Journal of the Serbian Chemical Society
JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS
*J. Serb. Chem. Soc.* *87* (10) 1185–1202 (2022)
*JSCS–5587*
*Original scientific paper*
Published 1 August 2022

# Forced degradation studies and structural characterization of related substances of bisoprolol fumarate in finished drug product using LC–UV–MS/MS

ELENA LAZAREVSKA TODEVSKA[1,2], MARJAN PIPONSKI[1] and MARINA STEFOVA[2]*

[1]*Replek Farm Ltd., Quality Control Department, Skopje, North Macedonia and* [2]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje, North Macedonia*

(Received 4 February, revised 20 June, accepted 21 June 2022)

*Abstract*: Methods for determination of bisoprolol and related substances mostly use UV detection and a phosphate buffer and are not suitable for MS detection. In this study, LC–UV–MS/MS for separation and characterization of bisoprolol related substances was developed, validated and applied for studying the degradation products of bisoprolol when exposed to hydrolytic stress, heat and light. The method uses a C18 column, formic acid in water and acetonitrile as mobile phases, gradient elution and UV and MS detection. Forced degradation revealed that acid hydrolysis produces the most intensive transformation of bisoprolol to its impurity A, along with impurities L and D. Alkaline hydrolysis produced impurities A, L, Q, G and K; oxidative and thermal degradation produced impurities A, L and K, while photodegradation produced impurities A, L, G and K, all characterized by their mass spectral data. The developed method using two detection systems was demonstrated as efficient since mass spectra allowed identification of the related substances of bisoprolol and quantification was possible using absorbance measurements at 270 nm. The obtained results will fill in the lack of data on the fragmentation patterns of bisoprolol and related substances that could be used by researchers and practitioners in research and quality control laboratories.

*Keywords*: bisoprolol; impurities; tablets; forced degradation study; fragmentation pathways.

## INTRODUCTION

Bisoprolol fumarate is a synthetic beta-1-adrenergic blocker, which is commonly used in clinical practice for the treatment of hypertension and ventricular arrhythmias. Chemically, bisoprolol is 1-(propan-2-ylamino)-3-[4-(2-propan-2-

* Corresponding author. E-mail: marinaiv@pmf.ukim.mk
https://doi.org/10.2298/JSC220204053L

-yloxyethoxymethyl)phenoxy]propan-2-ol and its common form in solid pharmaceutical dosage forms is bisoprolol fumarate, which is very soluble in water.[1] Its wide use implies the need for efficient and reliable analytical methods for its quality control, including methods for characterization of its related substances.

Literature data revealed analytical methods for the determination of bisoprolol in pharmaceuticals and biological fluids, as a single active substance or in combined dosage forms that are mainly chromatographic as the most widely used. Chromatographic methods can be thin layer chromatographic,[2] and HPLC methods,[3–14] as well as ones that use HPLC with mass spectrometric detection especially for pharmacokinetic studies.[15–21]

On the other hand, published data for determination of related substances of bisoprolol are limited, especially data on the characterization of its degradation products (DPs), which have been listed in European Pharmacopoeia as impurity A, B, C, D, E, F, G, K, L, N, Q, R, S, T and U.[22,23] Several published studies have been developed for the determination of related substances by using hydrophilic interaction liquid chromatography (HILIC) coupled with UV-DAD and MS detection[24,25] and an HPLC–MS study for characterization of one unspecified impurity in a finished drug product[26] for bisoprolol or in combination with amlodipine.[27] Kasagic *et al*.[25] performed a kinetic study of the degradation of bisoprolol fumarate in order to follow its stability for 72 h in water, acid, base and oxidation environments. Pandey *et al.* also studied the degradation of bisoprolol fumarate as an active substance after severe acidic, alkaline, oxidative, thermal and light stress and monitored the results by LC–UV and identified the products by HPLC–TOF MS.[28] A very useful stability study using LC–MS/MS for characterization of the degradation products of acebutolol, an active compound from the same group, has been published giving an overview of degradation pathways as well as fragmentation patterns of the degradation products.[29] Having such data of the active substance and its related substances is a valuable tool in every quality control laboratorys. Forced degradation studies and obtaining such experimental data for bisoprolol and its degradation products was the rationale of this study.

The International Conference of Harmonization in its guidelines suggests stress testing to confirm and elucidate the critical factors that affect stability of the active principle. It is of great importance to identify the eventually formed degradation products through different pathways because it gives important information for the stability of the final product.[30,31] Information obtained during forced degradation studies gives data for both stability of the tested product and the suitability of the analytical method.[32]

HPLC with UV detection is routinely used for quality control of finished drug products in the pharmaceutical industry,[2–21] whereas mass spectrometry is used as an additional tool for structure elucidation of degradation pro-

ducts.[25,26,28,29] Often, the pharmacopoeial methods propose non-volatile mobile phase constituents and cannot be transferred to LC–MS.[22,23]

Therefore, the main objective of this study was to introduce the method for determination of related substances of bisoprolol fumarate described in the British Pharmacopoeia/European Pharmacopoeia and then develop an analogous method compatible with mass spectrometry by optimizing the mobile phase composition and gradient elution. The developed method was then used for characterization of the products from a forced degradation study of the finished drug products using their mass spectra and also the corresponding mass spectra of these ions that have been selectively fragmented and analyzed by a second stage of mass spectrometry to generate $MS^2$ spectra consisting of their ion fragments (designated also as MS/MS). The mass spectrometer was used as an additional detection tool to the HPLC–UV method. In that way, a systematic overview of the degradation pathways and fragmentation patterns of the degradation products of bisoprolol is provided that can assist in their identification for regular quality control in laboratories in the pharmaceutical industry.

## EXPERIMENTAL

### *Chemicals, reagents, materials*

Bisoprolol fumarate working standard (WS) used for quantification was standardized *versus* a valid batch of bisoprolol fumarate certified reference substance according to EP (EPCRS), supplied from the European Directorate for the Quality of Medicines (EDQM). Bisoprolol for system suitability EPCRS and bisoprolol for peak identification EPCRS were from EDQM.

Bisoprolol film-coated tablets 2.5 mg were a product of ReplekFarm, Skopje, North Macedonia.

Acetonitrile (HPLC grade), phosphoric acid, formic acid, hydrochloric acid (analytical reagents), and hydrogen peroxide solution 30 % were from Carlo Erba Reagents, sodium hydroxide was from Honeywell, Riedel-de Haën™.

### *Instrumentation*

An Agilent 1100 HPLC/MS system coupled with a diode array and a mass detector was used with an Agilent G2449A ion-trap mass spectrometer with an electrospray ionization interface. Nitrogen was used as a nebulizing gas at 50 psi and 12 L $min^{-1}$. Capillary temperature was 325 °C and the voltage was 3500 V. MS data were collected in the positive mode of ionization. The full scan covered the *m*/*z* range from 70–800. The detection wavelength when using the diode array detector was 270 nm.

The pharmacopoeial analytical method was transferred to using a volatile mobile phase instead of the nonvolatile phosphate containing one. For that purpose, formic acid was added to both mobile phases and mobile phase A was 1 vol. % formic acid in water, and mobile phase B was 1 vol. % formic acid in acetonitrile. The stationary phase was Zorbax C18 SB 250 mm×4.6 mm (5 μm particle diameter), the flow rate was 1.0 mL/min, the injection volume was 20 μL, and the operating temperature was 25 °C. The gradient elution program was the same as in the pharmacopoeial method that uses *o*-phosphoric acid.

*Preparation of standard solutions and test solutions*

Bisoprolol for peak identification (containing fumaric acid, impurity A, bisoprolol and impurity E) was prepared by dissolving the content of the vial bisoprolol for peak identification CRS in 1 mL solvent. Bisoprolol for system suitability (containing fumaric acid, bisoprolol and impurity G) was prepared by dissolving the content of the vial bisoprolol for system suitability CRS 1 mL solvent.

Test solution was prepared by transferring weighed powdered tablet mass containing 25 mg of bisoprolol (as fumarate) into a 25 mL volumetric flask, then solvent composed of 20 vol. % acetonitrile in water was added, the sample was mechanically shaken for 15 min and filled to volume. Reference solution A was prepared by dilution of 1 mL of the test solution to 100 mL with the solvent, and additional dilution of 2 mL of this solution to 10 mL with the same solvent.

Samples for linearity testing were prepared by dilution of test solution prepared as described above, to cover the range 0.52–3.10 μg $mL^{-1}$. For recovery testing, 1325 mg placebos were weighed and spiked with known concentrations of bisoprolol covering the range from 1 to 3 μg $mL^{-1}$ and prepared as test sample. A sample for system repeatability was prepared as Reference solution A. Samples for method repeatability were prepared as five separate test solutions described above from the same homogenized tablet mass.

*Validation of the HPLC–UV method*

The developed HPLC method with UV diode array detection at 270 nm was validated according to ICH guideline Q2(R1) recommendations. The tested validation parameters were linearity, accuracy, system repeatability, method repeatability, and selectivity/specificity. The mass spectrometer was included as an additional detection system to confirm the selectivity/specificity of the HPLC–UV diode array analytical method for determination of related substances of bisoprolol in the finished drug product.

Linearity of the method was established in the range from 0.52–3.10 mg/mL. Accuracy of the method was tested by a recovery study in three concentration levels. Specificity and selectivity were demonstrated by injecting system suitability solution, peak identification solution, placebo solution, standard solution, and test solution, as well as by conducting forced degradation studies. System repeatability was investigated by multiple injections of the reference solution and method repeatability was tested by repetition of analysis on 5 test solutions, prepared according to the previously described analytical procedure.

*MS/MS studies*

MS/MS studies were conducted on samples that had been stressed under different conditions as described above. The samples were analysed using an MS spectrometer with ESI in the positive ionisation mode. Separation was achieved using the HPLC method with a MS compatible mobile phase. Structure elucidation was performed using the obtained MS/MS data.

*Forced degradation studies*

Forced degradation studies were performed in solution with the application of heat, as well as exposure to UV radiation, temperature and humidity. Test solutions and placebo solutions prepared under normal conditions were used as control. Forced degradation studies in this research were performed in order to demonstrate that the method is stable as indicated during the development and validation.

All samples including controls, samples for hydrolytic stress studies, oxidative degradation studies, as well as samples for thermal degradation and photodegradation were prepared by weighting 1350 mg powdered tablet mass and 1325 mg placebo powder that were trans-

ferred to 25 ml volumetric flasks. All samples were prepared as described before in *Preparation of standard solutions and test solutions*.

Hydrolytic forced degradation studies were performed on weighed powdered tablet mass and placebo powder that were treated with 2 mL 1 M hydrochloric acid at 60 °C for 1 h, and with 2 mL 1 M sodium hydroxide at 60 °C for 3 h. After the specified time intervals, samples for acid and alkaline forced degradation study were cooled and neutralised with 2 mL 1 M sodium hydroxide and 1 M hydrochloric acid, as required. Thereafter, samples were prepared as described in *Preparation of standard solutions and test solutions*.

Oxidative degradation was performed on weighed powdered tablet mass and placebo powder with the addition of 2 mL of 3 vol. % hydrogen peroxide at 60 °C for 3 h. After the specified period and treatment, sample was cooled and prepared as in *Preparation of standard solutions and test solutions*.

Thermal degradation studies were performed in a temperature-controlled oven, by placing the powdered tablet mass and placebo powder on an open quartz dish and kept at 105 °C for 5 h. Thereafter, samples were prepared and analysed according to the described method.

Photodegradation studies require exposure of the sample to UV radiation and temperature in a special chamber with an overall illumination of not less than 1.2 million lux h (UV energy not less than 200 W h $m^{-2}$). Samples of powdered tablet mass and placebo powder were put on an open quartz dish and kept for a period of 10 days in the chamber. Thereafter, the samples were analysed according to the described analytical procedure.

For both thermal degradation and photodegradation, after the exposure period, samples were prepared and analysed according to the described method under in *Preparation of standard solutions and test solutions*.

## RESULTS AND DISCUSSION

### *Method transfer and optimization*

Both European and British Pharmacopoeia have issued monographs for bisoprolol fumarate that include determination of related substances using a mobile phase with phosphoric acid and UV detection at 225 nm. To enable mass spectrometric detection and characterization of the related substances of bisoprolol, the method was modified to make it MS compatible. In that way, the fragmentation patterns of bisoprolol related substances were elucidated and applied for the characterization of the products of the forced degradation studies.

Zorbax C18 SB 250 mm×4.6 mm, 5 μm, was used as the stationary phase with a wide pH operation range. In the monograph for bisoprolol in European/ /British Pharmacopoeia, acidifying the mobile phase was made with *o*-phosphoric acid (10 g $L^{-1}$), but to transfer this method to MS, 1 vol. % formic acid was used instead since it is the preferred acidic component in the mobile phase in many HPLC applications with MS detection for enhancing both separation and ionization.[33] Bisoprolol has two absorbance maxima at 220 and 270 nm with different absorption coefficients, and the second had to be used upon changing the mobile phase to one with formic acid (the first one is recommended in the EP and BP methods that use phosphoric acid in the mobile phase).[33] The gradient elution program and the flow rate were the same as the ones described in the

official monographs. The obtained results confirm a good correlation between the relative retention times obtained with both methods, and almost the same values for the resolution between bisoprolol and bisoprolol impurity G. The chromatograms obtained with both methods are presented in Fig. 1 showing good separation between bisoprolol and bisoprolol impuirty G with resolution 2.24 and relative retention time of the peak from bisoprolol impuirty G of 1.05 in the official method, whereas the corresponding values in the alternative method are 2.24 for the resolution and 1.03 for the RRT of bisoprolol impuirty G. In the chromatograms obtained for the peak identification CRS, the RRT of bisoprolol impuirty A and bisoprolol impuirty E calculated *vs*. the retention time of bisoprolol are 0.50 and 1.13 with the official method and 0.52 and 1.13 with the alternative method. These data demonstrate the suitability of the alternative method for further studies of the related substances to bisoprolol with HPLC coupled to mass spectrometric detection.

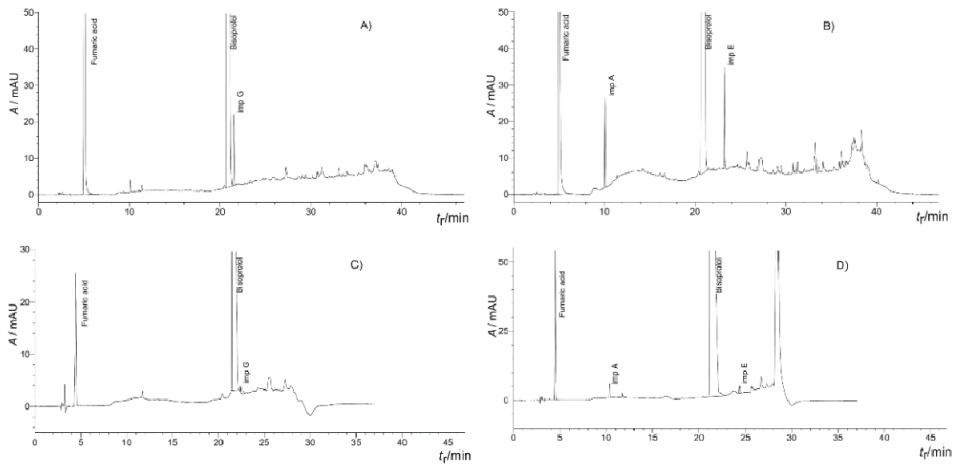


Fig. 1. UV DAD chromatograms of system suitability CRS (A and C) and of peak identification CRS (B and D) obtained with the official method (A and B) and with the alternative MS compatible method (C and D).

To confirm its performance to be used for characterization of related substances to bisoprolol in a forced degradation study, it was validated according to ICH requirements, tested for its selectivity and then applied for characterization of the samples of bisoprolol film-coated tablets subjected to forced degradation.

*Validation of the HPLC–UV method for analysis of bisoprolol and related substances*

To confirm its performance to be used for characterization of related substances to bisoprolol obtained in a forced degradation study, it was first validated

according to the ICH requirements with emphasis on testing its selectivity and suitability for the identification of bisoprolol and its related substances using their mass spectra and their quantification by UV detection at 270 nm. For this purpose, standards for system suitability and for peak identification were used, and then samples from the forced degradation studies were characterized in order to evaluate the suitability of the HPLC–UV method for its purpose: characterization of substances related to bisoprolol.

*Selectivity: bisoprolol and impurities G, A and E*

Specificity and selectivity were confirmed by injecting the system suitability solution, peak identification solution, placebo solution, standard solution, and test solution, as well as by conducting forced degradation studies, and additional confirmation of the specified impurities and bisoprolol with MS data. The chromatograms of the placebo, test solution and standard solution confirmed that there was no interference from the excipients with the main peak of bisoprolol and the specified impurities (the chromatograms are given in the Supplementary material to this paper, Fig. S-1).

The selectivity of the method was tested with a solution for system suitability and a solution for peak identification. In the obtained chromatograms and mass spectra, peaks of bisoprolol and bisoprolol impurity G were detected at about 23.7 and 24.3 min, respectively, *i.e.*, the relative retention time for impurity G was about 1.03 min.

Mass spectral data showed two peaks with high abundance for positively charged ions at *m*/*z* 326 and 356, confirming the presence of bisoprolol ($M_r$ = = 325) and bisoprolol impurity G ($M_r$ = 355). Fragmentation pathways of these two compounds are given in Fig. 2 and their MS and $MS^2$ spectra in the Supplementary material, Fig. S-2.

As suggested by Steckel and Schlosser,[34] the initial site of protonation was the basic $sp^3$-hybridized nitrogen atom, and after collision-induced activation, the resulting product ions that were detected for both compounds at *m*/*z* 222 and 116 are the result of inductive cleavages: the one at *m*/*z* 222 by cleavage of the [2-(1--methylethoxy)ethoxy]-side chain and the one at *m*/*z* 116 by cleavage of the side chain containing the amino group moiety. Since the first product ion was obtained by a loss of the side chain that is different for both compounds, and the second ion is the product obtained by cleavage of the side chain that is identical for both compounds, they give the same main peaks in $MS^2$. However they differ in their molecular ions and retention and the method is selective so that bisoprolol and its impurity G can be distinguished.

In the chromatogram and mass spectra obtained from the standard for peak identification, peaks that confirm the presence of bisoprolol impurity A, bisoprolol and bisoprolol impurity E were detected at about 12.1, 23.3 and 26.3 min, res-

pectively, *i.e.*, the relative retention time for bisoprolol impurity A is about 0.52 min and impurity E at about 1.13 min. The identity of these peaks were also confirmed by their mass spectra, as well (Supplementary material, Fig S-3).

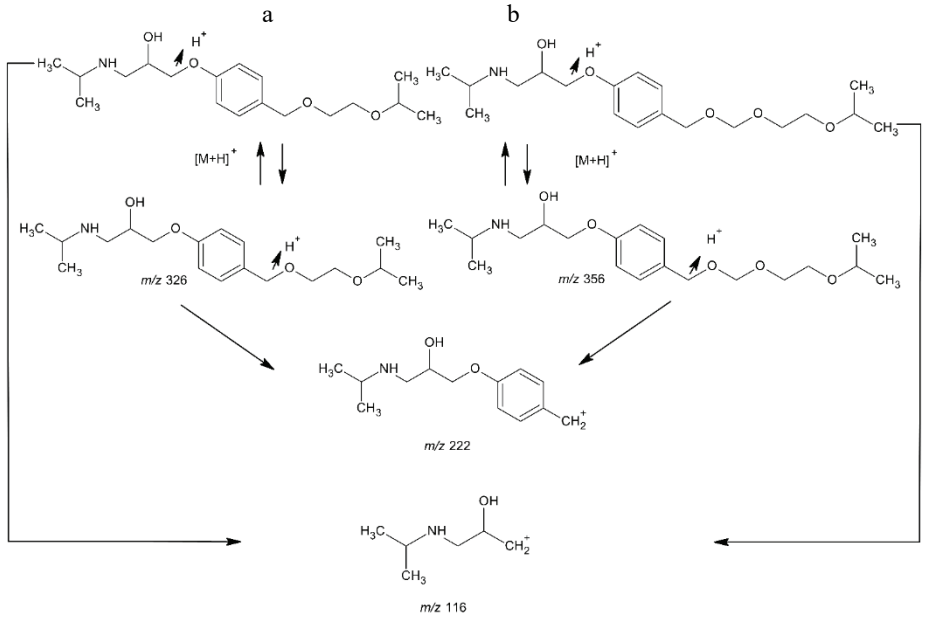


Fig. 2. Fragmentation pathways of a – bisoprolol ($C_{18}H_{31}NO_4$), and b – bisoprolol impurity G ($C_{19}H_{33}NO_5$).

The protonated molecular ion of bisoprolol impurity A ($M_r$ = 239) was detected at *m*/*z* 240 with specific fragment ions at *m*/*z* 222, 198, 163, 133, 116, 98 and 74 in $MS^2$ and the fragmentation pathways are shown in Fig. 3.

The ion at *m*/*z* 222 is obtained by a loss of a water moiety in the longer side chain. The product ion is then prone to further fragmentation with a loss of 59 Da that could be attributed to cleavage of the C-N bond, resulting in a product ion with *m*/*z* 163, which is the most intensive peak in the $MS^2$. This ion may further lose the short side chain and give a product ion with *m*/*z* 133. The observed product ion at *m*/*z* 116 is due to cleavage of the side chain that contains nitrogen (as for impurities A and G) and may undergo further cleavage of the propyl moiety producing a fragment with *m*/*z* 74, but can also lose water, thus producing the ion at *m*/*z* 98. Another fragmentation pathway that was encountered was by a cleavage of the isopropyl group in the side chain producing the ion at *m*/*z* 198 after a neutral loss of 42 Da.

On the other hand, the protonated molecular ion of bisoprolol impurity E ($M_r$ = 307) was detected at *m*/*z* 308. This impurity is a dehydration product of bisoprolol obtained by loss of water in the side chain. The molecular ion $[M+H]^+$

at *m*/*z* 308 showed a fragmentation pattern with two main product ions in $MS^2$ at: *m*/*z* 249 obtained by loss of 59 Da (isopropylamine from the side chain), and at *m*/*z* 145 by further loss in the other side chain due to cleavage of the ether bond, as shown in Fig. 4.

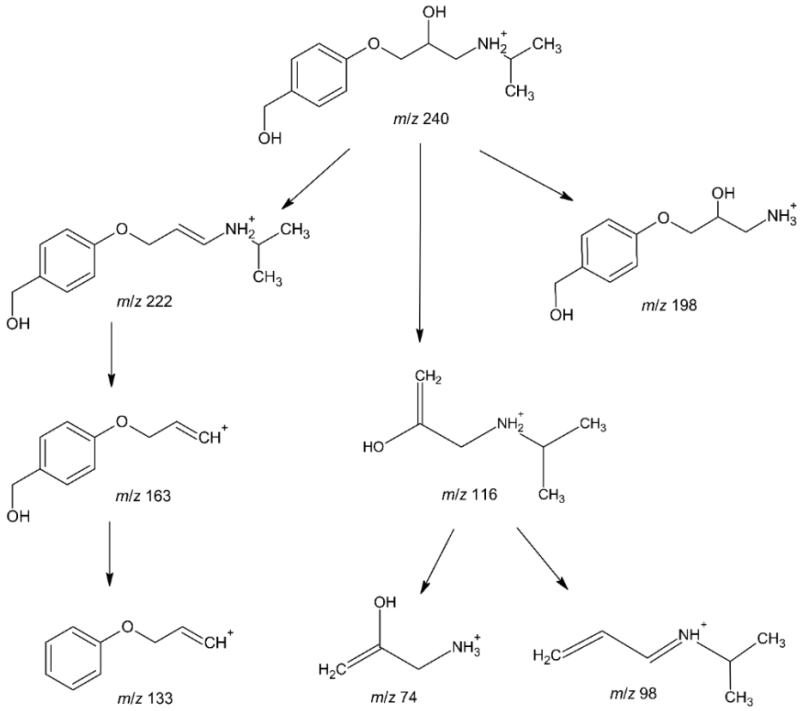


Fig. 3. Fragmentation pathways of bisoprolol impurity A ($C_{13}H_{21}NO_3$).

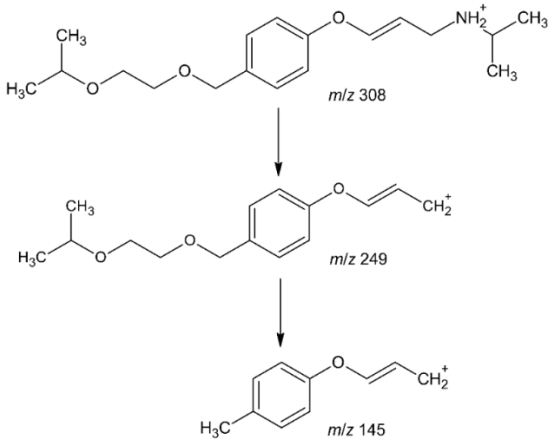


Fig. 4. Fragmentation pathway of bisoprolol impurity E ($C_{18}H_{29}NO_3$).

*Linearity, accuracy, repeatability*

Acceptability of the linearity data are defined by the coefficient of determination, *y*-intercept (%) (ratio between intercept and calculated *y*-value at a certain concentration level, more precisely target level), as well as residual standard deviation of the response factors.[35]

Linearity of the method was tested in the range from 0.52–3.10 μg $mL^{-1}$, with a target level of about 2 μg $mL^{-1}$ that corresponds to 0.2 % of the possibly present impurity. The calibration equation was $y = 3202x - 54.488$ with satisfactory value for the coefficient of determination $R^2$ of 0.9998 and an *RSD* of the response factor of 1.52 %. A coefficient of determination better than 0.998 is considered acceptable, since it represents the fit of the data with the regression line. The acceptable value for the *y*-intercept is less or equal to 5.0 % when compared to the *y*-value at a concentration level of 0.2 %,[34] which in the present case was 0.667 %. Nevertheless, the regression line is not ideal, and the intercept stands for the signal of zero concentration of active substance, which is here a peak area of 54.448 as absolute value, and if it was ideal, it should be zero. By determining the *p*-value of the intercept, the statistical significance of the obtained value of 54.448 was evaluated. To confirm that it is statistically not significant, the *p*-value should be much less than 0.05, which was found in the present case since the *p*-value was $9.93\times10^{-7}$.

The accuracy of the method was confirmed by a recovery study at three concentration levels (1.00, 2.00 and 3.00 μg $mL^{-1}$) with obtained values for analytical recovery that were within the limits of 90 and 110 %.

System repeatability tested by multiple injections of the reference solution confirmed good reproducibility with an *RSD* < 10 % and method repeatability tested by 5 test solutions prepared from powdered film-coated tablets gave *RSD* <20 %,[34] confirming its adequacy.

*Application of the HPLC–UV–MS/MS method for analysis of bisoprolol and related substances obtained in forced degradation experiments*

*Acid degradation*. During the acid degradation experiment (chromatogram on Fig. S-4 of the Supplementary material) degradation products (DPs) were generated demonstrating the sensitivity of bisoprolol to acid treatment and almost complete degradation. High amounts of impurity A and other DPs were identified using MS data demonstrating that the method could be used for their tentative identification even when the standards for all the 15 specified impurities of bisoprolol are not available. After the acid treatment, the DPs were identified as follows:

– The peak with relative retention time 0.49 (to bisoprolol) exhibited a protonated molecular ion $[M+H]^+$ at *m*/*z* 240 further fragmented into product ions at

*m*/*z* 222, 198, 163, 133, 116, 98 and 74, which is consistent with its identification as bisoprolol impurity A (Fig. S-9a of the Supplementary material).

– The peak with a RRT 0.55 gave a protonated molecular ion $[M+H^+]^+$ at *m*/*z* 238 with the corresponding product ions in $MS^2$ and *m*/*z* 220, 196, 178, 161, 149, 133, 116, 98 and 74 (Fig. S-9b). The ion at *m*/*z* 220 is due to water loss in the side chain that may further undergo a loss of the isopropylamine moiety giving a product ion at *m*/*z* 161, and a further loss of the aldehyde group in the other side chain giving a product ion at *m*/*z* 131 (Fig. 5). The other fragmentation pathway could be explained by a loss of the isopropyl moiety giving a product ion at *m*/*z* 196 (most abundant in $MS^2$), and further loss of water leading to an ion at *m*/*z* 178 (Fig. 5). These data suggest that it could be attributed to the bisoprolol impurity L.

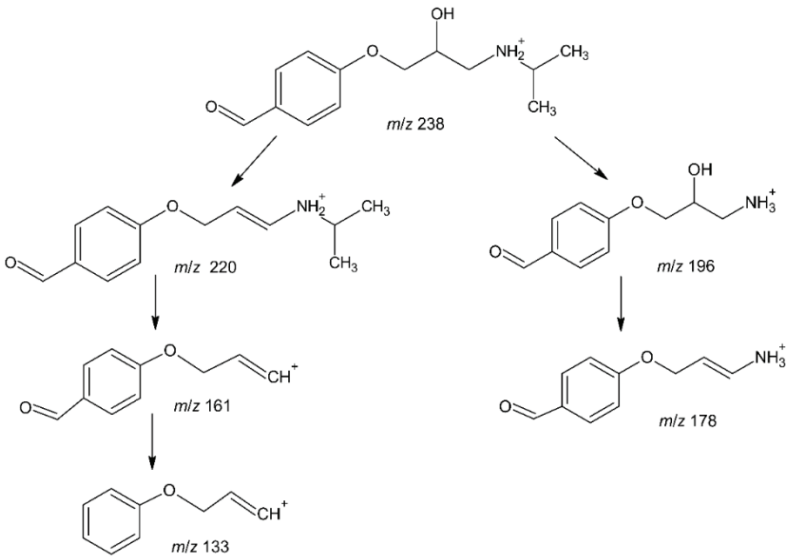


Fig. 5. Fragmentation pathway of bisoprolol impurity L ($C_{13}H_{20}NO_3$).

– The peak with relative retention time 0.56, with $[M+H]^+$ at *m*/*z* 461 was detected together with a more intensive peak at *m*/*z* 231, which is due to the same molecule ($M_r$ = 460) that when monoprotonated gives the $[M+H^+]^+$ ion at 461, and when diprotonated gives the $[M+2H^+]^{2+}$ ion at *m*/*z* 231 (Fig. S-9c). The protonated molecular ion gave product ions in MS2 at *m*/*z* 443, 385 and 328. The first ion at *m*/*z* 443 is due to water loss, and a further loss of isopropylamine from the side chain gave the product at *m*/*z* 385. The loss of the second isopropylamine resulted in the product ion detected at *m*/*z* 328 (Fig. 6). All these arguments led to the conclusion that this peak could be attributed to bisoprolol impurity D.

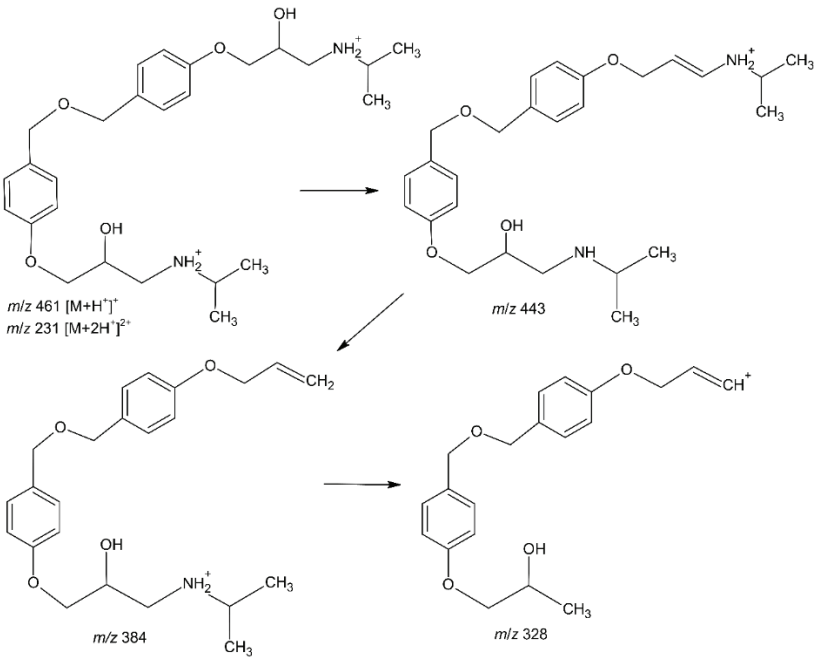


Fig. 6. Fragmentation pathway of bisoprolol impurity D ($C_{26}H_{40}N_2O_5$).

*Alkaline degradation*

Upon alkaline treatment, these degradation products were detected (Supplementary material, Fig. S-5 of the Supplementary material):

– The peak with relative retention time 0.51 was characterized with the protonated molecular ion at *m*/*z* 240 that gave the product ions in $MS^2$ at *m*/*z* 222, 198, 163, 133, 116, 98 and 74, which is consistent with its identification as bisoprolol impurity A (Fig. S-10a of the Supplementary material).

– The peak with relative retention time 0.58, with $[M+H]^+$ at *m*/*z* 238 and product ions at *m*/*z* 220, 196, 178, 161 and 133 was characterized as bisoprolol impurity L (Fig. S-10b).

– The peak with RRT 0.84, exhibited the protonated molecular ion at *m*/*z* 298 and product ions in $MS^2$ at *m*/*z* 280, 222 and 116 (Fig. S-10c). The fragmentation pathway is described in Fig. 7 with the product ion at *m*/*z* 280 due to a loss of water, and the ions at *m*/*z* 222 and 116 are typical product ions for bisoprolol. Following these experimental data, it could be concluded that this peak was due to bisoprolol impurity Q.

– The peak with RRT 1.03, with a protonated molecular ion at *m*/*z* 356 was observed with product ions at *m*/*z* 222 and 116 ($MS/MS^2$ of impurity G is given in Fig. S-10d) that suggested it is due to bisoprolol impurity G (fragmentation pattern described in Fig. 2b).

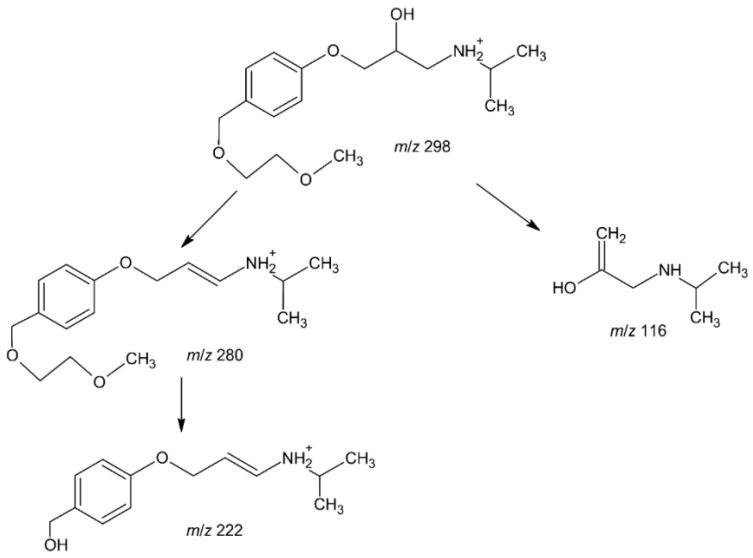


Fig. 7. Fragmentation pathway of bisoprolol impurity Q ($C_{26}H_{40}N_2O_5$).

– The peak at RRT 1.04, had a $[M+H^+]^+$ at *m*/*z* 340 with its product ions in $MS^2$ at *m*/*z* 322, 298, 263, 218, 205, 194, 176, 161, 121, 116 with different intensities (Fig. S-10e). The ion at *m*/*z* 322 is due to the loss of water molecule and is further fragmented by loss of the radical next to the carbonyl C-atom leading to a stable product ion at *m*/*z* 218. Another product ion is obtained by loss of the isopropylamine moiety leading to a product ion 263 that was subjected to further cleavage of the side chain giving the product ion with the most intensive peak in $MS^2$ at *m*/*z* 205. A signal at *m*/*z* 116, characteristic for the bisoprolol side chain, was also detected in $MS^2$, suggesting this compound has the same side chain in the structure (Fig. S-11 of the Supplementary material). According to all the discussed data for this detected compound, it may be concluded that the peak at RRT 1.04 is due to the presence of bisoprolol impurity K.

*Oxidative degradation*

After oxidative treatment (chromatogram in the Supplementary material, Fig. S-6), three DPs were detected (Fig. S-12a, b and c), two of them, impurity A and impurity L, already detected after acid and alkaline degradation. The third was detected as a peak at RRT 1.04 and exhibited a protonated molecular ion at *m*/*z* 340 due to presence of bisoprolol impurity K, and it was also observed after alkaline degradation.

*Thermal degradation*

The analysis of the sample following thermal degradation showed three degradation products (chromatogram in the Supplementary material, Fig. S-7): with

relative retention time 0.50, $[M+H]^+$ at *m*/*z* 240 that was identified as impurity A (Fig. S-13a), another with RRT 0.55, $[M+H]^+$ at *m*/*z* 238, analogous to the one found in the acid, alkaline as well as in oxidative and was attributed to bisoprolol impurity L (Fig. S-13b), and with RRT 1.04, $[M+H]^+$ at *m*/*z* 340, that followed the same fragmentation pattern as described for bisoprolol impurity K (Fig. S-13c).

*Photodegradation*

The analysis of the sample after photodegradation gave degradation products as found in thermal degradation (chromatogram in the Supplementary material, Fig. S-8, $MS/MS^2$ in Fig. S-14a, b, c and d), and also impurity G. Four peaks were detected and identified as the specified impurities: with RRT 0.50 for impurity A ($[M+H]^+$ at *m*/*z* 240), with RRT 0.57 for impurity L ($[M+H]^+$ at *m*/*z* 238), with RRT 1.04 for impurity G ($[M+H]^+$ at *m*/*z* 356), and with RRT 1.05 for impurity K ($[M+H]^+$ at *m*/*z* 340).

*Summary of forced degradation study*

A summary of the results obtained for all detected compounds in the forced degradation study that were characterized by their mass spectral data are given in Table I.

TABLE I. Overview of the results from forced degradation studies with relative retention times (RRT) and mass spectral data (MS and $MS^2$ spectra) for the detected related substances of bisoprolol

| Imp. | Normal condition/Control | | Acid degradation | | Alkaline degradation | |
|---|---|---|---|---|---|---|
| | RRT | *m*/*z* | RRT | *m*/*z* | RRT | *m*/*z* |
| A | 0.50 | MS: 240<br>$MS^2$: 222, 198, 163, 145, 116, 98, 74 | 0.49 | MS: 240<br>$MS^2$: 222, 198, 163, 145, 116, 98, 74 | 0.51 | MS: 240<br>$MS^2$: 222, 198, 163, 145, 116, 98, 74 |
| L | 0.60 | MS: 238<br>$MS^2$: 220, 196, 178, 161, 133, 98, 74 | 0.55 | MS: 238<br>$MS^2$: 220, 196, 178, 161, 133, 98, 74 | 0.58 | MS: 238<br>$MS^2$: 220, 196, 178, 161, 133, 98, 74 |
| D | – | – | 0.56 | MS: 461<br>$MS^2$: 443, 385, 328 | – | – |
| Q | – | – | – | – | 0.84 | MS: 298<br>$MS^2$: 280, 204, 116 |
| G | 1.02 | MS: 356<br>$MS^2$: 222, 116 | – | – | 1.03 | MS: 356<br>$MS^2$: 222, 116 |
| K | 1.03 | MS: 340<br>$MS^2$: 322, 298, 263, 218, 205, 194, 176, 161, 121, 116 | – | – | 1.04 | MS: 340<br>$MS^2$: 322, 298, 263, 218, 205, 194, 176, 161, 121, 116 |

TABLE I. Continued

| Imp. | Oxidative degradation | | Thermal degradation | | Photodegradation | |
|---|---|---|---|---|---|---|
| | RRT | *m/z* | RRT | *m/z* | RRT | *m/z* |
| A | 0.50 | MS: 240<br>$MS^2$: 222, 198, 163, 145, 116, 98, 74 | 0.50 | MS: 240<br>$MS^2$: 222, 198, 163, 145, 116, 98, 74 | 0.51 | MS: 240<br>MS2: 222, 198, 163, 145, 116, 98, 74 |
| L | 0.56 | MS: 238<br>$MS^2$: 220, 196, 178, 161, 133, 116 | 0.55 | MS: 238<br>$MS^2$: 220, 196, 178, 161, 133, 116 | 0.57 | MS: 238<br>$MS^2$: 222, 196, 178, 161, 133, 116 |
| G | – | – | – | – | 1.04 | MS: 356<br>$MS^2$: 222, 116 |
| K | 1.04 | MS: 340<br>$MS^2$: 322, 298, 263, 218, 205, 194, 176, 161, 121, 116 | 1.04 | MS: 340<br>$MS^2$: 322, 298, 263, 218, 205, 194, 176, 161, 121, 116 | 1.05 | MS: 340<br>$MS^2$: 322, 298, 263, 218, 205, 194, 176, 161, 121, 116 |

As could be seen, acid hydrolysis caused the most intensive transformation of bisoprolol to its related substance impurity A, along with other degradation products, impurities L and D. Alkaline hydrolysis also gave products characterized by MS as impurities A, L, Q, G and K, whereas oxidative and thermal stress produced impurities A, L and K, and photodegradation gave rise to four impurities characterized by MS as impurities A, L, G and K.

According to its structure and functional groups, bisoprolol is likely to produce degradation products when oxidative stress is applied.[25] Literature data showed forced degradation studies that confirmed that the percentage of degradation products were highest when oxidative treatment was applied, and also they revealed the fact that when acid hydrolysis was applied, bisoprolol impurity A was obtained at the highest level.[25] Pandey *et al.* performed force degradation studies of bisoprolol fumarate as an active substance and detected only three peaks: fumaric acid, bisoprolol and an unknown impurity at *m/z* 749 after thermal degradation, for which they suggested a structure and formation pathway. This degradation product was not detected in the present experiments. This study revealed the presence of six known impurities of bisoprolol and demonstrated the suitability of the method to characterize them by their mass spectra and quantified them by the HPLC–UV method.

## CONCLUSIONS

The developed method that combines the two detection systems was demonstrated as very efficient since the mass spectra allowed identification of the related substances of bisoprolol by studying the protonated molecular ions and their corresponding product ions, even when not all reference standards were avail-

able, and quantification was then possible by using UV absorbance measurements at 270 nm.

The mass spectral data obtained in this study of bisoprolol and its degradation products that are also its specified impurities, will be of great value and will contribute to fill in the lack of data on the fragmentation patterns of bisoprolol and its related substances that could be applied in quality control laboratories for their identification.

## SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/11609, or from the corresponding author on request.

## ИЗВОД

## СТУДИЈА ФОРСИРАНЕ ДЕГРАДАЦИЈЕ И СТРУКТУРНЕ КАРАКТЕРИЗАЦИЈЕ СУПСТАНЦИ СРОДНИХ БИСОПРОЛОЛ-ФУМАРАТУ У ФИНАЛНИМ ЛЕКОВИТИМ ПРОИЗВОДИМА ПРИМЕНОМ LC–UV–MS/MS

ELENA LAZAREVSKA TODEVSKA[1,2], MARJAN PIPONSKI[1] и MARINA STEFOVA[2]

[1]*Replek Farm Ltd, Quality Control Department, Skopje, North Macedonia и* [2]*Institute of Chemistry, Faculty of Natural Sciences and Mathematics, Ss. Cyril and Methodius University, Skopje, North Macedonia*

Методе за одређивање бисопролола и сродних супстанци применом UV детекције и фосфатног пуфера нису погодне за MC детекцију. У овој студији је развијена и валидирана LC–UV–MS/MS метода за раздвајање и карактеризацију супстанци сродних бисопрололу и примењена за испитивање деградационих производа бисопролола, при излагању хидролитичком стресу, топлоти и светлости. У методи се користи C18 колона, мравља киселина у води и ацетонитрил као мобилна фаза, градијентна елуција, UV и MS детекција. Форсирана деградација киселом хидролизом изазива најинтензивније трансформације бисопролола до његових нечистоћа A, L и D. Алкална хидролиза генерише нечистоће A, L, Q, G и K; оксидативна и термална деградација генерише нечистоће A, L и K, а фотодеградација A, L, G и K. Све су нечистоће окарактерисане масеном спектралном анализом. Развијена метода, која користи два детекциона система, показала се ефикасном с обзиром да масени спектри омогућују идентификацију супстанци сродних бисопрололу, а квантификација је могућа мерењем апсорбанције на 270 nm. Добијени подаци ће допунити недостатак података о фрагментацији бисоролола и сродних супстанци, који ће истраживачи и практичари моћи користити у истраживањима и лабораторијама контроле квалитета.

(Примљено 4. фебруара, ревидирано 20. јуна, прихваћено 21. јуна 2022)

## REFERENCES

1. H. Bakheit, R. Ali, A. D. Alshahrani, A. S. El-Azab, *Profiles Drug Subst. Excip. Relat. Methodol.* **46** (2021) 51 (https://doi.org/10.1016/bs.podrm.2020.07.006)
2. Witek, H. Hopkala, G. Matysik, *Chromatographia* **50** (1999) 41 (https://doi.org/10.1007/BF02493615)
3. L. Logoyda, S. Kovalenko, A. M. Abdel-Megied, I. Zhulkevych, I. Drapak, I. Demchuk, O. Netsyuk, *Int. J. Appl. Pharm.* **11** (2019) 186 (https://doi.org/10.22159/ijap.2019v11i3.32391)

4. D. Panainte, L. Agoroaei, N. Bibire, G. Tantaru, M. Apostu, M. Vieriu, F. A. Spac, F. A. *Rev. Chim.* **66** (2015) 1791 (https://www.researchgate.net/publication/299723693_A_HPLC_Method_for_the_Determination_of_Bisoprolol_in_Tablets_and_its_Application_to_a_Bioequivalence_Study)
5. E. Caudron, S. Laurent, E. M. Billaud, P. Prognon, *J. Chromatogr., B* **801** (2004) 339 (https://doi.org/10.1016/j.jchromb.2003.11.009)
6. S. Shaikh, O. A. Thusleem, M. S. Muneera, J. Akmal, A. V. Kondaguli, K. Ruckmani, *J. Pharm. Biomed. Anal*. **48** (2008) 1055 (https://doi.org/10.1016/j.jpba.2008.08.009)
7. R. J. Eastwood, J. C. Jerman, R. K. Bhamra, D. W. Holt, *Biomed. Chromatogr.* **4** (1990) 178 (https://doi.org/10.1002/bmc.1130040415)
8. L. Patel, B. Suhagia, P. Shah, R. Shah, *Ind. J. Pharm. Sci.* **68** (2006) 635 (https://doi.org/10.4103/0250-474X.29633)
9. S. J. Joshi, P. A. Karbhari, S. I. Bhoir, K. S. Bindu, C. Das, *J. Pharm. Biomed*. *Anal*. **52** (2010) 362 (https://doi.org/10.1016/j.jpba.2009.10.021)
10. S. Mahu, F. A. Spac, C. Ciobanu, M. Hancianu, L. Agroroaei, E. Butnaru, *Rev. Chim.* **67** (2016) 414 (https://www.researchgate.net/publication/299537958_Quantitative_Determination_of_Bisoprolol_Fumarate_by_HPLC_I_Method_validation)
11. G. Arjun, S. Dinakaran, B. Madhavi, M. M. Naga, R. Ramalingam, R. N. Anisetti, *Ind. Drugs* **46** (2009) 39 (https://www.researchgate.net/publication/306218289_A_simple_HPLC_method_for_quantitation_of_bisoprolol_fumarate_in_tablet_dosage_form#fullTextFileContent)
12. W. Wang, L. Gao, T. Shi, *Chin. J. Pharm. Anal.* **19** (1999) 308 (https://www.ingentaconnect.com/content/jpa/cjpa/1999/00000019/00000005/art00006)
13. Vora, A. Kadav, *Ind. J. Pharm. Sci*. 70 (2008) 542 (https://doi.org/10.4103/0250-474X.44616)
14. T. Lazarevska, M. Piponski, M. Stefova, *Maced. J. Chem. Chem. Eng.* **40** (2021) 263 (https://doi.org/10.20450/mjcce.2021.2430)
15. L. Logoyda, S. Kovalenko, M. Gaafar, A. M. Abdel-Megied, F. A. Elbarbry, *Microchem. J*. **155** (2020) 104700 (https://doi.org/10.1016/j.microc.2020.104700)
16. R. M. Turner, V. Fontana, M. Bayliss, S. Whalley, A. Santoyo Castelazo, M. Pirmohamed, *J. Pharm. Biomed. Anal.* **159** (2018) 272 (https://doi.org/10.1016/j.jpba.2018.06.062)
17. M. Liu, D. Zhang, Y. Sun, Y. Wang, Z. Liu, J. Gu, *Biomed. Chromatogr.* **21** (2007) 508 (https://doi.org/10.1002/bmc.785)
18. S. Li, G. Liu, J. Jia, Y. Liu, C. Pan, C. Yu, Y. Cai, J. Ren, *J. Chromatogr., B* **847** (2007) 174 (https://doi.org/10.1016/j.jchromb.2006.10.013)
19. G. Peste, C. Oniscu, A. Vlase, *Rom. Biotechnol. Lett.* **15** (2010) 5140 (https://www.rombio.eu/rbl2vol15/11%20%20PESTE.pdf)
20. G. Hemavathi, S. M. Hipparagi, *Asian J. Pharm. Clin. Res.* **10** (2017) 477 (https://doi.org/10.22159/ajpcr.2017.v10i4.16829)
21. L. Ding, X. Zhou, X. Guo, Q. Song, J. He, G. Xu, *J. Pharm. Biomed. Anal.* **44** (2007) 520 (https://doi.org/10.1016/j.jpba.2007.03.001)
22. *British Pharmacopoeia*, *Monograph for Bisoprolol fumarate in British Pharmacopoeia*, Stationary Office on Behalf of the Medicines and Healthcare Products Regulatory Agency (MHRA), London, 2018
23. *European Pharmacopoeia, Monograph for Bisoprolol fumarate 01/2012:171*, European Directorate for the Quality of Medicines and Healthcare, Council of Europe, Strasbourg, 2014

24. I. Kasagic-Vujanovic, B. Jancic-Stojanovic, D. Ivanovic, *Arh. Farm.* **64** (2014) 230 (https://doi.org/10.5937/arhfarm1403230K)
25. I. Kasagić-Vujanović, B. J. Stojanović, D. Ivanović, in *CMBEBIH 2017. IFMBE Proceedings*, Vol. 62, A. Badnjevic, Ed., Springer, Singapore, 2017, p. 415 (https://doi.org/10.1007/978-981-10-4166-2_64)
26. I. Mitrevska, E. Kikovska-Stojanovska, G. Petrusevski, M. Chachorovska, S. Memed-Sejfulah, S. Ugarkovic, *Adv. Chem.* **2017** (2017) 3047517 (https://doi.org/10.1155/2017/3047517).
27. T. Rakic, M. Jovanovic, A. Tumpa, B. Jancic-Stojanovic, M. Medenica, *Arh. Farm.* **64** (2014) 95 (https://doi.org/10.5937/arhfarm1402095R)
28. S. Pandey, R. Pandey, S.S. Shukla, *Ind. J. Pharm. Educ. Res.* **56** (2022) 272 (https://doi.org/10.5530/ijper.56.1.32)
29. U. Rakibe, R. Tiwari, A. Mahajan, V. Rane, P. Wakte, *J. Pharm. Anal.* **8** (2018) 357 (https://doi.org/10.1016/j.jpha.2018.03.001)
30. *ICH Topic Q1B*, *Photostability Testing of New Active Substances and Medicinal Products Step 5 Note for guidance on the photostability testing of new active substances and medicinal products*, 1998 (http://www.emea.eu.int)
31. *ICH Topic Q 1A (R2)*, *Stability Testing of new Drug Substances and Products Step 5 Note for guidance on stability testing: stability testing of new drug substances and products*, 2003 (http://www.emea.eu.int)
32. D. W. Reynolds, K. L. Facchine, J. F. Mullaney, K. M. Alsante, T. D. Hatajik, M. G. Motto, *Pharm. Technol.* **26** (2002) 48 (https://cdn.sanity.io/files/0vv8moc6/pharmtech/cef968de93fced22f668d7a89ec492e8dc542e12.pdf/article-9668.pdf)
33. J. A. Dolan, *Guide to HPLC and LC-MS Buffer Selection*, ACE HPLC Columns, Aberdeen, 2006 (https://www.hplc.eu/Downloads/ACE_Guide_BufferSelection.pdf)
34. A. Steckel, G. Schlosser, *Molecules* **24** (2019) 611 (https://doi.org/10.3390/molecules24030611)
35. Y. Kazakevich, R. LoBrutto, Eds, *HPLC for pharmaceutical scientists*, John Wiley & Sons, Inc., Hoboken, NJ, 2007, p.462 (https://doi.org/10.1002/0470087951).