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Investigation of the stability of aromatic hydrazones in plasma and related biological material

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Abstract

Novel aromatic hydrazones derived from pyridoxal isonicotinoyl hydrazone (PIH) are interesting compounds from the viewpoint of their pharmacodynamic activity. However, they were recently shown to suffer from relatively short biological half-lives. The purpose of the present study was to investigate the stability of novel aroylhydrazones in plasma and related biological media in order to reveal its potential involvement in the pharmacokinetics of these drugs. Three different aroylhydrazones (pyridoxal isonicotinoyl hydrazone – PIH, salicylaldehyde isonicotinoyl hydrazone – SIH and pyridoxal 2-chlorobenzoyl hydrazone – *o*-108) were incubated in plasma from different species, plasma ultrafiltrate, bovine serum albumin, RPMI cell medium and phosphate buffer saline (PBS) at 37 °C. Stability of these compounds was determined using precise, selective and validated HPLC methods. Although the aroylhydrazones were relatively stable in PBS, they underwent rapid degradation in plasma. Plasma proteins as well as low molecular weight components were involved in this matter. Furthermore, the products of hydrazone bond splitting revealed in this study were also found in the chromatograms from pharmacokinetic experiments. In the light of short biological half-lives determined *in vivo*, these *in vitro* findings strongly suggest that hydrolysis of investigated aromatic hydrazones in plasma could have a significant impact on their pharmacokinetics. Furthermore, these results also suggest that plasma stability of other novel drug candidates containing the hydrazone bond deserves to be considered.

Keywords: Hydrazone; Stability; Plasma; Iron chelator; HPLC

1. Introduction

Novel analogues of pyridoxal isonicotinoyl hydrazone (PIH) are currently extensively investigated from the viewpoint of novel drug development. Many *in vitro* as well as *in vivo* studies have revealed a wide variety of their remarkable biological effects such as mobilization of iron from cell, antioxidative and cardioprotective activities, antiproliferative and antimicrobial effects [1–5]. Hence, novel aroylhydrazones seem to be promising drug candidates with potential to be used in the treatment of several human pathologies.

The subjects of the present study are three different compounds derived from the group of aroylhydrazone iron chelators. The first one, pyridoxal isonicotinoyl hydrazone (Fig. 1), is a lead compound of this group which has passed through detailed investigations in nearly all stages of drug development (*in vitro*, *in vivo* as well as stage I of clinical trial) with main focus on its potential for the treatment of iron overload disorders. Although PIH had been demonstrated to have a significant efficacy in majority of these preclinical experiments, the pilot clinical evaluation suggested lower response than it was originally expected [6]. Therefore, current research is aiming mainly on its novel analogues that appear to be significantly more effective in the treatment of iron overload and particularly, these compounds were also shown to possess a number of other intriguing pharmacological effects (e.g., antiproliferative, antimicrobial, antioxidative and cardioprotective). For example the di-2-pyridylketone isonicotinoyl hydrazones have been identified as strong antiproliferative agents effective in many *in vitro* and *in vivo* studies [7–9]. The analogues derived from isonicotinoyl hydrazide were patented as antituberculotic agents [10]. Other two compounds involved in this study (salicylaldehyde isonicotinoyl hydrazone – SIH and pyridoxal 2-chlorobenzoyl hydrazone – *o*-108; Fig. 1) are the most interesting analogues

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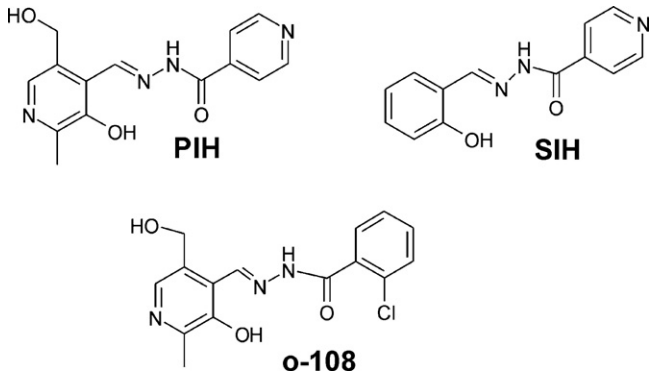

Fig. 1. Chemical structures of the investigated aroylhydrazones.

of PIH. Besides being effective in mobilization of iron in several iron overload models, both have been demonstrated to have strong antioxidative and cytoprotective activities. Moreover, both have been also shown to afford significant cardioprotection *in vivo* against chronic anthracycline cardiotoxicity [11–13]. In addition, these chelators were proven to be relatively non-toxic and well tolerated even after repeated administration to animals [14,15].

In spite of these intriguing pharmacodynamic findings, the pharmacokinetic (PK) experiments performed recently disclosed quite short half-lives of both latter compounds (cca. 17 and 30 min for SIH and *o*-108, respectively), which might be a certain limitation from the standpoint of their further development. Although the reason for this observation remains elusive, during the development and validation of the analytical methods for the purpose of PK experiments some indices suggested that these compounds might be labile in plasma [16,17]. Importantly, all aroylhydrazone analogues have been reported to be sensitive to hydrolysis in both acid and alkaline environment but significantly more stable at physiological pH with only slow hydrolysis being apparent [18,19]. The amino acid catalyzed hydrolysis of PIH and some other analogues studied using UV spectroscopy has been also reported in the literature [20].

The aim of this study was to employ validated HPLC methods for in depth investigation of the stability of three different aromatic hydrazones (PIH, SIH and *o*-108) in plasma and other relevant media (solution of bovine serum albumin (BSA), rabbit plasma ultrafiltrate as well as cell culture medium—RPMI) under physiological pH and temperature to reveal whether this issue might be involved in rather short biological half-lives of these compounds.

2. Experimental

2.1. Materials and chemicals

SIH, *o*-108 and PIH were prepared according to the procedures described previously [21]. Isoniazide (IN), pyridoxal hydrochloride (PL), salicylaldehyde (SA), 2-chlorobenzoyl hydrazide (BH), bovine serum albumin (purity ≥98%), RPMI-1640 (with 20 mM Hepes and L-glutamine, without sodium bicarbonate), acid alpha-glycoprotein (AAG) from bovine plasma and phosphate buffer saline (PBS) were purchased from Sigma–Aldrich (Munich, Germany). Methanol, acetonitrile, DMSO, phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$), EDTA and phosphoric acid were received from Merck (Darmstadt, Germany). Water was purified employing reverse osmosis. Drug-free rabbit, bovine and porcine plasma (obtained from several animals in each case) was received from the ZOO Servis (Dvur Kralove, Czech Republic). Plasma was obtained by harvesting animal blood to tubes containing citrate (alternatively heparin or EDTA) as anticoagulants with subsequent centrifugation at 4000 rpm. Centrifree Amicon Ultrafiltration device were provided by Millipore (Schwalbach, Germany).

2.2. Preparation of stock

The stock solutions of each analyte (2 mg/ml) as well as IS (1 mg/ml) were prepared by dissolving an appropriate amount of substance in either DMSO (SIH) or methanol (*o*-108 and PIH). These solutions were used to prepare both the spiking solutions for validation proposes and to spike the drug-free plasma samples used in the stability study.

2.3. Preparation of solution of bovine serum albumin (BSA) and plasma ultrafiltrate

Bovine serum albumin was dissolved in PBS to get a concentration of 50 mg/ml. Plasma ultrafiltrate was obtained by ultrafiltration of rabbit drug-free plasma (1000 × *g* for 15 min) using Centrifree Amicon Ultrafiltration device.

2.4. Analytical procedures

2.4.1. Sample preparation

Plasma and BSA samples (200 μl) were treated by addition of 0.3 ml of either acetonitrile (SIH and *o*-108) or mixture (1:1) of methanol/acetonitrile (PIH) followed by vortex (2 min) and centrifugation (1000 × *g*/4 min). The supernatant was either directly injected onto the column (SIH and *o*-108) or at first diluted with PBS (0.4 ml of supernatant and 0.2 ml of PBS) and thereafter analyzed (PIH).

In the case of ultrafiltrate, RPMI and PBS, the 50 μl of each sample were diluted with 450 μl of the mixture of methanol/PBS (1:1). This dilution was proven to prevent any further degradation of the compounds before analysis.

2.4.2. Chromatographic system

The chromatographic system LC 20A (Shimadzu, Duisburg, Germany) consisted of a DGU-20A3 degasser, two LC-20 AD pumps, a SIL-20 AC autosampler, a CTO-20AC column oven, a SPD-20AC UV–VIS detector, and a CBM-20AC communication module was used in this study.

2.4.3. Chromatographic conditions

Chromatographic conditions for the analysis of all compounds were based on an analytical method previously developed and validated for the analysis of SIH in plasma for

the purpose of PK experiment [16]. All experiments were performed on an analytical chromatographic column 250 × 4.6 (LiChrospher 100, RP-18, 5 μm) protected with a guard column (Purospher RP-18, 5 μm) purchased from Merck (Darmstadt, Germany). The column oven was set at 25 °C; the flow rate was 1.0 ml/min. The autosampler was cooled to 10 °C to prevent artificial degradation of the samples prior analyses. The composition of mobile phase used for the analysis of particular aroylhydrazone, wavelengths of UV detection as well as internal standards are given below. Injection volume of 30 μl was used for the analysis.

2.4.3.1. Analysis of SIH and o-108. The mobile phase was composed of phosphate buffer (0.01 M NaH_2PO_4; with addition of 2 mM EDTA, pH 6.0 adjusted with 1 M NaOH) and methanol; 53:47 (v/v). Signal was monitored at both 288 and 254 nm. Analyses of SIH utilized *o*-108 as an internal standard while in the case of *o*-108 SIH was analogically employed.

2.4.3.2. Analysis of PIH. Analysis of PIH utilized the phosphate buffer (the same pH and composition as in the previous case) and a mixture of methanol and acetonitrile (1:1, v/v) in a ratio 50:50 (v/v) as a mobile phase. UV detector was set at 297 and 254 nm and *o*-108 was used as an internal standard.

2.4.4. Validation procedure

The analytical methods optimized for the analysis of all aroylhydrazones were tested with respect to following validation parameters: selectivity, linearity, accuracy, precision, recovery and stability. In the case of SIH, beside quantification of this compound, the particular attention was also paid to determination of its degradation product (SA).

2.4.4.1. Preparation of the spiking solutions. The spiking solutions were prepared by appropriate dilution of the stock solutions with methanol to get a different concentration of each analyte. The particular concentrations were chosen to be suitable for spiking the drug-free plasma and BSA to get a set of standard samples of six different concentrations of analyte (2–100 or 5–100 μM in the case of SIH/PIH and *o*-108, respectively) and one concentration of IS (50 μM).

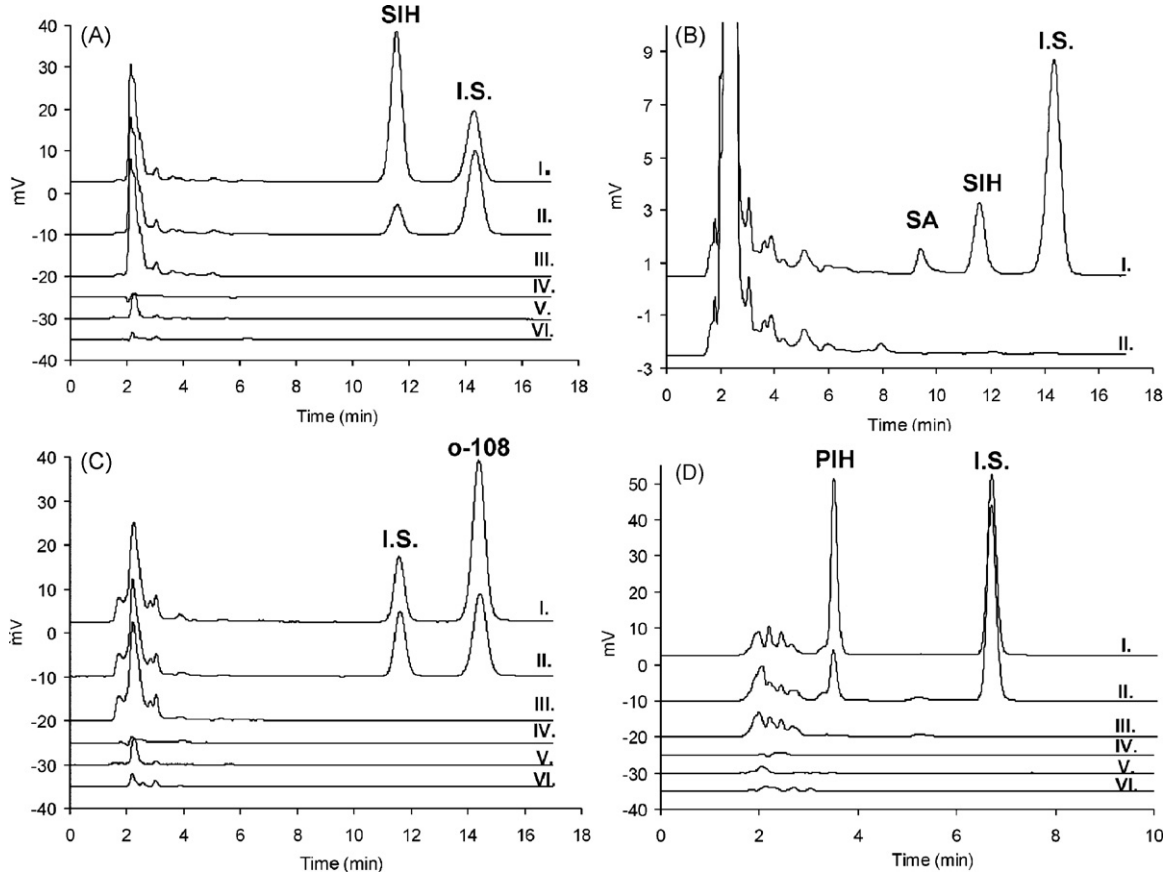

Fig. 2. Chromatograms from the stability experiments. (A) SIH, stability of the compound in rabbit plasma: (I) sample taken at the beginning of the experiment; (II) sample taken in the end of the experiment (180th minute); (III) blank, blanks of other biological materials: (IV) solution of BSA; (V) rabbit plasma ultrafiltrate; (VI) cell medium (RPMI) (the signal is monitored at 288 nm). (B) SIH, the chromatograms documenting the presence of SA in plasma: (I) sample taken at 180th minute of the experiment and (II) blank (the signal is monitored at 254 nm). (C) *o*-108, stability of the compound in rabbit plasma: (I) sample taken at the beginning of the experiment; (II) sample taken in the end of the experiment (240th minute); (III) blank, blanks of other biological materials: (IV) solution of BSA; (V) rabbit plasma ultrafiltrate; (VI) cell medium (RPMI) (the signal is monitored at 288 nm). (D) PIH, stability of the compound in rabbit plasma: (I) sample taken at the beginning of the experiment; (II) sample taken in the end of the experiment (180th minute); (III) blank, blanks of other biological materials: (IV) solution of BSA; (V) rabbit plasma ultrafiltrate; (VI) cell medium (RPMI) (the signal is monitored at 297 nm). The detailed chromatographic conditions are given in Section 2.4.3.

2.4.4.2. Linearity. Linearity was tested within the range of either 2–100 μM (SIH and PIH) or 5–100 μM (*o*-108) in plasma and BSA and in the range of 2–40 μM (all aroylhydrazones) for other media. In the case of SA, the linearity was evaluated over the range 10–50 μM in plasma and BSA and 1–20 μM in other media.

Drug-free plasma or BSA samples were spiked with particular spiking solution to get standard samples of six different concentrations of analyte over the tested range and one concentration of IS (50 μM). Thereafter the samples were treated as described above (Section 2.4.1).

In other cases (plasma ultrafiltrate, RPMI and PBS) the linearity of the detector response was evaluated via analysis of standard samples containing six different concentrations of each analyte (over the range 2–40 μM for aroylhydrazones and 1–20 μM for SA) and one concentration of 5 μM of IS. These standard samples were prepared by appropriate dilution of stock solution with a mixture of methanol/PBS (1:1).

2.4.4.3. Precision and accuracy. Precision and accuracy of the methods were tested by analysis of individually prepared standard samples ($n = 5$) at three different concentration levels (2, 50 and 100 μM for SIH and PIH and 5, 50 and 100 μM for *o*-108) of each analyte in plasma/BSA. The following concentration levels 2, 20 and 40 μM were used for other media.

In the case of SA, the precision and accuracy of the method were checked at concentrations of 10, 20 and 50 μM in plasma/BSA and 1, 10, 20 μM in other media.

2.4.4.4. Selectivity, recovery and stability. Selectivity of the methods was checked by analyses of each drug-free material after passing through the same sample preparation procedure as the analyzed samples. The recovery of the sample preparation procedure was evaluated by comparing the peak area of analyte in the samples treated as described above (Section 2.4.1) with the peak area of analytes in the appropriately diluted standards. The stability of the samples was tested in autosampler (10 °C) over the period of 18 h.

2.5. In vitro stability of aroylhydrazones in plasma and related biomaterial

The drug-free samples (preincubated at 37 °C for 10 min) were spiked with each stock solution to get a final concentration of either 10 μM (rabbit plasma) or 100 μM (other media tested) of each aroylhydrazone studied. Thereafter, these samples were incubated at 37 ± 0.5 °C in a water bath. During whole stability experiment the samples were constantly stirred (130 spin/min). In the different time periods 200 μl (plasma and BSA containing samples) or 50 μl of other media were transferred into the test tube containing 5 μl of IS, vortexed for 30 s and treated immediately as described above (Section 2.4.1). The samples were injected onto the column in triplicate. The stability of aroylhydrazones was expressed as a fall of the concentration in time. The half-lives of degradation were calculated according to the slope of the linear segment ($r^2 \geq 0.99$) of the curve ($\ln c$ versus time) using the equation for first-order kinetic. In the case of PIH and SIH the $t_{0.5}$ values were calculated from the segment of 40 and 60 min, respectively, while the half-lives of degradations of *o*-108 were calculated from the time of 240 min. All stability experiments were performed four times and the data are expressed as a mean ± S.D.

2.6. Statistical analysis

For the purpose of the statistical analysis the SigmaStat for Windows, Version 3.0 was used. To evaluate the statistical differences between results obtained under different conditions (analyte concentrations and different media) ANOVA test was used, while paired *t*-test was employed to compare the values within each group to their initial values. The threshold for statistical significance used in this study was $p < 0.05$, unless specified otherwise. The results are expressed as means ± S.D. ($n = 4$).

3. Results

3.1. Analytical methodology

HPLC method previously developed and validated for the purpose of PK experiment aiming at SIH [16] was modified for the purpose of this stability experiment. This method enabled to simultaneously detect SIH and its degradation product (SA) at retention times of 9.4 and 11.6 min, respectively while IS (*o*-108) was eluted at 14.3 min (Fig. 2A and B). The analysis of both SIH and *o*-108 were performed using the same chromatographic conditions (as described in Section 2.4.3.1, Fig. 2C). The HPLC method modified for the analysis of PIH allowed to detect this compound at the retention time of 3.5 min while its IS (*o*-108) was monitored at 6.7 min (Fig. 2D).

The validation parameters (linearity, precision, accuracy, recovery and stability samples) of these methods are given in the Tables 1–4. The selectivity of the methods is illustrated on the chromatograms of blanks for each medium (Fig. 2).

Table 1
The linearity of the methods used in this study

Compound	Medium	Concentration range (μM)	Linearity	
			Regression equation	Correlation coefficient (r^2)
SIH	Plasma	2–100	$y = 0.0114x + 0.0055$	0.998
	BSA	2–100	$y = 0.0209x + 0.0068$	0.999
	Other	2–40	$y = 0.1319x - 0.0595$	0.997
PIH	Plasma	2–100	$y = 0.0073x - 0.0005$	0.999
	BSA	2–100	$y = 0.0089x - 0.0077$	0.999
	Other	2–40	$y = 0.3341x - 0.1751$	0.995
o-108	Plasma	5–100	$y = 0.0314x - 0.027$	0.999
	BSA	5–100	$y = 0.0352x - 0.0077$	0.999
	Other	2–40	$y = 0.1176x - 0.0882$	0.998
SA	Plasma	10–50	$y = 0.0019x - 0.0057$	0.999
	BSA	10–50	$y = 0.0094x - 0.0558$	0.998
	Other	1–20	$y = 0.0241x - 0.0014$	0.999

Table 2
The precision and accuracy of the methods

Compound	Medium	Concentration (μM)		Precision (R.S.D.)	Accuracy (%)
		Added	Found ± S.D.		
SIH	Plasma	2.08	1.87 ± 0.31	16.41	107.35
		52.07	48.38 ± 3.39	7.01	100.15
		104.15	105.94 ± .60	0.57	101.27
	BSA	2.08	1.54 ± 0.09	5.87	80.03
		52.07	53.12 ± 0.82	1.55	102.16
		104.15	103.57 ± 4.34	4.19	98.98
	Other	2.05	1.90 ± 0.20	10.34	105.76
		20.50	18.64 ± 0.35	1.89	91.22
		41.00	41.15 ± 2.44	5.93	106.30
PIH	Plasma	2.03	2.23 ± 0.26	11.67	109.72
		50.58	50.15 ± 1.96	3.91	99.15
		96.04	94.94 ± 5.29	5.58	98.86
	BSA	2.01	2.02 ± 0.07	3.52	100.25
		50.35	48.49 ± 3.84	7.92	96.30
		100.70	97.50 ± 5.04	5.17	96.82
	Other	2.13	1.99 ± 0.07	3.71	93.43
		19.57	19.34 ± 0.49	2.51	98.83
		38.67	40.29 ± 0.62	1.53	104.19
o-108	Plasma	4.96	4.80 ± 0.24	5.04	96.75
		49.65	47.69 ± 1.19	2.49	96.05
		99.30	100.11 ± 3.67	3.66	100.82
	BSA	5.02	5.08 ± 0.20	3.95	101.23
		50.16	48.66 ± 1.26	2.59	97.01
		100.31	94.51 ± 1.77	1.87	94.22
	Other	1.97	2.86 ± 0.06	2.00	109.84
		19.70	20.20 ± 0.99	4.88	102.54
		39.40	40.41 ± 1.44	3.55	102.55
SA	Plasma	10.0	9.42 ± 0.52	5.51	94.17
		25.10	22.86 ± 1.27	5.57	91.07
		47.50	43.39 ± 0.82	1.88	91.35
	BSA	10.78	11.44 ± 0.27	2.40	106.11
		32.34	31.32 ± 0.86	2.75	96.87
		53.89	55.01 ± 1.31	2.37	102.08
	Other	1.08	1.02 ± 0.04	3.81	94.60
		11.58	12.28 ± 0.08	0.66	106.08
		21.56	21.61 ± 0.64	2.98	100.23

3.2. Stability of aroylhydrazones in vitro

3.2.1. Stability in rabbit plasma

SIH, PIH and *o*-108 were relatively stable in PBS under physiological temperature and pH since their concentrations dropped less than 5% after 3 h and calculated half-lives exceeded 24 h (Table 5). On the other hand, SIH, PIH as well as *o*-108 were significantly and quite rapidly decomposed in plasma under the same conditions. The concentration–time profiles for all compounds are given in Figs. 3–5A and half-lives ($t_{0.5}$) of degradation are displayed in Table 5.

At higher concentration of SIH, less than 10% of the initial amount remained at the end (3 h) of the study (Fig. 3A). The calculated half-life (approximately 0.5 h) derived from the linear part of the kinetic plot pointed out on very rapid degradation of this compound. The typical chromatograms of the samples from the stability experiments are shown in Fig. 2A. The fall of the concentration of SIH was accompanied by the gradual gain of the SA peak area. However, the SA peak area culminated at 60th minute of the experiment and thereafter; only minor increase was observed. The chromatogram from the 180th minute of stability experiment which documents the presence of SA is displayed in Fig. 2B.

PIH also underwent significant degradation in plasma. After 3 h of incubation the concentration of the compound decreased approximately to the 30% of the initial amount (Fig. 4A). The calculated $t_{0.5}$ value pointed out on the close degradation rate of PIH and SIH at a concentration of 100 μM (no statistical differ-

Table 3
The recovery of the precipitation procedures.

Compound	Medium	Recovery (%) (mean ± S.D.)
SIH	Plasma	75.60 ± 7.69
	BSA	100.05 ± 4.81
PIH	Plasma	83.49 ± 2.55
	BSA	99.58 ± 4.50
o-108	Plasma	83.92 ± 2.3
	BSA	99.36 ± 2.52
SA	Plasma	50.80 ± 1.13
	BSA	80.94 ± 3.51

Results are expressed as a mean ($n = 9$).

ence between their $t_{0.5}$ values was observed—$p > 0.05$). Unlike in the case of SIH, this method did not allow evaluation of the likely degradation products of PIH (isoniazide and pyridoxal). The typical chromatograms from these experiments (0, 180th minute and blank) are shown in Fig. 2D.

Concentration–time profile of degradation of *o*-108 in rabbit plasma (Fig. 5A) clearly documented that although *o*-108 (100 μM) was also apparently labile in plasma, its decomposition was significantly slower than in the case of both compounds mentioned earlier (47% of the initial concentration was determined after 240 min of incubation). The appropriate chromatograms documenting these experiments are shown in Fig. 2C.

The experiments focused on the stability of aroylhydrazones in rabbit plasma at a concentration of 10 μM have revealed an interesting phenomenon. The degradation of SIH at 10 μM was apparently slower than at 100 μM ($p < 0.001$). Moreover, there was a statistical difference between the values determined in both concentrations in all time points, with exception of the first interval (Fig. 3A). The similar concentration-dependent pattern of decomposition rate (characterized by $t_{0.5}$; Table 5) was also found in the case of *o*-108, although the individual concentrations were determined to be significantly different only in about one half of the time points (Fig. 5A). On the other hand, inverse pattern of the concentration-dependence of this process was observed in the case of PIH where the lower concentration of the compound tended to elicit more rapid decomposition as documented by the $t_{0.5}$ values. This was further supported by the statistical comparison of individual concentrations in each time interval revealing the same significance in majority of cases (Fig. 4A).

Table 4
Stability of the compounds in autosampler

Compound	Medium	% remaining	R.S.D.
SIH	Plasma	99.53	0.33
	Plasma ultrafiltrate	100.60	0.44
	BSA	98.83	0.75
	RPMI	99.04	0.68
	PBS	98.57	0.19
PIH	Plasma	101.93	1.35
	Plasma ultrafiltrate	99.02	0.53
	BSA	98.29	0.03
	RPMI	98.86	0.66
	PBS	99.22	0.54
o-108	Plasma	100.97	0.69
	Plasma ultrafiltrate	100.50	0.59
	BSA	99.11	0.02
	RPMI	100.97	0.06
	PBS	98.69	0.41
SA	Plasma	98.80	0.85
	Plasma ultrafiltrate	99.16	0.02
	BSA	98.19	2.21
	RPMI	98.09	0.84
	PBS	98.05	0.67

Results are shown as a mean ($n = 3$).

Table 5
The half-lives of degradation of aroylhydrazones in different media, calculated according to the first-order kinetic equation

Compound	Medium	*c* (μM)	Half-life (h) ± S.D.
SIH	Rabbit plasma	100	0.52 ± 0.10
		10	1.00 ± 0.06
	Plasma ultrafiltrate	100	1.21 ± 0.24
	BSA	100	3.59 ± 0.23
	RPMI	100	7.24 ± 0.45
	PBS	100	52.73 ± 9.04
	Porcine plasma	100	1.47 ± 0.07
	Bovine plasma	100	1.30 ± 0.09
PIH	Rabbit plasma	100	0.66 ± 0.13
		10	0.46 ± 0.08
	Plasma ultrafiltrate	100	1.11 ± 0.17
	BSA	100	2.54 ± 0.18
	RPMI	100	4.00 ± 0.47
	PBS	100	28.35 ± 0.25
o-108	Rabbit plasma	100	3.83 ± 0.19
		10	5.19 ± 0.11
	Plasma ultrafiltrate	100	4.99 ± 1.25
	BSA	100	6.54 ± 0.15
	RPMI	100	12.19 ± 0.91
	PBS	100	94.07 ± 6.94

Results are shown as a mean ($n = 4$) ± S.D.

3.2.2. *Stability of aroylhydrazones in other relevant media*

Beside relatively fast degradation of all aroylhydrazones in rabbit plasma, these compounds were also prone to degradation in rabbit plasma ultrafiltrate (Figs. 3–5B). The calculated $t_{0.5}$ values indicated the close rate of hydrolysis of SIH and PIH (Table 5), while in the case of *o*-108 $t_{0.5}$ value was significantly higher. In the case of SIH and PIH the rate of hydrolysis in rabbit plasma ultrafiltrate was significantly slower than in the rabbit plasma. However, interestingly, this was not the case of *o*-108 where $t_{0.5}$ values from both these experiments (rabbit plasma and plasma ultrafiltrate) were not statistically different ($p = 0.080$). The degradation of all aroylhydrazones in other media under the study (BSA solution and RPMI) proceeded more slowly than in rabbit plasma or plasma ultrafiltrate (Figs. 3–5B), however in all cases it was still significantly more rapid in comparison with decomposition in PBS (Table 5). The experiment focused on the addition of AAG to the solution of BSA did

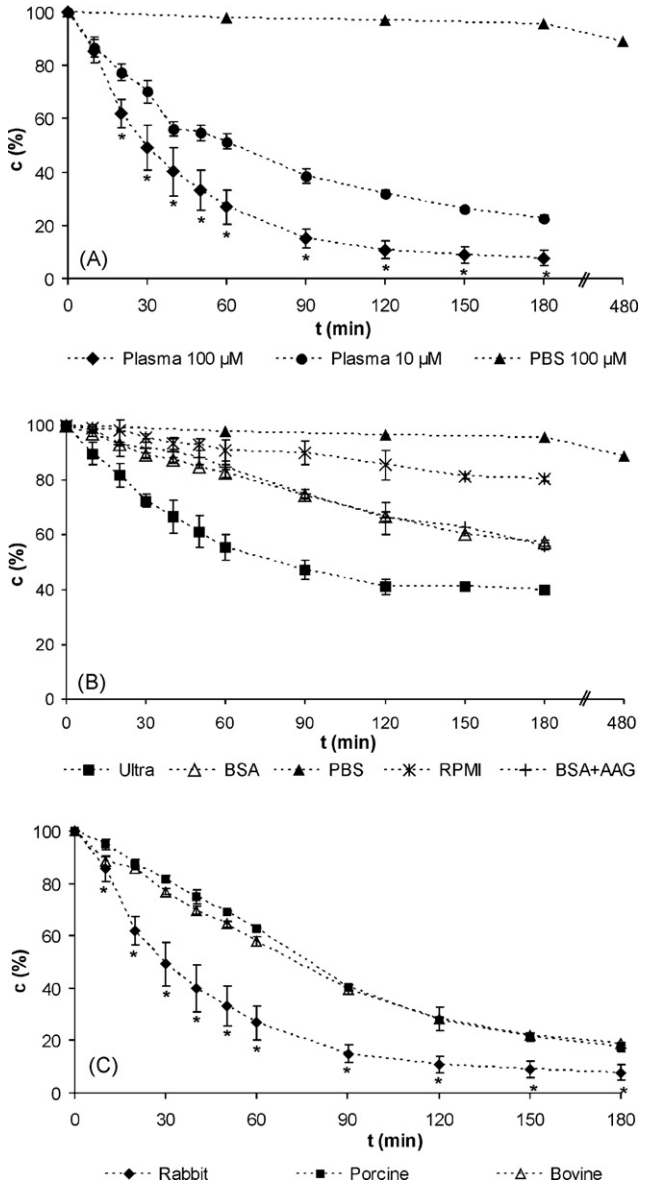

Fig. 3. Concentration–time profile of the degradation of SIH in plasma and other relevant media at 37 °C *in vitro*. (A) Rabbit plasma; (B) other media (rabbit plasma ultrafiltrate—Ultra; solution of BSA; PBS; cell culture medium—RPMI; solution of bovine serum albumin with addition of acid alpha-glycoprotein—BSA + AAG); (C) plasma of different animal species (rabbit, porcine and bovine). *The time points where the statistically significant differences between the results obtained at plasma at two concentrations tested (100 and 10 μM) were proven ($p < 0.05$). Results are shown as a mean ± S.D. ($n = 4$).

not indicate significant change in the degradation behavior of SIH.

3.2.3. Interspecies differences in plasma stability

The stability of SIH (100 μM) was evaluated in bovine and porcine plasma to reveal possible interspecies variability. The concentration–time profiles of SIH degradation in plasma of different species are shown in Fig. 3C, $t_{0.5}$ values are given in Table 5. Although the SIH degradation in plasma of the larger animal species was significantly slower, the decline of plasma concentrations was still marked and significant in both cases.

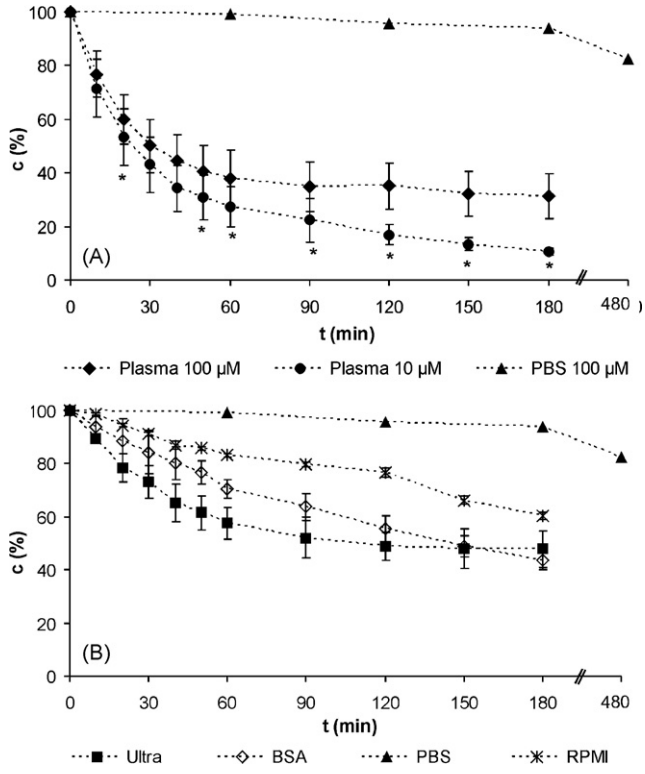

Fig. 4. Concentration–time profile of the degradation of PIH in rabbit plasma and other relevant media at 37 °C *in vitro*. (A) Rabbit plasma and (B) other media (rabbit plasma ultrafiltrate—Ultra; solution of BSA; PBS; cell culture medium—RPMI). *The time points where the statistically significant differences between the results obtained at plasma at two concentrations tested (100 and 10 μM) were proven ($p < 0.05$). Results are shown as a mean ± S.D. ($n = 4$).

3.2.4. Retrospective analysis of the chromatograms obtained from the study of pharmacokinetics of SIH and *o*-108 in rabbits

The retrospective analysis of the chromatograms obtained from the experiments focused on SIH (10 mg/kg, i.v.) pharmacokinetics in adult male rabbits [16], revealed one peak of the putative metabolite at a retention time of 11.3 min (Fig. 6A). This peak was detectable only at the lower wavelength from both 254 and 288 nm used in this PK study. As it is documented in Fig. 6A and B the retention times as well as peak shape corresponds well with the peak of SA standard (it was also verified using different chromatographic conditions). Hence, it is very likely, that SA as a putative metabolite/decomposition product appears in plasma after the i.v. administration of SIH to rabbits. In the similar PK study focused on the *o*-108, the UV detection was set only at 288 nm which did not allow sensitive detection of its putative metabolites (pyridoxal and 2-chlorobenzoyl hydrazide). Furthermore, the peak of pyridoxal (RT 2.7 min) was likely to be hidden in the background of biological material. However, a little peak at the retention time corresponding to 2-chlorobenzoyl hydrazide (3.9 min) appeared in the samples taken from 3rd to 7th minute of the PK experiment. Fig. 6C and D shows the chromatograms of the analysis of the sample from PK experiment (7th minute), corresponding blank as well as the analysis of the mix of standards.

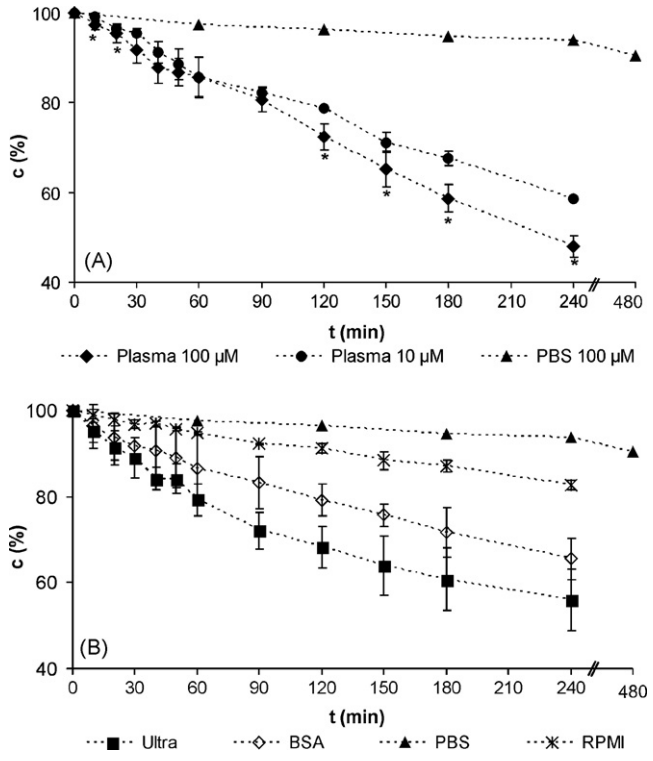

Fig. 5. Concentration–time profile of the degradation of *o*-108 in rabbit plasma and other relevant media at 37 °C *in vitro*. (A) Rabbit plasma and (B) other media (rabbit plasma ultrafiltrate—Ultra; solution of BSA; PBS; cell culture medium—RPMI). *The time points where the statistically significant differences between the results obtained at plasma at two concentrations tested (100 and 10 μM) were proven ($p < 0.05$). Results are shown as a mean ± S.D. ($n = 4$).

4. Discussion

The stability of three different aroylhydrazones in plasma was evaluated at two different concentrations (100 and 10 μM) selected with respect to both the results of the PK experiments and the quantification limits of the analytical methods. The higher concentration (100 μM) is close to c_{max} of SIH after i.v. administration, whereas the lower one (10 μM) is a concentration relevant to the elimination phase of the drug. The majority of our experiments utilized rabbit plasma—a biological material relevant to the experimental model employed in the PK studies. However, to assess the impact of the interspecies variability on this matter, bovine and porcine plasma were also used.

Although it is well known that hydrazones are prone to the acid and alkaline catalyzed hydrolysis, the outcomes of this study showed that aroylhydrazones are relatively stable in PBS under physiological pH and temperature with only slow hydrolysis being apparent. This is in line with other studies utilizing different non-separative analytical approaches [18,20]. Therefore, it was originally anticipated that these compounds shall be quite stable in *in vivo* environment. However, in this study we have revealed that this assumption need not be true. The stability experiments disclosed very significant and surprisingly fast degradation of all investigated aroylhydrazones in rabbit plasma. This phenomenon was especially marked in the cases of SIH and PIH, where the calculated half-lives at higher concentration were shorter than 40 min. Even though *o*-108 was significantly more stable than SIH and PIH, its degradation was still very marked and certainly significant enough to be considered as a factor with potential impact on *in vivo* pharmacokinetics of the compound. The hydrolysis of *o*-108 in plasma was also apparently much faster than it was in PBS. To rule out the contribution of anticoagulative agents remaining in plasma, besides citrate, the same experiments were also performed with plasma containing heparin or EDTA. However, this modification did not have any impact on the results obtained (data not shown). The same outcome was obtained when the hydrazones were used as their HCl salts instead of free bases.

Interestingly, the results of the present study suggest that the rate of hydrolysis might be affected by the concentration of parent aroylhydrazone. The similar pattern of this concentration-dependent effect was observed in the case of SIH and *o*-108, whereas in contrast, opposite tendency was found with PIH. It is uneasy to clarify this discrepancy. Although SIH and *o*-108 does not share any specific structural features which are not present in PIH, both the earlier mentioned compounds are significantly more lipophilic. It is plausible that these compounds might differ in plasma protein bound/free fraction which could have an impact on the amount of the compound available for the decomposition. However, so far, there are no experimental data concerning protein binding of these compounds. Moreover, it might be anticipated that affinity of drug to bind to plasma proteins is only one of factors being involved in this phenomenon.

The analytical methodology used for the analysis of SIH allowed simultaneous monitoring of parent compound and its degradation product (SA). Since until 60th minute of the experiment it was observed that the gradual gain of SA peak area was stoichiometrically proportional to the amount of SIH decomposed, it is strongly suggested that hydrolytic splitting of the hydrazone bond is a chemical reaction being responsible for the decline in the concentration of the aromatic hydrazones in this study. After that time point the peak area of SA further increased slowly and the little discrepancy between SIH and SA stoichiometry was observed. This might be caused by the degradation of SA in plasma which was suggested by the pilot experiment focused on SA stability in plasma (data not shown).

The SIH kinetic curve showed linear behavior only up to 60th minute of the study. After that point more than 70% of the parent compound was decomposed and therefore the concentration of degradation products was likely high enough to affect the rate of SIH hydrolysis by means of backward reactions. As the backward reactions are unlikely to play a significant role in an open biological system (*in vivo*), where the degradation products can be freely distributed to the tissues or undergo elimination, the half-lives for all compounds in this study were calculated from the linear segment of degradation curve, as this is assumed to be relevant to the *in vivo* conditions.

In the lights of all findings discussed above we decided to retrospectively analyze the chromatograms of the samples from both PK experiments performed previously with SIH and *o*-108 [16,17], in order to reveal whether the products of the hydrazone bond splitting were also present there. Importantly, in both cases we have found peaks with retention times corresponding

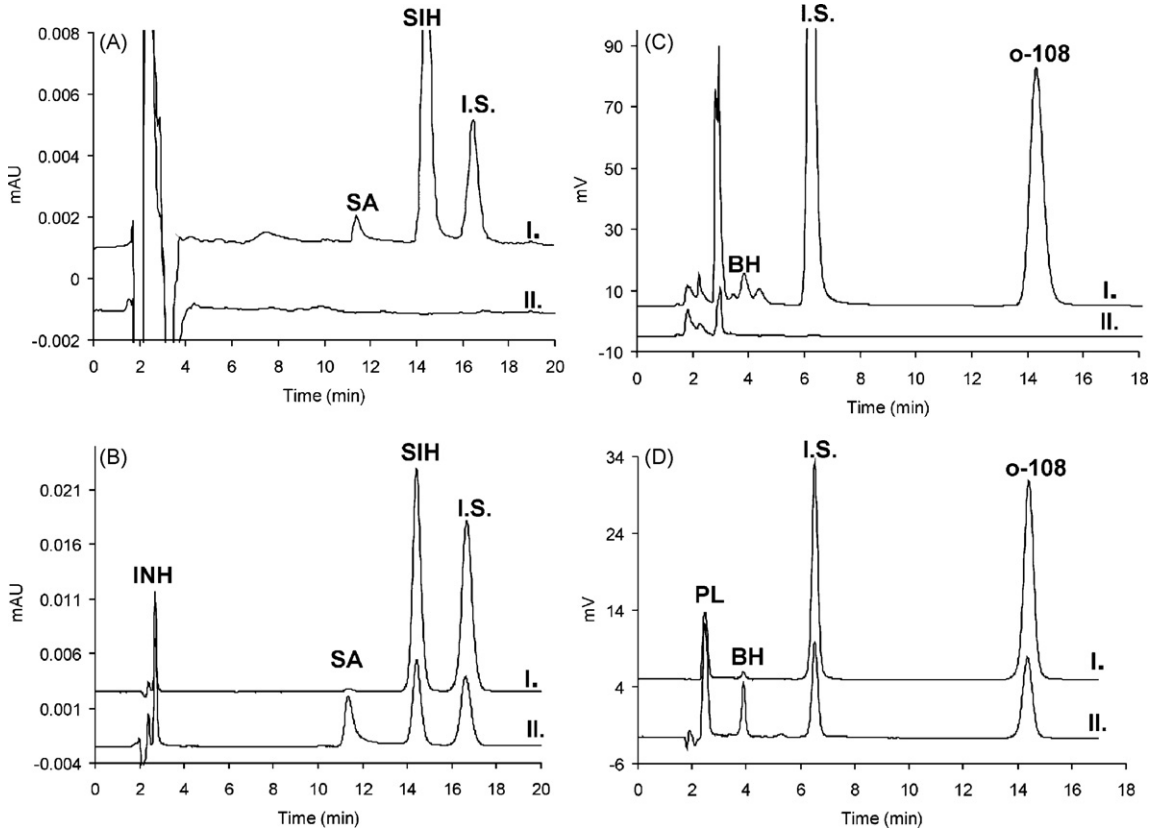

Fig. 6. Chromatograms of the analysis of the samples from the PK experiments (SIH and *o*-108) and corresponding standards. (A) SIH, analysis of the samples taken before (II), at 3rd minute (I) after i.v. application of SIH (10 mg/kg) to male rabbits. The chromatograms were detected at 254 nm. (B) SIH, chromatograms of the mix of standards (50 μM) monitored at both 288 nm (I) and 254 nm (II). (C) *o*-108, analysis of the samples taken before (II) and at 7th minute (I) after i.v. administration of *o*-108 (10 mg/kg) to male rabbits. The chromatograms are monitored at 288 nm. (D) *o*-108, chromatograms of the mix of standards (20 μM) monitored at both 288 nm (I) and 254 nm (II). INH, isoniazide; SA, salicylaldehyde; SIH, salicylaldehyde isonicotinoyl hydrazone; IS, pyridoxal 2-chlorobenzoyl hydrazone (in the case of SIH); BH, 2-chlorobenzoyl hydrazide; PL, pyridoxal; IS, pyridoxal isonicotinoyl hydrazone (in the case of *o*-108); *o*-108, pyridoxal 2-chlorobenzoyl hydrazone. The detailed analytical conditions are given in Refs. [16,17].

to SA (in the case of SIH) and 2-chlorobenzoyl hydrazide (in the case of *o*-108). However, since the analytical methods were not set up and validated properly to evaluate these compounds, it is very likely that their detection was suboptimal. This handicap is particularly evident in the case of 2-chlorobenzoyl hydrazide which was eluted close to plasma background and above all, its detection suffered from very low absorbance at the wavelength used in the PK experiment (288 nm). The other products of hydrazone bound splitting (isoniazide and pyridoxal) were not detected in these studies since they were most likely hidden in the matrix background. Although the products of hydrolysis of hydrazone bond warrants the particular study focused on this matter, these findings strongly suggest that decomposition of aroylhydrazones in plasma environment might also occur *in vivo* after the administration of the drug into the living organism. Since the degradation of the compounds in plasma is extensive, it is very likely that this phenomenon could have an impact on the fate of these compounds in the organism *in vivo* and therefore, it could influence the PK parameters (particularly the short half-life).

The wider applicability of this phenomenon was tested in experiments involving the plasma samples originated from two other different animal species (porcine and bovine). These experiments showed that the hydrolysis of aroylhydrazones in plasma is not a feature specific for small laboratory animals only but it seems to be a common phenomenon being involved also in the larger animal species and therefore, it might be expected in humans. Although the calculated $t_{0.5}$ for hydrolysis of SIH in porcine and bovine plasma were significantly lower than in rabbit plasma (approximately three times), these values are obviously still very significant and they definitely deserve consideration.

In order to get more complex insight into the investigated matter, the impact of main plasma components on aroylhydrazone stability was investigated. Albumin is a most abundant plasma protein; hence, the stability of the aroylhydrazones in BSA solution at the physiologically relevant concentration was examined. The calculated $t_{0.5}$ values indicated that all aroylhydrazones were more stable in BSA than in rabbit plasma (approximately seven, four and nearly two times for SIH, PIH and *o*-108, respectively). Experiments utilizing the bovine plasma recognized SIH to be nearly three times more stable in solution of BSA than in bovine plasma. However, the degradation rate in BSA was still significantly higher than in PBS (in all cases). Since AAG is one of the other important plasma proteins particularly responsible for binding of compounds of alkaline nature, potential contri-

bution of AAG was studied as well (added at a physiological concentration to the solution of BSA). However, AAG evidently did not have any impact on the investigated matter. The results of these experiments suggested that the most abundant plasma protein is likely to participate in the degradation of the investigated compounds, although it is probably not the main factor involved.

Experiments utilizing rabbit plasma ultrafiltrate revealed that low molecular plasma components (with molecular weight less then 30,000 Da) significantly catalyzed the hydrolysis of all the investigated compounds. In the case of SIH and PIH, the rate of hydrolysis in rabbit plasma ultrafiltrate was approximately only two times slower than in rabbit plasma. The close $t_{0.5}$ values of degradation in plasma ultrafiltrate for SIH and PIH pointed out to the nearly similar sensitivity of these compounds to decomposition in this medium. Analogically as in other environments discussed above, *o*-108 was apparently more stable in plasma ultrafiltrate than both SIH and PIH. Interestingly, comparing $t_{0.5}$ values of *o*-108 assessed in plasma and plasma ultrafiltrate it is obvious, that all these values are relatively close to each other (Table 5) and the statistical evaluation did not confirm any significant difference. In the light of these findings it seems to be plausible that the low molecular weight components participate in the degradation of *o*-108 to a higher degree than in the case of other compounds under the study.

Since *in vitro* experiments employing cell cultures are today an integral part of drug research, in the present study we also focused on the stability of the aroylhydrazones in one of the common cell culture medium (RPMI-1640). Moreover, this medium with defined composition helps to analyze the involvement of common low molecular bioorganic compounds in the investigated matter. Although all aroylhydrazones were much more stable in RPMI than in plasma, the hydrolysis was apparently more rapid and significant than in PBS (more than seven times higher for all compounds). The half-lives of degradation of the aroylhydrazones in RPMI determined in the present study were even higher than it was estimated previously using simple UV–VIS spectrophotometry [20].

In the study of Buss and Ponka [20] authors have suggested that degradation of hydrazones in RPMI cell culture medium could be catalyzed by amino acids. Our results are generally in accordance with these previous findings, but they also suggest much higher degree of complexity of this phenomenon. Although degradation of the aroylhydrazones in RPMI was significant it was still several times slower than in plasma ultrafiltrate. Hence, other low molecular weight plasma components, which are not present in cell culture medium, are likely to significantly contribute to the instability of the studied compounds. Furthermore, albumin and most likely some other abundant plasma protein are also involved in instability of these compounds in plasma and their contribution appears even more significant than that of the components of defined cell culture medium. Hence, it seems that the issue of instability of aroylhydrazones in biological material and particularly plasma is of much more complex nature and considerably more important than it was previously believed. In the light of these findings, it seems to be plausible that limited stability of drug in plasma might be one of the factors being involved in the failure of PIH in the phase I clinical trial [6]. On the other hand, it could be expected that the ketone-derived aroylhydrazones or thiosemicarbazones might be different in this way which, however, deserves further studies.

Apart from the particular group of aroylhydrazones included in this study, the outcomes of these experiments could have a wider applicability to other drug candidates containing the hydrazone bond. Importantly, so far, number of various aromatic hydrazones were synthesized and tested for the different biological activities [22–30]. The findings of the present study suggest that incorporation of routine *in vitro* plasma stability testing (under the physiologically relevant conditions) into the experiments should be considered in order to accelerate the process of novel drug development. Furthermore, the possibility to improve stability of aromatic hydrazones by means of minor modification of their chemical structure deserves further investigation.

5. Conclusion

In this study using validated analytical methodology we have for the first time shown, that, although three different aroylhydrazones (SIH, *o*-108 and PIH) can be considered as stable in the saline of physiological pH and temperature, they are considerably prone to the decomposition in animal plasma. Furthermore, detailed investigation has revealed that low molecular weight compounds as well as plasma proteins are involved in this matter. The decomposition of these compounds proceeds through the hydrolysis of hydrazone bond. Furthermore, the products of this reaction have been detected in chromatograms from the pharmacokinetic experiments. These findings, together with very short half-lives determined *in vitro*, strongly suggest that plasma instability could have a significant impact on the pharmacokinetics (particularly biological half-lives) of the studied compounds. Since several different aromatic hydrazones are currently under study as drug candidates, their plasma stability should be tested to accelerate their further development.

Acknowledgement

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