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Spectrophotometric Determination of Vancomycin Hydrochloride (Batch and Flow-Injection) Using O-Nitroaniline as diazotized Chromogenic Reagent

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Abstract

New, simple and sensitive batch and nFIA spectrophotometric methods for the determination of Vancomycin Hydrochloride in pure form and in pharmaceutical preparations were proposed, These method's were based on diazotization and coupling reaction between Vancomycin Hydrochloride and diazotized O-nitroaniline in alkaline medium to form Orange water-soluble dye that is stable and has a maximum absorbance at 465nm. Acalibration graph shows that aBeer's law is obeyed over the concentration range of 0.8-60 and 5-400 $\mu g.mL^{-1}$ of Vancomycin Hydrochloride with detection limit's of 0.16 and 1.666 $\mu g.mL^{-1}$ of Vancomycin Hydrochloride for batch and nFIA methods, respectively. The FIA procedure sample throughput was 80 h^{-1}. All different chemicals and physical experimental parameters affecting on the development and stability of the colored product were carefully studied, and the proposed methods were successfully applied for the determination of Vancomycin Hydrochloride in pharmaceutical preparations.

Keywords: Vancomycin Hydrochloride, Spectrophotometric determination, O-nitroaniline, Diazotization and coupling, Flow injection.

تقدير الفانكومايسين هيدروكلورايد بالطرائق الطيفية (الدفعة والحقن الجرياني) باستخدام اورثو نايتروانيلين كعامل ازوتة

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الخلاصة

يتضمن البحث تطوير طرائق طيفية جديدة وبسيطة للتقدير الكمي لمقادير ضئيلة من الفانكومايسين هيدروكلورايد في المحاليل المائية والمستحضرات الصيدلانية باستخدام طريقتي الدفعة والحقن الجرياني،تعتمد الطريقتين على تفاعل الازوتة والاقتران للفانكومايسين هيدروكلورايد مع كاشف اورثونايترو انيلين المؤزوت في وسط قاعدي حيث تتكون صبغة برتقالية مستقرة وذائبة في الماء والتي اعطت اعلى امتصاص عند طول موجي 465 نانوميتر. تشير منحنيات الامتصاص مقابل التركيز بان قانون بير ينطبق ضمن مدى التراكيز 0.8– 60 و 5– 400 مايكروغرام.مل$^{-1}$ من الفانكومايسين وبحد كشف 0.16 و1.66 مايكروغرام.مل$^{-1}$ من الفانكومايسين هيدروكلورايد لطريقتي الدفعة والحقن الجرياني على التوالي وبمعدل نمذجة 80 نموذج في الساعة بطريقة الحقن الجرياني، تم دراسة الظروف المثلى للتفاعل وجميع المتغيرات الكيمائية والفيزيائية بدقة، طبقت الطريقتين بنجاح على المستحضرات الصيدلانية الحاوية على الفانكومايسين.

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Introduction:

Vancomycin Hydrochloride(VHC) is a glycopeptidic antibiotic very efficient against a number of gram positive microorganisms [1]. Vancomycin hydrochloride (VHC) consists principally of the monohydrochloride[2].VHC is officially recognized in B.P . It was introduced in 1958 as an antibiotic active against Gram-positive cocci, particularly streptococci, staphylococci and pneumococci. Although it is not active against Gram-negative bacteria, VHC is recommended for use when infections fail to respond to treatment with the more common antibiotics [3]. Its molecule shows a complex tricycle structure containing amino acids and sugars as shown in Figure-1. Its mode of action is inhibition of cell wall synthesis of susceptible bacteria. The main target of this antibiotic, is the (L-Lys) -D-alanyl-Dalanine terminal peptide of the cell wall precursor. In addition VHC alters the bacterial cell membrane permeability and RNA synthesis. VHC is used clinically as a result of high activity against gram positive pathogens such as many coagulates negative Staphylococcus(CNS), Corynebacterium, Clostridium difficile, multi-resistant Staphylococcus aureus and gentamicin resistant Enterococcus which are refractory to established drugs [4].

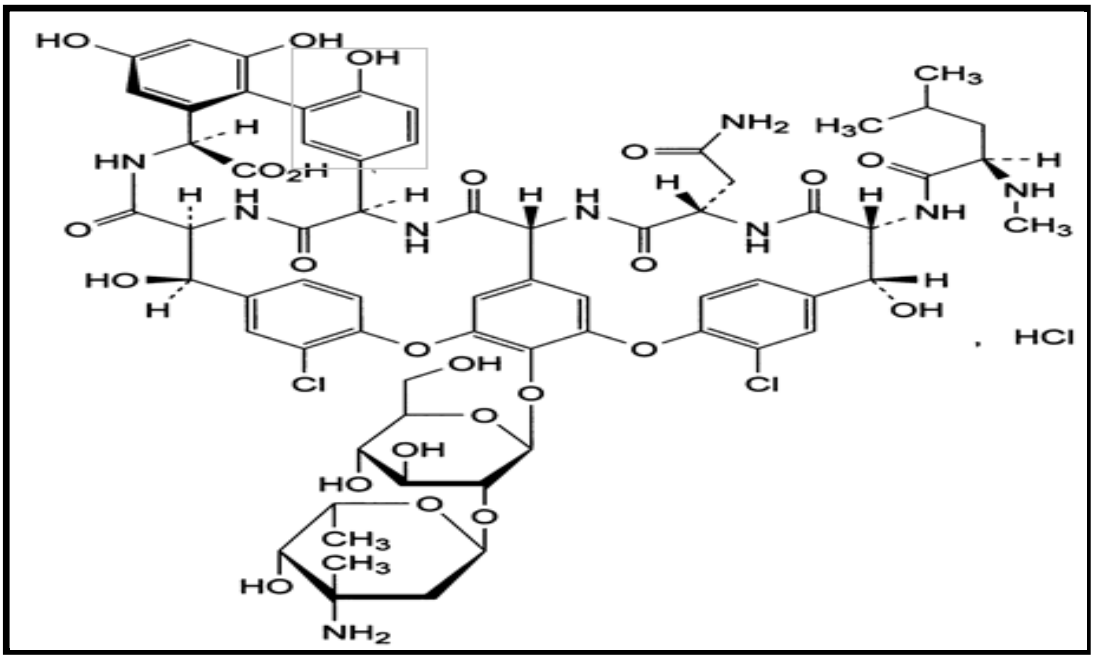


Figure 1-Vancomycin hydrochloride (VHC)

A survey of literature revealed that few methods based on visible spectrophotometry for VHC [5-8] have been reported. Other methods include HPLC [9-11], HPLC-tandem mass spectrometry [12], flow injection analysis [13], Polarography [14], Radioimmunoassay [15], Fluorescence polarization immunoassay [16]. This paper describes spectrophotometric methods for determination of VHC by the coupling reaction with O-nitroaniline in alkaline medium. O-nitroaniline was found to be a useful new coupling reagents for diazotization reaction, because they produced a stable and rapid coupling organic products furthermore, these reagent is easily obtainable, highly purified and are soluble in ethanol therefore the proposed methods are considered as a green methods. In addition these methods have been satisfactorily applied for the determination of VHC in pure and pharmaceutical preparations.

Materials and Methods

Apparatus

All spectral and absorbance measurements were carried out by using a shimadzu UV – visible –260 digital double beam recording spectrophotometer (Tokyo –Japan) and using 1 cm quarts cells. A quartz flow cell with 50 µL internal volume and 1 cm bath length was used for the absorbance measurements. A two channel manifold was employed for the FIA spectrophotometer determination of VHC. A peristaltic pump (Ismatec Lobortechnik–Analytic, CH – 8512, Glatbragg–Zurich, Switzerland, sixchannels) was used to transport the reagent's solution's. Injection valve (Rheodyne,Altex 210, supeko use) was employed to provide appropriate injection volume's of

standard solution and sample.Flexible vinyl tubing of 0.5mm internal diameter was used for the peristaltic pump. Reaction coil (RC) was of Teflon with internal diameter of 0.5 mm. The diazotized O-nitroaniline (ONA) (A) stream was combined with injected sample (VHC) and they merged with sodium carbonate (B) stream at T –link then mixed in reaction coil (RC) with length (75 cm), injection loop (200 µL), total flow rate 2.5 $mLmin^{-1}$, the absorbance was measured at 465 nm at temperature 25 C^o.

Chemicals:

Standard vancomycin hydrochloride (VHC) solution

Stock solution (500 $\mu g.mL^{-1}$) was prepared daily by dissolving 0.05 g of the pure VHC in 100 mL of distilled water and serial dilutions with distilled water were made.

Hydrochloric acid solution (2M) was prepared by diluting 86 mL of 11.64 M of concentrated hydrochloric acid (BDH) with distilled water in 500mL volumetric flask. The solution was standardized using standard solution of sodium carbonate.

Diazotized O-nitroaniline solution (ONA) (1×10^{-2}M)

Prepared daily by dissolving 0.138 gm of o-nitroaniline (Fluka) in 5 ml ethanol, 20 ml distilled water and 3 ml of 1M hydrochloric acid in a 100 ml volumetric flask. Cool the mixture to 0-5°C for 5 min using an ice-bath, add 0.069 gm amount of sodium nitrite and stir the mixture. After 5min the volume is made up to the mark with addition of cooled distilled water. More dilute solutions were prepared by suitable dilution with distilled water.

Sodium Carbonate anhydrous (BDH) solution: stock solution of 1M was prepared by dissolving 26.5 g of Na_2CO_3 in 250 mL distilled water, and working solutions were prepared by appropriate dilution of the stock solution.

Sample vancomycin hydrochloride VHC solution

The contents of five vials were mixed (two commercial sources vancolon VHC injection Julphar company UAE 500mg and vancorin VHC injection CheilJedang corporation company Republic of Korea 1g). An aliquot corresponding to 0.05 g of VHC was diluted to 100 mL with distilled water in a volumetric flask to obtain 500 $\mu g.mL^{-1}$ of VHC. More dilute solutions of pharmaceutical preparations for batch and FIA procedures were made by simple dilution with distilled water.

General procedure for calibration

a. General batch procedure

2mL of (5×10^{-3} M) Diazotized O-nitroaniline solution was transferred into a series, of 25 mL calibrated flask. Then, An aliquot of a standard solution (500 µg mL^{-1}) (3.36×10^{-4}M) of VHC was transferred into this series of 25 mL calibrated flasks and 2mL of 0.1 M sodium carbonate anhydrous solutions was added and the contents were diluted to the mark with distilled water and mixed well, After 5 min, the absorbance of the colored was measured at 465nm against the corresponding reagent blank. And the step worked with sodium hydroxide solution, the absorbance of the colored was measured at 449nm against the corresponding reagent blank. The regression equations obtained from a series of VHC standard solutions.

b. General FIA procedure

A Vancomycin Hydrochloride solution in the range of 5-400$\mu g.mL^{-1}$ was prepared from the standard working solution of 500 $\mu g.ml^{-1}$. A 200 µl portion of VHC was injected into the stream of diazotized O-nitroaniline (5×10^{-3}M) then the mixture combined with (0.1 M) Na_2CO_3 at T-link with a total flow rate of 2.5ml min^{-1} for the two channels, the resulting absorbance of the Orange product was measured at 465 nm and a calibration graph was constructed. Optimization of conditions was carried out on 40 $\mu g.ml^{-1}$ of VHC.

Results and Discussion

Batch spectrophotometric determination

The factor's affecting on the sensitivity and stability of the colored diazotization coupling reaction between diazotized O-nitroaniline and VHC in an alkaline medium were carefully studied. A typical spectrum for the azo dye formed was measured versus reagent blank which has negligible absorbance at λmax 465 nm Figure-2. The experimental conditions for the determination of VHC were established. The diazotization coupling reaction occurred in an acidic medium and a hydrochloric acid of concentration 1M was selected, the effect of different volumes of 1 M of HCl were studied and 2 ml volume seems to be optimum for an intense azo dye color. Effect variation of the volumes of reagent (O-nitroaniline 5×10^{-3} M) was studied in the range of 0.5-5 ml and 2 ml was found to be optimum. The

absorbance of the dye formed increased and became more stable in alkaline medium; therefore, the effect of different alkaline solutions (0.1M) on the colored product was studied such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, sodium acetate and sodium carbonate. Maximum sensitivity and stability were obtained only when the reaction was carried out in the presence of sodium carbonate solution. The effect of different volumes (0.25-5 ml) of Na_2CO_3 (0.1 M) was studied, A volume of 2mL was found enough to obtain a maximum absorbance, Experimental results revealed that the color intensity reach maximum after diazotized O-nitro aniline solution had been Reacted with VHC in alkaline medium for 5 min, therefore. a 5 min development time was suggested as the optimum reaction time and remain stable for 120 min. The order of addition of the reagents is an essential part of the experiment, it was found that the order of addition of the reagent cited under general procedure gave maximum color intensity and the minimum absorbance of the blank and was used in all subsequent experiments. Table-1 summarized the studied optimum conditions.

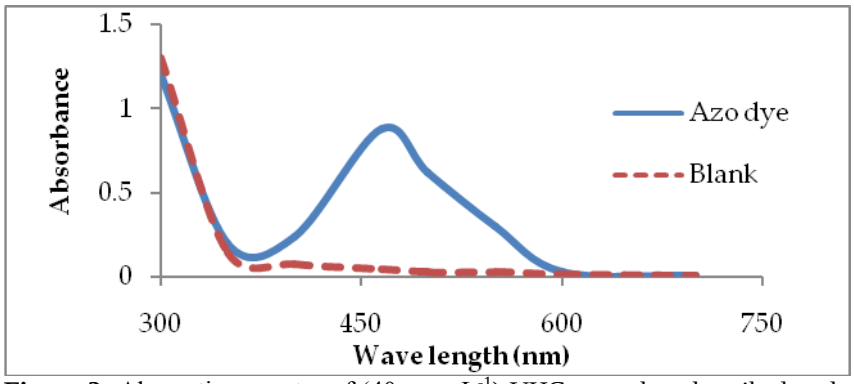
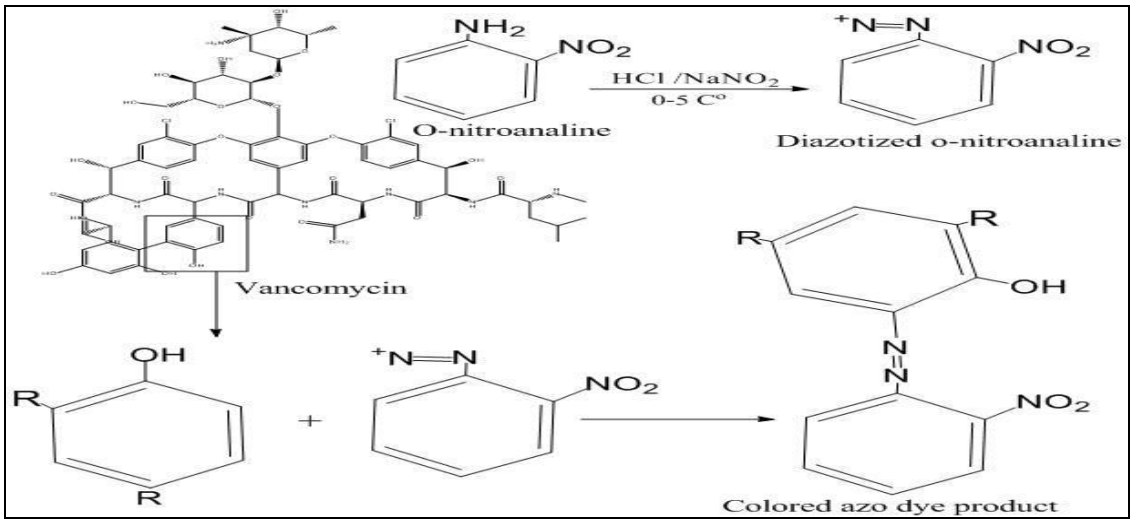


Figure 2- Absorption spectra of (40 µg.mL^{-1}) VHC treated as described under procedure and measured against reagent blank (Diazotized O-nitroaniline and sodium carbonate anhydrous) and the reagent blank measured against distilled water.

Table 1- Optimum conditions established in batch method

Parameter	Range selected	Optimum Conditions in procedure
λmax (nm)	350 – 700	465
Effect of volume of (5x10^{-3}M) O-nitroaniline solution required	0.5 - 5 mL	2 mL
Effect of volume of (1M) HCl solution required	0.5 - 5 mL	2 mL
Effect of volume of (0.1M) Na_2CO_3 solution required	0.25 - 5 mL	2 mL
Type of reaction medium	Alkaline, acidic, and neutral	Alkaline
Type of alkaline medium	NaOH, NH_4OH, Na_2CO_3, CH_3COONa	Na_2CO_3
Effect of Addition Order	DONA , VHC , Na_2CO_3	DONA +VHC+ Na_2CO_3
Effect of temperature	0 - 45°C	25°C
Stability period after final dilution	1 - 120 min	The colored product is formed immediately and becomes stable after 5 min and remains for more than 120 min.

The stoicheiometry of the reaction between VHC and diazotized O-nitroaniline was investigated using continuous variation method. The result obtained Figure-3 shows that a (1:1) azo dye was formed between VHC and diazotized O-nitroaniline according to scheme-1.



Scheme 1- Proposed mechanism of the reaction between VHC and DONA in alkaline medium.

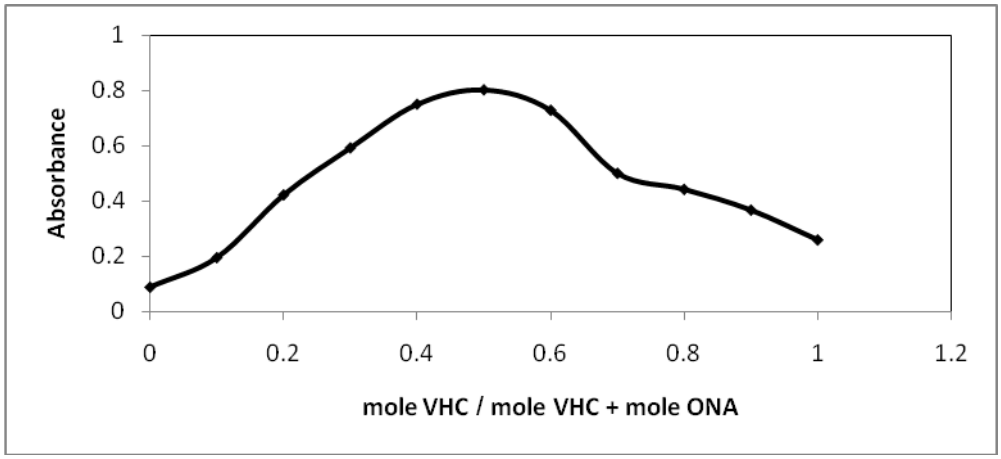


Figure 3- Continuous variation plot of the reaction between VHC and diazotized O-nitroaniline ($5x10^{-3}$M)

FIA-spectrophotometric determination

The batch method for the determination of VHC was adopted as a basis to develop a FIA procedure. The manifold used for the determination of VHC was designed to provide different reaction conditions for magnifying the absorbance signal generated by the reaction of the diazotized O-nitroaniline with VHC in sodium carbonate medium. Maximum absorbance intensity was obtained when the sample (VHC 40 μg.ml^{-1}) was injected into a stream of diazotized O-nitroaniline and then mixed with sodium carbonate as given in Figure-4. The influence of different chemical and physical FIA parameters on the absorbance of the colored product was optimized as follows:

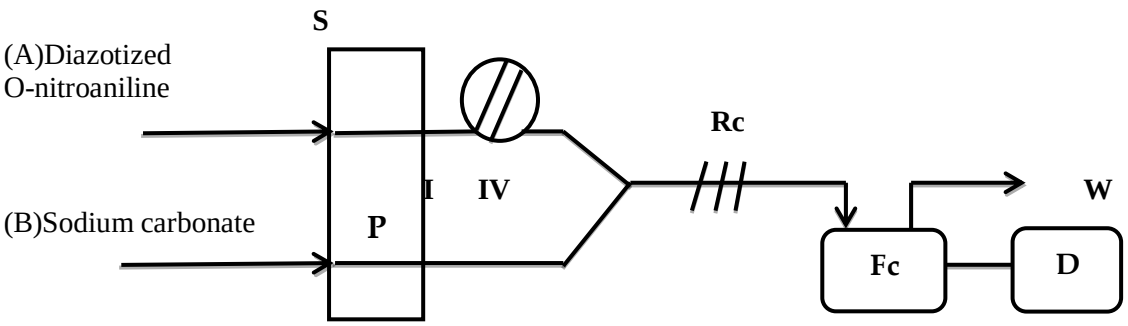


Figure 4- A schematic diagram of FIA manifold Where: (A) and (B), solutions of diazotized O-nitroaniline and sodium carbonate respectively; P =peristaltic pump; S= injection sample VHC; IV= injection valve; Rc= reaction coil; Fc= flow cell; D= detector; W= waste.

Optimization of chemical parameters

The effect of various concentrations of O-nitroaniline was investigated. A concentration of ($5x10^{-3}$M) O-nitroaniline, gave the highest absorbance and was chosen for further experiments as shown in Figure-5. It was observed that the reaction between diazotized O-nitroaniline and VHC depends on alkaline medium, therefore the effect of different concentrations of sodium carbonate was studied and 0.1M was found to be the optimum as shown in Figure-6.

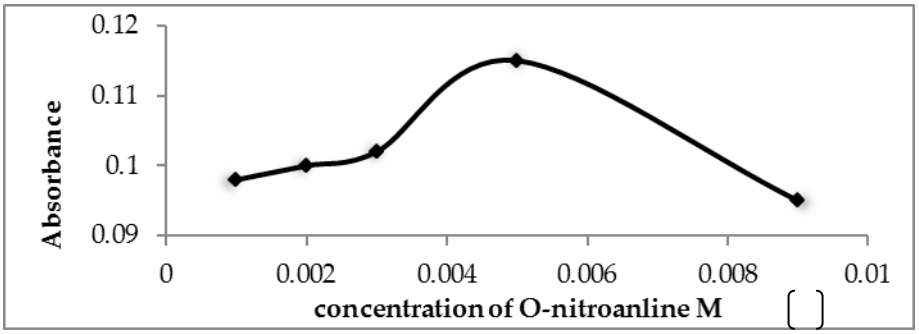


Figure 5- Effect of the concentration of O-nitroaniline reagent

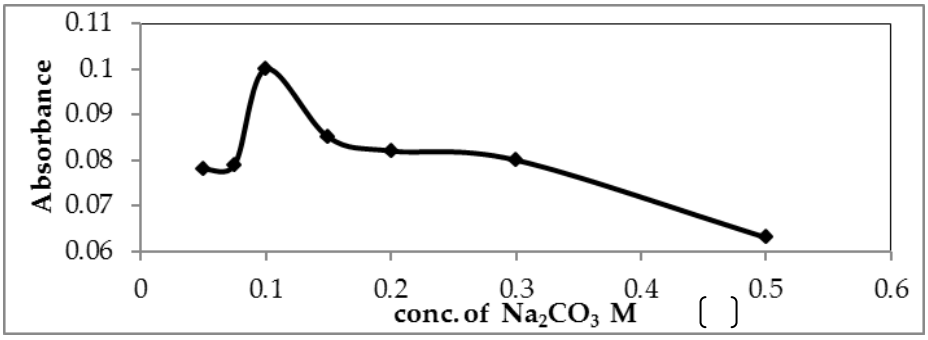


Figure 6- Effect of the concentration of Na_2CO_3 in (M)

Optimization of manifold parameters

The effect of total flow rate on the Absorbance of the colored reaction's product was investigated in the range of 0.6-4 ml min^{-1}. The results obtained showed that a total flow rate of 2.5 ml min^{-1}, (1.25 ml min^{-1} in each line) gave the highest absorbance as shown in Figure-7 and was used in all subsequent experiments. The volume of the injection sample was varied between (100 and 250 µl) using different lengths of sample loop Figure-8. A sample volume of 200 µl was selected for showing better engagement between sensitivity and analytical frequency, and was used in all subsequent experiments

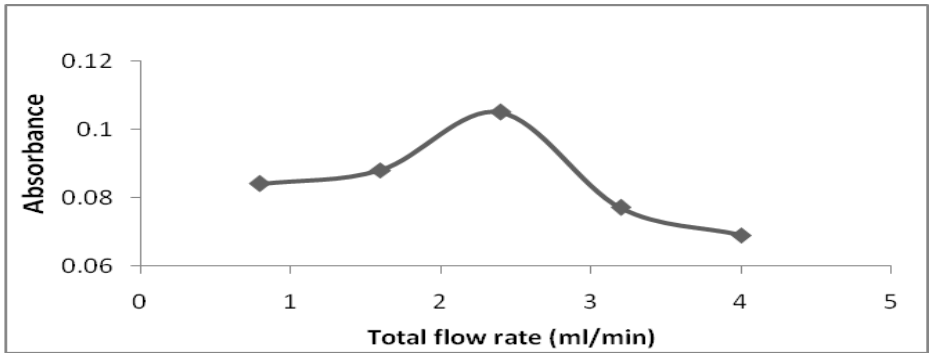


Figure 7- Effect of total flow rate

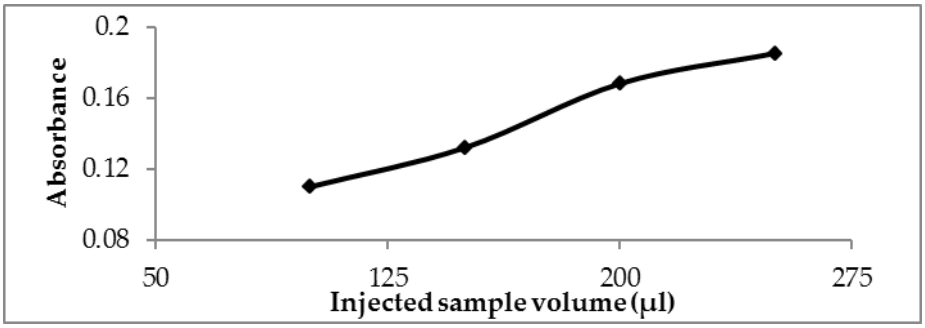


Figure 8- Effect of injection sample volume (µl)

The coil length is an essential parameter's that affects on the sensitivity of the colored reaction product and was investigated, in the range of 25-250 cm. the results obtained showed that a coil length of 75 cm gave the highest absorbance as shown in Figure-9 and was used in all subsequent experiments.

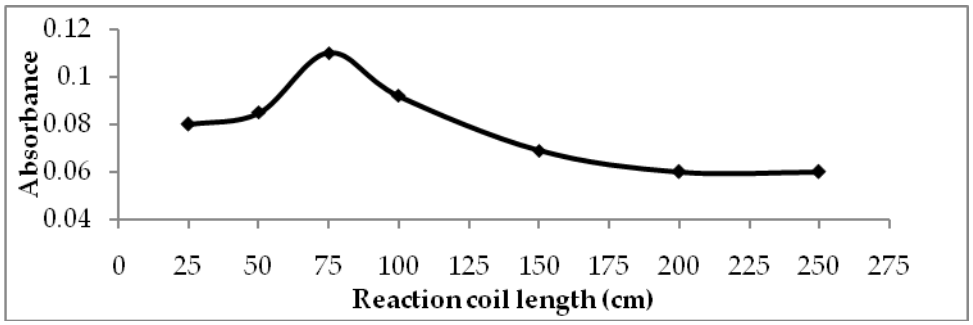


Figure 9-Effect of reaction coil (cm).

The reaction time is also an important parameter's that affected on the sample throughput and was investigated by calculate the interval time between the sample injection and the appearance of the end of the signals. The sample through put was 80 samples per hour.

Calibration graphs

After fixing the optimum conditions of both batch and FI methods for the determination of VHC, calibration graphs were constructed (Figure-10 and Figure-11). The analytical values of statistical treatments [17, 18] for the calibration graphs are summarized in Table-2. The accuracy of the methods was evaluated by analyzing pure samples of VHC and a good recovery was obtained Table-2.

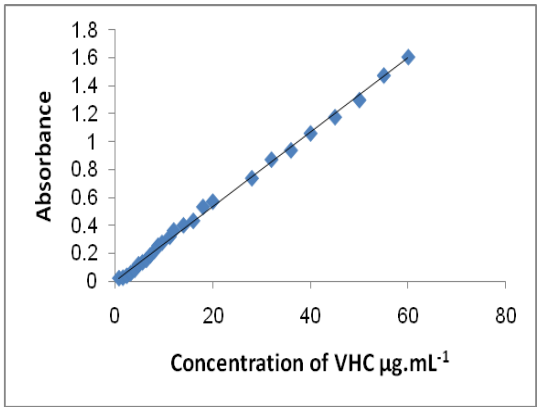


Figure 10- Calibration graph of Batch

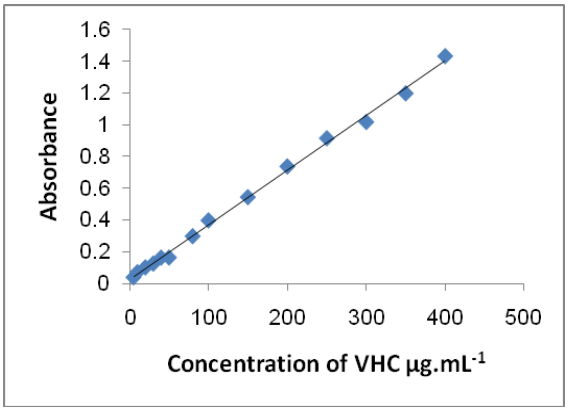


Figure 11- Calibration graph of nFIA

Table 2- Analytical values of the calibration graphs for the determination of VHC.

Parameter	Batch procedure	nFIA procedure
Regression equation	y = 0.0267x – 0.0014	y = 0.0034x + 0.0295
Molar absorption coefficient ($L.mol^{-1}.cm^{-1}$)	3.9668×10^4	5.051×10^3
Linearity range ($\mu g.mL^{-1}$)	0.8 – 60	5 – 400
Correlation coefficient	0.9976	0.9975
Linearity percentage r^2%	99.76	99.75
Sandell's sensitivity($\mu g\ cm^{-2}$)	0.0374	0.2941
Reproducibility (%)* (RSD %)	1.861	1.332
Average of Recovery%*	99.02	100.04
Limit of detection**($\mu g.mL^{-1}$)	0.48	0.5
Through-put (1/h)	6	80

*****The Average of reproducibility, recovery and error of each method was tested by analyzing five replicate samples containing 8, 16, 28 $\mu g.mL^{-1}$ of pure VHC for batch method and 40,80,100 $\mu g.mL^{-1}$ of pure VHC for FIA method.

RSD Relative standard deviation

******Limit of detection=$3SD_B/b$, SD_B is the standard deviation of the blank (n=10) of the blank determinations ($SD_B=4.272\times10^{-3}$ and 5.66×10^{-4} for batch and FIA methods respectively), b is the slope of the corresponding calibration curve).

Analytical application

The proposed methods were applied successfully to the analysis of some pharmaceutical preparations containing VHC (Injection), and they gave a good Reproducibility and Recovery as shown in Table-3. The results obtained by the proposed and reference methods [2, 19] for dosage forms were compared statistically by means of the F-test and t-test [20] and the proposed methods and the reference methods were found no significant differences in precision and accuracy between the proposed methods and the reference methods Table-4.

Table 3- Application of the proposed methods to the determination of VHC in dosage forms**.**

Pharmaceutical preparation	Proposed methods	Conc. $\mu g.mL^{-1}$		E%	Rec.%	RSD%
		Present Conc. $\mu g.mL^{-1}$	Found Conc. $\mu g.mL^{-1}$			
Vancolon Vancomycin Hydrochloride Injection Julphar UAE 500 mg	Batch	8	7.88	-1.5	98.5	1.91
		16	15.97	-0.18	99.82	0.55
		28	27.8	-0.71	99.29	0.94
	nFIA	40	40.14	0.35	100.35	2.4
		80	79.85	-0.18	99.81	1.99
		100	102.5	2.5	102.5	1.06
Vancorin Vancomycin Hydrochloride Injection CheilJedang corporation Republic of Korea 1g	Batch	8	8.1	1.25	101.25	1.14
		16	16.3	1.87	101.87	1.4
		28	28.18	0.64	100.64	0.82
	nFIA	40	39.85	-0.37	99.62	1.82
		80	79.85	-0.18	99.81	0.66
		100	98.67	-1.33	98.67	1.37

Table 4- The comparison of the proposed method with standard method

Pharmaceutical preparation	Proposed methods						Standard method Rf.
	Batch			nFIA			Rec.%
	Rec.%	t*	F*	Rec.%	t	F	
VHC pure	99.02	0.697	1.677	100.04	0.304	2.227	100.000
Vancolon	99.2			100.88			102.239
Vancorin	101.25			99.37			99.451
	S^2_1=1.534 S^2_2 =2.582			S^2_1= 1.155 S^2_2 =2.582			
	S =1.433			S =1.366			

*values at 95% confidence level, $n_1 = n_2 = 3$, $\mathbf{t_{tab}} = \mathbf{t_{0.05/2\,,n-1}} = 2.123$ where **t** has $\nu = n_1 + n_2 - 2$ degrees of freedom = 4, **F** = 19.0 where **F** has $\nu 1 = n_1 - 1$, $\nu 2 = n_2 - 1$ degrees of freedom = 2.

The results obtained by the proposed and standard methods for dosage forms were compared statistically by means of the F-test and t-test at 95% confidence level [20] and were found the calculated t and F-values (Table 4) did not exceed the theoretical values, which indicates that there is no significant difference between either methods in terms of accuracy and precision F-test, which is defined as testing differences between standard deviations (S_1, S_2) of data sets, provides a simple method for comparing the precision of two sets of measurements using the following equation:

$$F = \frac{S_1^2}{S_2^2} \text{ or } = \frac{S_2^2}{S_1^2} \quad (F > 1)$$

$$S_1^2 = \frac{\sum (Xi - \bar{X}_1)^2}{n_1 - 1} \text{ and } S_2^2 = \frac{\sum (Xi - \bar{X}_2)^2}{n_2 - 1}$$

Where S_1^2 = variation or Square standard deviation.

$(n_1 - 1)$ and $(n_2 - 1)$ = number of degrees of freedom of proposed method and standard method, respectively.

Student's t-test is a convenient way of comparing the mean one set of measurements with another. The value of t-test is chosen based on the desired confidence level. A 95% confidence level test is generally used.

This test is calculated using the following equations:

$$S = \sqrt{\frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{n_1 + n_2 - 2}}$$

$$t = \frac{\left|\bar{X}_1 - \bar{X}_2\right|}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

S=pooled standard deviation.

(n_1+n_2-2) =number of degrees of freedom.

Conclusion:

The application of diazotization–coupling reaction of diazotized o-nitroaniline in sodium carbonate medium to the spectrophotometric determinations of the vancomycin hydrochloride in pharmaceutical preparation's was described by batch and nFIA systems, Although the batch system has the advantages of higher sensitivity and lower limit of detection over the nFIA system, the nFIA system has several advantages over the batch system simplicity, reproducibility time saving, low reagent consumption need of small sample volume, large dynamic range and high sample throughput (80 sample h^{-1} for VHC) is important feature of the nFIA system.

The proposed methods offer a good linearity and precision and can be applied to the analysis of a wide concentration range of VHC in real samples with satisfactory results. The proposed methods are simple and inexpensive since it requires simple instrumentation.

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