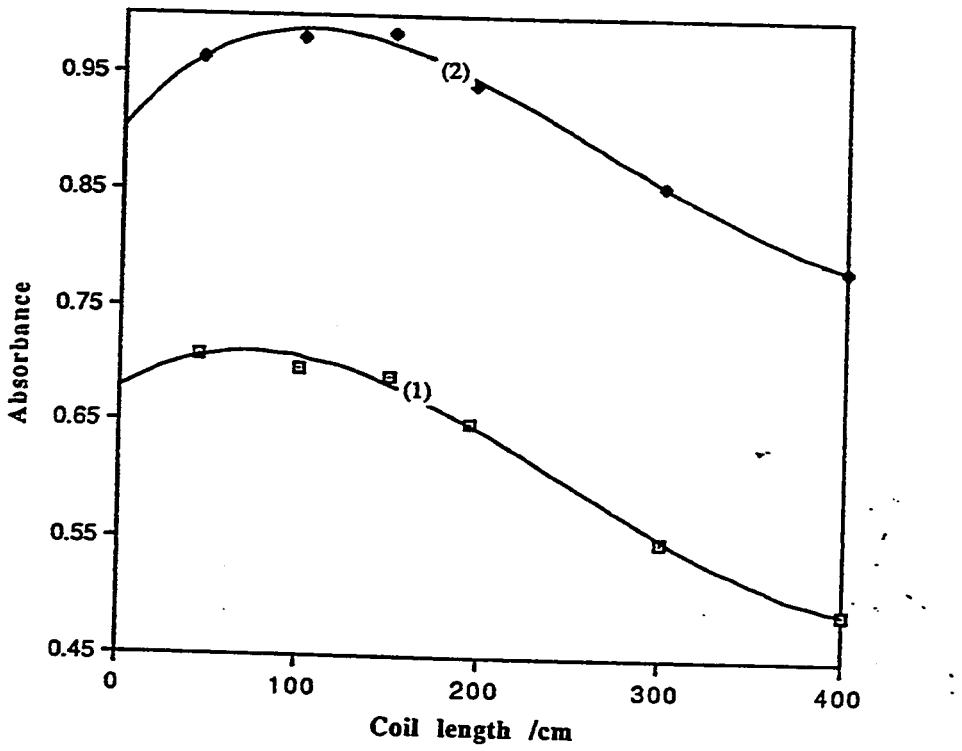


Fig 4.3.3. Variation of coil length for chlorotetracycline (1) first cycle where sulphuric acid concentrationis 0.001M, flow rate is 3.9 ml/min, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, flow rate is 3.79 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

4.3.2.3. Variation of Flow Rate

The conditions for variation of flow rate were as follows; in the first cycle, the sulphuric acid concentration 0.001M, coil length 45, sample loop size 110 μl, iron(III) concentration at 50 ppm. In the second cycle, the acid concentration is the same as in the first cycle, coil length 140, sample volume at 157 μl, and iron(III) concentration at 600 ppm. The flow rate was varied by changing the speed of the peristaltic pump, the relationship between the pump speed in ramps and the flow rate in ml/min could be obtained by Eq. (4.1). The results obtained are presented in Fig. 4.3.4. The same behaviour was observed for both cycles, that an increase in peak absorbance, until a maximum, followed by a decrease in peak absorbance towards higher flow rates. 3.79 ml/min was found to be the optimum flow rate in the first cycle, where as 3.81 ml/min was found to be the optimum flow rate in the second cycle. An improvement in peak absorbance was observed in the second cycle.

4.3.2.4. Sample Volume Variation

The sample volume was varied as in the case of the previous two compounds, between 110 μl and 204 μl. In the first cycle the conditions

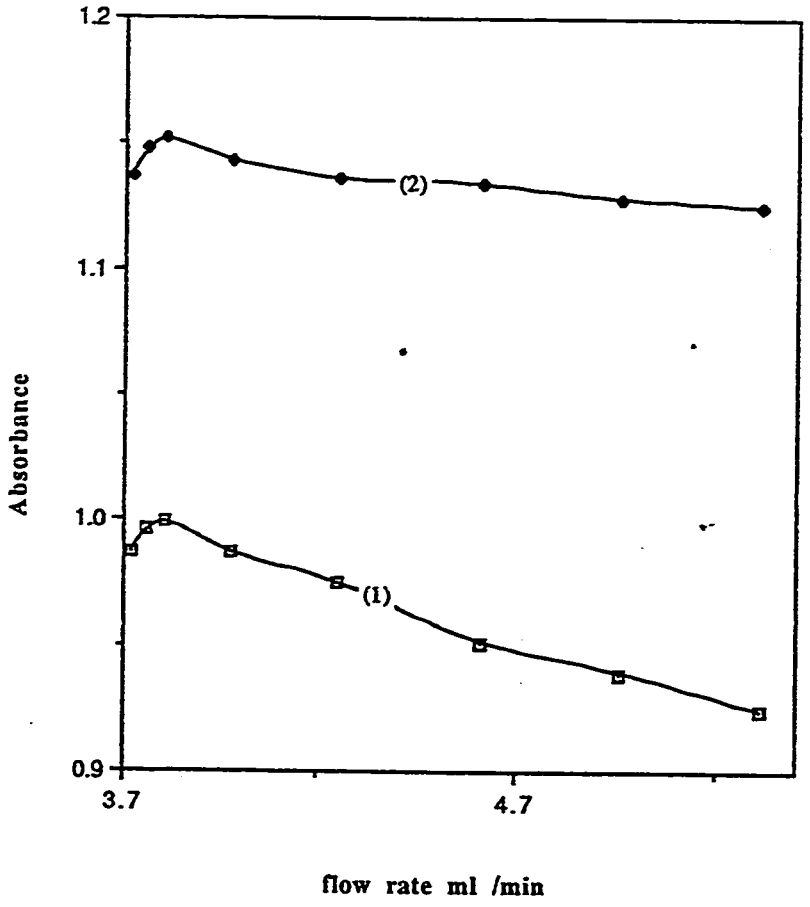


Fig 4.3.4. Variation of flow rate for chlorotetracycline (1) first cycle where sulphuric acid concentration is 0.001M, coil length is 45 cm, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 140 cm, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

were, sulphuric acid 0.001 M, coil length at 45 cm, flow rate at 3.79 ml/min, iron(III) concentration 50 ppm. In the second cycle, the other variables were kept constant, sulphuric acid concentration at 0.001M, coil length at 140 cm, flow rate at 3.81 ml/min iron(111) concentration at 600 ppm, The results obtained are presented in Fig. 4.3.5. The peak absorbance increases with the increase in the sample loop size, in both the first and second cycle, but it is not significant in the second cycle. Therefore, the optimum sample loop size in both cycles was found to be 157, loop size greater than this gave irreproducible peak heights.

4.3.2.5. Variation of Fe(III) Concentration

This factor was also varied, between 50 - 2000 ppm, In the first cycle, the other variables were kept constant, sulphuric acid concentration at 0.001M, coil length at 45 cm, flow rate at 3.79 ml/min and sample loop size at 157 μl. In the second cycle, the sulphuric acid concentration 0.001M, coil length 140 cm, flow rate 3.81 ml/min, and sample size at 157 μl. The results obtained were presented in Fig. 4.3.6. An increase in peak absorbance with an increase in iron(III) concentration was observed, reaching a maximum at 600 ppm for both cycles, then it remains constant. This indicates that the excess of reagent needed in

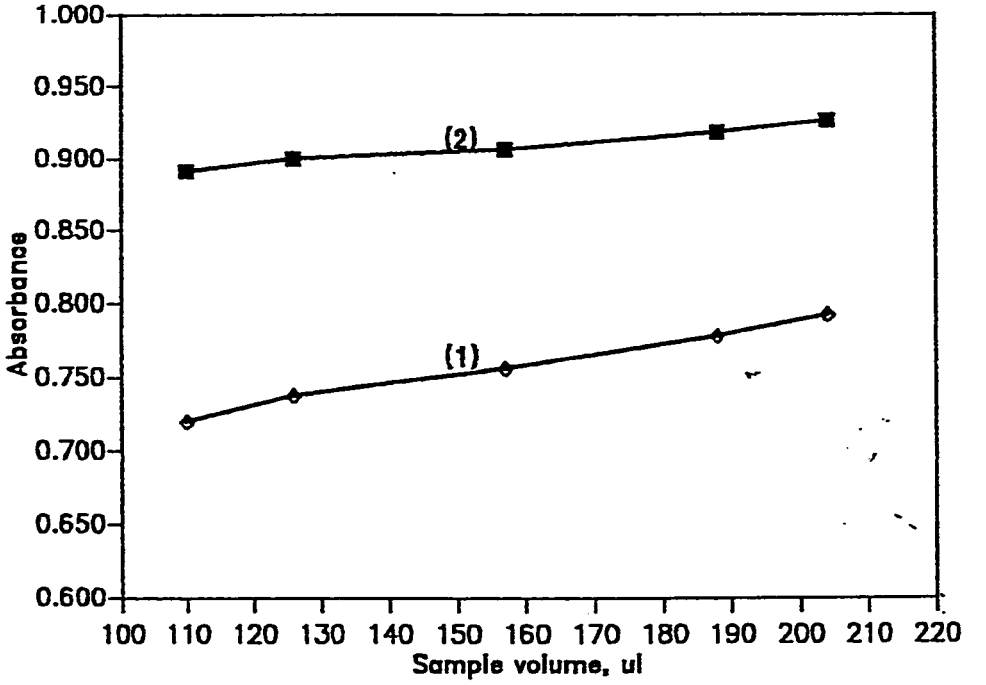


Fig 4.3.5. Variation of sample loop size for chlorotetracycline (1) first cycle where sulphuric acid concentration is 0.001M, coil length is 45 cm, flow rate is 3.79 ml/min, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 140 cm, flow rate is 3.81 ml/min, and iron(III) concentration is 600 ppm.

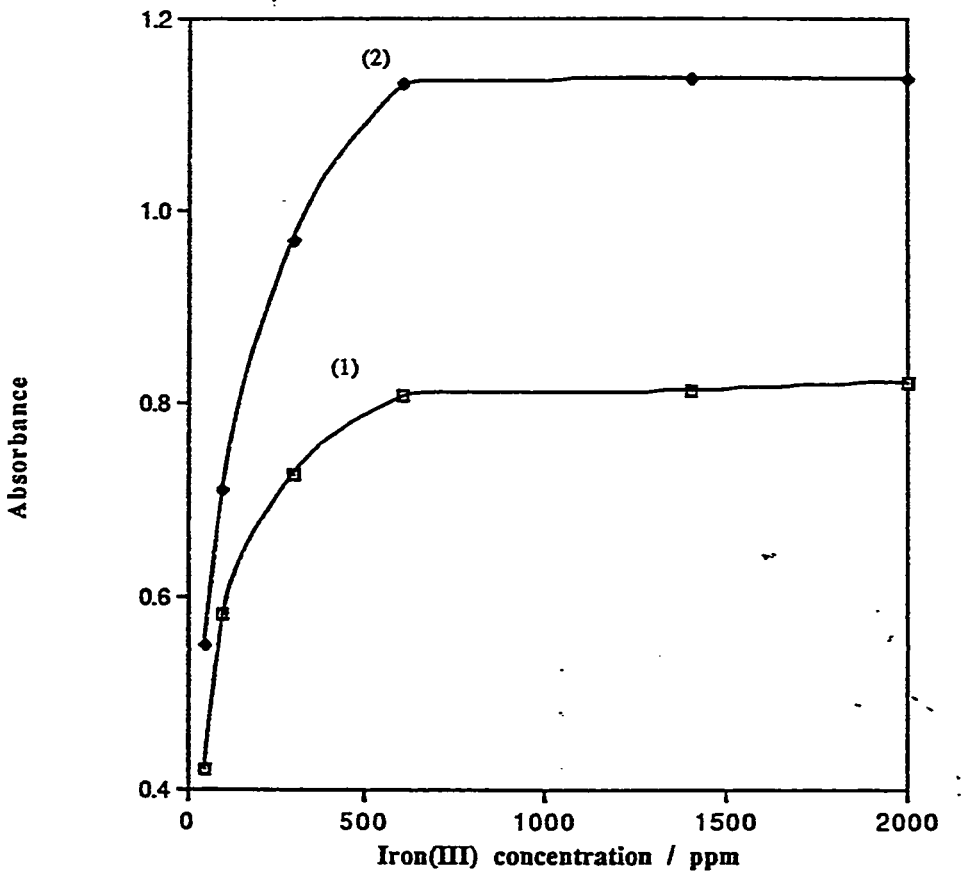


Fig 4.3.6. Variation of iron(III) concentration for oxytetracycline (1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 45 cm, flow rate is 3.79 ml/min and sample volume is 110 μl, (2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 140 cm, flow rate is 3.81 and sample volume is 157 μl.

spectrophotometric methods is reached at 600 ppm. in this method, therefore further additions of the reagent have no effect on the peak absorbance. Therefore the optimum iron(III) concentration for both cycles was found to be 600 ppm

From the previous discussion, the optimum conditions for the quantitative determination of chlorotetracycline, was found to be as follows: sulphuric acid concentration at 0.001M, coil length at 140 cm, flow rate at 3.81 ml/min, sample loop size at 157 μl, and iron(III) concentration at 600 ppm

4.3.3. Simplex Optimization Method

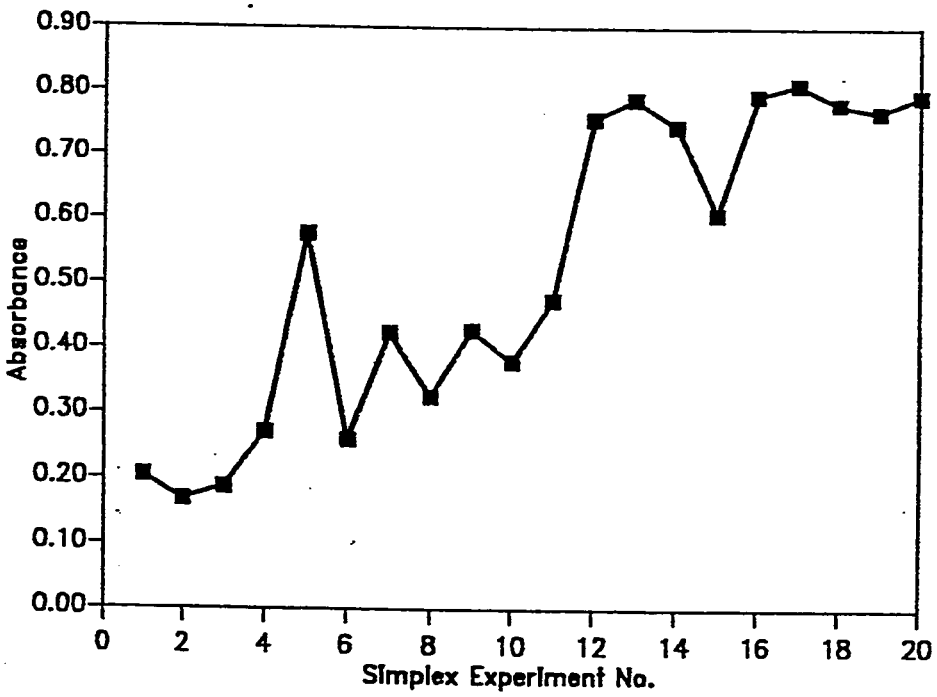
The simplex method of optimization was approached in the same way as in the case of the two previous compounds. The loop size used for all simplex optimization experiments was 157 μl, this sample loop size was used, because as it was mentioned that, there was no significant influence on the peak absorbance by the loop size, besides the economy of the sample solution was a considerable concern to the experimenter. The results obtained by the simplex method were presented in Table 4.3.1. and plotted in Fig. 4.3.7. From Table 4.3.1. point 2 is the worst point with a peak absorbance of 0.168, therefore this point is

Table 4.3.1: Simplex optimization of chemical and FIA variables for chlorotectracycline

EXP #	Coil Length/cm	Flow Rate /ml min^{-1}	$[H_2SO_4]$ /M	[iron(III)] / ppm	Peak absorbance
(1)	100	4.87	0.0500	100	0.204
(2)	400	4.87	0.0500	100	0.168
(3)	45	5.31	0.0500	100	0.186
(4)	45	3.90	0.1000	300	0.27
(5)	45	3.90	0.0100	600	0.576
(6) R	45	4.61	0.0750	200	0.258
(7) C	96	4.32	0.0360	375	0.423
(8) R	98	3.89	0.0680	337	0.324
(9) C	58	4.25	0.0350	409	0.426
(10) R	50	3.80	0.0600	492	0.378
(11) C	60	4.11	0.0330	448	0.474
(12) R	85	4.40	0.0017	466	0.756
(13) E	104	4.65	0.0010	549	0.786
(14) R	65	4.08	0.0049	552	0.744
(15) C	83	4.40	0.0100	515	0.606
(16) R	76	4.29	0.0010	566	0.792
(17) E	85	4.37	0.0010	626	0.810
(18) R	91	4.46	0.0010	534	0.780
(19) C	86	4.40	0.0023	575	0.768
(20) R	91	4.47	0.0013	612	0.792
(21) C	86	4.40	0.0012	583	0.780

R = Reflection, C = Contraction, E = Expansion

Fig.4.3.7 Simplex optimization for chlorotetracycline.



reflected through the centroid, to give the reflection point 6. The peak absorbance at the reflection point was found to be 0.423. A contraction point was then obtained with a peak absorbance of 0.324, which is less than the absorbance at the reflection point. Therefore, point 2 was rejected and replaced by point 6. The maximum peak absorbance was obtained by the expansion at point 17, another four additional experiments were done, but without any further improvement. The optimum conditions obtained by the simplex method were 0.001M, 85 cm, 4.37 ml/min and 626 ppm, sulphuric acid concentration, coil length, flow rate and iron(III) concentration respectively. The peak absorbance for the same batch of solution, obtained when the conditions stated by the simplex method were used was found to be 0.810, whereas in the case of the univarient conditions were used, the peak absorbance was found to be 0.694. This could be due to increased dispersion in the case of the univarient method, where coil length is 140 cm, and flow rate is 3.81ml/min Therefore, the conditions reached by the simplex method were used for the quantitative analysis of chlorotetracycline.

In Fig. 4.3.8 - 4.3.11, the modified simplex data are presented as a series of a scatter diagram. Fig. 4.3.8. which represents the simplex investigation of the sulphuric acid concentration, has an appearance similar to the iterative univarient investigation of this variable. Clus-

Fig.4.3.8 Simplex variation of acid concentration for chlorotetracycline.

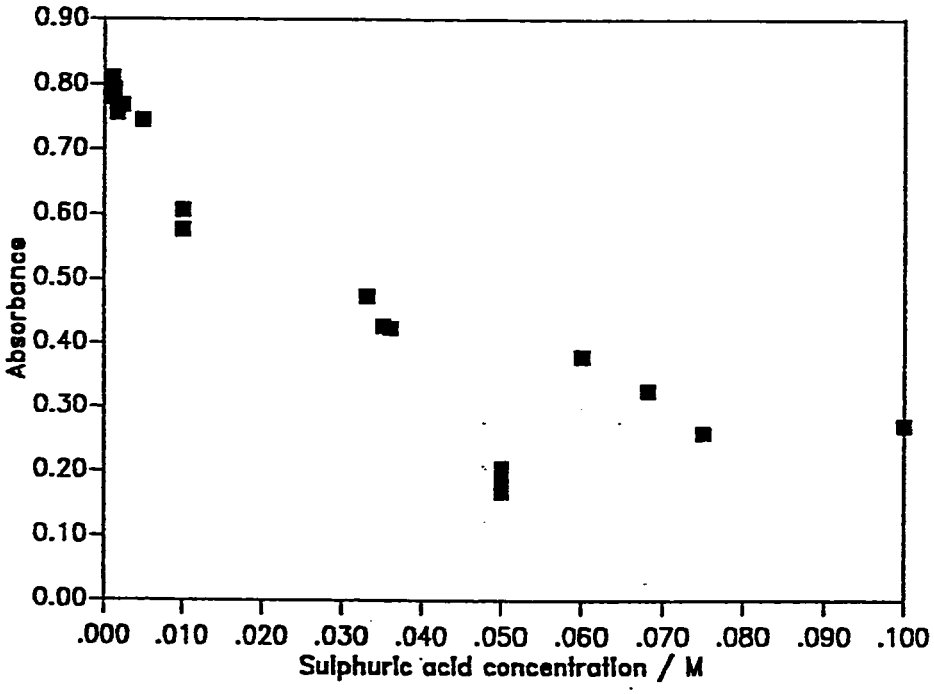


Fig.4.3.9 Simplex variation of coil length for chlorotetracycline.

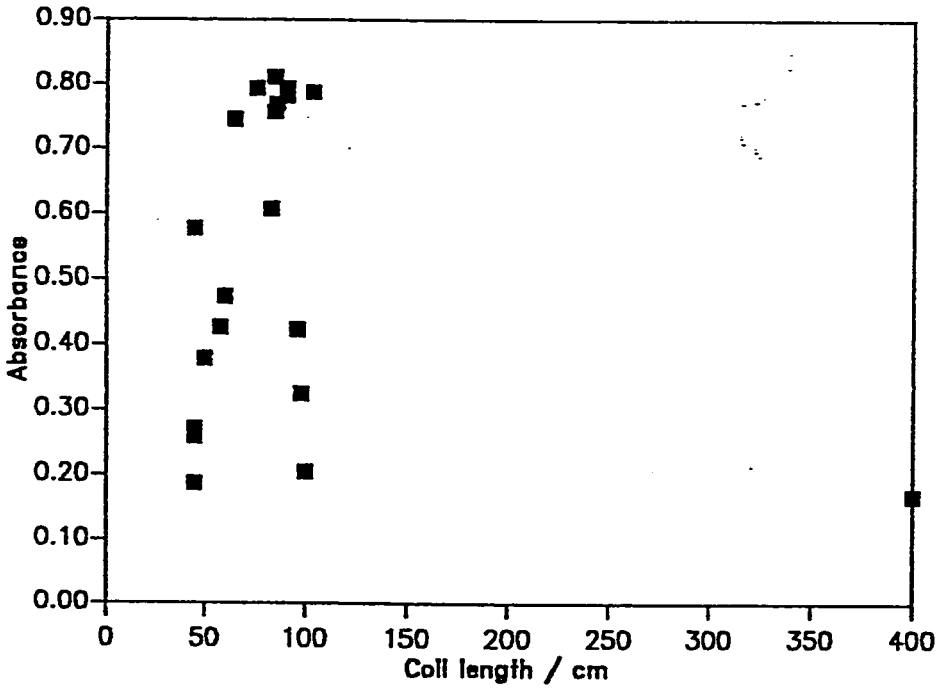


Fig.4.3.10 Simplex variation of flow rate for chlorotetracycline.

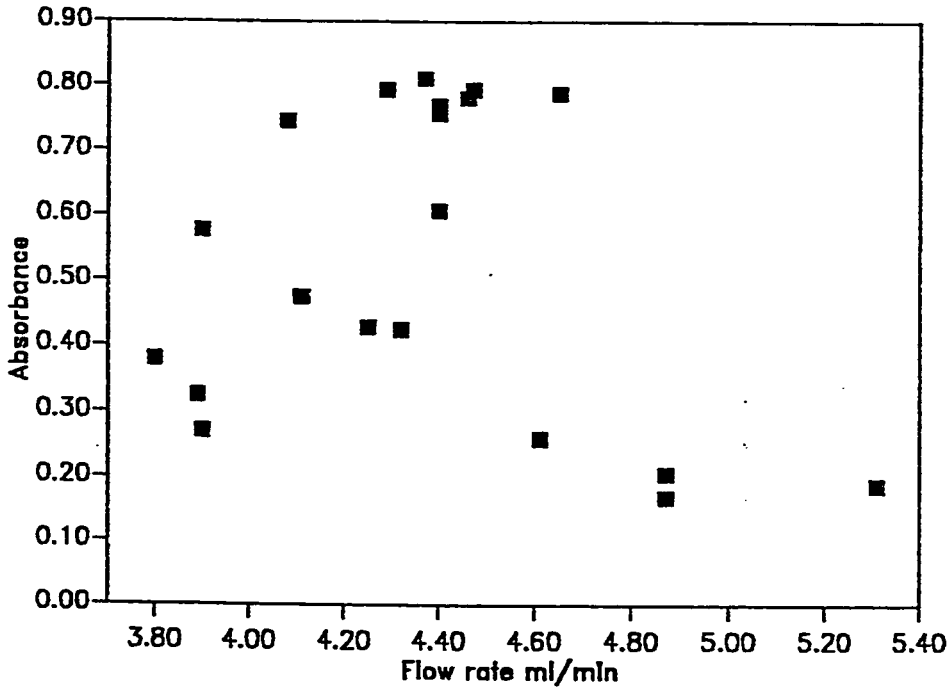
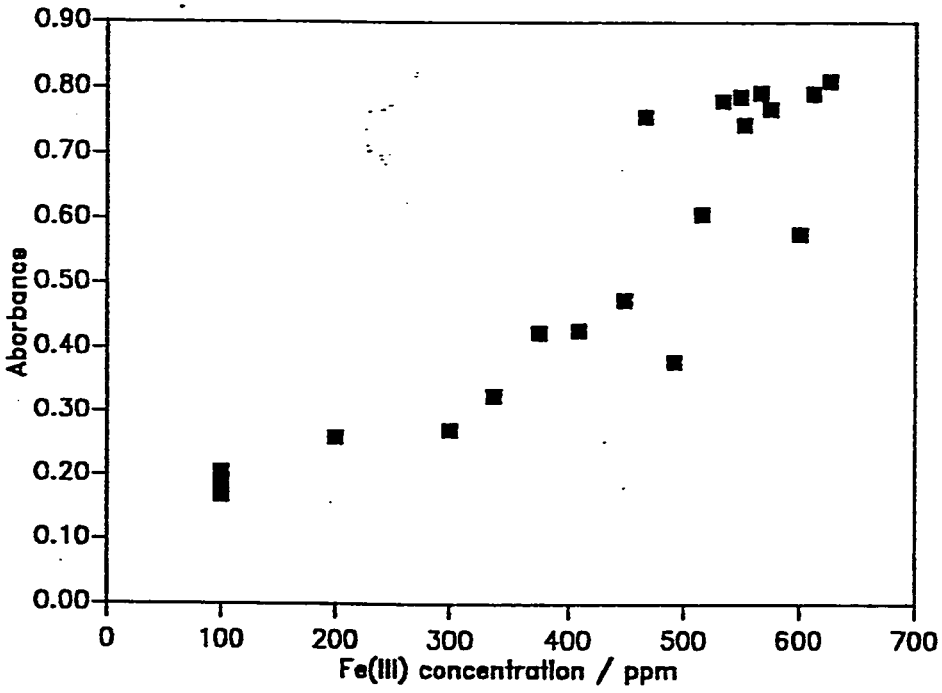


Fig.4.3.11 Simplex variation of Fe(III) concentration for chlorotetracycline.



tering of the points at the high peak absorbance region, where the range of the acid concentration is 0.001 - 0.05M. In Fig. 4.3.9. the coil length was investigated by the simplex program in the range of 45 - 100 cm. The flow rate was investigated over a wider range, this is shown in Fig. 4.3.10. In Fig. 4.3.11, the iron(III) concentration was investigated in the range 100-700 ppm. The same behaviour observed previously in the univarient method could be observed here, where a peak absorbance increase is observed towards high iron(III) concentration and low acid concentration.

4.3.4 Analytical Appraisals

The Beer's law was found to be valid in the range 40 - 300 $mg\ ml^{-1}$. The lower detection limit was found to be 20 $mg\ ml^{-1}$. Accurately about 0.25000 of chlorotetracycline is weighed using the technique of weighing by difference, dissolved in 0.001 M sulphuric acid and diluted to 250 ml with 0.001M sulphuric acid. Different standard chlorotetracycline solutions were prepared by adding an appropriate volume of stock solution to a 50 ml volumetric flasks, and then these flasks were completed to volume with 0.001M sulphuric acid.

The standard solutions were run in triplicate by injecting a volume of 157 μl into the carrier stream. The carrier stream, flowing at 4.37 ml/min is composed of 626 ppm iron(III) in 0.001M sulphuric acid. The absorbance of the resulting complex was at 435 nm. The results of the proposed method are presented in Table 4.3.2. A typical recorder tracing for these results are presented in Fig. 4.3.12. and the measured peak absorbancies are plotted versus different chlorotetracycline concentrations as shown in Fig. 4.3.13. and Fig. 4.3.14. The straight line was obtained in the range 40 - 300 $\mu g\ ml^{-1}$, with a correlation coefficient (r) of 0.9990 and an intercept of 0.0152. Above 300 $\mu g\ ml^{-1}$, there is a deviation towards the concentration axis. There-

Table 4.3.2: Beer's law validation for chlorotetracycline with the proposed FIA method.

concentration ppm	Absorbance
40	0.060
60	0.096
80	0.144
100	0.186
120	0.228
140	0.282
160	0.318
180	0.367
200	0.396
220	0.432
240	0.474
260	0.510
280	0.552
300	0.582
320	0.588
340	0.618

* Itercept for the slope = 0.016.
** Correlation coefficient for 14 different determinations in the range 40 - 300 ppm is 0.999.

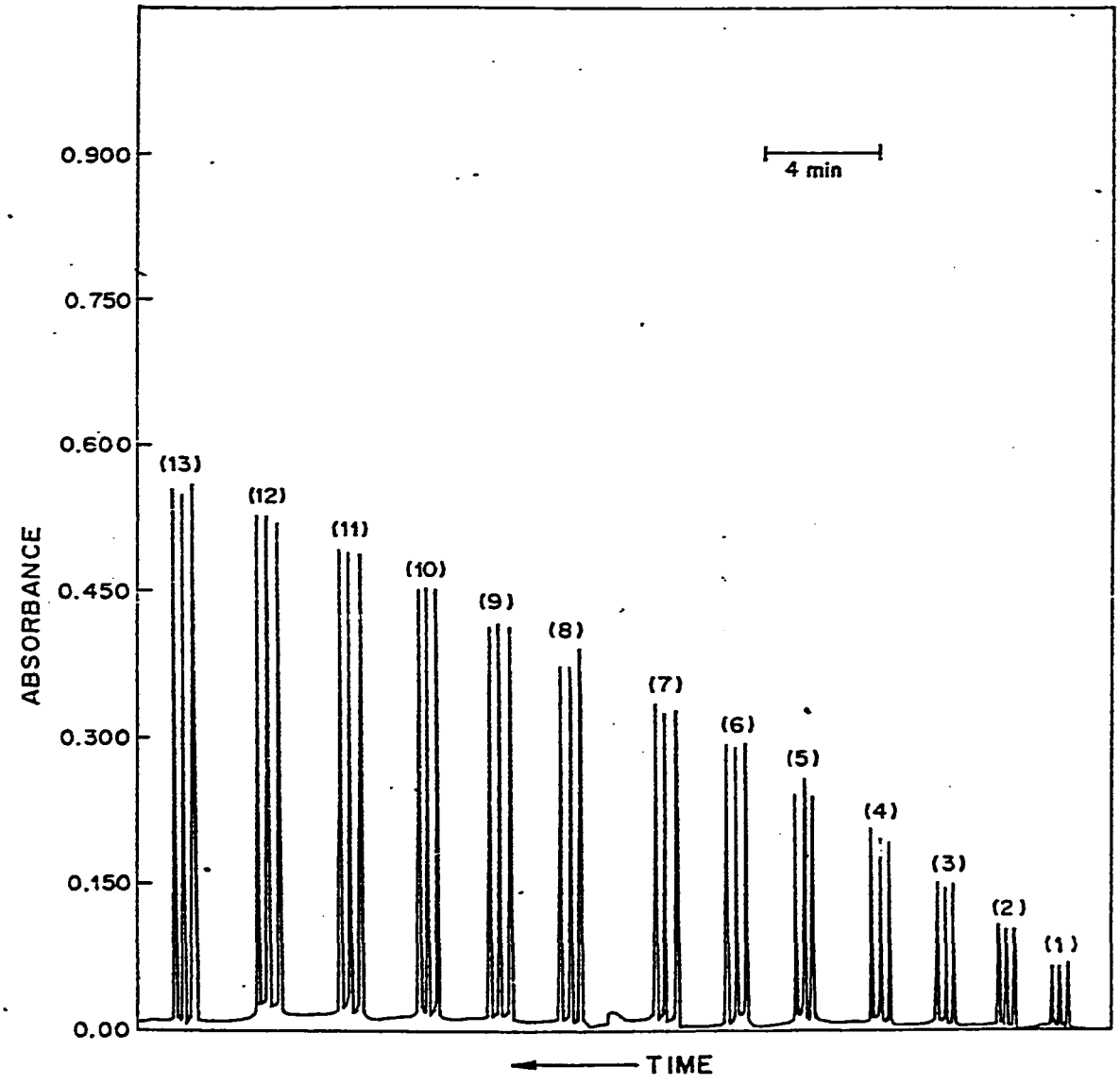


Fig.4.3.12. Typical FIA results (n = 3) for chlorotetracycline standard solutions of : (1)40 (2)60 (3)80 (4)100 (5)120 (6)140 (7)160 (8)180 (9)200 (10)220 (11)240 (12)260 (13)280 (14)300 ppm.

520

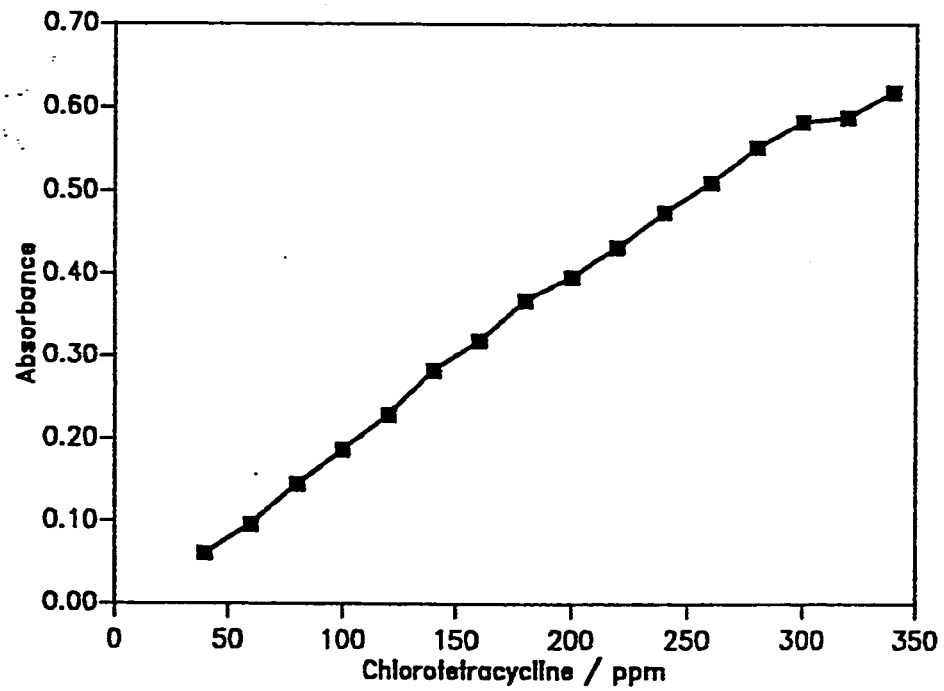


Fig.4.3.13 A typical computer plot of absorbance vs concentration of chlorotetracycline with the FIA proposed method.

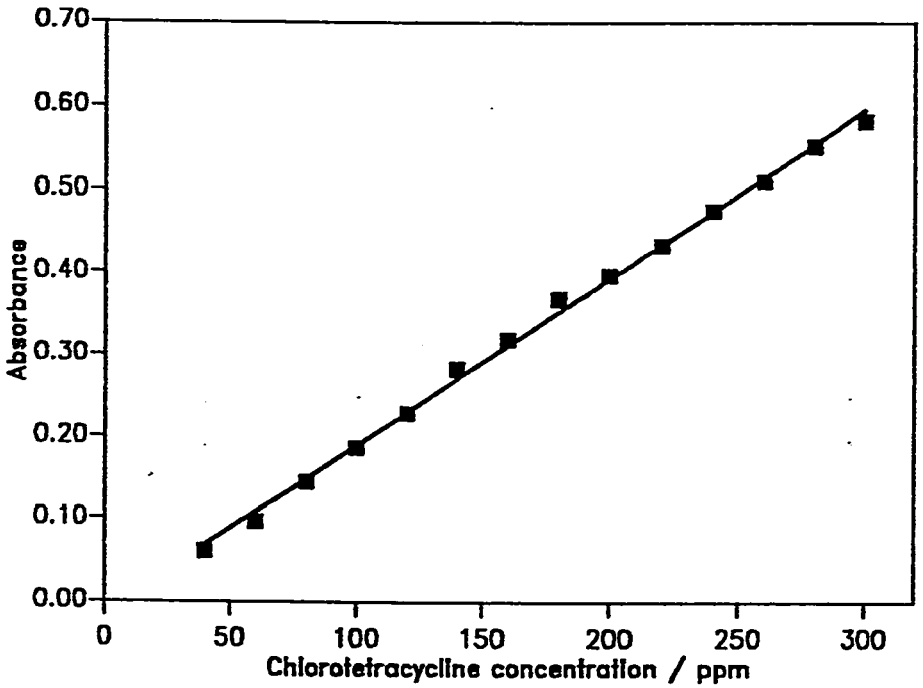


Fig.4.3.14 Calibration curve for chlorotetracycline in the range 40 - 300 ppm for chlorotetracycline.

fore, a calibration equation of the following type was obtained:

$$A = 0.016 - 0.0023\ C \qquad (4.3)$$

where

A = peak absorbance at 435

C = concentration of chlorotetracycline in $\mu g\ ml^{-1}$

The peak width at the base line was found to be 18.4 sec, from which the maximum sample frequency was found to be 195 s/h, and the relative standard deviation for six injections was found to be 0.73.

DETERMINATION OF DEMECLOCYCLINE

4.4.1 Introduction

Demeclocycline is dimethylchloro-tetracycline, its molecular weight is 464.88, its structural formula is shown in Fig. 4.4.1. The absence of the (CH_3) group from the ring C and the presence of (Cl) anion which is an electron withdrawing group on ring D, increases the stability of iron(111) - demeclocycline complex, that a stronger chelation in the case of demecocycline is expected, compared to tetracycline and the other derivatives.

The spectrum of pure demeclocycline dissolved in water shows a characteristic λ_{max} at 365.2 nm. A brown soluble complex is formed when demeclocycline is reacted with iron(III) in 0.001M sulphuric acid. This complex shows a characteristic λ_{max} at 435 nm.

As in the case of the previous compounds, the chemical and FIA variables were optimized by a modified simplex method and an iterative univarient method, so as to obtained the best conditions for the quantitative determination of demecocycline.

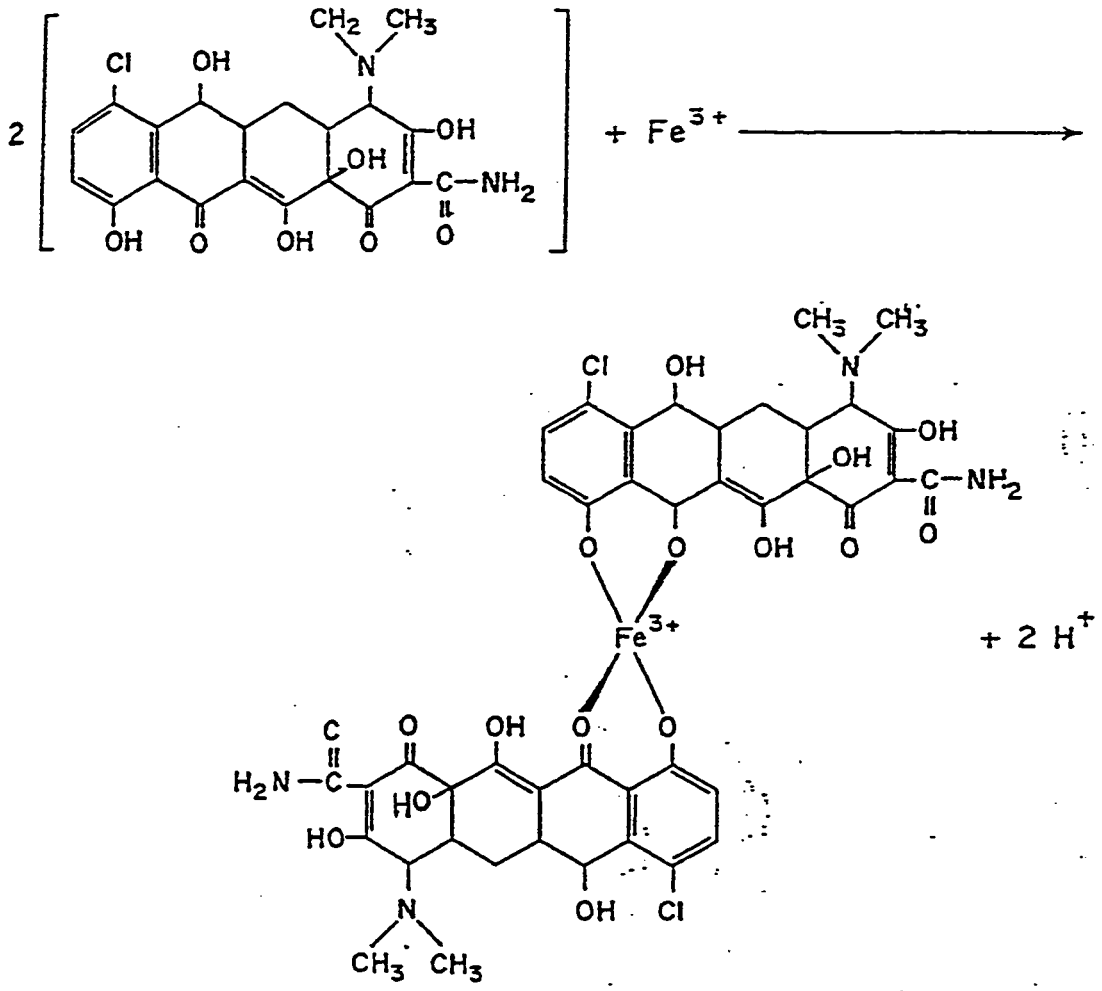


Fig. 4.4.1 Reaction scheme of demeclocycline with iron(III).

4.4.2. Univarient Optimization

4.4.2.1. Variation of Sulphuric Acid Concentration

Like the other compounds the acid concentration was varied in the range 0.001 - 0.50M, while other variables were kept constant. In the first cycle, coil length at 45 cm, flow rate at 3.90 ml/min sample loop size at 110 μl, and iron(III) at 50 ppm. In the second cycle, the values of these variables are, 45 cm, 3.86 ml/min 157 μl, and 600 ppm respectively. The results obtained were shown in Fig. 4.4.2. The effect of the acid concentration on the peak absorbance was the same as in the previous compound. Increasing acid concentration resulted in a rapid decrease in peak absorbance. At acid concentration more than 0.10M the peak absorbance was found to be constant, that disproportionation of the complex, and protonation of demeclocycline is expected. The optimum acid concentration was found to be 0.001 M, in both cycles, with an improved peak absorbance in the second cycle, as in the previous compounds.

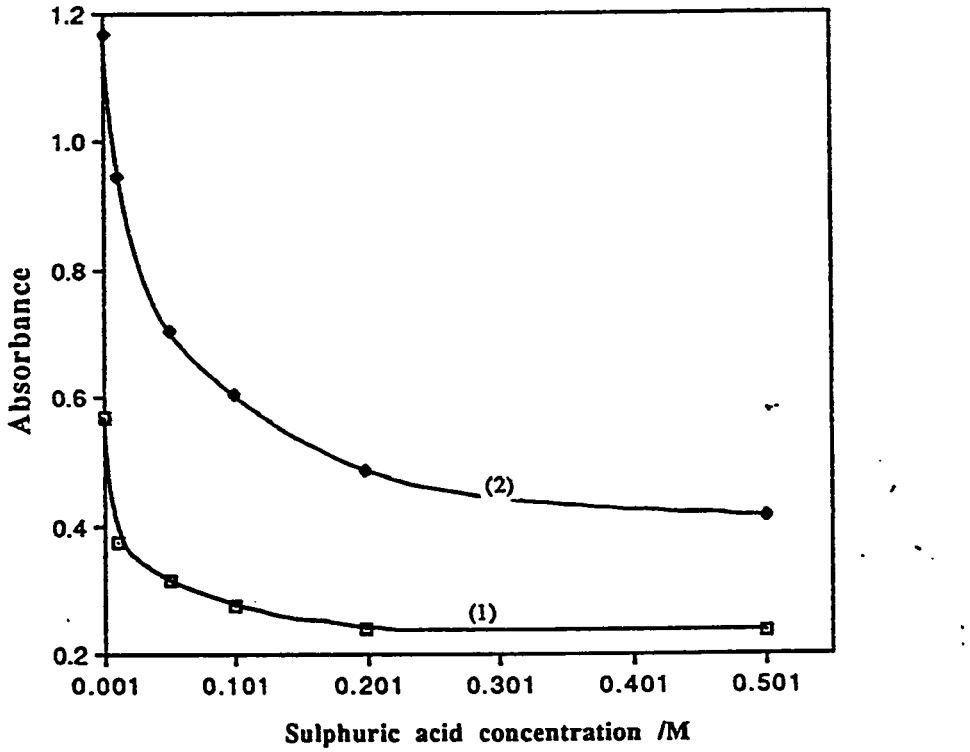


Fig 4.4.2. Variation of sulphuric acid concentration for demeclocycline (1) first cycle where coil length is 45 cm, flow rate is 3.90 ml/min and sample volume is 110 μl, and iron(III) concentration is 50 ppm (2) second cycle, where coil length is 45 cm, flow rate is 3.86 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

4.4.2.2. Variation of Reaction Coil Length

Also the coil length was varied between 45 - 400 cm, by using tubing, of 0.5 mm id, of different lengths, while the other variables were kept constant. In first cycle, sulphuric acid concentration at 0.001M, flow rate at 3.9 ml/min sample volume at 110 μl, and iron(III) concentration at 50 ppm. In the second cycle, the sulphuric acid concentration is at 0.001M, flow rate at 3.86 ml/min, sample volume at 157 μl and iron(III) concentration at 600 ppm. The results obtained were presented in Fig. 4.4.3. The dispersion factor dominate in the first cycle, where the peak absorbance decreases with increasing the coil length. In the second cycle interaction with other variable is expected, where the peak absorbance increases with coil length, attaining a maximum value at 100 cm, then decreases as the coil length is increased. In the second cycle, the peak absorbance was improved, therefore a coil length of 100 was found to be the optimum.

4.4.2.3. Variation of Flow Rate

The flow rate was varied between 3.70 and 5.5 ml/min. In the first cycle, the sulphuric acid concentration is at 0.001M, coil length at 45 cm, sample volume at 110 μl, and iron(III) at 50 ppm. In the second

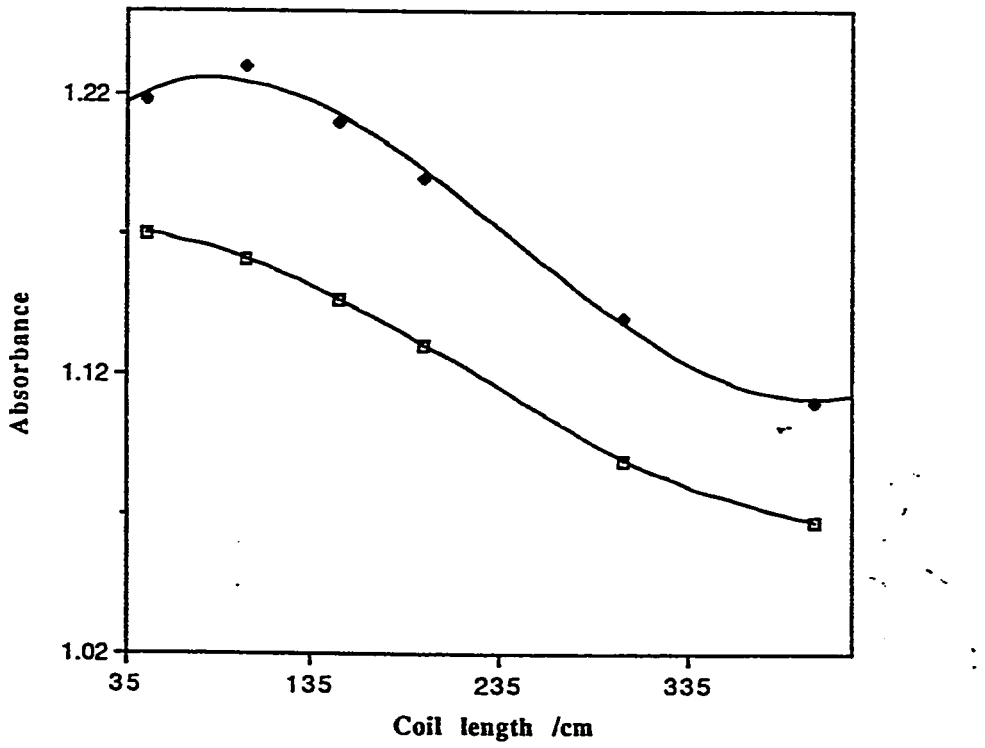


Fig 4.4.3. Variation of coil length for demeclocycline (1) first cycle where sulphuric acid concentrationis 0.001M, flow rate is 3.9 ml/min, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, flow rate is 3.86 ml/min, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

cycle, the conditions were 0.001M sulphuric acid, 100 cm coil length, 157 μl sample volume, 600 ppm iron(III) concentration. The results obtained were presented in Fig. 4.4.4. The optimum flow rates obtained were 3.86 and 3.77 ml/min for first and second cycles respectively. Improvement of peak absorbance in the second cycle, due to improved optimization conditions, was observed in the second cycle. Therefore, the optimum flow rate was found to be 3.77 ml/min

4.4.2.4. Variation of Sample Volume

The sample volume was varied, as in the previous compounds, between 110 - 204 μl, by changing sample loop size. In the first cycle the conditions for varying sample volume, were sulphuric acid at 0.001 M, coil length at 45 cm, flow rate at 3.86 ml/min, and iron(III) concentration at 50 ppm. In the second cycle, the conditions were as follows, 0.001M sulphuric acid, 100 cm coil length, 3.77 ml/min flow rate and 600 ppm iron(III) concentration. The results obtained were presented in Fig. 4.4.5. An insignificant increase in the peak absorbance was observed with increasing sample loop size, also the peak absorbance obtained at higher sample loop size were not reproducible. In both cycles, the optimum sample loop size was found to be 157 μL.

4.4.2.5. Variation of Iron(III) Concentration

Iron(III) concentration dissolved in 0.001M was varied in the range 50 - 2000 ppm. In the first cycle, the iron(III) concentration was varied by keeping other variables constant, sulphuric acid concentration at 0.001M , coil length at 45, flow rate at 3.86 ml/min, and sample volume at 157 μl. In the second cycle, the values of these variables are 0.001M, 100 cm, 3.77 ml/min and 157 μl, respectively.

The results obtained were shown in Fig. 4.4.6. The optimum iron(III) concentration was found to be 600 ppm in both cycles. Concentrations of iron(III) greater than 600 ppm has no effect on the peak absorbance. Therefore, 600 ppm was found to be the optimum iron(III) concentration for determination of demeclocycline.

From the previous discussion, the optimum conditions for the quantitative determination of demeclocycline, were found to be as follows; sulphuric acid concentration at 0.001M, coil length at 100 cm, flow rate at 3.77 ml/min, sample loop size at 157 μl, iron(III) concentration at 600 ppm

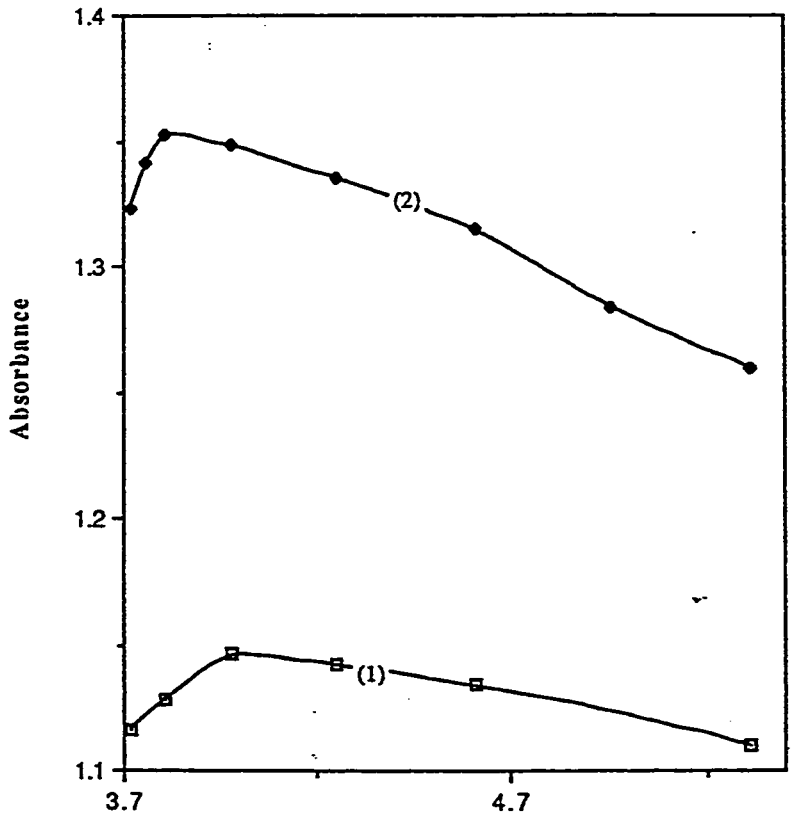


Fig 4.4.4. Variation of flow rate for demeclocycline (1) first cycle where sulphuric acid concentration is 0.001M, coil length is 45 cm, sample volume 110 μl, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 100 cm, sample volume is 157 μl. and iron(III) concentration is 600 ppm.

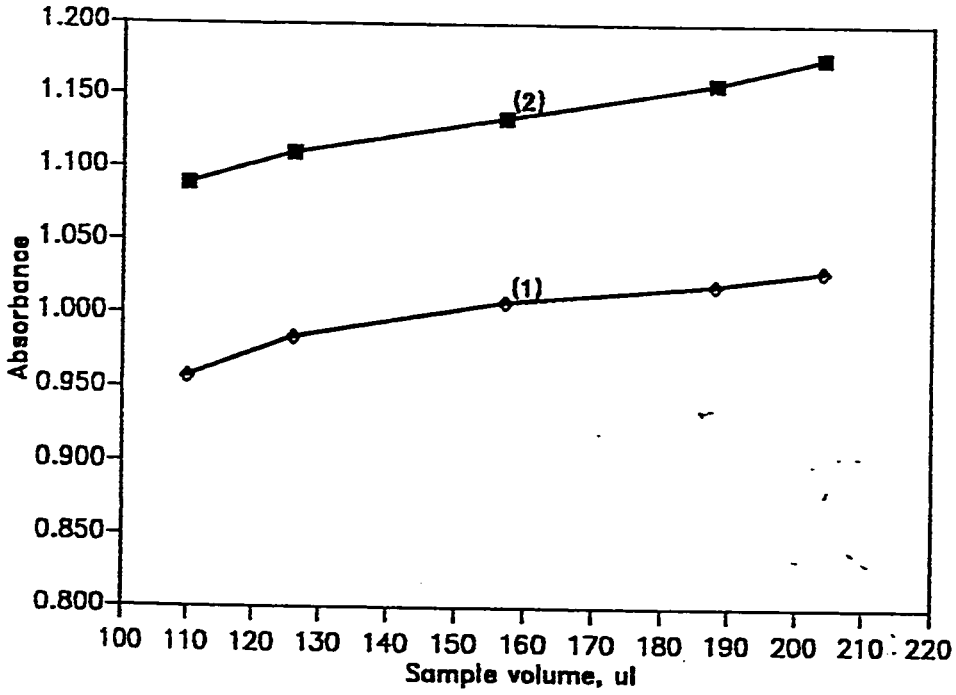


Fig 4.4.5. Variation of sample loop size for demeclocycline (1) first cycle where sulphuric acid concentration is 0.001M, coil length is 45 cm, flow rate is 3.86 ml/min, and iron(III) concentration is 50 ppm.(2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 100 cm, flow rate is 3.77 ml/min, and iron(III) concentration is 600 ppm.

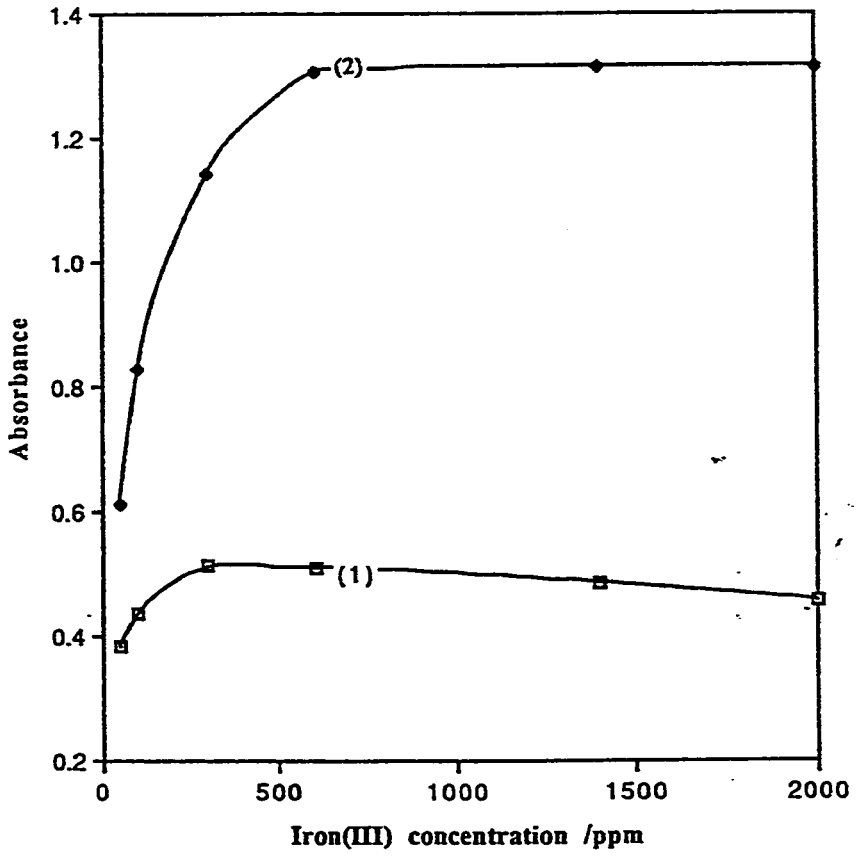


Fig 4.4.6. Variation of iron(III) concentration for demeclocycline (1) first cycle where sulphuric acid concentrationis 0.001M, coil length is 45 cm, flow rate is 3.86 ml/min and sample volume is 110 μl, (2) second cycle, where sulphuric acid concentration is 0.001M, coil length is 100 cm, flow rate is 3.77 and sample volume is 157 μl.

4.4.3. Simplex Optimization

The modified simplex method was performed with the procedure described earlier (Chapter 3). The results were shown in Table 4.4.1, and plotted in Fig. 4.4.7. The five first experiments (1-5) describe the initial simplex. The worst point is point 3, and the best point is point 6, with peak absorbancies 0.252 and 0.780 respectively. The first contraction was not better than the preceding reflection, therefore, point 3, which is the worst point was rejected and replaced by point 6. Point 15, is a reduction point, and it is the first time to get reduction. The reduction was obtained because the peak absorbance at point 15 is less than that at the worst point in the simplex cycle preceded the reduction. The optimum conditions were obtained by the expansion at point 20. Another additional six simplex experiments were performed. Seeking further improvement, but without success. The maximum sample throughput at this point was found to be 170 s/h. The results obtained by the simplex method are comparable to those obtained by the univarient method. As in the other compounds, shorter coil length 45 cm, was obtained by the simplex method, compared to 100 cm by the univarient method. Unlike other compounds a lower iron(III) concentration (447 ppm) is obtained by the simplex method, whereas 600 ppm iron(III) was obtained by the univarient method.

Table 4.4.1: Simplex optimization of chemical and FIA variables for demeclocycline

EXP #	Coil Length /cm	Flow Rate/ ml min^{-1}	$[H_2SO_4]$ /M	[iron(III)] / ppm	Peak absorbance
(1)	100	4.78	0.0500	100	0.312
(2)	400	4.78	0.0500	100	0.324
(3)	45	5.31	0.0500	100	0.252
(4)	45	3.90	0.0100	300	0.780
(5)	45	3.90	0.1000	600	0.570
(6) R	250	3.72	0.0550	450	0.546
(7) C	96	4.12	0.0310	288	0.570
(8) R	193	3.72	0.0460	544	0.714
(9) C	96	4.04	0.0290	311	0.654
(10) R	45	3.72	0.0350	649	0.774
(11) C	58	3.94	0.0270	337	0.720
(12) R	45	3.77	0.0520	487	0.660
(13) C	53	3.92	0.0260	345	0.738
(14) R	80	4.00	0.0010	50	0.264
(15) Red	45	3.90	0.0550	450	0.546
(16) R	80	4.00	0.0010	196	0.63
(17) C	54	3.93	0.0160	312	0.72
(18) R	45	3.81	0.0100	335	0.87
(20) E	45	3.72	0.0010	447	1.098
(21) R	45	3.79	0.0010	314	1.080
(22) C	47	3.79	0.0070	336	0.954
(23) R	45	3.74	0.0060	352	0.984
(24) C	46	3.78	0.0060	340	0.972
(25) R	45	3.72	0.0010	318	1.080
(26) C	45	3.76	0.0040	339	0.954

R = Reflection, C = Contraction, E = Expansion, Red = Reduction

Fig.4.4.7 Simplex optimization for demeclocycline.

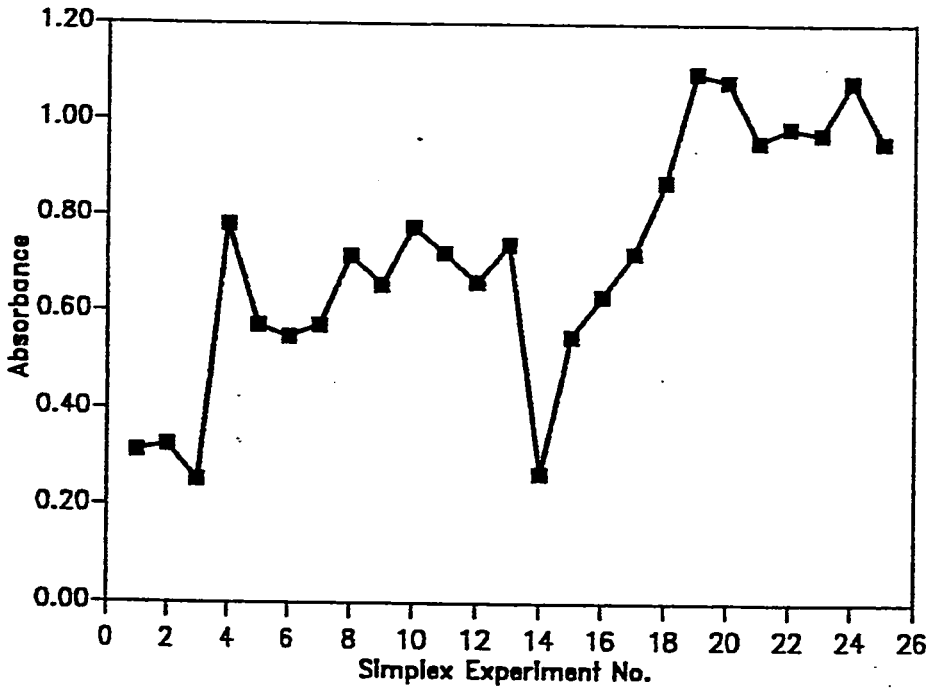


Fig.4.4.8 Simplex variation of sulphuric acid concentration for demeclocycline.

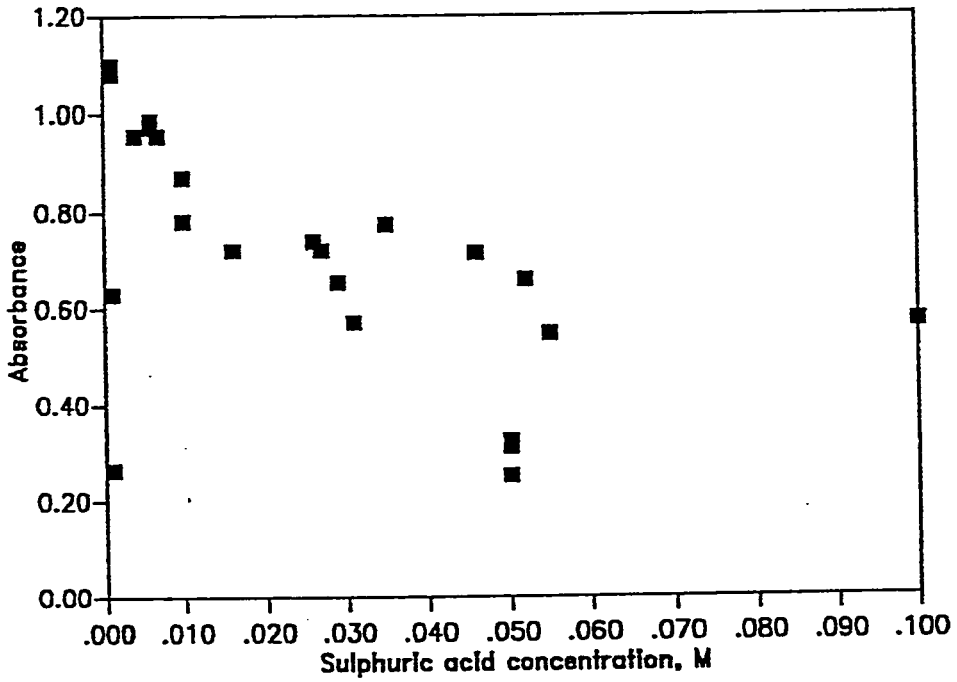


Fig.4.4.9 Simplex variation of coil length for demeclocycline.

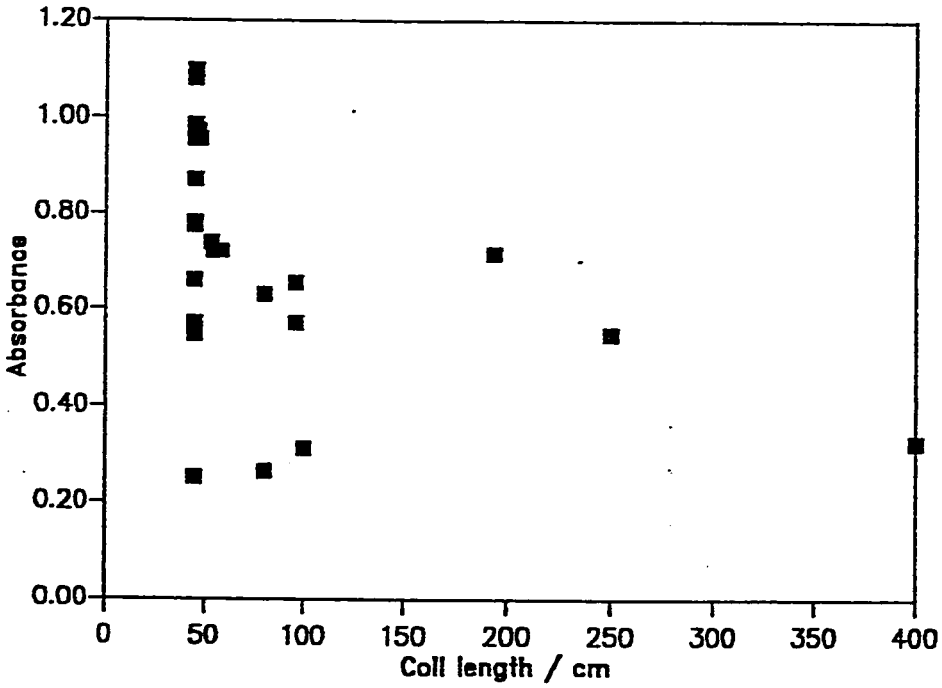


Fig4.4.10 Simplex variation of flow rate for demeclocycline.

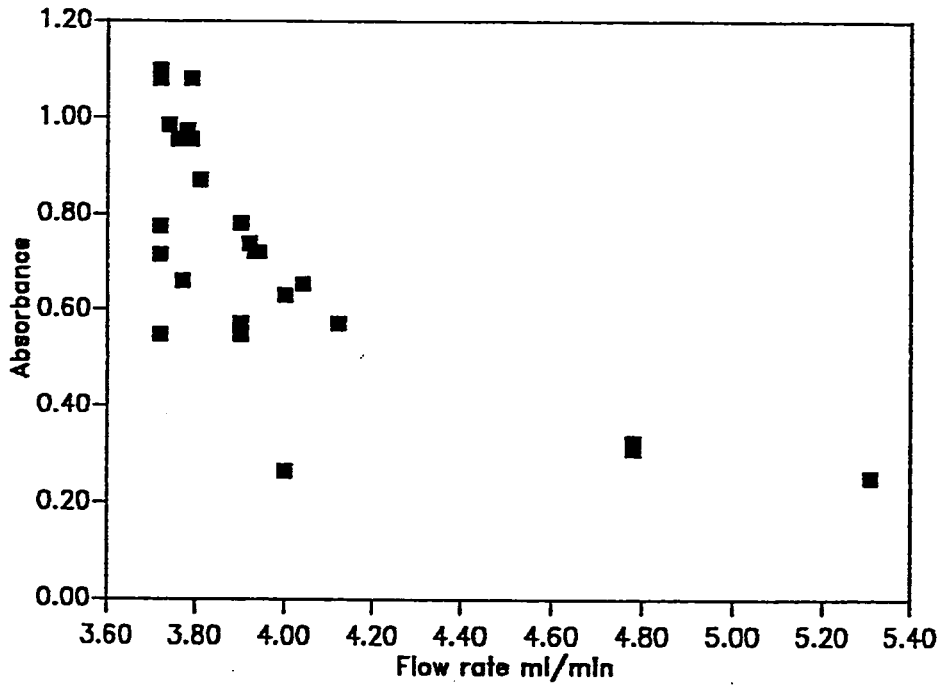
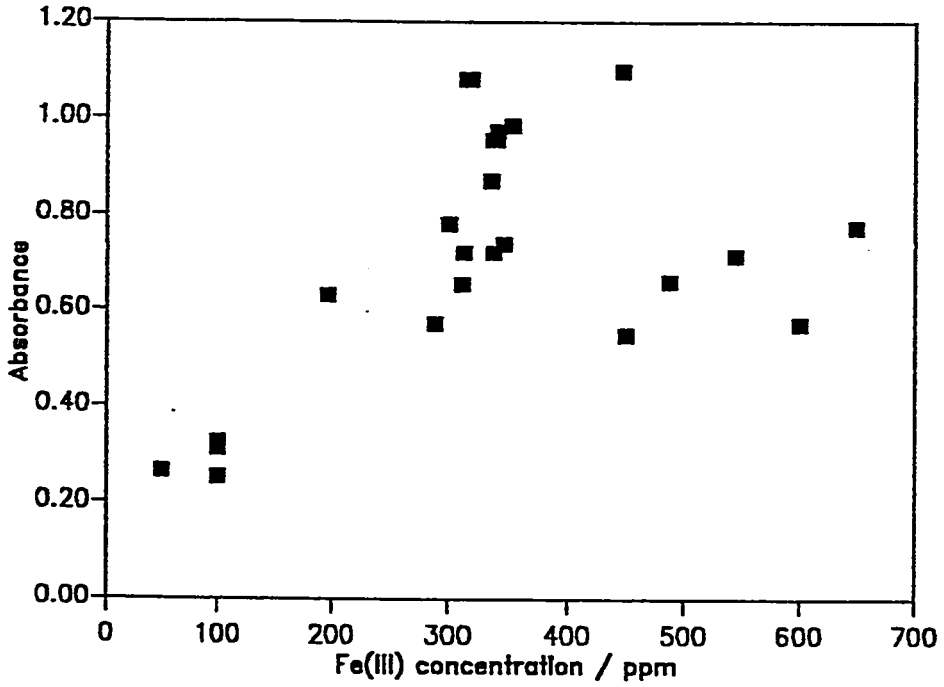


Fig.4.4.11 Simplex variation of Fe(III) concentration for demeclocycline.



As in other tetracyclines, the simplex method shows narrower range than do the univarient method, for the variation of variables to obtain the optimum conditions. This is clearly represented by the scattered diagrams, Figs. 4.4.8. - 4.4.11. The shorter coil length, and lower iron(III) concentration obtained by simplex procedure, favour the conditions obtained by the simplex method; therefore, a coil length of 45 cm, a sulphuric acid concentration of 0.001M, a flow rate of 3.72 ml/min and an iron(III) concentration of 447 ppm were used for the determination procedure of demeclocycline.

4.4.4 Analytical Appraisal

The Beer's law was found to be valid in the range 20 - 220 $\mu g\ ml^{-1}$. Accurately about 0.25000 g of pure demeclocycline is weighed, dissolved in 0.001M sulphuric acid, and completed to volume to 250 ml with 0.001M sulphuric acid. Different standard demeclocycline solutions were prepared by adding an appropriate volume of stock solution to 50 ml volumetric flasks, and the volume of these flasks was completed to volume with 0.001 M sulphuric acid.

The standard solutions were run in triplicate, by injecting a successive volumes of 157 μl into the carrier stream. The carrier stream con-

sists of 447 ppm iron(III) and 0.001M sulphuric acid. The absorbance of the resulting iron(III)-demeclocycline complex was monitored at 435 nm. The results of the proposed method are presented in Table 4.4.2. A typical record tracing for these results are presented in Fig. 4.4.12. The measured peak absorbancies are plotted versus different demeclocycline concentration as shown in Figs. 4.4.13. and 4.4.14.

The straight line was obtained in the range 20 - 220 $\mu g\ ml^{-1}$, with a correlation coefficient (r) of 0.998 and intercept of 0.0087. Above 220 $\mu g\ ml^{-1}$, there is a deviation towards the concentration axis, therefore, a calibration equation of the following type was obtained:

$$A = 0.0087 + 0.0027\ C \qquad (4.4)$$

where

A = peak absorbance at 435

C = concentration of oxytetracycline in $\mu g\ ml^{-1}$

This equation was then used for determination of unknown concentrations of oxytetracycline by the proposed procedure.

The peak width at the base line was found to be 21sec, from which the maximum sample frequency was found to be 170s/h. The relative

Table 4.4.2: Beer's law validation for demeclocycline with the proposed method.

concentration ppm	Absorbance
20	0.055
30	0.072
40	0.108
50	0.150
60	0.174
70	0.206
90	0.246
100	0.286
120	0.348
140	0.390
160	0.444
180	0.534
200	0.588
220	0.594
240	0.618
260	0.630

* Intercept for the slope = 0.0087.
** Correlation coefficient for 14 different determinations in the range 20 - 220 ppm is 0.998.

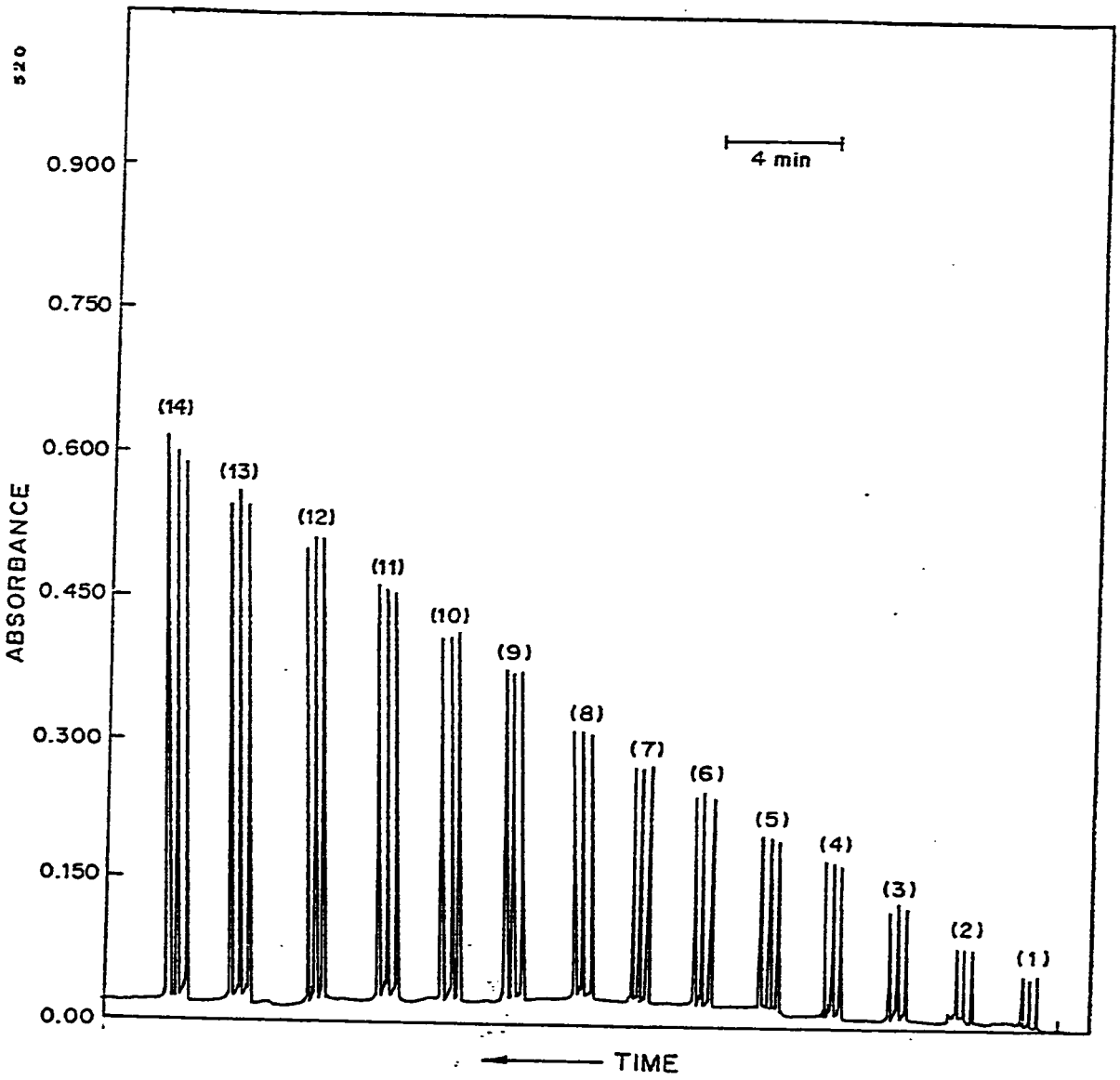


Fig.4.4.12. Typical FIA results (n = 3) for demeclocycline standard solutions of : (1)30 (2)40 (3)50 (4)60 (5)70 (6)90 (7)100 (8)120 (9)140 (10)160 (11)180 (12)200 (13)220 ppm.

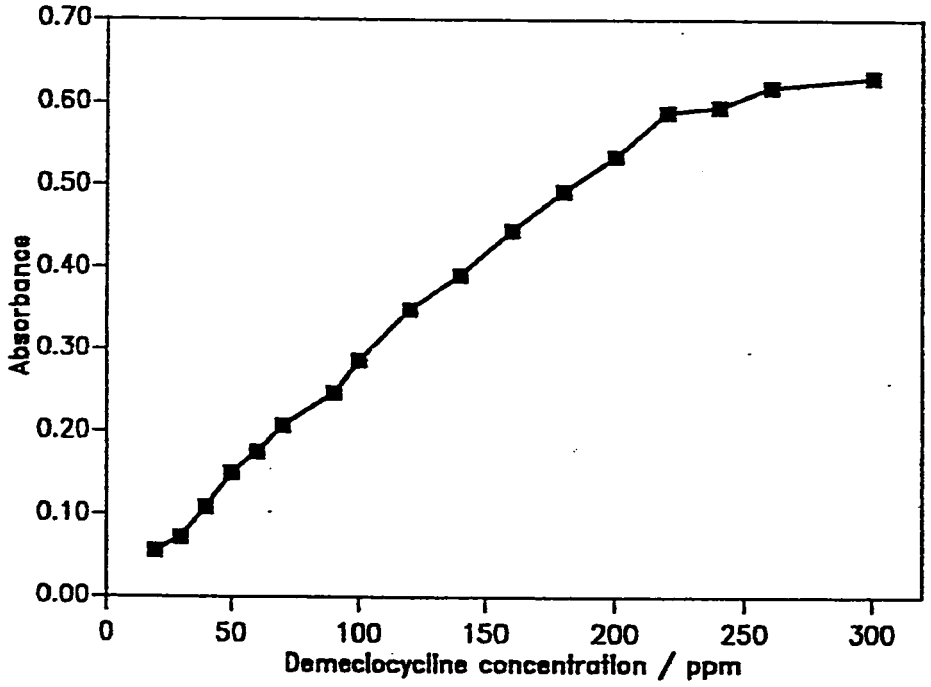


Fig.4.4.13 A typical computer plot of absorbance vs demeclocycline concentration by the FIA proposed method .

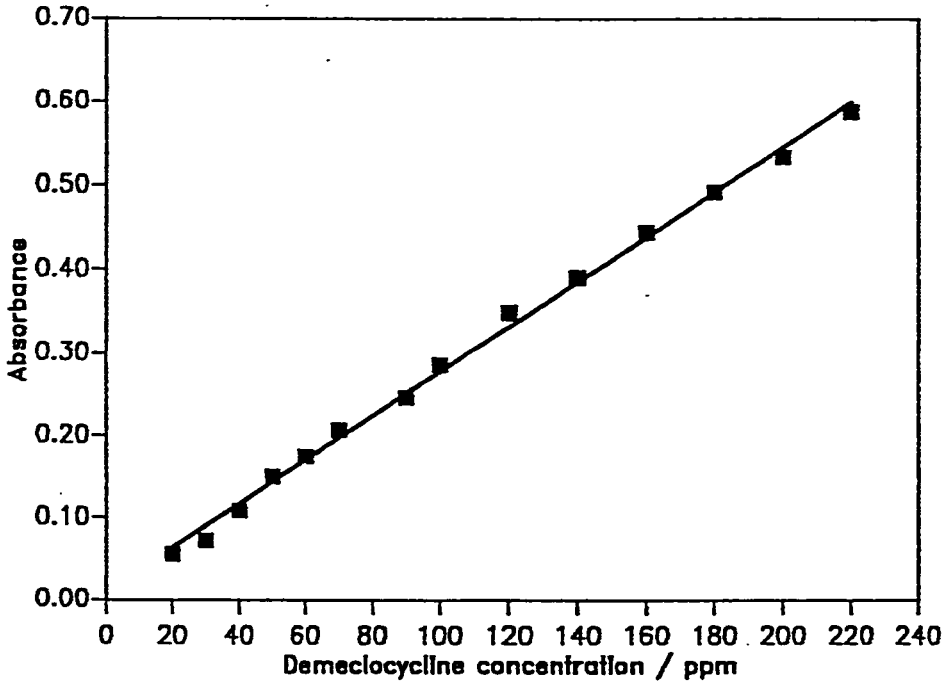


Fig.4.4.14 Atypical calibration curve for demeclocycline in the range 20 - 220 ppm by the proposed method.

standard deviation (RSD) was found to be 0.562 for seven injections of the oxytetracycline solution.

4.5 Applications

In order to establish the validity of the proposed method, the proprietary drugs containing tetracycline HCl and oxytetracycline, listed in Table 4.5.1, were analyzed. The proposed method was also compared to the conventional spectrophotometric method[1]

4.5.1 Dumocycline Capsules

The contents of 10 capsules were emptied carefully, mixed, and a quantity of powder equivalent to about 250 mg tetracycline-HCl was accurately weighed out. This quantity is dissolved in 200 ml of 0.001M sulphuric acid. Then this was warmed for 10 minutes, and shaken. After cooling to room temperature, the flask was diluted to 250 ml with 0.001M sulphuric acid. Both the conventional spectrophotometric method[1], and the proposed method were applied for the assay of this solution. The results of the assay of tetracyclineHCl obtained by this method were compared with the results obtained by the conventional spectrophotometric method.

Table 4.5.1: Proprietary Drugs Investigated by the Propose Method

Drug	Composition	Supplier
Dumocycline	TetracyclineHCl 250 mg	Dumex Denmark
Hostacy line P	TetracyclineHCl 500 mg Sodium hexa- metaphosphate 220 mg	Hoeschst,Germany
Latycin G	Tetracycline HCl 250 mg Glucosamine HCl 250 mg	Biochemie Austria
Balkacycline	Tetracycline HCl 250 mg	Arab pharmaceutical Jordan
Terramycin	Oxytetracycline HCl 250 mg	Pfizer Belgium
Oxytetracycline Syrup	Oxytetracycline 200 mg ml	Dopharma Holland

4.5.1.1 Results Obtained with the Conventional Spectrophotometric Method

To determine different concentrations of tetracyclineHCl in the range (10 - 200) $\mu g\ ml^{-1}$, in eight 25 ml volumetric flasks, 10 ml of 1 $mg\ ml^{-1}$ iron(III) dissolved in 0.001 M sulphuric acid were placed in each flask. Then different concentrations of tetracycline-HCl solution (10 - 200 $\mu g\ ml^{-1}$) were added to each flask, and the flasks were diluted to the mark by 0.001 M sulphuric acid. The absorbance of the coloured species of each mixture was monitored at 423 nm versus a reagent blank. The results obtained were presented in Table 4.5.2.

The same batch of samples "Dumocycline" were analyzed; repeated six times, by this method, and results are demonstrated in Table 4.5.3

4.5.1.2 Results Obtained with the Proposed Method

For the determination of the tetracycline-HCl, in the range 20 - 200 $\mu g\ ml^{-1}$, 100 μL aliquot of the sample (dumocycline) solution (140 $\mu g\ ml^{-1}$) was injected into the carrier stream (composition was previously specified), and the peak absorbance of the resulting coloured complex was measured at 423 nm. Figure 4.5.1. shows the FIA

Table 4.5.2: **Results Obtained with the Conventional Spectrophotometric Method using Different Concentrations of Tetracycline HCl.**

Volume Taken ml	Absorbances at 423 nm	Concentration mg/ ml
0.3	0.124	11.738
0.8	0.354	31.302
1.3	0.576	50.866
2.0	0.861	78.256
3.1	1.340	121.297
3.6	1.420	140.861
4.1	1.773	160.425
5.1	2.100	199.553

Correlation coefficient = 0.998

Intercept for the slope =0.0252

Table 4.5.3: Results Obtained with the Conventional Spectrophotometric Method using the same Concentration of Tetracycline HCl (140 $\mu g\ ml^{-1}$) for "Dumocycline" Capsules.

A	Found ppm	Found mg	Recovery %	Error
1.512	141.5	253.24	101.30	1.31
1.521	142.3	254.67	101.87	1.87
1.515	141.8	253.77	101.51	1.51
1.531	143.3	256.46	102.58	2.58
1.525	142.7	255.39	102.15	2.15

Standard deviation (n = 5) = 0.42

Table 4.5.4: Results Obtained with the FIA Proposed Method by using the same Concentrations of Tetracycline Hydrochloride (140 $\mu g\ ml^{-1}$) for "Dumocycline" Capsules.

A	Found ppm	Found mg	Recovery %	Error
0.336	138.81	248.40	99.36	-0.64
0.335	138.42	247.73	99.09	-0.91
0.340	140.34	250.34	100.14	-0.14
0.339	139.96	249.66	99.86	0.14
0.340	140.34	250.34	100.14	0.14

RSD (n = 5) =0.46

Student-t test value = 1.15

Theoretical t value = 2.31

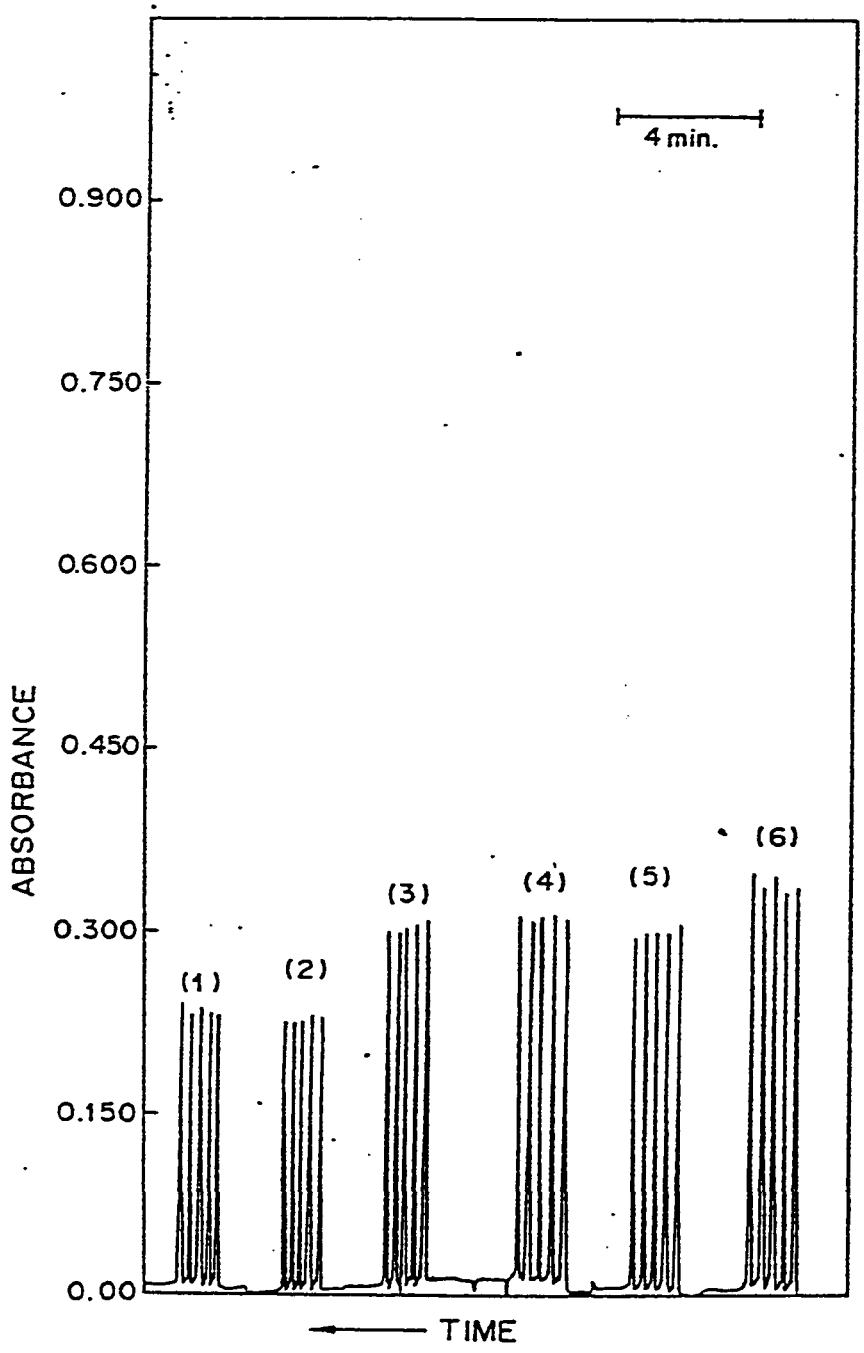


Fig. 4.5.1 Typical FIA recorder tracing (n = 5) for : (1) Oxytetracycline syrup 200 mg ml^{-1} (2) Terramycin capsules, 250 mg (3)Balkacycline capsules, 250 mg (4) Latycin G capsules, 250 mg. (5)hostacycline capsules, 500 mg (6) Dumocycline capsukes, 250 mg.

recorder tracing for this drug as curve 6. The results of assay of dumocycline were presented in Table 4.5.4.

4.5.2 Hostacycline Capsules

The contents of 10 capsules were emptied carefully, mixed, weighed and a quantity of powder equivalent to exactly about 250 mg tetracyli-neHCl was accurately weighed out. Then the same procedure for dumo-cycline capsules is followed.

4.5.2.1 Results Obtained with the Conventional Spectrophotometric Method

Analysis of the hostacycline solution by the conventional spectropho-tometric method was carried out, and results obtained were presented in Table 4.5.5.

4.5.2.2 Results Obtained with the Proposed Method

The same batch was analyzed by the proposed method. The same procedure for the analysis of dumocycline capsules was repeated for hostacycline, and the results obtained were presented in Table 4.5.6.

Table 4.5.5: **Results Obtained with the Conventional Spectrophotometric Method by using the same Concentrations of Tetracycline Hydrochloride (120 $\mu g\ ml^{-1}$) for "Hostacycline" Capsules.**

A	Found ppm	Found mg	Recovery %	Error
1.577	147.65	609.89	121.98	21.98
1.590	148.89	615.02	123.00	23.00
1.591	148.98	615.39	123.08	23.08
1.588	148.70	614.23	122.85	22.85
1.596	149.46	617.37	123.47	23.47

RSD (n = 5) = 0.55

Table 4.5.6: Results Obtained with the Proposed Method by using the same Concentrations of Tetracycline Hydrochloride (120 $\mu g\ ml^{-1}$) for "Hostacycline" Capsules.

A	Found ppm	Found mg	Recovery %	Error
0.300	125.13	516.85	103.37	2.60
0.295	123.04	508.24	101.65	1.65
0.296	123.42	509.81	101.96	1.96
0.297	123.81	511.42	102.28	2.28
0.297	123.81	511.42	102.28	2.28

RSD (n = 5) = 0.65

A typical FIA recorder tracing (fig.4.5.1) was shown for this drug as curve 5.

4.5.2.3 Interferences

The results of analysis of the components in the drug hostacycline, indicate that the presence of sodium metaphosphate in this drug exhibit great interference with positive error encountered with the determination of tetracycline with the conventional spectrophotometric method. This could be to attributed to the fact that phosphate equlibrates in acidic media and buffers the solution raising its pH leading to higher absorbances and hence positive error was obtained. When the same batch was assayed by the FIA proposed method, there is no interference, this could be due to the fact, that the amount of the sample injected is very small (157 μL).

4.5.3 Latycin-G

The contents of 10 capsules were emptied carefully by a micro-brush, mixed, weighed and a quantity of powder equivalent to exactly about 250 mg tetracycline-HCl was accurately weighed out. A stock solution of (1 mg ml^{-1}) was prepared and used for both the conventional spectrophotometric method and the proposed FIA method.

4.5.3.1 Results Obtained with the Conventional Spectrophotometric Method

The same procedure for the analysis of the two previous drugs by this method was used here. The concentration of the drug used was 120 $\mu g\ ml^{-1}$ and the procedure was repeated six times. Results obtained were presented in Table 4.5.7

4.5.3.2 Results Obtained with the Proposed Method

The same batch was analyzed by the proposed method. The procedure used was the same as in the previous drugs. The results obtained were presented in Table 4.5.8. For this drug, the FIA recorder tracing for five runs were shown in Fig. 4.5.1. as curve 4.

4.5.4 Balkacycline Capsules

The same procedure was carried out for the treatment of these capsules, as in the previous capsules. Then both, the conventional spectrophotometric method and the proposed method were used to analyze the contents of these capsules. The results obtained together with the statistical parameters are presented in Table 4.5.9. for the conventional

Table 4.5.7: **Results Obtained with the Conventional Method by using the same Concentrations of Tetracycline Hydrochloride (120 $\mu g\ ml^{-1}$) for "latycin G" Capsules.**

A	Found ppm	Found mg	Recovery %	Error
1.280	119.39	248.90	99.56	-0.44
1.281	119.39	248.90	99.56	-0.44
1.268	118.25	246.55	98.62	-1.38
1.274	118.82	247.74	99.10	-0.90
1.257	116.90	243.73	97.98	-1.46
1.265	117.97	245.97	98.39	-1.61

RSD (n = 5) = 0.73

Table 4.5.8: **Results Obtained with the Proposed Method by using the same Concentrations of Tetracycline Hydrochloride (1240 $\mu g\ ml^{-1}$) for "Latycin G" Capsules.**

A	Found ppm	Found mg	Recovery %	Error
0.301	125.35	261.36	104.54	4.54
0.302	125.76	262.22	104.89	4.54
0.300	124.96	260.54	104.22	4.22
0.299	124.58	259.75	103.90	3.90
0.298	124.19	258.94	103.58	3.58

RSD (n = 5) = 0.56

Student-t value = 1.71

Theoretical student-t value = 2.31

Table 4.5.9: **Results Obtained with the Conventional Method by using the same Concentrations of Tetracycline Hydrochloride (120 $\mu g\ ml^{-1}$) for "Balkacycline" Capsules.**

A	Found ppm	Found mg	Recovery %	Error
1.315	122.72	256.56	102.62	2.62
1.320	123.20	257.61	103.04	3.04
1.330	124.15	259.60	103.84	3.84
1.320	123.20	257.61	103.04	3.04
1.325	123.67	258.47	103.39	3.39

RSD (n = 5) = 0.46

Table 4.5.10: **Results Obtained with the Proposed Method by using the same Concentrations of Tetracycline Hydrochloride (120 $\mu g\ ml^{-1}$) for "Balkacycline" Capsules.**

A	Found ppm	Found mg	Recovery %	Error
0.294	122.69	256.54	102.62	2.62
0.292	121.92	254.93	101.97	1.97
0.291	121.54	254.14	101.66	1.66
0.289	120.77	252.53	101.01	1.01
0.288	120.38	251.71	101.69	0.69

RSD (n =5) = 0.58

Student-t value = 1.63

Theoretical t value = 2.31

spectrophotometric method, and in Table 4.5.10. for the proposed method.

4.5.5 Terramycin Capsules

Each capsule was claimed, containing 250 mg oxytetracycline. As in the previous capsules, the same procedure was followed for treatment of capsules. The conventional and FIA methods were used for the analysis of the prepared terramycin solution. The results of the assay of oxytetracycline obtained with the proposed method were compared to those obtained with the conventional spectrophotometric method.

4.5.5.1 Results Obtained with the Conventional Spectrophotometric Method

To determine different concentrations of oxytetracycline in the range (10 – 200 $\mu g\ ml^{-1}$), in eight calibrated flasks of 50 ml volume, 10 ml of iron(111) 1 $mg\ ml^{-1}$ dissolved in 0.001 M sulphuric acid were placed in each flask. Then different concentrations of oxytetracycline solution (10 – 200 $\mu g\ ml^{-1}$) were added to each flask, and the flasks were diluted to the mark by 0.001 M sulphuric acid. the absorbance of the

coloured complex of each mixture was measured versus a reagent blank at 435 nm. The results obtained were presented in Table 4.5.11. The same batch of samples "Terramycin" were analysed; repeated five times, by this method and results are shown in Table 4.5.12.

4.5.5.2 Results Obtained with the Proposed Method

For the determination of the oxytetracycline in the range (40 – 260 $\mu g\ ml^{-1}$), 157 μL of the sample "Terramycin" solution (120 $\mu g\ ml^{-1}$) were injected into the carrier stream (composition previously specified) and the resulting peak absorbance was recorded. Fig. 4.5.1 shows the FIA recorder tracing for this drug as curve 2. The results of assay of terramycin were presented in Table 4.5.13.

4.5.6 Oxytetracyline Syrup

One ml containing 200 mg oxytetracycline was pipetted and converted to a 500 ml calibrated flask, then 50 ml sulphuric acid 0.01 M were added. The volume was completed to the mark with the distilled water. The conventional spectrophotometric method was then used for the analysis of the solution.

Table 4.5.11: **Results Obtained with the Conventional Spectrophotometric Method using Different Concentrations of Oxytetracycline.**

Volume Taken ml	Absorbances at 423 nm	Concentration ppm
0.50	0.114	9.708
1.60	0.318	31.05
2.70	0.542	52.39
4.30	0.876	83.44
6.40	1.301	124.19
7.50	1.503	145.53
8.60	1.713	166.87
10.70	2.023	207.62

Intercept for the slope = 0.0310

Correlation coefficient = 0.9992

Table 4.5.12: Results Obtained with the Conventional Method by using the same Concentrations of Oxytetracycline (120 $\mu g\ ml^{-1}$) for " Terramycin " Capsules.

A	Found ppm	Found mg	Recovery %	Error
1.347	124.16	260.39	104.16	4.16
1.350	124.45	260.97	104.39	4.39
1.344	123.88	259.78	103.91	3.91
1.332	122.75	257.41	102.96	2.96
1.335	123.03	257.99	103.20	3.20

RSD (n = 5) = 0.62

Table 4.5.13: Results Obtained with the proposed Method by using the same Concentrations of oxytetracycline (120 $\mu g\ ml^{-1}$) for "Terramycin " Capsules.

A	Found ppm	Found mg	Recovery %	Error
0.228	119.87	251.37	100.55	0.55
0.228	119.87	251.37	100.55	0.55
0.224	117.77	246.96	98.78	-1.22
0.222	116.72	244.76	97.90	-2.10
0.223	117.24	245.85	98.34	-1.66

RSD (n = 5) = 0.87

Student-t value = 1.99

Theoretical t value = 2.31

4.5.6.1 Results Obtained with the Conventional Spectrophotometric Method

In five calibrated flasks of 50 ml volume, 10 ml of iron(III) 1 mg ml^{-1} dissolved in 0.001M sulphuric acid were placed in each flask. Then 15 ml of the prepared solution were added to each flask, mixed with the contents of the flask, and diluted to the mark with 0.001M sulphuric acid. The absorbance of the formed coloured complex was monitored at 435 nm versus a reagent blank. The results obtained were presented in Table 4.5.14.

4.5.6.2 Results Obtained with the Proposed Method

To determine oxytetracycline in the range 40 – 260 $\mu g\ ml^{-1}$, 157 μL of the sample "oxytetracycline syrup" solution (120 $\mu g\ ml^{-1}$) was injected into the carrier stream, and the resulting peak absorbance, measured at 435 nm, was recorded. Figure 4.5.1 shows the FIA recorder trace for this drug as curve 1. The results of assay of oxytetracycline were presented in Table 4.5.15.

Table 4.5.14: Results Obtained with the Conventional Method by using the same Concentrations of oxytetracycline (120 $\mu g\ ml^{-1}$) for " Oxytetracycline syrup "

A	Found ppm	Found mg	Recovery %	Error %
1.327	122.28	204.20	102.10	2.10
1.340	123.50	206.25	103.12	3.12
1.321	121.71	203.26	101.63	1.63
1.324	121.99	203.72	101.86	1.86
1.329	122.46	204.51	102.25	2.25

RSD (n = 5) = 0.56

Table 4.5.15: Results Obtained with the proposed Method by using the same Concentrations of oxytetracycline (120 $\mu g\ ml^{-1}$) for "oxytetracycline syrup "

A	Found ppm	Found mg	Recovery %	Error %
0.222	116.72	194.92	97.46	-2.54
0.225	118.29	197.55	98.78	-1.22
0.228	119.87	200.19	100.09	-0.09
0.222	116.70	194.92	97.46	-2.54
0.226	118.82	198.43	99.23	-0.78

RSD (n = 5) = 0.63

Student-t value = 1.23

4.6 Conclusion

The present method is accurate compared to the other reported methods but quite suitable for the assay of tetracyclines in drug formulations without suffering interferences from excipients and other common additives in such compounds.

Being flow injection method (FIA), it has a distinct advantadge of providing high sampling throughput and less time required for the analysis.

Also the chemical system permitted a successful application of the modified simplex procedure of optimization that helped in developing chemical intution, achieving maximum response with optimum operating conditions reached faster and with less number of experiments than with the iterative univarient method thus preserving excessive labour, time, drug and reagent expenditure.

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