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HPLC-DAD and MS/MS analysis of novel drug candidates from the group of aromatic hydrazones revealing the presence of geometric isomers

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Abstract

Salicylaldehyde isonicotinoyl hydrazone (SIH) is an iron-chelating aromatic hydrazone with promising pharmacological properties. However, it suffers from relatively short biological half-life. Hence, two novel derivates of SIH, HAP-INH and HPP-INH were synthesized in order to overcome this pharmacokinetic drawback. The aim of the present study was to employ HPLC-DAD and HPLC–MS/MS methods to investigate the identity of the putative impurities of these newly prepared substances, which are being formed in aqueous environment. At first, it was shown that their retention times as well as UV spectra did not correspond to any expected synthetic precursor, by-product or degradation product. HPLC-DAD analysis confirmed purity of peaks and revealed close but not identical UV spectra of putative impurities and corresponding hydrazones. The subsequent HPLC–MS/MS analyses using ESI and the ion trap mass analyzer showed the identical molecular ions (in both modes) as well as their fragmentation, which implicated presence of geometric isomers. This suggestion was further supported by the NMR analyses. Since the Z/E isomers can have different biological activities, results of this study might be of great importance for further development of the aroylhydrazones as novel drug candidates as well as from the theoretical point of view.

Keywords: Geometric isomers; Aroylhydrazones; HPLC–MS; HPLC-DAD; Iron chelators

1. Introduction

Novel iron chelating agents belonging to the group of aromatic hydrazones are currently investigated as potential drugs for the treatment of various human pathologies, including iron overload-associated diseases (such as β-thalassemia), cancer, anthracycline-induced cardiotoxicity or tuberculosis [1–4]. Salicylaldehyde isonicotinoyl hydrazone (SIH) is among the most promising agents of this group. Besides its ability to reduce cellular-iron burden in iron-overload models, it has been also shown to possess significant cardioprotective activity against oxidative stress-induced injury [5] as well as against anthracycline-induced cardiotoxicity both *in vitro* and *in vivo* [6]. Moreover, it has been demonstrated to be relatively non-toxic and well tolerated even after repeated administration to rabbits [7].

Despite these interesting pharmacodynamic findings, pilot pharmacokinetic study of SIH revealed its relatively short biological half-life (approximately 17 min), apparently due to the limited stability of the compound in plasma [8].

Therefore, we focused on the modification of the chemical structure of SIH to improve its stability. Two novel derivatives of SIH (Fig. 1) related to the corresponding aromatic ketones were synthesized: 2′-hydroxyacetophenone isonicotinoyl hydrazone

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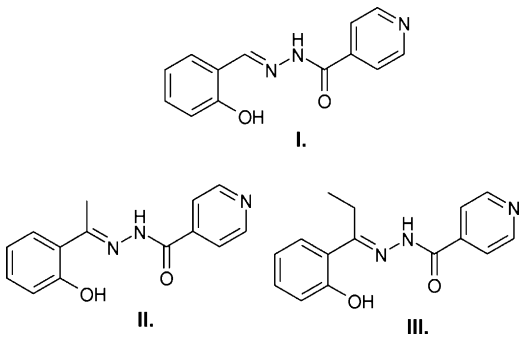

Fig. 1. The chemical structures of the investigated hydrazones. I. Salicylaldehyde isonicotinoyl hydrazone (SIH, MW 241); II. 2′-hydroxyacetophenone isonicotinoyl hydrazone (HAP-INH, MW 255); III. 2′-hydroxypropiophenone isonicotinoylhydrazone (HPP-INH, MW 269).

(HAP-INH) and 2′-hydroxypropiophenone isonicotinoylhydrazone (HPP-INH).

Interestingly, during an HPLC method development for the determination of these compounds, an unexpected peaks of suspected impurities, which did not correspond to either synthetic precursors or expected degradation products was observed. Therefore, the aim of the present study was to employ HPLC-DAD and HPLC–MS/MS methods to characterize the identity and the origin of these unknown substances.

2. Experimental

2.1. Material

Salicylaldehyde isonicotinoyl hydrazone (SIH) was prepared according to the procedure described previously [9]. Methanol and acetonitrile (HPLC grade), DMSO, EDTA, $NaH_2PO_4{\cdot}2H_2O$ were purchased from Merck (Darmstadt, Germany). Water was purified employing reverse osmosis. The synthetic precursors and other chemicals used for the synthesis were purchased from Sigma–Aldrich (Schnelldorf, Germany). The drug-free porcine plasma was obtained from ZOO service (Dvur Kralove, Czech Republic).

2.2. Synthesis, structure elucidation and determination of the chelation efficacy of HAP-INH and HPP-INH

The novel compounds were prepared by the Schiff base condensation of 2′-hydroxyacetophenone or 2′-hydroxypropiophenone, respectively, with 1 equiv. isonicotinoyl hydrazide in aqueous ethanol with catalytic amount of acetic acid. HAP-INH was prepared under conventional reflux for 9 h, while the less reactive propiophenone derivative was prepared under microwave irradiation (400 W), which significantly decreased the reaction time to 1 h. The product was recrystallized from ethanol. The structure and purity of the compounds were confirmed by FTIR (Nicolet Impact 400 spectrophotometer) and ^{1}H and ^{13}C NMR spectra (Varian Mercury-Vx BB 300 instrument, operating at 300 MHz for ^{1}H, 75 MHz for ^{13}C, 5 mm tubes, standardized to internal tetramethylsilane). Melting points were measured using the Koffler apparatus and are uncorrected.

2.2.1. HAP-INH

Yellowish crystals; m.p. 240–242 °C; yield 77%. IR (KBr): ν_{max} 3436; 3116; 1681; 1642; 1610; 1535; 1495; 1304; 1298 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 13.19 (s; OH); 11.59 (s; NH); 8.79 (2H; d; J = 4.4 Hz; Py); 7.84 (2H; d; J = 5.3 Hz; Py); 7.65 (1H; d; J = 7.6 Hz; Ph); 7.32 (1H; dd; J = 5.9 Hz; J = 7.6 Hz; Ph); 6.92 (1H; d; J = 8.2 Hz; Ph); 6.89 (1H; d; J = 7.8 Hz; Ph); 3.36 (3H; s; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 163.2; 159.7; 159.0; 150.4; 140.3; 131.8; 129.0; 122.2; 119.4; 118.8; 117.6; 14.5 ppm. These data are in accordance with Maurya [10].

2.2.2. HPP-INH

Yellowish crystals; m.p. 244–246 °C; yield 48%. IR (KBr): ν_{max} 3436; 3139; 1683; 1603; 1528; 1488; 1279 cm^{-1}; ^{1}H NMR (300 MHz, DMSO): δ 13.28 (s; OH); 11.61 (s; NH); 8.79 (2H; d; J = 5.9 Hz; Py); 7.80 (2H; d; J = 5.5 Hz; Py); 7.64 (1H; d; J = 7.5 Hz; Ph); 7.31 (1H; dd; J = 8.4 Hz; J = 7.2 Hz; Ph); 6.92 (1H; d; J = 8.3 Hz; Ph); 6.89 (1H; d; J = 7.2 Hz; Ph); 3.01 (2H; q; J = 7.6 Hz; CH_2); 1.14 (3H; t; J = 7.6 Hz; CH_3); ^{13}C NMR (75 MHz, DMSO): δ 163.6; 162.7; 159.5; 150.3; 140.5; 131.7; 128.6; 122.4; 118.9; 117.9; 117.9; 19.6; 11.6 ppm.

Iron chelation efficiency of novel chelators was determined using the fluorimetric calcein assay as described previously [11]. Both novel chelators were shown to rapidly and effectively remove Fe^{3+} from the fluorescence-quenched iron–calcein complex. Their iron chelation efficacies (97.84 ± 0.54% for HAP-INH and 97.78 ± 1.34% for HPP-INH) were similar to that of the reference agent SIH (100%).

2.3. Preparation of the stock and working solutions

The stock solution of investigated aroylhydrazones was prepared in DMSO (0.5 mg/mL). This solution was further diluted with a mixture of water and methanol (1:1, v/v) to get the working solutions (10 and 1 μg/mL) for the HPLC-DAD and HPLC–MS analyses, respectively.

2.4. HPLC-DAD analysis

2.4.1. Chromatographic system

HPLC analyses were performed on the chromatographic system LC 20A Prominence (Shimadzu, Duisburg, Germany) consisting of a DGU-20A3 degasser, two LC-20 AD pumps, a SIL-20 AC autosampler, a CTO-20AC column oven, UV or SPD-M20A photodiode array detector, and a CBM-20AC communication module. The chromatographic data were processed using an LC solution software, version 1.21 SP1 (Schimadzu, Duisburg, Germany).

2.4.2. Chromatographic conditions for purity evaluation

The purity of both compounds was evaluated on an analytical column 250 mm × 4.6 mm (LiChrospher 100, RP-18, 5 μm) purchased from Merck (Darmstadt, Germany). Both compounds were analyzed using the mixture 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 (40:60; v/v) as a mobile phase. The flow rate of 1.0 mL/min was utilized. The column oven was

set at 25 °C. The UV spectra were scanned in the range of 200–400 nm and the samples were injected in the volume of either 20 or 40 μL for the analyses of HPP-INH and HAP-INH, respectively.

2.5. HPLC–MS analysis

2.5.1. HPLC–MS system and settings

HPLC–MS analyses were performed using a chromatographic system LC 20A Prominence (DGU-20A3 degasser, LC-20 AD pumps, SIL-20 AC autosampler, CTO-20AC column oven, CBM-20AC communication module- Shimadzu, Duisburg, Germany) coupled with LCQ Max advantage mass spectrometer (Thermo Finnigan, San Jose, U.S.A.) equipped with ESI source and an ion trap mass analyzer. The samples were analyzed in both positive and negative modes. In the positive mode, the following parameters were set: spray voltage 4 kV, capillary temperature 250 °C, capillary voltage 5 V, tubes lens offset 5 V, sheet/auxiliary gas flow 60/30 units. Same setting was also employed in negative ionization mode. The mass chromatograms were recorded in total ion current (TIC) within a range of either 50–1000 *m/z* (the initially experiment) or 100–600 *m/z* (the later experiments) as well as in single ion monitoring (SIM) on $[M+H]^+$. In MS^2 experiments the fragmentation of molecular ions was carried out using collision energy (CE) of 37–40%. The data were analyzed using Xcalibur software (Thermo Finnigan, San Jose, U.S.A.).

2.5.2. Chromatographic conditions

HPLC–MS analyses were performed on a chromatographic column Luna, phenyl-hexyl, 150 mm × 30 mm, 5 μm (Phenomenex, Aschaffenburg, Germany). A mixture of water and methanol (1:1, v/v) was employed as a mobile phase in the initial experiments, while the later analyses utilized 50% acetonitrile. The column oven was set at 25 °C. A flow rate of 0.3 mL/min and an injection volume of 20 μL were used.

2.6. In vitro experiment

Either drug-free plasma or PBS (1 mL) was spiked with the stock solution of HPP-INH to get a concentration of 100 μM. Thereafter, these samples were incubated at 37 °C for 1 h. At the beginning and at the end of this experiment, 0.3 mL of the sample was either precipitated using 0.5 mL of acetonitrile (plasma) or diluted with methanol (1:1; v/v) and immediately analyzed (the chromatographic conditions are given in the Section 2.4).

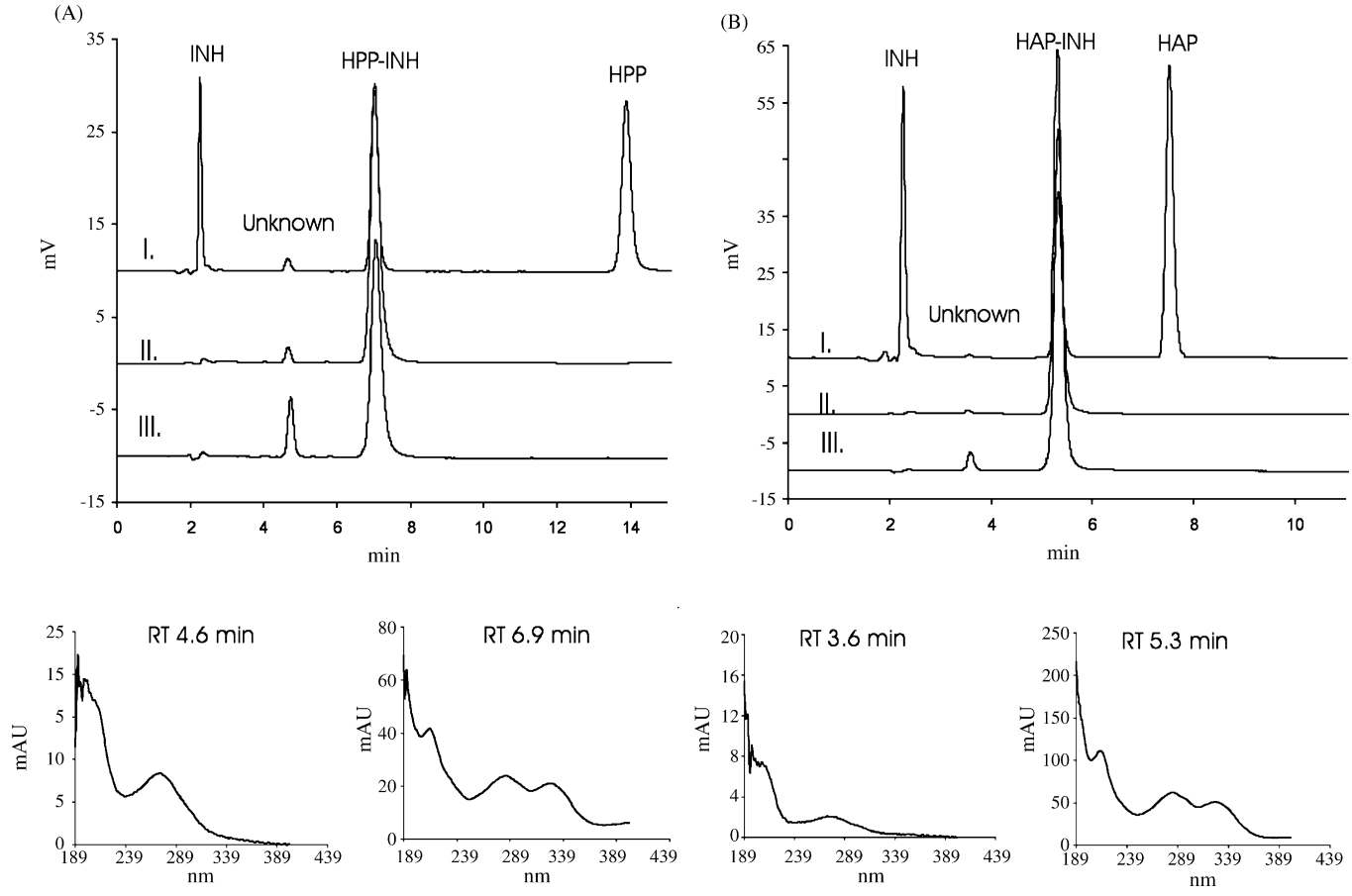

Fig. 2. HPLC-DAD analyses (chromatograms and UV spectra) documenting the presence of the putative impurities. (A) HPP-INH; (B) HAP-INH. I. Mixture of the each aroylhydrazone and the corresponding synthetic precursors/degradation products; II. Analyses of aroylhydrazones (10 μg/mL) dissolved in the mixture of methanol water (1:1, v/v), time 0 min, III. Analyses of aroylhydrazones (10 μg/mL) dissolved in the mixture of methanol water (1:1, v/v), time 12 h. INH: isoniazide; HPP-INH: 2′-hydroxypropiophenone isonicotinoylhydrazone; HPP: 2′-hydroxypropiophenone; HAP-INH:2′-hydroxyacetophenone isonicotinoylhydrazone; HAP: 2′-hydroxyacetophenone (The detailed HPLC conditions are given in the Section 2.4).

3. Results and discussion

During the HPLC evaluation of the purity of newly prepared SIH analogues (HAP-INH and HPP-INH) an interesting phenomenon was observed. In contrast to SIH, the HPLC analyses of the novel ketone derivatives revealed the presence of additional peaks with lower retention times than the parent compounds (Fig. 2). Furthermore, in aqueous solution the area of this unexpected peak tended to increase, while the peak area of the parent compound decreased. Although this behavior was observed mainly in the case of HPP-INH, a small peak corresponding to a similar "impurity" was detected on the chro-

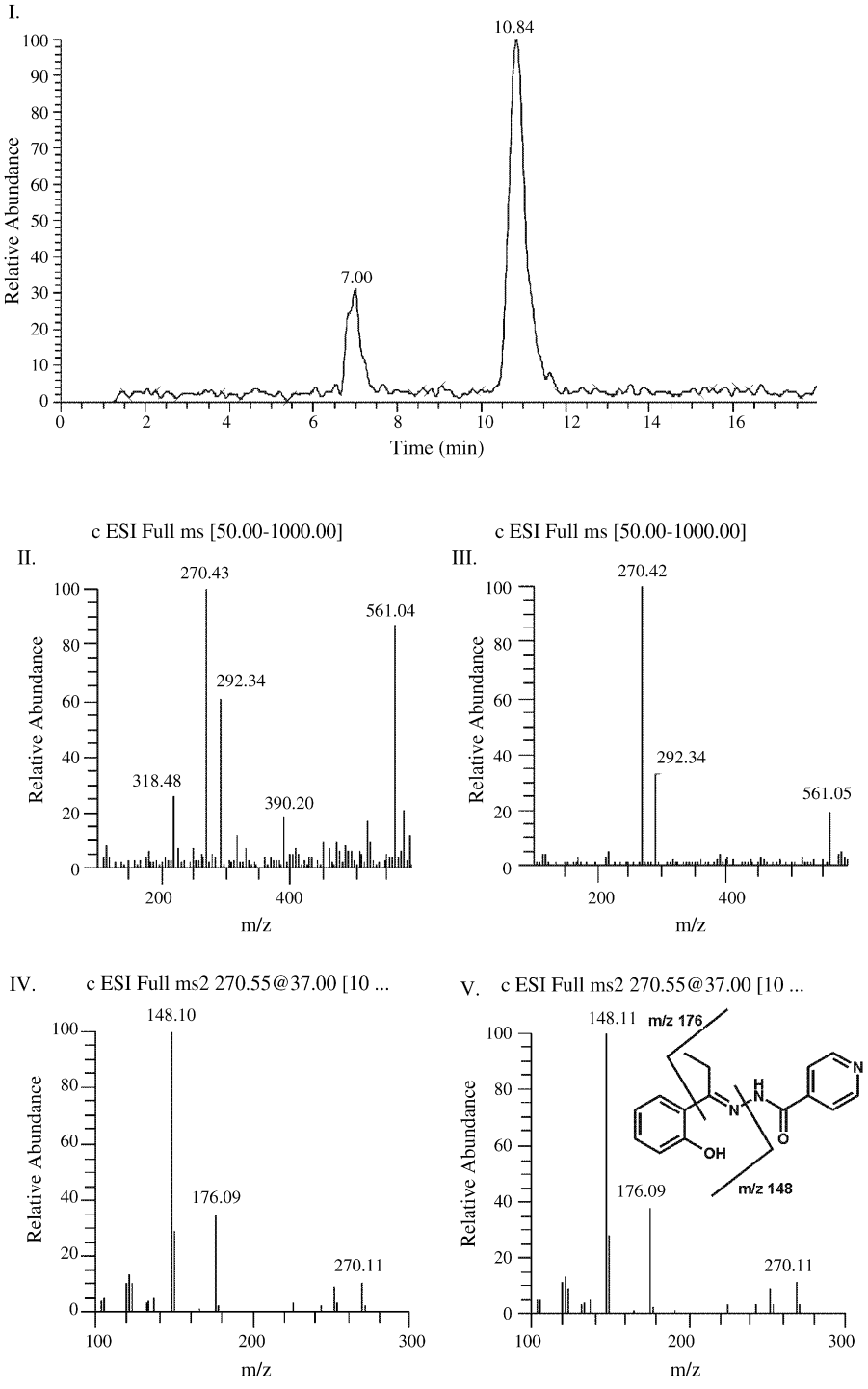

Fig. 3. Initial HPLC/MS/MS analysis of 2-HPP-INH using positive mode and 50% methanol as a mobile phase (Detailed chromatographic conditions are given in the Section 2.4). I.: Selected ion monitoring (SIM) at $m/z = 269.5–270.5$ extracted from full scan; putative impurity – RT 7.00 min, 2-HPP-INH – RT 10.84 min; II. putative impurity: MS (full scan) at RT 6.6–7.4 min; III. 2-HPP-INH: MS (full scan) at RT 10.5–11.3 min; IV. putative impurity: MS^2 from molecular ion $[M+H]^+$- 270 *m/z* (CE 37%); V. 2-HPP-INH: MS^2 from molecular ion $[M+H]^+$- 270 *m/z* (CE 37%).

matograms of HAP-INH as well. Importantly, retention times of the observed impurities corresponded to neither synthetic precursors nor expected degradation products (Fig. 2).

Hence, two preliminary hypotheses were proposed: The first supposed that the unknown peak could be an impurity arising from the degradation of the compounds in aqueous environment, while the second suggested it to be an unexpected synthetic by-product The latter hypothesis was rejected as the NMR spectra measured prior the HPLC analysis confirmed high purity of the prepared hydrazones while the peak areas still tended to increase. Regarding the possible degradation of the investigated compounds in aqueous media the most likely degradation pathway would be the hydrolysis of the hydrazone bond as it was shown previously with aldehyde-derived hydrazones [12,13]. However, this was excluded by the analysis of the standards of the appropriate degradation products (Fig. 2).

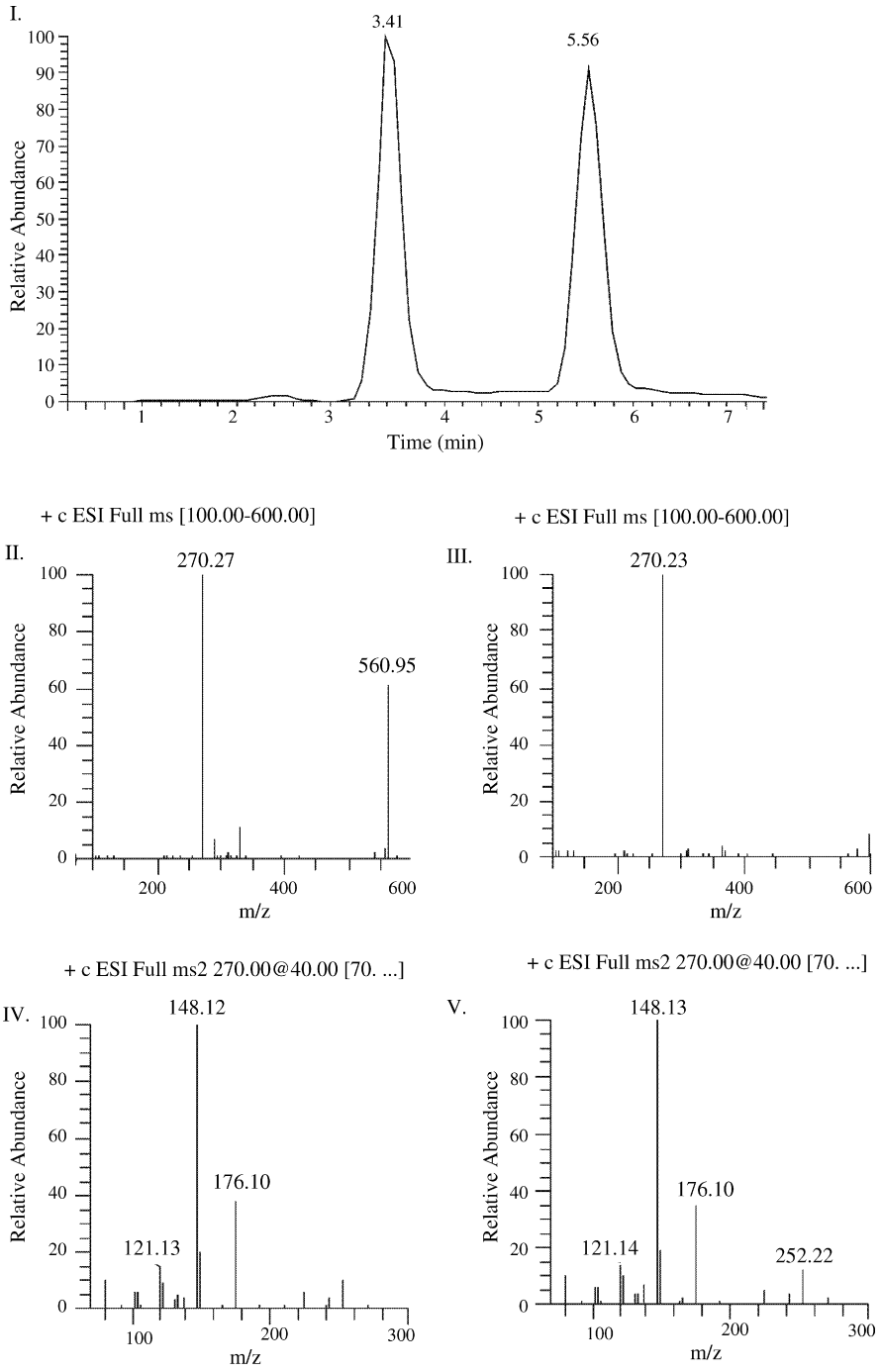

Fig. 4. HPLC/MS/MS analysis of 2-HPP-INH at positive mode using 50% acetonitrile as a mobile phase (Detailed chromatographic conditions are given in the Section 2.5). I.: Selected ion monitoring (SIM) at $m/z = 269.5–270.5$ extracted from full scan; putative impurity – RT 3.41 min, 2-HPP-INH – RT 5.56 min; II. putative impurity: MS (full scan) at RT 3.1–3.8 min; III. 2-HPP-INH: MS (full scan) at RT 5.2–6.0 min; IV. putative impurity: MS^2 from molecular ion $[M+H]^+$- 270 m/z (CE 40%); V. 2-HPP-INH: MS^2 from molecular ion $[M+H]^+$- 270 m/z (CE 40%).

In order to obtain more information about the origin and identity of the unknown impurities, HPLC-DAD analysis was performed. First, using this approach we documented the peak purity of all peaks in the study, which excluded the possibility of co-elution. Thereafter, DAD spectra of the putative impurity were compared with those of the parent compounds (Fig. 2). Although certain similarity could be observed, marked differences were apparent, particularly the missing absorption maximum at 325 nm. This suggested a change in the conjugated double bond system of the hydrazone molecule in an aqueous environment. The inconclusive outcomes of HPLC-DAD study encouraged us to employ HPLC–MS/MS to step further in the investigation of the putative impurities.

The chromatographic conditions used previously for the purity evaluation had to be adjusted to be compatible with MS detection. Mass spectrometer was tuned using a direct injection of the freshly prepared solution (1 μg/mL) of each compound and the mass spectra and fragmentation of main ions were

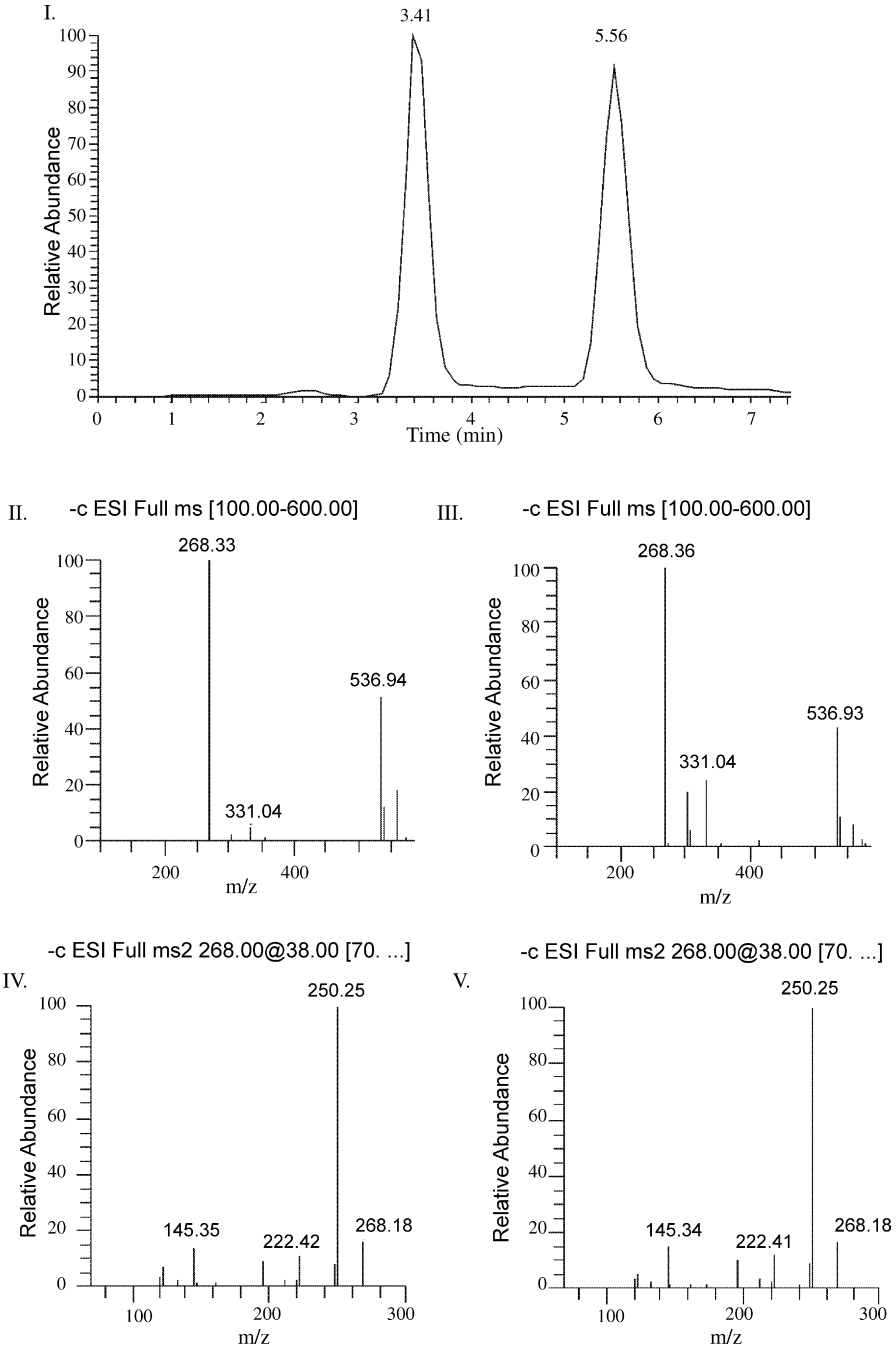

Fig. 5. HPLC/MS/MS analysis of 2-HPP-INH at negative mode using 50% acetonitrile as a mobile phase (Detailed chromatographic conditions are given in the Section 2.5). I.: Selected ion monitoring (SIM) at $m/z = 267.5–268.5$ extracted from full scan; putative impurity – RT 3.41 min, 2-HPP-INH – RT 5.56 min; II. putative impurity: MS (full scan) at RT 3.1–3.8 min; III. 2-HPP-INH: MS (full scan) at RT 5.2–6.0 min; IV. putative impurity: MS^2 from molecular ion $[M+H]^+$ - 270 m/z (CE 38%); V. 2-HPP-INH: MS^2 from molecular ion $[M+H]^+$ - 270 m/z (CE 38%).

found. In the positive mode HPP-INH (Fig. 3) yielded protonated molecular ion $[M+H]^+$ at 270 *m/z* and an ion adduct $[M+Na]^+$ at 292 *m/z*, however $[2M+Na]^+$ at 561 *m/z* was clearly seen as well. The fragmentation of the molecular ion (CE 37%) gave two main fragments $[M+H\text{-}C_6H_5N_2O]^+$ at 148 *m/z* and $[M+H\text{-}C_6H_5O]^+$ at 176 *m/z* (Fig. 3). In the case of HAP-INH, the analogical spectra and the fragmentation were seen: $[M+H]^+$ at 256 *m/z*, $[M+Na]^+$ at 278 *m/z*, $[2M+Na]^+$ at 533 *m/z*; MS^2 of $[M+H]^+$ at 256 *m/z* (CE 37%) gave $[M+H\text{-}C_6H_5N_2O]^+$ at 134 *m/z* and $[M+H\text{-}C_6H_5O]^+$ at 162 *m/z*.

In the negative mode HPP-INH yielded $[M-H]^-$ at 268 *m/z* and $[2M-H]^-$ at 537 *m/z*. Several other ions of lower abundance were detected as well (e.g. at 559 and 331 *m/z*). MS^2 of the parent ion (CE 38%) gave the loss of water $[M-H\text{-}H_2O]^-$ detected at 250 *m/z*. In the case of HAP-INH, the molecular ion $[M-H]^-$ was detected at 254 *m/z*, however the presence of dimer $[2M-H]^-$ was not observed. Analogically, MS^2 of the parent ion gave $[M-H\text{-}H_2O]^-$ at 236 *m/z*.

Since our attempts to prepare and isolate the putative impurity were unsuccessful, we decided to employ HPLC–MS/MS for further characterization of the unknown peak. The initial analyses were performed in the positive mode using 50% methanol as a mobile phase. In the case of HPP-INH, the impurity and the parent compound were detected at the retention times of 7.0 and 10.8 min, respectively. However, the mass spectra of both peaks showed the same protonated molecular ions—$[M+H]^+$. Furthermore, subsequent MS^2 analyses pointed out on the identical fragmentation pathways of the molecular ions in both the hydrazone and the unknown compound. In addition, MS spectra of both peaks revealed also the presence of similar adduct ions $[M+Na]^+$ and $[2M+Na]^+$, although they differed in abundance. The mass chromatograms from these analyses as well as the MS spectra of both peaks are shown in Fig. 3. In the case of HAP-INH, the analogical results were found (not shown).

As seen in Fig. 3, the initial analyses suffered from relatively high background noise. Since ESI ionization in acetonitrile is known to provide lower background noise in comparison with methanol, in further experiments 50% acetonitrile was utilized as the mobile phase to improve the analysis and potentially add a discriminative value. Moreover, MS analysis was performed both in positive and negative modes. In the case of HPP-INH, the putative impurity was eluted at a retention time of 3.4 min while parent compound was detected at 5.6 min. The mass chromatograms of these analyses as well as mass spectra obtained for both peaks are displayed in Fig. 4 (positive mode) and Fig. 5 (negative mode). As in the previous case (separation using 50% methanol), the same molecular ions and their fragmentation were revealed in both peaks. Interestingly, in the spectra of putative impurity the adduct ion $[2M+Na]^+$ at 561 *m/z* was detected, whereas it was missing in the spectra of parent compound. In the negative mode, the MS spectra of both peaks were similar (Fig. 5). In the case of HAP-INH, analogical mass spectra were obtained (data not shown).

As stated above, HPLC–MS/MS analyses indicated that the putative impurities have an identical molecular ion, MS^2 fragmentation and generally very close mass spectra to their corresponding parent compounds (HPP-INH, HAP-INH). From that point, the original assumption considering the presence of degradation products or by-products became implausible. Indeed, these results strongly suggested that both peaks represent compounds with the same chemical constitution. The chemical structure of the compounds together with their elution in two pure and well-separated peaks with similar MS spectra pointed out on the role of geometric (Z/E) isomers. According to the chemical shifts of the methyl (3.36 ppm) and methylene (3.01 ppm) hydrogens in the vicinity of the hydrazone carbon in HPP-INH and HAP-INH, respectively, together with the unusually deshielded singlets at around 13 and 11 ppm corresponding to the OH and NH, respectively, an E isomer was suggested to prevail in the non-aqueous media. Similar spectral characteristics, fully assigned to the E isomer were found in 2′-hydroxyacetophenone nicotinoyl hydrazone [14] and salicylaldehyde benzoyl hydrazone [15]. In the case of SIH, which is an aldehyde-derived hydrazone, the Z isomer was clearly observed in the NMR spectrum as indicated by NH, OH and aldehydic hydrogen singlets at 12.0; 10.0 and 8.4 ppm, respectively, in addition to those of an E isomer at 12.3; 11.1 and 8.7 ppm. In NMR spectrum of salicylaldehyde benzoyl hydrazone [15] a

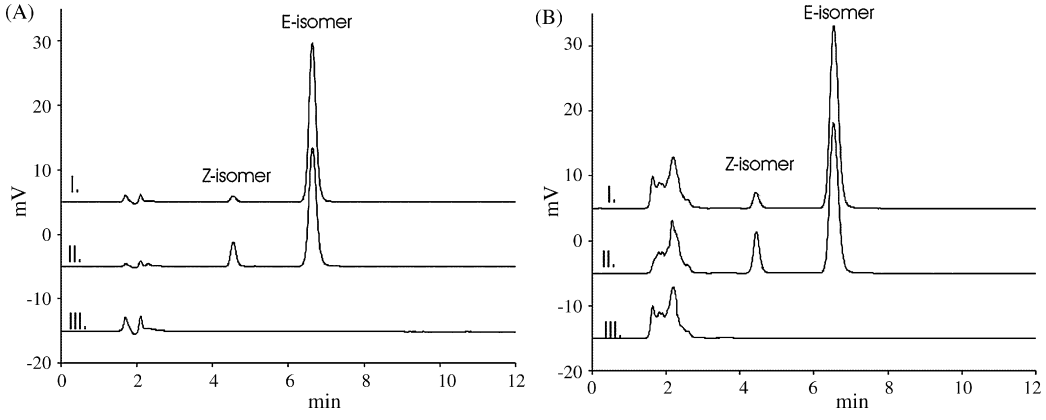

Fig. 6. HPLC-DAD analyses of the samples from *in vitro* experiment (HPP-INH) (A) Phosphate buffered saline (PBS); (B) Porcine plasma. I.: Analysis of the samples taken at the beginning of the experiment; II.: Analysis of the samples taken after 1 h of incubation; III.: Blank (The detailed HPLC conditions are given in the Section 2.4).

minor form, representing about 1% of the sample with upfield NH, OH and aldehydic H chemical shifts was found and assigned to the Z isomer. The NMR spectra of the hydrazones prepared in the present study revealed similar minor form with a weak singlet at 10.8 ppm, which corresponds to the Z isomer. Therefore, the putative impurities detected on the chromatograms of the novel aroylhydrazones (HPP-INH, HAP-INH) are very likely their Z isomers formed upon the addition of water. This also explains why our attempts to isolate the putative impurity failed. The Z isomer is less stable and converts in the solid state into the E-isomer [16], which is stabilized via an intramolecular hydrogen bond between the phenolic hydroxyl and N1.

Of note, the difference in UV spectra of the isomers might be considered as quite marked, taking into account the similarity of their chemical structures. The differences in UV spectra of Z/E isomers have been shown also by others [18,19], although rather than absence of an absorption maximum they observed their shifting. Furthermore, although the majority of MS data were in very good agreement in both isomers, the difference in the intensity of dimers and adduct ions in their mass spectra remain an interesting phenomenon. The different geometry of the isomer might have an impact on the susceptibility of the compound to dimerization or formation of adducts, however, this definitely deserves further study.

Interestingly, no separation of the two isomers of SIH was observed under similar conditions. On the other hand, in the case of either HPP-INH or HAP-INH, the effective separation of the geometric isomers was achieved on the chromatographic columns packed with different reverse phase sorbents (e.g., phenylhexyl, C18, RP-amide). The robustness of the separation was proven using different chromatographic conditions. This difference in the chromatographic behavior of the geometric isomers of the aldehyde and ketone-derived hydrazones may be caused by the steric hindrance of the latter or a difference in hydrogen bonding ability. Furthermore, several reports documenting the RP-HPLC separation of different geometric isomers including aromatic hydrazones can be found in literature [17–21], which further support the outcomes of the present study.

The preliminary *in vitro* experiment performed in this study demonstrates that the hydrazone isomerization can occur in PBS and plasma under physiologically relevant conditions (pH and temperature). As it can be seen from Fig. 6, the peak area of Z-isomer tended to increase in time while the peak area of E-isomer decreased. The results of this experiment pointed out that the hydrazone isomerization might have a relevance to the physiological in *vivo* conditions and thus it deserves to be considered in further investigation focused on aroylhydrazones.

4. Conclusion

HPLC-DAD and HPLC–MS/MS methods were employed to investigate the identity and origin of putative impurities of novel aromatic hydrazones HPP-INH and HAP-INH, being formed particularly in aqueous environment. The results of the present study strongly suggest that these substances are the Z isomers. This is supported by the NMR spectra of the investigated compounds. The results are in agreement with other studies describing separation of geometric isomers including hydrazones. Since the Z/E isomers could possess different chelating properties as well as pharmacodynamic activities; these findings can be of substantial importance from both the theoretical point of view as well as for the future studies focused on aroylhydrazones.

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