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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-boronyl salicylaldehyde isonicotinoyl hydrazone-BSIH 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 μM, 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 μM, 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 its 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 H_2O_2. The observed integrity of BSIH in plasma samples from the *in vivo* study suggests that the concept of prochelation might be a promising strategy for further development of aroylhydrazone cytoprotective agents.

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

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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 it is 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 boronyl ester. Previous *in vitro* studies demonstrated by UV/vis spectrophotometry that BSIH releases SIH when activated by H_2O_2 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.

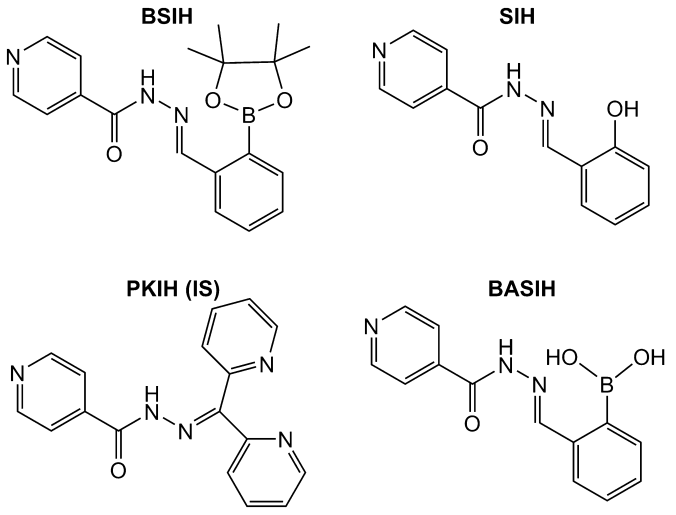

Fig. 1. The chemical structures of the analytes and internal standard.

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 ($NaH_2PO_4{\cdot}H_2O$), 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 the analysis of BSIH and SIH in plasma.

All analyses were performed on a Zorbax Bonus-RP (150 mm × 3 mm, 3.5 μm) column protected by a guard column (Zorbax Bonus-RP, 20 mm × 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.