Original Article

Stability-indicating spectrofluorimetric method with enhanced sensitivity for determination of vancomycin hydrochloride in pharmaceuticals and spiked human plasma: Application to degradation kinetics

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ABSTRACT

Based on investigating the relative fluorescence intensity of vancomycin hydrochloride (VCM) in methanol, a simple, highly sensitive, time-saving and specific spectrofluorimetric method was developed and validated. VCM fluorescence was measured at 335 nm when excited at 268 nm. Excellent linearity is obeyed in the concentration range 1–100 ng/mL with a detection limit of 5.94 pg/mL, a quantitation limit of 18.03 pg/mL and a very good correlation coefficient (r = 0.9999). Our method was applied to analyze VCM in pharmaceuticals as well as spiked human plasma. Moreover, VCM stability was studied when exposed to various degradation conditions such as oxidative, alkaline as well as acidic stress. Acidic and alkaline degradation kinetics of VCM was studied for the first time. The degradation follows pseudo-first-order kinetics. The apparent rate constants and half-life times were calculated. The Arrhenius equation was assessed and the activation energies of the degradation were also calculated. The developed method can be easily applied in quality control laboratories due to its sensitivity, specificity, simplicity and low cost.

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1. Introduction

Vancomycin hydrochloride (VCM, Fig. 1) is a glycopeptide antimicrobial agent [1] with a primarily bactericidal action against different species of Gram-positive bacteria. It is used to treat dangerous staphylococcal and some Gram-positive infections when other antibacterial can not be used due to patient intolerance or resistance. It is considered a drug of last resort, when treatment with other antibacterial is inefficient. This is because vancomycin can not be absorbed orally. It must be given intravenously because of the highly polar groups in its structure [1]. The literature revealed some different spectrophotometry [2–4], HPLC [5–10], Mass Spectrometry [11,12], fluorescence polarization immunoassay (FPIA) [13] and radioimmunoassay [14] methods for determination of VCM in different matrices.

As far as we know, VCM in its bulk powder or dosage forms has not been previously estimated by fluorimetric technique. Recently, fluorescence measurement is extensively applied in forensics, genetic analysis and biotechnology. Spectrofluorimetry was widely applied in different fields of pharmaceutical analysis due to its high sensitivity and selectivity. Furthermore, spectrofluorimetry has a lot of merits over other different analytical techniques. Overall, it's known inherent sensitivity, less time consumption and economical property on comparison with other techniques like ultraviolet (UV) spectrophotometry, HPLC, LC/MS or other hyphenated techniques. So that, we are promoted to study the use of native fluorescence of VCM to develop a highly sensitive fluorimetric method for VCM analysis in bulk, pharmaceuticals and spiked human plasma. VCM stability was studied when exposed to various degradation stress such as oxidative, alkaline as well as acidic stress. Acidic and alkaline degradation kinetics of VCM was studied for the first time.

2. Experimental

2.1. Instrumentation

Fluorescence measurements were made on Perkinelmer Ensight Instrument. A Docu pH-meter (Sartorius, USA) was used for pH measurement. A vortex (Scientific industries, INC, MODEL NO. SI-0286), and an ultrasonic bath (S 100 H, Elmasonic, Germany) were used.

2.2. Material and reagents

Analytical Reagent Grade chemicals and HPLC grade solvents were used. Vancomycin hydrochloride (VCM) pure sample, methanol, ethanol, acetonitrile, 2-propanol, (HPLC grade), hydrochloric acid (HCl), sodium dodecyl sulphate (SDS), methyl-β-cyclodextrin, tween 80, hydrogen peroxide (H_2O_2, 30%, w/v), sodium hydroxide (NaOH), sodium acetate
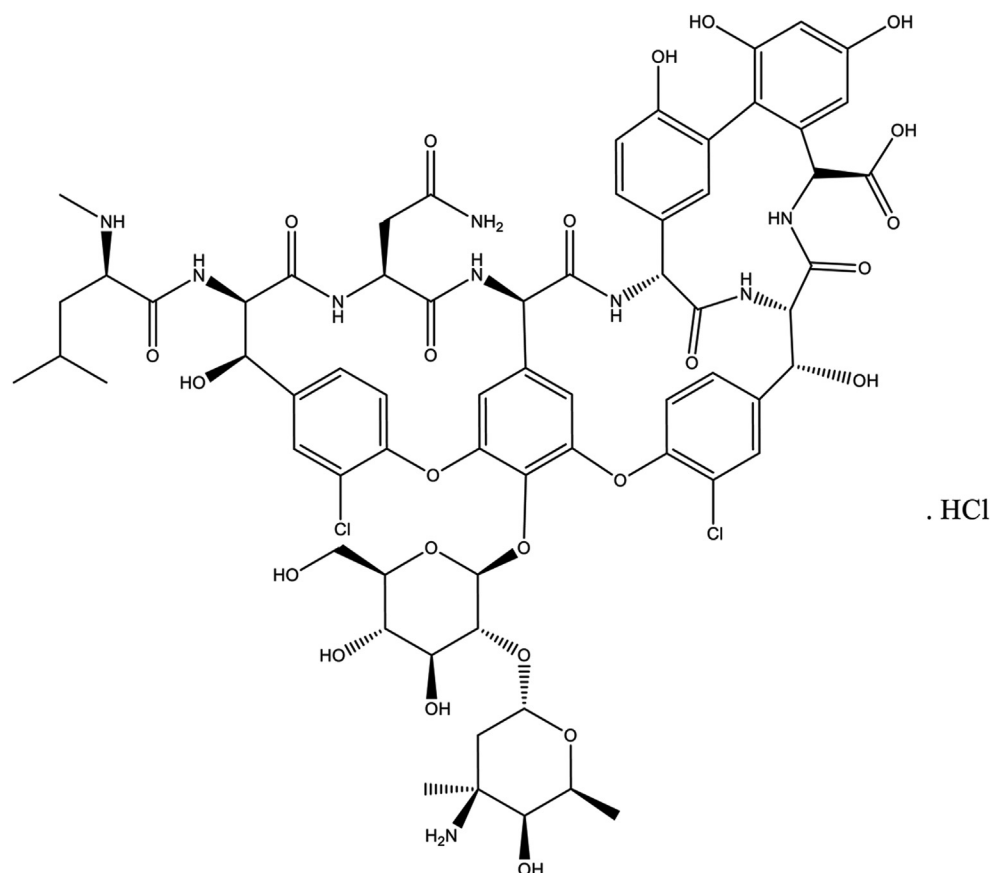

Fig. 1 – Structural formula of vancomycin hydrochloride.

trihydrate, acetic acid 96%, boric acid and sulfuric acid (H_2SO_4) were purchased from Wako Pure Chemical industries (Osaka, Japan). Vancomycine® vial formulation (batch no. #156704) labeled as containing 500 mg vancomycin hydrochloride, product of Vianex S.A., plant C-16th km Marathons Avenue 153 51 Pallini Attiki-Athens-Grece, was purchased from Egyptian pharmacies. Plasma samples were obtained from Biopredic International Company (France) and kept frozen at −20 °C until use.

2.3. Standard solution

Stock solution (100 μg/mL) of VCM was prepared in methanol. To prepare working solutions, further dilution of the stock solution was made with methanol. VCM solutions were protected from light by covering with aluminum foil throughout the work [15]. The solution was stored at 4 °C in a refrigerator and was stable for minimum 7 days.

2.4. Procedure

2.4.1. Construction of calibration curve
Aliquots of VCM working solution over the range of 1.0–100.0 ng/mL were transferred to a set of volumetric flasks and completed with methanol. The RFI was measured at 335 nm when excited at 268 nm against a solvent blank. The RFI was plotted *versus* the final drug concentration (ng/mL) to construct a calibration curve and then the corresponding regression equation was derived.

2.4.2. Analysis of VCM in raw material
Aliquots of VCM working solution over the whole linearity range (1.0–100.0 ng/mL) were transferred to a set of volumetric flasks. Procedure in 'Construction of calibration curve' section was then followed. Percentages found were then calculated from the regression equation.

2.4.3. Analysis of VCM in Vancomycine® vial
An accurate weight (100.0 mg) VCM from Vancomycine® vial was transferred to 100-mL volumetric flask, dissolved in methanol to get stock solution (1.0 mg/mL), sonicated for 30 min and then filtered. Aliquots of the solutions in the range of 1.0–100.0 ng/mL were transferred into a set of 10 mL volumetric flasks. Procedure in 'Construction of calibration curve' section was followed and percentages found were calculated using the corresponding regression equation.

2.4.4. Analysis of VCM in spiked human plasma
To a set of centrifugation tubes, accurately measured volumes of human plasma (0.5 mL) were transferred and then spiked with different aliquots of VCM stock solution. The solutions were mixed with vortex for 5 min, completed to 5.0 mL with methanol for complete protein precipitation and then centrifuged at 2500 rpm for 20 min. The upper layers were aspirated and filtered. After that, accurate volumes (constant volume of 1.0 mL) of the filtrate were transferred to a set of 10 mL volumetric flasks and completed with methanol to obtain final concentrations in the range of 10.0–50.0 ng/mL. A blank plasma experiment was prepared simultaneously. Procedure in 'Construction of calibration curve' section was applied.

Alternatively, the corresponding regression equation was derived.

All the experimental procedures involving biological fluids were performed in compliance with the relevant laws and institutional guidelines, with the approval from Kyushu University Institutional Review Board.

2.4.5. Procedure for the stability studies
For both acidic and alkaline degradation: Aliquots of VCM methanolic stock solutions were transferred to a set of screw-capped tubes. Then an accurate volume (4.0 mL) of NaOH or HCl (0.5 M). The tubes were heated at (60, 70, 80 or 90 °C) for different times (10, 20, 30, 40, 50 or 60 min). At the specified time, solutions were cooled, neutralized and then transferred into a series of volumetric flasks. Procedure in 'Construction of the calibration curve' section was then followed.

For oxidative degradation: Aliquots of VCM methanolic stock solution were transferred to a series of screw-capped glass tubes; different volumes of (0.5% w/v) of H_2O_2 were added. The fluorescence was then measured at room temperature. Procedure in 'Construction of calibration curve' section was then applied.

3. Results and discussion

VCM exhibits a native fluorescence in methanol at 335 nm when excited at 268 nm as indicated in Fig. 2. The study aimed to enhance the fluorescence intensity, for developing a highly sensitive method for VCM analysis in pure form, pharmaceuticals and spiked human plasma. So, different experimental parameters were investigated, including: the pH, different organized media and type of diluting solvents.

3.1. Optimization of the experimental conditions

3.1.1. The influence of different buffers at different pH
For studying the influence of different buffer solutions at different pH on the RFI of VCM, different buffer systems were used, 0.2 M acetate buffer (pH range 3.6–5.6) and 0.2 M borate buffer (pH range 6.5–9.5). The use of buffer solutions was observed to decrease the fluorescence and maximum RFI was achieved without using buffers as shown in Fig. 3. So, the study was completed without buffer.

3.1.2. The influence of organized media
The possibility of further enhancing of VCM fluorescence by different organized media was also examined using anionic surfactant (SDS), nonionic surfactant (Tween 80) and a macromolecule, methyl-β-cyclodextrin. But the use of organized media has a negligible or even a negative effect on RFI of VCM (Fig. 4). Therefore, no organized medium was included in this study.

3.1.3. The influence of diluting solvent
Upon dilution with different solvents like ethanol, methanol, water, acetonitrile, 0.2 M acetic acid, 0.2 M H_2SO_4, 2-propanol and 0.2 M NaOH, methanol was observed to give the highest RFI value (Fig. 5). So, methanol was the diluting solvent of choice during the work.

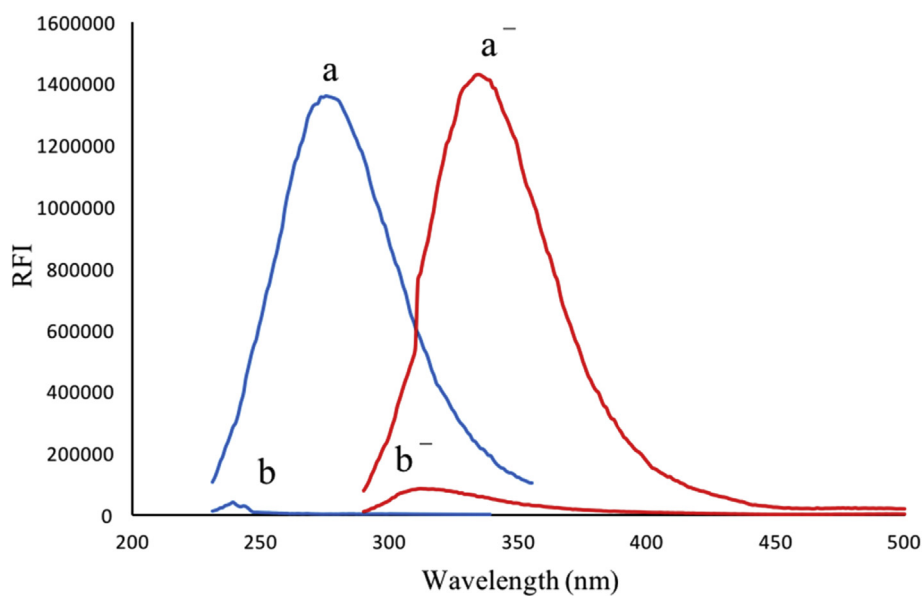Fig. 2 – Fluorescence spectra: excitation and emission spectra of (a and a⁻) VCM (50 ng/mL), while (b and b⁻) blank.
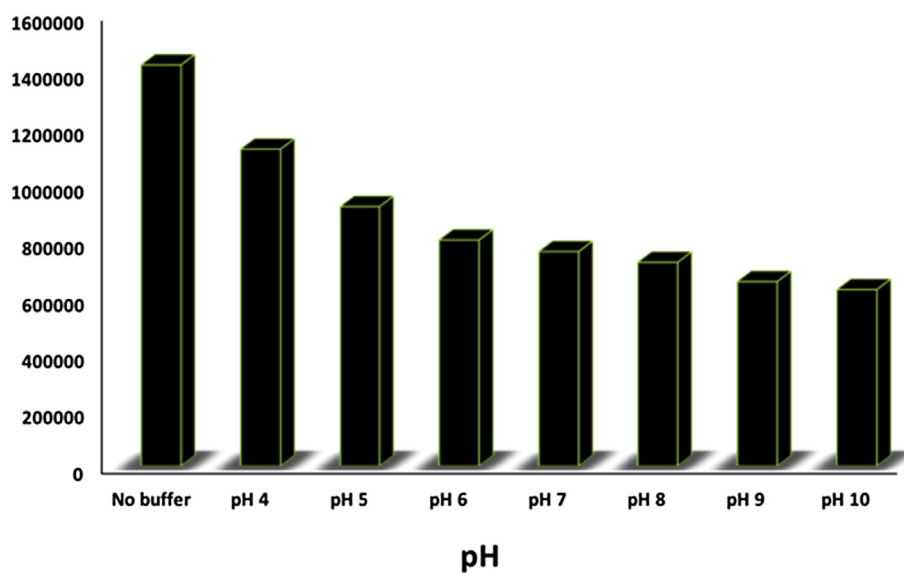

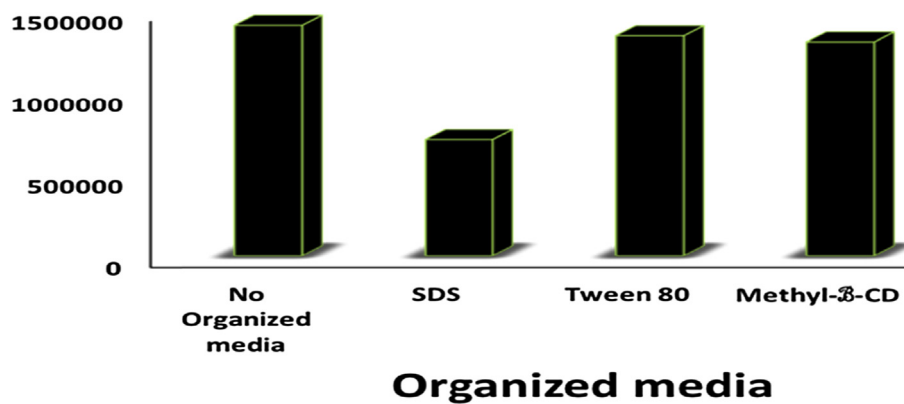Fig. 3 – Effect of different pH on RFI of VCM (50 ng/mL).

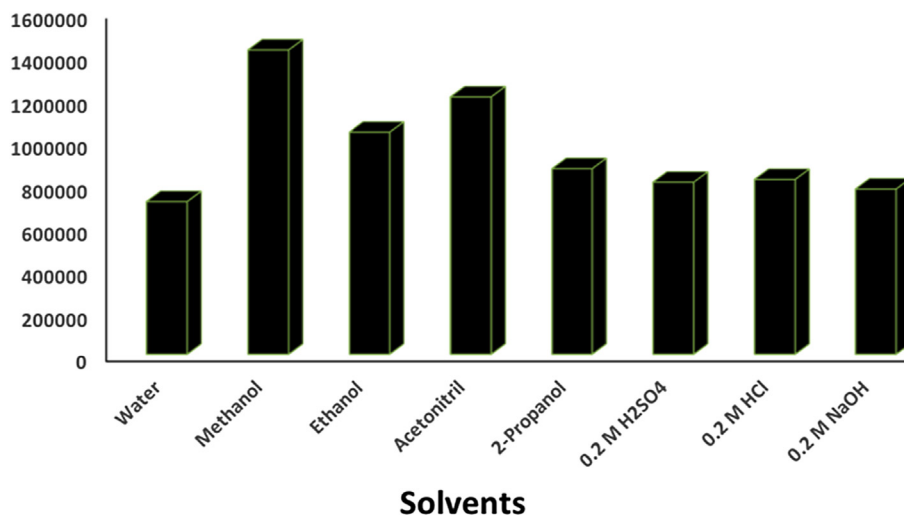Fig. 4 – Effect of different organized media on RFI of VCM (50 ng/mL).

Fig. 5 – Effect of different diluting solvents on RFI of VCM (50 ng/mL).

3.2. Validation of the method

Our developed spectrofluorimetric method was fully validated following (ICH) Q2 (R1) guidelines [16]. Validation parameters including linearity and range, limit of detection (LOD) and limit of quantitation (LOQ), precision, accuracy, and specificity were studied.

Calibration curve was rectilinear over the concentration ranges 1.0–100.0 ng/mL. The intercept, slope and correlation coefficient were also calculated. The intercept was of low value and the correlation coefficient ($r = 0.9999$) was high which prove the excellent linearity of the calibration curve. Regression data summarized in Table 1. Furthermore, LOD and LOQ were calculated (Table 1) following the ICH [16] guidelines using the equations, (LOD = 3.3 Sa/b) and (LOQ = 10 Sa/b). Where Sa is the standard deviation of the intercept and b is slope of the calibration curve.

Our method was applied to analyze VCM in pure samples over the cited concentration range (Table 1) for the evaluation of its accuracy. No significant differences between the performance of both developed and comparison method was detected after statistical evaluation of the results of both methods [2] using the Student t-test and the variance ratio F-test [17], Table 2.

Repeatability (intra-day) and intermediate precision (inter-day) were tested at three different concentrations of VCM in triplicate in one day and in three successive days, respectively. % RSD values were less than 2% (Table 3). This proves the acceptable precision of the developed method.

Excipients and additives in VCM dosage form did not show any interference with our method which confirms the method specificity for VCM analysis in pharmaceuticals (Table 4).

3.3. Applications

3.3.1. Pharmaceutical application

Our method is simple, selective and time-saving. So that, it was applied to VCM analysis in Vancomycine® vial. The

Table 1 – Performance data for the determination of VCM using the proposed method.

Parameter	VCM
Linearity range (ng/mL)	1.0–100.0
Intercept (a)	1.3×10^6
Slope (b)	1.5×10^3
Correlation coefficient (r)	0.9999
SD of residuals ($S_{y/x}$)	57.31
SD of intercept (S_a)	2.78
SD of slope (S_b)	0.61
SD	0.81
% RSD	0.81
% Error	0.33
LOD (pg/mL)	5.94
LOQ (pg/mL)	18.03

Table 2 – Comparison of assay results for the determination of VCM in pure form using the proposed and reference methods.

Parameter	Proposed method			Comparison method [2]
	Conc. taken (ng/mL)	Conc. found (ng/mL)	% Found[a]	% Found[a]
VCM	1.0	0.992	99.20	99.24
	5.0	4.974	99.48	100.32
	10.0	10.069	100.69	99.80
	50.0	50.300	100.60	
	80.0	79.103	98.88	
	100.0	100.56	100.56	
Mean			99.90	99.79
SD			0.81	0.54
t-test			0.22 (2.364)[b]	
F-test			2.23 (19.296)[b]	

[a] Each result is the mean of three individual determinations.
[b] Values between parentheses are the tabulated t and F values at $P = 0.05$.

Table 3 — Precision data for the determination of VCM using the proposed method.

Parameter		VCM concentration (ng/mL)		
		1.0	50.0	100.0
Intra-day	Mean	99.42	100.25	100.17
	±SD	0.74	1.75	0.56
	% RSD	0.75	1.75	0.56
	% Error	0.43	1.01	0.32
Inter-day	Mean	99.60	99.39	99.74
	±SD	0.51	0.41	0.77
	% RSD	0.51	0.41	0.78
	% Error	0.29	0.24	0.45

Table 5 — Assay results for the determination of VCM in spiked human plasma.

Parameter	Proposed method		
	Conc. taken (ng/mL)	Conc. found (ng/mL)	% Found[a]
Spiked plasma	10.0	9.338	93.38
	30.0	30.882	102.94
	50.0	49.510	99.02
Mean ± SD			98.45 ± 4.806
% RSD			4.882
% Error			2.819

[a] Each result is the mean of three individual determinations.

results of the proposed method were statistically compared with those of the comparison method [2] (Table 4) using Student's *t*-test and variance ratio *F*-test [17]. No significant difference between the performance of the two methods was observed.

3.3.2. *Preliminary* in vitro *application*

Owing to the high selectivity, sensitivity, simplicity and rapidness of our method, the method was applied to determine VCM in spiked human plasma using a simple method using methanol avoiding the tedious multi-step extraction procedure. The results obtained were satisfactory as shown in Table 5.

3.3.2.1. *Validation of the proposed method for the determination of VCM in spiked human plasma.* **Linearity, range, LOD and LOQ**: The RFI of VCM in spiked plasma was plotted against its concentration (ng/mL) to obtain the calibration curve. A rectilinear relationship was confirmed over the concentration range 10.0—50.0 ng/mL. The following equation resulted from linear regression analysis of the data:

$$RFI = 447.39\,C + (131.4 \times 10^4) \quad (r = 0.99745)$$

where C is VCM concentration in ng/mL, *r* is the correlation coefficient.

LOD and LOQ for VCM analysis in human plasma were also calculated according to ICH guidelines and found to be 0.879 and 2.664 ng/mL, respectively.

Accuracy: The accuracy of the developed method was evaluated by analyzing plasma samples spiked with different concentrations of VCM. The mean % recoveries of VCM in

Table 6 — Precision data of the developed method for VCM analysis in spiked human plasma.

Parameter		VCM concentration (ng/mL)		
		10.0	30.0	50.0
Intra-day	Mean	98.02	99.90	100.43
	±SD	0.33	0.86	1.27
	% RSD	0.33	0.86	1.27
	% Error	0.19	0.49	0.73
Inter-day	Mean	97.53	100.72	99.29
	±SD	0.60	1.45	0.92
	% RSD	0.61	1.44	0.93
	% Error	0.35	0.35	0.54

spiked human plasma samples based on the average of three independent determinations were 98.45 ± 4.806% at 350 nm (Table 5).

Precision: Intra-day and inter-day precision were evaluated through replicate analysis of plasma samples spiked with VCM at three times within the same day and on three successive days. The small values of % RSD and % error proved the high precision of the developed method for the determination of VCM in plasma (Table 6).

Specificity: The specificity of the developed method for the determination of VCM in human plasma was proved by the absence of any significant interference from plasma matrix and endogenous components at the wavelength selected for analysis of VCM (335 nm).

Table 4 — Assay results for the determination of VCM in Vial dosage form.

Parameter	Proposed method			Comparison method [2]
	Conc. taken (ng/mL)	Conc. found (ng/mL)	% Found[a]	% Found[a]
Vancomycine® vial	1.0	1.013	101.30	99.04
	50.0	49.973	99.95	99.51
	100.0	100.013	100.01	101.94
Mean			100.42	100.16
SD			0.76	1.55
t-test			0.256 (2.776)[b]	
F-test			4.164 (19.0)[b]	

[a] Each result is the mean of three individual determinations.
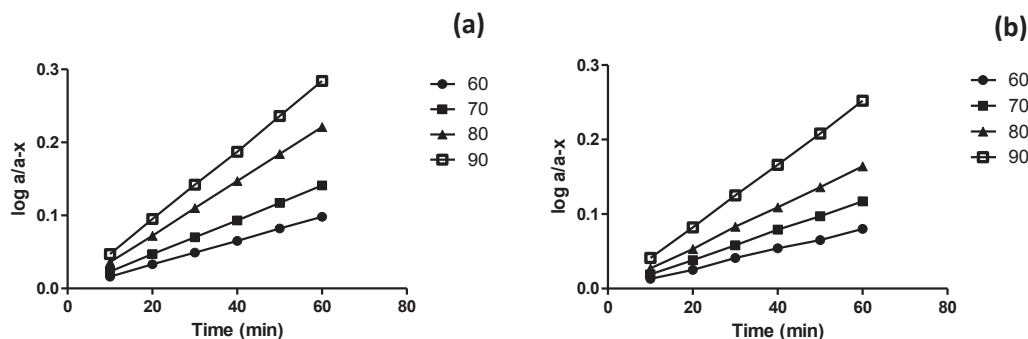[b] Values between parentheses are the tabulated t and F values at P = 0.05.

Fig. 6 – Semi-logarithmic plot of VCM (100 ng/mL) *versus* different heating times with (a) 0.5 M NaOH, (b) 0.5 M HCl at different temperature setting.

3.3.3. Stability study of VCM

Different stress conditions including acidic, alkaline and oxidative degradation were studied. VCM was liable to acidic and alkaline degradation upon heating with 0.5 M HCl and 0.5 M NaOH solutions and a consequent decrease of the RFI was observed. Additionally, it is liable to oxidative degradation (0.5% v/v, H_2O_2) at room temperature resulting in nearly 83% degradation after 10 min. Moreover, reaction of VCM solution with 3% H_2O_2 at room temperature or heating at different temperatures resulted in a complete degradation of VCM. Hence, the degradation was too fast to obtain reliable kinetic results.

3.3.4. Degradation kinetics

The kinetics of both acidic and alkaline degradation of VCM was studied using 0.5 M HCl and NaOH at various temperatures (60, 70, 80 and 90 °C). The concentration of VCM was regularly decreased with increasing time intervals. The degradation was observed to follow pseudo-first-order kinetics (Fig. 6a and b). The apparent rate constants (K) and half-life times ($t_{1/2}$) were calculated (Table 7) from the following equation:

$$Kt = 2.303 \log (a/a - x)$$

where a, a-x are VCM concentrations at zero time and at a given time t, respectively.

Rate constants were then subjected to fitting in Arrhenius equation:

$$\text{Log K} = \log A - Ea/2.303 RT$$

where A is the frequency factor, Ea is the activation energy (Kcal mol^{-1}), R is the gas constant (1.987 cal/K mol) and T is the absolute temperature.

An Arrhenius plot was constructed by plotting the log of the rates of degradation (log K) against the reciprocals of the absolute temperature (1/T) as indicated in Fig. 7. Activation energies for both alkaline and acidic degradation were then calculated and the results are summarized in Table 7.

4.   Conclusion

The developed method is a highly sensitive, time-saving and inexpensive analytical method for VCM analysis in bulk, pharmaceuticals and spiked human plasma in nano-concentration range, owing to its high sensitivity and selectivity. Moreover, the method was applied in VCM stability studies as per the ICH guidelines and the kinetics of both

| Table 7 – Pseudo-first-order rate constants (k) and half-life times ($t_{1/2}$) for alkaline and acidic degradation of VCM at different temperatures.			
	Temperature (°C)	Reaction rate constant, K (min^{-1})	Half-life time, $t_{1/2}$ (h)
0.5 M NaOH	60	0.0038	3.04
	70	0.0054	2.13
	80	0.0085	1.36
	90	0.0109	1.06
Activation Energy E_a (Kcal mole^{-1})	7.13		
0.5 M HCl	60	0.0031	3.73
	70	0.0046	2.56
	80	0.0063	1.83
	90	0.0096	1.20
Activation Energy E_a (Kcal mole^{-1})	7.69		
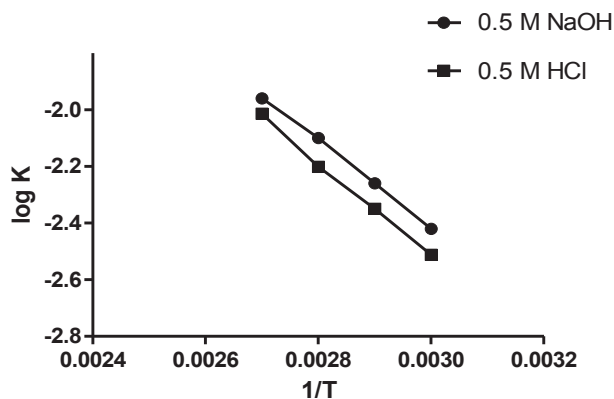
Fig. 7 – Arrhenius plot for the alkaline and acidic degradation of VCM (100.0 ng/mL) with 0.5 M NaOH and 0.5 M HCl.

acidic and alkaline degradation of VCM was studied for the first time. The degradation process followed pseudo-first-order kinetics. The apparent rate constants and half-life times were calculated. The Arrhenius equation was assessed and the activation energies of the degradation were also calculated. The high sensitivity, analysis speed and selectivity are the substantial advantages of direct spectrofluorimetry in comparison with the reported chromatographic techniques for VCM determination. These advantages make the developed method convenient for routine quality control in the pharmaceutical industry.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgment

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