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Stability of new potential ACE inhibitor in the aqueous solutions of different pH

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ABSTRACT

Angiotensin-converting enzyme (ACE) inhibitors are a group of active substances binding to an active site of ACE. Many authors who studied the structure activity relationship suggested the structural elements needed for a potent ACE inhibitor. While many authors studied the activity of ACE inhibitor substances only a few structure stability studies have been presented. In this paper the stability properties of molecule xPRIL were studied by determination of degradation path and rate of degradation in aqueous solutions with different pH (2.0, 6.8 and 12.0) and temperatures (40, 60 and 80 °C). The degradation of molecule through two main degradation paths was identified and confirmed by liquid chromatography and mass spectroscopy (LC–MS). Stability properties of xPRIL were determined in a stability study evaluated by high-performance liquid chromatography (HPLC). The first order kinetics of degradation reaction of xPRIL and Arrhenius equations for each pH were determined at observed conditions. xPRIL showed the highest stability at pH 2 solution. The degradation kinetics of xPRIL was compared to the degradation kinetics of enalapril maleate (EM) and perindopril (PER) in bio relevant solutions with pH 2.0 and 6.8. In addition to the stability study of xPRIL the forced degradation study of all three molecules at rigorous conditions was conducted. From the obtained results the structural element having the highest influence on stability properties of the studied molecules was identified. The fragmentation paths of xPRIL, its cyclization degradation product and its hydrolysis degradation product were identified and confirmed by MS/MS method.

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1. Introduction

Angiotensin-converting enzyme (ACE) is an important enzyme of the renin–angiotensin–aldosterone system. It converts inactive decapeptide angiotensin-I (AI) to biologically active octapeptide angiotensin-II (AII), which raises blood pressure by vasoconstriction as well as by triggering the formation of sodium and water retaining steroidal hormone, aldosterone, in the human body [1–3].

Increased serum ACE levels have been associated with hypertension and hypertension-related target organ disorders such as congestive heart failure, left ventricular hypertrophy, acute myocardial infarction as well as with some nephrological and pulmonary disorders [4–6].

The role of ACE inhibitors is to inhibit the last step of the biosynthesis of angiotensin II, a potent vasoconstrictor resulting in lower blood pressure [7]. ACE is a metallopeptidase having three hydrophobic ($S_{1'}$, $S_{2'}$ and S_1) and binding Zn^{2+} active sites as presented in Fig. 1 together with ACE inhibitor in its binding site [8].

Angiotensin I (X–Phe_8–His_9–Leu_{10}) binds in the active site of ACE with the last three amino acids on C terminal end of peptide. Amino acids with its structural elements bind with $S_{1'}$, $S_{2'}$ and S_1 hydrophobic binding sites [9]. The functional group that forms coordination bond with Zn^{2+} is also important for the binding of Angiotensin I into the active site of ACE [10].

Based on the structure of ACE many ACE inhibitor peptidomimetics were developed having the structure that suits the active site of the enzyme. Already in 1982 Hassall et al. discovered the main structural elements needed for a ACE inhibitor [10]. The field of peptidomimetics is progressing at a rapid pace and is now offering solutions to the age-old issues of bioavailability and oral activity [11]. Many authors have studied the structure activity relationship (SAR) with different potential ACE inhibitors [8].

The structure of ACE inhibitors, the monographs in European Pharmacopoeia of the known ACE inhibitors [12,13], and other published papers [14–16] indicate that these molecules degrade in general by two main degradation paths. The first is hydrolysis of ester and the second is intramolecular cyclization.

In the present work the stability properties of molecule xPRIL having all structural elements needed for potent ACE inhibitor were studied. xPRIL is a maleate salt of 1-{2-[1-carbethoxybutyl)amino]-1-oxopropyl}-pyrrolidin-2-carboxilic acid. xPRIL contains struc-

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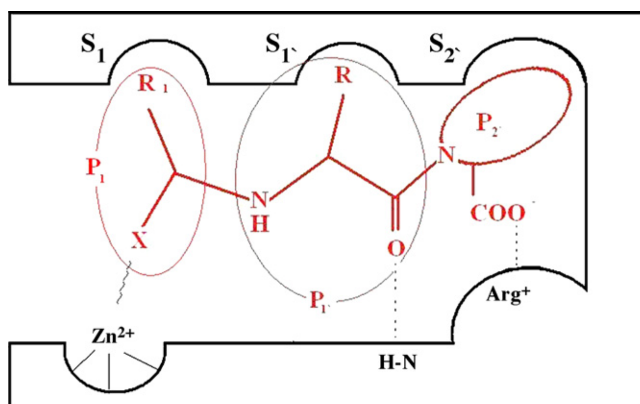

Fig. 1. Model of ACE active site and binding of ACE inhibitor.

tural elements of enalapril maleate (EM) and perindopril (PER), both known and potent ACE inhibitors. Structures of all three molecules are presented in Fig. 2.

The aim of the study was to elucidate the degradation paths of the molecule, to determine the rate of each degradation path of xPRIL in solutions with different pH and at different temperature and to elucidate the fragmentation pattern of xPRIL and its main degradation products. Furthermore, the degradation kinetics of xPRIL was compared to the degradation kinetics of EM and PER in bio relevant solutions with pH 2.0 and 6.8. In additional forced degradation study the stability properties of xPRIL were compared to EM and PER at rigorous conditions with the aim to detect the structure stability relationship.

2. Experimental

2.1. Samples, solvents and reagents

xPRIL, EM and PER were produced by Krka, d.d., Novo mesto, Šmarjeska cesta 6, Novo mesto, Slovenia. Studied samples of xPRIL, EM and PER in phosphate buffers solutions with pH 2.0, pH 6.8 and pH 12.0 were used for identification of degradation products and for stability study. Initial concentration of samples used for stability study was 1 mg/ml.

Phosphate buffers with pH 2.0, 6.8 or 12.0 were prepared by dissolving 136 mg of potassium dihydrogenphosphate (Merck, Darmstadt, Germany) in 800 ml of water, adjusting the pH to 2.0, 6.8 or 12.0 with phosphoric acid or KOH and diluting with water to 1000 ml.

For HPLC and LCMS assays the following reagents were used: acetonitrile (HPLC grade), and methanol (HPLC grade) obtained from Sigma–Aldrich Chemie (Steinheim, Germany); NaOH (p.a.), HCl (p.a.), H_2O_2 (p.a.), triethylamine (p.a.), formic acid (LC–MS grade) and orthophosphoric acid (p.a.) obtained from Merck (Darmstadt, Germany).

2.2. Apparatus and equipment

HPLC system Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) with on-line degasser (G1332A), binary pump (G1312A), autosampler (G1329A), column thermostat (G1316A), diode array detector (G1315A) and controlled by Chemstation (ver. A.10.2.) was used. This system is optionally equipped also with fraction collector (G1364C) for sample collection purposes.

LC–MS analyses were performed on a 1200L triple quadrupole MS/MS (Varian, Walnut Creek, CA, USA) coupled to a HPLC sys-

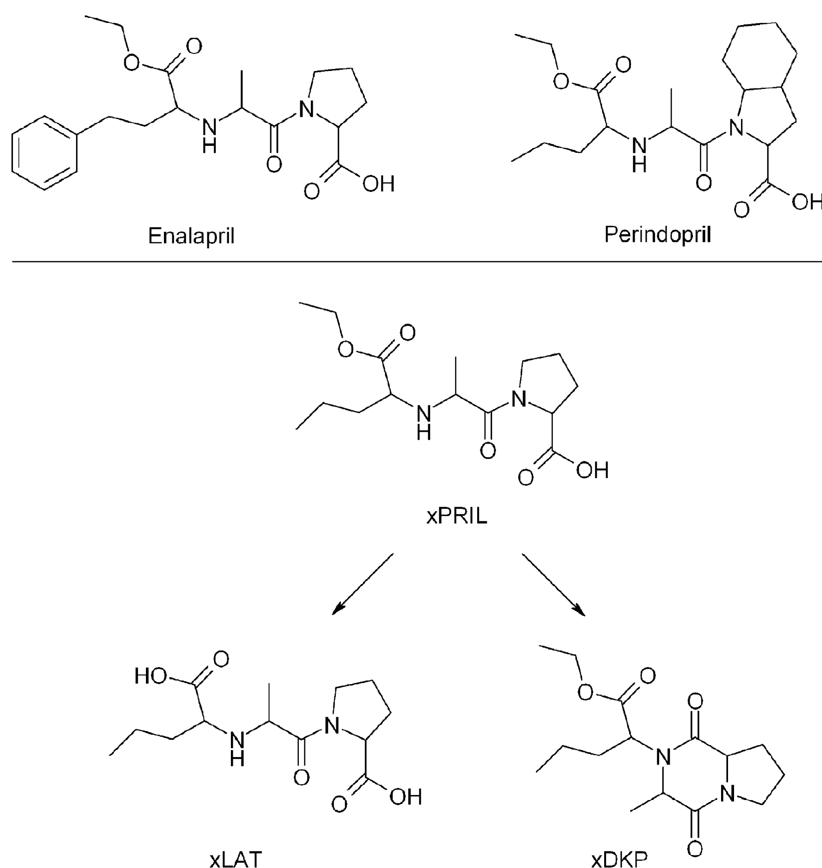

Fig. 2. Structure of EM, PER and xPRIL with its degradation products (xLAT and xDKP).

tem. The LC part consisted of degasser (Degassit, MetaChem, USA) two isocratic pumps (Prostar 210,Varian), autosampler (Prostar 420, Varian) column thermostat (Prostar 510, Varian) and photo-diode array detector (Prostar 330, Varian). MS equipped with electrospray ionization source operated at positive ionization mode. The whole LC–MS system was operated by a Varian MS Workstation, Ver. 6.5. For direct injections of samples into MS (MS/MS analyses) infusion pump Harvard Apparatus 11+ (MA, USA) was used.

For stability studies, temperature controlled water baths were used (Kambic, Slovenia). Other equipments used were pH meter MP 220 (Mettler Toledo, Schwerzenbach, Switzerland), precision analytical balance AG 245 (Mettler Toledo, Schwerzenbach, Switzerland) and auto pipettes (Eppendorf; Hamburg, Germany).

2.3. Methods

2.3.1. HPLC

2.3.1.1. HPLC method used for determination of xPRIL and its degradation products. HPLC instrument (Agilent 1100 Series) with diode array detector and column thermostat was used. This system is optionally equipped with fraction collector for sample collection purposes. Analyses were performed under the following conditions: Synergi Hydro, 4 μm, 250 mm × 4.6 mm (Phenomenex, CA, USA) column at 60 °C, flow rate 1 ml/min and mobile phase 70% of triethylamine aqueous solution (1% aqueous solution of triethylamine adjusted to pH 2.3 with orthophosphoric acid) and 30% methanol. UV detection was performed at 215 nm.

2.3.1.2. HPLC method used for determination of EM and PER and its degradation products. Two similar HPLC area percent methods were used for the determination of the contents of EM, PER and its degradation products. The first method was used for samples containing EM and its degradation products enalaprilat (ET) and diketopiperazine originating from enalapril structure (eDKP) and the second method for determination of PER and its degradation products perindoprilat (PAT) and diketopiperazine originating from perindopril structure (pDKP). HPLC instrument (Agilent 1100 Series) with a variable UV detector and column thermostat was used for both methods. The analyses were performed under the following conditions: Hypersil ODS, 5 μm particles, 250 mm × 4 mm i.d. column (Thermo Scientific, Waltham, USA) at temperature 70 °C. The mobile phases used in each method were phosphate buffer (pH 2.0): acetonitrile (58:42, v/v) in the EM method and phosphate buffer (pH 2.0): acetonitrile in gradient flow in the PER method. The UV detection was performed at 215 nm.

2.3.2. HPLC/mass spectrometry (MS)

LC–MS analyses were performed on a 1200L triple quadrupole MS/MS (Varian) coupled to a Prostar 210 liquid chromatograph (Varian). The chromatographic conditions were the same as in the case of HPLC method for determination of xPRIL (Section 2.3.1). The only adjustment was the use of 0.1% formic acid instead of triethylamine solution in mobile phase (70% formic acid and 30% methanol). The output flow from the column was split in the ratio of 1:3, where one part entered the MS detector and three parts were passed to waste.

Other parameters of MS and MS/MS are presented in Table 1.

For MS/MS analyses infusion pump for samples at flow rate 0.04 ml/min was used. MS/MS breakdown was performed automatically by a Varian MS Workstation, Ver. 6.5.

Table 1
Parameters of MS and MS/MS.

Drying gas	Nitrogen with pressure 145 kPa at 350 °C
Spray voltage	5.0 kV
Shield voltage	600 V
Detector voltage	1300 V
Full scan mode	*m*/*z* 90–360
Mass resolution	1 unit
Collision gas	Argon at 0.2 Pa

2.3.3. Sample preparation

2.3.3.1. Identification of degradation products of xPRIL. Samples of xPRIL (1 mg/ml) in pH 2.0 and 6.8 solutions were prepared and incubated at 80 °C. These stressed samples were diluted with 0.1% formic acid/methanol (70/30, v/v) prior to the LC–MS analyses. For MS/MS analyses xPRIL and its degradation products were separated by HPLC (see chromatographic conditions in Section 2.3.1) and individual chromatographic peaks were collected by fraction collector. Before injection into MS/MS interface, fractions were diluted with 0.1% formic acid/methanol (50/50, v/v).

2.3.3.2. Forced degradation studies of xPRIL, EM and PER. Untreated samples were prepared by weighing approximately 10 mg of xPRIL or 10 mg of EM into a 10 ml volumetric flask, adding the diluent (H_2O) to volume and well mixed. The PER sample was prepared using 60 mg of PER in a 20 ml volumetric flask and treated in the same manner as for xPRIL.

Samples heated in H_2O were prepared by weighing approximately 10 mg of xPRIL or 10 mg of EM into a 10 ml volumetric flask, adding 6 ml of H_2O and heating for about 15 min on a boiling water bath. After boiling samples were cooled diluted to volume and mixed well. The PER sample was prepared using 60 mg of PER in a 20 ml volumetric flask and treated in the same manner as for xPRIL.

Samples heated in H_2O_2 were prepared by weighing approximately 10 mg of xPRIL or 10 mg of EM into a 10 ml volumetric flask, adding 5 ml of H_2O, 1 ml of 3% H_2O_2 and heating for about 15 min on the boiling water bath. After boiling samples were cooled diluted to volume and mixed well. The PER sample was prepared using 60 mg of PER in a 20 ml volumetric flask and treated in the same manner as for xPRIL.

Samples heated in NaOH were prepared by weighing approximately 10 mg of xPRIL or 10 mg of EM into a 10 ml volumetric flask, adding 5 ml of H_2O, 1 ml of 1 M NaOH and heating for about 15 min on the boiling water bath. After boiling samples were cooled, neutralized with 1 ml of 1 M HCl diluted to volume and mixed well. The PER sample was prepared using 60 mg of PER in a 20 ml volumetric flask and treated in the same manner as for xPRIL.

Samples heated in HCl were prepared by weighing approximately 10 mg of xPRIL or 10 mg of EM into a 10 ml volumetric flask, adding 5 ml of H_2O, 1 ml of 1 M HCl and heating for about 15 min on the boiling water bath. After boiling samples were cooled, neutralized with 1 ml of 1 M NaOH diluted to volume and mixed well. The PER sample was prepared using 60 mg of PER in a 20 ml volumetric flask and treated in the same manner as for xPRIL.

2.3.3.3. Stability study of xPRIL, EM and PER. Three sample solutions (a, b and c) of 1 mg/ml of xPRIL were prepared by transferring 250 mg of xPRIL to 250 ml volumetric flask and diluting to mark with buffer pH 2.0 for sample solution a, with buffer pH 6.8 for sample solution b and with buffer pH 12.0 for sample solution c. From each of the sample solutions three aliquots of 50 ml were withdrawn. First aliquot of each sample solution was incubated at temperature 40 °C, second at temperature 60 °C and third at temperature 80 °C.

After incubation of aliquots at different temperatures (40, 60 and 80 °C) samples of 1 ml were withdrawn at different time points. At each time point of stability study two parallel samples were withdrawn (time points used for the withdrawal of the samples are presented in Table 2). The withdrawn samples were stored in

Table 2
Time of sample withdrawal at different temperatures for all three pH solutions.

	pH 2.0 and 6.8	pH 12.0[a]
40 °C	0, 4, 16, 24, 48, 72, 144, 192, 312 and 480 h	0, 10, 20, 30,65, 130, 185 and 270 min
60 °C	0, 4, 16, 24, 48, 72, 144, 192, 312 and 480 h	0, 15, 30, 45, 70, 80, 95, 105 and 125 min
80 °C	0, 4, 16, 24, 48, 72, 144, 192, 312 and 480 h	0, 10, 20, 30, 40 and 50 min

[a] Due to fast degradation of xPRIL in solutions with pH 12.0 the time plan was amended to shorten time period.

refrigerator till the beginning of HPLC analysis. Samples were then directly injected into HPLC system.

The same principle was also used for stability study of PER and EM.

3. Results and discussion

3.1. Validation of developed HPLC stability-indicating methods

3.1.1. HPLC method used for determination of xPRIL and its degradation products

The development of HPLC method used for the evaluation of stability study was based on known published HPLC methods of other ACE inhibitors available in European Pharmacopoeia monographs [12,13]. The main adjustment was in composition of mobile phase. Method was properly validated for the parameters selectivity, linearity, precision and accuracy, limit of quantification and limit of detection. The method was shown to be linear in range from 5×10^{-3} to 1.25 mg/ml with correlation coefficient higher than 0.99. Accuracy (relative error) and precision (relative standard deviation) were less than ±5%. The limit of detection was 3.3×10^{-4} and the limit of quantification was 1.0×10^{-3} mg/ml. Since standards of degradation products were not available the above parameters were not determined for xLAT and xDKP.

The method was shown to be selective with peaks of xPRIL, xLAT and xDKP completely separated from each other. The sample chromatograms are presented in Fig. 8. Peak purity factors were higher than the requested limit of 990 (the individual purity factors were higher than 998).

During the stability study the samples were also analysed by the LC–MS method and individual peaks were identified as xPRIL (t_r = 6.6 min), xLAT (t_r = 2.7 min) and xDKP (t_r = 9.7 min). These results confirmed the suitability of the applied HPLC method.

3.1.2. HPLC method used for determination of EM and its degradation products

HPLC method for determination of EM and its degradation products ET and eDKP was developed based on EM method from European Pharmacopoeia [12]. It was properly validated as required under ICH guidelines Q2(R1) [17] for the parameters repeatability, selectivity, linearity, precision and accuracy, limit of quantification and limit of detection. The method was shown to be linear in ranges from 0.2×10^{-3} to 100.3×10^{-3} mg/ml for EM, from 0.051×10^{-3} to 51.3×10^{-3} mg/ml for ET, and from 0.051×10^{-3} to 51.3×10^{-3} mg/ml for eDKP with individual correlation coefficients higher than 0.999. The limits of detection were 0.05 μg/ml for EM, 0.013 μg/ml for ET and 0.013 μg/ml for eDKP. The limits of quantification were 0.20 μg/ml for EM, 0.05 μg/ml for ET and 0.05 μg/ml for eDKP. The method was shown to be accurate for EM, ET and eDKP. The recoveries were within ±5% of the expected value (results were from 95% to 104%). The method was shown to be selective with peaks of EM, ET and eDKP completely separated from each other. The chromatogram of solution for selectivity control is presented in Fig. 3. Peak purity factors were higher than the requested limit of 990 (the individual purity factors were higher than 998).

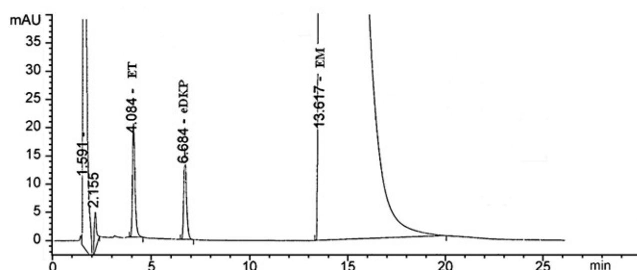

Fig. 3. Chromatogram of solution for selectivity control of EM HPLC method.

3.1.3. HPLC method used for determination of PER and its degradation products

HPLC method for determination of PER and its degradation products PAT and pDKP was developed based on PER method from European Pharmacopoeia [13]. It was properly validated as required under ICH guidelines Q2(R1) [17] for the parameters repeatability, selectivity, linearity, precision and accuracy, limit of quantification and limit of detection. The method was shown to be linear in ranges from 0.3×10^{-3} to 93.6×10^{-3} mg/ml for PAT, from 0.15×10^{-3} to 101.0×10^{-3} mg/ml for pDKP, and from 0.15×10^{-3} to 97.1×10^{-3} mg/ml for PAT with individual correlation coefficients higher than 0.999. The limits of detection were 0.075 μg/ml for PER, 0.150 μg/ml for PAT and 0.075 μg/ml for pDKP. The limits of quantification were 0.150 μg/ml for PER, 0.300 μg/ml for PAT and 0.150 μg/ml for pDKP. The method was shown to be accurate for PER, PAT and pDKP. The recoveries were within ±5% of the expected value (results were from 98% to 102%). The method was shown to be selective with peaks of PER, PAT and pDKP completely separated from each other. The chromatogram of solution for selectivity control is presented in Fig. 4. Peak purity factors were higher than the requested limit of 990 (the individual purity factors were higher than 998).

3.2. Degradation path of xPRIL

Degradation path of xPRIL was expected to be similar to known ACE inhibitors (degradation to xLAT by hydrolysis of ethyl ester and to xDKP by intramolecular cyclization). The degradation mechanism of the two main degradation paths as foreseen and confirmed by stability study is presented in Fig. 2.

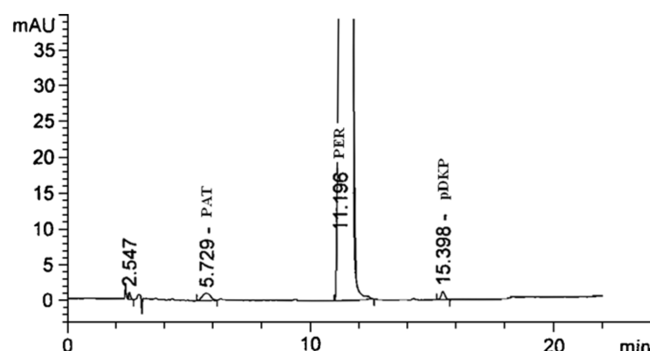

Fig. 4. Chromatogram of solution for selectivity control of PER HPLC method.

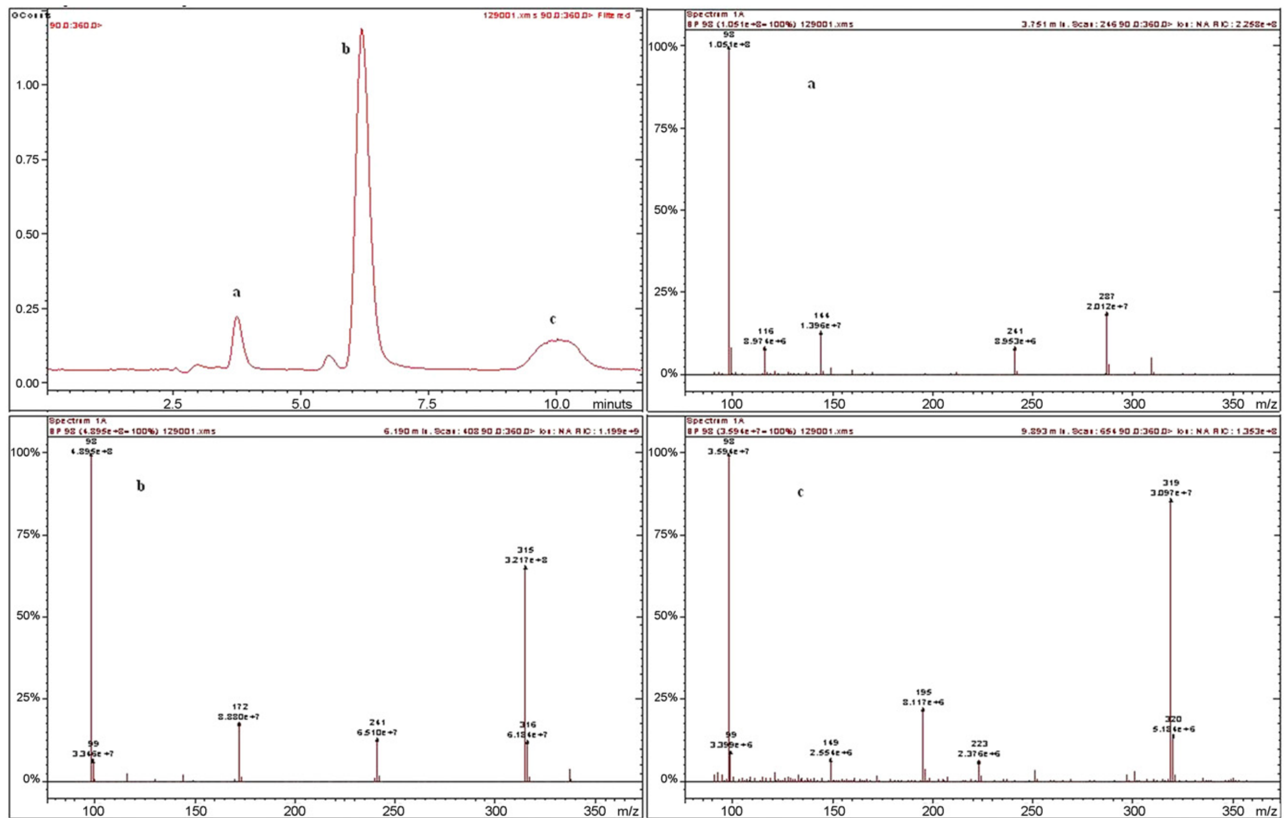

Fig. 5. LC and MS spectra of individual peaks obtained on LC–MS system ((a) xLAT, (b) xPRIL, and (c) xDKP).

During the development of the HPLC method for xPRIL several samples of partially degraded xPRIL were analysed. Due to the nature of HPLC column and polarity of xPRIL and the two main degradation products (xLAT and xDKP) the sequence of the elution was anticipated and confirmed for all three molecules. Three eluted peaks were identified by LC–MS as presented in Fig. 5. The first eluted peak with the shortest retention time was identified as xLAT with signals m/z 309 (xLAT + Na^+), 287 (xLAT + H^+), and other xLAT fragments with m/z 241, 144 and 98. The second peak was identified as xPRIL with clear signal m/z 315 (xPRIL + H^+) and its fragments 241, 172 and 98. The last peak belonging to the most lipophilic molecule was identified as xDKP with m/z 319 (xDKP + Na^+) and its fragments with m/z 223, 195 and 98.

With the aim to determine fragmentation of each individual molecule (xPRIL, xLAT and xDKP) the fractions of each individual peak were collected and MS/MS analysis of these samples was performed. An MS/MS breakdown spectrum of xDKP presented in Fig. 6 is in agreement with MS spectrum of LC–MS analysis (Fig. 5c). With MS/MS analysis some low intensity fragmentation to m/z 172 was additionally observed. Good agreement between both spectra was found also in case of xPRIL and xLAT.

Results of MS/MS analysis confirmed the proposed fragmentation paths (as presented in Fig. 7). The major fragmentation route of xPRIL and xLAT includes elimination of ethylformate from xPRIL and formic acid from xLAT resulting in fragment with m/z value of 241.3. Second fragmentation route that was identified for xPRIL and

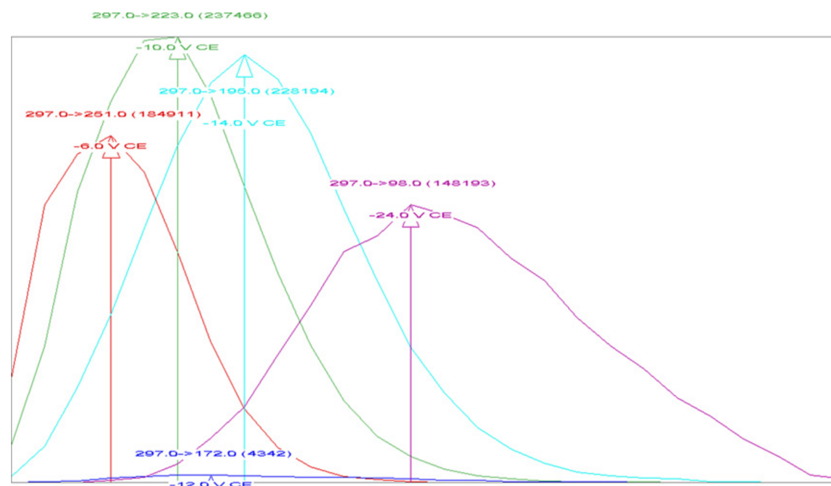

Fig. 6. Fragmentation of ion with m/z 297 belonging to xDKP (MS/MS breakdown analysis).

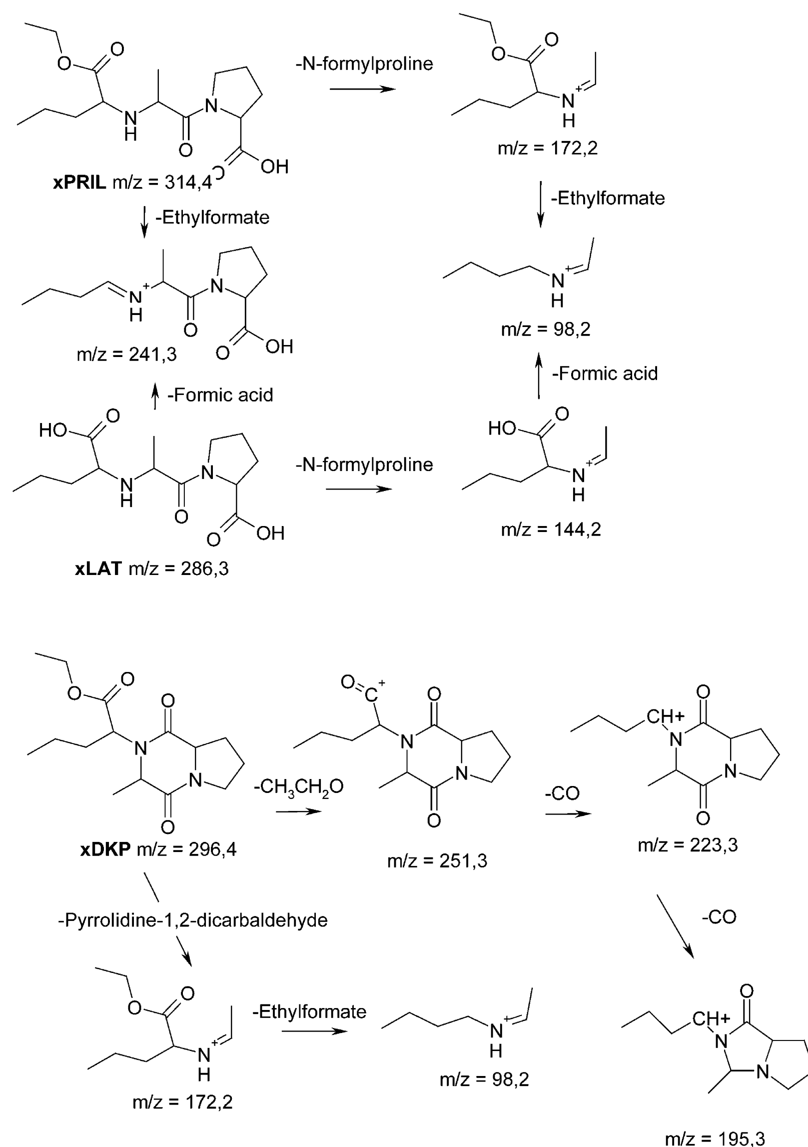

Fig. 7. Fragmentation path of xPRIL and its degradation products.

xLAT involves elimination of N-formylproline resulting in the formation of fragments with *m*/*z* values of 172.2 and 144.2. These two ions can further fragment loosing ethylformate or formic acid into *m*/*z* 98.2 ion.

The degradation product xDKP follows a separate fragmentation route compared to xPRIL and xLAT as outlined in Fig. 7. One of the routes includes elimination of CH_3CH_2O resulting in *m*/*z* 251.3 fragment that after two sequential eliminations of CO results in fragments with *m*/*z* 223.3 and *m*/*z* 195.3. Second fragmentation path of xDKP can be explained by elimination of pyrrolidine-1,2-dicarbaldehyde resulting in *m*/*z* 172.2 fragment that afterwards by elimination of ethylformate results in *m*/*z* 98.2 fragment. Observed fragmentation pattern with present *m*/*z* 172.2 of xDKP is somehow different from already known fragmentation of similar ACE inhibitors [18] and presents new fragmentation pattern characterized in this study.

3.3. Forced degradation studies of xPRIL, EM and PER

xPRIL, EM and PER were exposed to various stress conditions with the aim to obtain and compare information on their individual basic stability properties (intrinsic stability). The exposed stress conditions were very rigorous and cannot be directly compared to the conditions from stability study described below. The stability-indicating HPLC methods as described in Section 2.3 were used for determination of each individual substance and its degradation products. The results are presented in Table 3.

In the forced degradation study under the conditions mentioned in Section 2.3.3, it was shown that active drug substance xPRIL and EM are stable in water since less than 1% of decomposition occurred after heating. PER showed higher degradation since almost 9% of the substance hydrolyzed to PAT. From these facts we can conclude that perhydroindole which is a structural element of PER induces hydrolysis of ester bond in water medium in higher degree than proline heterocycle present in xPRIL and EM.

Forced degradation of all three substances in H_2O_2 mainly resulted in degradation through other degradation paths (not hydrolysis or cyclization) that resulted in many peaks observed in HPLC chromatogram. Amount of these additional degradation products was the highest in PER sample (approximately area 7%). PER was also the only molecule that hydrolyzed in H_2O_2 into PAT in higher extent. Since in stability study of xPRIL only xDKP and xLAT were observed as degradation products the degradation through other degradation paths was not studied in details.

Table 3
HPLC results of samples exposed to forced degradation study.

xPRIL	EM	PER
Untreated sample		
xPRIL – .2%	EM – 99.8%	PER – 99.8%
xLAT – bld[a]	LAT – 0.03%	PAT – 0.07%
xDKP – 0.77%	DKP – 0.03%	DKP – 0.08%
	Add peak[b]	Add peak[b]
Sample heated in H_2O		
xPRIL – 99.1%	EM – 99.7%	PER – 91%
xLAT – 0.15%	LAT – 0.06%	PAT – 8.7%
xDKP – 0.77%	DKP – 0.12%	DKP – 0.08%
	Add peak[b]	Add peak[b]
Sample heated in H_2O_2		
xPRIL – 96.7%	EM – 98.9%	PER – 88%
xLAT – 0.37%	LAT – 0.08%	PAT – 4.6%
xDKP – 0.84%	DKP – 0.10%	DKP – 0.08%
Add peak[b]	Add peak[b]	Add peak[b]
Sample heated in NaOH		
xPRIL – bld[a]	EM – 1.5%	PER – 4%
xLAT – 100%	LAT – 98%	PAT – 95%
	DKP – 0.03%	DKP – 0.08%
	Add peak[b]	Add peak[b]
Sample heated in HCl		
xPRIL – 98.5%	EM – 99.3%	PER – 97.5%
xLAT – 0.65%	LAT – 0.43%	PAT – 1.9%
xDKP – 0.77%	DKP – 0.13%	DKP – 0.5%
Add peak[b]	Add peak[b]	Add peak[b]

[a] Bld – below limit of detection.
[b] Add peaks – additional peaks were present on the chromatogram.

Samples of xPRIL and EM treated in boiling NaOH were fully degraded and xLAT and ET as the main degradation products were formed. In PER sample the degradation was also high but a small portion of PER was still present. Based on this fact one could propose that PER would be more resistant to higher pH and would therefore have somewhat higher stability in environments with higher pH compared to EM and xPRIL. Again the only reasonable explanation would be the influence of perhydroindole heterocycle as the only structural difference on stability properties. All three substances were quite stable in neutral and acidic environment.

Comparing the results and chemical structures of all three substances it can be concluded that PER is the substance with the lowest stability in all tested samples and on the other side EM is the substance with the highest stability in all tested samples. For the comparison we excluded the results from NaOH solution since the degradation of all three molecules was almost complete after the study. xPRIL built of structural elements of both, EM and PER showed that its stability properties in studied conditions are in between the stability properties of EM and PER. The nature of degradation products and degradation kinetics of PER through different degradation paths can be in qualitative manner somehow differentiated from the other two molecules (i.e. xPRIL and EM).

We could conclude that among different structural elements studied, the heterocycle of ACE inhibitor has higher influence on stability properties compared to the side chain of the ACE inhibitor. For a clearer picture of the role that the individual structural element has on the stability properties of the ACE inhibitor some additional studies would be necessary.

3.4. *Stability study of xPRIL in solutions and comparison of drug degradation kinetics between xPRIL, EM and PER*

After obtained general picture of xPRIL, EM and PER stability properties we studied the degradation kinetics of xPRIL in aqueous solutions and compared it with degradation kinetics of EM and PER in same medium and conditions.

The most important factors that affect stability of substances in solutions are pH and temperature. The aim of our stability study in solutions was therefore to determine stability properties of xPRIL at three different temperatures (40, 60 and 80 °C) and at three different pH (2.0, 6.8 and 12.0). HPLC method was used to measure the concentration of xPRIL and its degradation products in treated samples as a function of time.

In pH 2.0 degradation products xDKP and xLAT were formed in a similar extent. In pH 6.8 and 80 °C the degradation of xPRIL was approximately 6 times faster than in pH 2. The degradation to xLAT prevailed even though the degradation to xDKP was still present in a certain extent. The degradation was the fastest at pH 12 and was limited to the formation of xLAT only. The chromatograms of samples stored in solutions with different pH are presented in Fig. 8. The degradation of xPRIL at observed conditions was shown to follow the first order kinetics. The Arrhenius equations for each pH were determined and are presented in Table 4.

Table 4
First order kinetic constants for degradation of xPRIL determined in stability study of xPRIL in solutions with different pH at different temperatures.

pH 2.0	pH 6.8	pH 12.0
k (determined from the stability study)		
40 °C		
$k = 8.3 \times 10^{-5}$ h^{-1}	$k = 7.1 \times 10^{-4}$ h^{-1}	$k = 3.3 \times 10^{-1}$ h^{-1}
$r^2 = 0.9723$	$r^2 = 0.9975$	$r^2 = 0.9972$
60 °C		
$k = 7.6 \times 10^{-4}$ h^{-1}	$k = 5.2 \times 10^{-3}$ h^{-1}	$k = 9.2 \times 10^{-1}$ h^{-1}
$r^2 = 0.9717$	$r^2 = 0.9993$	$r^2 = 0.9903$
80 °C		
$k = 4.4 \times 10^{-3}$ h^{-1}	$k = 2.7 \times 10^{-2}$ h^{-1}	$k = 3.1$ h^{-1}
$r^2 = 0.9766$	$r^2 = 0.9878$	$r^2 = 0.9913$
k[a] (calculated)		
25 °C		
$k = 1.4 \times 10^{-5}$ h^{-1}	$k = 1.4 \times 10^{-4}$ h^{-1}	$k = 1.2 \times 10^{-1}$ h^{-1}
Arrhenius equation for degradation of xPRIL		
$\ln k = -11099 \times 1/T + 26.065$	$\ln k = -10146 \times 1/T + 25.154$	$\ln k = -6151.2 \times 1/T + 18.486$
$r^2 = 0.9974$	$r^2 = 0.9996$	$r^2 = 0.9974$
E_a (kJ mol^{-1})		
92.3	84.4	51.1

[a] k was calculated from presented Arrhenius equation.

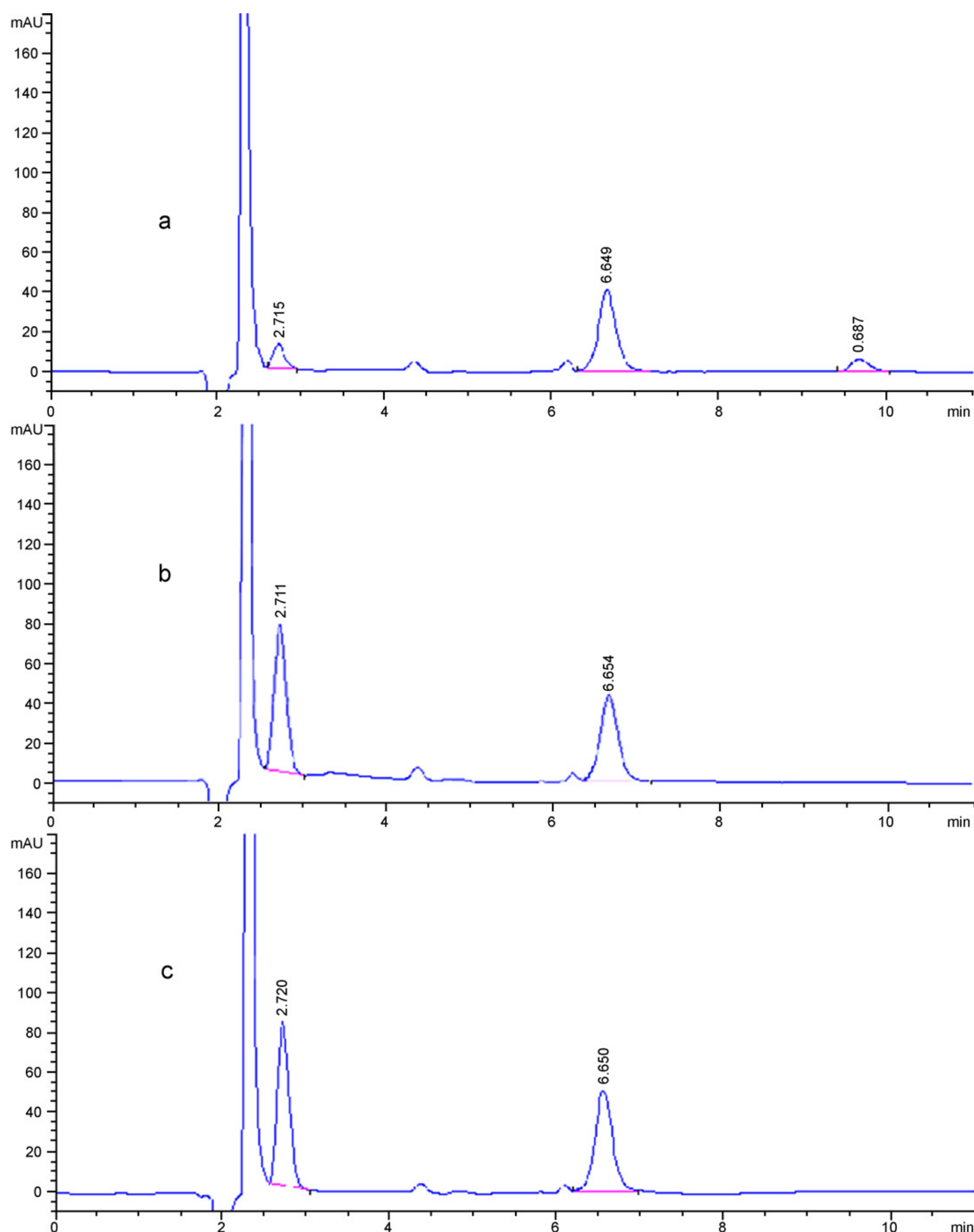

Fig. 8. Typical degradation chromatograms of the xPRIL samples stored in pH 2.0 at 80 °C for 144 h (a), in pH 6.8 at 80 °C for 24 h (b) and in pH 12.0 at 80 °C for 20 min (c).

According to the results originating from our study and described above it is evident that degradation of xPRIL is pH and temperature dependant. With increasing temperature the amount of degraded xPRIL increases at all three pH. Comparison of degradation kinetics at different pH indicates that with increasing pH (within individual pH 2.0, 6.8 and 12.0) also the rate of degradation of xPRIL increases. Using the results of individual degradation kinetics of xPRIL at different temperatures the Arrhenius equations were determined and the activation energies (E_a) for the degradation of xPRIL were calculated at each pH. Comparison of these results indicated that the activation energy was the highest at pH 2.0. This means that the temperature has the highest influence on degradation of xPRIL at the lowest pH. With Arrhenius equations the kinetic constants for all three pH solutions at room temperature (25 °C) were also determined. At room temperature, t_{90} (time required for 10% loss of starting material) values are 313 days, 31 days and 52 min for solutions with pH 2.0, 6.8 and 12.0, respectively.

In second part of the stability study we compared xPRIL degradation kinetics in biorelevant pH solutions (pH 2.0 and 6.8) with degradation kinetics of PER and EM. Values of determined degradation kinetic constants are presented in Table 5. From the results it is evident that the kinetics of degradation of individual ACE inhibitor is influenced by its structure. Comparing stability at room temperature and different pH EM showed higher stability in pH 6.8 then in pH 2.0 solution whereas PER and xPRIL showed higher stability in pH 2.0 solution.

When comparing the individual degradation kinetics in pH 2.0 we concluded that by the kinetics and by Arrhenius parameters PER somehow differ from EM and xPRIL. Lower activation energy (E_a) of PER is a result of lowest influence of temperature on degradation

Table 5
First order kinetic constants for degradation of xPRIL, EM and PER determined in stability studies of xPRIL, EM and PER in bio relevant solutions with pH 2.0 and 6.8 at different temperatures with calculated Arrhenius equation and kinetic constant at room temperature.

pH 2.0	pH 6.8
80 °C	
xPRIL	
$k = 4.4 \times 10^{-3}\ h^{-1}$, $r^2 = 0.977$	$k = 2.7 \times 10^{-2}\ h^{-1}$, $r^2 = 0.988$
EM	
$k = 7.3 \times 10^{-3}\ h^{-1}$, $r^2 = 0.991$	$k = 4.3 \times 10^{-3}\ h^{-1}$, $r^2 = 0.992$
PER	
$k = 2.6 \times 10^{-3}\ h^{-1}$, $r^2 = 0.992$	$k = 3.1 \times 10^{-2}\ h^{-1}$, $r^2 = 0.996$
60 °C	
xPRIL	
$k = 7.6 \times 10^{-4}\ h^{-1}$, $r^2 = 0.972$	$k = 5.2 \times 10^{-3}\ h^{-1}$, $r^2 = 0.999$
50 °C	
EM	
$k = 3.9 \times 10^{-4}\ h^{-1}$, $r^2 = 0.996$	$k = 2.3 \times 10^{-4}\ h^{-1}$, $r^2 = 0.998$
PER	
$k = 2.6 \times 10^{-4}\ h^{-1}$, $r^2 = 0.719$	$k = 2.7 \times 10^{-3}\ h^{-1}$, $r^2 = 0.999$
40 °C	
xPRIL	
$k = 8.3 \times 10^{-5}\ h^{-1}$, $r^2 = 0.972$	$k = 7.1 \times 10^{-4}\ h^{-1}$, $r^2 = 0.998$
EM	
$k = 1.0 \times 10^{-4}\ h^{-1}$, $r^2 = 0.940$	$k = 3.6 \times 10^{-5}\ h^{-1}$, $r^2 = 0.799$
PER	
$k = 1.5 \times 10^{-4}\ h^{-1}$, $r^2 = 0.840$	$k = 8.3 \times 10^{-4}\ h^{-1}$, $r^2 = 0.987$
25 °C[a]	
xPRIL	
$k = 1.4 \times 10^{-5}\ h^{-1}$	$k = 1.4 \times 10^{-4}\ h^{-1}$
EM	
$k = 1.7 \times 10^{-5}\ h^{-1}$	$k = 5.8 \times 10^{-6}\ h^{-1}$
PER	
$k = 5.2 \times 10^{-5}\ h^{-1}$	$k = 1.7 \times 10^{-4}\ h^{-1}$
Arrhenius equation	
xPRIL	
$\ln k = -11100 \times 1/T + 26.1$, $r^2 = 0.997$	$\ln k = -10150 \times 1/T + 25.2$, $r^2 = 0.999$
EM	
$\ln k = -11700 \times 1/T + 28.3$, $r^2 = 0.998$	$\ln k = -12770 \times 1/T + 30.8$, $r^2 = 0.984$
PER	
$\ln k = -7320 \times 1/T + 14.7$, $r^2 = 0.986$	$\ln k = -9910 \times 1/T + 24.6$, $r^2 = 0.996$
E_a (kJ mol^{-1})	
xPRIL	
92.3	84.4
EM	
97.3	106.2
PER	
60.9	82.4

[a] Calculated based on Arrhenius equation.

kinetics. At the same time the rate of degradation of PER at room temperature is the highest among all three. Based on known structural differences between all three molecules we can conclude that perhydroindole being part of PER somehow influence and accelerate the degradation in pH 2.0 solution but at the same time lowers the influence of temperature on degradation in comparison to proline heterocycle that is present in xPRIL and EM.

Contrary, in pH 6.8 solutions similar degradation kinetics and Arrhenius parameters were observed between xPRIL and PER. Degradation of both was faster compared to EM. From these results and the fact that the only structural difference between EM and the other two molecules is in presence of aromatic phenyl group in EM it was concluded that this group has the highest impact on stabilising the EM and decelerating the degradation in pH 6.8 solution.

4. Conclusion

This paper presents the degradation path and stability properties of molecule xPRIL. The determined degradation products of xPRIL were xLAT that was formed by hydrolysis of ethyl ester and xDKP that was formed by intramolecular cyclization. Both degradation products were identified with MS. For all three molecules (xPRIL, xLAT and xDKP) the fragmentation patterns were elucidated. Comparison of xPRIL, EM and PER stability properties obtained from the forced degradation studies at rigorous conditions was performed. The results indicated that xPRIL stability properties in qualitative manner were more alike to the more stable EM than to the less stable PER. Based on the results it was concluded that the structural element having the highest influence on stability properties among the tested elements was perhydroindole heterocycle since it reduced the stability of PER molecule.

Furthermore, the stability study of xPRIL in solutions at different pH and at different temperatures was performed and stability properties were compared with known ACE inhibitors, EM and PER. It was found that the mechanism and the rate of degradation of xPRIL are highly pH dependent. In pH 2.0 degradation products xDKP and xLAT were formed in a similar extent. In pH 6.8 the degradation of xPRIL was approximately 6 times faster than in pH 2.0. The degradation to xLAT prevailed even though the degradation to xDKP was still present in a certain extent. The degradation was the fastest at pH 12.0 and was limited to the formation of xLAT only.

Comparison of degradation kinetics among xPRIL, EM and PER in two bio relevant pH (2.0 and 6.8) indicated some impact of structural elements on stability properties of ACE inhibitors. In pH 2.0 solution xPRIL and EM showed slower degradation compared to PER. This fact indicated that perhydroindole heterocycle which is a structural element present only in PER caused faster degradation compared to proline that is structural element present in xPRIL and EM. Contrary, in pH 6.8 similar degradation rates of xPRIL and PER were noticed and were much higher than degradation rate of EM. Based on this fact we concluded that at pH 6.8 aromatic phenyl group is the structural element with the highest influence on stability properties. It caused slower degradation of EM compared to aliphatic propyl element which is part of xPRIL and PER.

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