Extraction and Determination of Sildenafil Citrate by Spectrophotometry: Classical Versus Factorial Design Optimization

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Abstract

In this work, the complexation and extraction processes of sildenafil citrate (SC) were evaluated by using classical optimization versus factorial design methodology. The selected factors were based on the reaction of sildenafil with methyl orange (MO) as a chromogenic reagent via the formation of ion-association complex in acidic buffer media, followed by extraction with chloroform and quantitatively measured by visible spectrophotometry at λ_{max} of 427 nm. The optimization step was first carried out by classical one-factor-at-a-time (OFAT) from which the extracted results were exploited in the experimental design optimization by using 2^{4-1} fractional factorial and 3^2 full factorial designs for the chosen variables such as pH, concentration of reagent, reaction time, extraction time and temperature. Results obtained from fractional factorial design 2^{4-1} showed that only the variables pH, MO concentration and their interaction based on analysis of variance (ANOVA) in term of Pareto chart, were statistically significant at 95% confidence level. Under the optimized conditions, the assay method of SC was validated in term of the analytical figures of merit. SC can be determined in concentration range of 0.5-40 µg mL^{-1} with correlation coefficient of 0.9991, detection limit (S/N) of 0.15 µg mL^{-1}, RSD (n=10) of 1.43%, accuracy as $\%E_{rel}$ of -2.23% and mean percent recovery of 97.77±0.87. The proposed method was applied for the determination of SC in commercial medicaments without fear from excipients interferences with the assay procedure.

زهير عبد الامير خماس

الملخص

جرى في هذا العمل تقويم التدبير التقليدي في الحصول على الظروف الفضلى مقابل طرائق التصميم العاملي في عمليات تكوين معقد عقار سترات السيلدينافيل واستخلاصه المذيبي، إذ تم اعتماد اختيار المتغيرات او العوامل المؤثرة على تفاعل عقار السيلدينافيل مع المثيل البرتقالي ك كاشف لوني عبر تكوين معقد المزدوج الايوني في الوسط الحامضي للمحاليل المنظمة ومن ثم استخلاصه بالكلوروفوم وتقديره كميا بالمطيافية المرئية عند الطول الموجي الاقصى (λ_{max} =427 nm). تم اجراء الحصول على الظروف الفضلى اولا باستخدام التدبير التقليدي التي استغلت نتائجه في وضع فرضيات طرائق التصميم التجريبي إذ جرى استخدام التصاميم العاملي الجزئي من نوع (2^{4-1})والتام من نوع (3^2) باختيار العوامل مثل الدالة الحامضية وتركيز الكاشف اللوني واوقات التفاعل والاستخلاص ودرجة الحرارة . دلت النتائج المستخلصة من التصميم العاملي الجزئي ان المتغيرات مثل الدالة الحامضية وتركيز الكاشف وتداخلهما هما العاملان الاكثر تأثيرا او معنوية مقارنة بالعوامل الاخرى استنادا الى تحليل التباين من خلال مخطط باريتو عند مستوى 95% من الثقة .جرى اثبات صحة طريقة تقدير دواء سترات السيلدينافيل طيفيا عند الظروف الفضلى من استخراج ارقام الاستحقاق التحليلة إذ تبين من خلال تحليل الارتداد لمنحني المعايرة إن المدى الخطي الذي يطيع قانون بير كان 0.5-40 مايكروغرام مل $^{-1}$ بمعامل ارتباط 0.9991 وحد كشف 0.15 مايكروغرام مل $^{-1}$ والانحراف القياسي النسبي المئوي (n =10) 1.43% والخطأ النسبي المئوي (-2.23%) ومعدل الاسترداد بالمئة (97.77±0.87) . طبقت الطريقة الطيفي ة على تقدير عقار السيلدينافيل في المستحضرات الصيدلانية كميا بدون تاثير اية تداخل لمكونات العقار الاخرى على طريقة تقديره.

Introduction

Through the scrutiny for the papers that published in the chemical literatures, the great majority of analytical procedures or inorganic synthesis that deal with the formation of complexes depend on a number of experimental factors, employing a classical optimization which is known as one-factor-at-a-time (OFAT). In this approach, the effect of one factor at a time on an experimental response is observed. While only one factor is changed, others are kept at a constant level [(1)]. but this procedure does not ensure at all that the real optimum will be conformed because this approach would be valid only if the variables or factors to be optimized would be totally independent from each other. In addition, it is time-consuming, expensive due to more consumption of reagents and materials [(2)].

To avoid these dilemmas, the optimizations based on the experimental designs become a must in this domain. In these designs, simultaneous optimization of the levels of the selected variables is carried out to attain the best conditions of system performance. One of the important phases in experimental design is a screening phase which allows determining the variables that significantly (major) affect the response. For this purposes, full or fractional two levels fractional designs can be used to "screen out" insignificant factors that have a tiny effect on response. This in turn gives the possibility to move on to response surface methodology (RSM) as the most relevant multivariate techniques used in analytical optimization to have a design with variables at three levels or more [(3)]. RSM consists of a group of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design. Toward this objective, linear or square polynomial functions are employed to describe the system studied and, consequently, to explore (modelling and displacing) experimental conditions until its optimization [(4)]. RSM as a full factorial three-level designs can be formalized in the same way as known for two-level designs , i.e. , the number of experiments required for this design calculated by expression $N=3^k$ where N is the experiments number and k is factor number. The most often used RS methodologies for three-level optimization to avoid large number of experimental points (N) such as the Box-Behnken, central composite and Doehlert designs [(2)]. Sildenafil citrate (SC) has marketed as impotent drug in 1998, since then it has approved for this purpose by the USA Food and Drug Administration. SC acts as a selective inhibitor of enzyme PDE5 (phosphodiesterase type) and thereby relaxes the muscles to allow healthy erection [(5-6)]. Sildenafil citrate chemically (IUPAC) named as 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo [4, 3-*d*] pyrimidin-5-yl) phenylsulfonyl]-4 methylpiperazine citrate (666.33 g mol^{-1}), its structural formula is shown in Fig.1[(7)] . Although, the drug is official in USP but no pharmacopoeia method has been found for the assay of SC in pure and dosage forms. However, several works have been reported for the assay of sildenafil citrate in pharmaceuticals and other matrices include, HPLC [(8-10)] LC-MS and LC/MS/MS/MS [(11-12)] ESI-MS-MS [(13)] HPTLC [(14)] GC [(15)] potentiometry [(16)] voltammetry [(17-18)] and FIA using UV detection [(19)]. In recent years, more attention have been paid toward using dyes as the chromogenic reagents which are capable of forming high stable ion-pair complexes with many medicaments, in an attempt to establish the spectrophotometric assay procedures satisfying the requirements of quality control in pharmaceutical industries. In this respect, few extractive spectrophotometric methods have been suggested based on the formation of ion-pair complexes of SC with such chromogenic reagents for quantification of Sc in pure and pharmaceuticals [(20-26)].

The aim of this work was firstly to study the effective factors, namely pH, reagent concentration, reaction time, extraction time and temperature on SC: MO ion-pair complex formation and extraction by using a classical

optimization approach. Secondly, to use the RSM) for simply achieving the optimum conditions of the variables studied, confirming the results of the classical optimization. This work was extended and exploited to validate the potential of experimental design (screening and proposed method via the analytical figures of merit beside its application in the determination of SC in the pharmaceuticals by using UV-Vis spectrophotometic method.

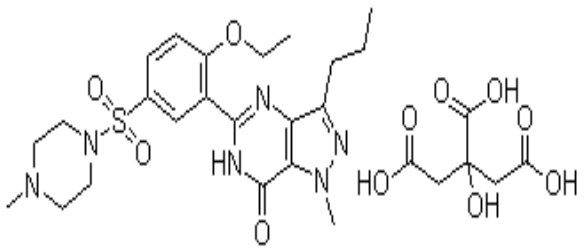

Fig. (1):- Chemical Structure of Sildenafil citrate (molecular formula $C_{28}H_{38}N_{11}O_4S$ **)**

Materials and Instruments Materials

All reagents and chemicals used were of analytical or pharmaceutical grade and double distilled water (DDW) was used throughout the experiments. Methyl orange (Hopkin and Williams LTD), chloroform (BDH), KCl, HCl, CH_3COONa and CH_3COOH (Fluka AG) were used without further purification. Pharmaceutical pure grade of Sildenafil citrate (> 99.9% purity) was generously supplied by the Drug Industries and Medical Appliances (SDI), Samarra /Iraq. Viagra tablets and jell formulations were purchased from markets; Vegon(Atlantis Life Sciences, India) , Excegra (Excel Life sciences, UK) each tablet has claim labelled to contain SC of 100 mg , KAM-GRA (Ajanta Pharma Limited , India) oral jell contains SC of 50 mg and VINAGRA tablet (N.D.I. Iraq) contains SC of 50 mg.

A stock standard solution (1000 µg mL^{-1}) of sildenafil citrate was prepared by dissolving 100 mg of pure drug (pharmaceutical grade) in 5.0 mL of 0.05 N HCl and made up to 100 mL with DDW and kept in refrigerator before use. Working standard solutions were then prepared by suitable dilutions of the stock solution with DDW. Methyl orange solution of 1000 µg mL^{-1} (or 0.1% w/v) was prepared by approximately dissolving 0.1g of MO (327.33 g mol^{-1}) in small amount of warm water and dilute to 100 mL with water. Two series of buffer solutions of pH (0.5-2) and (3-5) were prepared according to the procedure described elsewhere [27] by mixing an appropriate volume of 0.1M solution of HCl-KCl and CH_3COOH-CH_3COONa respectively .

Instruments

A double-beam Shimadzu UV-Visible spectrophotometer model UV-1650PC(Japan) connected to a computer with PC software was used for recoding spectra and all absorbance measurements with wavelength accuracy of ± 0.3nm , fast scan speed , scan range of 200-1100 nm and matched fused silica cell of 1.0-cm (capacity of min. volume 3.5 mL). The pH measurements were carried out by using pH-meter model HI98150 (HANNA, Romania).

Optimization strategies Classical optimization

The effect of several factors such as pH, MO concentration (C_R), reaction time (R_t), extraction time (E_t) and temperature (T) were studied for the extraction system of ion-pair complex by OFAT procedure. For assessment of pH effect, to 25 mL volumetric flasks containing 25 µg SC mL^{-1} and 9 mL of 100 µg

mL^{-1} MO, 5 mL of buffer solutions at varying pH(0.5-5) were added and kept aside for 2 min for complete ion-pair complex formation.Then, the content of each flask was extracted after shaking for 2 min with 5 mL $CHCl_3$ at temperature of 25±5 ºC and a responses in term of absorbance were measured at a specified wavelength. The same procedure was also carried out for the other variables; MO concentration (5-60 µg mL^{-1}), reaction time (1-6 min), extraction time (0.5-3.5 min) and temperature (25- 80 ºC).

Experimental design

In first stage, a fractional factorial design (FFD) was carried out to screen the effect of four factors that play an important role in the processes of ion-paring formation and extraction processes. The factors selected are; pH, MO concentration (C_R), reaction time (R_t), extraction time (E_t) and keeping the fifth factor (T) constant at room temperature. It worth noting that the factor levels for experimental design were chosen based on the results obtained from the above OFAT optimization procedure. For screening, a FFD was adopted based on 2^{k-p} design without replicate ,where 2 stands for number of factor levels , k expresses number of factors under study and p=1. Consequently, in 2^{4-1} design, eight experiments instead of sixteen can be performed for four factors at two levels in order to analyze which effects from factors and their interactions that possibly to most significant. The factor levels are coded at (-) for low level and (+) for high level and actual values of each factor in 2^{4-1} design for this study are given in Table 1.

Table (1):- Actual and corresponding coded values of factors in 2^{4-1} fractional factorial design for SC-MO extraction system.

Level of factor	pH		MO Conc. (C_R)		Reaction time (R_t)		Extraction time (E_t)	
	actual	coded	actual	coded	actual	coded	actual	coded
Low level	1	−	5	−	2	−	2	−
High level	5	+	60	+	4	+	3.5	+

The experimental matrix along with actual and coded values taken out according to the output of statistical software [28] is shown in Table 2. In the second stage, full factorial design based at three levels of each factor (Table 3) which proved to be the most significant variable resulting from the fractional factorial design was adopted and analyzed by using response surface methodology (RSM). Very often RSM is aimed at judging relationship between the selected factors graphically and the consequences are drawn from its plot. All statistical calculations, design matrices, mathematical modelling and graphical representation were performed using Minitab 14 software.

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Table (2):- Experimental 2^{4-1} design matrix.

Run	pH		C_R		Rt		Et		Response
	actual	coded	actual	coded	actual	coded	actual	coded	
1	5	+	60	+	2	−	2	−	R1
2	5	+	60	+	4	+	3.5	+	R2
3	5	+	5	−	4	+	2	−	R3
4	1	−	60	+	4	+	2	−	R4
5	1	−	5	−	4	+	3.5	+	R5
6	1	−	5	−	2	−	2	−	R6
7	5	+	5	−	2	−	3.5	+	R7
8	1	−	60	+	2	−	3.5	+	R8

Table (3):-Coding the two factors at three levels.

Factor	Level		
	+1	0	-1
pH	5	3	1
C_R	60	32.5	5

Recommended procedure

Aliquots of SC (0.125-10 mL, 100 µg mL^{-1}, corresponding to 0.5- 40 µg mL^{-1}) solution were transferred into a series of 25 mL volumetric flasks. Then 9.0 mL of 100 µg mL^{-1} and 5.0 mL of buffer solution (pH 3) were added and dilute to mark with water. The solutions were kept aside for 2 min for the complete ion-pair complex formation. Each solution was transferred into 50 mL separating funnel and extracted once with 5.0 mL CH_3Cl after shaking well for 2 min at 25±5 ºC. After the two phases were allowed to separate, the chloroform layer was transferred into a cuvette (1.0-cm) and the absorbance of the complex solution was measured at 427 nm against the corresponding reagent blank. A calibration graph was constructed and the amount of SC in drug samples (prepared under identical conditions) was calculated from linear regression equation.

Preparation of drug samples

Ten tablets were weighted and finely powdered. An amount of accurately weighted equivalent to 100 mg and/or 50 mg was transferred into 100 mL beaker and dissolved in 50 mL 0.05 N HCl. The mixture was shaked well at magnetic stirrer for about 20 min and then filtered. The filtrate was transferred to 100 mL volumetric flask and the volume was made up to mark with the same acid to obtain a concentration of 1000 and /or 500 µg mL^{-1}. Further dilutions were made for the sample solution when analyzed by recommended procedure or for accuracy measurement by standard additions method.

Mole-ratio method

An aliquot (2-mL) of a solution $2x10^{-4}$ M of SC solution was added to a series of 25-mL volumetric flask containing 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mL of $2.0x10^{-4}$M of MO reagent. To each flask 5-mL of buffer solution (pH 3) was added and dilute to the mark with water. The complex formed in each flask was extracted with chloroform and the absorbance of the extract was measured at λ_{max} (427 nm). The absorbance versus the volume ratio of MO/SC was plotted (Fig.11) from which the stoichiometry of ion-pair complex was determined.

Results and Discussion Absorption spectra

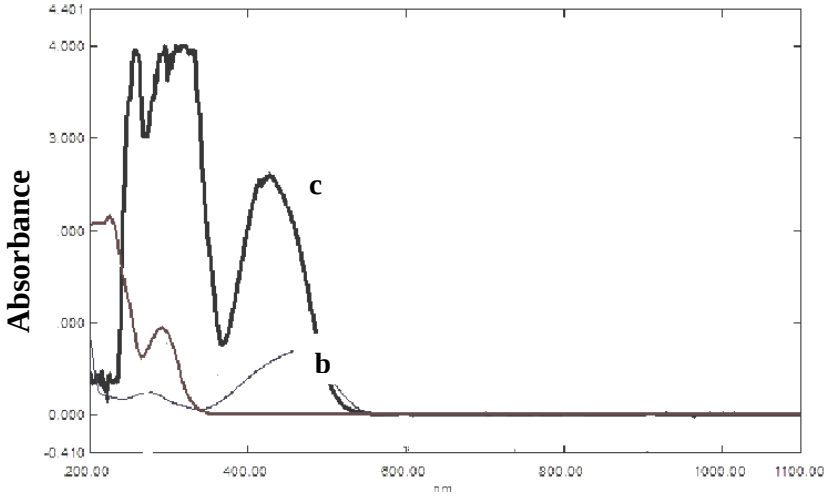
UV-Vis spectra of the pure Sc drug, pure MO and the complex SC-MO were scanned using Shimadzu model UV-1650 PC equipped with 1.0-cm matched quartz cell for recoding the spectra to verify of the formation of complex. It was shown that the pure drug gave two absorption maxima at 225 and 292 nm and the spectrum of the pure MO showd two distinctive absorption maximum at 273 and 464 nm, while the SC-MO complex gave a smooth absorption maximum at 427 nm (Fig.2) indicating the formation of complex between the drug and chromgenic reagent in $CHCl_3$.

Optimization by OFAT strategy

The effects of several experimental parameters which impact the extraction efficiency were carried out by classical optimization (one factor-at-a-time). In this respect, the effects of pH, concentration of MO, reaction time, extraction time, temperature were selected in this study.

Effect of pH

The effect of pH was studied by extracting the colored ion-pair complex in the presence of two buffers such as KCl-HCl (pH 0.5-2) and CH_3COONa-CH_3COOH (pH 3-5). The results revealed that the maximum colour intensity and hence maximum absorbance at 427 nm were perceived in NaOAc-AcOH buffer of pH 3 with buffer volume of 5.0 mL for final 25 mL solution. At higher pH values, the absorbance decreases suddenly as shown in Fig.3, and this most probably due to decrease in the protonation power on SC molecule or the interference of the H_3O^+ with MO [29] leading to completely dissociation of SC-MO complex. Thus the pH 3 was selected as optimum in further studies.



a **Wavelength (nm)**

Fig. (2):- The absorption spectra of (a) SC (50 mg L^{-1});blank water, (b) 40 mg L^{-1} MO; blank water, and (c) SC-MO complex (25 mg L^{-1} + 40 mg L^{-1} MO + buffer solution, pH 3) in $CHCl_3$; blank $CHCl_3$

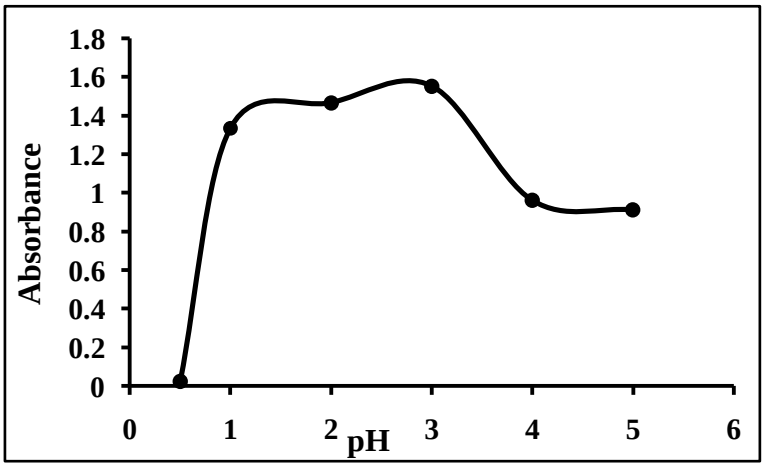


Fig. (3):- Effect of pH on the formation of the SC-MO complex (Conditions: 25 µg SC mL^{-1}+ 40 µg mL^{-1} MO + 5.0 mL of buffer at variable pH)

Effect of MO concentration

Fig.4 shows the influence of the concentration of the MO solution on the formation of the extracted SC-MO complex containing 25 µg SC mL^{-1} and in the presence of various concentration of MO separately at optimum pH. The results indicate that the absorbance values of complex formed increase linearly with the increasing concentration of MO and become constant between 36 to 56 µg MO $mL^{-1.}$ Accordingly, a concentration of 36 µg mL^{-1} MO was chosen as optimal for complete formation of the extracted ion-pair complex because further excess of the reagent has no considerable effect on complex formation.

Effect of reaction time

To ensure the complete formation of ion-pair complex before the extraction process, the absorbance was recorded versus the reaction time which varied from 0.5 to 6 min at room temperature with remaining other variables at optimum conditions. Fig.5 depicts that the response was highest at 2 min was adopted for all experiments.

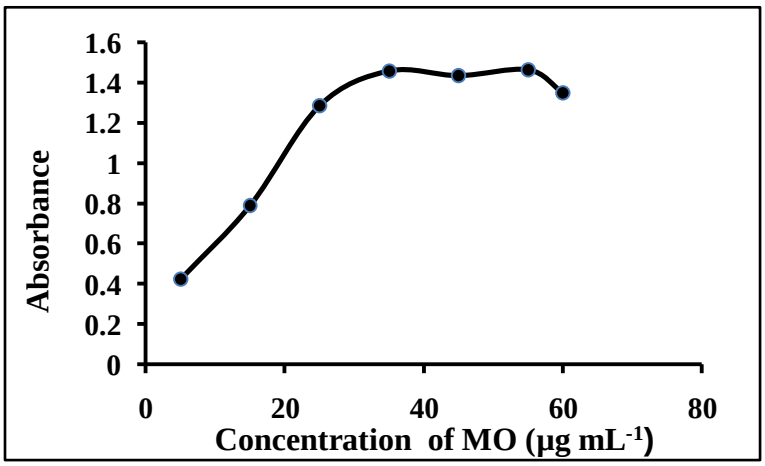


Fig.(4):- Effect of concentration of MO on the determination of SC (Conditions: 25 µg SC mL^{-1}+(x) µg MO mL^{-1} + 5.0 mL buffer pH 3)

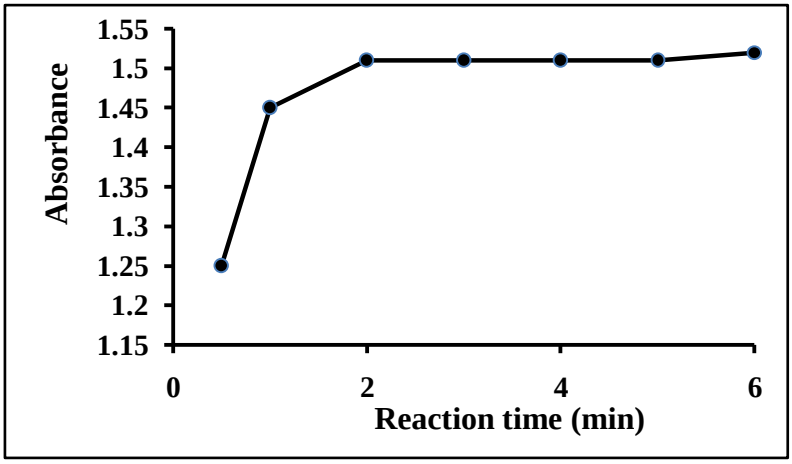


Fig.(5):- Effect of the reaction time on the formation of the SC-MO complex. (Conditions: 25 µg SC mL^{-1}+ 36 µg mL^{-1} MO + 5.0 mL buffer pH 3)

Effect of extraction time

The extraction system of the complex was studied under the previously optimum conditions by varying the shaking time for 0.5-3.5 min for the formed ion-pair complex with chloroform. It was appeared that the absorbance remained invariable between 2-3.5 min (Fig.6). Thus 2 min was chosen as an optimum value throughout the experiments.

Effect of temperature

The effect of temperature (25-80 ºC) on the reaction between SC and MO for the formation of ion-pair complex is shown in Fig.7., which revealed that the coloured ion-pair complex remained stable up to 30 ºC at fixed reaction time of 2 min . Beyond this temperature, the absorbance decreased drastically, indicative of occurrence in the decomposition of the complex. For this reason, all experiments were carried out at room temperature (25± 5 ºC) which is considered the most suitable for the reaction between the drug and reagent.

Effect of order of addition

Under the established optimum experimental conditions, the effect of order of addition on the absorbance of the ion-pair complex was also studied. The results indicated that this complex is formed with high sensitivity by the following order; Drug + MO reagent + acetate buffer solution.

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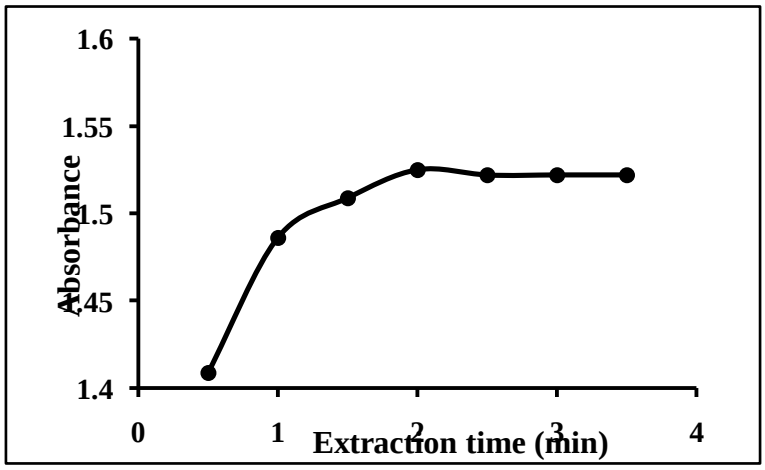

Fig. (6):- Effect of extraction time for the SC-MO complex (Conditions: 25 µg SC mL^{-1}+ 36 µg mL^{-1} MO + 5.0 mL buffer pH 3)

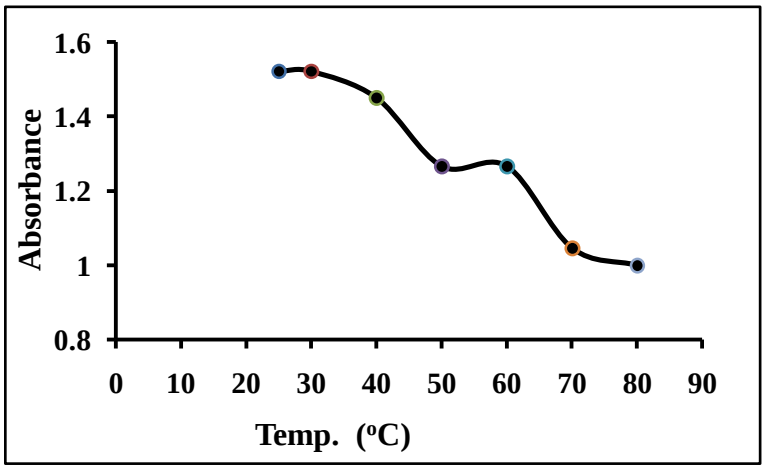

Fig. (7):- Effect of temperature for the SC-MO complex (Conditions: 25 µg SC mL^{-1}+36 µg mL^{-1} MO+ 5.0 mL buffer pH 3)

Experimental design strategy

Fractional factorial 2^{4-1} design (FFD) was firstly used in screening experiment in order to dentify the main factors and their interactions that are most active to maximize the absorbance response. The factors and levels (+ and−) used in this design (Table 1) . For each test, related response (i.e. absorbance) was obtained and the data of this experimental design is shown in Table 4. The predicated absorbance is obtained from the output of the treated data by Minitab

program. The significance of the effects was checked by analysis of the variance (ANOVA) and using p-value significance levels. The ANOVA results are presented in Pareto chart of main effects, as shown in Figure 8. In Pareto chart, the length of each bar is proportional to the absolute value of its associated estimated effect (Table 5). The bars that exceed a reference line mean the factor is significant with respect to the response at p=0.05.

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Consequently, the results revealed that the variation in pH (A) and MO (B) concentration was most significant ($p< 0.05$) but their interaction is statistically less significant(AB), while the factors such as reaction (C) and extraction times(D) were insignificant (i.e. there is a minimal influence by these two variables) at the selected levels in this experiment.

Table (4):- Design matrix and the results of the two-level fractional factorial design .

run	pH	C_R	R_t	E_t	Abs	Predicted Abs
1	+	+	−	−	0.480	0.504
2	+	+	+	+	0.480	0.502
3	+	−	+	−	0.760	0.730
4	−	+	+	−	0.840	0.824
5	−	−	+	+	1.000	1.024
6	−	−	−	−	1.004	1.026
7	+	−	−	+	0.720	0.704
8	−	+	−	+	0.828	0.798

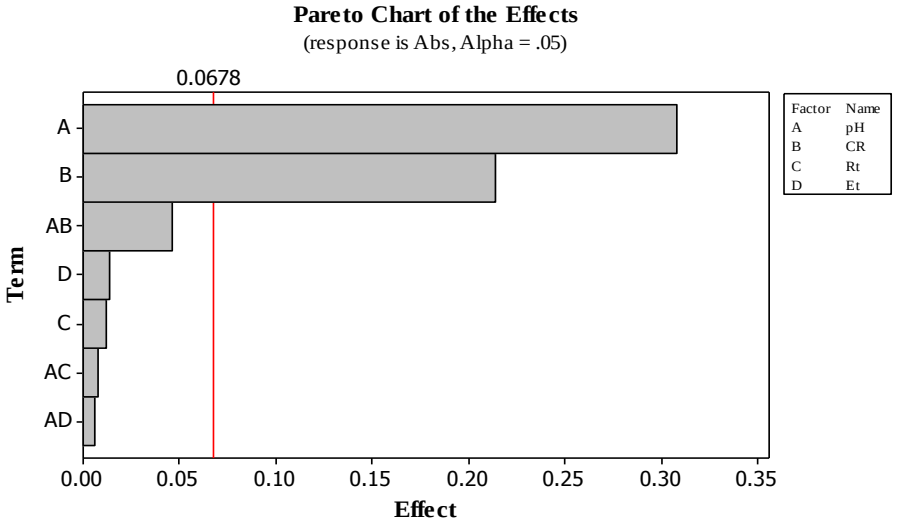

Fig. (8):- Pareto chart for net absorbance

Table (5):- Estimated effects and coefficients for

Absorbance (coded units).

term	effect	Coefficient
Constant		0.7640
pH	-0.3080	-0.1540
C_R	-0.2140	-0.1070
R_t	0.0120	0.0060
Et	0.0140	-0.0070
pH.C_R	-0.0460	-0.0230
pH.R_t	0.0080	0.0040
pH.E_t	-0.0060	-0.0030

The same conclusion can be drawn from the normal plot (Fig.9) of effect estimated for the factors studied which also exhibits strongly the splitting between unimportant and important effects of each factor. Again, only two factors such as pH (A) and C_R (B) appear important and the remaining factors appear insignificant as they cluster around zero and can be approximately joined by a simple near vertical line as shown in Fig.9. From fractional factorial design, maximum response (i.e. increasing in sensitivity) can be achieved for ion–pair formation when all factors were at low levels (Table 4).

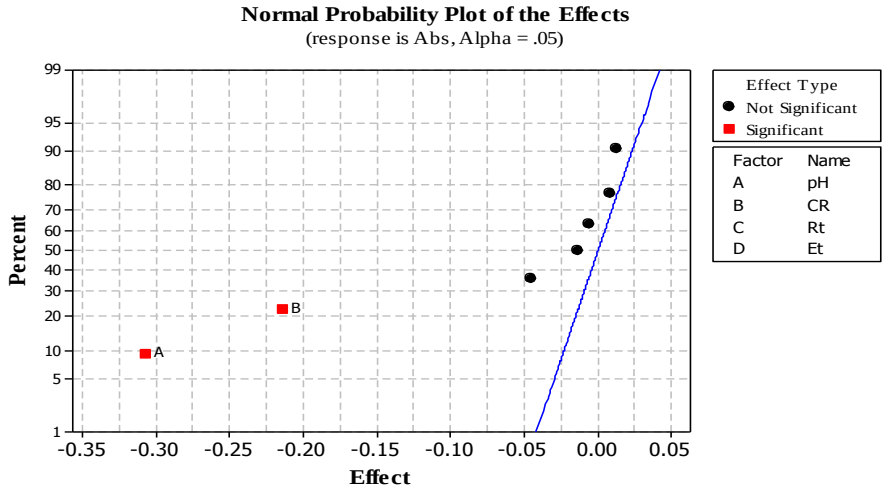

Fig.(9):- Normal plot of effect estimates

Analysis of variance indicates the model terms of coded factors were linear with respect to the absorbance and can be described by the following linear regression;

Abs= 0.764- 0.154 pH – 0.107 C_R + 0.006 Rt – 0.006 E_t – 0.023 pH*C_R +0.004 pH*R_t-0.003 pH* E_t

It can be also seen from the above equation that there is no significant variation in the response or on the extractability of ion-pair complex if we keep the reaction and extraction times between low and high levels. Full factorial design for the significant factors (pH and C_R) at three levels (Table 3) is conducted to evaluate the model and best optimum conditions of these

variables. The results are shown in Table 6. From data in Table 6, the response surface was drawn graphically as three dimensional (3D) and counter plots (Fig.10 a and b). It can be seen that dependences in the direction of both factors lead to a maximum absorbance at uncoded level of pH and C_R to the range close to the optimal values. Then, the surface starts to fall-off slightly in the case of increasing factor value from the optimal limit. However, the response surface was observed to be depressed extremely toward the least factor value, hence, inferring that it is necessary to maintain the pH at level higher than 1 and lower than 5 with optimum value of pH 3 which is exactly coincide to that obtained with classical optimization. The same situation for the amount of MO reagent (C_R) was predicated by RSM which gave the final optimum value of 32.5 µg mL^{-1} and being close to the amount that achieved by traditional optimization (36 µg mL^{-1}).

Table (6):- Full factorial design at three levels for two factors (3^{2-}design).

Run	Factor				Response
	Coded value		Uncoded value		
	pH	C_R	pH	C_R	(Absorbance)
1	+1	+1	5	60	0.760
2	+1	-1	5	5	0.912
3	-1	+1	1	60	0.680
4	+1	0	5	32.5	0.900
5	-1	0	1	32.5	0.680
6	0	+1	3	60	0.880
7	0	-1	3	5	1.004
8	-1	-1	1	5	0.680
9	0	0	3	32.5	1.012

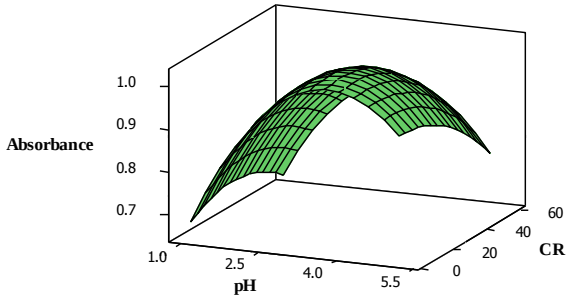

Fig (10a):- Three dimensional (3-D) RSM plot showing the effect of pH and MO concentration and their mutual effect on the absorbance

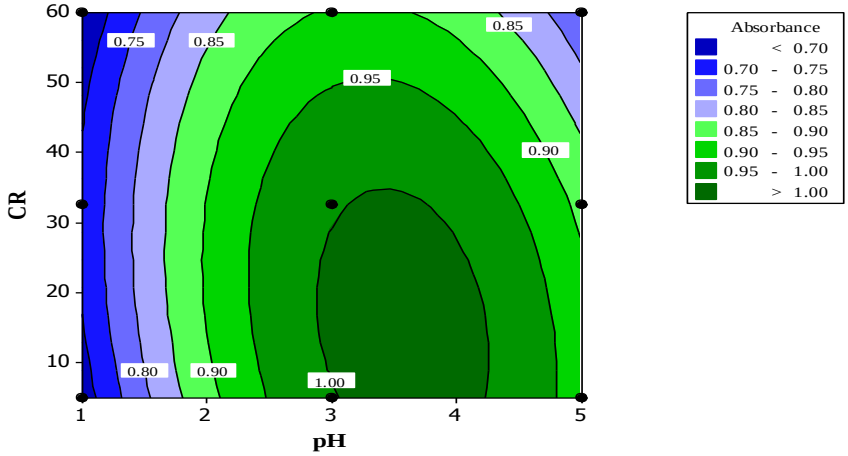

Fig (10b):- Contour plot of absorbance vs pH and MO concentration

Stoichiometry of ion-pair complex

Under the optimum conditions, the stoichiometry of the reaction between SC and MO was investigated by mole ratio method using equimolar solutions (2×10^{-4}M) of the drug and reagent, at the wavelength of maximum absorption. The results obtained in Fig.11 show that the stoichiometric ratio of the ion-pair complex is 1:1 (SC: MO).The stability constant was estimated by using the following equation [30]:

where K is stability constant, A_1, A_2, and A_3 refers to the absorbances of intersect points of the two slopes, at constant absorbance and first point absorbance on the Fig. 11, respectively and C is the molar concentration of complex vs. A_1. The stability constant was found to be $1.13\text{x}10^8$ M^{-1} at λ_{max} 427 nm. This indicates that this complex is completely stable.

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$$K = \frac{(A_1 - A_3)(A_2 - A_3)}{(A_2 - A_1)^2 C}$$

Sildenafil molecule (pK_a 8.7) exhibits a basic property due to the presence of 6 nitrogen atoms (Fig.1) which are distributed between basic functional groups like pyrimidine, pyrazol and piperazine rings and behaves as monovalent species. It would be more expected that the protonation could occur on the nitrogen (N4) bonded to electron-donating methyl group in the piperazine ring rather than in the substituted and fused rings of pyrimidine and pyrazol due to the resonance and steric effect [22]. Since the MO molecules become anionic in acidic buffer it can form ion-pair complex with protonated SC with 1:1mole ratio as calculated aforesaid by the above method. Thus, according to Coulomb's law, ions of opposite charge are naturally attracted to each other by an electrostatic force. Consequently, the most probable mechanism of the ion-pair complex formation is illustrated in Fig.12.

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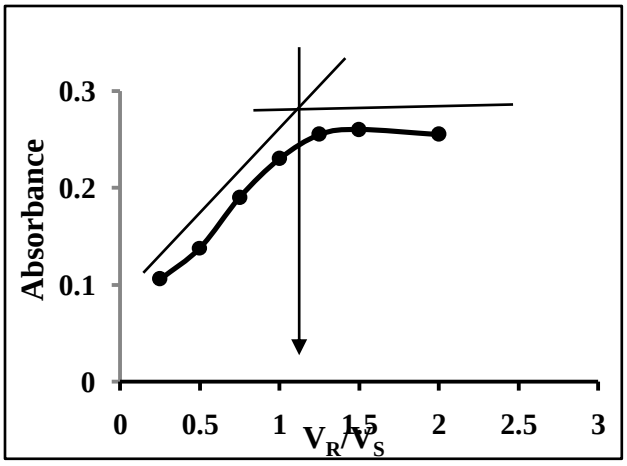

Fig .11 Mole ratio method for the compositions of SC-MO complex (V_R= volume of MO, Vs= volume of SC)

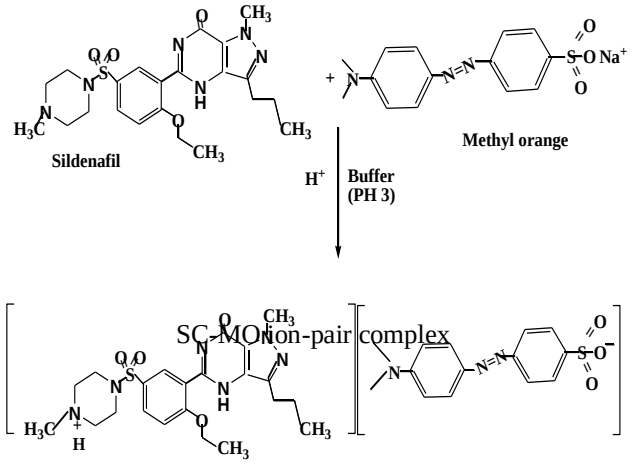

Fig.12 Probable mechanism for the formation of 1:1 SC-MO

Method validation

Under the established optimized conditions, linear calibration graph was obtained by plotting the AA signal versus the concentration of SC. The optical characteristics and analytical figures of merit are summarized in Table 7.

Linear relationship was found between absorbance at λ_{max} and the concentration of the SC and found to be in the range 0.5-40.0 µg mL^{-1}. The statistical evaluation for the calibration graph has shown that a strong correlation between absorbance and concentration may exist (R^2=99.8%). On the other hand, the analysis of variance (ANOVA) also proved the linear regression equation [y= (0.0457±0.00057)x–(0.022±0.0097)] was statistically valid. This because of the ratio (MS_{reg}/MS_{error}) for 1 and 11 DOF, larger than the critical value ($F_{1, 11}$=4.84 at 95% CI), indicating that the predication based on the regression line was satisfactory (Table 8) .

The high molar absorptivity (Table 7) of the resulting coloured ion-pair complex indicates the high sensitivity of the method which was better than that obtained by Ashour and Alkourdi [31] which was of $7.5x10^3$ L mol^{-1} cm^{-1} using oxidative coupling reaction for the assay of SC by spectrophotometric method, but it was in harmony with the results that obtained by Harikrishna et al [23] which were of $12.08x10^4$ and $3.28x10^4$ L mol^{-1} cm^{-1} by using BBG and BCP and by Danish et al [22] which was of $1.58x10^4$ L mol^{-1} cm^{-1} with BCG as chromgenic reagents for the detection of SC in pharmaceuticals. Limit of detection was found to be of 0.15 µg mL^{-1} which was better than that obtained by other workers [23, 31], but in harmony with that found by Danish et al [22].

Table (7):- Analytical statistics data for the determination of Sc by proposed method .

Parameter	value
Colour	orange
λ_{max} (nm)	427
Range of concentration (µg mL^{-1})	0.5-40
Limit of detection (µg ml^{-1}) for n=10	0.15
Regression line	y=0.0457x-0.022
Correlation coefficient (r)	0.9991
Coefficient of determination (R^2)	99.8%
C.L. for the slope($b\pm ts_b$) at 95%	0.0457± 0.00057
C.L. for the intercept($a\pm ts_a$) at 95%	-0.022±0.0097
RSD %(n=10 at 10 µg SC mL^{-1})	1.43%
Molar absorptivity (L mol^{-1} cm^{-1})	$2.72x10^4$
Sandell's sensitivity (µg.cm^{-2})	0.0244
Extraction Efficiency (%E)	94.42
Distribution ratio (D)	84.61
Stability of complex (h)	24

Table (8):- Analysis of variance (ANOVA) of regression line.

Source	dof	SS	MS	F	P
Regression	1	3.4637	3.4637	6309.44	0.00
Error	11	0.0060	0.0005		
Total	12	3.4697			

dof=degrees of freedom; SS= sum of squares; MS= mean of sequares; F=Fisher test; p=probability

Recovery Percent

The accuracy was evaluated through the recovery test. This procedure was performed by spiking known amounts of standard solutions (5, 10, 20 µg mL^{-1}) into the two selected samples having pre-analyzed definite concentrations, followed by analyses of mixture using the proposed method. The results were summarized in Table 9. The mean recovery percentage obtained for the two selected commercial drug were quantitative, indicating

good accuracy of the method. This highlights that the excipients such as talc, lactose, starch,

dextrose, gum and magnesium stearate did not interfere with the analysis of Sc in the drug, since the formation of an ion-pair complex with anionic dyes needs a basic moiety.

Determination of Sc in pharmaceuticals

The proposed method was applied to the assay of sildenafil citrate in the three dosage forms available in market and one locally produced by SDI. The sample solution prepared was diluted such that final concentration of the drug within the limits of linearity of the constructed calibration graph, from which the amount of SC in mg /tablet was calculated. The results for the determination of SC are summarized in Table 10. The amount of SC in the samples analyzed was mostly in a good agreement with claimed values, with mean percent relative error of (-0.71%). In order to determine the precision of the method, five replicate analysis of each sample were made and the average found to be 2.89±0.47%. The small values of the relative standard deviation and the mean relative error can be considered acceptable for the quality control analyses of medicaments.

Table (9):- Accuracy of the proposed method.

Sample	Amount Sc taken (μ mL^{-1})	Amount SC found (μg mL^{-1})	Recovery* (%)	E_{rrol} (%)	Mean Rec% ±t.s$\sqrt{n}$ (at 95% Cl)
Vegon (tablet)	-	15	-		
	5	19.84	96.80	-3.20	97.77±0.87
	10	24.80	98.00	-2.00	with mean
	20	34.35	96.75	-3.25	$\%E_{rel}$ =-2.23
Kam-Gra (Oral jelly)	-	10	-		
	5	14.92	98.40	-1.60	
	10	19.79	97.90	-2.10	
	20	29.75	98.75	-1.25	

* R = [(C_F – C_S) / C_A] x 100 where C_F represents the concentration of analyte measure in spiked test sample; C_S , the concentration of analyte measure in test sample; and, C_A , the concentration of analyte added to the test sample.

Table (10):- Determination SC in pharmaceutical preparations by the proposed method.

Sample type	Manufacturer	Labelled claim mg/ tablet or Jelly	Amount found* (mg)	Recovery (%)	% E_{rel}
Vinagra (tablet)	**SDI, Iraq**	50	51.22	102.44	2.44
Kam-Gra (Jelly)	**Ajanta pharma limited, India**	50	48.99	97.98	-2.02
Excegra (tablet)	**Excel life science London, U.K**	100	101.11	101.11	1.11
VeGon (tablet)	**Atlantis life sciences, India**	100	96.35	96.35	-3.65

* Average of five determinations

The results of the proposed method for three type of samples in this study were compared statistically with reported method that used metacresol purple (MCP) as a chromogenic reagent and spectrophotometric determination

of SC [32]. The results are summarized from Table 11.

The statistical analysis performed by the paired t- test for comparison of means between the proposed and the reported methods for the selected samples (Table 11) have revealed that the p value [P(T<t; 0.05< 0.361) two tailed] based on the 5% critical value of 4.30 was more than the |t| calculated value(1.18), indicating acceptance of null hypothesis (Ho) which specify there is insufficient evidence to suggest the accuracy (i.e. systematic errors) of the proposed method differs to that of reported method. On the other hand, Fisher F-test for the comparison of precision (variances) between the two methods has also shown that the p value [P (F<f; 0.05<0.946) two-tailed] based on the 5% significance level of 39 was much more than the f calculated (1.13) .It would be therefore conclude that there is no significant difference between variances (precision) of the two methods at the 5% level.

Table (11):- The statistical comparison (paired t-teat) of this work with reported method[32]

Sample type	This work (mg/tablet)	Reported method (mg/tablet)	$\overline{X_d}$	S_d (SE)	t_{cal} (n=3)	$t_{crit.}$ at 95% dof=2	P value
Kam-Gra **(jelly)**	48.99	49.55	1.53	2.25502 (1.30193)	1.18	4.30	0.361
Excegra **(tablet)**	101.11	99.88					
VeGon (tablet)	96.35	92.43					

where $\overline{X_d}$= mean differences, S_d= standard deviation, ,dof=degrees of freedom, SE= standard error of mean, t=test, P=probability.

Conclusions

The application of fractional factorial or full factorial designs as the simplest statistical tools of experimental designs have proven somewhat suitable for simplifying optimization for analytical procedures. They are rapid and economic optimization across reducing number of experiments and successfully applied for the extractive spectrophotometric determination of SC in pharmaceuticals compared with classical optimization. However, more studies are needed for employing other designs such as Box- Behnken design (BBD), central composite design (CCD) or Doehlert design to establish correctly the mathematical models for more understanding the relationship between the response and individual factors with their interaction effects which would be very useful for determining the optimal parameters values that maximize the analytical response in the extraction process of the complex under study. Anyhow, the determination of Sc using MO as pairing chromogenic ion showed low detection limit and good sensitivity compared with other chromogenic reagents. The analytical results obtained for the determination of SC in some pharmaceutical compounds showed good agreement with the given–labeled quantity thus proved to be suitable for the quality control of the raw materials and formulations. The proposed method was further compared with an UV-Vis spectrophotometric method reported and proved to be with acceptable accuracy and precision. Finally, further work is needed to apply this method for the analysis of Sc drug in biological samples (i.e. blood, urea etc) rather than the pure or pharmaceutical preparations.

References

1. Leardi R., Experimental design in chemistry: A tutorial, *Anal Chim Acta* 2009; 652: 161–172.
2. Bezerraa MA, Santelli RE , Oliveiraa EP,Villar LS and Escaleiraa LA,Response

surface methodology (RSM) as a tool for optimization in analytical chemistry, *Talanta* 2008;76: 965-977.

3. Kellner R, Mermet JM, Otto M , Widmer HM, Analytical chemistry –the approved text to the FECS curriculum analytical chemistry , Wiley-VCH Verlag GmbH, 1998, pp.759.
4. Teofilo RF and Ferreira MM., Chemometrics II: Electronic spreadsheets for experimental design calculation , a tutorial, *Quim.Nova* 2006;29:338-350
5. Boolell M, Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM, Osterloh IH and Gingell C, Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int. J. Import. Res.* 1996;8 : 47–52.
6. Morales A, Gingell C, Collins M, Wicker P A and Osterloh IH, Clinical safety of oral sildenafil citrate (VIAGRA) in the treatment of erectile dysfunction, *Int. J. Import. Res.*, 1998; 10:69-73.
7. Clarke's Analysis of Drugs and Poisons, electronic version(CD), 3rd edition, UK 2005.
8. Abd-Elbary A, Foda NH, El-Gaazayerly ON, Stability high performance liquid chromatography assay for the determination od sildenafil citrate in bulk and in formulations, *Chromatographia* 2004; 59: 561-566 **.**
9. N. Kannappan N, Yada D, Shashikanth DY and Mannavalan. R , Method development and validation of stability indicating methods for assay of tadalafil and sildenafil citrate by HPLC, *Int. J. Chem. Tec. Res.* 2010; 2: 329-333.
10. Vijay Kumar R, Yarkala S, Rao VU, Karra UM, Radhika B and Naresh K, Analytical method development and validation of Sildenafil Citrate by RP-HPLC, *J. Sci Res. Pharm.* 2012 ;1: 13-14.
11. Zhua X, Xiaoa S, Chena B, Zhanga F, Yaoa S,Wanb Z, Yang D and Hongwei H, Simultaneous determination of sildenafil, vardenafil and tadalafil as forbidden components in natural dietary supplements for male sexual potency by high-performance liquid chromatography–electrospray ionization mass spectrometry, *J. Chromatogr. A* 2005;1066 :89–95.
12. Lee S , Kim Y, Kim T, Im G,Lee B, Kim D, Jin C and Yoo H, Determination of mirodenafil and sildenafil in the plasma and corpus cavernous of SD male rats, *J.Pharm. Biomed. Anal.*2009; 49 : 513-518.
13. Abdel-Hamid ME, Determination of Sildenafil, Tadalafil, and Vardenafil in tablets and adulterated herbal products by ESI-MS-MS *J. Liq Chromatogr. Related Technol*. 2006; 29:591 – 603.
14. Abourashed EA, Abdel-Kader MS and Habib AM, HPTLC determination of sildenafil in pharmaceutical products and aphrodisiac herbal preparations, *J. Planar Chromatogr.*2005; 18:372-276.
15. Berzas JJ , Rodriguez J, Villasenor M J , Contento AM and Cabello MP, Validation of a capillary gas chromatographic method for the determination of Sildenafil Citrate in its pharmaceutical formulations (Viagra). Experimental design for evaluating the ruggedness of the method, *Chromatographia* 2002;55:601-606.
16. Hassan S.S.M.,Elnemma E.M, Mahmoud W.H.,Mohammed A.H.K., "Continuous potentiometric monitoring of viagra (sildenafil) in pharmaceutical preparations using novel membrane sensors" J. Appl. Electrochem. 2006; 36:139–146 .
17. Rodriguez J, Berzas JJ.,Castaneda G and Rodriguez N, Determination of sildenafil citrate (Viagra) and its metabolite (UK-103,320) by square-wave and adsorptive stripping square-wave voltammetry. Total determination in biological samples, *Talanta* 2004; 62: 427-432
18. Tyszczuk K and Korolczuk M, Voltammetric method for the determination of sildenafil citrate (Viagra) in pure form and in pharmaceutical formulations, *Bioelectrochemistry* 2010; 78: 113-117.
19. Altıokka G, Atkosar Z, Sener E and Tunçel M, FIA of sildenafil citrate using UV-

detection, *J. Pharm. and Biomed. Anal*. 2001; 25: 339-342.

20. Reddy MN, Murthy TK , Rao YS and Sankar DG, Spectrophotometric determination of Sildenafil citrate in pharmaceutical dosage forms, *Indian J. Pharm. Sci*. 2002; 64: 253-9
21. Reddy MN, Murthy TK, Rao YS, Sushmak K and Sankard DG, Extractive spectrophotometric determination of sildenafil citrate in pharmaceutical dosage forms, *Indian drugs* 2002 ;39 :106-109.
22. Dinesh ND, Nagaraja P, Made Gowda NM and Rangappa KS, Extractive spectrophotometric methods for the assay of sildenafil citrate (Viagra) in pure form and in pharmaceutical formulations, *Talanta* 2002; 57: 757-764.
23. Harikrishna K, Nagaralli BS and Seetharamappa J, Extractive spectrophotometric determination of Sildenafil Citrate (Viagra) in pure and pharmaceutical formulations, *J. Food Drug Anal*. 2008;, 16:11-17.
24. Amin AS, Moustafa ME and El-Dosoky, R, Colorimetric determination of sildenafil citrate (Viagra) through ion-associate complex formation, *J AOAC Int* 2009; 92: 125-130 .
25. Verma, JK and Syed, HA, Spectrophotometric method for determination of Methotrexate, Sildenafil Citrate and Trimetazidine Dihydrochloride in pharmaceutical *formulations J. Pharm Res*. 2010; 3:615-617.
26. Issa YM, El- Hawary WF, Youssef AFA and Senosy AR, Spectrophotometric determination of Sildenafil Citrate in pure form and in pharmaceutical formulation using some chromotropic acid azo dyes, *Spectrochim. Acta Part A* 2010;75 : 1297-1303.
27. Perrin DD and Dempsey B, Buffers for pH and metal ion control, 3rd ed. Champman and Hall 1989, pp 128-139.
28. Minitab® Statistical Software 14, State College, Pennsylvania, USA,2011.
29. Hassan WS, El-Henawee MM., Gouda, AA, Spectrophotometric determination of some histamine HI-antagonists drugs in their pharmaceutical preparations, *Spectrochim. Acta A* 2008; 69:245-244.
30. Meyer Jr AS and Ayers GH, The mole ratio method for Spectrophotometric determination of complexes in Solution' *J. Am. Chem. Soci.*1957;79:49-53.
31. Ashour S and Alkourdi K, Application of Oxidative Coupling Reactions for the Estimation of Sildenafil Citratein Bulk Sample and Dosage Forms, *Arabian J. Chem*. 2008;1:137-144 .
32. Muhamad YH, A Simple Spectrophotometric Assay of Sildenafil in Pure and Pharmaceutical Preparations, *Journal of Al-Nahrian Unversity-Science*, 2012; 15:18-24.