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Diazepam influences urinary bioindicator of occupational toluene exposure

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ABSTRACT

In the present study, we investigated the influence of diazepam (DZP) on the excretion of TOL by examining their urinary metabolites, hippuric acid (HA) and *ortho*-cresol (*o*-C). Male Wistar rats were exposed to TOL (20 ppm) in a nose-only exposure chamber (6 h/day, 5 days/week for 6 weeks) with simultaneous administration of DZP (10 mg/kg/day). Urinary *o*-C levels were determined by GC–MS, while HA, creatinine (CR), DZP and its metabolite, nordiazepam, were analysed by HPLC-DAD. The results of a Mann-Whitney *U* test showed that DZP influenced the urinary excretion of *o*-C ($p < 0.05$). This pioneering study revealed that there was an interaction between DZP and TOL, probably by the inhibition of the CYP isoforms (CYP2B6, CYP2C8, CYP2E1, and CYP1A2) involved in the oxidative metabolism of the solvent. This is relevant information to be considered in the biomonitoring of occupational toluene exposure.

1. Introduction

Toluene (TOL) is a volatile organic solvent that is largely used in industry and in a number of commercial products such as detergents, dyes, paints, adhesives, glues, and pharmaceuticals. Inhalation is the main route of human exposure to TOL, although it can be absorbed through the skin and the gastrointestinal tract (EPA, 2005). The occupational exposure limit for TOL is 78 ppm (294.1 mg/m^3) in Brazil (MTE, 2014), while the updated threshold limit value-time weighted average (TLV-TWA) is 20 ppm (75.4 mg/m^3), as recommended by the American Conference of Governmental Industrial Hygienists in the United States (ACGIH, 2015). After inhalation, TOL readily crosses the blood-brain barrier and produces neurological effects comparable to those of sedative-hypnotics such as alcohol and benzodiazepines (Win-Shwe and Fujimaki, 2010). Previous studies have suggested that TOL has anxiolytic and anticonvulsant effects by modulating γ-aminobutyric acid (GABA) release and $GABA_A$ receptor function. It is important to mention that GABA is the main inhibitory neurotransmitter in the central nervous system (Beckley and Woodward, 2011; Paez-Martinez et al., 2013).

In the human liver, TOL is metabolized by cytochrome P450 (CYP), predominantly by CYP2E1 (followed by CYP2B6, CYP2C8, CYP1A2, and CYP1A1) to benzyl alcohol, which is easily converted to benzoic acid via aldehyde dehydrogenase and is excreted into urine as hippuric acid (HA). *Ortho*-cresol (*o*-C) and *para*-cresol (*p*-C) are formed by the catalytic action of CYP2E1, CYP1A2, and CYP2B6 as minor metabolites from TOL (EPA, 2005; Lee and Yang, 2008; Nakajima and Wang, 1994; Nakajima et al., 1997). Biological monitoring of occupational exposure to this solvent is based on unaltered urinary and blood TOL and their metabolites, HA and *o*-C. This approach has been adopted in most countries. For many years, HA has been the marker of choice. However, this metabolite is nonspecific and may arise from other sources, such as certain fruits and foods, which contain benzoic acid. The HA excreted in urine cannot be solely attributed to TOL exposure in concentrations below 50 ppm (Cosnier et al., 2013; Fustinoni et al., 2000).

As an alternative, urinary *o*-C has been adopted as a determinant both in the biological exposure indices (BEI) and in the biological tolerance values for occupational exposures (BAT). A value of 0.3 mg/g of creatinine (CR) for samples collected at the end of the shift is suggested as a BEI, and a BAT of 1.5 mg/L for samples collected at the end of the shift after long-term exposure are listed (ACGIH, 2015; DFG, 2015). The "B" notation reported by ACGIH indicates that *o*-C is usually present in a significant amount in

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biological specimens collected from non-occupationally exposed subjects (ACGIH, 2015).

According to Truchon et al. (1999), *o*-C performs similarly to HA as a marker at high exposure levels. In contrast, *o*-C appears to outperform HA at lower TOL concentrations (below 50 ppm). Urinary and blood TOL themselves are more specific to access the exposures; hence, the analysis of TOL in urine is most convenient due to its non-invasive sampling and higher volume available (Fustinoni et al., 2000; Netto et al., 2008). A BEI of 0.02 mg/L in blood and 0.03 mg/L in urine has been adopted to monitor TOL exposure (ACGIH, 2015).

Most contemporary regulations and risk assessments regarding toxic chemicals are still single-compound-oriented, even though in the general environment most workers are exposed to complex mixtures (Chang et al., 2005). Interactions between ethanol and solvents are well-known, but there have been few reports on the interactions between solvents and drugs (Døssing et al., 1984; Ernstgård et al., 1999; Wang and Nakajima, 1991, 1992). With the increasing consumption of therapeutic drugs in many countries, workers are often exposed to occupational chemicals and drugs. Adequate knowledge and/or sufficient assessment of situations affecting the levels of bioindicators may be necessary to avoid incorrect interpretations of the results of biological monitoring (Campbell et al., 1988; Døssing et al., 1984).

Diazepam (DZP) has been used in a wide spectrum of disorders due to its anticonvulsant, anxiolytic, sedative, muscle relaxant, and amnestic properties. It is also routinely prescribed as the standard first-line treatment for acute convulsions and prolonged *status epilepticus* (Alldredge et al., 2001; Sweetman, 2009). The metabolic pathway of DZP involves a variety of CYPs, which are responsible for their three major active metabolites: *N*-desmethyldiazepam or nordiazepam (NOR), oxazepam (OX), and temazepam (TZ). DZP C3-hydroxylation is mediated by CYP2C19, 3A4, and 3A5 and leads to TZ, whereas *N*-demethylation leading to NOR is mediated by CYP2B6, 2C8, 2C9, 2C18, 2C19, 3A4, and 3A5. NOR and TZ, the primary metabolites of DZP, are oxidized further to OX, which is excreted mainly in the urine, predominantly as glucuronide conjugates. In time, the metabolite NOR is metabolized by CYP3A4, 3A5, and 2C19 via C3-hydroxylation to form OX. In contrast, TZ is demethylated by CYP3A4, 2C19, 3A5, 2C8, 2C9, and 2B6 to create OX (Acikgöz et al., 2009; Yang et al., 1998).

Metabolic interactions may have implications for biological monitoring when *o*-C and HA are used to assess TOL uptake in exposed workers. Thus, taking into account the high frequency of BZP utilization in clinical practice and the large number of workers who are exposed to solvents, the present study was conducted with rats to investigate the effects of DZP on TOL metabolism by examining their metabolites.

2. Materials and methods

2.1. Chemicals

The study was performed with the following commercially available standards: toluene - 99.% Sigma-Aldrich®, Steinheim, Germany; isopropylbenzene - 98% Sigma-Aldrich®, Steinheim, Germany; hippuric acid - 99% Sigma-Aldrich®, Saint Louis, USA; creatinine - >98% Sigma-Aldrich®, Saint Louis, USA; *ortho*-cresol-d_8 - >98% Sigma-Aldrich®, Steinheim, Germany; *ortho*-cresol - 99.9% Sigma-Aldrich®, Steinheim, Germany; nitrazepam 100 μg/mL - 99.97% Sigma-Aldrich®, Saint Louis, USA; diazepam - 100% União Química Farmacêutica Nacional S/A©, Pouso Alegre, Brazil; and nordiazepam - 100% União Química Farmacêutica Nacional S/A©, Pouso Alegre, Brazil. The following analytical-grade reagents were used: PEG 400 (polyethylene glycol) - Sigma-Aldrich®, Saint Louis, USA; carbon disulfide - Sigma-Aldrich®, Saint Louis, USA; potassium dihydrogen phosphate - Proquímica®, Rio de Janeiro, Brazil; acetonitrile - Lichrosolv®, Darmstadt, Germany; sulfuric acid – Vetec®, Rio de Janeiro, Brazil; Na_2CO_3 (sodium carbonate) - CAAL®, São Paulo, Brazil; diisopropyl ether - Honeywell®, Seelze, Germany; sodium hydrogen phosphate - Caal®, São Paulo, Brazil; *n*-hexane - Honeywell®, Morristown, USA; ethanol - Química Moderna®, Barueri, Brazil; potassium dihydrogen phosphate - Proquímica®, Rio de Janeiro, Brazil.Ultra-pure water (18.2 MΩ/cm) was obtained from a Millipore® system.

2.2. Animals

Male Wistar rats weighing approximately 250 g were used for the study. The animals' room was maintained at a temperature of 20 ± 1 °C and 60 ± 20% relative humidity, with a 12-h light-dark cycle. Rat chow (Presence®, Paulínia, Brazil) and tap water were available *ad libitum*, except during exposure procedures. The rats were divided into 4 groups (5 rats per group): control, GDZP, GTOL, and GTOL/DZP groups.

2.3. Experimental design

The animals used in this study were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* adopted by the Brazilian Association for Laboratory Animal Science (COBEA). The protocol that was followed was approved by the Ethics Committee for Use of Animals (27/2015) of the Faculty of Pharmaceutical Sciences, São Paulo State University, Araraquara.

In the first group (GTOL), rats ($n = 5$) treated with diluent (PEG 400:saline solution - 70:30, vol/vol) were exposed to TOL (99.9% pure) at 20 ppm (TLV-TWA) in a nose-only inhalation chamber for 6 h/day, 5 days/week for 6 weeks (Cardoso et al., 2015). For the 6-h exposure period, the rats did not have access to food or water. In the second group of animals (GTOL/DZP), 20 min prior to TOL exposure (as previously mentioned), 10 mg/kg DZP dissolved in diluent was administered by gavage to rats ($n = 5$).

In the third group (GDZP), rats ($n = 5$) were exposed to filtered air in a nose-only inhalation chamber (6 h/day, 5 days/week for 6 weeks). Additionally, 20 min before TOL exposure, 10 mg/kg DZP dissolved in diluent was administered by gavage. A control group with unexposed rats ($n = 5$) was treated with diluent and exposed to filtered air.

2.4. Nose-only inhalation exposure

The nose-only inhalation chamber consisted of two planes (top and bottom) with 24 ports each, as previously described (Cardoso et al., 2015). Before the exposures, the generation of the controlled atmosphere of TOL was validated by examining fluctuations in TOL concentration between doors (homogeneity) and for the 6-h exposure period (stability). Air samples from the inhalation chamber were collected using glass tubes packed with activated charcoal Anasorb CSC 100/50 mg (SKC®, Blandford Forum, UK). The TOL collected was desorbed from the activated charcoal with carbon disulfide. Isopropylbenzene was added as an internal standard (IS), and samples were analysed on a Perkin Elmer® Clarus 680 gas chromatograph (GC) (Massachusetts, USA) equipped with a flame ionization detector. The TOL samples were assayed on a 30 m × 0.25 mm × 1.4 μm film thickness Elite-624 capillary column (Perkin Elmer®). The column oven temperature was programmed at 60 °C for 1 min, then increased to 110 °C at a rate of 20 °C/min. Subsequently, the column temperature was raised to 180 °C at a rate of 10 °C/min, then maintained at 180 °C for 1 min. The injector