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High resolution-ion mobility mass spectrometry as an additional powerful tool for structural characterization of mycotoxin metabolites

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ABSTRACT

This work was designed as a proof of concept, to demonstrate the successful use of the comparison between theoretical and experimental collision cross section (CCS) values to support the identification of isomeric forms. To this purpose, thirteen mycotoxins were considered and analyzed using drift time ion mobility mass spectrometry. A good linear correlation (r^2 = 0.962) between theoretical and experimental CCS was found. The average ΔCCS was 3.2%, fully consistent with the acceptability threshold value commonly set at 5%. The agreement between theoretical and experimental CCS obtained for mycotoxin glucuronides suggested the potential of the CCS matching in supporting the annotation procedure.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by a large number of fungal species, potentially infesting foodstuffs at all stages of food production, processing and storage. Therefore, humans and animals can be simultaneously exposed through the diet to “cocktails” of mycotoxins (Assunção, Silva, & Alvito, 2016; Hove, Van Poucke, Njumbe-Ediage, Nyanga, & De Saeger, 2016; Smith, Madec, Coton, & Hymery, 2016). However, the possible extent and the combined effects of co-exposure in humans and animals are still to be defined (Alassane-Kpembi et al., 2017; Shirima et al., 2015). Current risk assessment involves the estimation of the exposure, based on the combination of estimation food consumption data with co-occurrence data collected by control agencies (Heyndrickx et al., 2014). Although very useful for risk management, this approach is based on a severe approximation, as average co-occurrence data obtained for each food category are projected on food consumption data averaged throughout the EU population. Therefore, this under/over-estimation may affect the regulatory action, leading to less effective policies.

In the era of personalized healthcare, a more individual-focused exposure assessment could be obtained by measuring the levels of relevant biomarkers in urine (Ali, Muñoz, & Degen, 2017; Föllmann, Ali, Blaszkewicz, & Degen, 2016; Heyndrickx et al., 2015; Wallin et al., 2015).

Glucuronidation is one of the main phase II metabolic pathways, whereby mycotoxins, in a similar way to other natural compounds occurring in the diet and drugs, are biotransformed into polar conjugates, before excretion in bile or urine (Warth, Sulyok, Berthiller, Schuhmacher, & Krska, 2013). The reaction is mediated by a family of membrane-bound enzymes, UDP-glucuronosyltransferases (UGTs), which catalyze the transfer of glucuronic acid to a nucleophilic functional group of the target compound. In humans, up to 20 different UGT isoforms have been characterized, following expression of their corresponding cDNA in heterologous cells (Ritter, 2000). Studies have shown that these isoforms present distinct, but frequently overlapping, substrate specificities, leading therefore to the formation and excretion of a

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range of isomeric glucuronides of the same xenobiotic (Dong, Ako, Hu, & Wu, 2012; Tripathi, Bhadauriya, Patil, & Sangamwar, 2013; Wu, Basu, Meng, Wang, & Hu, 2011).

Besides their urinary excretion as biomarkers, regio- and stereo-specificity in isomers formation are relevant also in consideration of the possible differences in their biological activity. Although glucuronidation is indeed often regarded as a detoxification process, in some cases the xenobiotic glucuronidation may elicit biologically active or toxic metabolites, i.e. morphine-6-glucuronide is a more potent opioid agonist than morphine itself (Ritter, 2000). Similarly, DON-15-glucuronide has been predicted, according to computational calculation, to be more ribotoxic than its parent compound (Dellafiora, Galaverna, & Dall'Asta, 2017).

Concerning mycotoxins, the glucuronidation pathways of deoxynivalenol (DON), zearalenone (ZEN), ochratoxin A (OTA), alternariol (AOH) and its methyl ether (AME), have been partially or fully elucidated so far, also in terms of the activity of different human UGT isoforms (Maul et al., 2012, 2015; Pfeiffer, Hildebrand, Mikula, & Metzler, 2010; Pfeiffer et al., 2009). Besides inter-species variability (Maul et al., 2012, 2015; Welsch & Humpf, 2012), inherent factors, such as gender, age and epigenetics, may affect the glucuronidation, and therefore have an impact on the pattern of urinary biomarkers (Dluzen et al., 2014; Videmann, Koraichi, Mazallon, & Lecoeur, 2012; Yasar, Greenblatt, Guillemette, & Court, 2013).

Glucuronidation studies are often performed using liver microsomes or microsomal fractions from different animals, to explore possible inter-species differences leading to a different biomarker pattern in urine and/or plasma (Welsch & Humpf, 2012). Specific enzymes can be isolated, as well. Once different glucuronides are obtained upon incubation, further steps of isolation and purification are required before structural elucidation, which is usually performed by NMR analysis. However, although efficient for evaluating isomeric heterogeneity, the main limitation of this approach is the need for a considerable amount of pure analyte.

In this frame, a new analytical technique, ion mobility spectrometry (IMS), is gaining wider recognition as a promising approach that can overcome the above-mentioned NMR limitations, making it an ideal candidate for improving confidence in the identification and separation of structurally closely related isomers (Righetti, Paglia, Galaverna, & Dall'Asta, 2016). IMS is a gas-phase electrophoretic technique that provides a new dimension (3D) of separation based on size, shape, and charge of ions (Cumeras, Figueras, Davis, Baumbach, & Gràcia, 2015; Paglia, Angel et al., 2015; Paglia, Kliman, Claude, Geromanos, & Astarita, 2015). So far, three major IMS-MS separation approaches are currently commercially available: drift-time IMS (DT-IMS), traveling-wave IMS (TW-IMS), and high field asymmetric waveform IMS (FAIMS), also known as differential-mobility spectrometry (DMS) (Cumeras et al., 2015; Paglia, Kliman et al., 2015; Paglia, Angel et al., 2015). In DT-IMS (May et al., 2014), ions move through a homogeneous, continuous electric field in a drift tube in the presence of neutral gas molecules. DT-IMS consists of a series of stacked-ring electrodes where a near-uniform electric field is created along the axis of the drift tube. The carrier gas and the gaseous sample are introduced into the ionization region, while a counter current flow of a neutral gas (mostly nitrogen, helium or argon), is introduced from the side of the detection region (Cumeras et al., 2015). Thus, species with the same mass-to-charge ratio (i.e., isomers) can be separated according to their ability to pass through a tube filled with a gas under the influence of an electric field.

Since isomers, such as those obtained upon glucuronidation, have the same mass-to-charge ratio, but a different three-dimensional (3D) conformation, the time taken for each parent ion to drift through the tube will be significantly different. Since the ion drift time depends principally on the collision frequency between the ions and the buffer gas (Paglia, Angel et al., 2015), it allows the measurement of the collision cross section (CCS) values, according to the Mason-Schamp equation (May et al., 2014). On the other side, being related to the unique structural conformation of the molecule, the CCS value can be calculated by theoretical computation (Boschmans et al., 2016; Lapthorn et al., 2015), offering therefore a powerful tool for structural imputation in the absence of reference compounds.

To prove the effectiveness of IMS-MS in supporting the structural identification of isomeric glucuronides through the comparison of theoretical and experimental CCS values, this study was aimed at the HRMS characterization of the glucuronides obtained by incubation of four possible co-occurring mycotoxins, i.e. DON, ZEN, AME, and AOH, with human liver microsomes.

2. Materials and methods

2.1. Chemicals and reagents

Mycotoxin standards of AOH, AME and ZEN were purchased from Sigma (Stuttgart, Germany). Analytical standard of DON was purchased from Romer Labs® (Tulln, Austria). HPLC-grade solvents methanol, acetonitrile and acetic acid were obtained from Sigma (Stuttgart, Germany). Double-distilled water was obtained by a Milli-Q purification system (Millipore, Bedford, MA). Human liver microsomes were purchased from Sigma Aldrich and stored at −80 °C. Uridine 5′-diphosphoglucuronic acid (UDPGA), uridine 5′-diphospho-*N*-acetylglucosamine (UDPGN), Tris-HCl and $MgCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Glucuronidation assay

Human liver microsomes were individually incubated with AOH, AME, ZEN and DON, following the protocols already reported in the literature (Maul et al., 2012; Pfeiffer et al., 2009, 2010; Uhlig, Ivanova, & Fæste, 2013). Briefly, the incubation mixture contained 3.75 mM of each mycotoxin, 7.4 mM UDPGA, 50 mM Tris-HCl buffer (pH 7.4), 8 mM $MgCl_2$, 0.3 mM UDPGN, and 100 µL of liver microsomes in a total volume of 0.5 mL. The incubation was performed at 37 °C for 60 min in a shaking water bath and stopped by adding acetonitrile (1:1; *v/v*). Protein was precipitated after centrifugation (4000*g* for 5 min) and the supernatant was evaporated to dryness and reconstructed in methanol. Glucuronides were purified from the unreacted parent mycotoxins by means of semi-preparative HPLC (1525 Binary HPLC; Waters) equipped with UV detector (998; Waters) using a Synergi fusion C18 column (80 Å, 150 × 10 mm) and double-distilled water and methanol (both acidified with 0.2% formic acid) as mobile phases.

Measurements of the exact mass and the fragmentation patterns of glucuronide isomers were performed by UHPLC-IM-QTOFMS.

2.3. UHPLC-IM-QTOF analysis

Agilent 1290 Infinity UHPLC system coupled to commercial prototype IM-MS, which incorporates a drift tube coupled to a quadrupole time-of-flight mass spectrometer (IM-QTOFMS, Agilent Technologies, Santa Clara, CA) was employed. An orthogonal electrospray ionization (ESI) source (Agilent Jet Stream) was used.

For the chromatographic separation of ZEN, AOH and AME glucuronides, a reversed-phase C18 Acquity HSS T3 column (2.1 × 100 mm and a particle size of 1.8 µm; Waters) heated to 40 °C was used. Gradient elution was performed by using double-distilled water (eluent **A**) and acetonitrile (eluent **B**), both acidified with 0.5% acetic acid. From the initial conditions set at 20% **B**, eluent **B** was increased to 40% in 5 min and to 95% in 1 min. After an isocratic step (2 min), the system was re-equilibrated to initial conditions for 2 min. The total run time was 10 min and flow rate was set at 0.300 mL/min.

By contrast, for the separation of DON-GlcAs an Acquity UPLC BEH Amide column (1.7 µm; 2.1 mm × 100 mm) was used; gradient elution was performed as previously described (Zachariasova, Vaclavikova,