

ANALYSIS

MECHANISM OF SOLVOLYSIS OF *N*-[2-(4-*o*-FLUOROPHENYLPIPERAZIN-1-YL)ETHYL]-2,5-DIMETHYL-1-PHENYLPYRROLE-3,4-DICARBOXIMIDE (PDI)AGNIESZKA SOBCZAK^{1*}, MARIANNA ZAJĄC¹, WIESŁAW MALINKA²
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Abstract: The stability of *N*-[2-(4-*o*-fluorophenylpiperazin-1-yl)ethyl]-2,5-dimethyl-1-phenylpyrrole-3,4-dicarboximide (PDI; a derivative with an analgesic activity) was studied in order to investigate its degradation mechanism and identify its degradation products in aqueous-organic solutions. The stability of PDI and its two degradation products (**A** and **B**) was performed with an HPLC method: (LiChrospher C18 column (250 × 4 mm I.D., dp = 5 μm), mobile phase: 3.5 g/L solution of sodium lauryl sulfate and 1.6 mL of phosphoric acid(V) - acetonitrile (40:60, v/v), UV detector: 240 nm, flow rate: 1 mL/min). The identification of products **A** and **B** was conducted using HPLC-ES-MS and ¹H- and ¹³C-NMR methods.

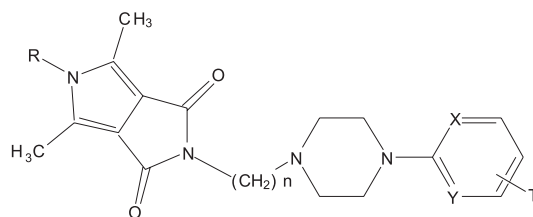
Keywords: kinetics of degradation, stability, derivatives of pyrroledicarboximide, HPLC-ES-MS, ¹H- and ¹³C-NMR methods

Cyclic imides, because of their various chemical structure and pharmacological activity, have been extensively used in medicine. Their chemical structure is often the basis for designing new groups of biologically active compounds.

Derivatives of imides applied in medicine include the following groups: derivatives of barbituric acid, hydantoins with anticonvulsant activity or new generation anxiolytics, i.e. buspirone, tandospirone and gepirone. The fact that anxiolytic activity of buspirone results from its reaction with receptor 5-HT_{1A} contributed to seeking new ligands for this receptor. Many newly synthesized groups of compounds have been designed based on the buspirone structure by modification of its three main fragments: substituents in aryl- or heteroaryl piperazine, the length of the central alkanyl chain and a fragment at the imide group. Most of the obtained derivatives are characterized by serotonergic, anxiolytic and antidepressive activity (1). Furthermore, some compounds show additionally analgesic or antiinflammatory properties. The example of buspirone analogs are derivatives of pyrroledicarboximide (Fig. 1). Most of them demonstrate moderate acute toxicity (LD₅₀), suppress spontaneous and

amphetamine-induced locomotor activity in mice (central nervous system depressive action). Some of them show additionally analgesic activity. Furthermore, in preliminary screening none of the investigated compounds had anxiolytic or anticonvulsive properties, influenced arterial blood pressure or pulse in rats (2).

While investigating the structure-activity relationship in pyrroledicarboximide derivatives, Malinka et al. demonstrated that substituents R, T, X, Y (Fig. 1) and changes in the central alkanyl



IA R = Ar, *n*-C₄H₉, X, Y = CH or N, T = H
IB R = Ar, *n*-C₄H₉, X, Y = CH, T = *o*-Cl, *o*-CH₃O, *m*-Cl, *m*-CF₃
IC R = Heteroatom, X = N, Y = N or CH, T = H

Fig. 1. Structure of pyrrole-3,4-dicarboximide derivatives (3).

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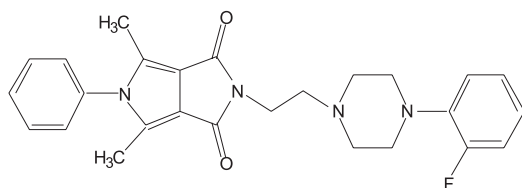


Figure 2. Structure of *N*-[2-(4-*o*-fluorophenyl)piperazin-1-yl]ethyl]-2,5-dimethyl-1-phenylpyrrole-3,4-dicarboximide (PDI).

chain (*n*) were essential for toxicity and pharmacological activity. It was found that *N*-[2-(4-*o*-fluorophenyl)piperazin-1-yl]ethyl]-2,5-dimethyl-1-phenylpyrrole-3,4-dicarboximide (PDI) displays the desired properties (Fig. 2). The analgesic activity of PDI ($ED_{50} = 9.35$ mg/kg) was approx. 4 times greater than that of acetylsalicylic acid ($ED_{50} = 39.15$ mg/kg) in the "writhing test" and its toxicity was low ($LD_{50} > 2000$ mg/kg) (2, 3).

In our previous studies we investigated the kinetics of the degradation of PDI in aqueous-organic solutions. The solvolysis of PDI was a pseudo-first order reaction in which two degradation products were formed. The specific acid-base catalysis of PDI involved the following reactions:

- solvolysis of the undissociated molecules of PDI catalyzed by hydroxyl ions;
- spontaneous solvolysis of the undissociated molecules and monoprotonated forms of PDI under the influence of solvent (4, 5).

The purpose of these studies is to identify the degradation products of PDI with MS and NMR methods and to investigate the kinetics of their formation and degradation using an HPLC method. These studies allow for establishing the degradation pathway of PDI. The knowledge of the chemical structure of degradation products is important in the design of potential drugs. Due to it, it is possible to reject compounds which have toxic degradation products in the initial stage of research or to modify their structure.

EXPERIMENTAL

Materials and reagents

The compound, *N*-[2-(4-*o*-fluorophenyl)piperazin-1-yl]ethyl]-2,5-dimethyl-1-phenylpyrrole-3,4-dicarboximide, ($C_{26}H_{27}FN_4O_2$; molecular mass: 446.21; melting point: 158–160°C) designated as PDI in this paper, was synthesized in the Department of Chemistry of Drugs, Wrocław Medical University, Poland. All other chemicals and solvents were of analytical or high-performance liquid chromatographic grade.

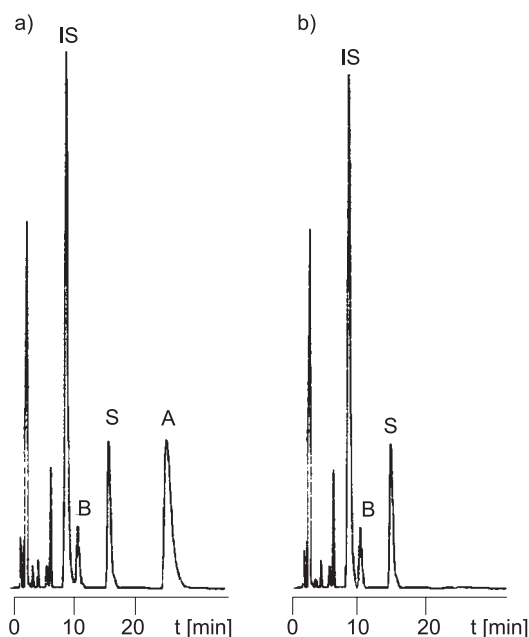


Figure 3. Chromatograms of PDI (S), its products of degradation (A, B) and internal standard (IS), after solvolysis in: a) acetone – ethanol – water solution of 0.02 mol/L sodium hydroxide at 308 K, for $t = 10$ min, b) acetone – water solution of 0.02 mol/L sodium hydroxide at 308 K, for $t = 40$ min.

Chromatographic conditions

The compound to be analyzed and its products of degradation were determined by means of a high-performance liquid chromatograph equipped with an LC-6A pump (Shimadzu), a UV-VIS detector SPD-6AV (Shimadzu) and a Rheodyne 7120 (50 μ L fixed-loop injector). A Merck analytical column LiChrospher 100 C-18 (250 \times 4 mm ID, $dp = 5$ μ m) was used as the stationary phase. The method was conducted using a reversed-phase technique. The mobile phase consisted of 3.5 g/L solution of sodium lauryl sulfate and 1.6 mL phosphoric acid(V) - acetonitrile (40:60, V/V); the flow rate was 1 mL/min. Detection was performed at 240 nm. The internal standard (IS) was antazoline sulfate in a mixture of ethanol and water (1:1, v/v) at a concentration of 0.3 mg/mL.

Validation of the HPLC method

The HPLC method was validated with regard to: selectivity, linearity, precision, limits of detection and quantitation and robustness. The method is selective for PDI, degraded products A and B and internal standard, as shown in Figure 3. In the chromatograms taken over a period of 0 – 30 min, the following peaks emerged:

- S, corresponding to compound PDI, with $t_R = 16.23$ min

- A, corresponding to the degradation product **A** with $t_R = 26.44$ min
- B, corresponding to the degradation product **B** with $t_R = 11.07$ min
- IS, corresponding to internal standard, with $t_R = 9.13$ min.

The linearity between P/P_{IS} (P and P_{IS} = peak areas of PDI and IS, respectively) and concentrations of PDI ranging from 20 to 140 mg/mL were evaluated. The parameters of regression were as follows: $y = (15.36 \pm 0.60)x$; $r = 0.9984$; $n = 11$ (b value = -0.0271 calculated from the equation $y = ax + b$ was statistically insignificant).

The intra-day precision of the measurements was good because the relative standard deviation (RSD) was: 1.29% for 20 mg/mL, 0.76% for 40 mg/mL, 1.02% for 80 mg/mL and 0.74% for 120 mg/mL ($n = 6$ for each concentration).

The limits of detection (LOD = 6.83 mg/mL) and quantitation (LOQ = 20.70 mg/mL) were calculated from the formulas $LOD = 3.3S_y/a$ and $LOQ = 10S_y/a$, where S_y is the standard deviation of the blank signal and a is the slope of the corresponding calibration curve.

The influence of changes in the quantitative composition of the mobile phase (concentration of sodium salt of lauryl sulfate ranging from 3.0 g/L to 4.0 g/L and of acetonitrile from 55 to 65%, V/V) on chromatographic separation was investigated. It was found that these changes affected the shape and symmetry of the peaks.

Kinetic procedures

The stability of PDI and its degradation products investigated in three aqueous-organic solutions:

- acetone-ethanol-water solution of 0.02 mol/L sodium hydroxide (4:46:50, $v/v/v$) pH 11.92 at 308 K (solution I),
- acetone-water solution of 0.02 mol/L sodium hydroxide (50:50, v/v) pH 11.92 at 308 K (solution II),

- acetone-ethanol-water solution of Britton-Robinson's buffer (4:45:50, $v/v/v$) pH 7.23 at 333 K (solution III).

The ionic strength, $\mu = 0.5$ mol/L, was adjusted for each solution by adding a calculated amount of sodium chloride (4.0 mol/L). The pH values for sodium hydroxide solutions were calculated from the equation: $pH = pK_w + \log f_{NaOH} [NaOH]$. The activity coefficients f_{NaOH} were taken from the literature [6]. The pH value of the buffers was measured potentiometrically (a CD-401 pH-meter, Elmtron, Zabrze, Poland) at reaction temperature. The degradation was initiated by adding a dissolved sample of PDI (5 mg in 1.0 mL of acetone) to an aqueous-organic solution of specified pH, equilibrated at 308 K in a stoppered flask.

The initial concentration of PDI in the samples to be examined was 0.2 mg/mL. At specified time intervals 0.5 mL of the reaction solution was collected, neutralized and instantly cooled with a mixture of ice and water. To each sample, 0.25 mL of the IS solution was added. 50 mL of the so obtained solutions was injected onto the column.

HPLC-ES-MS and NMR

The HPLC-ES-MS and NMR spectra were obtained at the Faculty of Chemistry at Adam Mickiewicz University, Poznań, Poland.

The NMR spectra were recorded with a Varian Mercury 300 spectrophotometer, operating at 300 MHz for 1H -NMR and 75 MHz for ^{13}C -NMR. For the 1H -NMR analysis, the following parameters were used: aquisition time at = 3.5 s, number of transition, $nt = 64$, power width, $pw = 8.8$; for ^{13}C -NMR analysis: at = 1.5 s, current of transition, $ct = 40876$ (PDI) or 40856 (B), $pw = 8.8$ (PDI) or 9.3 (B). Samples for the NMR study were dissolved in deuterated methanol and tetramethylsilane was used as the internal reference.

Isolation

PDI was stored in solution II (1.2 mg/mL) at 308 K until only product **B** was present in the solu-

Table 1. The gradient profile of the HPLC method.

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0 – 30	100 → 0	0 → 100
30 – 31	0 → 100	100 → 0
31 – 40	100	0

Table 2. The observed rate constants for the degradation of PDI and for the formation and degradation of products A and B.

Solution I			
Compound	$k \pm Dk$	-r	n
PDI	$k_d = (3.16 \pm 0.04) \times 10^{-3} [s^{-1}]$	0.9999	9
A	$k_d = (8.02 \pm 0.89) \times 10^{-6} [s^{-1}]$	0.9939	8
	$k_f = (3.58 \pm 0.78) \times 10^{-3} [s^{-1}]$	0.9827	7
B	$k_f = (7.97 \pm 0.44) \times 10^{-6} [s^{-1}]$	0.9977	10
Solution II			
PDI	$k_d = (4.64 \pm 0.29) \times 10^{-4} [s^{-1}]$	0.9965	11
B	$k_f = (4.64 \pm 0.21) \times 10^{-4} [s^{-1}]$	0.9989	8
Solution III			
PDI	$k_d = (2.23 \pm 0.08) \times 10^{-5} [s^{-1}]$	0.9991	10
A	$k_f = (1.29 \pm 0.10) \times 10^{-5} [s^{-1}]$	0.9959	10
B	$k_f = (1.01 \pm 0.10) \times 10^{-5} [s^{-1}]$	0.9920	10

k_d – observed rate constants of degradation

k_f - observed rate constants of formation

Table 3. ¹H- and ¹³C-NMR chemical shifts for PDI and product B (solvent CD₃OD).

Group	PDI		Product B	
	¹ H	¹³ C	¹ H	¹³ C
-CH ₃	2.15	11.89	2.14	12.88
	-	-	2.24	13.30
-CH ₂ -N	2.66	54.31	2.67	54.43
	3.72	57.33	3.5	58.49
-CH ₂ -	2.7	35.61	2.7	37.60
	3.09	51.57	3.1	51.50
Ar	6.98-7.57	116.83-158.81	6.98-7.61	114.18-139.12
-OH	-	-	11.22	-
-C=O	-	166.98	-	169.31
	-	-	175.49	-

tion. Then, after the sample was neutralized, the solvent of this solution was evaporated in cool air stream. The dried sample was dissolved in 20 mL of acetone. Then, it was filtered through a filter paper and the acetone was removed by evaporation. The action was repeated twice. The samples of PDI and degraded PDI in solution II were examined using the NMR method.

The HPLC-ES-MS system consisted of a Waters 2690 high-performance liquid chromatograph (20 μ L fixed-loop injector) with a Waters 936 diode array detector and a mass-spectrometer (Waters-micromass ZQ) with ES ionization. Chromatographic separation was conducted with a Symmetry C18 col-

umn (150 \times 4.6 mm ID, dp = 5 μ m; Waters). The mobile phase A was water and B acetonitrile. The gradient profile is presented in Table 1. The flow rate was 0.5 mL/min. For the MS analysis, the following parameters were used: capillary voltage 3 kV, cone voltage 30 V, desolvation cartridge temperature 300°C, source temperature 120°C, desolvation gas 300 L/h, cone gas 100 L/h, extractor 4 V. The samples of PDI, degraded PDI in solution I and II were examined using the HPLC-ES-MS method.

Thin-layer chromatography

Plates coated with silica gel 60 F₂₅₄ (0.2 mm, Merck) were used as the stationary phase. The chro-

matographic solvent consisted of a mixture of ethyl acetate - ethanol - water (6:3:0.5, v/v/v). The chromatograms were examined under ultraviolet light at 254 nm. The above conditions allowed for a separation of PDI and its degradation products in solution I and III ($R_{\text{PDI}} = 0.85$; $R_{\text{fA}} = 0.62$; $R_{\text{fB}} = 0.34$), in solution II ($R_{\text{PDI}} = 0.85$; $R_{\text{fB}} = 0.34$).

RESULTS AND DISCUSSION

First-order rate constants

Under the conditions applied (solution I – III) the degradation of PDI, as a result of solvolysis, is a pseudo-first-order reaction described by the following equation:

$$\ln P_t = \ln P_0 - k_{\text{obs}} \times t$$

where: P_0 , $P_t = p_{\text{PDI}}/p_{\text{IS}}$ - peak areas of PDI at time zero and time t , respectively; P_{IS} - peak area of IS; k_{obs} - observed pseudo-first-order reaction rate constant of the degradation of PDI.

Kinetic of products

In acetone-ethanol-water solution of 0.02 mol/L sodium hydroxide (solution I), the two decomposition products **A** and **B** ($t_{\text{R}} = 26.44$ min for **A**; $t_{\text{R}} = 11.07$ min for **B**) were formed. The chromatogram obtained suggests that product **B**, which has a short retention time, could be a highly polar

(hydrophilic) compound, whereas product **A**, which elutes after the main peak (PDI), is relatively lipophilic.

No statistically significant differences were found between the values of the observed rate constants (degradation and formation) of PDI and its degradation products (Table 2). These results indicate that **A** is an intermediate product. In the period of time from t_0 to t_{max} its concentration increased from $(P = p_{\text{A}}/p_{\text{IS}})_0 = 0$ to $(P = p_{\text{A}}/p_{\text{IS}})_{\text{max}}$. Then, from t_{max} to t_{∞} its concentration decreased from $(P = p_{\text{A}}/p_{\text{IS}})_{\text{max}}$ to 0. At the same time, an increase of product **B** was observed from $(P = p_{\text{B}}/p_{\text{IS}})_0 = 0$ to $(P = p_{\text{B}}/p_{\text{IS}})_{\infty}$. The PDI degradation in solution I can be described as follows: $S \rightarrow A \rightarrow B$.

The pseudo-first-order rate constants k_{obs} were calculated from the following equations:

$$\ln P_{tA} = \ln P_{0A} - k_{\text{obs}} \times t; \text{ for } t > t_{\text{max}}$$

$\ln (P'_t - P_{tA}) = \ln (P'_0 - P_{0A}) - k_{\text{obs}} \times t; \text{ for } t < t_{\text{max}}$
for product **A**

$$\ln (P_8 - P_t)_B = \ln (P_8 - P_0)_B - k_{\text{obs}} \times t$$

for product **B**

where: $P = p/p_{\text{IS}}$; P' is a theoretical value calculated from the equation in the time range t to t_{max} ; t_{max} - the time corresponding to P_{max} ; P_{∞} is the value that P reaches at time t_{∞} .

In acetone-water solution (solution II) only one degradation product is observed. The retention time

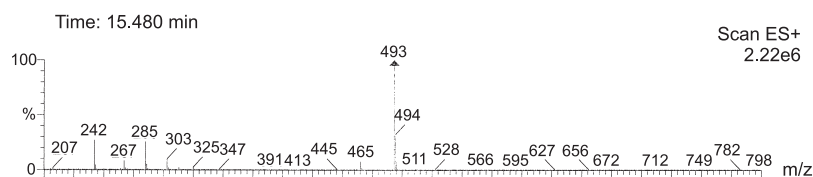


Figure 4. Mass spectrum (MS-ES⁺) of product **A** ($M = 492$).

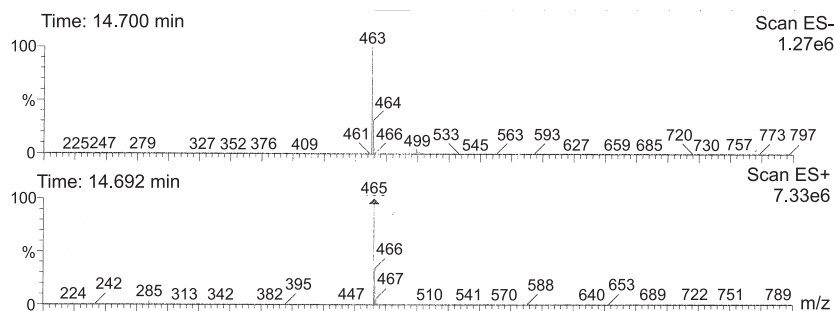
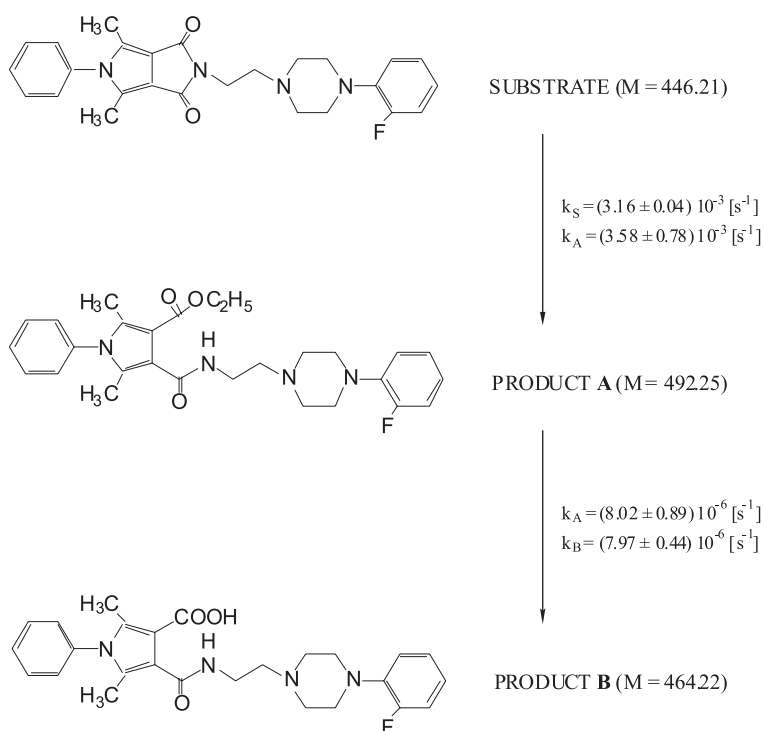
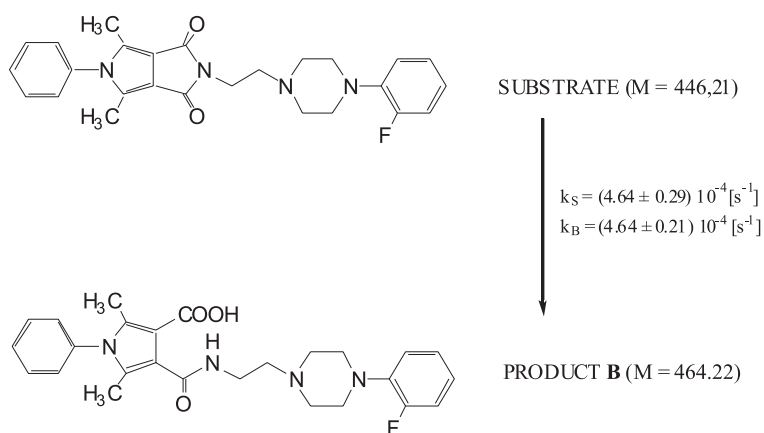


Figure 5. Mass spectra (MS-ES⁺ and MS-ES⁻) of product **B** ($M = 464$).



Scheme 6. Proposed mechanism of degradation of compound PDI in solution I.



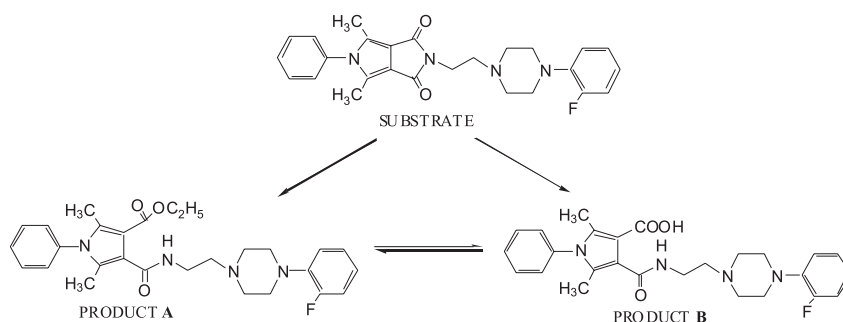
Scheme 7. Proposed mechanism of degradation of compound PDI in solution II.

of this product is equal to the retention time of product **B** obtained from solution I ($t_R = 11.07$ min). The reaction rate constants of the degradation of PDI and of the formation of product **B** have the same value, which suggests that product **B** is produced directly from PDI (Table 2). The concentration of product **B** in time interval from t_0 to t_∞ increased from 0 to (P

$= p_B/p_{IS})_\infty$. This process can be described as follows: $S \rightarrow B$.

The degradation of PDI in solution II occurs more slowly than in solution I. It can be concluded from these results that ethanol plays a part in the solvolysis of PDI in solution I.

At pH 7.23, in acetone-ethanol-water solution



Scheme 8. Proposed mechanism of degradation of compound PDI in solution III.

(III), two products were formed. The retention times of these products were equal to the retention times of products **A** and **B**, respectively. The concentrations of product **A** and **B** in time interval from t_0 to t_∞ increased from 0 to $(P = p_{A(B)}/p_{IS})_\infty$ (Table 2). The pseudo-first-order rate constant was calculated from the following equation:

$$\ln(P_\infty - P_{I(A \text{ or } B)}) = \ln(P_\infty - P_0)_{A \text{ or } B} - k_{\text{obs}} \times t$$

The reaction rate constants of the formation of products **A** and **B** are not equal to the reaction rate constant of the degradation of PDI, what indicates that these products are formed in a parallel reaction but they next undergo the reversible reactions of hydrolysis and esterification.



Identification of products **A** and **B** chemical structure

In the structure of PDI the imide bond in position 3 or 4 of the pyrroledicarboximide ring is the most susceptible to degradation. Thus, it was assumed that in the first stage of the degradation of PDI in solution I, this bond is broken and intermediate product **A** (ethyl ester) is formed. In the next stage this ester hydrolyzes to an acid (product **B**). In solution II, in the absence of ethanol, product **B** (an acid) is produced directly.

The presence of two products in solution I and III and only one product in solution II was confirmed also by TLC method. The comparison, the values R_F of products in solution applied, shows:

- product with $R_F = 0.62$ is corresponding with product **A** ($t_R = 26.44$ min),
- product with $R_F = 0.34$ is corresponding with product **B** ($t_R = 11.07$ min).

In order to confirm the assumed structures of the degradation products, HPLC-ES-MS studies and the isolation and identification of product **B** by ^1H - and ^{13}C -NMR methods were performed.

In the first stage, the identification of the degradation products was performed in the degraded sample with an HPLC-ES-MS method. On the chromatogram of undegraded PDI the peak at $t_R \sim 27.7$ min is observed and the ES^+ spectra associated with this peak show a number of ions including the protonated PDI m/z 447 [$\text{PDI} + \text{H}^+$] and m/z 469 [$\text{PDI} + \text{Na}^+$].

On the chromatogram of solution I the following main peaks were detected: **A** $t_R \sim 15.5$ min; **B** $t_R \sim 14.7$ min. The ES-mass spectrum associated with the HPLC peak at $t_R = 15.5$ min contains the ion m/z 493 [$\text{A} + \text{H}^+$], which is attributed to degradation product **A** (Fig. 4). The ES^+ spectrum associated with the HPLC peak at $t_R = 14.7$ min contains the ions: m/z 463 [$\text{B} - \text{H}^+$] and m/z 465 [$\text{B} + \text{H}^+$], which are derived from product **B** (Fig. 5).

The chromatogram of solution II shows the main peak at $t_R = 14.7$ min. The ES mass spectra associated with this peak contain the ions: m/z 463 and m/z 465, which corresponds to the molecular weight of product **B**: [$\text{B} - \text{H}^+$] and [$\text{B} + \text{H}^+$], respectively.

The spectra obtained indicate the correctness of the model of degradation established.

The structure of product **B** was also confirmed by NMR spectroscopy. Product **B** was isolated from the acetone – water solution of 0.02 mol/L sodium hydroxide of PDI, heating at 308 K. The ^1H - and ^{13}C -NMR spectra were interpreted by comparing the chemical shifts of the substrate with the degradation product, as shown in Table 3.

The chemical shifts are in good agreement with the proposed structures. The ^1H -NMR spectrum of product **B** shows several characteristic signals. An

amide proton was detected as a singlet at $\delta = 8.54$ ppm and a carboxyl proton appeared at $\delta = 11.22$ ppm. This indicates the hydrolysis of the imide bonds in the pyrroledicarboximide ring. The hydrolysis of this group causes the methyl groups in the pyrrole ring to be no longer equivalent, thus in the spectrum two signals ($\delta = 2.14$ and $\delta = 2.18$ ppm) are observed, as opposed to the spectrum of PDI with one signal from two equivalent methyl groups ($\delta = 2.15$ ppm). The other protons present in the molecule of PDI and product **B** show similar chemical shifts (Table 3).

In the ^{13}C -NMR spectrum of product **B** signals from the carbonyl group in the amide group at $\delta = 169.31$ ppm and from the carbonyl group in the carboxyl group at $\delta = 175.49$ ppm were detected. Under the same conditions, PDI showed one signal from the two carbonyl groups at $\delta = 166.98$ ppm.

Additionally, the possibility of breaking the amide bond in products **A** or **B** and formation of a dicarboxylic derivative and *o*-fluorophenylpiperazinethylamine was considered. No signals from such molecules were detected in solutions I and II by using the HPLC-ES-MS method. While using the TLC method and ninhydrine as a detecting reagent no amine derivative was observed.

In conclusion, the solvolysis pathway established of PDI in aqueous-organic solution proceeds according schemes 6, 7 and 8.

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