Normal and Reverse Flow Injection- Spectrophotometric Determination of Vancomycin Hydrochloride in Pharmaceutical Preparations Using 2, 4-Dinitrophenylhydrazine

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Abstract

Simple and sensitive batch and flow injection methods (normal and reverse flow injection analysis (nFIA and rFIA)) for spectrophotometric determination of vancomycin hydrochloride (VHC) in pharmaceutical preparations were proposed and optimized. Both methods are based on the oxidative - coupling reaction between vancomycin hydrochloride and 2,4-Dinitrophenylhydrazine (DNPH) in the presence of sodium periodate in alkaline medium to form a yellow water-soluble product that is stable and has a maximum absorption at 461 nm. Beer's law was obeyed over the range of 1- 40, 0.5-120 and 0.5-150 µg.mL^{-1}; the limits of detection were 0.537, 0.0823 and 0.233 µg.mL^{-1} for batch, normal and reverse flow injection methods respectively. The sampling rates were 124 and 120 injections per hour for normal and reverse flow injection methods respectively. The effects of chemical and physical parameters have been carefully considered and the proposed procedures were successfully applied to the determination of vancomycin hydrochloride in pharmaceutical preparations.

Keywords: Vancomycin hydrochloride, Oxidative – coupling reaction, 2,4-dinitrophenylhydrazine, Spectrophotometric determination, Flow injection.

التقدير الطيفي – الحقن الجرياني الاعتيادي و العكوس للفانكومايسين هايدروكلورايد في المستحضرات الصيدلانية باستخدام 4,2 – ثنائي نايتروفنيل هيدارزين

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الخلاصة:

يتضمن البحث تطوير طريقة طيفية جديدة وبسيطة للتقدير الكمي للمقادير الضئيلة من الفانكومايسين هيدروكلورايد في المحاليل المائية والمستحضرات الصيدلانية باستخدام المطياف الضوئي–الحقن الجرياني الاعتيادي و العكوس. تعتمد الطريقة على تفاعل الازدواج التاكسدي بين الفانكومايسين مع 4,2 – ثنائي نايتروفنيل هيدارزين بوجود بيرايودات الصوديوم وفي وسط قاعدي حيث يتكون ناتج اصفر غامق مستقر وذائب في الماء يعطي اعلى قمة امتصاص عند طول موجي 461 نانوميتر. تشير منحنيات الامتصاص مقابل التركيز بان قانون بير ينطبق ضمن مدى التركيز1 – 40 و 0.5 – 120 و 0.5 – 150 مايكروغرام.مل$^{-1}$من الفانكومايسين و بحد كشف 0.537 و 0.0823 و 0.233 مايكروغرام.مل$^{-1}$من

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الفانكوماسين لطريقة الدفعة والحقن الجرياني الاعتيادي و العكوس على التوالي وبمعدل نمذجة 124 و 120 نموذج بالساعة لطريقتي الحقن الجرياني الاعتيادي و العكوس على التوالي. تمت دراسة الظروف المثلى للتفاعل وجميع المتغيرات الكيميائية والفيزيائية بعناية وطبقت الطريقتين بنجاح على المستحضرات الصيدلانية الحاوية على الفانكومايسين.

Introduction

Vancomycin is a glycopeptide antimicrobial substance or mixture of glycopeptides produced by the growth of certain strains of Amycolatopsis orientalis (Nocardia orientalis, Strptomyces orientalis), or by any other means [1]. Vancomycin hydrochloride (VHC) consists principally of the monohydrochloride of (3S, 6R,7R,22R,23S,26S,30aSa,36R,38aR)-3-(2-amino-2-oxoethyl)-44-[[2-O-(3-amino-2,3,6-trideoxy-3-O-methyl-α-L-*lyxo*-hexopyranosyl)-β-D -glucopyranosyl]oxy]-10,19-dichl-oro-7,22,28,30,30,32-pentahydroxy-6-[[(2R)-4-methyl-2-(mthylamino) pentanoyl]amino]-2,5,24,38,39 -pentaoxo-2,3,4,5,6,7,23,24,25,26,36,37,38,38a-tetradecahydro-22*H*-8,11:18,21-dietheno-23,36(imin-omethano)-13,16:31,35-dimtheno-1*H*,13*H*[1,6,9]oxadiazacyclohexadecino[4,5*m*][10,2,16]benzoxadia-zacyclotetracosine-26-carboxylic acid [2]. Vancomycin was introduced in 1958 as an antibiotic active against Gram-positive cocci, particularly streptococci, staphylococci and pneumococci. It is not active against Gram-negative bacteria, Vancomycin hydrochloride is recommended for use when infections fail to respond to treatment with the more common antibiotics [3]. VHC is officially recognized in B.P [2] and U.S.P [4]. A survey of literature revealed that few methods based on visible spectrophotometry for VHC [5-7] have been reported. Other methods include HPLC [8-11], Polarography [12], Capillary electrophoresis [13], Radioimmunoassay [14], Fluorescence polarization immunoassay [15] and Flow injection analysis [16, 17]. All the reported methods are either not sufficiently sensitive or tedious and require highly sophisticated instrumentation. This paper describes the batch and flow injection methods (normal and reverse) for spectrophotometric determination of vancomycin hydrochloride (VHC) by the oxidative-coupling reaction between vancomycin hydrochloride and 2,4-Dinitrophenylhydrazine (DNPH) with sodium periodate, where an electrophilic intermediate (diazonium salt of the reagent) is produced, which couples with VHC in the presence of sodium hydroxide to form a yellow water-soluble product that is stable and has a maximum absorption at 461 nm. The analytical procedure is simple, fast, and accurate, it has been satisfactorily applied for the determination of vancomycin hydrochloride in pure and injections preparations. The reaction can be carried out in batch and FIA and the two approaches were compared.

Experimental

Apparatus

All spectral and absorbance measurements were carried out using a digital double beam spectrophotometer (shimadzu, UV-vis 260). A silica cells were used for the absorbance measurements of the batch procedure. A flow cell 50 µL internal volume and 1 cm bath length was used for the absorbance measurements of FIA. A peristaltic pump (Ismatec, Laborechnik Analytik, CH8152, Zurich, Switzerland) was used to transport the solution. In addition, an injection valve (Rheodyne,Altex 210, Supelco, USA) was employed to provide appropriate injection volumes of standard solutions and samples while a flexible vinyl tubing (0.5 mm internal diameter) was used for the peristaltic pump. The reaction coil (RC) was of Teflon material with an internal diameter of 0.5 mm. The solutions were propelled by peristaltic pump with initial total flow rate of 1.5mL.min^{-1} in the normal and reverse flow injection methods, and the absorbance was measured at 461nm.

Reagent and materials

All the chemicals used were of analytical grade and all the solutions were prepared with distilled water, freshly prepared solutions were always used.

Standard vancomycin hydrochloride VHC solution

Stock solution (500 µg.mL^{-1}) was prepared daily by dissolving 0.05 g of the pure compound (Molecular weight of VHC is 1486 g.mol^{-1}) in 100 mL of distilled water and serial dilutions with distilled water were made.

Sample vancomycin hydrochloride VHC solution

The contents of five vials (three commercial sources) were mixed. An aliquot corresponding to 0.05 g of VHC was diluted to 100 mL with distilled water in a volumetric flask to obtain 500 µg.mL^{-1}

of VHC. More dilute solutions of pharmaceutical preparations for batch and FIA procedures were made by simple dilution with distilled water.

2,4-dinitrophenylhydrazine DNPH (BDH) solution

Stock solution (5 mM) was prepared daily by dissolving 0.0990 g of DNPH (Molecular weight is 198.14 $g.mol^{-1}$) in 2 mL concentrated sulphuric acid, transferred in to 100 mL volumetric flask and diluted to the mark with distilled water, and working solutions were prepared by appropriate dilution of the stock solution.

Sodium periodate $NaIO_4$ (BDH) solution

Stock solution (5 mM) was prepared daily by dissolving 0.1069 g of $NaIO_4$ (Molecular weight is 213.89 $g.mol^{-1}$) in 100 mL distilled water, and working solutions were prepared by appropriate dilution of the stock solution.

Sodium hydroxide NaOH (Merck) solution

Stock solution of 1 M was prepared by dissolving 10 g of NaOH (Molecular weight is 40 $g.mol^{-1}$) in 250 mL distilled water, and working solutions were prepared by appropriate dilution of the stock solution.

Procedures

General batch procedure

Aliquots of standard VHC solution containing 25 -1000 µg of VHC was transferred into a series of 25 mL standard flasks. A volume of 0.5 mL of 5 mM of DNPH, 1.5 mL of 5 mM of $NaIO_4$ and 3 mL of 0.5M NaOH solution were added. The contents of the flasks were diluted to mark with distilled water, mixed well. The absorbance was measured at 461 nm (at room temperature 25°C) against reagent blank containing all materials except VHC. A calibration graph was drawn and the regression equation was calculated. For the optimization of conditions and in all subsequent experiments, a solution of 500 µg was used in a final volume of 25 mL (20$\mu g.mL^{-1}$).

General nFIA procedure

Working solutions of VHC in a range of 0.5 – 120 $\mu g.mL^{-1}$ were prepared from the stock solutions (500 $\mu g.mL^{-1}$). A 200 µL portion of VHC was injected into the stream of 0.7 M NaOH and was then combined with a stream of mixture of DNPH and $NaIO_4$ (0.3 mM + 0.3 mM) solution with a total flow rate of 2.5 $mL.min^{-1}$ and reaction coil length of 75 cm figure-1(a). The resulting absorbance of the produced was measured at 461 nm. Moreover, optimization of conditions was carried out using 100 $\mu g.mL^{-1}$ of VHC.

General rFIA procedure

Working solutions of VHC in a range of 0.5 – 150 $\mu g.mL^{-1}$ were prepared from the stock solutions (500 $\mu g.mL^{-1}$). A 200 µL portion of mixture of DNPH and $NaIO_4$ (0.6 mM + 0.9 mM) solution was injected into the stream of VHC solution and was then combined with a stream of 0.3 M NaOH solution with a total flow rate of 2 $mL.min^{-1}$ and reaction coil length of 100 cm figure-1(b). The resulting absorbance of the produced was measured at 461 nm. Moreover, optimization of conditions was carried out using 100 $\mu g.mL^{-1}$ of VHC.

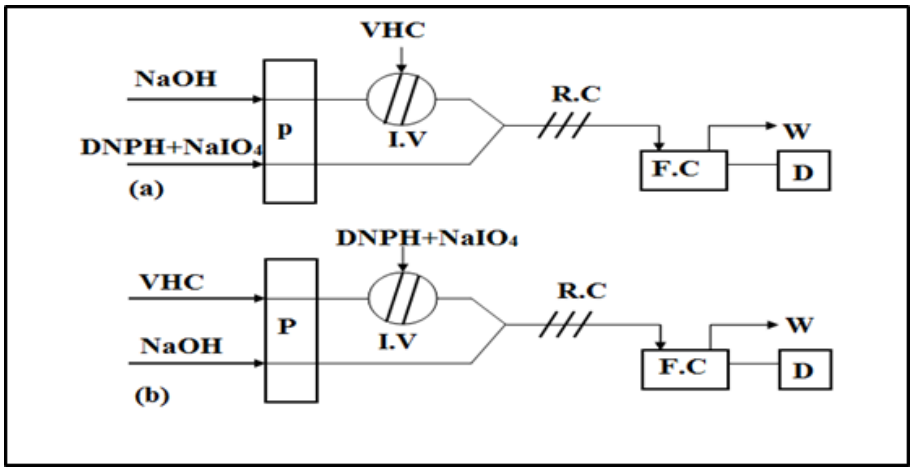


Figure 1- Schematic diagram of flow injection-Spectrophotometric analysis **P**, Peristaltic pump; **I.V**, Injection valve; **R.C**, Reaction coil; **F.C**, Flow cell; **D**, Detector (vis-spectrophotometric); **W**, Waste.

Results and discussion

Absorption spectra

VHC forms a yellow-colored product (λ_{max} of 461 nm with a molar absorption coefficient of 36690 $L.mol^{-1}.cm^{-1}$) with DNPH in the presence of sodium periodate in alkaline medium. The absorption spectra of the colored product are given in figure-2. The reaction is based on the oxidation of DNPH with sodium periodate to produce diazonium cation (I); the intermediate of DNPH undergoes electrophilic substitution in alkaline medium with the Phenolic group of VHC to form a colored product (II).

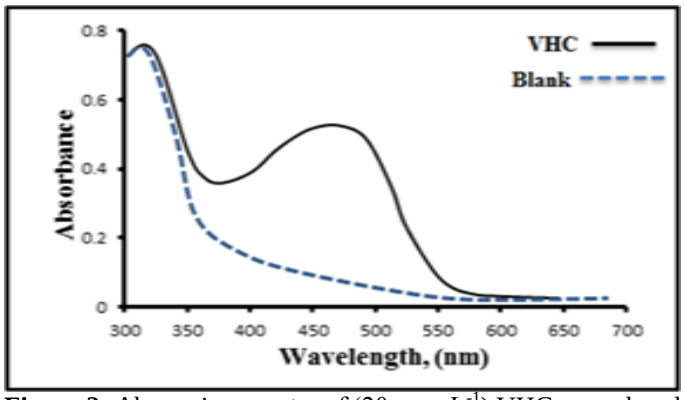


Figure 2- Absorption spectra of (20 $\mu g.mL^{-1}$) VHC treated as described under procedure and measured against reagent blank and the reagent blank measured against distilled water.

The stoichiometry of the reaction between each VHC and DNPH was investigated under the recommended optimum conditions by Job's method [18]. The figure-3 reached a maximum value at a mole fraction of 0.5 indicating that the reaction proceeds with mole ratio 1:1 for DNPH:VHC. The proposed mechanism of the reaction between VHC and DNPH showed in Scheme 1.

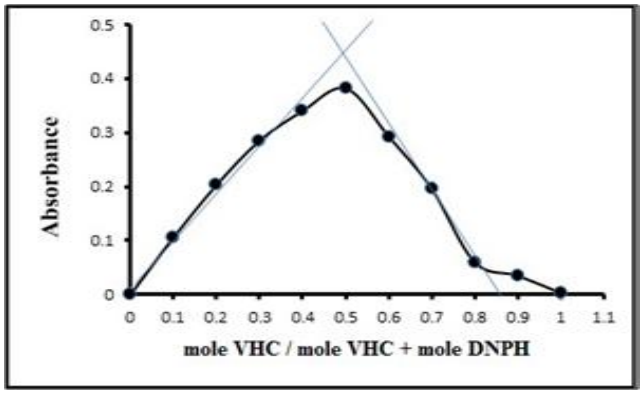
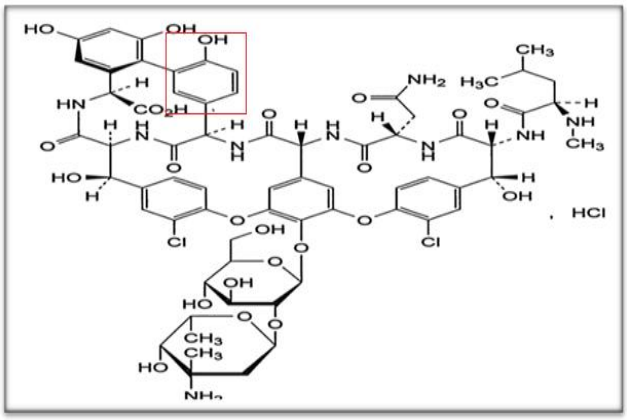
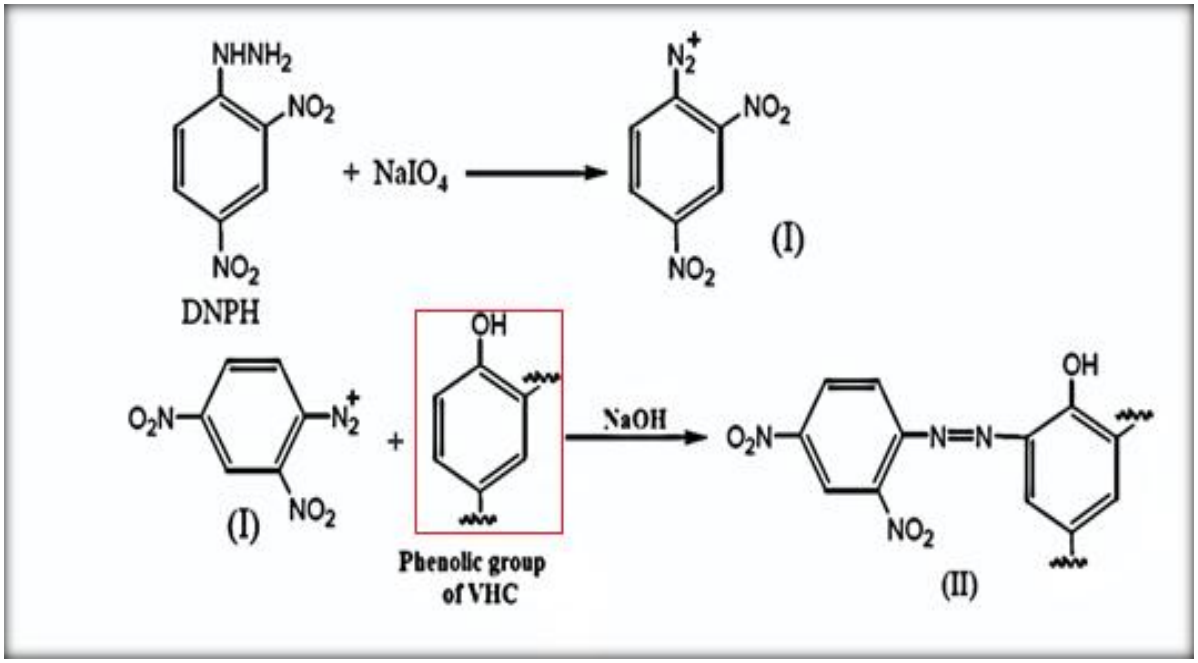


Figure 3- The mole ratio of the reaction between VHC and DNPH



Vancomycin hydrochloride (VHC)



Scheme 1- Proposed mechanism of the reaction between DNPH and VHC

Batch spectrophotometric determination

The parameters affecting mainly the sensitivity and stability of the colored product were studied and optimized. Optimum conditions were established by changing one-factor-at-a-time (OFAT) and keeping the others fixed by observing the effect produced on the absorbance of the colored species. the yellow-colored product which was formed between VHC and DNPH had developed only in alkaline medium; therefore, the effects of different alkaline solutions were studied such as sodium acetate, sodium carbonate, ammonium hydroxide and sodium hydroxide.

The maximum sensitivity and stability were obtained only when the reaction was carried out in the presence of sodium hydroxide solution. The best experimental conditions for the determination of VHC were established for DNPH 5 mM (from 0.1 to 3 mL), sodium periodate 5 mM (from 0.1 to 4 mL) and sodium hydroxide 0.5 M (from 0.5 to 7 mL) by varying OFAT to a fixed concentration of VHC (20 $\mu g.mL^{-1}$) while the others were kept constant in a final volume of 25 mL and measuring the absorbance at 461 nm. Colored product is formed immediately and remains stable for about 3 hr.

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 (2.3.1) gave maximum color intensity and a minimum absorbance of the blank and was used in all subsequent experiments. The effect of temperature on the color intensity of the dye was studied. A high absorbance was obtained when the color is developed at room temperature (25°C) than the calibrated flasks were placed in an ice bath at (0 $^{\circ}$C) or in a water bath at (45 $^{\circ}$C). The stability constants of the dye products were calculated [19] by comparing the absorbance of a solution containing stoichiometric amount of VHC and DNPH with that of solution containing five-fold excess of DNPH reagent.

The stability constant of the dye products in water under the described experimental conditions was 2.6753×10^4 $L.mol^{-1}$. The optimum conditions for batch method are incorporated in table-1, and the effect of excipients on the recovery of VHC is incorporated in table-2. The regression equation obtained from a series of VHC standards, and the analytical figures of merits of this procedure are summarized in table-3 as shown latter.

Table 1- Optimum conditions established in batch method

Parameter	**Range selected**	**Optimum Conditions in procedure**
λ_{max} (nm)	350 - 700	461
Effect of volume of (5mM) DNPH solution required	0.1 - 3 mL	0.5 mL
Effect of volume of (5mM) $NaIO_4$ solution required	0.1 - 4 mL	1.5 mL
Effect of volume of (0.5M) NaOH solution required	0.5 - 7 mL	3 mL
Type of reaction medium	Alkaline, acidic, and neutral	Alkaline
Type of alkaline medium	NaOH, NH_4OH, Na_2CO_3, CH_3COONa	NaOH
Effect of Addition Order	VHC, DNPH, $NaIO_4$ and NaOH	VHC + DNPH + $NaIO_4$ + NaOH
Effect of temperature	0 - 45 ˚C	25 ˚C
Stability period after final dilution	1 - 200 min	The colored product is formed immediately and becomes stable after 1 min and remains for more than 180 min.

Influence of excipients

Despite the fact that vancomycin is more used in the pure form, however, in order to assess the possible analytical applications of the proposed method, the influence of four common excipients: starch, talc, lactose and poly vinyl pirrolidone (pvp) was studied by analyzing synthetic sample solutions containing 20 μg.mL-^{1} of VHC and excess amounts (10-fold excess) of each excipient, none of these substances interfered seriously in the determination of VHC by the proposed methods table-2.

Table 2- Effect of excipients (200 μg.mL^{-1}) on the recovery of VHC (20 μg.mL^{-1})

Excipient	**Conc. μg.mL^{-1}**	**Error %**	**Recovery %**
Starch	19.951	- 0.242	99.757
Talc	19.573	- 2.132	97.867
Lactose	20.032	+ 0.161	100.161
PVP	20.396	+ 1.983	101.983

Spectrophotometric determination for nFIA and rFIA

The batch method for the determination of VHC was adopted as a basis to develop nFIA and rFIA procedures. Both manifolds used for the determination of VHC were designed to provide different reaction conditions for magnifying the absorbance signal generated by the reaction of VHC with DNPH and sodium periodate in sodium hydroxide medium. For minimizing the lines of FIA manifold figure-1 (a, b), many mixtures of reagents solutions were tested between the reagent of DNPH, oxidant and also the solution of sodium hydroxide. The results show that a mixture solution of DNPH and $NaIO_4$ gave the maximum absorbance, and was chosen for further use. Maximum absorbance intensity was obtained when the VHC solution was injected into a stream of sodium hydroxide and was then combined with the stream of mixed DNPH with sodium periodate in nFIA figure-1 (a), but in rFIA, the maximum absorbance intensity was obtained when the mixed DNPH with sodium periodate solution was injected into a stream of VHC and was then combined with the stream of sodium hydroxide figure-1 (b). The influences of different physical and chemical parameters on the intensity of the colored product were optimized as follows:

Optimization of reagents concentration

The effects of various concentrations of DNPH in the range (0. 02-1 mM) and $NaIO_4$ in the range (0.1-1.2 mM) in the mixture of DNPH and $NaIO_4$ solution were investigated for both kinds of FI. A concentration of 0.3 mM of DNPH and 0.3 mM of $NaIO_4$ in mixture gave the highest absorbance for nFIA and 0.6 mM of DNPH and 0.9 mM of $NaIO_4$ in mixture gave the highest absorbance for rFIA, and was chosen for further use figure-4(a,b). Therefore, the effect of various concentrations of NaOH was studied in the concentration range of 0.04-1M and the greatest absorbance intensity was obtained with 0.7 M and 0.3 M for normal and reverse flow injection manifolds, respectively figure-4(c).

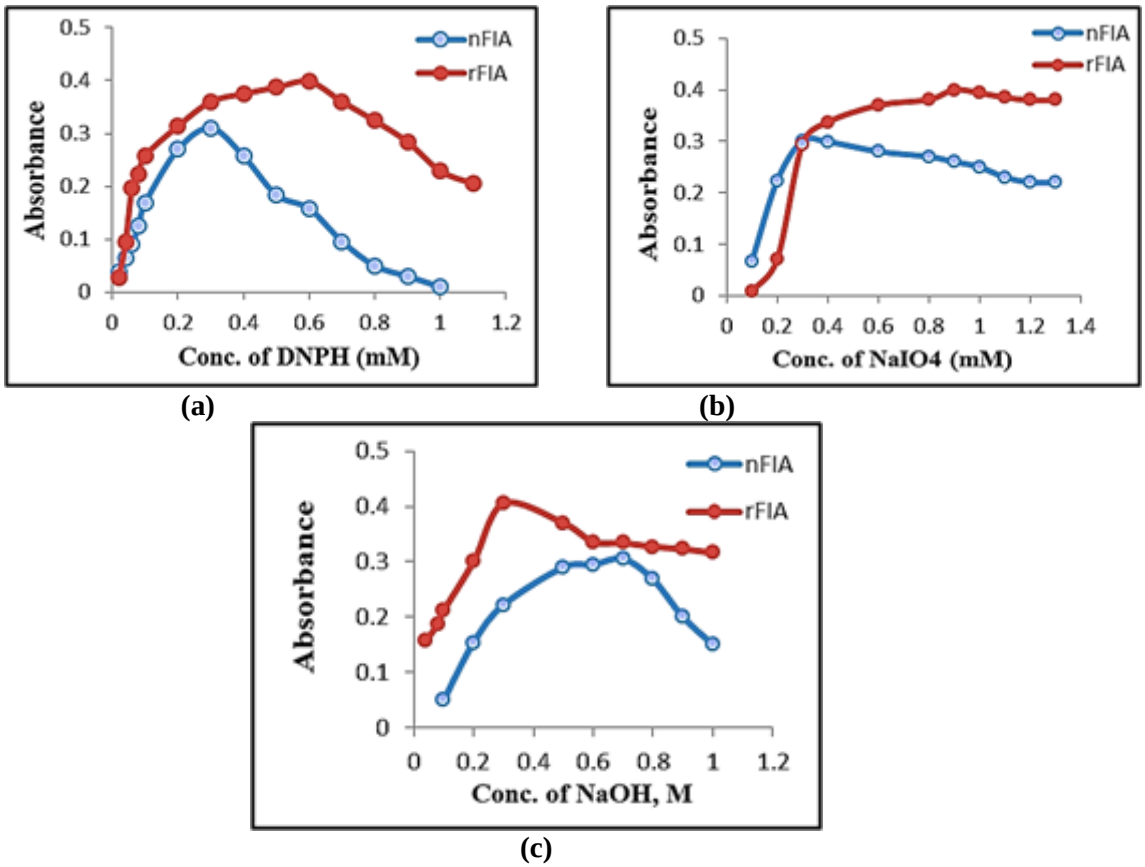


Figure 4- Effect of reagents concentration on nFIA and rFIA **(a)** Effect of concentration of DNPH **(b)** Effect of concentration of $NaIO_4$ **(c)** Effect of concentration of NaOH**.**

Optimization of manifold parameters

The variables studied under the optimized reagents concentrations were the flow rate, the injected sample volume and the reaction coil length. The results showed that a total flow rate of 2.5 and 2mL.min^{-1} gave the highest absorbance for nFIA and rFIA, respectively, figure-5(a) and they were used in all subsequent experiments. The volume of the sample was varied between 50 and 250 µL using different lengths of sample loop and showed that a sample of 200 µL gave the best absorbance for both methods figure-5(b). Moreover, a coil length of 75 cm and 100 cm gave the highest absorbance for both nFIA and rFIA respectively figure-5(c) and was used in all subsequent experiments. A standard calibration graph, obtained from a series of VHC standards and the main analytical figures of merits of the developed procedures are indicated and compared in table-3.

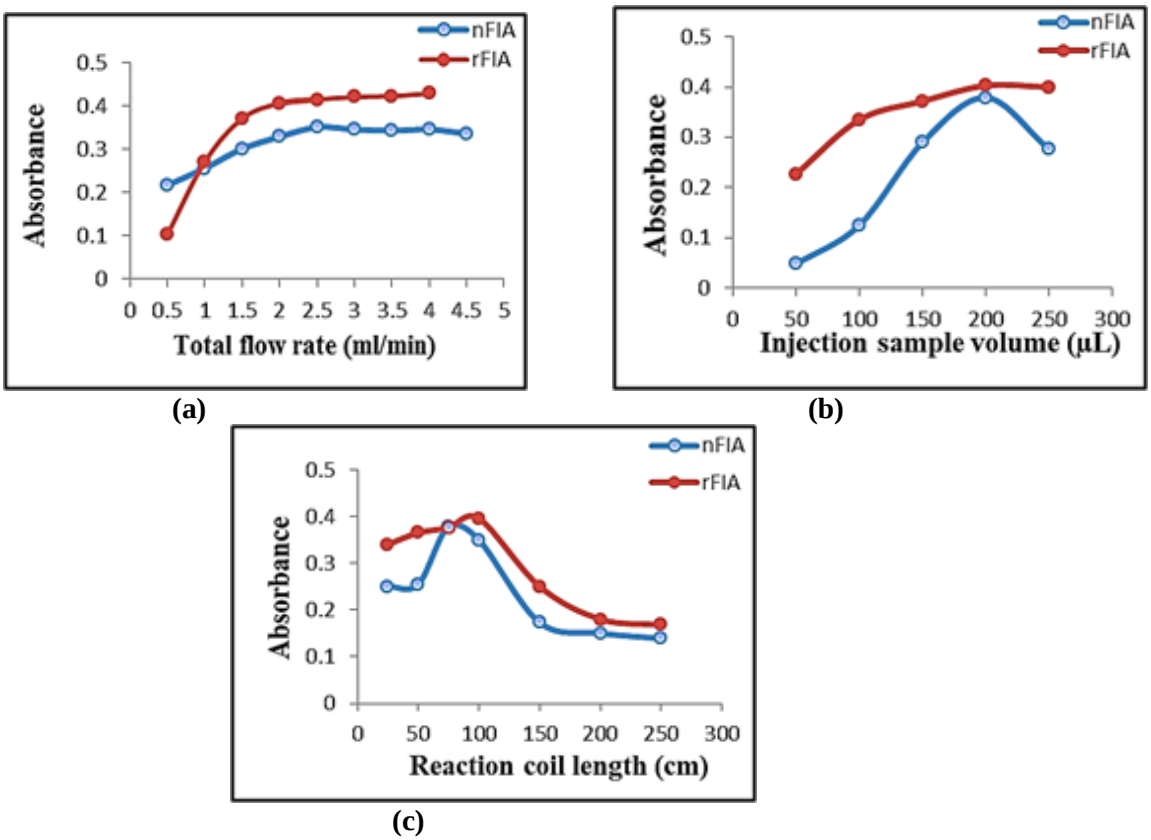


Figure.5- Effect of manifold parameters on nFIA and rFIA **(a)** Effect of total flow rate **(b)** Effect of injection sample volume **(c)** Effect of reaction coil.

Analytical application

The accuracy of the methods was evaluated by analyzing pure samples of VHC and a good recovery was obtained table-3. The proposed methods were applied successfully to the analysis of some pharmaceutical preparations containing VHC (Injection and oral use), and they gave a good accuracy and precision as shown in table-4. The results obtained by the proposed and reference methods [2, 4] for dosage forms were compared statistically by means of the F-test and t-test [20] and were found no significant differences in precision and accuracy between the proposed methods and the reference methods table-5.

Table 3- Compared between three different methods for the determination of VHC.

Parameter	Batch procedure	nFIA procedure	rFIA procedure
Regression equation	y = 0.0247 x - 0.0138	y = 0.0031x + 0.0705	y = 0.0038x + 0.0204
Molar absorption coefficient ($L.mol^{-1}.cm^{-1}$)	3.669×10^{4}	4.605×10^{3}	5.645×10^{3}
Linearity range ($\mu g.mL^{-1}$)	1 – 40	0.5 - 120	0.5 - 150
Correlation coefficient	0.99957	0.9997	0.9999
Sy/x	1.1657×10^{-2}	3.495×10^{-3}	2.463×10^{-3}
Sa	6.3567×10^{-3}	1.840×10^{-3}	1.250×10^{-3}
Sb	2.8475×10^{-4}	2.830×10^{-5}	1.727×10^{-5}
Sandell's sensitivity ($\mu g\ cm^{-2}$)	0.04048	0.3225	0.2631

Reproducibility (%)* (RSD %)	1.233	1.863	0.920
Recovery%*	101.029	100.806	99.602
Limit of detection** ($\mu g.mL^{-1}$)	0.5378	0.0823	0.2334
Through-put (1/h)	10	124	120

*The reproducibility, recovery and error of each method was tested by analyzing five replicate samples containing 5, 20, 35 $\mu g.mL^{-1}$ of pure VHC for batch method and 10, 50,100 $\mu g.mL^{-1}$ of pure VHC for nFIA and rFIA method.

RSD Relative standard deviation.

Sy/x Standard deviation of the residuals.

Sa Standard deviation of the intercept.

Sb Standard deviation of the slope.

** Limit of detection = $3SD_B/b$, SD_B is the standard deviation of the absorbance (n=10) of the blank determinations ($SD_B = 4.427 \times 10^{-3}$, 8.504×10^{-5}, 2.956×10^{-4} for batch, nFIA, and rFIA methods respectively), b is the slope of the corresponding calibration curve)

Table 4- 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	**Found***			
Vancomycin[(1)] Hydrochloride For Solution For Infusion Wockhardt UK 500mg and 1g	Batch	5	4.931	- 1.376	98.623	1.618
		20	19.708	- 1.457	98.542	1.528
		35	34.323	- 1.931	98.068	0.518
	nFIA	10	10.161	+ 1.612	101.612	1.703
		50	50.806	+ 1.612	101.612	0.601
		100	99.516	- 0.483	99.516	0.870
	rFIA	10	9.894	- 1.052	98.947	1.952
		50	49.456	- 1.087	98.912	0.970
		100	98.736	- 1.263	98.736	1.498
Vancolon[(2)] Vancomycin Hydrochloride Injection Julphar UAE 500 mg and 1 g	Batch	5	4.971	- 0.566	99.433	0.722
		20	19.773	- 1.133	98.866	0.483
		35	34.900	- 0.283	99.716	0.204
	nFIA	10	10.354	+ 3.548	103.548	1.940
		50	51.451	+ 2.903	102.903	0.684
		100	100.806	+ 0.806	100.806	0.523
	rFIA	10	10.157	+ 1.578	101.578	1.360
		50	50.596	+ 1.192	101.192	0.778
		100	101.824	+ 1.824	101.824	1.395
Vondem[(3)] Vancomycin Hydrochloride For Solution For Infusion DEMO S.A. Greece 500 mg	Batch	5	5.133	+ 2.672	102.672	2.216
		20	20.145	+ 0.728	100.728	1.810
		35	35.484	+ 1.384	101.384	0.259
	nFIA	10	10.064	+ 0.645	100.645	2.654
		50	50.483	+ 0.967	100.967	1.496
		100	98.225	- 1.774	98.225	0.570
	rFIA	10	9.947	- 0.526	99.473	1.349
		50	49.039	- 1.921	98.078	0.693
		100	98.842	- 1.157	98.842	0.824

*Mean of five measurements of each method, (1), (2), (3) three commercial sources of VHC

Table 5- The comparison of the proposed method with standard method.

Pharmaceutical preparation	**Proposed methods**									**Standard method Rf.**
	Batch			**nFIA**			**rFIA**			
	Rec.%	**t***	**F***	**Rec.%**	**t**	**F**	**Rec.%**	**t**	**F**	**Rec. %**
VHC pure	101.029	0.210	1.090	100.806	0.760	2.244	99.602	0.618	1.459	100.000
Vancomycin[(1)] Hydrochloride	98.411			100.913			98.865			98.506
Vancolon[(2)]	99.338			102.419			101.531			102.239
Vondem[(3)]	101.594			99.945			98.797			100.523

*Theoretical values at 95% confidence limit, $n_1 = n_2 = 4$, **t** = 2.45 where **t** has $\nu = n1 + n2 - 2$ degrees of freedom = 6, **F** = 9.277 where **F** has $\nu_1 = n_1 - 1$, $\nu_2 = n_2 - 1$ degrees of freedom = 3.

Conclusions

The proposed methods are simple and cost-effective for determination of VHC. They are adequate in aqueous solution and in pharmaceutical samples at a concentration level of traces ($\mu g.mL^{-1}$) without the need for previous separation steps, temperature or pH control. The procedures have also good linearity, rapid, through-put 124 sample of nFIA and 120 sample of rFIA at hour, sensitivity and economical value compared to other methods.

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