Spectrophotometric Analysis of Vancomycin Hydrochloride in Pure and Pharmaceutical Injections via Batch and Cloud Point Extraction Techniques

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Abstract:

Development of a precise and delicate reaction has been acquired for the determination of vancomycin hydrochloride using batch and cloud point extraction (CPE) methods. The first method is based on the formation of azo dye as a result of diazotized dapsone coupled with vancomycin HCl (VAN) in a basic medium. The sensitivity of this reaction was enhanced by utilizing a nonionic surfactant (Triton X-114) and the cloud point extraction technique (second method). The azo dye formed was extracted into the surfactant-rich phase, dissolved in ethanol and detected at λ_{max} 446 nm spectrophotometrically. The reaction was investigated using both batch and CPE methods (with and without extraction), and a simple comparison between the two developed methods was made. The conditions that affect the extraction process and the sensitivity of the methods have been carefully examined. The linearity of the calibration curves was in the range of 3-50 and 0.5- 25 µg.mL^{-1} with limits of detection of 0.806 and 0.214 µg.mL^{-1} for VAN in both batch and CPE procedures, respectively. The percentage of relative standard deviation (R.S.D.%) for the two methods was better than 2.54% and 2.83%, respectively. The recommended procedures have been effectively used to assay VAN in commercial injections.

Keywords: Cloud point extraction, Dapson, Diazotization and coupling reaction, Spectrophotometry, Vancomycin hydrochloride.

Introduction:

Vancomycin hydrochloride (VAN) has a molecular formula of ($C_{66}H_{75}Cl_2N_9O_{24}.HCl$), with molecular mass of 1486 g/moL[1]. Its chemical structure is shown in Fig. 1.
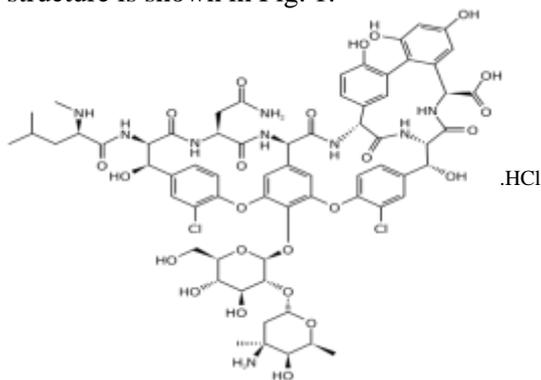

Figure 1. Structure of vancomycin HCl [1]

Vancomycin is an antibiotic belonging to the class of glycopeptides, and has been used for the past few decades as an effective solution for the treatment of infections caused by methicillin-resistant pathogens, particularly Staphylococcus spp.[2].

Various methods for determining VAN using different techniques have been described in the literature, spectrofluorimetry [3,4], UV-spectrophotometry [5-8], IR-spectrometry [9], electrochemical detection [10], flow injection spectrometry [11-13], solid phase extraction including adsorbents magnetic nanoparticles [14], LC-MS spectroscopy [15], spectrophotometric oxidation methods [16-17] and high performance liquid chromatography methods [18-20]. Most of the methods described above are sensitive but require expensive instrumentation and time consuming. In contrast,

spectrophotometric methods were chosen due to their economy and simplicity.

Dapsone (DP) [1] which has a chemical name of 4,4'-sulfonyldianiline, is a major therapy for herpetiform dermatitis and an antibiotic used to treat leprosy. It has been determined using different spectrophotometric methods based on different reactions such as cargo transfer-complex formation reaction [21], diazotization-coupling reactions [22, 23], other methods such as polarography [24] and HPLC [25, 26] were also used.

Cloud Point Extraction (CPE) as a separation and extraction technique still offers attractive properties in the routine analysis of different compounds with surfactants instead of organic solvents. CPE depends on a well-known surfactant phenomenon. Adoption uses micellar systems for extraction and pre-concentration. It has received a lot of attention due to its consistency with the principles of green chemistry. CPE offers many advantages over traditional extraction methods, such as: Simplicity, using an aqueous medium instead of large amounts of toxic organic solvents, and achieving higher efficiency with a large enrichment factor. CPE is comprised of subsequent steps, including solubilization of analytes in surfactant micelles, followed by heat for clouding, and finally two-phase separation for analysis [27].

CPE was used for the determination of many drugs and metals [28-35]. In the present work, a diazotization reaction of VAN with a drug (dapsone) as a reagent was adopted for sensitive assay of trace amounts of VAN in pharmaceutical forms. The formation of azo-dye was determined spectrophotometrically at λ_{max} 446 nm using both batch and CPE methods (with and without extraction). The proposed CPE method was simple, very sensitive for the accurate assay of trace amounts of VAN in pharmaceutical applications.

The aim of the present work is an attempt to establish a new method for the extraction and preconcentration of VAN drug based on the diazonium and coupling reaction using dapson drug as a chromogenic reagent in pure form and pharmaceutical formulations using CPE followed their determination using spectrophotometry.

Materials and Methods:
Materials and Procedures:
Instruments used:

Measurements of absorbance were performed by a Shimadzu UV-Visible 1240 single-beam digital spectrophotometer, on the other hand, the spectra measurements were performed by a Shimadzu UV-1800 digital double beam spectrophotometer using a quartz cell (1 cm bath length). For cloud point extraction, absorbance measurements, was carried out using a quartz cuvette semi-micro cell "QS" optical path length of 10 mm / 1 ml. For the CPE technique, a Gemmy Industrial Corp., Taiwan, YCW-40 M thermostatic water bath [15-90 °C] model was used. For separation of the two phases, a centrifuge (Hermle labortechnik, Z 200 A Model-Germany) was used with calibrated centrifuge tubes of 10 mL capacity.

Preparation of chemicals and reagents:

All reagents used in this experiment were of analytical grade.

Pure vancomysin hydrochloride (VAN) (purity 99.9%) was kindly donated by the state company for drug industries and medical appliances (SDI-Samarra/Iraq).

VAN-containing pharmaceutical injections were obtained from local pharmacies and analyzed by using the developed methods: Vancolon 1g-Vancomycine hydrochloride/Julphur-U.A.E, Voxin 1g-Vancomycine hydrochloride/Vianex-Greece and vancomycine hydrochloride REIG JOFRE 1g Vancomycine hydrochloride/ REIG JOFRE S.A.-Spain.

Vancomycin standard solution 500 ($\mu g. mL^{-1}$), 50 mg of standard VAN was dissolved in 100 mL distilled water. A concentration of 100 $\mu g. mL^{-1}$ (working standard solutions) was made up by diluting concentrated VAN solution with a suitable volume of distilled water.

Diazotized Dapsone (DDP) (Merck) (2mM), using the equal molar procedure, the DDP was freshly prepared every day by dissolving 0.04966 g of dapsone in 2 mL of (1M) hydrochloric acid and cooling the mixture to 0-5 °C for 5 minutes using an ice bath. A weight of 0.0138 g of sodium nitrite (Merck) was added to the prepared mixture above for 5 minutes while constantly stirring. The volume was completed to 100 mL volumetric flask with cold distilled water. More working solutions (4 x 10^{-4} M) working solutions were prepared by dilution with distilled water.

Hydrochloric acid solution (BDH) (1 M), the diluted solution of acid was prepared by diluting 21.5 mL of concentrated HCl (11.638 M) with distilled water to make the volume up to 250 mL in a volumetric flask.

Sodium hydroxide solution (Merck) (0.1 M), in a 250 mL volumetric flask, 1 g of NaOH was dissolved in distilled water and the volume was completed to the mark with the same solvent.

Triton X-114 (10%) v/v, 10 mL of surfactant (purity> 99.9%, Sigma-Aldrich) was dissolved in a 100 mL volumetric flask with distilled water.

Preparation of pharmaceutical forms solutions

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VAN was obtained in pharmaceutical forms from three different sources; three vials (from each source) had been mixed together. An aliquot corresponding to 50 mg of VAN was dissolved in a mount of distilled water and diluted to the mark of 100 mL volumetric flask with the same solvent, to get 500 µg.mL^{-1} of VAN. Three different concentrations of each pharmaceutical solution were analyzed in three replicates using both spectrophotometric procedures (with and without extraction).

The general procedure for the batch method (without extraction)

An increasing volume of the standard VAN solution was transferred into a series of volumetric flasks (10 mL) to cover the concentration range of the calibration graph (3-50 µg. mL^{-1}), 2 mL of DDP (0.4 mM) and 1 mL NaOH solution (0.1 M) were added gradually to the mixture. The flask contents were diluted to volume using distilled water, shaken well, and stand up for 10 min at room temperature. The absorbance of the sample and the blank were then measured at λ_{max} of 446 nm (the blank containing all components except the VAN).

The general cloud point extraction procedure (CPE)

An aliquot of the standard VAN solutions was transferred into a series of volumetric flasks (10 mL) to cover the concentration range of the calibration graph (0.5–25 µg. mL^{-1}). 1 ml of DDP (0.4 mM), 1.5 ml of NaOH (0.1 M), and 1 ml of Triton X-114 (10%) were added, shaked well, and made up to volume with distilled water. Using a 10 mL centrifuge tube, each mixture formed was transferred and kept in a thermostatic water bath for 20 min at 65 °C. The step of separation of the aqueous and surfactant-rich phases was accelerated by centrifuging the sample for 10 min at 3000 rpm. In an ice bath, the mixture was then cooled for 1 minute to increase the surfactant phase's viscosity and by reversing the tube, the aqueous phase was decanted. The colored product's absorbance was measured at 446 nm after the surfactant phase was dissolved in 1 mL of ethanol.

Results and Discussion:
Absorption spectra

In an alkaline medium, a diazotization-coupling reaction between DDP and VAN drug produced an orange-colored azo dye (Fig. 2) with maximal absorbance at 446 nm compared to the blank. Preconcentration of VAN in pharmaceuticals was carried out using the CPE procedure, which involves the addition of non-ionic surfactant Triton-X114 to reach its cloud point in a thermostatic water bath, followed by phase separation.
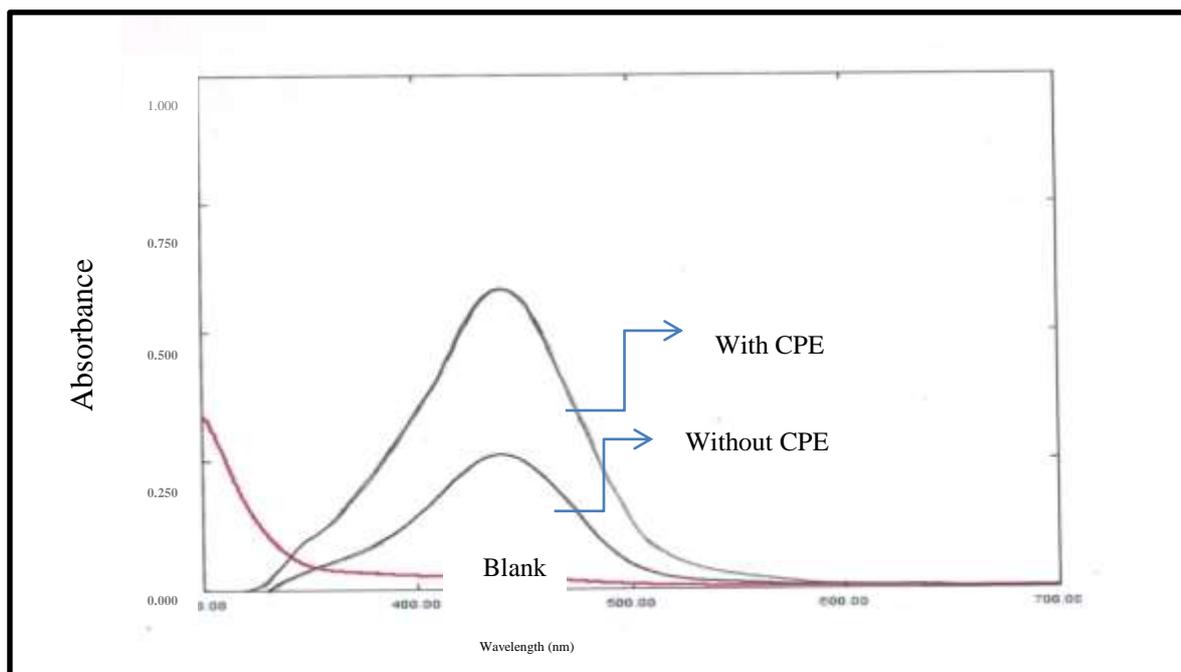

Figure 2. Spectra of absorption of VAN (10 µg.mL^{-1}), the orange product was measured against a reagent blank with and without CPE, and the reagent blank was measured against the distilled water.

The double aromatic amino group in dapsone was diazotized with nitrous acid (NaNO$_2$/HCl) in an ice bath at 0-5 °C and the resulting diazonium salt was coupled with VAN at room temperature. Job's and mole ratio methods [36], were used to investigate the stoichiometry of the

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reaction between VAN and DDP under the recommended optimum conditions. The results showed that a 2:1 (VAN:DPP) ratio product is formed between the drug and DDP at 446 nm (Figs. 3 and 4).

The stability constant (K) of the product formed was evaluated as follows: Equimolar concentrations of both drug and DDP were prepared. Two sets of solutions were prepared, first set of solutions were formulated to contain a twofold excess amount of the drug (DPH) and the second set was formulated to contain tenfold excess of drug and the stability constant (K) was calculated as:

$$\propto = \frac{Am - As}{Am} \qquad k = \frac{1-\propto}{4\propto^3 C^2}$$

Where: $\propto$ is the dissociation constant. Am is the absorbance of the product in the presence of an optimum amount of DDP, as is the absorbance of product in the presence of a stoichiometric amount of DDP and C is the molar concentration of the drug.

Accordingly, to the previous equations it was found that the stability constant of the dye product was 8.9792×10^{12} $L^2.mol^{-2}$ indicate the high stability of the product and the reaction was carried out as described in Scheme 1.
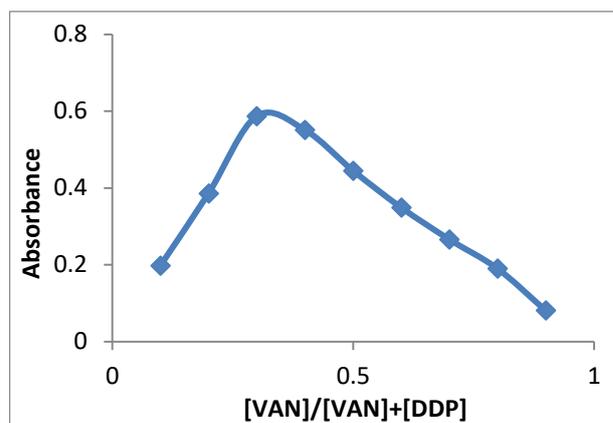

Figure 3. Job's method of continuous variation plot for the reaction of VAN with DPP
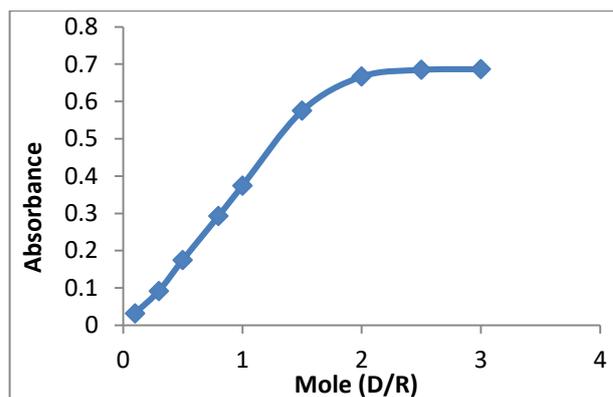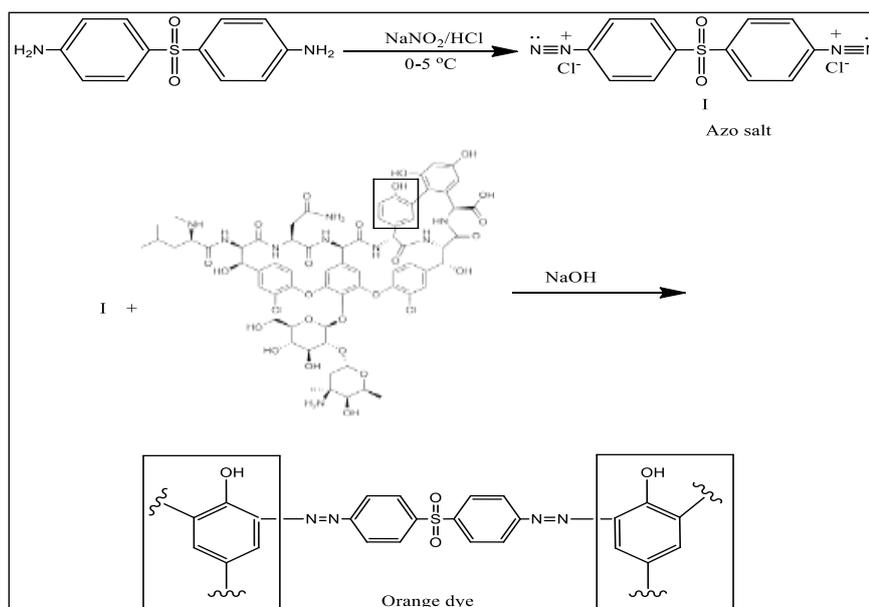

Figure 4. Mole ratio method plot for the reaction of VAN with DPP

Scheme 1. Proposed mechanism of the reaction between VAN and DDP.

Batch and CPE techniques optimization

All factors that affect the sensitivity of the proposed methods (with and without extraction) were evaluated to reach the optimum procedure conditions and increase the sensitivity of the colored product. Various experimental parameters were investigated by changing one variable over time (OVOT), while the others remained constant. In all subsequent experiments. A 100 μg of VAN in 10 mL volumetric flask was employed in all subsequent experiments, with λmax at 446 nm measured against a reagent blank.

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Optimization of stability time, order of addition and Temperature

The stability of the product was studied, and it was found that the color intensity of the product reached its maximum after 10 minutes and stayed up to 45 minutes. The effect of addition order was also investigated, with several addition orders being used and the findings analyzed (Fig. 5), the sequence of addition of reagents (Drug + DDP + Base) was found to give the maximum absorbance. Four different temperatures (15, 25, 55, and 75 ℃) were used to investigate the effect of temperature on the product's color intensity for batch method, the results in Fig. 6 showed that, a high absorbance was obtained when the calibrated flasks were placed at room temperature and a slightly decreases in absorbance when the flasks placed in a water bath (45 ℃) but a loss in intensity was obtained at 75 ℃.
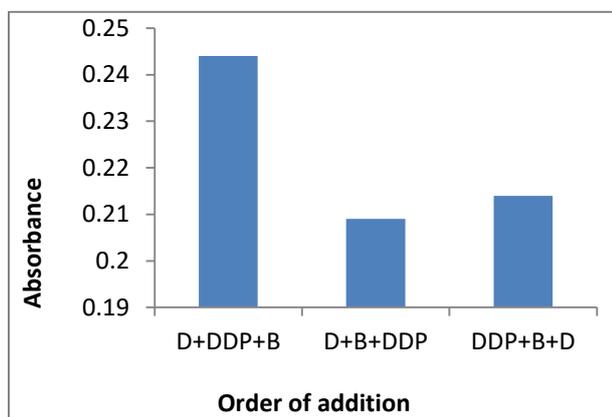

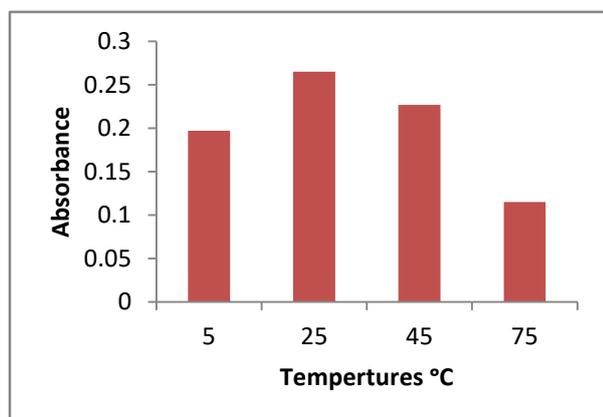**Figure 5. Effect of order of addition for the reaction of VAN with DPP**

Figure 6. Effect of temperatures for the reaction of VAN with DPP

Optimization of chemical parameters
Effect of the volume of NaOH

The reaction medium type has an effect on the formation of the dye because the reaction occurred in a basic medium. Basicity is the most essential parameter affecting the reaction complement and extraction efficiency. Basicity also has an impact on missile formation and extraction effectiveness. Different alkalis such as NaOH, NH₄OH, and Na₂CO₃, were tested to find the type of alkaline medium. Sodium hydroxide has a great effect on the azo-dye formation and was utilized in this investigation. The pH effect was studied by adding different volumes of 0.1M NaOH (0-3 mL) and (0.1-3 mL) for both batch and CPE methods. It was found that 1 mL and 1.5 mL of NaOH (0.1M) gave a maximum absorbance for batch and CPE methods, respectively (Fig.7).
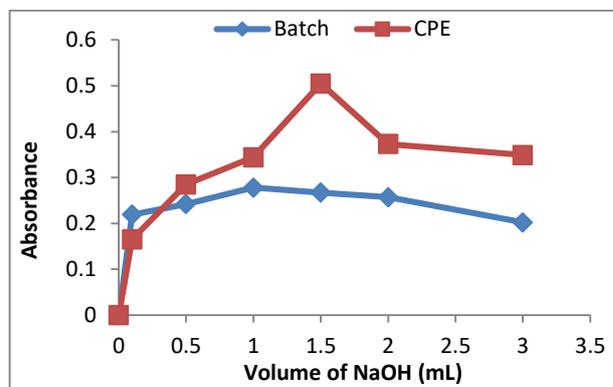

Figure 7. Effect of the Volume of NaOH (0.1 M solution) for the reaction of VAN with DPP

DDP volumes effect

Different volumes effects of DDP were investigated using different volumes (0.1- 4 mL) of DDP (4×10^{-4} M) while keeping other conditions constant. The absorbance increased as the DDP volume increased and reached a maximum at 2 mL and 1 mL for batch and CPE methods respectively, then a decrease in absorbance was observed when increased volumes of DDP were added, the results are shown in Fig. 8.
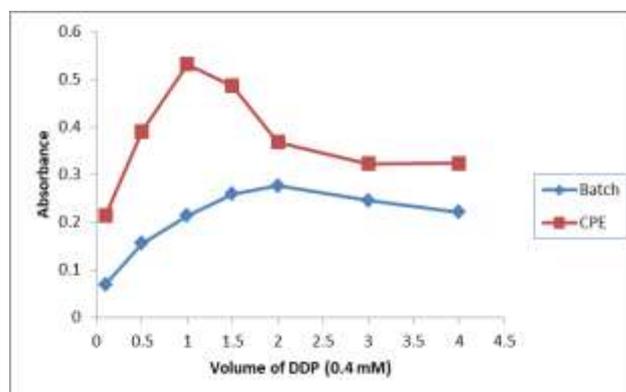

Figure 8. The volume effect of DDP (0.4 mM) for both batch and CPE methods Optimization of the CPE method

Effect of surfactant concentration (Triton X-114)

In this approach, Triton X-114 was used as a CPE surfactant. Due to its essential effect on increasing extraction effectiveness by decreasing the ratio of the phase volume, the concentration of Triton X-114 has a significant impact on the extraction effectiveness under the cloud point extraction method [33]. To study the effect of Triton X-114 concentration on the absorbance of the extracted complex, a range of volumes (0.1-3 mL) of surfactant were used and all results were presented in Fig 9, and because of its high viscosity, Triton X-114 must be utilized under heating at a temperature of about 65 °C.
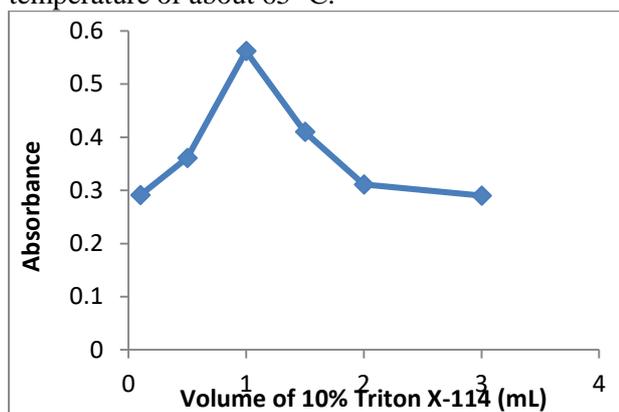
Figure 9. Effect of volume of (10%) Triton X-114 for the reaction of VAN with DPP by CPE method

Effect of temperature and time of heating

Temperature and time of incubation are important factors in assisting surfactant to reach its cloud point and form the viscose rich-surfactant phase and to achieve equilibrium between the two phases for the separation procedure. Temperature and time of heating effect were investigated in the ranges of (25-85°C) and (5-60 min), respectively. The findings indicated (Figs 10 and 11) that the temperature was set to 65°C and the time of heating was set to 20 minutes for maximum absorbance.
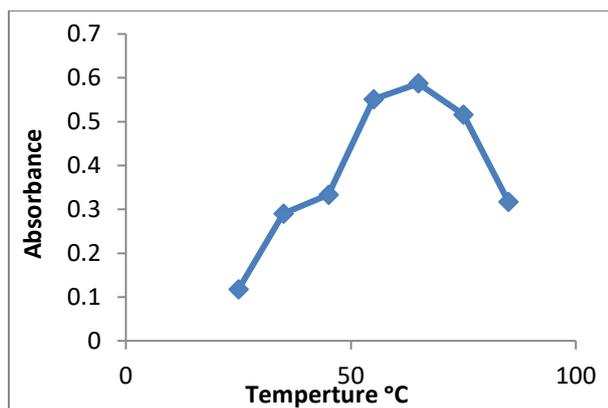
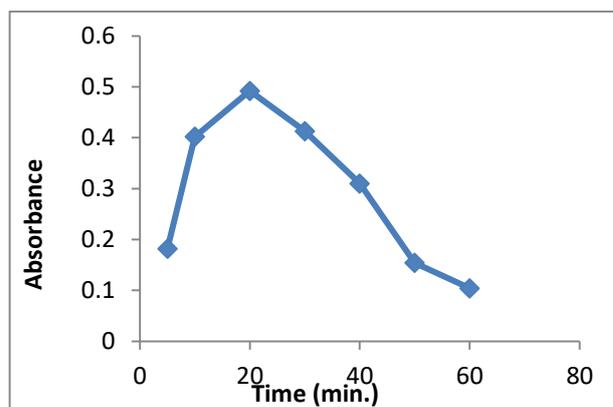**Figure 10. The effect of temperature for the reaction of VAN with DPP by CPE method**

Figure 11. The effect of extraction time for the reaction of VAN with DPP by CPE method

Effect of separation time

Time of centrifugation was investigated in the range of 2-30 min at 3000 rpm (revolutions per minute) for improving the completed separation of two phases, rich-surfactant and aqueous phase. The results obtained are shown in Fig. 12, indicating that the separation was done at 10 min, because this time was sufficient for complete separation between the aqueous phase and surfactant phase, and thus was selected as an optimal parameter. Fig. 13 shows that a centrifuge speed of 3000 rpm was chosen as the best speed since a complete separation was achieved in this period and no substantial improvements were noticed for higher speeds.
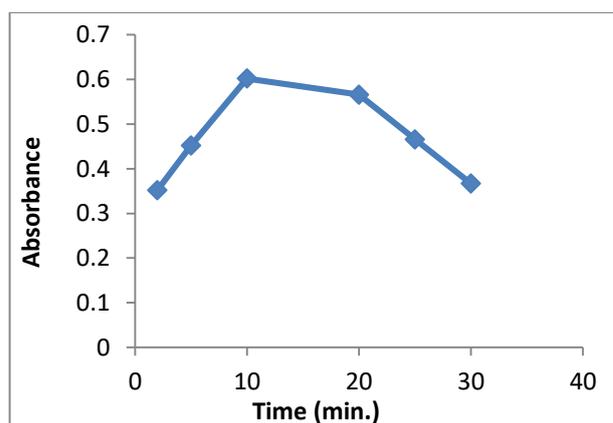
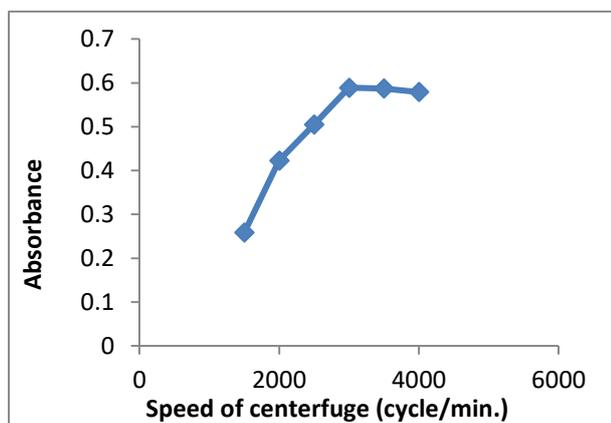**Figure 12. Effect of separation time**

Figure 13. Effect of centrifuge speed

Analytical characteristics

Using the optimized conditions for assay of VAN (with and without extraction). For both procedures, calibration curves were created., The linearity ranges were 3-50 and 0.05-25 µg. mL^{-1} of VAN for both batch and CPE methods (Fig. 14), respectively. The regression equations for both techniques were developed using the least-square method, and the analytical characteristics such as slope, intercept, correlation coefficient, and molar absorptivity values are summarized in Table 1. The sensitivity of both methods was demonstrated by LOD=3SD/b (where SD is the standard deviation of 10 replicates of the blank and b is the slope of the calibration graph) values of 0.803 and 0.214 µg. mL^{-1} for batch and CPE procedures, respectively.

The small values of the standard deviation of the residual ($S_{y/x}$), slope (S_b) and intercept (S_a) are suggested that there was a little scattering of points on the calibration curve and that the current procedures were precise. The enrichment factor (the slope ratio of the analyte's calibration graph with extraction (CPE method) to the slope of the analyte's calibration graph without extraction (batch method)) was calculated to be 2, while the preconcentration factor (the ratio of the volume of the aqueous solution to the volume of the surfactant-rich phase) was calculated to be 20.
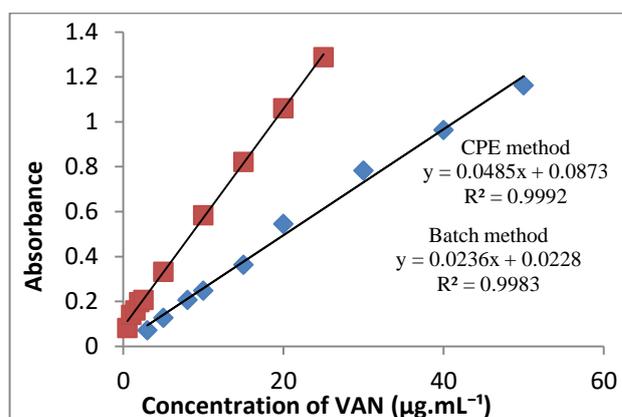

Figure 14. Calibration graphs of batch and CPE methods

Table 1. The statistical data of VAN determination by batch and CEP methods.

Parameter	Value	
	Batch	**CPE**
Regression equation	y = 0.0236x+0.0228	y = 0.0485x+0.0873
Correlation coefficient, r	0.9991	0.9996
Linearity percentage, % R^2	99.83	99.92
Dynamic range (µg.mL^{-1})	3-50	0.5-25
Molar absorptivity, ε (L.mol^{-1} cm^{-1})	3.5069×10^4	7.2071×10^4
Sandell sensitivity (µg.cm^{-2})	0.0423	0.0206
Slope, b (mL.µg^{-1})	0.0236	0.0485
Intercept, a	0.0228	0.0873
$S_{y/x}$	0.0328	0.0132
S_b	0.0007	0.0004
S_a	0.042	0.012
Detection limit (µg.mL^{-1})	0.803	0.214
Quantitation limit (µg.mL^{-1})	2.679	0.715
Preconcentration factor	-	20
Enrichment factor	-	2

Accuracy and precision

The precision test was done for three different concentrations of VAN solutions assessed by two suggested procedures (batch and CPE) in three replicates to investigate the reliability and accuracy of the offered methods. The acceptable relative error and relative standard deviation values provided in (Table 2) demonstrated that the proposed approaches were accurate and repeatable.

Table 2. Values of accuracy and precision for suggested methods.

Suggested methods	Conc. µg.mL^{-1}		E%	Rec.%	R.S.D.%
	Present value	Establish value*			
Batch method	5	5.064	+1.280	101.280	2.548
	15	14.965	-0.233	99.766	1.022
	40	41.180	+2.950	102.950	1.476
CPE	1.5	1.512	+0.800	100.800	2.837
method	5	5.086	+1.720	101.720	2.079
	20	20.027	+0.135	100.135	0.516

* Mean of three determinations

Analytical applications for the developed methods

The suggested methods were applied for the extraction and spectrophotometric analysis of vancomycin in pharmaceutical injections, the efficiency and practicality of the method were evaluated. Three concentration levels of three types of injections were analyzed according to the optimum procedures. The relative recoveries for each injection were estimated as the average of three determinations (Table 3). Statistical results using student's t and F-tests [37], were compared between the findings produced by the recommended procedures and those obtained by UV method [1]. The findings show that the two methods (calculated t- and F-values vs. theoretical t- and F-values) have no significant differences in precision and accuracy (Table 4).

Table 4. The suggested methods applications on the analysis of VAN in injection pharmaceutical types.

Injections	developed methods	Conc. µg.mL^{-1}		E. %	Rec. %	R.S.D. %
		Present value	Establish value*			
Vancolon		5	5.064	+1.289	101.286	2.031
1g	Batch	15	15.036	+0.240	100.240	1.137
Vancomycin		40	39.584	-1.040	98.960	1.843
hydrochloride		1.5	1.4711	-1.927	98.073	1.524
Julphar-U.A.E,	CPE	5	5.196	+3.920	103.920	0.598
		20	20.295	+1.475	101.475	0.391
Voxin		5	5.157	+3.140	103.140	3.985
1g	Batch	15	15.152	+1.011	101.011	1.645
Vancomycin		40	40.077	+0.192	100.192	0.750
hydrochloride		1.5	1.567	+4.466	104.466	3.845
Vianex-Greece	CPE	5	5.113	+2.260	102.260	2.204
		20	20.467	+2.335	102.335	1.122
Vancomycin		5	5.135	+2.710	102.710	2.459
hydrochloride 1g	Batch	15	14.863	-0.980	99.020	2.731
REIG JOFRE S.A.-		40	39.683	-0.792	99.208	0.728
Spain.		1.5	1.560	+4.000	104.000	3.452
	CPE	5	5.127	+2.540	102.540	1.752
		20	20.378	+1.890	+101.890	0.421

* Three measurements mean of each developed method.

Table 5. The evaluation between the developed method and the standard method

Injection preparations	developed methods						Standard method
	Batch			CPE			
	Rec.%	t*	F*	Rec.%	t	F	Rec.%
Pure VAN	101.332			100.885			99.980
Vancolon	100.162			101.156			101.350
Voxin	101.446	0.356	4.268	103.020	1.602	1.541	98.870
Vancomycin hydrochloride	100.313			102.810			101.93

* 95% confidence limit (theoretical values) , n_1 and n_2 are equaled to 4,
t = 2.132 where, t represented; n_1+n_2-2, the degrees of freedom = 6
F = 9.277 where, F represented; n_1-1, n_2-1, the degrees of freedom = 3

Conclusions:

In comparison to previously reported methods of VAN, a green, simple, and highly sensitive CPE and spectrophotometric methods for determining vancomycin HCl drug in pure and injection forms has been proposed, with non-ionic

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surfactant used for the enrichment of extraction of VAN drug. These suggested methods are considered as a new reliable and highly sensitive method with no toxic solvents used. Its analytical features indicate good reproducibility, wider dynamic range and lower detection limit, successfully applied for estimation of VAN drug in its pharmaceutical injections.

Authors' declaration:
- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' contributions statement:

H. S. A. design, acquisition of data, analysis, drafting the Manuscript. M. Q. A. drafting the Manuscript, analysis, revision and proofreading. M. R. A. drafting the Manuscript, revision and proofreading.

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التقدير الطيفي الحساس لهيدروكلوريد الفانكومايسين بصورته النقية وفي الحقن الصيدلانية بوساطة طريقتي الدفعة والاستخلاص بنقطة الغيمة

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الخلاصة:

تم تطوير تفاعل بسيط وحساس لتقدير هيدروكلوريد الفانكومايسين باستخدام طرائق الدفعة واستخلاص نقطة الغيمة (CPE) وتعتمد الطريقة الأولى على تكوين صبغة الآزو الناتجة عن تفاعل اقتران الدابسون المؤزوت مع هيدروكلوريد الفانكومايسين في الوسط القاعدي وتم تطوير حساسية هذا التفاعل باستخدام المادة الفعالة غير الأيونية (Triton X-114) وبتقنية نقطة الغيمة. تمت إذابة صبغة الآزو المستخلصة في الطور الغني بمادة الشد السطحي في الإيثانول وقياسها طيفيًا عند الطول الموجي الاعظم 446 نانومتر. تم تقدير التفاعل باستخدام كل من الطرائق الدفعة وطريقة CPE (أي مع الاستخلاص وبدونه) وتم إجراء مقارنة بسيطة بين الطريقتين. وقد درست جميع الظروف الكيميائية والفيزيائية لطريقتي الدُفعة والاستخلاص بنقطة الغيمة بعناية في ظل الظروف المثلى ، كانت مديات الخطية من 3 إلى 50 و 25-0.5 ميكروغرام. مل[-1] لهيدروكلوريد الفانكومايسين بينما كانت حدود الكشف 0.806 و 0.462 ميكروغرام.مل[-1] لطريقتي الدفعة و التحليل بالاستخلاص بنقطة الغيمة على التوالي. تمت مقارنة قيم الاستعادية التي تم الحصول عليها مع تلك التي تم الحصول عليها من تطبيق طريقة الأشعة فوق البنفسجية. استخدمت التقنيات المقترحة بفعالية عالية في تقدير الفانكومايسين في الحقن الصيدلانية.

الكلمات المفتاحية: استخلاص نقطة الغيمة، دابسون، تفاعل ازوتة وازدواج، المقياس الطيفي، هيدروكلوريد الفانكومايسين .