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HPLC analysis, isolation and identification of a new degradation product in carvedilol tablets

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ABSTRACT

Carvedilol (CV) is an antagonist of α_1 and β_1,β_2 membrane adrenoceptors and also a modulator of cardiac electrophysiological properties. It is widely prescribed for the treatment of cardiovascular diseases. During stability testing of CV solid dosage forms an unknown degradation product referred as UP, exceeded the identification thresholds of ICH Q3B guidelines. The HPLC analysis of the detected unknown product was performed by a newly, developed, specific and validated method, also suitable for the quantitative determination of the known CV impurities (imp B, C, E and F) and the other degradation products. The separation was achieved with an X-terra C_{18} column, using acetonitrile–phosphate buffer pH 2.5 as mobile phase. The isolation of UP was carried out by semi-preparative chromatography method, followed by deep freezing of the collected fractions until the organic and the aqueous phases were separated. Chromatographic behaviour of CV and UP was compared, in mobile phases of different pH and gave valuable information concerning the dissimilarities of their ionization. UP was further studied by MS and ^{1}H NMR spectrometry, revealing structural similarities with the parent molecule. Finally, the unknown peak of degradation product was attributed to a new compound generated from the interaction of CV molecule and polyvinyl pyrrolidone (PVP) in the presence of water molecules. Moisture and temperature was proved to affect the formation of UP and its concentration in CV tablets. Appropriate modifications of the packaging of CV tablets can be made in order to reduce UP concentration down to the accepted levels, during the tablets' shelf life.

1. Introduction

Carvedilol (CV) (Fig. 1) is an antagonist of α_1 and β_1,β_2 membrane adrenoceptors and also a modulator of cardiac electrophysiological properties via interaction with K^+ and Ca^{2+} ion channels [1–3]. Due to its anti-oxidative capacity, it reduces the oxidative stress and protects mitochondria from oxidative phosphorylation. CV is widely prescribed for the treatment of cardiovascular diseases such as hypertension, ischemic heart diseases, myocardial infractions and congestive heart failures, in tablets of 3.125, 6.25, 12.5 and 25 mg. Both prototype and generic products contain the racemic mixture of *R*[+] and *S*[−] CV, since neither enantiomer alone has the pharmacologic profile of the racemic mixture [4].

CV is generally considered to be a quite stable molecule. Nevertheless, during our stability studies of generic CV tablets three unknown degradation products were observed. One of them exceeded the identification thresholds (ICH guideline Q3B [5]) when tablets were stored at accelerated (ACC) conditions. According to the guideline Q1A [6] for stability studies, each product batch should be stored both, under long-term (LT) conditions and ACC conditions. Other quality parameters of the drug should also be monitored [7].

The metabolic and pharmacokinetic properties of CV have been extensively investigated in several studies and many chromatographic methods have been reported for the determination of CV as racemic mixtures [8–10] or as enantiomers [11–13], in biological samples.

The European Pharmacopoeia method for CV purity test identifies two synthesis by-products and one potential degradation product (impurity B). When this method was used for generic tablets' stability studies, a new peak of unknown origin appeared, that was not well resolved and could not be monitored accurately.

In the present study, we report the development and validation of a new HPLC method, enabling the determination of CV potential impurities and other degradation products. With the proposed method and until the end of the tablets' shelf-life, three new degra-

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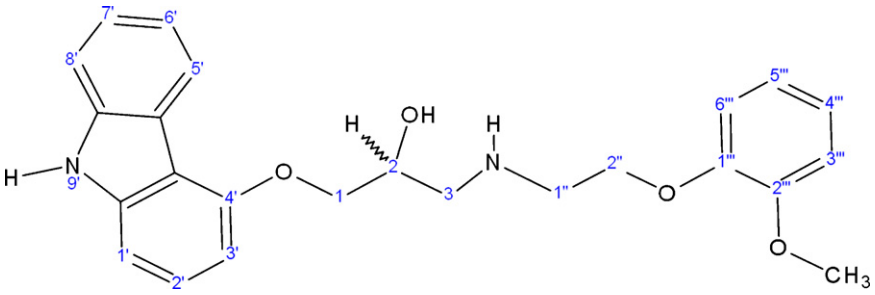

Fig. 1. Chemical structure of carvedilol: ((2*RS*)-1-(9*H*-carvazol-4-yloxy)-3-{[2-(2-methoxyphenoxy) ethyl] amino} propan-2-ol).

dation products of CV were detected, one of which exceeded the identification threshold level and was further investigated. It was isolated by a semi-preparative chromatography method, followed by deep-freezing of the collected fractions for phase separation [14]. This process has been proposed by Gu for protein separations [15,16]. ^{1}H NMR and MS spectra were used in an attempt to characterize the isolated unknown degradation product (UP), since identification of degradation products is considered as an important step for compliance with specifications and for the safety and efficiency of drug products.

Finally, the storage parameters enhancing the formation of UP were thoroughly studied in order to achieve the reduction of UP to accepted levels.

2. Experimental

2.1. Materials

CV, its impurities and synthesis by-products, along with all the excipients used in this study, were offered by ELPEN Pharm. Co Inc. (Attica, Greece) and were stored protected from light. All phosphate salts used for buffer solutions were of analytical grade and were purchased from Merck KGaA (Damstadt, Germany). Water for HPLC was obtained from Milli-Q Plus water purifying system, Millipore (Bedford; MA, USA). All other solvents were HPLC grade from Mallinckrodt Baker Inc. (Netherlands). The mobile phases were vacuum filtered and degassed through a 0.45-μm PTFE Millipore membrane. Deuterated dimethylsulfoxide (DMSO-d_6) was purchased from Euriso-top (Gif-sur-Yvette, France).

2.2. Instruments

2.2.1. Analytical chromatography

The samples were analyzed by a high-performance liquid chromatograph, Alliance 2690 Separation Module, equipped with a PDA detector, Waters Co. (Milford, MA, USA). Empower-1 was used as software facility.

2.2.2. Semi-preparative chromatography

504 Pump, equipped with LC481 detector (Waters Co.) was used for the collection of the fractions.

2.2.3. MS spectrometry

It was performed using Finnigan AQA mass spectrometer (Thermo Quest, Manchester, UK), equipped with electrospray ionization (ESI) source operating in the positive ion mode. The capillary voltage was set at 3.0 kV and the source block voltage (AQAmax) was set at 20 V. A Nitrox-N_2 Model UHPLCMS12E nitrogen generator, domnick hunter (Gateshead, England) was used to provide highly pure nitrogen utilized as sheath and nebulizing gas. Data acquisition and analysis were performed using Xcalibur (v. 1.2) IBM data system running under Windows NT (v. 4.0).

2.2.4. ^{1}H NMR spectroscopy

^{1}H NMR spectra were recorded on a Bruker DRX-Avance 400-MHz spectrometer with operating frequency 400.13 MHz, equipped with a direct and an inverse 5-mm broadband probe and B_0 gradients. Probe temperature was regulated at 298 K through a BVT-3000 Bruker control unit.

2.2.5. Accelerated conditions cabinet

Climatic Test Cabinet, Vötsch VC0057 (Reiskirchen-Lindenstruth, Germany), was used for the stability studies at ACC conditions. The temperature was set at $40 \pm 2\,^{\circ}$C and the relative humidity at $75 \pm 5\%$.

2.3. Liquid chromatography

2.3.1. Analytical chromatography

The chromatographic separation was performed on a 5-μm X-Terra C_{18} column (Waters Co), length 100 mm, i.d. 4.6 mm. The mobile phase, acetonitrile–phosphate buffer (pH 2.5; 0.01 M) (40:60, v/v) and the flow rate was 0.8 ml min^{-1}. The column temperature was regulated at 30 °C. UV detector was set at 240 nm and the injection volume at 10 μl.

2.3.2. Semi-preparative chromatography

The above-described analytical method was scaled up to semi-preparative chromatography, keeping the same mobile phase and using a Symmetry C_{18} column, of 100 mm length and 7.8 mm i.d. (Waters Co). The flow rate was increased from 0.8 (analytical chromatography) to 2.30 ml min^{-1}, according to the following equation:

$$\text{flow rate}_{\text{semi-prep.}} = \text{flow rate}_{\text{anal.}} \left(\frac{\text{diameter}_{\text{semi-prep.}}}{\text{diameter}_{\text{anal.}}} \right)^2 \quad (1)$$

Loading capacity of the column was increased 2.87 times, according to the following equation:

$$\text{scale up factor} = \left(\frac{\text{diameter}_{\text{semi-prep.}}}{\text{diameter}_{\text{anal.}}} \right)^2 \times \frac{\text{length}_{\text{semi-prep.}}}{\text{length}_{\text{anal.}}} \quad (2)$$

and the injection volume was increased to 25 μl.

2.3.3. Chromatographic behaviour of CV impurities and degradation products

By modifying the pH of the aforementioned mobile phase and injecting the CV and UP solutions separately, their chromatographic behaviour was examined. At the same time, the behaviour of the known impurities and other degradation products of CV was also examined. For this purpose, phosphate buffers in the range of

pH 1.6–8.0 were prepared as different mixtures of H_3PO_4 0.1 M, NaH_2PO_4 0.11 M and $Na_2HPO_4{\cdot}7H_2O$ 0.15 M.

2.4. Samples preparation for the HPLC method validation

2.4.1. Specificity

CV tablets contain lactose, sucrose, povidone (polyvinyl pyrrolidone, PVP), cross-povidone, aerosil and magnesium stearate as excipients. A mixture of CV and all these excipients, according to the tablets' composition, was prepared and spiked with degradation products. A quantity of the mixture was dissolved in mobile phase and analysed.

2.4.2. Linearity

11 CV solutions in the range of 2.5–7.5 μg ml^{-1} were prepared.

2.4.3. Precision

Two series of 10 CV solutions in the concentration range of 2.5–7.5 μg ml^{-1} were prepared. In each solution, an appropriate quantity of the excipients' mixture was added, to obtain a ratio equivalent to the tablets' formula. The final solutions were filtered through a Whatman No. 41 filter.

2.4.4. Accuracy

A stock solution of CV and all excipients' mixture was used as matrix for standard addition method. In a series of equal aliquots of the matrix, increasing amounts of CV solution were added, to obtain a range of CV concentrations from 2.5 to 7.5 μg ml^{-1}.

2.4.5. Limit of detection/quantitation

CV solutions of 10, 15, 20, 100, and 200 ng ml^{-1} were prepared to obtain a 3.3 signal to noise ratio for LOD and 10 for LOQ.

2.4.6. Linearity at LOQ

Nine CV solutions in the concentration range of 100–120% of the LOQ were prepared.

2.4.7. Precision at LOQ

Two series of 12 CV solutions at the LOQ level, in the presence of tablets' excipients were prepared.

2.4.8. Accuracy at LOQ

The accuracy at the LOQ was examined with the standard addition method. Stock solution of CV and the tablets' excipients was used as matrix. In a series of equal aliquots of the matrix, CV solution was added in order to obtain the concentration of the LOQ.

2.4.9. Robustness

Small deliberate variations of the experimental conditions were applied, in order to determine the effect on retention time and resolution: (a) three different C_{18} columns of the same dimensions were tested; (b) the mobile-phase composition was changed to 58:42 and 62:38 (v/v); (c) the flow rate changed to 0.6 and 1.2 ml min^{-1}.

2.5. Procedures

2.5.1. Stability studies of carvedilol tablets

2.5.1.1. Protocol A: 3-months stability period. Four batches of CV tablets were manufactured, one of each dosage strength (3.125, 6.25, 12.5 and 25 mg), packed in PVDC–aluminium blisters and stored for 3 months at ACC and at LT conditions (25 ± 2 °C/RH 60 ± 5%). The degradation products were determined at zero time and at the end of each month, for samples at ACC conditions, using the chromatographic method described in European Pharmacopoeia for CV impurities (not described here, for brevity reasons). At LT conditions samples were analysed at the end of the storage period.

2.5.1.2. Protocol B: 2-year stability period. A total of 12 pilot batches, three from each one of the dosage strength, were manufactured, packaged and stored at LT conditions. Degradation products were determined after 3, 6, 9, 12, 18 and 24 months using the method described in Section 2.3.

2.5.2. Isolation of the unknown product

UP, appeared after the 3-month stability storage, was isolated with the aid of the semi-preparative method. Fractions containing UP were collected in a glass container immersed in ice-water bath, sealed and then stored in sub zero temperature. The lower solidified aqueous phase was separated from the upper liquid organic phase, under cooling at −20 °C for 24 h. Analysis of both phases showed that, UP was determined only in the organic phase. The freezing temperature necessary for phase separation differs as the percentage of acetonitrile in the sample changes [16]. In our case, −20 °C was proved sufficient for phase separation.

2.5.3. Sample preparation for spectrometry studies

2.5.3.1. MS. 500 μl of the above solution of UP in acetonitrile was acidified with 10 μl formic acid and was used for MS spectrometry study. 10.0 mg of CV were diluted in 10.0 ml of acetonitrile. In 500 μl of this solution 10 μl of formic acid were added.

2.5.3.2. ^1H NMR. Acetonitrile from the isolated UP solution was eliminated under N_2 stream. The residue was dried over P_2O_5 for ∼24 h and then was diluted with 700 μl DMSO-d_6. Furthermore, a solution of 6.5 mg of CV in 700 μl DMSO-d_6 was prepared.

2.5.4. Factors affecting the formation of the unknown product

2.5.4.1. Tablets' excipients. Equal masses of CV and lactose were homogenized and transferred in a container. The same procedure was followed with each one of the tablets' excipients. Finally, a mixture of all excipients, according to the tablets composition, was prepared and transferred into a glass container (10.0 ml). All samples were stored in ACC conditions for 30 days. At the end of the storage period, aliquots from the samples were diluted in mobile phase (final concentration 2 mg ml^{-1}) and were analyzed.

2.5.4.2. Carvedilol:PVP ratio. Samples of CV and PVP in ratios 1:1, 1:5 and 1:20 (w/w) were homogenized, transferred into separate glass containers (10.0 ml), sealed with glass stoppers and stored at 40 °C for 30 days. Two series of samples were prepared each with PVP from different batch of the same manufacturer.

2.5.4.3. Storage conditions, temperature and humidity. Three samples, CV, PVP and PVP/CV 1:1 (w/w) mixture were stored for 6 days at the following conditions: (a) at 40 °C, with low humidity level (in the presence of desiccant), (b) at 25 °C, in a vapour saturated chamber and (c) at ACC cabinet. Two series of samples, each with different batch of PVP, were studied.

2.5.4.4. Moisture content of the matrix. Accurately weighed equal masses of CV and PVP, were combined and homogenized. 100.0 mg from this mixture were separately transferred in eight glass containers. Various quantities of water ranging from 0 to 150 μl were added in each container (Table 1). Samples were well blended and the containers were sealed with glass stoppers. Samples were stored at 50 ± 5 °C for 6 days. Two more series of samples were similarly prepared and stored at the same conditions for 12 and 18 days.

Table 1
Samples used for the study of moisture effect on the formation of the unknown degradation product

	Sample no.							
	1	2	3	4	5	6	7	8
1:1 mixture of CV:PVP (mg)	100	100	100	100	100	100	100	100
Added water (μl)	0	2	5	10	20	50	100	150
Estimated water content (%)	1.79	3.71	6.45	10.69	18.12	34.47	50.83	60.66
UP formed (%)								
6 days	0.08	0.15	0.72	2.74	1.10	0.69	0.40	0.21
12 days	0.09	0.14	2.19	3.08	3.34	1.93	1.10	
18 days	0.10	0.13	1.14	3.94	4.20	1.54	1.76	0.91

At the end of the storage period, samples were diluted in 10.0 ml of mobile phase and analyzed.

3. Results

After the 3-month stability period, the comparison of chromatograms from CV tablets of different strength showed that certain unknown peaks appeared and increased with time, especially at ACC conditions and 3.125 mg tablets. The same results were also obtained at the end of 2-year stability study, at LT conditions. The maximum level of the unknown peak was 0.25 and 0.41% for tablets of 6.25 and 3.125 mg, respectively. In the present investigation this degradation product (UP) was separated, quantitated and isolated. Two more unknown peaks appeared in the same study, but they were below the identification levels of ICH guidelines.

3.1. Liquid chromatography

3.1.1. HPLC method

The final chromatographic conditions were selected after several experiments with C_8 and C_{18} columns, of different length and from different manufacturers. Acetonitrile was selected as organic modifier, as it was found to improve both the peak shape and the system backpressure. The pH 2.5 for the aqueous phase (Section 2.3) was selected in order to maintain CV in ionized form (reported pK_a values of CV are 7.9 [17] and 8.25 [18]).

3.1.2. Chromatographic behaviour of CV, impurities and degradation products

The retention time (Rt) changes of CV and UP in the different buffer solutions used in the mobile phase are depicted in Fig. 2. In the same figure, the Rt of the other two degradation products (degr. prod. 1 and degr. prod. 2) was also monitored. In low and high pH values, CV and UP almost co-elute, while in the intermediate values their chromatographic behaviour diverged significantly. The Rt of CV increased abruptly at pH values just below its pK_a, while UP showed a similar increase at much lower pH (around 3).

The Rt profile of the known impurities of CV in relation to the pH was also compared with the profile of CV (Fig.3). Impurities B, C and F are synthesis by-products and E is a synthesis residual; impurity B is also known as a degradation product (their structure is presented in Fig. 4). By increasing the pH of the mobile phase, elution of impurities B, C and E was retarded. pH 2.5 combined the distinct resolution of all peaks and small run times and was selected for all the chromatographic studies.

3.2. Liquid chromatography method validation

3.2.1. Specificity

The resolution between CV and impurity E, which have similar structures, was not less than 1 (Fig. 4). All the other peaks shown in Fig. 4, are adequately resolved. Therefore, the method is specific for CV, its impurities and degradation products.

3.2.2. Linearity

CV showed good linearity within the range 50–150% (10 concentration levels) of the target concentration (5 μg ml^{-1}). The equation was: area = 79,171(±1138.1)$C_{\mu g/ml}$ − 4770.3(±5682.2). The r^2 value was 0.9996 and the sum of residuals zero.

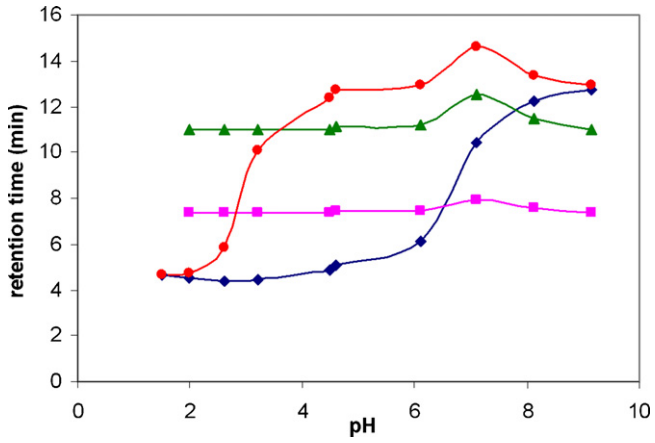

Fig. 2. Chromatographic behaviour in different pH of the mobile-phase buffer solutions: (♦) carvedilol, (●) the unknown degr. prod. (UP), (■) unknown degr. prod. 1, (▲) unknown degr. prod. 2.

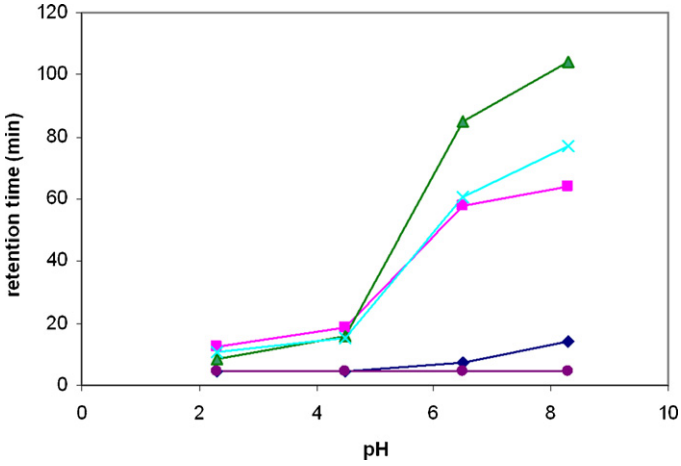

Fig. 3. Comparison of chromatographic behaviour of four known impurities of CV, in different pH of the mobile-phase buffer solutions: (♦) carvedilol, (■) imp B, (▲) imp C, (×) imp E and (●) imp F.

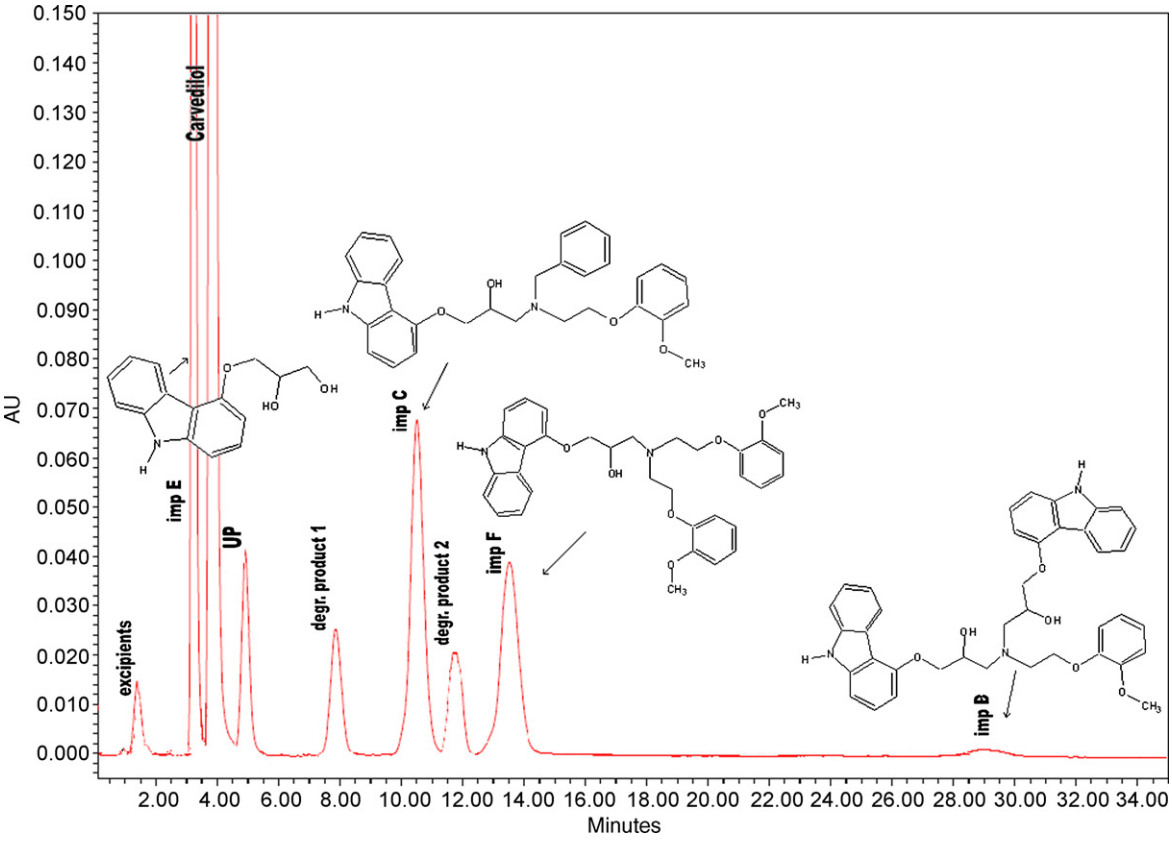

Fig. 4. Spiked chromatogram showing the resolution of carvedilol, its known impurities, imp E (synthesis residual), imp C and imp F (synthesis by-products) and degradation products, imp B, unknown product (UP), degr. prod. 1 and degr. prod. 2.

3.2.3. Precision

S.D. of the first series of solutions was ±1.78% and R.S.D. 1.34%. S.D. of the second series was ±1.67% and R.S.D. 1.65%. For the two series, a two-tailed *F*-test was applied, which showed that $S.D._1$ and $S.D._2$ do not present statistically significant difference. The R.S.D of the total samples, at three concentration levels (2.5, 5 and 7.5 μg ml^{-1}) was 1.61%.

3.2.4. Accuracy

The mean error was found to be 0.83%. No one-sided error was detected.

3.2.5. Limit of detection/quantitation

The limit of detection was 20 ng ml^{-1} (S/N = 3.3), while the limit of quantitation was 100 μg ml^{-1} (S/N = 10).

3.2.6. Linearity at LOQ

The equation of the linear regression was: area = 79.47(±1.10) $C_{\mu g/ml} - 294.63(\pm 628.66)$, the r^2 value was 0.9998 and the sum of residuals zero.

3.2.7. Precision at LOQ

S.D. of the first series of samples was ±1.90% and R.S.D. 1.38%. S.D. of the second series was ±1.60% and the R.S.D. 1.26%. A two-tailed *F*-test was applied, which showed that $S.D._1$ and $S.D._2$ do not present statistically significant difference. The R.S.D. of the total samples was 1.30%.

3.2.8. Accuracy at LOQ

The mean error was found to be 1.14%. No one-sided error was detected.

3.2.9. Robustness

The comparison of different C_{18} columns showed that any stationary phase with strongly deactivated silica could be used. Changes in mobile-phase composition (±2%) or the flow rate did not affect significantly the chromatographic method.

3.3. Factors affecting the formation of the unknown product

3.3.1. Tablets' excipients

After 30 days of storage of CV and excipient mixtures at ACC conditions, the peak of UP appeared only in the presence of povidone. In the case of plain, linear PVP an intense peak was seen, which was smaller in the sample with cross-povidone (linked PVP).

3.3.2. Carvedilol:PVP ratio

Increasing the ratio of PVP in its mixtures with CV (CV:PVP—1:1, 1:5 and 1:20, w/w) the formation of UP also increased. More specifically, mixtures with the first PVP batch produced 0.005, 0.049 and 0.195% (w/w) UP, while with the second 0.006, 0.024 and 0.315% UP, at 1:1, 1:5 and 1:20 (w/w) ratios, respectively. These findings were in agreement with tablets' 3-month stability data, where the formation of UP was more intense in tablets with the lowest CV content.

3.3.3. Storage conditions, temperature and humidity

ACC conditions combine elevated temperature with increased humidity. It was examined which one of these two factors promoted UP formation. In CV and PVP samples, stored separately at 40 °C/low humidity, at 25 °C/high humidity or at ACC conditions, UP was not detected. On the contrary, in their mixtures stored at conditions with high humidity levels remarkable quantity of UP was

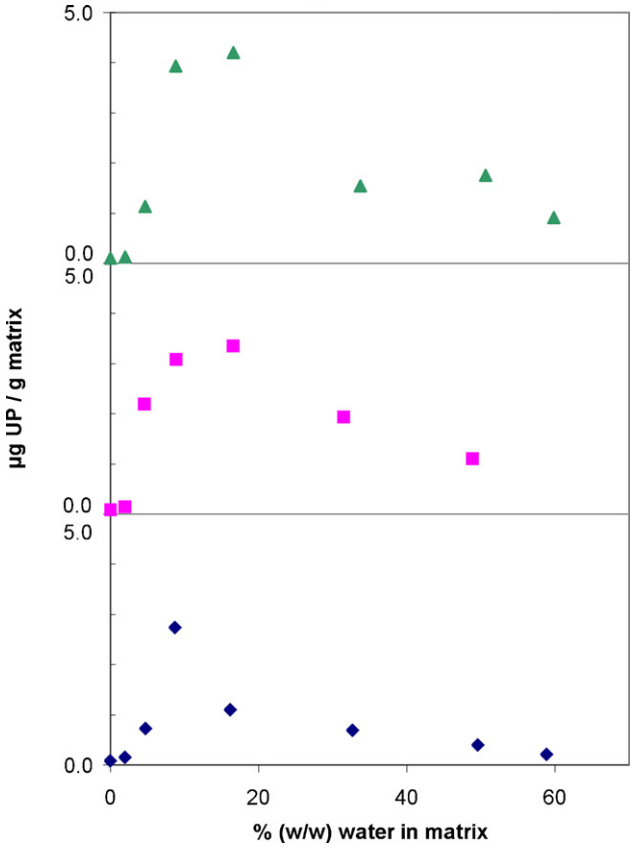

Fig. 5. Unknown degradation product (UP) concentration vs. moisture content of the matrix, after sample storage at accelerated conditions for (a) ♦ 6 days, (b) ■ 12 days and (c) ▲ 18 days.

formed. These results demonstrate that the presence of humidity was required for the appearance of UP, while the elevated temperature was of minor importance.

3.3.4. Moisture of the matrix

Samples with different water content were comparatively studied. It is easily concluded (data presented at Fig. 5) that, the % concentration of UP increased with time. Also, at increased moisture the concentration of UP also increased, until a maximum was reached (moisture 5–20%, w/w). With further increase of the sample moisture (over 30%, w/w) the obtained UP concentration was reduced. Apparently, the formation of UP was favoured within a specific range of moisture content.

It is widely known that, up to five molecules of water are distributed around each free carbonyl group of PVP, forming a solvation sphere. According to Xiang and Anderson [19], in PVP matrices with 0.5% (w/w) moisture the water molecules are mostly isolated and uniformly distributed. With 10% (w/w) moisture, their distribution is markedly heterogeneous; they form strands of water molecules that occupy channels between the polymer chains. In our experiments the matrix was not dried before use. Intrinsic moisture was 0.13 and 3.44% for CV and PVP, respectively, and since equal masses of both materials were combined (Table 1), the estimated water content of the mixture was around 1.78%. This low percentage of water was probably adequate to cause the formation of small portions of UP (Fig. 5). It could be suggested that water molecules, even in low concentrations, are already distributed in the matrix, forming extended intermolecular hydrogen bond networks.

3.4. Prevention of the unknown product formation

In order to restrict or better to avoid the UP formation during the shelf life of CV tablets, several precautionary measures must be considered: (1) Excipients used for tablet formulations, especially PVP, should have the lowest possible water content. (2) The tablets' manufacturing conditions, mainly the air humidity, should be strictly controlled. (3) Finally, tablets must be packed in aluminium blisters, which are considered adequate water barriers.

After the current study, new batches of CV tablets were manufactured stored in both LT and ACC conditions and a new 2-year stability study was scheduled. Results of regularly checked samples showed that UP formed did not exceed any safety limit (0.1%) under any storage condition, until the end of the stability period.

4. Discussion

4.1. ^{1}H NMR spectroscopy

The spectra of CV and UP were acquired in an attempt to determine the structure of isolated UP. The assignment of CV protons, presented in Table 2, is in accordance with the data reported from Marques et al. [18] and Almeida et al. [20]. Comparison of ^{1}H NMR spectra of both substances showed that, all signals of CV protons existed also in spectrum of the UP. This points out that the principal structural features of CV are included in UP, even though molecules are not identical. In more detail, the signals ascribed to aromatic protons were found slightly displaced ($\Delta\delta$ values from −0.01 to +0.04 ppm), while peaks assigned to protons of aliphatic chain moiety of CV revealed pronounced deshielding. $\Delta\delta$ values from +0.08 to +0.11 ppm were detected for methin and methyl groups. In addition the signal attributed to the NH group proton at 5.20 ppm does not exist in the spectra of UP. The slight displacement of the aromatic protons could be attributed to molecular modifications, such as conformational changes, affecting long-range intramolecular interactions. The strong downfield shifts of the aliphatic chain protons and the lack of the NH group proton are indicative of the differences in the chemical structure of UP. The above data led us to the suggestion that, the part of CV molecule bearing amino and hydroxyl groups underwent a kind of degradation modifying the electronic environment of 1, 3, 1″, 2″ protons.

Table 2
^{1}H NMR spectra data (DMSO-d_6)

Protons	$\delta_{(ppm)}$ CV	$\delta_{(ppm)}$ UP	$\Delta\delta$ ($\delta_{UP} - \delta_{CV(ppm)}$)
8′	8.21 (d, 1H)	8.25 (d, 1H)	+0.04
5′	7.43 (d, 1H)	7.43 (d, 1H)	–
7′	7.31 (t, 1H)	7.32 (t, 1H)	+0.01
2′	7.28 (t, 1H)	7.28 (t, 1H)	–
6′	7.11 (t, 1H)	7.12 (t, 1H)	+0.01
1′	7.06 (d, 1H)	7.05 (d, 1H)	−0.01
3‴, 6‴	6.93 (m, 2H)	6.92 (m, 2H)	−0.01
4‴, 5‴	6.86 (m, 2H)	6.85 (m, 2H)	−0.01
3′	6.67 (d, 1H)	6.66 (d, 1H)	−0.01
NH	5.20 (d, 1H)	–	
1	4.17 (m, 2H)	4.27 (m 2H)	+0.10
2	4.13 (m, 1H)	4.15 (m.1H)	+0.02
2″	4.01 (t, 2H)	4.09 (t, 2H)	+0.08
CH_3	3.71 (s, 3H)	3.70 (s, 3H)	−0.01
1″	2.92 (m, 2H)	3.03 (m, 2H)	+0.11
3	2.85 (m, 2H)	2.93 (m, 2H)	+0.08

The chemical shifts in δppm of protons of carvedilol (CV) and the unknown product (UP).

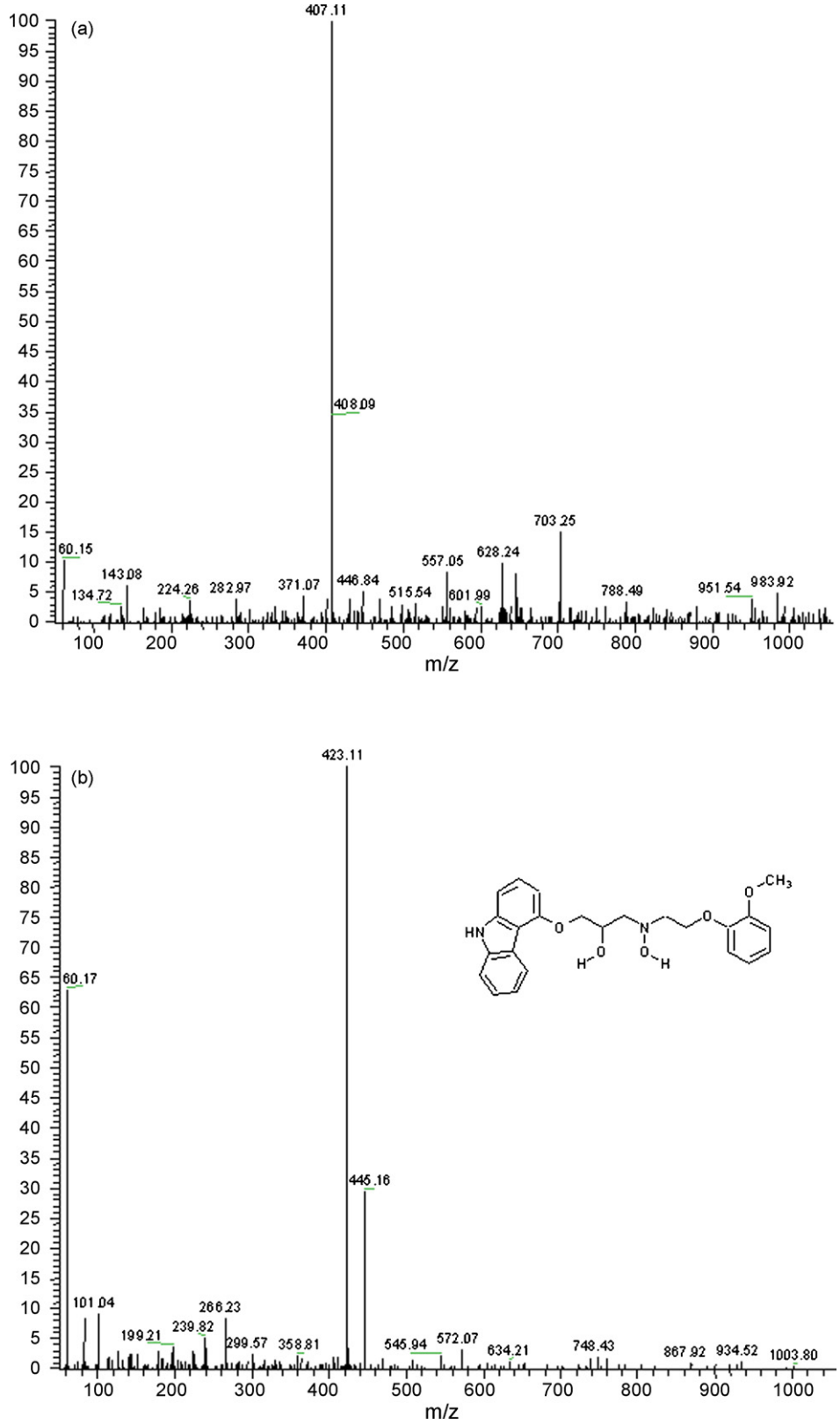

Fig. 6. MS result (a) of carvedilol protonated form (407.1 *m*/*z*) and (b) of the unknown degradation product (UP), *N*-[(2*RS*)-3-(9*H*-carbazol-4-yloxy)-2-hydroxypropyl]-*N*-[2-(2-methoxyphenoxy) ethyl] hydroxylamine.

4.2. Chromatographic behaviour

CV and UP retention times in different pH media (Fig. 3) present different profiles. The strong change of Rt corresponds to the protonation of the aliphatic amino group, which in the case of CV molecules occurs at mobile phase pH 7–8 while in the case of UP it occurs at pH 3–4. The protonation of UP, at much lower pH, clearly demonstrate the decreased basic strength of its aliphatic nitrogen (it requires low pH to be ionized).

4.3. Proposed structure of UP

According to the literature, secondary amines could undergo mild oxidation leading to hydroxylamines, after rearrangement of the initially derived N-oxides. This reaction could also be observed in solvent-less environment, with the aid of matrices (silica) acting as catalysts [21]. Therefore, the oxidative degradation of CV secondary amine group to hydroxylamine group in the presence of PVP and moisture could be proposed as a rational explanation for the above findings. CV dispersed in PVP matrices (tablet formulation) can undergo oxidation in a particular environment consisting of pyrrolidone moiety functional groups (carbonyl groups) and their solvation spheres of water molecules, in the presence of atmospheric oxygen. The extended networks of PVP and water molecules, probably provide the electronic environment that facilitates the addition of one oxygen atom leading to the conversion of CV to the respective hydroxylamine.

4.4. MS spectrometry

The ^{1}H NMR data of the UP spectrum led to the suggested structure (Fig. 6b). This approach was further verified by MS spectrometry results. The relative molecular mass of CV ($C_{20}H_{26}O_4N_2$) is 406.5 and the main molecular ion obtained from MS spectrometric analysis of pure CV was m/z 407.1. The m/z value of the main molecular ion of UP was 423.1, just 16 units more than CV, which is in agreement with the carvedilol hydroxylamine relative molecular mass.

5. Conclusions

Moisture must be carefully monitored in solid pharmaceutical dosage forms, in order to avoid the unwanted product formation. In the present work, the water content of CV tablet was proved to be a critical factor in the appearance of the new degradation product. Parameters enhancing its formation were studied using a new specific HPLC method and appropriate modifications were made in order to assure low levels of UP formation, within threshold limits (selection of the appropriate packaging).

Similar results could be expected in formulations of many other active substances with structural similarities to CV (secondary aminoalcohol), especially when PVP is one of the formulation excipients.

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