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Suspect and untargeted screening of bisphenol S metabolites produced by *in vitro* human liver metabolism

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ABSTRACT

Bisphenol S (BPS) is increasingly used as substitute for bisphenol A, resulting in higher potential of human exposure to this compound. Yet, information on the human metabolism of BPS is limited. Hence, current biomonitoring studies rely only on the measurement of BPS itself, leading to a potential underestimation of assessing human exposure to this emerging contaminant. The aims of this study were to investigate the *in vitro* metabolic pathways of BPS using human liver microsomes and cytosol fractions and propose *in vitro* metabolites for evaluation in pharmacokinetics studies. Liquid chromatography coupled to quadrupole time-of-flight high-resolution mass spectrometry was used for the screening, identification, and structural elucidation of Phase I and II metabolites of BPS for the first time. Metabolite identification was performed using two complementary workflows: suspect and untargeted screening. Two Phase I metabolites were formed through hydroxylation of the phenolic rings. Four Phase II metabolites were formed through conjugation with glucuronic acid or sulfate. Three of these metabolites, namely dihydroxy-BPS, hydroxy-BPS-glucuronide and hydroxy-BPS-sulfate were identified and structurally elucidated for the first time. As such, we provide an expanded set of *in vitro* biotransformation products of BPS, which can potentially support a reliable assessment of BPS exposure in future biomonitoring studies.

1. Introduction

Bisphenol A (4-[2-(4-hydroxyphenyl)propan-2-yl]phenol, BPA), a widely used industrial chemical, is being produced in high quantities over five decades, with yearly global production of 8 million tonnes, and is mainly used as a monomer in the production of polycarbonate plastic and epoxy resins (Asimakopoulos et al., 2016; Brandon et al., 2003). Many studies have associated exposure to BPA with adverse effects in humans (Eladak et al., 2015). Due to its potential harm as an endocrine disrupting chemical, more stringent regulations on the production and application of BPA are in force (EFSA, 2015; EU, 2011). To comply with legislation, BPA has been gradually replaced with other structurally related compounds, including bisphenol S (4,4′-sulfonyldiphenol, BPS).

BPS is one of the main alternatives to BPA in a number of applications such as the manufacturing of plastic, epoxy resins and as a developer in thermal paper (Skledar et al., 2016). Recent studies have shown its presence in indoor dust, foodstuff, paper products, sludge, sediments (Wu et al., 2018), wastewater influent and effluent (Sun et al., 2017) and surface water (Chen et al., 2016). In accordance with BPA, the mechanism by which the population is exposed to BPS is mainly by ingestion (food, dust) and in lower extent through inhalation (air, particles) and dermal adsorption (dust) (Caballero-Casero et al., 2016; Wu et al., 2018). Handling thermal paper is a possible additional way of exposure (Liao et al., 2012b). In 2012, the study by Liao et al. (Liao et al., 2012b) reported the presence of BPS in human urine for the first time, in a concentration range from < 0.02 to 21.0 ng/mL, with a detection frequency of 81%. Recent biomonitoring studies confirmed the presence of BPS in human urine at similar (Wu et al., 2018) or even higher concentrations of BPS in comparison to BPA (Asimakopoulos et al., 2016).

Since international production and application of BPS are not regulated, BPS levels in the environment and therefore human exposure to this compound are steadily increasing. While human metabolism for BPA has been studied extensively (Gramec Skledar and Peterlin Mašič, 2016; Thayer et al., 2015; Völkel et al., 2002), only a few studies have

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used *in vitro* and *in silico* experiments to provide a preliminary evaluation of human metabolism of BPS (Gramec Skledar et al., 2015; Grignard et al., 2012; Le Fol et al., 2015; Skledar et al., 2016). Two *in vitro* studies have described the influence of metabolism on the endocrine activity of BPS, reporting divergent results (Kang et al., 2014; Skledar et al., 2016). So far, no study has performed a full screening of Phase I and Phase II *in vitro* metabolites of BPS using accurate mass high-resolution mass spectrometry, which allows chemical identification and characterisation. Well-designed *in vitro* experiments followed by the analysis using liquid chromatography combined with accurate mass spectrometry could enhance confidence in detecting and identifying metabolites formed in the *in vitro* metabolism (Erratico et al., 2015; Lai et al., 2015; Mortelé et al., 2018; Negreira et al., 2015; Van den Eede et al., 2013).

Pooled human liver microsomes (HLMs) contain a wide variety of drug metabolising enzymes and are commonly used for *in vitro* human metabolism studies. They particularly contain the cytochrome P450 s (CYPs) and UDP-glucuronosyltransferases (UGTs), which are the major enzyme systems responsible for the metabolism of exogenous compounds (Brandon et al., 2003; Zhang et al., 2016). In a previous *in vitro* study, the hepatic human UGT enzyme UGT1A9 was found to conjugate BPS with glucuronic acid more extensively than the homologous intestine enzyme (Gramec Skledar et al., 2015). Conjugation with glucuronic acid or sulfate is generally regarded as the main detoxification pathway for most bisphenols (Gramec Skledar and Peterlin Mašič, 2016). Although BPS is structurally related to other bisphenols, it cannot be assumed that their metabolism is interchangeable (Oh et al., 2018).

The aims of this study were to elucidate the *in vitro* metabolic pathway of BPS using HLMs and human liver cytosol fractions (HLCYT) to produce Phase I and Phase II metabolites and to propose a set of potential additional biomarkers for use in future biomonitoring studies. To our best knowledge, this study was the first to use liquid chromatography coupled to high-resolution quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS) to perform a full screening, identification, and structural elucidation of *in vitro* BPS metabolites.

2. Materials and methods

2.1. Chemicals and reagents

The analytical reference standard of BPS (4,4′-sulfonyldiphenol, purity 98%) was purchased from Sigma-Aldrich (Missouri, USA). The analytical reference standards of valsartan-d3 and BPS-glucuronide were acquired from Toronto Research Chemicals (Toronto, Canada). Pooled human liver microsomes (HLMs, mixed gender, n = 50) were obtained from Tebu-Bio (Boechout, Belgium). Pooled human liver cytosol (HLCYT), 2,5-uridinediphosphate glucuronic acid (UDPGA), adenosine-3′-phosphate 5′-phosphosulfate (PAPS, > 60%) lithium salt hydrate, alamethicin (neat, > 98%), dimethyl sulfoxide (DMSO), and 4-nitrophenol (4-NP) were acquired from Sigma-Aldrich (Missouri, USA). NADPH tetrasodium salt hydrate (> 96%) was purchased from Acros (Geel, Belgium). Acetonitrile (ACN, HPLC-grade) was obtained from Fisher Chemical (Loughborough, United Kingdom), formic acid (> 98%) from Merck KGaA (Darmstadt, Germany). A 100 mM TRIS-buffer was prepared by dissolving 12.1 g Trizma base (Janssen Chimica, Beerse, Belgium) and 1.0 g $MgCl_2$ (Merck KGaA, Darmstadt, Germany) in 1 L ultrapure water. The pH was adjusted to 7.4 by adding 1 M HCl solution. Ultra-pure water was produced in-house with a PURELAB-purifier system of Elga Labwater (Tienen, Belgium).

2.2. In vitro metabolism assay

This study employed the *in vitro* human metabolism assay optimised and used in our previous studies (Erratico et al., 2015; Lai et al., 2015; Mortelé et al., 2018; Negreira et al., 2015; Van den Eede et al., 2013). The experimental setup in a schematic summary can be found in the Supporting Information (Fig. SI-1.1). All sample sets consisted of three replicates. Briefly, Phase I metabolites were generated using pooled HLMs. The reaction mixture (1 mL), which consisted of 945 µL of TRIS-buffer (pH 7.4, 100 mM), 25 µL of HLM (20 mg/mL) and 10 µL of BPS stock solution (0.5 mM), was incubated in a 1.5 mL Eppendorf tube at 37 °C for 1 and 3 h. At 5, 60 and 120 min of incubation, 10 µL of NADPH (0.1 M in buffer) was added. Three negative control samples (*i.e.*, either without BPS, HLM or NADPH) were included in the experiment. Also, a positive control sample, using 10 µL phenacetin (5 µg/mL) as substrate, was prepared and incubated. The reaction was stopped after 1 and 3 h incubation by adding 250 µL ice-cold ACN with 1% (v/v) formic acid and the internal standard valsartan-d3, at a concentration of 1 µg/mL.

Following the *in vitro* Phase I metabolism, samples were subject to Phase II conjugation *via* either glucuronidation (GLU) or sulfation (SUL). Phase I samples were centrifuged for 5 min at 8000 rpm. For the GLU samples, 935 µL of the supernatant was transferred and incubated with 25 µL of HLM and 10 µL of alamethicin (1 mg/mL in DMSO) at 37 °C. At 5, 60 and 120 min of incubation, 10 µL of UDPGA (100 mM in buffer) was added. For the SUL samples, 965 µL of the supernatant originating from the Phase I samples was transferred and incubated with 25 µL of HLCYT (20 mg/mL). At 5, 60 and 120 min of incubation, 10 µL of PAPS (10 mM in buffer) was added. Negative control samples were prepared for both sets of samples by omitting BPS and the cofactor (UDPGA and PAPS). Another sample was prepared by subjecting BPS only to Phase II metabolism (GLU or SUL respectively). Positive control samples were prepared by spiking the reaction mixture with 4-NP (10 mM) as the substrate. The reaction was stopped after 3 h incubation by adding 250 µL of ice-cold ACN with 1% (v/v) formic acid and the internal standard valsartan-d3, at a concentration of 1 µg/mL.

Before the concentration step, all the samples were centrifuged for 5 min at 8000 rpm. The supernatant was then transferred to a clean glass tube and evaporated under nitrogen at 37 °C. Subsequently, samples were reconstituted in 200 µL of a 10:90 (v/v) MeOH/water solution.

2.3. LC-QTOF-MS analytical method

Analysis of the samples was conducted using an Agilent 1290 Infinity UPLC coupled to an Agilent 6530 Accurate-Mass QTOF (Agilent, Santa Clara, US). Chromatographic separation was achieved on a Poroshell 120 C18 column (50 x 3 mm; 2.7 µm particle size, Agilent), using a mobile phase composed of ultra-pure water with 1 mM NH_4F (A) and MeOH with 1 mM NH_4F (B). The injection volume was 5 µL and column temperature was kept constant at 40 °C. All samples were analysed in positive and negative ionisation mode. Both methods used the same chromatographic conditions; the run started with an isocratic part for 2 min at 10% B, then a gradient was applied to raise the percentage of B to 50% at 12.8 min. B remained at this percentage until 13 min. A second gradient was used to increase the level of B to 95% at 14 min. Subsequently, the column was rinsed with 95% B for six minutes and re-equilibrated at 10% B for five minutes. The flow rate was 0.4 mL/min.

For all analyses, the QTOF-MS instrument was operated in the 2 GHz (extended dynamic range) mode, providing a Full Width at Half Maximum (FWHM) resolution of approximately 5400 at *m/z* 112.9856 and 11,000 at *m/z* 1033.9881. The ions 121.0508 and 922.0097 for positive mode and 112.9856 and 980.0164 for negative mode were selected for a continuous recalibration during the run to ensure mass accuracy. The eluting compounds were ionised using Agilent Jet-Stream electrospray ionisation (AJS-ESI) under the following parameters for both positive and negative ionisation. Drying gas temperature was set at 300 °C and the flow was 8 L/min. The sheath gas temperature was 400 °C at a flow of 11 L/min. Nebulizer pressure was set at 25 psig. Capillary, nozzle and fragmentor voltages were set at 2000 V, 0 V and 120 V, respectively. Acquisition parameters were set for the *m/z* values