Application of Factorial Design for Optimization of Flow-Injection Spectrophotometric Determination of Tetracycline in Some Pharmaceutical Formulations

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(Received: 21/ 10 / 2012 ---- Accepted: 9 / 12 / 2012)

Abstract
A simple and rapid flow injection (FI) spectrophotometric procedure for the determination of tetracycline (TC) has been developed. The method is based on the injection of 150 µl standard or sample solution into a distilled water carrier stream which merged at the merging zone with ferric nitrate solution with the optimum flow rate of 1.5 mL/min, subsequently the reaction will occur inside the reactor coil, forming brown color product that monitored at 418 nm. The flow injection experimental conditions were optimized by means of full factorial design. Under the optimum conditions, calibration graph was obtained over the range 2.0-120 µg/ml with a detection limit of 1.0 µg/ml. The correlation coefficient and molar absorptivity were 0.9994 and $4.343x10^3$ L/mol.cm respectively. The results showed a good precision and accuracy. The method was successfully applied to the determination of tetracycline in pharmaceutical formulations and showed good agreement with those obtained by the standard method at 95% confidence level, with a sample throughput of 60/h.

Key words: flow injection, tetracycline, factorial design

Introduction
Tetracyclines (TC) are a family of antibiotics highly employed in the treatment of human and animal diseases. Tetracycline molecules comprise a tetracyclic nucleus to which various groups are attached[1]. Although their different kinds differ on the substations they have, all the molecules from this family present a common structure which owns antibiotic activity when there is a dimethyl-amine in one of their carbons, whereas the elimination of this group, reduces these properties and increases their non-antibiotic action [2].

Tetracycline having a chemical name [4s, 4as, 5as, 12as]-4-(Dimethylamino)-1,4,4a,5,5ab,11 12a octahydro 3,6,10,12,12a-pentahydroxy-6-methyl-1,11 dioxonapthacene carboxamide [3,4] (as shown in Figure 1). It is a broad-spectrum antibiotics widely used for treatment of infections. Recently it was found that tetracycline antibiotics have some other important functions[5]. The medicines give new hopes for ailing heart attack and ulcer patients. A novel single tetracycline-regulative adenoviral vector was investigated by Fang group for tumer-specific Bax gene expression and cell killing in vitro and vitro which may become a potential therapeutic agent for the treatment of cancer. The tetracycline hydrochloride inducible gene expression system has become a commonly used approach to experiments-controlled expression of agents for functional evaluation in mammalian cells. TC is used to switch gene activity on and off [5,6] .

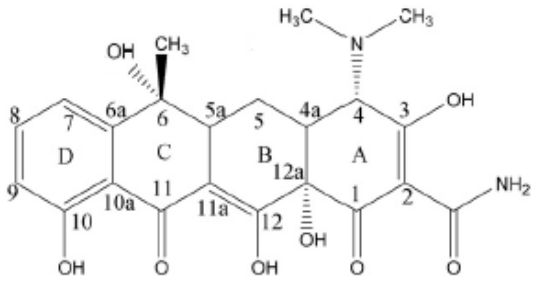

Figure 1: Chemical structure of tetracycline

Tetracyclines represent a class of antibacterial compounds which are broad-spectrum agents [7,8] against a wide range of gram-positive and gram-negative bacteria, a typical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites [9]. It is used for many different infections, such as respiratory tract infections, urethritis and severe acne, it also has a role in the treatment of multidrug resistant malaria [10, 11] .

Although the tetracyclines retain important roles in both human and veterinary medicine, the emergence of microbial resistance has limited their effectiveness. Undoubtedly the use of tetracyclines in clinical practice has been responsible for the selection of resistant organisms. Nevertheless, as we enter the new millennium, the use of tetracyclines and other antibiotics as animal growth promoters is becoming increasingly controversial because of concerns that this practice may be contributing to the emergence of resistance in human pathogens [12].

Various methods have been developed for determination of tetracycline in pharmaceutical preparations and biological samples including UV-visible spectrophotometry [2,6,13,14], fluorimetry [15,16], capillary electrophoresis [5,17], chemiluminescence [18-21], liquid chromatography [7,22-24], Voltammetry [8], flow injection analysis [9,25-31].

The main purpose of this work was to develop a simple, fast and low-cost flow injection procedure for determination of tetracycline based on the spectrophotometric detection of the colored product formed between the tetracycline and ferric nitrate to evaluate the drug content in capsule dosage forms using full factorial design program.

Exerimental

Apparatus

The proposed FI system is depicted in Figure 2. It consists of two channels (A Watson-Marlow 501Z multichannel peristaltic pump was employed for fluid propelling). All measurements were performed with a CECIL CE 3021, 3000 series UV-Visible spectrophotometer connected to a FUJITSU SIEMENS laptop. The samples were pumped through Tygon pumping tube of 0.9 mm i.d., the sample loop and reaction coils were made of poly tetrafluoroethylene tubes (PTFE) of 1.0 mm i.d. The sample or standard solution was injected via a six-way PTFE loop valve (Omnifit) with a 150 µL sample loop. The absorbance of the produced color was monitored at 418 nm.

Reagents and analytical solutions

Solutions of ferric nitrate $Fe(NO_3)_3$. $9H_20$ (Fluka AG) 3% and 5% (w/v) were prepared in distilled water. Pharmaceutical grade tetracycline hydrochloride was obtained from Scharlau (purity 99.0%). Stock solution of tetracycline (1000 µg/mL) was prepared by dissolving 0.1080 g of the tetracycline hydrochloride in a total volume of 100 ml distilled water. Standard solutions for linearity study were prepared by diluting the calculated volumes of the stock solution with distilled water. The standard solutions of tetracycline are stable for more than 1 month when kept at 40C and protected from direct light throughout the analysis, due to the photosensitivity of tetracycline to the light [11].

Factorial design

The optimum reaction conditions for tetracycline determination with spectrophotometric flow-injection analysis were investigated by full factorial design. A two-level (2^4) full factorial design with 16 runs was developed in order to determine the influence of the factors and their interactions on the system response. Four factors were studied: ferric nitrate concentration, flow rate, reaction coil, and loop size.

Two-level factorial designs are screening designs that combine the low and high levels of each factor (Table 1). These designs require only a reduced number of experiments to find those trends that are helpful to understand the behavior of the system. In addition, they can be easily upgraded to surface response designs in order to perform further optimizations.

Recommended procedure

A volume of 150µl of the tetracycline solution was injected into the sample loop by means of a syringe. Samples or standards were injected into the carrier stream (distilled water) and merged at the merging point with ferric nitrate solution (both of them pumped at a 1.5 ml/min flow rate), subsequently the reaction will occur inside the reactor coil, forming brown color products and finally passed through the flow injection spectrophotometric cell(FC), resulting a signal and recorded absorbance at 418 nm which is proportional to the tetracycline content in the sample. When the baseline was reached, another sample was injected.

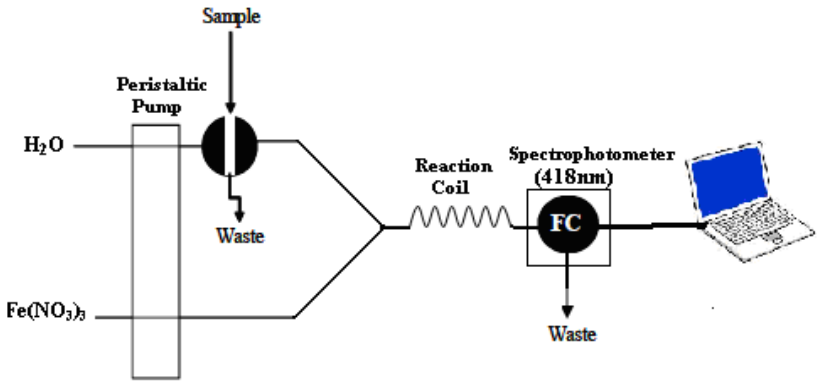

Figure 2: Flow diagram of the FI system used

Samples preparation

Four commercial samples of pharmaceutical formulations (capsules) containing 500 mg and 250mg of tetracycline hydrochloride different brands and companies were analysed by the proposed method.

Preparation of the samples involved the following procedure; ten capsules were opened and their contents were weighed and mixed well. Then a powder, equivalent to the weight of one capsule was accurately weighed into a 100ml volumetric flask, 50 mL of distilled water was added, shaken continuously to dissolve, filled and mixed totally. The solution was then filtered through Whatman 41 filter-paper. The first few milliliters of the filtrate were discarded and an aliquot of 2.0 mL of the filtrate was transferred to a 100 mL volumetric flask and the volume was

completed with distilled water. All samples were analyzed according to the recommended procedure.

Results and Discussion

The idea of the present work is based on the reaction of tetracycline with ferric nitrate to form a stable colored product which has maximum absorption at 418nm as shown in the absorption spectra (Figure 3), using 50µg of tetracycline and 2.5% ferric nitrate. This method was adapted for the determination and automation of tetracycline in different pharmaceutical drugs using spectrophotometric flow-injection analysis (FIA) technique.

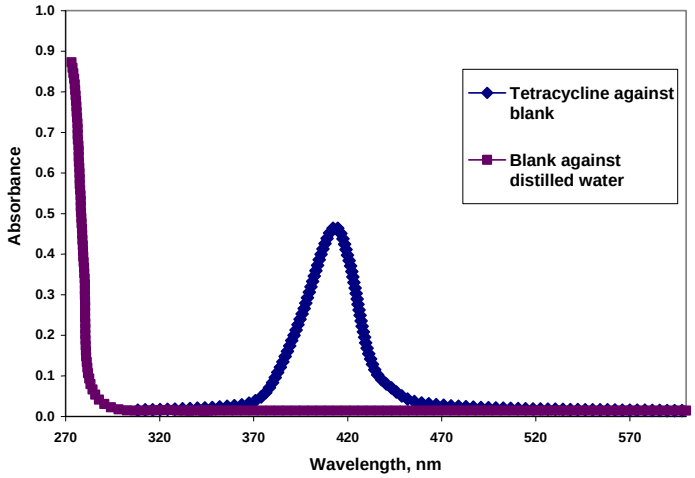

Figure 3: Absorption spectra of the reaction product between TC and ferric nitrate.Tetracycline concentration =50µg/ml

Optimization of the chemical and FI variables

Consequently, in order to achieve reasonable sampling rate, reproducible measurements and low detection limits, several factors must be optimized. These factors include the chemical variables and the FI variables.

Due to the fact that more than one variable is potentially important, and that it would be difficult to optimize the conditions through a uni-variant optimization procedure, the experimental conditions were obtained using a Factorial designs which are widely used in experiments involving several factors to investigate the effects of the factors on the response.

It is desirable to develop an acceptable pharmaceutical formulation determination in shortest possible time and low chemical concentration. Traditionally pharmaceutical formulation determinations are developed by changing one variable at a time approach. The method is time consuming in fact and requires a lot of imaginative efforts. Moreover, it may be difficult to develop an ideal formulation using this classical technique since the joint effects of independent variables are not considered. It is therefore very essential to understand the complexity of pharmaceutical formulations by using established statistical tools such as factorial design. In addition to the art of formulation, the technique of factorial design is an effective method of indicating the relative significance of a number of variables and their interactions[32].

The factorial methods are straightforward to implement and their results can be very easily interpreted; these methods are created to measure the additive effects on a response for each of the input factors. In addition, the effects of interactions between factors can also be investigated. Two level factorial designs have many advantages in analytical procedures. In this method, there are comparisons available for each main effect on the experimental results. The total number of runs in factorial design is much less as compared to the "one variable at a time" approach. Estimation of the interaction of effects is an additional advantage over the "one variable at a time" approach [33].

The design determines which factors have important effects on a response as well as how the effect of one factor varies with the level of the other factors[34]. The determination of factor interactions could only be attained using statistical designs of experiments, since it can not be observed when the system optimization was carried-out by varying just one factor at the time and fixing the others[35].

Initially the influence of the variables (ferric nitrate concentration, flow rate, reaction coil and loop size) were examined by 2^4 full factorial design in which four factors each having two levels are run to study the effect of the factors on the response . The two levels of each factor are general coded as "-1" for the "low" level and "+1" for the "high" level of the factor, for this design, 16 experiments were necessary. The factors and levels used in the factorial design are summarized in Table 1.

Table 1: Factors and levels used in the factorial design experiment

Factor	Low level (-1)	High level (+1)
Flow rate(ml/min)	0.8	1.5
Loop size(µl)	100	150
Reaction coil(cm)	80	120
Ferric nitrate(%)	3	5

Pareto chart (Figure 4) was plotted to check the influence of the factors and their interactions in the system using analysis of variance (ANOVA) and *p*-values ($p > 0.05$). As can be seen from the Pareto chart, the factors ferric nitrate concentration, reaction coil length are statistically significant at the 95% confidence level. Pareto chart illustrates the order of significance of the variables affecting the response of the system (absorbance).

Pareto chart is a horizontal bar-chart. The length of each bar on the chart and their signs are proportional to the absolute value of its associated estimated effect or the standardized effect. The importance of each variable depends on its sign and value. Positive signs indicate that the absorbance signal is increased with an increase of the value of the respective variable (directly proportional) within the range studied, while negative signs indicate that the absorbance signal is favored with a decrease of the variable (inversely proportional). If the effect is smaller than 5% (i.e. the resulting graph does not go over the vertical line), the variation of the response caused by changing the variable is smaller than the experimental error, and therefore, the variable is considered not to be significant.

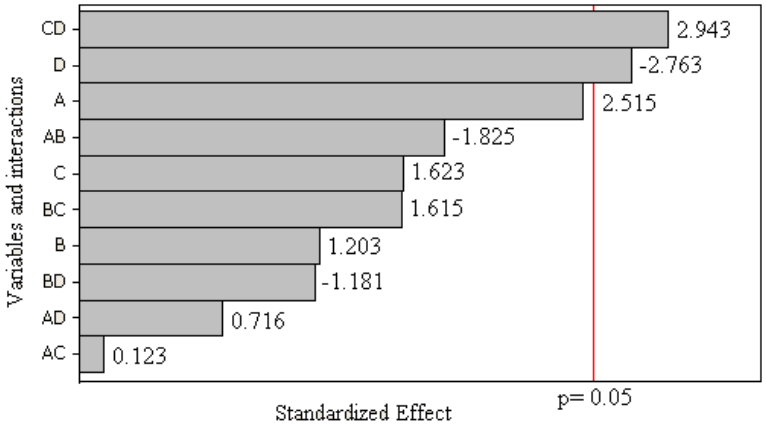

Figure 4: Pareto chart of standardized effects for the FI system variables using integrated absorbance as analytical response. A: flow rate(ml/min), B: loop size(µl), C: reaction coil(cm), D: ferric nitrate(%)

As seen from Pareto chart the flow rate, loop size and the interactions between variables are under the vertical line so they are not significantly important, so the flow rate(A) and loop size(B) were fixed at 1.5ml/min and 150µl (high level) respectively due to the positive sign on the variable bar.

The variables most significant ferric nitrate concentration(D) and the interaction between reaction coil length(C) and ferric nitrate concentration(D) were optimized by response surface methodology. These variables were studied in 14 runs for statistical validity within range -1.41 to +1.41, which corresponds to the ferric nitrate concentration with a range of 1.5 to 3.0% (w/v) and reaction coil with a length range 120 to 160cm.

Response surface methodology (RSM) is an empirical statistical technique employed for multiple regression analysis by using quantitative data. It solves multivariable data which is obtained from properly designed experiments to solve multivariable equation simultaneously [36]. The graphical representation of their functions is called Response Surface, which was used to describe the individual and cumulative effect of the test variables and their subsequent effect on the response. Easy way to estimate Response Surface, Factorial designs is the most useful scheme for the optimization of variables with a limited number of experiments [37].

Response surface methods are often employed after a screening of important factors, usually by performing a previous factorial design, which indicated a curvature [35]. This statistical design is used to examine the relation ship between one or more response variables and a set of quantitative experimental factors. After that, it is necessary to find the factor settings that optimize the response[38].

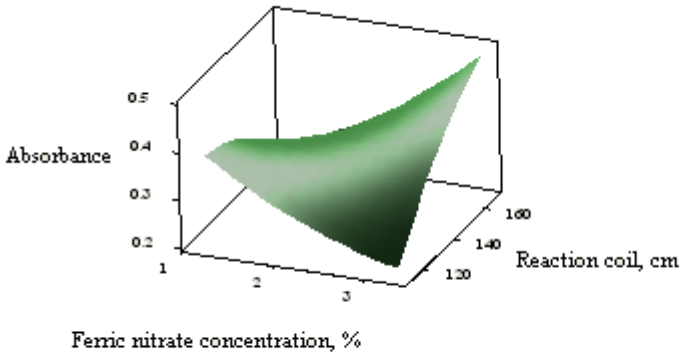

Figure 5: Response surface Plot of Absorbance against ferric nitrate concentration and reaction coil length

The response surface methodology program (Figure 5) showed an optimum value of 2.5% (w/v) and 156.2cm of ferric nitrate concentration and reaction coil length respectively. The optimum values of the variables obtained are summarized in Table 2

Table 2: Optimized values of chemical and flow injection variables in the proposed method

Variable	Optimum value
Flow rate (ml/min)	1.5
Loop size (µl)	150
Reaction coil (cm)	156.2
Ferric nitrate (%)	2.5

Calibration curve

Under the optimized experimental condition for determination of tetracycline, a linear calibration curve over the range of 2.0-120 µg/ml ($4.5*10^{-6}$ – $2.7*10^{-4}$ M) was established with the regression equation y = -0.0256 + 0.0098x (correlation coefficient 0.9994), where y represents the absorbance and x is the tetracycline concentration in parts per million. The detection limit (defined as 3 times standard deviation) and molar absorptivity were found to be 1.0 µg/ml ($2.25*10^{-6}$) and $4.343x10^{3}$ L/mol.cm respectively.

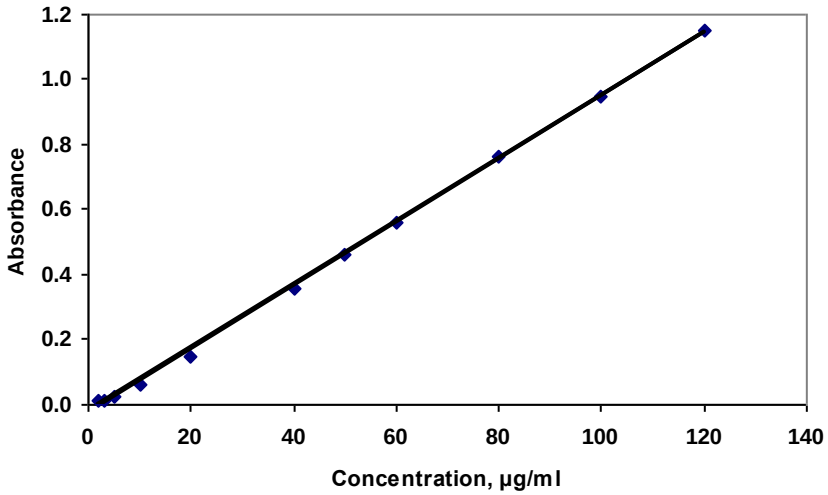

Figure 6: Calibration curve of determination of tetracycline treated according to the flow- injection analysis system

Precision and accuracy

The precision and accuracy for the determination of tetracycline were calculated depending upon the values of the relative standard deviation percentage (RSD %) or coefficient of variance (CV), and error percentage (Error %) for five replicates and three concentrations. Table 3 shows the results.

Table (3): Precision and accuracy of the proposed FI method.

Tetracycline concentration (µg/ml)	RSD%	Error %
2.0	1.984	2.222
50	0.6217	0.7391
120	0.4790	0.5041

Application of the method

The proposed method was applied successfully to the determination of tetracycline in different pharmaceutical formulations as listed in Table 4. The concentrations of tetracycline were calculated from the straight- line equation of the calibration curve.

The results were statistically compared with that obtained by the standard method of sodium molybdate method [39, 40] as summarized in Table 4. In all cases, the calculated t values are less than the theoretical ones (3.18) at 95% confidence level (for degree of freedom equal to 3), indicating that there is no significant difference between either methods concerning precision and accuracy in the determination of tetracycline in pharmaceutical formulations. The flow system proposed provides a sampling frequency of 60 samples h^{-1}

Table (4): Determination of tetracycline in pharmaceutical formulations

Commercial name of the drug	Composition	Proposed FI method Found (mg/cap.)	Standard method Found (mg/cap.)	Error%	t value
SAMACYCLINE	250mg tetracycline.HCl	253.25	252.45	0.3169	0.97
TETRAMIN	250mg tetracycline.HCl	247.80	246.45	0.5477	1.08
Tetrabact	500mg tetracycline.HCl	495.35	495.53	- 0.0363	0.70
Tetramac	500mg tetracycline	498.42	498.07	0.0702	0.96

Conclusions

The development of analytical methodologies can be further improved by the assistance of experimental design procedures. Even, the use of factorial experimental designs represents a key help in understanding not only the influence of several variables on analytical systems, but also to find their trends and behaviors. In this work application of factorial design allowed the optimization of parameters that influence the response of flow injection spectrophotometric method for determination of tetracycline. Employing the Pareto chart; it was possible to evaluate the influence of each variable and the combination of variables on the response of the system (absorbance). It was demonstrated that the most critical parameters for reaching the optimum values are ferric nitrate concentration and reaction coil length which were solved by response surface methodology. The proposed method is simple, unexpensive, allows rapid determination at low costs, and shows adequate selectivity, low limit of detection and very good precision and accuracy. The method was successfully applied for the determination of tetracycline in some pharmaceutical drug samples .

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تطبيق تصميم المضروب لإيجاد الظروف المثلى للحقن الجرياني المطيافية الضوئية لتقدير التتراسايكلين في بعض التركيبات الدوائية

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(تاريخ الاستلام: 21 / 10 / 2012 ---- تاريخ القبول: 9 / 12 / 2012)

الملخص

تم وصف طريقة سريعة وبسيطة لتقدير التتراسايكلين بواسطة حقن جرياني مطيافية ضوئية. تعتمد الطريقة على حقن 150مايكروليتر نموذج التتراسايكلين في تيار الماء المقطرالحامل والتي يندمج عند نقطة الالتقاء مع تيار محلول نترات الحديديك وبسرعة جريان مثلى عند 1.5 مل/دق، ويحدث التفاعل داخل انبوب التفاعل مكونا ناتجا ذو لون قهوائي والذي قيس عند 418 نم . تم ايجاد الظروف المثلى للحقن الجرياني بواسطة تصميم المضروب الكامل. وبأستخدام الظروف المثلى تم الحصول على منحني معايرة في المدى 2.0 –120 مايكوغرام/مل مع حد كشف 1.0 مايكوغرام/مل. وكانت معامل الارتباط والامتصاصية المولارية عبارة عن 0.9994 و $4.343*10^3$ لتر/مول.سم على التوالي. وتم تطبيق الطريقة لتقدير التتراسايكلين في بعض من التركيبات الدوائية بنجاح ، وقد اظهرت النتائج مطابقتها مع نتائج الطريقة القياسية عند مستوى ثقة 95%. وبتحليل نماذج 60 نموذج/ساعة.