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Simultaneous determination of the bilirubin oxidation end products *Z*-BOX A and *Z*-BOX B in human serum using liquid chromatography coupled to tandem mass spectrometry

Raphael A. Seidel[a,b], Marcel Kahnes[a], Michael Bauer[b], Georg Pohnert[a,*]

[a] *Institute for Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Lessingstrasse 8, 07743 Jena, Germany*
[b] *Department of Anesthesiology and Intensive Care Medicine/Center for Sepsis Control and Care, Jena University Hospital, Friedrich Schiller University Jena, Erlanger Allee 101, 07747 Jena, Germany*

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ABSTRACT

Bilirubin oxidation end products (BOXes) appear upon endogenous heme degradation and can be found in the cerebrospinal fluid after hemorrhagic stroke. BOXes are assumed to contribute to delayed cerebral vasospasm and secondary loss of brain tissue. Here, we present a validated LC–ESI-MS/MS method for the sensitive determination of the regio-isomers *Z*-BOX A and *Z*-BOX B in human serum. We found that *Z*-BOX A and *Z*-BOX B appear in serum of healthy volunteers. The sample preparation includes the addition of 5-bromonicotinamide as internal standard and protein precipitation with acetonitrile. Baseline-separation was achieved on a C-18 column with a binary solvent gradient of formic acid in water/acetonitrile at 1 mL/min within a total analysis time of 17 min. Using single reaction monitoring in the positive ion mode, the linear working ranges were 2.74–163 pg/μL (*Z*-BOX A) and 2.12–162.4 pg/μL (*Z*-BOX B) with $R^2 > 0.995$. Intra- and inter-day precisions were <10%. The inherent analyte concentrations of *Z*-BOX A (14.4 ± 5.1 nM) and *Z*-BOX B (10.9 ± 3.1 nM) in pooled human serum were determined by standard addition. The photolability of both analytes was demonstrated. This method enables to monitor *Z*-BOX A and *Z*-BOX B as a prerequisite to systematically study the biological significance of higher order metabolites of heme degradation.

1. Introduction

Bilirubin oxidation end products (BOXes) increasingly attract attention because they are suspected to play an important role in delayed cerebral vasospasm after subarachnoid hemorrhage [1–3]. In the course of blood clotting and hemolysis large amounts of hemoglobin are liberated. Heme is released from hemoglobin and accelerates its own enzymatic degradation *via* induction of heme oxygenase-1 (HO-1) [4,5]. The levels of the inducible isoenzyme HO-1 are increased under conditions of oxidative stress [6,7]. Neurons also express the non-inducible isoform HO-2 that contributes to the degradation of heme [4,5]. The reaction products of this rate-limiting enzymatic step are biliverdin, iron (Fe^{2+}) and carbon monoxide. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase [4]. In addition to the well-known hepatic biotransformation and excretion [8], bilirubin can be degraded oxidatively [9]. Among the variety of oxidation products 2-(4-methyl-5-oxo-3-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene)acetamide (BOX A), 2-(3-methyl-5-oxo-4-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene)acetamide (BOX B) and 4-methyl-3-vinyl-maleimide (MVM) were identified as potentially accumulating end products (see Scheme 1). These pyrroline derivatives have been found in the cerebrospinal fluid of patients suffering from subarachnoid hemorrhage [9,10]. The endogenous production of BOXes from heme and bilirubin was ascribed to the action of reactive oxygen species (ROS) that are generated in the reaction of iron with hydrogen peroxide or inside white blood cells around perivascular hematoma [1].

Abbreviations: BOX A, 2-(4-methyl-5-oxo-3-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene)acetamide; BOX B, 2-(3-methyl-5-oxo-4-vinyl-1,5-dihydro-2H-pyrrol-2-ylidene)acetamide; CE, collision energy; CSF, cerebrospinal fluid; ESI, electrospray ionization; EDTA, ethylenediaminetetraacetate; HPLC, high performance liquid chromatography; HO, heme oxygenase; IS, internal standard; LC, liquid chromatography; LLOQ, lower limit of quantification; MS/MS, tandem mass spectrometry; MVM, 4-methyl-3-vinyl-maleimide; PPT, protein precipitation; ROS, reactive oxygen species; RSD, relative standard deviation; SRM, selected reaction monitoring; ULOQ, upper limit of quantification; UV, ultra violet.

* Corresponding author. Tel.: +49 3641 948170.
E-mail addresses: Raphael.Seidel@uni-jena.de (R.A. Seidel), Georg.Pohnert@uni-jena.de (G. Pohnert).

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In a biomimetic approach BOXes can be produced by *in vitro* degradation of bilirubin with hydrogen peroxide [9]. Animal and *in vitro* experiments demonstrated vasoconstrictive effects of BOXes on rodent cerebral vessels and increased oxygen consumption in vascular smooth muscle cells [11,12]. This vascular reactivity was linked to delayed vasospasm which is a serious complication after subarachnoid hemorrhage and can cause secondary loss of brain tissue [1,13]. On a molecular level BOXes were shown to inhibit large-conductance Ca^{2+}- and voltage-dependent potassium channels (Slo 1 BK) that are expressed in vascular smooth muscle cells. Because the inhibition of Slo 1 BK by BOXes leads to increased intracellular potentials, depolarization and constriction of the vascular smooth muscle cells is facilitated [2].

Recently, the configuration of BOX A from oxidative bilirubin degradation was assigned. As expected from the structure of heme the *Z*-configuration of the exocyclic double bond could be confirmed by UPLC-co-injection experiments with a synthetic reference [14]. Rough estimations of BOXes concentrations in CSF were obtained with high performance liquid chromatography coupled to ultra violet spectroscopy (HPLC-UV). That method delivered appraised values of 1.2–1.4 μM BOXes in CSF after subarachnoid hemorrhage [10,15,16].

Despite the high interest in these bilirubin oxidation end products there is no method available to quantify *Z*-BOX A, or *Z*-BOX B. The lack of a suitable method to study these metabolites in any body fluid hampers the further investigation of their (patho)physiological role. Therefore we aimed to determine *Z*-BOX A and *Z*-BOX B in blood samples with a highly selective and sensitive quantification method. Since MVM is not stable and spontaneously forms hardly soluble products (unpublished results) this metabolite was not included in the present work. Here we report on the validated simultaneous determination of *Z*-BOX A and *Z*-BOX B in human serum by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The method will provide a basis to ascertain the role of *Z*-BOX A and *Z*-BOX B in illnesses related to heme and bilirubin metabolism.

2. Experimental

All procedures using *Z*-BOX A, *Z*-BOX B or human serum were carried out under light protection to avoid photoisomerization of the analytes (see Section 2.7.6).

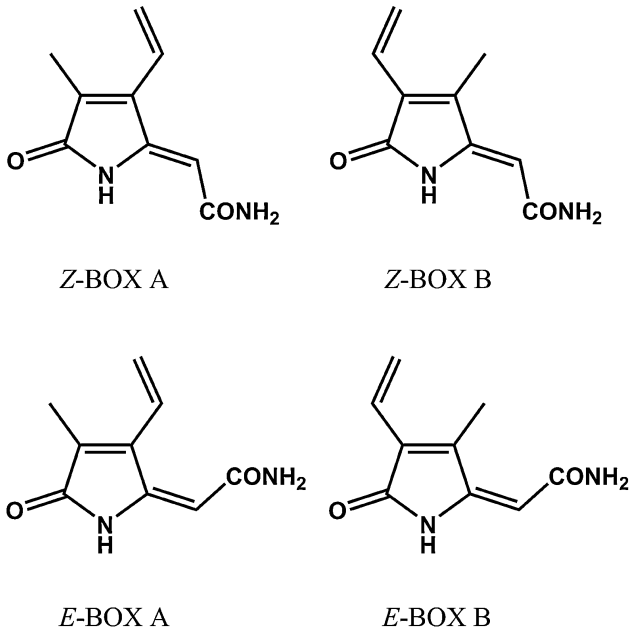
2.1. Materials

2.1.1. Chemicals

All chemicals were purchased and used without further purification: Bilirubin (Sigma–Aldrich, Germany); 5-bromonicotinamide (Alfa Aesar, Germany); formic acid and hydrochloric acid (Roth, Germany); sodium hydroxide (Chempol, Czech Republic); hydrogen peroxide and HPLC gradient grade acetonitrile (VWR Intl., France). HPLC water was obtained from a TKA microPure system (Thermo Electron, Niederelbert, Germany).

2.1.2. Bilirubin oxidation procedure

Z-BOX A and *Z*-BOX B were obtained following a previously published protocol by Wurster et al. [16]. Briefly, 1 g bilirubin was dissolved in 500 mL 5 N sodium hydroxide by stirring for 24 h. After neutralization with 37% hydrochloric acid, 50% hydrogen peroxide solution was added to obtain a final concentration of 10% hydrogen peroxide. The unbuffered solution was adjusted to pH 7.5 and stirred for 24 h at room temperature yielding the crude bilirubin oxidation mixture.



Scheme 1. Structures of the regio-isomeric bilirubin oxidation end products *Z*-BOX A and *Z*-BOX B and their corresponding isomers *E*-BOX A and *E*-BOX B.

2.1.3. Purification of analytes

The two regio-isomers *Z*-BOX A and *Z*-BOX B were purified from the crude bilirubin oxidation mixture according to Klopfleisch et al. [14]: The dried chloroform extract of the crude bilirubin oxidation mixture was suspended in acetonitrile/water (20/80 v/v) and centrifuged at 16,100 × *g*. The supernatant was purified on a Shimadzu LC-8A HPLC system (Kyoto, Japan) equipped with a SPD-10AV UV-VIS detector measuring at 310 nm. Isocratic separation was achieved on a HTEC C18 column (250 mm × 16 mm, 5 μm; Macherey-Nagel, Düren, Germany) equilibrated with acetonitrile/water (20/80 v/v) at a flow rate of 6 mL/min. Retention times were 18.8 and 23.3 min for *Z*-BOX A and *Z*-BOX B. The structures of both compounds were confirmed by NMR spectroscopy [9].

2.1.4. Pooled human serum

Human blood samples were collected from 10 healthy adult volunteers by peripheral venous puncture (approved by ethical vote No. 3548-08/12 of the Jena University Hospital). Blood samples from individuals taking any medicine or drugs except contraceptives were not considered for pooling. To exclude samples from individuals with potential malfunction in bilirubin metabolism immediate photometrical analysis of total bilirubin levels was carried out on an ARCHITECT *ci*16200 instrument (Abbott, Abbott Park, IL, USA) *via* azobilirubin formation. Only samples with total bilirubin levels within the reference range of <21 μM were chosen for pooling [17]. Serum was prepared using serum gel S-Monovetten (Sarstedt, Germany) according to the manufacturer's instruction. Pooled serum was constituted by mixing equal volumes of 8 appropriate individual sera (4 ♀, 4 ♂) and stored protected from light at −80 °C.

2.2. Preparation of standard solutions

Stock solutions of *Z*-BOX A and *Z*-BOX B were prepared by dissolving the accurately weighed compounds in acetonitrile/water (4/96 v/v) through vortex mixing and heating to 40 °C. The stock solutions (*Z*-BOX A, 129.5 μM; *Z*-BOX B, 111.4 μM) were mixed to obtain a combined stock solution with equimolar concentrations of both analytes (59.9 μM). The combined stock solution was diluted