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Identification of imatinib mesylate degradation products obtained under stress conditions

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Abstract

In this paper, the decomposition of imatinib mesylate (ImM) under hydrolytic (neutral, acidic, alkaline), oxidative and photolytic conditions was studied. The imatinib mesylate is practically photostable and stable under neutral conditions. The main degradation products under acidic and alkaline conditions are compounds: 4-methyl-N^3-(4-pyridin-3-yl-pyrimidyn-2-yl)-benzene-1,3-diamine (**2**) and 4-(4-methyl-piperazin-1-ylmethyl)-benzoic acid (**3**). The main degradation products under oxidation conditions, i.e. 4-[(4-methyl-4-oxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (**6**), 4-[(4-methyl-1-oxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (**7**) and 4-[(4-methyl-1,4-dioxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (**8**), were isolated from the reaction mixtures and identified by the HPLC, ^{1}H NMR and MS techniques. During stress study the suitability of the proposed HPLC method to control purity of the samples was verified.

Keywords: Imatinib mesylate; Glivec®; Gleevec®; Degradation products; Stress conditions

1. Introduction

Imatinib mesylate (ImM, **1**) (trade name Glivec® or Gleevec®), a new anti-cancer agent, is a protein tyrosine kinase inhibitor that inhibits the Bcr-Abl tyrosine kinase, the constitutive abnormal tyrosine kinase created by the Philadelphia chromosome abnormality in chronic myeloid leukemia (CML) [1]. It has also been found to be effective in the treatment of gastrointestinal stromal tumors (GISTs) [2].

ImM (**1**) is designated chemically as 4-(4-methylpiperazin-1-ylmethyl)-*N*-{4-methyl-3-[4-(pyridin-3-yl)pyrimidyn-2-ylamino]phenyl}benzamide methanesulfonate and its chemical formula is presented in Fig. 1.

In order to achieve a high level of safety and effectiveness of pharmacotherapy the requirements on quality of active substance are growing [3,4]. The investigation of the stability of drugs represents an important issue in drug quality evaluation. Many environmental conditions, for example heat, light, humidity as well as substance ability for hydrolysis or oxidation can play an important role in drug stability. Stress testing [5] of a drug substance can help identify the degradation products and provide important information about the inherent stability of the substance under hydrolytic, oxidative and photolytic conditions. Moreover, during a stress study the suitability of the proposed analytical procedure to control the purity of samples may be verified.

Several HPLC procedures for the determination of imatinib (Im **1a**, Fig. 1) and its *N*-desmethyl-metabolite **4** [6] (*N*-demethyl-metabolite [7]) (Fig. 2) have been reported in the literature. For the simultaneous quantification of Im (**1a**) and its *N*-desmethyl-metabolite **4**, in human or monkey plasma, methods using liquid chromatography coupled with tandem mass spectrometry were worked out [8,9]. Rapid and sensitive HPLC methods were developed to estimate of Im (**1a**) in human plasma using UV detectors [10]. Ivanovic et al. have reported the HPLC method for simultaneous determination of Im (**1a**) and its main metabolite **4** in pharmaceutical dosage forms [11].

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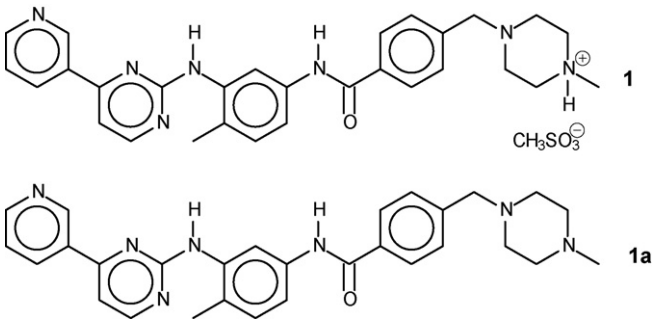


Fig. 1. Structure of imatinib (Im) and imatinib mesylate (ImM).

To the best of our knowledge, the degradation of ImM (**1**) under hydrolytic, oxidation and photolytic conditions has not been reported. The objective of the present study is stress tests of ImM (**1**) under various conditions. The HPLC method and NMR as well as MS techniques were used to monitor and identify the degradation products. The isolation procedure of main degradations products formed under oxidation conditions is described in Section 2.8.

2. Experimental

2.1. Materials and reagents

Sodium hydroxide, hydrochloric acid, hydrogen peroxide and all used solvents of HPLC grade were purchased from POCH (Gliwice, Poland). Water used was freshly prepared (18 μΩ, from Barnstead apparatus). 1-Octanesulfonic acid sodium salt monohydrate was obtained from Fluka (Buchs, Switzerland).

Im (**1a**), ImM (**1**) and 4-methyl-N^3-(4-pyridin-3-yl-pyrimidyn-2-yl)-benzene-1,3-diamine (**2**) were synthesized from 2-methyl-5-nitroaniline, 3-acetylpyridine, 4-(chloromethyl)-benzoyl chloride and *N*-methylpiperazine, offered by commercial suppliers. Using piperazine, instead of *N*-methylpiperazine, the possible impurities of Im (**1a**), i.e. *N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidyn-2-ylamino)-phenyl]-4-piperazin-1-ylmethyl-benzamide (*N*-demethyl-imatinib or *N*-desmethyl-imatinib, **4**) and 1,4-bis-{4-[4-methyl-3-(4-pyridin-3-yl-pyrimidyn-2-ylamino)-phenylcarbamoyl]-benzyl}-piperazine (**5**) as well as their monomesylate salts were also obtained. The known 4-(4-methyl-piperazin-1-ylmethyl)-benzoic acid **3** [12] (as its dihydrochloride [13]) was synthesized from commercially available 4-(4-methyl-piperazin-1-ylmethyl)-benzoyl chloride dihydrochloride by hydrolysis in water. The 4-[(4-methyl-4-oxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (**6**), 4-[(4-methyl-1-oxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (**7**) and 4-[(4-methyl-1,4-dioxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (**8**) were isolated as detailed in Section 2.8. Moreover, compound **8** was transformed into its monomethanesulfonate, i.e. 4-[(4-methyl-1,4-dioxido-piperazin-1-yl)-methyl]-*N*-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide methanesulfonate (**8a**). The chemical structures of compounds **2**–**5** are presented in Fig. 2 and those of **6**–**8a** in Fig. 3.

2.2. FT-IR spectroscopy

FT-IR spectra were recorded on Perkin-Elmer Spectrum BX (FT-IR system) apparatus as KBr pellets.

2.3. NMR spectroscopy

^{1}H NMR spectra were recorded at 200 MHz on Varian Gemini 2000 or at 500 MHz on Bruker DRX 500 spectrometer using DMSO-d_6 as solvent and TMS as internal standard of chemical shift.

2.4. MS spectrometry

EI–MS and EI–HRMS spectra were recorded on AMD 604 (AMD Intectra GmbH, Germany) spectrometer at 70 eV.

2.5. Melting points

Melting points were determined on a Büchi Melting Point B-540 apparatus and are uncorrected.

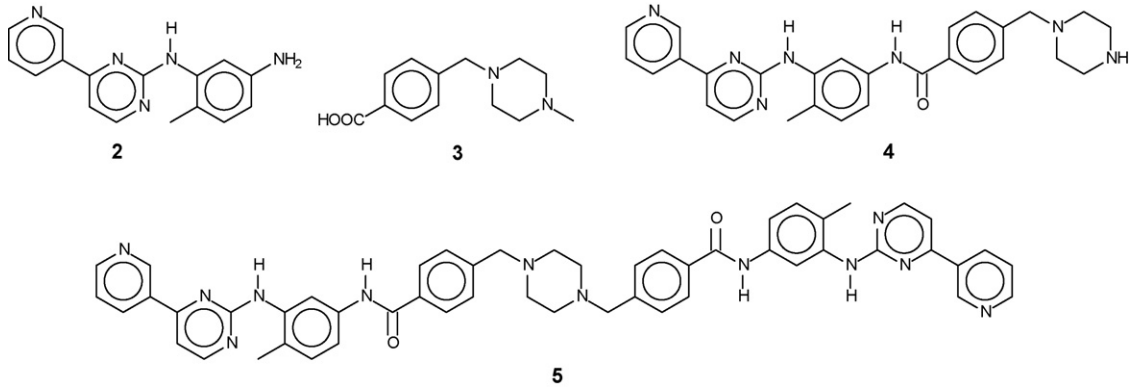


Fig. 2. Structure of compound **2**, **3**, *N*-desmethyl metabolite (main metabolite, CGP-74588) **4** and expected impurity **5**.

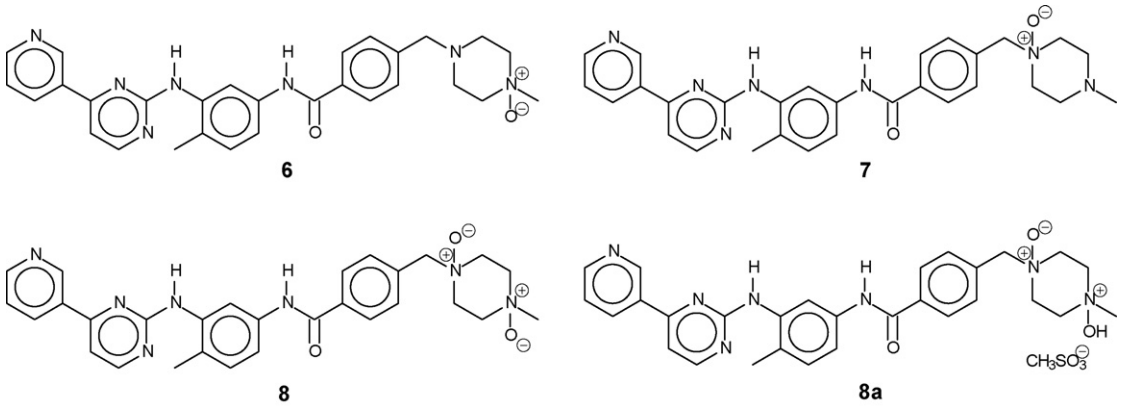


Fig. 3. Structure of degradation products obtained under oxidation conditions (compounds **6**, **7**, **8** and **8a**).

2.6. Chromatographic conditions

Chromatographic experiments were performed using a Shimadzu (Kyoto, Japan) chromatograph equipped with: pump Model—LC-10AD VP, auto sampler Model SIL 10AD VP (20 μl injection), a UV–vis detector Model SPD-10AV VP (detection: 269 nm), the temperature 25 °C was controlled using column oven Model CTO 10ASVP.

The chromatographic analyses were worked out on the basis of the analysis presented in the Patent [14].

The column used was: 150 mm × 4.6 mm I.D. (Waters) Symmetry C18 with 5 μm particle.

The mobile phases were prepared as follows: buffer solution—7.5 g 1-octanesulfonic acid sodium salt monohydrate was dissolved in 1000 ml water and adjusted with phosphoric acid to pH 2.6; the mobile phase A and B was a mixture of buffer with methanol in ratio 42:58 (v/v) for A and 4:96 (v/v) for B. The mobile phases were filtered through a 0.45 μm filters and degassed under vacuum prior to use. The flow rate was 1.2 ml/min. The gradient analysis was as follows:

Time	Phase B%
0–20	0
20–30	0 → 30
30–55	30
55–56	30 → 0

The HPLC method was employed to study decomposition of Im in reaction mixtures. The blanks consisting of equal volume of solvent and degradation medium were injected every single analysis.

The 5 ml of the reaction mixture from given stress conditions were diluted with MeOH:H_2O 55:45 (v/v) mixture in 25 ml volumetric flask.

2.6.1. Validation of the developed HPLC method

For the validation procedure the solutions of ImM (**1**), impurities **4** and **5** (as their monomethanesulfonates) and degradation products **2**, **3**, **6** and **7** were prepared by dissolving an appropriate amount of substance with MeOH:H_2O 55:45 (v/v) mixture. While compound **8**, as its monomethanesulfonate **8a**, was dissolved in MeOH. The concentrations of stock solutions were about 1 mg/ml and only for compound **8a** was about 0.2 mg/ml.

2.6.1.1. Selectivity of the method. Standard solution of the ImM (concentration about 1 mg/ml) was spiked by the solutions of impurities **4** and **5** as well as by solutions of degradation products **2**, **3**, **6**, **7**, **8a**. The concentration level of **2**, **4**, **5**, **6**, **7** was 0.15%, and that of compounds **3** and **8a**—1.5% and 0.1%, respectively.

2.6.1.2. Precision of the method and linearity. Six solutions containing ImM and impurity **4** were prepared by standard solutions dilution with MeOH:H_2O 55:45 (v/v) solution on the level of concentration 0.1% and 0.15%, respectively. Precision of the method was examined by three injections of the six test solutions. The means of the ratio: peak area to amount of component (mg) for six test solutions were measured ($\bar{X}_1, \ldots, \bar{X}_6$). Statistic parameters were calculated as standard deviation (S.D.), relative standard deviation R.S.D. (%) and confidence interval for mean value ($\bar{X} \pm \Delta X$).

For the linearity test the five solutions in the range of concentration from 0.02 to 0.2% of ImM and impurities **2**, **4**, **5**, **6**, **7** and **8a** were prepared. Each solution was injected three times. The linear correlation was checked by plotting the peak area versus corresponding concentration. The limits were established on the basis of the data from linearity examinations according to the formulas:

Detection Limit (LOD):

$$\mathrm{LOD} = \frac{3S_{yx}}{a}$$

Quantitation limit (LOQ):

$$\mathrm{LOQ} = \frac{10S_{yx}}{a}$$

where S_{yx} is standard deviation of response and a is the slope from linear regression.

For compound **3** the limits were estimated from the ratio between noise and signal.

2.6.1.3. Assay validation. The three stock solutions (1 mg/ml) were prepared by dissolving an appropriate amount of ImM with MeOH:H_2O 55:45 (v/v). The calibration curve was made using five standard solutions of different concentration (50, 250, 500, 750, 1000 μg/ml—in range 5–100%). The standard solutions were prepared by dilution of an appropriate volume of stocks solutions with MeOH:H_2O 55:45 (v/v). Each solution was injected three times. The linear correlation was checked by plotting the peak area versus corresponding concentration.

From the ratio between concentration and peak area the response factor was calculated. The value of the relative standard deviation of response factor was calculated to determine precision of the analysis. Accuracy was evaluated as a percentage of recovery obtained from analysis of samples of known amount of ImM (100, 200, 800 μg/ml).

2.7. Stress test conditions

All stress tests were carried out on a single batch of ImM. The drug concentration in solutions was about 5 mg/ml. The conditions of the stress studies are presented in the Table 1.

The photostability tests were carried out in chemically inert and UV–vis transparent quartz containers using Xenon lamp.

2.8. Isolation of oxidation products of ImM (*1*) under stress conditions

2.8.1. Isolation of 4-[(4-methyl-4-oxido-piperazin-1-yl)-methyl]-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (*6*) and 4-[(4-methyl-1-oxido-piperazin-1-yl)-methyl]-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (*7*)

A solution of ImM (**1**; 11.8 g) in a mixture of 30% hydrogen peroxide (80 ml) and water (160 ml) was stirred at r.t. for 30 h. The reaction mixture was diluted with water (360 ml), alkalified with 10% aqueous sodium hydroxide and extracted successively with chloroform (300 ml) and chloroform–methanol mixtures (3:1, 600 ml; 3:2, 500 ml and 1:1, 600 ml). The organic layers were separated, dried over anhydrous $MgSO_4$, filtered and evaporated to dryness. The obtained residue was chromatographed on silica gel column. The column was washed successively with mixtures chloroform–ethyl acetate–methanol–ammonia (60:30:10:0.5; 60:20:20:0.5; 60:10:30:1; 60:10:30:2; 60:0:40:2). Fractions containing the less polar product were combined and evaporated to dryness. The residue was treated with isopropanol, evaporated to dryness and then crystallized from methylene chloride to give crystals A. The filtrate was evaporated to dryness and crystallized from ethyl acetate to give crystals B.

Recrystallization of crystals A from methylene chloride afforded compound **6**; m.p. 182.5–183.9 °C (light yellow crystals); IR (KBr): 3412, 1654 (C=O), 1583, 1557, 1505, 1449, 1415, 1313, 971, 796 cm^{-1}; EI–MS (70 eV): 493 (22%), 451 (20%), 450 (59%), 423 (60%), 395 (100%), 394 (54%), 393 (47%), 277 (29%), 276 (19%), 262 (13%), 146 (41%), 119 (52%), 118 (58%), 99 (47%), 90 (61%), 56 (59%), 43 (39%); EI–HRMS: calcd. for $(M+H)^+$ $[C_{29}H_{32}N_7O_2]$ 510.2617, found 510.2637. Lit.: MS: 532 ($[M+Na]^+$), 510 ($[M+H]^+$), 450, 435, 423, 395, 394, 393, 277, 276, 262, 255.6 ($[M+2H]^{2+}$) [6]; LC–ESI^+–LTQ–MS for *N*-oxide metabolite: 492, 478, 463, 450, 436, 423, 410, 395, 393, 338 [7]; m.p. 154–158 °C (yellow crystals) [13]. ^1H NMR (see Table 5).

Recrystallization of crystals B from ethyl acetate afforded compound **7**; m.p. 191.8–193.6 °C; IR (KBr): 3296, 1667 (C=O), 1587, 1561, 1541, 1504, 1431, 1323, 798 cm^{-1}; EI–MS (70 eV): 493 (6%), 450 (13%), 423 (16%), 409 (33%), 396 (26%), 395 (84%), 394 (40%), 380 (26%), 276 (21%), 262 (8%), 261 (21%), 133 (19%), 119 (100%), 105 (21%), 99 (51%), 91 (60%), 90 (23%), 77 (21%), 70 (36%), 58 (42%), 56 (53%), 42 (43%); EI–HRMS: calcd. for $(M)^+$ $[C_{29}H_{31}N_7O_2]$ 509.25392, found 509.25343. ^1H NMR (see Table 5).

The combined filtrates were evaporated to dryness and crystallization from ethyl acetate gave a mixture (ca. 1:1) of compounds **6** and **7**.

2.8.2. Isolation of 4-[(4-methyl-1,4-dioxido-piperazin-1-yl)-methyl]-7-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide (*8*)

A solution of ImM (**1**; 5 g) in 30% hydrogen peroxide (100 ml) was stirred at r.t. for 8.5 h. Then 100 ml of 30% hydrogen peroxide were added and the solution was stirred at r.t. for 17.5 h. Additional portion of 30% hydrogen peroxide (60 ml) was added and stirring was continued for 5 h. The reaction mixture was alkalified with 10% aqueous sodium hydroxide and the formed crystalline product was filtered off. The alkaline filtrate was extracted with a mixture chloroform–methanol (2:1; 150 ml) and the organic layer was separated and dried over anhydrous $MgSO_4$ and then filtered. The crystalline product was dissolved in a mixture chloroform–methanol (1:1) and the obtained solution was combined with the dried extract and then evaporated. The obtained residue was chromatographed on silica gel column. The column was washed successively with mixtures chloroform–ethyl acetate–methanol–ammonia (60:20:20:0.2;

Table 1
The stress test conditions for hydrolysis (under neutral, acidic and alkaline conditions), oxidation and photolysis of ImM

Stress test conditions	Solvent	Time (h)	Temperature
Neutral	Water	12	Reflux
	Water	24	Reflux
	Water	48	Reflux
	Water	120	Reflux
Acidic	0.1 M HCl	6	Reflux
	1.0 M HCl	12	Reflux
Basic	0.05 M NaOH[a]	6	Reflux
	1.0 M NaOH[a]	12	Reflux
Oxidation	3% H_2O_2	6	Room temperature
	3% H_2O_2	24	Room temperature
	10% H_2O_2	24	Room temperature
	30% H_2O_2	24	Room temperature
Photolysis	UV light exposure	24	Room temperature
	UV light exposure	72	Room temperature

[a] Water:methanol, 1:1 (v/v).

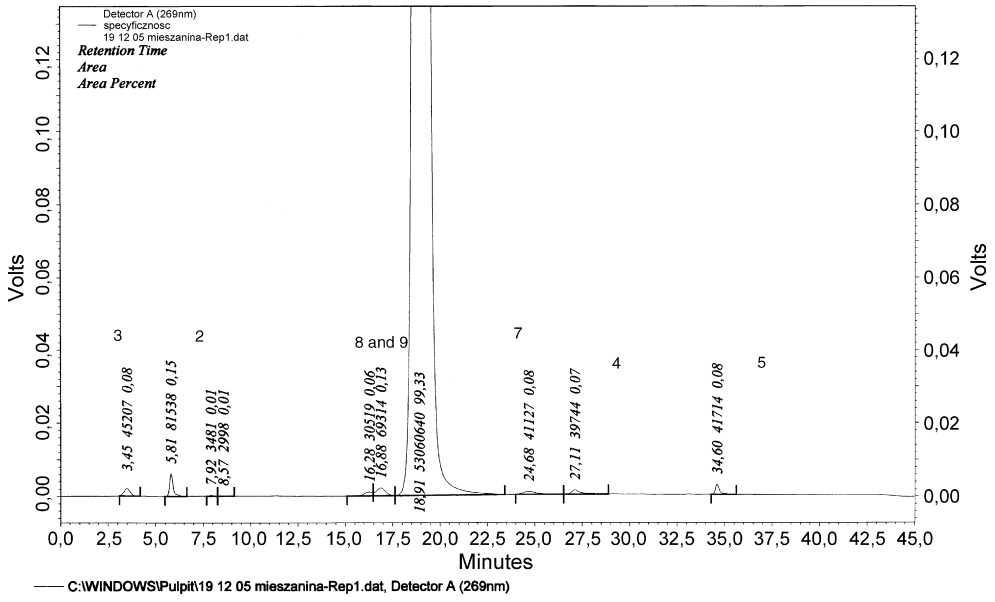


Fig. 4. Chromatograms of sample of ImM skipped by impurity **4** and **5**, and degradation products **2**, **3**, **6**, **7** and **8a**.

60:10:30:0.2; 60:10:30:0.3; 60:0:40:0.3; 60:0:40:1; 50:0:50:1) and methanol. Fractions containing the main polar product were combined and evaporated to dryness. The residue was crystallized from methylene chloride–isopropanol to give 2.94 g (66%) of compound **8**; m.p. 217.4–218.2 °C (white crystals); IR (KBr): 3284, 1648 (C=O), 1583, 1554, 1525, 1494, 1453, 1423, 1285, 953, 795 cm^{-1}; EI–MS (70 eV): 395, 394, 276, 222, 207, 177, 149, 119, 58, 40 (100%); EI–HRMS: calcd. for $(M+H)^+$ $[C_{29}H_{32}N_7O_3]$ 526.2567, found 526.2566. Lit.: m.p. 242–244 °C (yellow crystals) [1]. ^{1}H NMR (see Table 5).

2.8.3. Synthesis of 4-[(4-methyl-1,4-dioxido-piperazin-1-yl)-methyl]-N-[4-methyl-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-phenyl]-benzamide monomethanesulfonate (*8a*)

A stirred solution of **8** (2.613 g) in methanol (30 ml) was treated with methanesulfonic acid (0.3 ml). Methanol (20 ml) was added and the mixture was heated to 45 °C. Then the mixture was stirred at r.t. for 1.5 h. The formed product was filtered off, washed with methanol (20 ml) and ethyl acetate (15 ml) and then dried at r.t. under reduced pressure to give 2.893 g (93.6%) of compound **8a**; m.p. 209.9–211.3 °C; IR (KBr): 3439, 1677 (C=O), 1581, 1555, 1523, 1450, 1415, 1401, 1192, 1044, 797, 771 cm^{-1}. ^{1}H NMR (see Table 5).

3. Results and discussion

3.1. Chromatographic results

3.1.1. Validation results

The aim of the validation procedure was to present the selectivity, precision of the method, linearity and quantitative limits obtained for the determination of impurities **4**, **5** and degradation products **2**, **3**, **6**, **7**, **8** using HPLC method.

The method was checked for selectivity between Im (**1a**) and its metabolite **4**, expected impurity **5** and degradation products **2**, **3**, **6**, **7** and **8**. The chromatogram for the mixture of degradation products, compounds **4** and **5** and Im is shown in Fig. 4

As one can see the developed HPLC method is characterized by good selectivity between Im and the impurities.

The precision of the method was checked for Im (**1a**) and its metabolite **4**. The obtained results are collected in Table 2. The results indicated acceptable precision of the method. The used chromatographic method was validated with respect to linearity of the peak area versus low concentration (see experimental) of Im and compounds **2**, **4**, **5**, **6**, **7** and **8**. For the studied compounds in the studied concentration range method was linear ($r^2 > 0.99$). The limits of detection and quantification of the studied compounds are shown in Table 3. For the studied compounds quantification limit is lower or equal the reporting level (0.05%), only in the case of compound **3** the detection level is much higher (0.4%).

The used chromatographic method was also validated with respect to linearity of the peak area versus ImM concentration in the concentration range 5–100%, precision of the response factor and accuracy. The studied method was strictly linear in the studied concentration range (r^2 −0.9995). The R.S.D. for the response factor for the studied concentration was 0.4%. The recovery percentage for the three different levels of concentration was in the range 99.5–100.1%

3.1.2. Stress results

The chromatogram of the starting sample of ImM (**1**) taken to the stress tests is presented in Fig. 5. The obtained results are summarized in Table 4. The data presented in the Table were calculated from the normalization of the peak area.

Under the neutral conditions (even after 120 h of heating) and after 72 h of UV light exposure the samples were practically stable. Only a very small increase of peak (about 0.1%) corresponding to compound **2** was detected. Detection of compound **3** was not possible because of much higher detection limit.

Table 2
Method precision

	No. of experiment					
	1	2	3	4	5	6
(A) For ImM						
$\bar{X}$	48646	46402	48842	49466	50615	49394
$\bar{\bar{X}}$			48894			
S.D.			1401			
R.S.D. (%)			2.9			
ΔX			1470			
(B) For compound **4**						
$\bar{X}$	34739	34749	34546.	36318	36322	34039
$\bar{\bar{X}}$			35560			
S.D.			965.			
R.S.D. (%)			2.7			
ΔX			1013			

Table 3
Limits of detections and quantifications

Compound	LOD		LOQ	
	μg/ml	%	μg/ml	%
Im	0.09	0.01	0.29	0.03
4	0.15	0.02	0.51	0.05
5	0.12	0.01	0.40	0.04
6	0.09	0.01	0.31	0.03
7	0.15	0.02	0.50	0.05
8	0.16	0.02	0.53	0.05
2	0.07	0.01	0.23	0.02
3	1.19	0.12	3.93	0.39

As it has been expected, the main degradation products in the reaction mixture under acidic and alkaline conditions are compounds **2** and **3**, i.e. products of amide group hydrolysis. The structure of degradation products **2** and **3** (for chemical structures see Fig. 2) was confirmed by comparison with the retention times of pure substances **2** and **3**. To estimate the molar ratio of compound **2** and **3** in the reaction mixture, a sample of a known amount of the reference substance **2** and **3** was prepared. The obtained chromatograms show that the peak area (at 269 nm) of the same molar concentration for substance **2** is about 17 times greater than for substance **3**. After multiplication of the percentage for substance **3** by the factor 17, very similar values were obtained for both compounds (see Table 4).

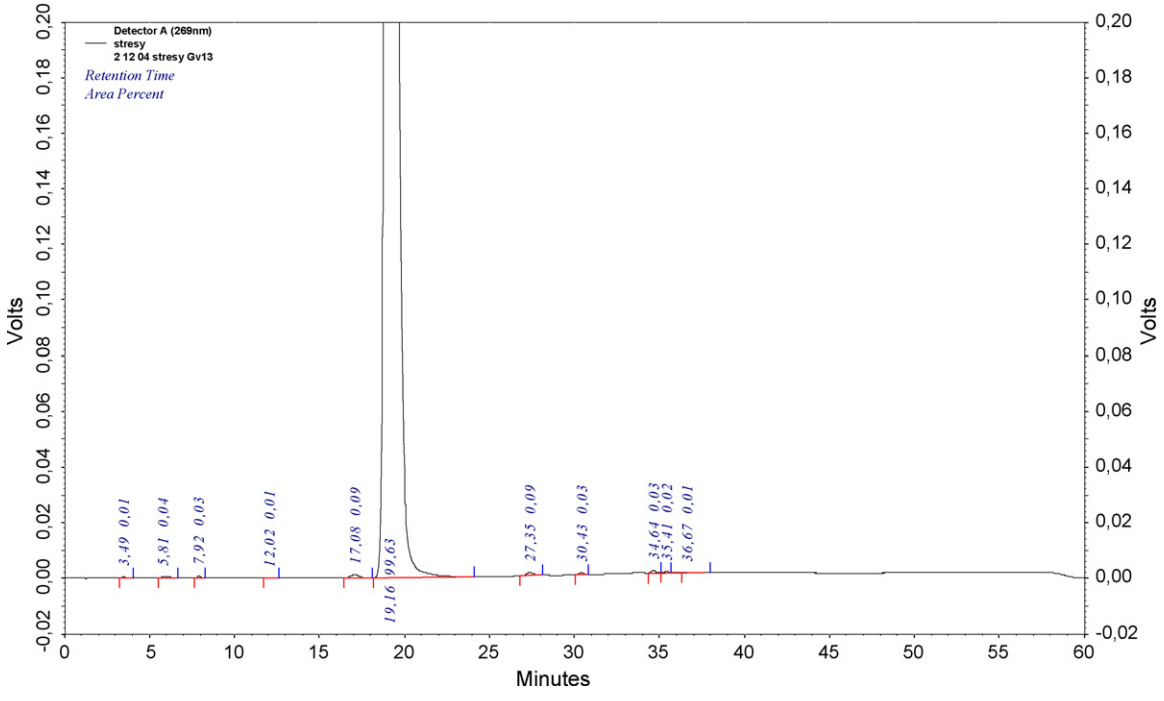
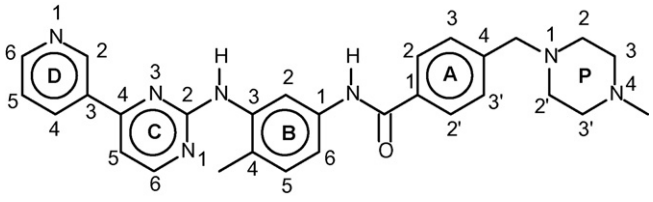


Fig. 5. Chromatogram of ImM samples before stress test.

Table 4
The results of the stress testing in various conditions (chromatographic conditions see Section 2)

Test time of reaction	HPLC results						
	Composition of reaction mixture in %						
	Im (**1**) RRT = 1.0	**3** RRT = 0.2	**2** RRT = 0.3	**6** + **8** RRT = 0.9	**7** RRT = 1.3	**4** RRT = 1.4	**5** RRT = 1.9
ImM starting material	99.7	<0.05	<0.05	0.1	<0.05	0.1	<0.05
H_2O 12 h	99.7	<0.05	0.1	0.1	<0.05	0.1	<0.05
H_2O 24 h	99.4	<0.05	0.1	0.1	<0.05	0.1	<0.05
H_2O 48 h	99.4	<0.05	0.1	0.1	<0.05	0.1	<0.05
H_2O 120 h	99.3	<0.05	0.2	0.1	<0.05	0.1	<0.05
UV light 24 h	99.3	<0.05	0.1	0.1	<0.05	0.1	<0.05
UV light 72 h	99.1	<0.05	0.2	0.1	<0.05	0.1	<0.05
0.1 M HCl 6 h	94.4	0.3	5.0	0.1	<0.05	0.1	<0.05
1.0 M HCl 12 h	0.8	5.1	94.0	<0.05	<0.05	<0.05	<0.05
0.05 M NaOH 6 h	98.0	0.1	1.8	0.1	<0.05	0.1	<0.05
1.0 M NaOH 12 h	25.0	3.6	70.7	<0.05	<0.05	<0.05	<0.05
3% H_2O_2 6 h	94.4	<0.05	0.1	3.1	2.2	0.1	<0.05
3% H_2O_2 24 h	87.8	<0.05	0.1	7.1	4.8	0.1	<0.05
10% H_2O_2 24 h	60.0	<0.05	0.1	25.3	14.3	0.1	<0.05
30% H_2O_2 24 h	7.2	<0.05	0.1	69.4	22.3	0.2	<0.05

Table 5
^{1}H NMR data for compounds **1**, **1a**, **6**, **7**, **8** and **8a**



Proton position (multiplicity)	Compound					
	Im (**1a**)	**6**	**7**	**8**	**8a**	ImM (**1**)
—CO—NH— (s)	10.16	10.28	10.29	10.20	10.23	10.19
—NH— (s)	8.96	9.06	9.06	8.91	8.93	8.96
—NH^+—	–	–	–	–	–	9.50
D2 (d)	9.29	9.28	9.28	9.27	9.27	9.29
D4 (dt)	8.48	8.48	8.48	8.47	8.46	8.48
D5 (dd)	7.51	~7.52[a]	~7.52[a]	~7.51[a]	~7.51[a]	~7.52[a]
D6 (dd)	8.69	8.69	8.69	8.68	8.68	8.69
C5 (d)	7.42	7.43	7.43	7.41	7.41	7.43
C6 (d)	8.51	8.51	8.51	8.51	8.51	8.52
B2 (d)	8.11	8.08	8.10	8.08	8.10	8.11
B5 (d)	7.21	7.21	7.21	7.21	7.21	7.22
B6 (dd)	7.50	~7.50[a]	~7.50[a]	~7.48[a]	~7.49[a]	~7.51[a]
[B4]—C**H$_3$** (s)	2.24	2.22	2.23	2.23	2.23	2.24
A2,2′ (d)	7.92	7.92	7.92	7.93	8.02 (+0.05)	7.97
A3,3′(d)	7.43	7.45	7.72 (+0.29)	7.79 (+0.36)	7.79 (+0.31)	~7.48[a]
P2,2′ (m)	2.35 [8H]	2.52	2.48	2.78 [4H]	3.20 [2H]	2.99
P3,3′ (m)		2.83	2.70	4.05 [4H]	3.57 [2H]	3.36
		3.35	3.36		4.19 [4H]	
[P1]—C**H$_2$**— (s)	3.52	3.61 (+0.09)	4.36 (+0.84)	4.47 (+0.95)	4.76 (+1.10)	3.66
[P4]—C**H$_3$** (s)	2.15	3.03 (+0.88)	2.18 (+0.03)	3.10 (+0.95)	3.51 (+0.71)	2.80
C**H$_3$**—SO_3^- (s)	–	–	–	–	2.35	2.39

In parentheses the calculated $\Delta\delta_H$ values are given [for compounds **6**, **7** and **8** $\Delta\delta_H = \delta_H$(compound) − δ_H(**1a**) and for compound **8a** $\Delta\delta_H = \delta_H$(compound) − δ_H(**1**)].
[a] overlapping signals.

Chromatograms showing the decomposition of ImM under oxidation stress are presented in Fig. 6. First of them, obtained for the first test solution showed the formation of three oxidation products (relative retention times (RRT): 0.85, 0.89 and 1.30, respectively). On the basis of this observation an attempt of isolation of primary oxidation products was undertaken. Oxidation of ImM with 10% hydrogen peroxide for 30 h led to isolation of a mixture of primary oxidation products. Both components of the mixture showed the same R_f values on TLC in dichloromethane-ethyl acetate-methanol-ammonia (6:1:3:0.2). Fractional crystallization of this mixture yielded compounds **6** and **7**, i.e. products of monooxidation of Im. The structures of compounds **6** and **7** were confirmed by their spectral data (MS and ^{1}H NMR). Using EI–HRMS, their molecular formula was deduced to be $C_{29}H_{31}N_7O_2$. The intense fragment ion peaks at *m/e* 394, 276 and 262 [6], present in EI-MS of both compounds, suggested that oxidation took place only at nitrogen atoms of the piperazine ring. Comparing with Im (**1a**), the ^{1}H NMR data (see Table 5 and point 3.2) of compound **6** showed that it is 4-oxido-derivative of Im whereas

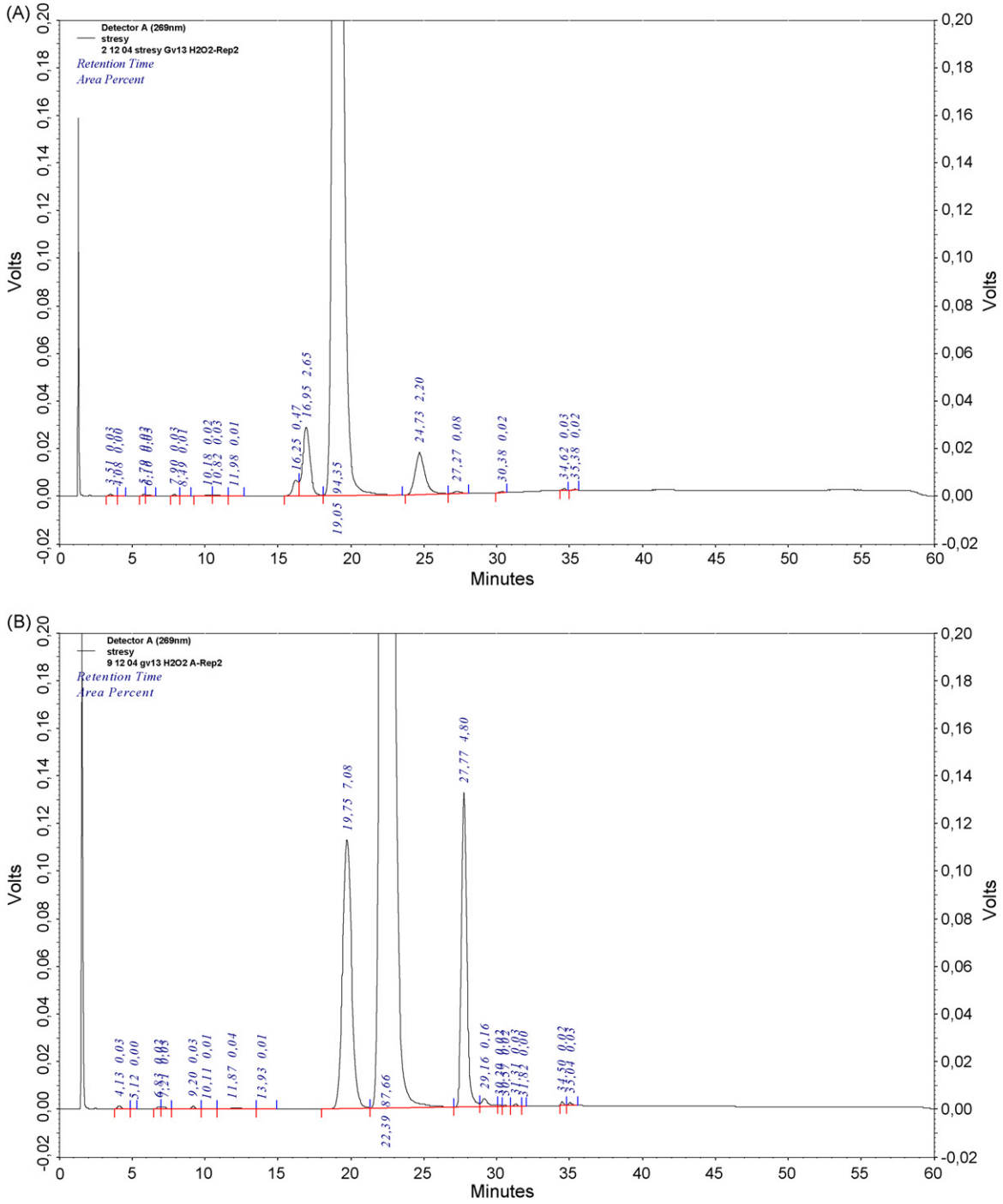


Fig. 6. Chromatograms of ImM after oxidation stress: (A) 3% H_2O_2 after 6 h; (B) 3% H_2O_2 after 24 h; (C) 10% H_2O_2 after 24 h; (D) 30% H_2O_2 after 24 h.

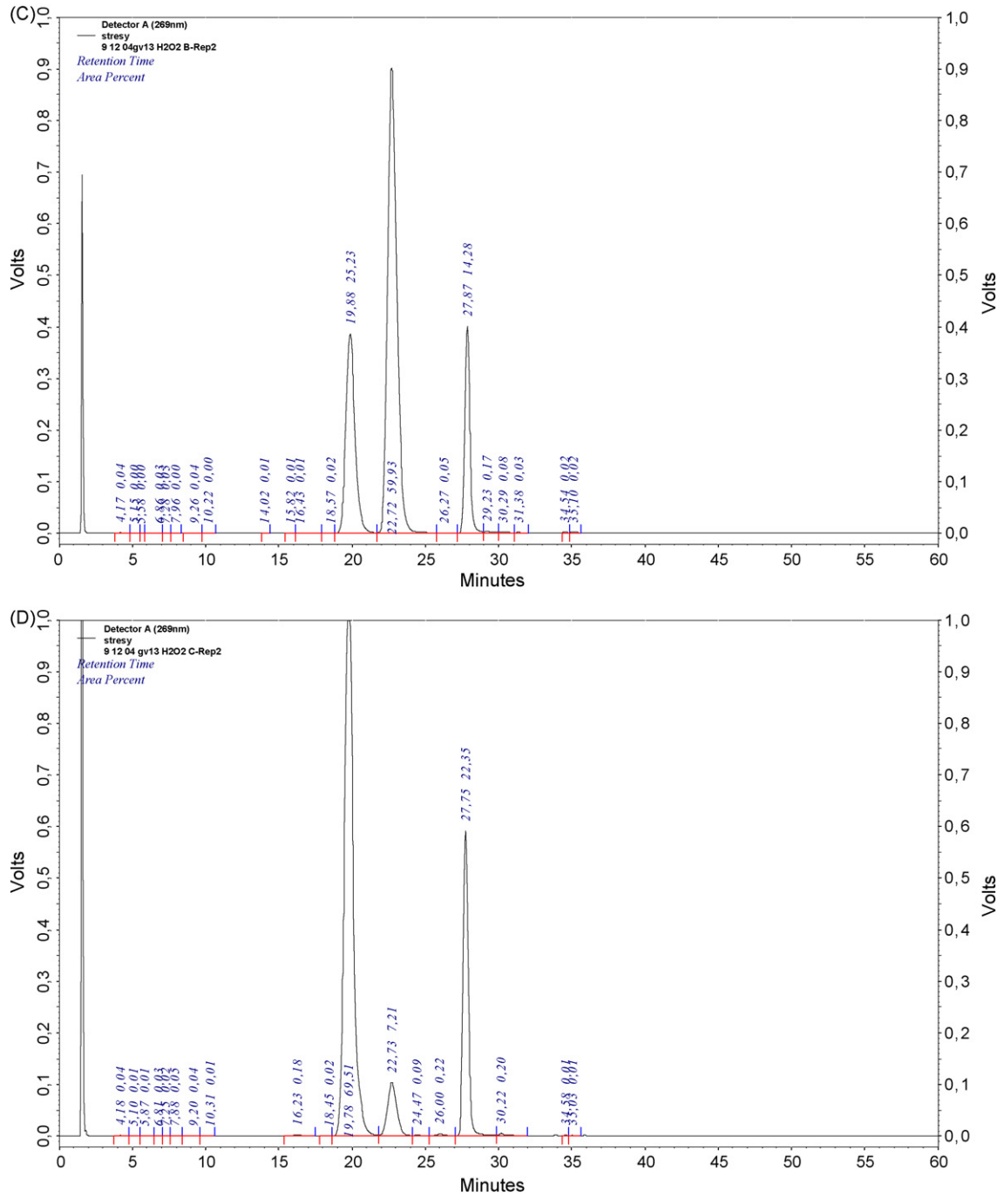


Fig. 6. (*Continued*).

those of compound **7** that it is 1-oxido-derivative of Im. Compound **8**, the final product of oxidation of ImM with hydrogen peroxide, was isolated as the main product when ImM was treated with an excess of 30% hydrogen peroxide for 31 h. From its EI–HRMS the molecular formula was deduced to be $C_{29}H_{31}N_7O_3$. The fragment ion peaks (although of low intensity) at m/e 394, 276 and 262 [6], present in EI–MS, suggested that oxidation also took place only at nitrogen atoms of the piperazine ring. Comparing with Im (**1a**), the ^{1}H NMR data (see Table 5 and point 3.2) of compound **8** showed that it is 1,4-dioxido-derivative of Im. The same compound was formed as third product in oxidation reaction of ImM with 10% hydrogen peroxide.

3.2. ^{1}H NMR results

The chemical formulas of the oxidation products are presented in Fig. 3 and the ^{1}H NMR data of these compounds are collected in Table 5.

Insertion of an oxygen atom at nitrogen atom No.4 of the piperazine ring of Im (compound **6**) results in weak shifting of the Ar–**CH$_2$**–N< signal (singlet) from 3.52 to 3.61 ppm

(+0.09 ppm) and, as expected, strong shifting of the >N–**CH$_3$** signal (singlet) from 2.15 to 3.03 ppm (+0.88 ppm). In the case of insertion of an oxygen atom at nitrogen atom No. 1 of the piperazine ring of the Im molecule (compound **7**) the downfield shifting of the following signals is observed:

- >N–**CH$_3$** signal (singlet), weak shifting from 2.15 to 2.18 ppm (+0.03 ppm).
- Ar–**CH$_2$**–N< signal (singlet), strong shifting from 3.52 to 4.36 ppm (+0.84 ppm).
- The signal corresponding to the aromatic protons of **CH** groups at the C3 and C3′ positions of the benzene ring A (doublet with coupling constant $^3J_{HH}$ ca. 8.1 Hz), moderate shifting from 7.43 to 7.72 ppm (+0.29 ppm). All these effects are slightly stronger in the molecule with oxygen atoms at both nitrogen atoms of the piperazine ring (compound **8**)—the aromatic **CH** doublet is shifted to 7.79 ppm (+0.36 ppm), the Ar–**CH$_2$**–N< singlet to 4.47 ppm (+0.95 ppm) and the >N–**CH$_3$** singlet to 3.10 ppm (+0.95 ppm).

Comparison of the chemical shifts of protons for ImM (**1**) and monomesylate **8a** shows comparable downfield shits of the same groups of protons as in the case of compound **8**. The aromatic **CH** doublet is shifted from ca. 7.48 to 7.79 ppm (+0.31 ppm), the Ar–**CH$_2$**–N< singlet from 3.66 to 4.76 ppm (+1.10 ppm) and the >N–**CH$_3$** singlet from 2.80 to 3.10 ppm (+0.71 ppm). Moreover, a weak downfield shift of the signal corresponding to the aromatic protons of **CH** groups at the C2 and C2′ positions of the benzene ring A (doublet with coupling constant $^3J_{HH}$ ca. 8.1 Hz) from 7.97 to 8.02 ppm (+0.05 ppm) is observed.

In all cases, the shapes and exact chemical shift values of signals corresponding to protons of **CH$_2$** groups of the piperazine ring vary depending on the conditions of the measurement (temperature and concentration in DMSO solution).

4. Conclusion

According to the stress stability study the ImM (**1**) is practically photostable and stable under neutral conditions. The main degradation products under acidic and alkaline conditions are compounds **2** and **3**. Under oxidation stress the main degradation products are compounds **6**, **7** and **8**.

According to the classification system proposed in "Guidance on Conduct of Stress Tests to Determine Inherent Stability of Drugs"; Pharmaceutical Technology On-Line, April 2000, it was found that ImM (**1**) is practically photostable and stable under neutral conditions, very stable under oxidation conditions, stable under acidic and alkaline conditions.

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