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Metabolic transformation as a diagnostic tool for the selection of candidate promutagens in effect-directed analysis

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ABSTRACT

To implement metabolic activation by S9 rat liver homogenate in the selection of candidate promutagens in effect-directed analysis, we critically assessed the capability of LC-HRMS measurements to detect depletion and formation of metabolites by S9 exposure. The exposure of a reference mixture to S9 led to a depletion by >70% for most compounds. Other processes than metabolism were excluded as significant contribution to compound depletion. Metabolites formed by S9 exposure were identified and S9 metabolism was incorporated in the identification of candidate promutagens in a wastewater treatment plant (WWTP) effluent with mutagenic activity only after metabolic activation by S9. The metabolism by S9 in the WWTP effluent was confirmed. Based on a candidate exclusion of all peaks not depleted, thus not activated by the S9 mix, the number of candidate promutagens was reduced by 40%. Selected remaining candidates were evaluated and identified, but could not be confirmed as promutagens.

1. Introduction

While chemical and bio-analytical tools allow for the sensitive detection of trace chemicals in complex environmental mixtures and of biological effects of these mixtures, the establishment of cause-effect relationships with individual mixture components is still a challenge. Effect-directed analysis (EDA) has been shown to be a promising tool to address this challenge (e.g. Brack et al., 2005; Higley et al., 2012; Lübcke-von Varel et al., 2012; Gallampois et al., 2013). EDA combines biological tests and physico-chemical separation procedures for the isolation of toxic fractions and compounds, which are typically subjected to GC–MS (e.g., Rostkowski et al., 2011; Lübcke-von Varel et al., 2012; Creusot et al., 2013) or in case of more polar compounds to LC-MS analyses (e.g., Schmitt et al., 2012; Gallampois et al., 2013). Using high resolution-high accuracy tandem mass spectrometry, molecular formulas can be obtained for most peaks. However, for many molecular formulas thousands of possible structures may be derived from structure generation (Schymanski et al., 2012) or searches in large compound databases such as ChemSpider or PubChem (Hug et al., 2014). In order to focus identification efforts on compounds with the potential to cause the effect of interest as much information as possible from chemical and biological analysis needs to be exploited and integrated. This may include acid-base properties (Gallampois et al., 2013), chromatographic retention prediction (Ulrich et al., 2011), fragmentation prediction (Wolf et al., 2010; Hug et al., 2014), and toxicity or mutagenicity prediction (Meinert et al., 2010).

The Ames test (Maron and Ames, 1983) and modifications thereof such as the Ames fluctuation test (AFT) (Reifferscheid et al., 2012; International Organization for Standardization, 2012) using different strains of the bacterium *Salmonella typhimurium* are among the most frequently used bioassays to detect mutagens in environmental samples and are important tools in the EDA of mutagens (Reifferscheid and Grummt, 2000; Ohe et al., 2004; Higley et al., 2012; Lübcke-von Varel et al., 2012; Reifferscheid et al., 2012). In contrast to other compounds, which might be deactivated by the S9 metabolism e.g. chlorinated furanones and dinitropyrenes (Meier et al., 1987; Shane and Winston, 1997) promutagens exhibit mutagenicity only after metabolic activation by enzymes which functionalize molecules to reduce their hydrophobicity and to allow for excretion (Shimada et al., 1989; Guengerich, 2001; Rendic and Guengerich, 2012). Bacteria may lack this metabolic capability. In order to include the activation of promutagens in bacterial assays vertebrate liver tissue homogenates containing the metabolic enzymes are added to the assay to simulate mammalian metabolism in the liver (Ames et al., 1973). These supernatants of an organ homogenate, usually liver

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homogenates, so-called S9 fractions contain cytosol and microsomes (Duffus et al., 2007). The S9 fraction is a very diverse and undefined mixture of enzymes, mainly cytochrome P450 isoforms (Mori et al., 2001), which simulate phase I metabolism of mammalians known to activate promutagens, but also catalyze diverse other reactions (Bauer et al., 1995; Guengerich, 2001; Connors et al., 2013). The stability of (mutagenic) metabolites formed by the S9 metabolism varies from rather stable epoxides (Glatt et al., 1991) to very reactive nitrenium cations, which diffuse as metabolic intermediates into the cell and react after further chemical or enzymatic reactions in the cell with the DNA (Shamovsky et al., 2012). The incorporation of a metabolic activation by the S9 mix is a standard procedure in mutagenicity assessment of samples and compounds (Ohe et al., 2004; International Organization for Standardization, 2012, Reifferscheid et al., 2012), but the potential of S9 metabolism has not been implemented in the identification of promutagens and their mutagenic metabolites in EDA before.

Our objective was to apply and critically assess the potential of the metabolism by S9 in the identification of promutagens and mutagenic metabolites in EDA. Therefore, we first checked the capability of LC-HRMS measurements to detect metabolic depletion and formed metabolites in a mixture of reference standard compounds with expected metabolism under S9 exposure. The contribution of non-metabolic processes to the depletion of reference compounds and the possibility to detect potential mutagenic metabolites were evaluated.

Furthermore, we characterized the impact of S9 on a complex fraction of a wastewater treatment plant (WWTP) effluent. The impact of S9 metabolism on all peaks was monitored and the depletion of previously identified compounds was compared between the complex sample and the mixture of reference standards. Finally, the depletion of compounds by S9 was used for candidate exclusion in the identification of promutagens in the WWTP effluent fraction.

2. Materials and methods

Details on reference standards and reagents are given in the Supplementary material (SM).

2.1. Sample collection and sample preparation

Grab samples from the effluent of a WWTP were collected during 4 day at the outlet of the WWTP Bitterfeld–Wolfen, Saxony-Anhalt, Germany, into 5 L aluminum containers and frozen at −20 °C until their extraction within four days. Samples from different dates were separately extracted by automated large volume solid phase extraction (LVSPE) in two steps at the original pH values of 6.9–7.5 and at a pH of 2.6 ± 0.5 adjusted by 10 vol.-% formic acid in bi-distilled water at a temperature of 4 °C (Schulze et al., 2012). Details on the extraction are given in the SM, section S1.2 and by Hug et al., 2014. All samples were pooled to a combined sample (CS) representative for the whole sampling period. A blank sample was prepared from tap water analogous to the wastewater extraction.

2.2. Fractionation procedure and candidate selection in environmental sample

The procedure for the separation of mutagenic from non-mutagenic fractions, the physico-chemical characterization of mutagens and the selection of candidates for the identification process is shown in Fig. 1. A reversed-phase fractionation was conducted at acidic (pH 2.6, FA 1–10) and neutral (pH 7.6, FN 1–10) conditions to gain information about lipophilicity and neutral or ionic status of mutagens at different pH values. The fractionation process is described in the SM, section S1.3. For an additional sample clean up to remove matrix components, the most mutagenic fractions (FN5 & FN6) were solvent-exchanged into dichloromethane (DCM) and back to methanol. The DCM insoluble residue, $FN5/6_{res}$, showed no mutagenic activity and was removed without any impact on mutagenicity of FN5 and FN6 ($FN5/6_{sol}$) (results not shown). The DCM insoluble residue, $FN5/6_{res}$, and the DCM soluble fraction were chemically analyzed after solvent exchange back to methanol for peak exclusion (see sec. 2.7).

2.3. LC-HRMS/MS analysis and data processing

After filtration of sample extracts by a PTFE syringe filter (0.45 μm, Macherey–Nagel) the LC separation was performed with an Agilent 1200 series UPLC system equipped with a Kinetex™ Core-Shell C18 column (100 mm × 3.0 mm; 2.6 μm; Phenomenex). A linear gradient elution with water (A) and methanol (B) both containing 0.1 vol.-% of formic acid was run with a flow rate of 0.2 mL/min. The injection volume was 10 μL. The LC system was connected to an ion trap-Orbitrap hybrid instrument (LTQ Orbitrap XL, Thermo Scientific) operated with electrospray ionization both in positive and negative ion mode (ESI±). Full scan HRMS chromatograms (m/z 100–1000) were acquired with a nominal resolving power of 60,000 (full width at half maximum) referenced to m/z 400. In a separate run after full scan measurements, data-dependent high-resolution product ion spectra (HRMS/MS) were recorded based on parent mass lists including masses selected for identification. Exact mass ion chromatograms of the reference standard compounds were extracted from full scan HRMS chromatograms with a mass deviation <5 ppm using the QuanBrowser of the Xcalibur software (Thermo Scientific). Peaks were automatically integrated and manually reviewed.

For the detection of nontarget peaks, accurate mass ion chromatograms of ESI+ and ESI− measurements of all samples and blanks were retrieved from full scan data by the software MZmine 2.10 (Pluskal et al., 2010) as described by Hug et al., 2014. Peak lists including the accurate mass, retention time, peak intensity and area were exported from MZmine to Microsoft Excel for further evaluation. For the characterization of general chemical changes by the S9 metabolism, m/z values were converted to neutral molecule masses assuming protonation in ESI+ and deprotonation in ESI−. Molecular formulas from accurate masses were calculated by the Seven Golden Rules (SGR) fast checker software (Kind and Fiehn, 2007). Carbon, hydrogen, nitrogen, oxygen, phosphor, sulfur, fluorine, chlorine, bromine, and silicon were considered for the determination of molecular formulas. The oxygen, nitrogen, and sulfur abundance were determined from the molecular formulas of detected peaks. Changes in the composition of samples were evaluated considering all peaks with an intensity $>5 \cdot 10^4$, because peaks with a lower intensity are typically not reliably detected by MZmine.

During the identification of candidate promutagens, peaks with a sample-to-blank intensity ratio <50 or an area ratio <10, with implausible mass defect for singly charged ions, without Lorentzian peak shape or out of the retention time window defined by the fractionation procedure (0–25 min) were excluded from the peak list, for details see Hug et al. (2014) and S 2.5. Selected candidates were further processed as described by Hug et al. (2014). HRMS/MS spectra were recorded in a second analysis run and evaluated by MetFrag with a score cut off of 0.8 at a mass accuracy of 5 ppm (MZppm) in combination with a search for candidates in ChemSpider (Wolf et al., 2010). The MetFrag score was evaluated in combination with the number of data sources in ChemSpider. The number of data sources given by ChemSpider was considered as an