Research article

Stability indicating liquid chromatography method for the analysis of Vecuronium bromide: study of the degradation profile

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ABSTRACT

Neuromuscular blocker agent namely; Vecuronium bromide (VEC) was quantified through developing a simple reversed phase liquid chromatographic (RP-LC) method, in drug substance and in drug product. The proposed method could quantify VEC in the presence of its degradation products produced from exposing VEC to different stress conditions as recommended by the International Conference on harmonization (ICH) guidelines. Acidic (2M HCl), basic (2MNaOH) hydrolysis, oxidation (3% H_2O_2), photolysis (UV light at 254nm), and thermal (135 °C) degradation were estimated by exposing the drug substances to different stress conditions. The separation of the drug from its degradation products was successfully conducted on Tracer Extrasil CN (150 × 4.6mm; 5μm) column using O-phosphoric acid (pH6; 0.05M)-acetonitrile (50:50v/v) as mobile phase. The detection and quantification was done with UV detection at210nm.The validation data were found to be acceptable over a concentration range10-120 μg/ml. The limit of quantification (LOQ) and detection (LOD) were 8.10 and 2.67 μg/ml, respectively. The proposed method met all criteria for validation in accordance with the International Conference on harmonization (ICH) guidelines. The presented work monitored the degradation profile for VEC under various stress conditions and provided a simple LC method for its routine analysis. The structures of the forced degradation products had been described in details using the MS data and the possible degradation pathways were outlined. Besides, the results obtained from the developed method compared statistically with that of the official method indicting high accuracy and precision.

1. Introduction

Vecuronium bromide (VEC) 1-[(2β, 3α, 5α, 16β, 17β)-3, 17-Bis(acetyloxy)-2-(1-piperidinyl)androstan-16-yl]-1-methylpiperidinium bromide Figure 1 [1], is an aminosteriodal competitive neuromuscular blocking agent. It was used in general anesthesia as it causes muscle relaxation facilitating endotracheal intubation and to aid controlled ventilation [1, 2]. VEC is official drug where, USP applies LC methods for its determination in presence of its related substances [3], while BP applies potentiometric method for determination of VEC [4].

To date, all techniques reported for the detection of VEC in biological specimens and drug product involved LC–MS [5, 6, 7, 8, 9], LC with electrochemical detection [10], LC with electrochemical and UVdetection [11], LC with post-column ion-pair extractionusing solid phase extraction followed by fluorimetric detection [12], ion-pair LC [13], LC and CZE comparative study [14], capillary electrophoresis [15], capillary gas chromatography using nitrogen sensitive detector [16] and mass spectrometry [17, 18, 19]. The specificity of the stability indicating assay methods is investigated by conducting forced degradation studies.

These studies afford a foresight into the possible degradation products of the drug and the degradation pathways and provide explication of the structure of the degradation products. These studies also explain the behavior of the drug towards various stress conditions that aids in the package and development of formulation [20]. No previously reported methods provided a complete degradation study of VEC or determined all the possible degradation products. The present work is concerned with the forced degradation behavior of VEC and the developing a specific stability indicating LC method for its quantificationin drug substance and drug product using the International Conference on harmonization (ICH) guidelines of stress-testing [21]. It also suggests the structures of all VEC possible degradation products and explained the expected pathway using the MS data.
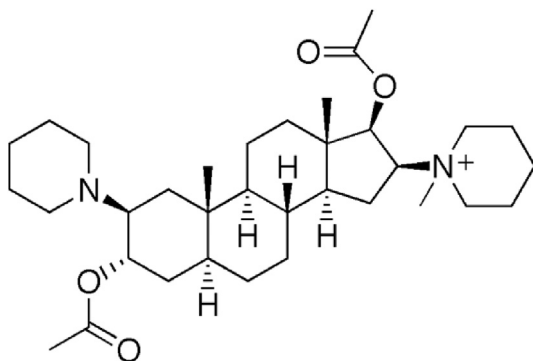

Figure 1. Chemical structure of Vecuronium.

2. Experimental

2.1. Apparatus

The LC system used was Agilent 1200 series (Germany) equipped with quaternary-pump, column oven, vacuum degasser, UV/VIS detector and 20 μl injector loop. The output signal was processed and monitored using Chemstation software. The column used was Tracer Extrasil CN (150 × 4.6mm; 5μm,China). Injection was done by the aid of Hamilton 50μl syringe (Sigma-Aldrich, Germany). UPLC MS/MS (Waters 3100, USA) equipped with auto sampler (Acquity ultra performance LC), TQ Detector (Acquity ultra performance LC), binary solvent manager pump (Acquity ultra performance LC), and Mass lynx V4.1 software (+ve mode) was used for identification and structure elucidation of the forced degradation products of VEC. Mineralight (UVGL-25) UV lamp 254nm (USA) and Heraeus UT 20 instrument oven (Germany) were used to prepare photolytic and thermal degradation products, respectively. Other apparatus were used as:Sartorius CP224S balance (Germany), Elma sonic P60 H ultrasonic processor (Germany), pH-meter Jenway 3510 (UK), Memmert water bath (Germany), water purification system Milli-Q^R Direct 8/16 system (France) and membrane filters 0.45μm from Teknokroma (Spain).

2.2. Materials and chemicals

Vecuronium bromide reference standard; was supplied by future pharmaceutical industries was certified to be 100.12% according to the official method. Pharmaceutical preparation was fortified at lab to contain 10 mg of VEC in 10 ml vial, 16.25 mg sodium phosphate dibasic anhydrous, 20.75 mg citric acid anhydrous, 97 mg mannitol (to adjust tonicity), phosphoric acid and/orsodium hydroxideto buffer and adjust to a pH of 4.

Analytical grade O-phosphoric acid (85%) was purchased from Sigma Aldrich (Germany). HPLC grade acetonitrile was purchased from Macron Advantor performance material Poland S.A. Sodium hydroxide, Hydrogen peroxide (30%), sodium phosphate dibasic anhydrous, citric acid anhydrous and mannitol were purchased from Adwic (El Nasr pharmaceutical chemicals, Cairo, Egypt). Hydrochloric acid was obtained from Riedel-de Haën (Germany). LC grade water was prepared in lab and was filtered through membrane filters 0.45μm.O-phosphoric acid (pH 6; 0.05M) was prepared by dissolving 3.5ml O-phosphoric acid (85%) in 1 L of water adjusting the pH to 6 with NaOH.

2.3. Standard solution

A stock standard solution of VEC (1 mg/ml) was prepared by dissolving an appropriate amount of drug in acetonitrile.

2.4. Forced degradation studies

Intentional degradation was attempted acidic (2MHCl) and basic hydrolysis (2MNaOH), oxidation (3% H_2O_2), photolysis (UV light at 254 nm) and thermal (135 °C). For acidic, basic hydrolysis and oxidative degradation, the drug was refluxed for 5, 10 and 1 h, respectively. For photolysis and thermal degradation, the study period was 3 h at 254 nm and 2.5 h at 135 °C, respectively. In the forced degradation studies, the drug solutions were prepared by dissolving the standard in a little volume of acetonitrile then complete the volume to 10 ml with either 2MHCl, 2MNaOH, 3% H_2O_2 or acetonitrile, to yield a concentration of 1 mg/ml of VEC. All solutions were then evaporated on water bath, dissolved in the mobile phase to obtain concentration of 1 mg/ml for VEC. The solutions of acidic and basic hydrolysis were neutralized before evaporation. Degradation products stock solutionswere derived from the degradation of 1 mg/ml VEC stock solution.

2.5. Chromatographic conditions

The chromatographic separation was performed on Tracer Extrasil CN (150 × 4.6 mm; 5μm) column maintained at 25 °C using a mobile phase composed of O-phosphoric acid (pH6; 0.05M)-acetonitrile (50:50 v/v). The mobile phase was filtered using a 0.45 μm membrane filter and was pumped at a flow rate of 1 ml/min. The signal was monitored at 210 nm. The run time was 4 min. The chromatographic system and the column were conditioned with the mobile phase for 45 min before injection.

2.6. Procedures

2.6.1. Construction of calibration curve
Into series of 10-ml volumetric flasks, volumes from VEC stock solution (1 mg/ml) were accurately transferred. Then the volume was completed with mobile phase to the mark to obtain a concentration range of (10–120 μg/ml). 20 μl of each concentration was injected three times. The injected concentrations of VEC were plotted against the corresponding peak areas. Linear plot was drawn from which the regression equation was obtained.

2.6.2. Analysis of VEC in pharmaceutical preparation
A concentration of 1 mg/ml was prepared from the vial as stock solution. Suitable aliquots were used to quantify the drug using the previous described procedure.

3. Results

3.1. Chromatographic conditions

Complete separation of VEC from all its forced degradation products was performed on Tracer Extrasil CN (150 × 4.6mm; 5μm) maintained at 25 °C. A mobile phase consisting of O-phosphoric acid (pH6; 0.05M)-acetonitrile (50:50 v/v) was used. The column was conditioned with mobile phase at flow rate of 1 ml/min. The detection based on peak area was conducted at 210 nm, where linear plot was obtained between VEC concentration and the corresponding peak area. Separation between VEC and its forced degradation products was attained in less than 4min. The retention times were as shown in Figure 2. Results were shown in TableI.

3.2. System suitability

System suitability parameters were studied in order to confirm system performance. These parameters are Resolution (R), capacity factor (k′), Tailing factor (T) andnumber of theoretical plates (N).
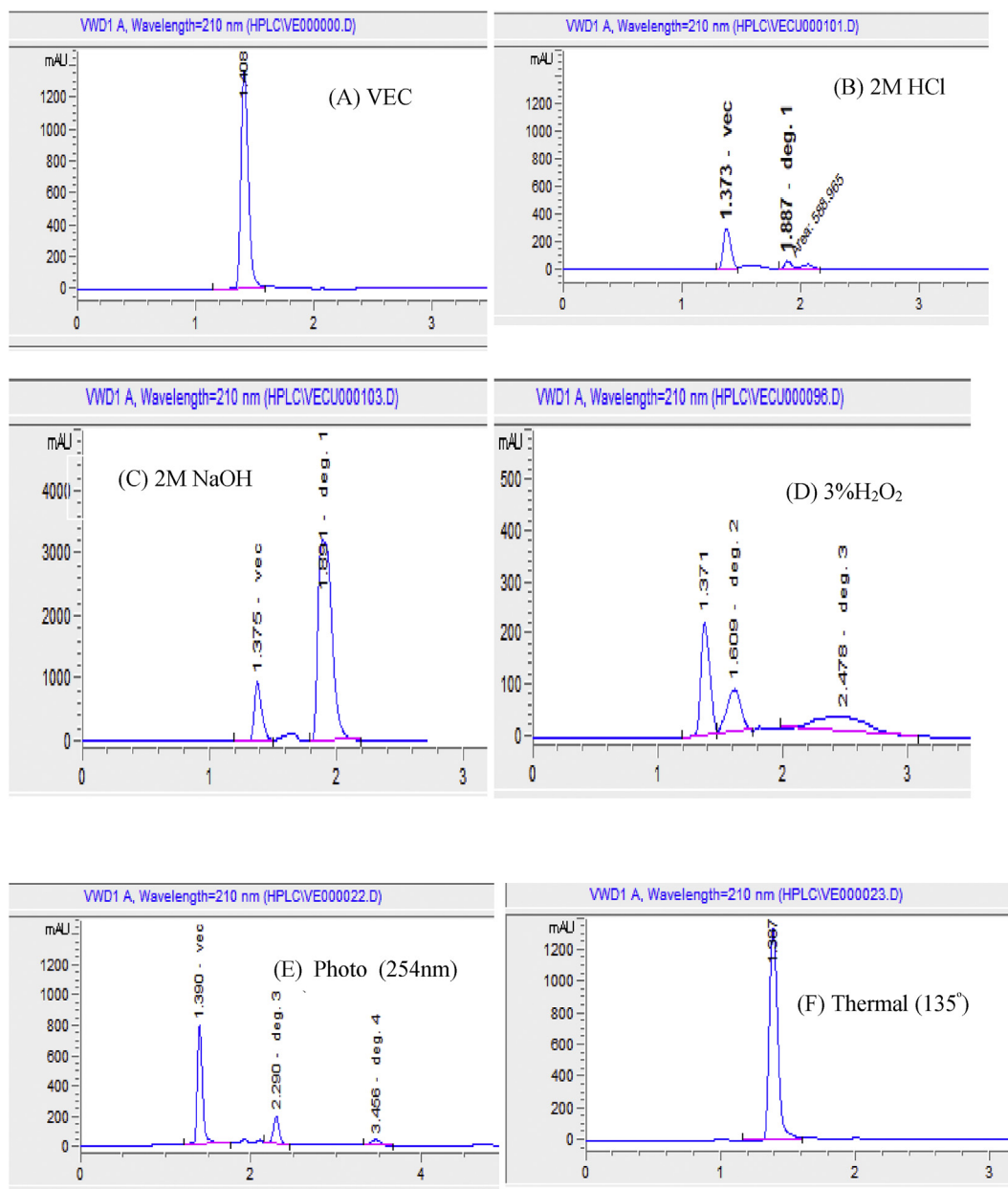
Figure 2. Typical LC chromatograms of Vecuronium bromide under stress conditions (A) VEC, (B) 2M HCl, (C)2MNaOH, (D) 3%H$_2$O$_2$, (E) Photo (254 nm)and(F) Thermal (135 °C).

System suitability tests were calculated and results were presented in TableII.

3.3. Identification of the degradation products

Degradation was observed for VEC sample during forced degradation conditions namely; Acidic (2MHCl), basic (2MNaOH) hydrolysis, oxidation (3%H$_2$O$_2$) and photolysis (UV light at 254nm), but it showed high stability towards thermal decomposition (135 °C)Figure 3. VEC was unstable towards Acidic (2MHCl), basic (2MNaOH) hydrolysis giving one degradation product (the same t$_R$) (Deg1). On forced oxidation VEC gave one oxidazible degradation product (Deg2). Photolytic degradation resulted into the formation of two degradation products (Deg 3, 4). The identification and structure elucidation of all VECpossible degradation products were conducted by the aid of the LC-MS data Figure 4 TableIII. The results of VEC stability studies are summarized in TableIV.

3.4. Application of the proposed LC method to vial analysis

The proposed LC method was applied for the determination of VEC in vials. The results obtained were found to be in agreement with labeled claimed. The obtained recoveries showed that there were no interferences from the excipients in the pharmaceutical product. The mean % recovery of VEC was found to be 100.06% as shown in TableI.

3.5. Method validation

3.5.1. Linearity

The linearity of the developed method was obtained over the concentration range of 10–120 µg/ml for VEC; the linear regression equation was y = 5.553x+115.4 with correlation coefficient 0.9996. The results showed that an excellent correlation was found between the peak area and the corresponding concentration of VEC TableI.

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Table I. Validation data for the determination of pure VEC using the proposed LC method.

Parameter	RP-LC method
Calibration range (μg/ml)	10–120
Linearity	
Slope	5.553
Intercept	115.4
Correlation coefficient(r)	0.9996
Accuracy (mean ± SD)	
Drug substance	100.32 ± 1.25
Drug product	100.06 ± 1.02
Standard added	98.50 ± 0.39
Repeatability (RSD%)	0.338
Intermediate precision (RSD%)	0.509
LOD (μg/ml)	2.67
LOQ (μg/ml)	8.01

Table II. System suitability test for proposed LC method.

Parameters	Obtained value	Reference value [23]
Resolution (R)		≥1.5
(VEC/Deg 1)	1.79	
(VEC/Deg 2)	1.50	
(VEC/Deg 3)	6.82	
(VEC/Deg 4)	7.86	
Tailing factor (T)	1.15	<2
Capacity factor (K′)	1	1–10 acceptable
Number of theoretical plates per column (N)	4499	Increases with efficiency of the separation

3.5.2. Accuracy

Percentage recovery of pure VEC expressed the accuracy of the proposed method, which ranged from 99.7-101.5% as shown in TableI. The accuracy of the assay method was also assessed onVEC in its drug product after spiking with pure VEC. The results obtained were listed in TableI. Statistical comparison between the developed method and the official method showed no significant difference TableV.

3.5.3. Precision

Intermediate precision (inter-day variation) and Repeatability (intra-day variation) were checked by analyzing three concentrations of (50, 60 and 70 μg/ml) on three different days and within the same day, respectively. The % RSD values were 0.338% and 0.509% for intra- and inter-day, respectively. This indicated that the proposed method was adequately precise TableI.

3.5.4. Specificity

The proposed method was able to completely detect and discriminate VEC in the presence of all its degradation productsformed undervarious stress conditionsas shown from the chromatograms in Figure 2. The proposed method showed a good resolution between VEC and its degradation products indicated the specificity of the proposed method. Besides, the chromatogram obtained by injecting VEC sample solution was found to be identical to the chromatogram obtained by injecting standard drug solution. These results demonstrated the specificity of the proposed method as no interference from the excipients in the pharmaceutical product was observed.

3.5.5. Robustness

The robustness of the developed LC method was examined by making analysisunder small variationsin experimental conditions.

These include; the organic strength percent (±1%), column temperature (±2 °C) and pH (±0.20 units) of the mobile phase. It was found that the method was robust when the organic strength, column temperature andpH of the mobile phasewere slightly varied. Results were shown in TableVI.

3.5.6. Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ and LOD were determined as described above to be 8.10 and 2.67 μg/ml, respectively. So, the method could be used to quantify and detect VEC over a very extensive range of concentrations.

4. Discussion

4.1. Optimization of chromatographic conditions

The main target of the proposed LC method was separating of VEC from all its possible degradation products. VEC, Deg1 and 2 were co-eluted because of their structural similarity. However Deg 3 and 4 were well separated and eluted away from VEC. In a trial to separate VEC, Deg 1 and 2, different mobile phases with various aqueous and organic ratios were tried. O-phosphoric acid (pH 6; 0.05 M)played a main role in attaining the separation between VEC, Deg 1and 2. The pH 6 was found to be suitable for determination of VEC (pka = 8.97) and its degradation products as basic compounds tended to be ionized (more polar) at pH below their pka value. So they were more retained on CN columns, which are strong dipoles that attract and separate polar molecules. The more basic compound was the most retained and eluted lastly. So CN (150 × 4.6 mm; 5μm) column was found to be the column for choice for this method. The high molar concentration of O-phosphoric acid (0.05 M) increased the concentration of hydrogen ion competed for the residual silanol groups on CN columns so reduced peak tailing and retention time. Acetonitrile was chosen as organic modifiersinceitwas a good solvent for VEC. The mobile phase ratio chosen attained the best resolution of all peaks within optimal run time. The detection was done at 210 nm as a compromise between the maximum absorption of the detected compounds and the very low concentrations of the degradation products with respect to VEC. A flow rate of 1 ml/min was found to be the best possible for the analysis as it provided good resolution of all peaks within less than 4min.

4.2. Forced degradation studies

Stability testing as a component of medication advancement procedure allows us to comprehend the component of a medication's deterioration. It further aides in acquiring data on chemical and physical factors that leads to instability. The resulting data would help in stabilizing VEC standard or drug product, resulting in expanded shelf-life or enhanced efficacy. According to ICH guideline Q1A (R2), different stress conditions (hydrolysis, oxidation, photolysis, and thermal degradation) were carried out to test the stability of the drug substance to prove the superiority of stability indicating analytical methods [21, 22, 24]. VEC was highly degraded when subjected to basic hydrolysis and oxidative degradation TableIV. So during drug formulation basic excipient must be avoided and the final product had to be packaged under inert atmosphere. Also opaque glass containers were recommended due to the proved drug photosensitivity.

5. Conclusion

In the present contribution, a validated stability indicating LC method was proposed to monitor the degradation profile of VEC under various stress conditions. The drug is found to be liable to acidic and basic hydrolysis, photolysis and oxidation, while it is stable towards thermal degradation. LC/MS was used for the identification and structure elucidation offourdegradation products. The proposed LC method providessensitive, accurate and specific stability-indicating method for the

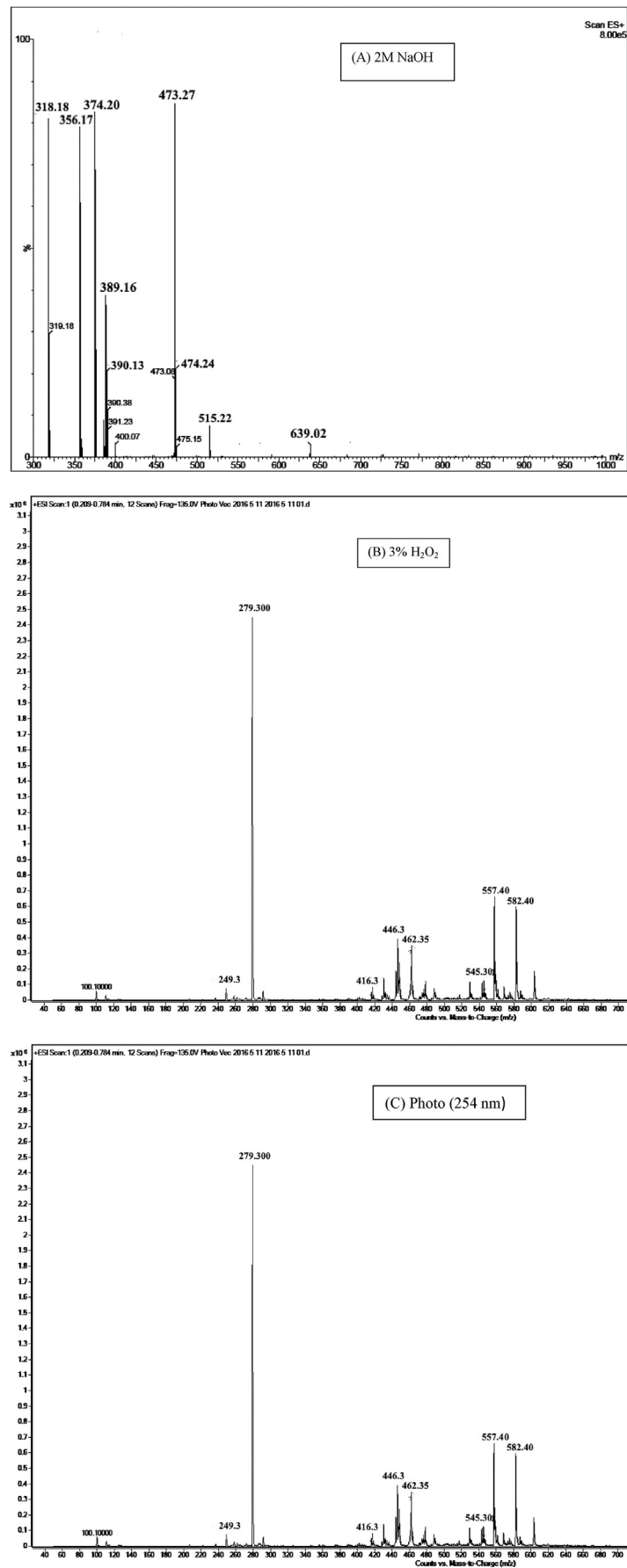**Figure 3.** The MS spectra of (A) 2M NaOH, (B) 3% H_2O_2 and (C) Photo (254 nm).
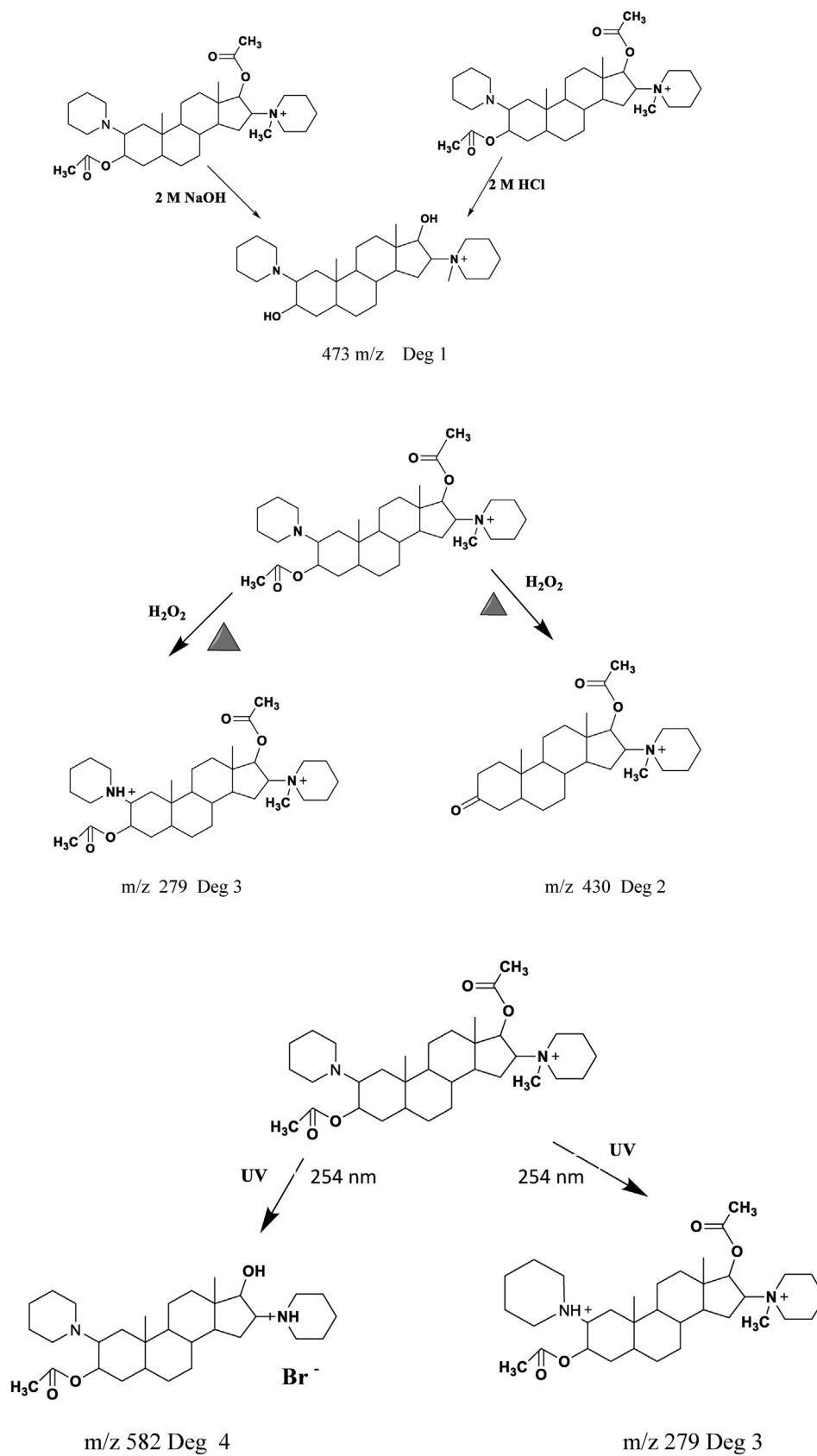
Figure 4. The Suggested chemical structure of all possible degradation products.

Table III. Structural properties, formulae and expected m/z values for VEC and its degradation products.

Name	Structural differences from VEC	Formula	Expected m/z
VEC	-	$C_{34}H_{57}BrN_2O_4$	637.73
Deg 1	Hydrolyzed acetate groups (2 groups)	$C_{30}H_{53}N_2O_2^+$	473.27
Deg 2	Loss of piperidine, Hydrolyzed acetate group	$C_{27}H_{44}NO_4^+$	430.3
Deg 3	Oxidation of nitrogen atom	$C_{34}H_{58}N_2O_{34}^{2+}$	279.3
Deg4	Hydrolyzed acetate groups, loss of methyl group	$C_{31}H_{53}BrN_2O_3$	582.4

Vec: 1-(3-acetoxy-10,13-dimethyl-2-(piperidin-1-yl)-17-(prop-1-en-2-yloxy)hexadecahydro-1H-cyclopenta[a]phenanthren-16-yl)-1-methylpiperidin-1-ium.
Deg1:1-(3,17-dihydroxy-10,13-dimethyl-2-(piperidin-1-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-16-yl)-1-methylpiperidin-1-ium.
Deg2:1-(17-acetoxy-10,13-dimethyl-3-oxohexadecahydro-1H-cyclopenta[a]phenanthren-16-yl)-1-methylpiperidin-1-ium.
Deg3: 1-(3,17-diacetoxy-10,13-dimethyl-2-(piperidin-1-ium-1-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-16-yl)-1-methylpiperidin-1-ium.
Deg4: 1-(3-acetoxy-17-hydroxy-10,13-dimethyl-2-(piperidin-1-yl)hexadecahydro-1H-cyclopenta[a]phenanthren-16-yl)piperidin-1-ium bromide.

Table IV. Summary of the forced degradation study.

Stress conditions	Number of degradation products/(t_R)	Time of degradation (hr)	Degradation (%)
HCl (2M)	1/(1.887)	5	32
NaOH (2M)	1/(1.891)	10	77
H_2O_2 (3%)	2/(1.666, 2.478)	1	95
Photo (254nm)	2/(2.290, 3.406)	3	42
Thermal (135 °C)	-	2.5	0

Table V. Statistical comparison between the results obtained by the proposed LC method and the official method.

Item	The proposed LC method	The official method[a][3]
Mean	100.32	100.12
SD	1.25	0.77
Variance	1.56	0.60
n	5	6
F (5.19)*[b]	2.58	-
t (1.83)*[b]	0.367	-

[a] HP LC method Mobile phase: mixture of Solution A and Solution B (3:2). Solution A: 8.0 g of sodium perchlorate to a 1000-mL volumetric flask, dissolve in 6.0 mL of water, dilute with acetonitrile to volume, mix, filter, and degas. Solution B: 3.2 g of ammonium chloride to a 2000-mL volumetric flask, dissolve in 16 mL of ammonium hydroxide, dilute with methanol to volume, mix, filter, and degas. Detection:215-nm. Column:4.6-mm × 25-cm, 5μm packing L3.
[b] Figures in parentheses represent corresponding tabulated values for F and t at $p = 0.05$.
* F ratio and t test are the most basic parametric statistical significance test used to compare two independent groups.

Table VI. Robustness study of the proposed LC method.

Factor	Retention time	Tailing factor	Symmetry	Recovery (%)
Acetonitrile (%)				
51	1.40	1.20	0.84	99.84%
49	1.42	1.15	0.83	100.35%
pH				
6.20	1.41	1.19	0.84	99.19%
5.80	1.41	1.17	0.86	100.43%
Temperature (°C)				
23	1.41	1.17	0.86	99.80%
27	1.40	1.18	0.86	99.80%
Mean ± SD	1.41 ± 0.008	1.18 ± 0.018	0.85 ± 0.013	99.90 ± 0.450

quantification of VEC in drug substance and in drug product, without any interference from the excipients. The method has been found to be better than previously reported methods asit is highly sensitivity, useavailable chemicals, UV detection, lack of extraction procedures, and short run time. So, it savestime and money and it can be used in routine analysis of VEC.

Declarations

Author contribution statement

Diana Fahem: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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