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LC-UV/MS methods for the analysis of prochelator - boronyl salicylaldehyde isonicotinoyl hydrazone (BSIH) and its active chelator salicylaldehyde isonicotinoyl hydrazone (SIH)

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Abstract

Salicylaldehyde isonicotinoyl hydrazone (SIH) is an intracellular iron chelator with well documented potential to protect against oxidative injury both *in vitro* and *in vivo*. However, it suffers from short biological half-life caused by fast hydrolysis of the hydrazone bond. Recently, a concept of boronate prochelators has been introduced as a strategy that might overcome these limitations. This study presents two complementary analytical methods for detecting the prochelator BSIH (boronyl salicylaldehyde isonicotinoyl hydrazone) along with its active metal-binding chelator SIH in different solution matrices and concentration ranges. An LC-UV method for determination of BSIH and SIH in buffer and cell culture medium was validated over concentrations of 7 – 115 and 4 – 115 μ M, respectively, and applied to BSIH activation experiments *in vitro*. An LC-MS assay was validated for quantification of BSIH and SIH in plasma over the concentration range of 0.06 – 23 and 0.24 – 23 μ M, respectively, and applied to stability studies in plasma *in vitro* as well as analysis of plasma taken after *i.v.* administration of BSIH to rats. A Zorbax-RP bonus column and mobile phases containing either phosphate buffer with EDTA or ammonium formate and methanol/acetonitrile mixture provided suitable conditions for the LC-UV and LC-MS analysis, respectively. Samples were diluted or precipitated with methanol prior to analysis. These separative analytical techniques establish the first validated protocols to investigate BSIH activation by hydrogen peroxide in multiple matrices, directly compare the stabilities of the prochelator and chelator in plasma, and provide the first basic pharmacokinetic data of this prochelator. Experiments reveal that BSIH is stable in all media tested and is partially converted to SIH by H₂O₂. The observed integrity of BSIH in plasma

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samples from the *in vivo* study suggest that the concept of prochelation might be a promising strategy for further development of aroylhydrazone cytoprotective agents.

Keywords

aroylhydrazone; boronyl salicylaldehyde isonicotinoyl hydrazone; prochelator salicylaldehyde isonicotinoyl hydrazone; pharmacokinetics

1. Introduction

Oxidative stress is a common denominator of a variety of diseases, such as ischemia-reperfusion myocardial damage, congestive heart failure, myocarditis, anthracycline induced cardiomyopathy and atherosclerosis [1, 2].

Iron (Fe) plays an indispensable role in essential life processes that include energy metabolism, DNA synthesis and cellular respiration, but under certain disease conditions unregulated Fe can catalyze Fenton reactions that transform H_2O_2 to extremely reactive and toxic hydroxyl radicals, thereby promoting oxidative stress and cellular and tissue damage. Thus the concept of Fe chelation seems to be a useful therapeutic approach in preventing Fe-mediated aggravation of oxidative stress [3].

Salicylaldehyde isonicotinoyl hydrazone (SIH, Fig 1) is a tridentate biocompatible metal chelator that easily penetrates cell membranes and firmly binds Fe of the intracellular labile pool [4]. SIH has been previously demonstrated to protect isolated cardiomyocytes and H9c2 cells against oxidative injury induced by H_2O_2 [4-6] and H9c2 cells against damage induced by *tert*-butyl hydroperoxide [7] or catecholamines and their reactive intermediates [8]. Marked protective potential of SIH has been also shown in anthracycline cardiotoxicity both *in vitro* using neonatal cardiomyocytes [9] and *in vivo* in a chronic model of daunorubicin-induced heart failure in rabbits [10]. Importantly, good inherent tolerability and low toxicity profile of SIH have been demonstrated following its 10-weeks repeated administration to rabbits [11].

Despite these promising pharmacodynamic outcomes, a pilot pharmacokinetic study has revealed a short biological half-life following single intravenous administration of SIH to rabbits, apparently due to the fast hydrolysis of its hydrazone bond in plasma [12, 13]. Additional study disclosed that this property is not specific for SIH, but rather a class effect of the hydrazones derived from aromatic aldehydes [13]. The concept of a pro-drug is one of the possible strategies to overcome this issue. Such a prochelator should be stable in plasma after drug administration but be easily converted to its active form at the site of disease. Boronyl-ester or boronic acid prochelators based on aroylhydrazone ligands were prepared for this purpose [14, 15]. These compounds have little affinity for metal ions until the protective mask, a boronyl ester or acid group that blocks metal binding, is conditionally removed by reaction with H_2O_2 under conditions that are unique for diseases associated with oxidative stress [14]. The reactive nitrogen species peroxynitrite is also able to activate aryl boronates into phenols [16].

Boronyl salicylaldehyde isonicotinoyl hydrazone (BSIH, Fig. 1) represents a first generation prochelator, where the phenol oxygen of SIH is replaced by boronylester. Previous *in vitro* studies demonstrated by UV/Vis spectrophotometry that BSIH releases SIH when activated by H₂O₂ and that the prochelator is more stable than SIH in cell culture medium [15, 17]. A pilot toxicity study indicated low toxicity and good tolerability of BSIH even after repeated treatment of retinal pigment epithelial cells [17].

Hence, the concept of prochelation might overcome limitations associated with aroylhydrazone chelators while maintaining their unique antioxidative and cytoprotective properties. However, further progress in this field strongly requires bioanalytical methods capable of quantitative analysis of prochelators along with their active forms in relevant biological media. Moreover, modern and appropriately validated bioanalytical methods are vital for determination of the concentration–time profile of a prochelator in plasma and are essential for estimation of pharmacokinetic parameters. Owing to short aroylhydrazones' elimination half-life, such data are crucial for further development of these prochelators.

The aim of this study was to develop and validate the first bioanalytical methods (LC-UV and LC-MS) capable of simultaneous determination of the prochelator BSIH and its active ligand SIH in different matrices (buffer, DMEM media and plasma). The applicability of these complementary methods was demonstrated by the analysis of samples from *in vitro* activation and stability experiments as well as from a pilot pharmacokinetic study *in vivo*. The LC-UV method developed in this study presents simple analytical conditions useful for routine BSIH activation studies *in vitro*, while its modification suitable for LC-MS offers a more sensitive and selective tool for analysis of samples from a pharmacokinetic study.

2. Experimental

2.1. Materials and Chemicals

SIH (purity 98%), di-2-pyridyl ketone isonicotinoyl hydrazone (PKIH, internal standard - IS), BSIH (purity 98%), and its acid form – BASIH were synthesized and characterized as described previously [15, 18, 19]. Methanol, acetonitrile, both gradient grade, dimethyl sulfoxide HPLC grade (DMSO), phosphate buffer (NaH₂PO₄·H₂O), ammonium formate, EDTA potassium salt, sodium hydroxide and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (Munich, Germany). ADS buffer was prepared according to the previously published procedure [7]. Milli-Q water was prepared using Millipore purification system (Merck-Millipore, Darmstadt, Germany).

2.2. Preparation of stock and working solutions

Stock solutions (2.85, 4.15 and 3.30 mM for BSIH, SIH and IS, respectively), were prepared by dissolving an appropriate amount of the substance in methanol. The stock solutions were further diluted with the same solvent to get working solutions used to spike ADS buffer, DMEM cell medium and plasma for validation purposes.

2.3. Chromatographic system and conditions

The chromatographic system Prominence LC 20A (Shimadzu, Duisburg, Germany) consisting of a DGU-20A3 degasser, two LC-20AD pumps, a SIL-20AC autosampler, a CTO-20AC column oven, a SPD-20AC detector and a CBM-20AC communication module was used for the analysis of the compounds (BSIH, SIH) in ADS buffer and DMEM medium. SPD-20AC detector was replaced with LCQ Advantage Max mass spectrometer (Thermo Finnigan, San Jose, USA) for analysis of BSIH and SIH in plasma.

All analyses were performed on a Zorbax Bonus-RP (150 × 3 mm, 3.5 μm) column protected by a guard column (Zorbax Bonus-RP, 20 × 3 mm, 3.5 μm) both purchased from Agilent Technologies (Santa Clara, USA). The column oven was set at 25 °C and the autosampler at 5 °C. The flow rate of 0.4 mL/min was used. PKIH was selected as an IS (Fig. 1). The mobile phase compositions and other chromatographic parameters employed in this study are specified below.

2.3.1. LC-UV analysis—A mobile phase composed of 2 mM EDTA in 7 mM phosphate buffer (pH 6.0) and a mixture of methanol and acetonitrile (40:60, v/v) was used in a ratio of 60:40 (v/v) for the analysis of the compounds in ADS buffer and DMEM medium. Fifty microliters of the samples were injected onto the column and UV detector was set at 297 nm.

2.3.2. LC-MS analysis—A mobile phase composed of 2 mM ammonium formate (pH not adjusted) and a mixture of methanol and acetonitrile (40:60, v/v), in a ratio of 60:40 (v/v) was used to determine BSIH and SIH in plasma. Ten microliters of the samples were injected onto the column. ESI in positive mode was employed for these analyses and MS detector was set as follows: source voltage of 1.5 kV, capillary voltage of 46 V, capillary temperature of 250 °C, sheath and auxiliary gas flows 60 and 30 arbitrary units, respectively. Quantification was performed in selected reaction monitoring (SRM) using a collision energy of 38% and the following fragments of the most abundant precursor ions for each analyte: m/z 252 → m/z 224 (BSIH); m/z 242 → m/z 123 (SIH) and m/z 304 → m/z 248 (IS).

2.4. Sample preparation

Two hundred microliters of ADS buffer and DMEM medium containing BSIH and SIH were spiked with 2 μl of IS stock solution (3.30 mM) and diluted with 200 μl of methanol. Diluted samples were vortexed (2000 rpm, 1 min) and analyzed.

Fifty microliters of plasma samples containing the analytes were spiked with 2 μl of IS working solution (3.30 μM) and precipitated with 100 μl of methanol, vortexed (2000 rpm, 1 min) and centrifuged (16,800 g, 10 min). Resulted supernatant was filtrated (0.45 μm) and injected onto the column.

2.5. Methods validation

Both LC-UV and LC-MS methods were validated according to FDA recommendations for validation of bioanalytical methods [20]. The following validation parameters were tested:

selectivity, linearity, precision, accuracy and stability. In addition, extraction recovery, matrix effect and dilution integrity were evaluated in the case of LC-MS analysis.

2.5.1. Selectivity and linearity (calibration range)—Selectivity was evaluated by the analysis of drug-free ADS buffer, DMEM medium with and without H₂O₂ and plasma samples obtained from six individual sources.

Linearity of the LC-UV method was tested in ADS buffer and DMEM medium within the concentration ranges of 7 – 115 µM and 4 – 115 µM of BSIH and SIH, respectively. The concentration ranges 0.06 – 23 µM (BSIH) and 0.24 – 23 µM (SIH) were used for LC-MS analysis of the compounds in plasma. Internal standard was added to the samples at the concentration of either 30 or 0.13 µM for LC-UV and LC-MS assay, respectively. The calibration ranges were identical with the concentration range used to test linearity of methods. The lower concentration of the analytes used in calibration curves represents a lower limit of quantification (LLOQ). The peak areas ratios (analyte/IS) obtained from the analysis of spiked plasma samples were plotted against the concentration of the analytes. Linearity was tested using least square regression and it is expressed as a coefficient of determination (r^2).

2.5.2. Precision and accuracy—Precision and accuracy of LC-UV method was assessed using quality control (QC) samples at three different concentration levels for the analytes: **1)** low either 7 (BSIH) or 4 (SIH) µM; **2)** medium 57 µM (both compounds) and **3)** high 115 µM (both compounds). For LC-MS assay, these parameters were tested at the following concentration levels: **1)** low either 0.06 (BSIH) or 0.24 (SIH) µM; **2)** medium 3 µM (both compounds) and **3)** high 23 µM (both compounds). The intra-day precision and accuracy were evaluated by the analysis of five aliquots of QC samples on the same day. For inter-day precision and accuracy, the same experiment was repeated the next day.

2.5.3. Stability—Post-preparative stability of the analytes in all tested matrices was assessed after sitting for 12 hours in the autosampler (5 °C). Long term stability of the analytes was tested after 7 days storage at –80°C. The stability was checked at low, medium and high concentration levels (three aliquots per each level).

2.5.4. Extraction recovery and matrix effect—Extraction recovery was evaluated by comparing the peak areas of the analytes and IS in extracted QC samples with the peak areas from the analysis of these compounds added to extracted blank. Matrix effect (expressed as absolute and normalized matrix factors – MF) was tested by comparing the peak areas of both analytes and IS recorded in the analysis of blank spiked with compounds after extraction and the peak areas of these compounds in standards dissolved in 50% methanol. Both parameters were tested at three concentration levels (low, medium and high) and one concentration of IS (0.13 µM).

2.5.5. Dilution integrity—Dilution integrity was tested in order to ensure capability of the method to properly determine both compounds in plasma at concentrations above upper limit of quantification (ULOQ). Plasma spiked with BSIH and SIH (100 µM) was diluted ten

times with pooled blank plasma and analyzed. Dilution integrity was expressed as the ratio of the amount of the analyte assayed in the sample versus its actual concentration.

2.6. In vitro activation experiment

The stock solution of BSIH and SIH in DMSO (100 mM) was used in these experiments. Final concentration of DMSO in all samples did not exceed 0.1%. ADS buffer and DMEM medium samples spiked with BSIH and SIH (100 μ M) were incubated in Petri dishes (37 °C, in dark) both with and without H₂O₂ (200 μ M). The samples were collected after 0, 1, 3, 6, 9, 12, 24 hours, immediately processed as described in section 2.4. and analyzed.

Concentrations of BSIH determined in ADS buffer and DMEM medium at each time point were compared and statistically analyzed using ANOVA (Microsoft Excel 2010). The threshold for statistical significance used in this study was $p < 0.05$.

2.7. In vitro stability experiment

Drug-free plasma was preincubated at 37°C for 10 minutes and then spiked with the stock solution of either BSIH or SIH to get a final concentration of 100 μ M. Plasma was incubated at 37°C in Thermomixer comfort (Eppendorf, Hamburg, Germany) for 10 hours and gently shaken (600 rpm) during the whole experiment. Fifty μ l of plasma were sampled every 1 hour, diluted 10 times with pooled blank plasma, immediately processed as described in section 2.4. and analyzed. Stability of both compounds was described by the decrease of their concentration in time and it is expressed as a mean $\pm$ S.D (n = 4).

2.8. In vivo pharmacokinetic experiment

Wistar Han male rats (average weigh 460 g, n = 5) obtained from MediTox (Konarovice, Czech Republic) were kept in an air-conditioned room under a 12 hours light-dark cycle, constant temperature and humidity, and had a free access to a standard laboratory pellet diet for rodents (Velaz, Czech Republic) and tap water. Rats fasting for 12 hours were anaesthetized with pentobarbital sodium *i.p.* (45 mg/kg, Sigma-Aldrich, USA). BSIH dissolved in the mixture of 96 % ethanol, PEG 300 and saline (1:1:1, v/v/v) was administered *i.v.* (10 mg/kg) into *vena saphena magna* using an infusion pump (1 mg BSIH/min; Kent Scientific Corporation, USA). At predefined time intervals (1, 10, 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min following the end of the infusion) whole blood was sampled into heparinized tubes from *arteria iliaca communis sinister*. Blood was immediately centrifuged (2,500 g, 10 min; MPW-52, MPW Med. Instruments, Poland) and the obtained plasma was stored at –80°C prior to the analysis. Fifty microliters of thawed plasma samples were processed as described in section 2.4. and analyzed.

The study had the approval of The Ethics Committee of Charles University in Prague, Faculty of Pharmacy in Hradec Králové, and it conformed to The Guide for the Care and Use of Laboratory Animals (The US National Institutes of Health).

Pharmacokinetic parameters were calculated using Kinetica 5.0 software (Thermo Fisher Scientific Inc., U.S.A.) by applying a non-compartmental model with the linear trapezoidal rule.

3. Results and Discussion

3.1. Characterization of BSIH in aqueous solution

The purity and elemental composition of BSIH was confirmed by liquid chromatography (LC) and high-resolution mass spectrometry (HRMS) of BSIH samples dissolved in methanol or acetonitrile (Fig S1 in Supp Info). Under these conditions, BSIH appeared as a clean peak with corresponding m/z values of 352.1827 and 374.1645 for the $[M+H]^+$ and $[M+Na]^+$ ions, respectively, matching the chemical structure shown in Fig 1. When dissolved in aqueous solution and analysed by LC, BSIH was markedly less retained on a RP column as compared with SIH, despite their similar estimated lipophilicity (log P 2.3 and 2.4 calculated for BSIH and SIH, respectively [14]). This phenomenon is explained by dissociation of the pinacol group (2,3-dimethyl-2,3-butanediol) of the aryl boronic ester to generate its boronic acid form (*i.e.* BASIH, Fig 1), as has been documented for structurally related compounds [21]. Conversion to BASIH in water was confirmed by mass spectral analysis of BSIH samples dissolved in water or organic/water mixtures and using direct infusion of BSIH solutions to the ion source. When analysed in the negative mode (Figure S2, Supp Info), the prochelator appeared at m/z 268.0907, which corresponds to the $[M-H]^-$ ion of the BASIH structure shown in Fig 1. Another prominent ion appeared at m/z 250.0801, which corresponds to the neutral loss of H_2O from BASIH. The fact that the ratio of m/z 250/268 ion intensities increased as a function of fragmentor voltage indicates that m/z 268.0907 represents the parent ion corresponding to the predominant species in solution, whereas the ion at m/z 250.0801 is a byproduct of the ionization procedure. When analysed in the positive mode (Figure S3, Supp Info), this neutral loss of water from BASIH appeared predominantly at m/z 252.0940. The similar MS behaviour was observed in MS analysis using ion trap instrument.

3.2. LC-UV method development and validation

Two different stationary phases (Ascentis C18 column, Zorbax Bonus-RP column) were tested for the simultaneous analysis of BSIH and SIH in ADS buffer and DMEM cell medium. It was found that the Ascentis C18 column suffered from an asymmetric peak of BSIH. This parameter was significantly improved employing Zorbax Bonus-RP column containing an embedded amide linkage in the C14-alkyl chain.

A mobile phase composed of phosphate buffer provided higher peak symmetry when compared to that containing ammonium formate. The mobile phase pH of 6.0 was found to be optimal as the lower pH resulted in peak tailing while higher pH caused a coelution of BSIH with a peak of cell medium. Buffer strength (5–20 mM) had only minor influence on separation parameters. Using acetonitrile instead of methanol resulted in lower retention as well as certain improvement of peak shapes, however insufficient separation of BSIH and DMEM medium components precluded utilization of this solvent. Finally, a mixture of methanol and acetonitrile in 40:60 ratio (v/v) was employed as a compromise between separation efficacy, peak shapes and analysis time. Mobile phase containing EDTA enabled improved reproducibility of peak areas of SIH, due to the restriction of ions chelating with the compound in the chromatographic system.

The sufficient separation within 10 minutes was finally achieved with a mobile phase composed of 2 mM EDTA in 7 mM phosphate buffer of pH 6.0 and a mixture of methanol and acetonitrile (40:60, v/v), in a ratio of 60:40 (v/v). As both ADS buffer and DMEM medium are protein free, these samples could be analyzed directly after simple dilution with methanol. The representative chromatogram of the separation of BSIH, SIH and IS in DMEM medium are presented in Fig 2.

3.2.1. Method validation—Selectivity of the method for determination of all the compounds in both ADS buffer and DMEM medium was clearly demonstrated by the analysis of corresponding blank samples (Fig. 2). Linearity has been proven over the range of 7.0–115 μ M and 4–115 μ M for BSIH and SIH, respectively, in both media ($r^2 = 0.997$). The typical regression equations and coefficients of determination as well as the intra- and inter-day precision and accuracy are presented in Table 1 and reached acceptable values [20]. LLOQ was determined as a concentration of 7 and 4 μ M for BSIH and SIH, respectively. The chromatogram of analysis of a sample at LLOQ is shown in Supplementary information (Fig S4).

No significant degradation of either BSIH or SIH was revealed under the tested conditions (in autosampler at 5 °C and in freezer at –80 °C) in ADS buffer and DMEM medium (Table 2).

3.3. In vitro activation experiment

Practical applicability of the LC-UV method has been demonstrated by the analysis of samples from an *in vitro* experiment focused on BSIH activation in ADS buffer and DMEM cell medium. Both SIH and BSIH were stable at 37°C in ADS buffer without H₂O₂ addition, where 97% and 99% of the initial amount of SIH and BSIH remained unchanged after 24 hours of incubation (Fig 3a). Hydrogen peroxide had no impact on stability of SIH alone (data not shown), whereas it caused a decrease in the amount of BSIH detected over time. Concomitant with the loss of BSIH was the appearance of SIH, although its gain was not equimolar to the fall of BSIH concentration (Fig 3a). This result suggests that SIH is not the only product of the activation process of this prochelator. Further analysis using UV detection at 254 nm revealed the presence of other unknown byproduct resulted from the reaction of BSIH with H₂O₂ that provide no signal at wavelength used to monitor activation (297 nm). The presence of the unknown peak was observed by setting detector to 254 nm (Fig S5). As our effort to identify this unknown compound by LC-MS failed we utilized HPLC for semipreparative isolation of this byproduct from the sample from the activation experiment in ADS buffer. The appropriate fraction of the sample was collected and subjected to GC-EI-MS analysis. The measured EI spectrum of the byproduct matches the spectrum of salicylaldehyde in EI spectrum library (NIST 11) and was also identical with those recorded from the GC-MS analysis of the chemical standard (please see Supplementary information Fig S6). In addition, we also found identical retention times of the chemical standard of salicylaldehyde and the byproduct of BSIH activation using both GC-MS and LC-UV which confirms identification furthermore. As it was proved in this study that SIH is stable in ADS buffer with H₂O₂ addition, salicylaldehyde detected after

activation could not be formed by direct hydrolysis of this chelator. Therefore, the mechanism of salicylaldehyde formation from BSIH requires further particular study.

BSIH incubation in DMEM cell medium without H₂O₂ resulted only into minor compound decomposition, as after 24 hours 85% of the initial amount of the compound remained unchanged (Fig 3b). On the contrary, SIH rapidly decomposed in this medium (Fig 3b), in agreement with a previous study where we found significantly faster decomposition of SIH in RPMI-1640 cell medium as compared to PBS buffer [13]. Degradation of aroylhydrazones in cell media has been previously suggested to be catalyzed by amino acids [22]. Hydrogen peroxide had no additional impact on SIH degradation (data not shown), whereas it accelerated the fall of BSIH over time. The pseudo-first order rate constants for the compound activation in both media calculated from the loss of BSIH ($0.0671 \pm 0.0051 \text{ h}^{-1}$ and $0.0934 \pm 0.0026 \text{ h}^{-1}$ for ADS and DMEM, respectively) suggested slightly higher activation rate in DMEM than in ADS. A significant difference (ANOVA, $p = 0.05$) between the concentrations of the prochelator after reaction with H₂O₂ determined in both media was found after the sixth hour of the experiment, where BSIH concentrations were lower in DMEM medium.

The amount of SIH resulting from BSIH and H₂O₂ reaction assayed in DMEM medium was similar to that found in ADS buffer, with the only exceptions of 12 and 24 hours where the concentrations were significantly lower in the cell medium, likely due to poor inherent stability of SIH in this environment. The salicylaldehyde detected as byproduct of BSIH activation in ADS buffer could not be detected in DMEM medium due to its coelution with matrix.

The amount of SIH resulting from BSIH activation with H₂O₂ in both ADS and DMEM was lower than expected based on the initial BSIH concentration. While it was found previously that BSIH and BASIH react *in vitro* with H₂O₂ in the same manner and rate to generate equimolar SIH, it should be noted that those experiments were conducted in methanol [17]. In addition, our current preliminary results suggest that the efficiency of BSIH-to-SIH conversion might be influenced by the content of an organic solvent in the incubated sample. Hence, this phenomenon deserves further detailed investigation. BSIH has been previously shown to protect cells against oxidative stress *in vitro* [23], which suggests that the amount of SIH released in a cellular context is sufficient for cytoprotection [17].

3.4. LC-MS method development and validation

To complement the LC-UV method, we aimed to modify the chromatographic conditions to enable sensitive and simultaneous LC-MS analysis of both compounds in plasma. While the same stationary phase could be used for both analyses, the mobile phase composition had to be modified to be MS compatible. Phosphate buffer with EDTA was therefore replaced by ammonium formate. The final mobile phase was composed of 2 mM ammonium formate and a mixture of methanol/acetonitrile (40:60, v/v) in the same ratio as for LC-UV measurements. ESI in positive mode provided the highest sensitivity as compared with negative ionization mode. PKIH served as an IS for LC-MS assay as well. The precursor ion at m/z 242 together with its product ion at m/z 123 were utilized for SIH quantification. The most abundant ion at m/z 252 corresponding to the loss of H₂O from the acid form of BSIH

(BSIH, m/z 270) along with a product ion at m/z 224 were chosen for quantification in selected reaction monitoring mode. High-resolution mass spectra taken over a range of fragmentor voltages confirmed that m/z 224.0995 is a fragment of m/z 252.0945. The mass difference of 27.9950 matches the exact mass of CO (Fig S3). The loss of CO has been already described in compounds contacting carbonyl group [24, 25]. The proposed identity and fragmentation mechanisms for the products ions are presented in Supplementary material (Fig S7 and S8).

In order to make a method as simple, fast and cost-effective as possible, protein precipitation was chosen as a sample pretreatment technique. Acetonitrile and methanol in different volumes (100–200 μ l) were tested for this purpose. Methanol provided higher extraction recovery as compared to acetonitrile. Methanol volume of 100 μ l was chosen as a compromise between the efficiency of protein precipitation and the enhanced sensitivity of minimal sample dilution. The representative SRM chromatograms of LC-MS separation of BSIH, SIH and IS in plasma and the analysis of blank are shown in Fig 4.

3.4.1. Method validation—Selectivity of the LC-MS method has been demonstrated by the analysis of blank plasma samples (Fig 4). Linearity of the method was evidenced over the range of 0.06 – 23 μ M for BSIH and 0.24 - 23 μ M for SIH, with the coefficients of determination 0.998. The mean regression equations and r^2 , together with intra- and inter-day precision and accuracy, are shown in Table 3 and reached acceptable values [20]. LLOQ was determined as a concentration of 0.06 and 0.24 μ M for BSIH and SIH, respectively. The chromatogram of analysis of a sample at LLOQ is shown in Supplementary information (Fig S9). Protein precipitation used in this study provided consistent extraction recovery for both analytes and IS (Table 4). The IS-normalized matrix factor reached an average value of $98.44 \pm 4.62\%$ for BSIH and $101.96 \pm 2.65\%$ for SIH, which indicates that matrix effect did not affect the assay significantly. The matrix factors are presented in Table 4. Long-term (-80°C , 7 days) and post-preparative (5°C , 12 h) stability experiments did not reveal any significant decomposition of either BSIH or SIH (Table 4). The analysis of ULOQ samples diluted with blank plasma determined a mean of 98.97% of the actual value of BSIH and 94.54% of SIH, which indicated an acceptable dilution integrity.

3.5. Stability of BSIH and SIH in plasma *in vitro*

The LC-MS method developed in this study was then used for investigation of stability of BSIH in plasma *in vitro* under physiologically relevant conditions (37°C). Stability of this prochelator was directly compared with stability of the active form – SIH. The prochelator was stable in rat plasma, as 93.21% of the initial concentration was detected after 10 hours of incubation (Fig. 5). Similar results were also found in rabbit plasma (data not shown) where 91.07% of the initial amount was assayed after the same time period. On the contrary, SIH quickly decomposed in plasma, as less than 2.5 % of the initial concentration of the compound was detected at the end of this experiment (Fig 5), in accordance with previously published results [13]. When comparing stability of BSIH and its active form SIH in plasma it is clear that the prochelator is significantly more stable in this matrix, which is beneficial for further development of this compound.

3.6. Application of the method to analysis of samples from a pilot pharmacokinetic study

The practical applicability of the LC-MS method was tested by analyzing plasma taken after single *i.v.* administration of BSIH to rats. The concentration-time profile of BSIH in plasma is shown in Fig. 6 and the main pharmacokinetic parameters estimated using a non-compartmental model are summarized in Table S1 (Supp. Info). The negligible difference between AUC estimated to the last measured point ($AUC_{0-t} = 1083 \pm 136 \mu\text{mol}\cdot\text{min/L}$) and those extrapolated to infinity ($AUC_{0-\infty} = 1088 \pm 135 \mu\text{mol}\cdot\text{min/L}$) indicates sufficient duration of the experiment as well as suitability of the method for determination of the plasma pharmacokinetics in rats. The elimination half-life ($t_{0.5} = 69 \text{ min}$) is apparently higher as compared to the aroylhydrazone chelators, where half-lives of 18 and 28 min were calculated for SIH and pyridoxal 2-chlorobenzoyl hydrazone, respectively, after single *i.v.* administration of these compounds to rabbits [12, 26]. However, possible interspecies variability might influence this comparison. The active form of the prochelator - SIH was not detected in plasma in any of the intervals. This result was expected because the *in vivo* experiment was performed under normal conditions without the oxidative stress, where minimal BSIH conversion would be anticipated. Besides the significant improvement of the compound stability in plasma *in vitro*, these results suggest that modification of the chemical structure of aroylhydrazone by introduction of boronyl containing groups might be a useful approach to improve the pharmacokinetic parameters of these agents.

4. Conclusion

In this study, two complementary LC-UV and LC-MS analytical methods that are capable of simultaneous quantification of the prochelator BSIH and its active chelating ligand SIH in different media were developed. The LC-UV method was validated over the concentration range of 7–115 μM and 4–115 μM for the simultaneous determination of BSIH and SIH in ADS buffer and DMEM cell medium, respectively and used in an *in vitro* bioactivation experiment. These *in vitro* experiments revealed that H_2O_2 activation of BSIH under both media conditions result in partial conversion to SIH, whereas approximately 75 % of the initial prochelator is lost to degradation. The LC-MS method was validated for the determination of BSIH and SIH in plasma over the ranges of 0.06 – 23 μM and 0.24 - 23 μM , respectively. The method applicability was demonstrated by analysis of samples from *in vitro* stability study in plasma as well as the analysis of plasma taken after *i.v.* administration of BSIH to rats. Unlike the active ligand – SIH, the prochelator was found to be stable in plasma *in vitro* and the estimated basic pharmacokinetic parameters showed an improvement in elimination half-life of BSIH over SIH. Hence, the validated analytical methods presented for the first time in this study allow monitoring of prochelator conversion to its active form using a separative analytical technique, enable direct comparison of the stability of these two compounds in plasma *in vitro*, and importantly, provide a suitable analytical tool to estimate basic pharmacokinetic parameters of BSIH. Results of this study are important for the development of this promising class of iron-binding oxidative stress inhibitors. The improved stability of BSIH compared to SIH in media and plasma suggests that incorporation of boronic ester or acid functionalities to the chemical structure of aroylhydrazones might be a promising strategy for improving their plasma half-lives. Furthermore, modifications to BSIH that could maximize the peroxide-induced conversion

to chelator by minimizing its non-productive degradation to salicylaldehyde could improve the efficacy of these agents to protect cells against oxidative damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Salicylaldehyde isonicotinoyl hydrazone (SIH) is an effective cytoprotective agent.

Boronyl salicylaldehyde isonicotinoyl hydrazone (BSIH) is activated to SIH.

LC-UV/MS methods were developed and validated for analysis of SIH and BSIH.

BSIH activation was studied *in vitro* using LC-UV.

LC-MS method was used in an *in vitro* stability experiment and pilot *in vivo* study.

Figure 1.
Chemical structure of the analytes

Figure 2.

Representative chromatograms of LC-UV analysis of BSIH, SIH and IS in DMEM medium at concentration levels of 100 μM for both BSIH and SIH and 30 μM for IS and corresponding blanks (DMEM medium, ADS buffer). The chromatograms were recorded at 297 nm.

Figure 3.
In vitro activation of BSIH in (a) ADS buffer and (b) DMEM media.

Figure 4.

Representative chromatograms of LC-MS analysis of (a) BSIH, SIH and IS in plasma at concentration levels of 5 μM for both BSIH and SIH and 0.13 μM for IS and (b) the corresponding blank. The chromatograms were recorded in SRM using following fragments of the most abundant precursor ions for each analyte: m/z 252 $\rightarrow$ m/z 224 (BSIH); m/z 242 $\rightarrow$ m/z 123 (SIH) and m/z 304 $\rightarrow$ m/z 248 (IS). The intensity of signal is presented as NL value (normalization level).

Figure 5.
Stability of BSIH and SIH in plasma *in vitro* at 37 °C (n = 4; mean ± SD).

Figure 6.
Concentration-time profile of BSIH in plasma obtained after *i.v.* administration to rats (n = 5; mean $\pm$ SD).

Table 1

Validation parameters of the LC-UV method for the analysis of BSIH and SIH. The linearity of the calibration curve was proved within the concentration range of 7-115 μM for BSIH and 4 – 115 μM for SIH.

ADS buffer								
Linearity	Slope	Intercept	r^2					
BSIH	0.0125	-0.0587	0.9989					
SIH	0.0227	-0.0364	0.9998					
Conc. level (μM)	Intra-day (n = 5)				Inter-day (n = 5)			
	Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Accuracy (%)	Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Accuracy (%)
BSIH								
115	113.96	115.20 $\pm$ 0.59	0.51	101.09	111.38	112.61 $\pm$ 4.17	3.70	101.10
57	56.98	55.05 $\pm$ 1.05	1.91	96.62	55.69	52.94 $\pm$ 0.94	1.78	95.05
7	7.12	7.89 $\pm$ 0.14	1.78	110.79	6.96	7.48 $\pm$ 0.10	1.39	107.48
SIH								
115	119.40	120.39 $\pm$ 0.42	0.35	100.83	118.49	116.82 $\pm$ 3.91	3.34	98.59
57	59.70	59.49 $\pm$ 0.96	1.62	99.64	59.24	58.42 $\pm$ 0.72	1.23	98.60
4	3.73	4.02 $\pm$ 0.17	4.31	107.73	3.70	3.85 $\pm$ 0.11	2.77	104.07
DMEM cell medium								
Linearity	Slope	Intercept	r^2					
BSIH	0.0140	-0.0778	0.9967					
SIH	0.0207	-0.0016	0.9997					
Conc. level (μM)	Intra-day (n = 5)				Inter-day (n = 5)			
	Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Accuracy (%)	Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Accuracy (%)
BSIH								
115	114.64	119.22 $\pm$ 0.17	0.14	103.99	111.68	114.80 $\pm$ 0.61	0.53	102.80
57	57.32	56.11 $\pm$ 0.36	0.65	97.89	55.84	53.52 $\pm$ 0.49	0.92	95.85
7	7.17	8.40 $\pm$ 0.06	0.73	117.28	6.98	8.24 $\pm$ 0.06	0.70	118.11
SIH								
115	113.76	114.67 $\pm$ 0.24	0.21	100.80	116.41	124.71 $\pm$ 1.74	1.40	107.13
57	56.88	62.26 $\pm$ 0.43	0.69	109.46	58.21	60.98 $\pm$ 0.91	1.49	104.77
4	3.56	3.63 $\pm$ 0.08	2.33	101.99	3.64	3.54 $\pm$ 0.09	2.63	97.18

Table 2

Stability of BSIH and SIH in ADS buffer and DMEM cell medium

Concentration level (μM)	Stability			
	ADS buffer		DMEM cell medium	
	Post-preparative (%)	Long term (%)	Post-preparative (%)	Long term (%)
BSIH				
115	100.31	99.91	96.01	100.82
57	101.90	98.39	93.77	99.28
7	99.24	102.99	87.38	95.89
SIH				
115	99.87	98.96	93.36	99.22
57	99.41	98.32	94.18	97.93
4	99.57	94.50	97.36	101.58

Table 3

Validation parameters of the LC-MS method for determination of BSIH and SIH in plasma. The linearity of the calibration curve was proved within the concentration range of 0.06-23 μM for BSIH and 0.24 – 23 μM for SIH.

Linearity	Slope	Intercept	r^2	
BSIH	0.9784	-0.0447	0.9982	
SIH	0.3027	-0.0031	0.9989	

Conc. level (μM)	Intra-day (n = 5)				Inter-day (n = 5)			
	Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Accuracy (%)	Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Accuracy (%)
BSIH								
23	23.02	24.94 $\pm$ 1.58	6.34	108.32	23.48	25.22 $\pm$ 1.65	6.54	107.44
3	2.88	2.71 $\pm$ 0.27	10.14	94.16	2.93	2.55 $\pm$ 0.23	9.02	87.06
0.06	0.058	0.054 $\pm$ 0.011	19.90	93.33	0.059	0.049 $\pm$ 0.003	5.57	81.47
SIH								
23	22.98	22.68 $\pm$ 2.72	11.99	98.69	22.84	22.09 $\pm$ 2.36	10.68	96.72
3	3.00	2.87 $\pm$ 0.19	6.62	95.67	2.98	2.75 $\pm$ 0.37	13.45	92.28
0.24	0.24	0.27 $\pm$ 0.03	11.11	112.50	0.23	0.19 $\pm$ 0.01	5.26	82.61

Table 4

Recovery, matrix factors and stability of BSIH and SIH in rat plasma.

Concentration level (μM)	Extraction recovery (%)	Absolute MF (%)	IS-normalised MF (%)	Stability	
				Post-preparative (%)	Long term (%)
BSIH					
23	77.51	79.42	97.29	94.02	99.44
3	80.38	83.52	102.32	97.28	98.78
0.06	77.67	78.03	95.59	95.42	97.05
SIH					
23	82.52	85.43	104.66	97.63	96.90
3	90.29	81.11	99.36	91.75	95.80
0.24	81.19	83.14	101.85	94.68	98.89
IS					
0.13	87.99	81.63			