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Determination of 5-hydroxythiabendazole in human urine as a biomarker of exposure to thiabendazole using LC/MS/MS

Eva Ekman*, Moosa H. Faniband, Margareta Littorin, Margareta Maxe, Bo A.G. Jönsson, Christian H. Lindh

Division of Occupational and Environmental Medicine, Department of Laboratory Medicine, Lund University, SE-221 85 Lund, Sweden

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ABSTRACT

Thiabendazole (TBZ) is widely used as a pre-planting and post-harvest agricultural fungicide and as an anthelminthic in humans and animals. TBZ is of toxicological concern, since adverse effects including nephrogenic, hepatogenic, teratogenic and neurological effects have been reported in mammals. Occupational exposure can occur among agricultural workers and the general public may be environmentally exposed to TBZ through the diet. The metabolite 5-hydroxythiabendazole (5-OH-TBZ) was chosen as biomarker of exposure to TBZ and a LC/MS/MS method for the quantification of 5-OH-TBZ in human urine was developed. The method includes enzyme hydrolysis, as 5-OH-TBZ is conjugated to glucuronide and sulphate in urine. Sample through put was optimised using 96-well plates for sample handling as well as for solid phase extraction (SPE). The method has excellent, within-run, between-run and between-batch precision between 4 and 9%. The limit of detection (LOD) of 0.05 and a limit of quantification (LOQ) of 0.13 ng 5-OH-TBZ/mL urine enable detection in environmentally exposed populations. When applying the method in a general Swedish population, 52% had levels above LOD. The method was also applied in one oral and one dermal human experimental exposure study in two individuals. After oral exposure, the excretion of 5-OH-TBZ in urine was described by a two-compartment model and both the first rapid and the second slower elimination phase followed first-order kinetics, with estimated elimination half-life of 2 h and 9–12 h. The recoveries in urine were between 21 and 24% of the dose. Dermal exposure was described by a one compartment model and followed first order kinetics, with estimated elimination half-life of 9–18 h. The recovery in urine was 1% of the administrated dose of TBZ. Although these studies are limited to two individuals, the data provide new basic information regarding the toxicokinetics of TBZ after oral and dermal exposure.

1. Introduction

Thiabendazole (TBZ) was introduced in the 1960s first as an anthelmintic in humans and animals and later also as an agricultural fungicide. It is currently widely used as a fungicide, registered mainly for pre-planting and post-harvest treatment of vegetables (potatoes) and fruits.

Agricultural workers may be occupationally exposed to TBZ, but studies on exposure are missing. In the general public, there is a potential source of exposure by residues of TBZ in food [1]. Also, for some, medical treatment is an obvious source of exposure.

In general, mammalian toxicity is low [2]. However, humans treated with TBZ as an anthelmintic, have experienced adverse effects, like abdominal pain and nausea, dizziness and other cognitive complaints [3–5]. Serious effects such as liver diseases have also been reported [6] and in animal studies adverse kidney and liver effects, as well as teratogenic and reproductive toxicity at high doses [7–10]. TBZ is very toxic to aquatic organisms, and release of TBZ-containing waste water into the environment is prohibited within the EU [2].

In the risk assessment of pesticides, dermal as well as, oral and inhalation routes of exposure should be considered [11]. However, studies on dermal uptake of TBZ are missing, a shortcoming in view of safety control among agricultural workers. Further, there is a need for epidemiological studies of exposure–response relationships; in such studies an accurate exposure assessment is

Abbreviations: ADI, acceptable daily intake; b.w, body weight; CID, collision induced dissociation; IS, internal standard; LC/MS/MS, liquid chromatography triple quadrupole mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; QC, quality control; SPE, solid phase extraction; SRM, selected reaction monitoring; TBZ, thiabendazole; 5-OH-TBZ, 5-hydroxy thiabendazole.

* Corresponding author. Tel.: +46 46 2221645.
E-mail address: eva.ekman@med.lu.se (E. Ekman).

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required. Biomarkers have many advantages in comparison with other methods of exposure assessment, i.e., all routes of exposure are taken into account. However, for the interpretation of exposure data, basic knowledge of the metabolic fate is important. After human oral exposure to 1 g ^{14}C radiolabeled TBZ, 87% of the radioactive dose was recovered in urine [12]. Of the recovered dose, 38% of the metabolites was identified as conjugates of 5-hydroxythiabendazole (5-OH-TBZ) and less than 1% was found as TBZ or unconjugated 5-OH-TBZ. Thus, to measure the total 5-OH-TBZ in urine samples enzymatic hydrolysis prior to measurement is suggested.

A biomarker of exposure should be selective and validated [13]. Thus, reliable analytical methods are needed. Methods for the analysis of TBZ and 5-OH-TBZ in serum, using liquid chromatography (LC) with fluorescence detection [14], and in urine, using LC-UV have been reported [15]. However, the limit of detection, and selectivity in these methods are not sufficent for the use in studies of environmental exposure. No mass spectrometry based methods have been presented previously.

The aim of this study was to develop an analytical method for the quantification of total 5-OH-TBZ as a biomarker of exposure to TBZ in human urine using LC/MS/MS. The method was applied in one oral and one dermal human experimental exposure pilot study in two individuals to validate 5-OH-TBZ as biomarker of exposure and to estimate some basic toxicokinetic data for TBZ. The method was also applied in samples from a Swedish general population.

2. Materials and method

2.1. Chemicals and materials

The standards 5-OH-TBZ (10 ng/μL in methanol) and TBZ were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Acetone (analytical grade ACS Reag Ph Eur) and acetic acid (glacial) were from Fisher Scientific (Loughborough, UK). Methanol and acetonitrile (hyper grade for LC-MS), ammoniumacetate (EMSURE ACS, Reag. Ph Eur), and ammonia (25%) (NH_3) were from Merck (Darmstadt, Germany) and the enzyme β-glucuronidase/arylsulfatase from *Helix pomatia* and β-glucuronidase from *E. coli* from Roche Diagnostics Scandinavia AB (Bromma, Sweden).

Formic acid (FA) was from Sigma–Aldrich Inc. (St. Louis, MO, USA). The IS [$^{13}C_2$, ^{15}N] 5-OH-TBZ was purchased from Toronto Research Chemicals (North York, ON, Canada). Purified water from a Millie-Q Integral 5 system (Millipore, Billerica, MA, USA) was used.

Polypropylene (PP) Riplate® Squarwell (SW) 2 mL 96-well-plates from Ritter (Schwabmünchen, Germany) were used and sealed during long term storage in −20 °C with an airtight sealing mat, 96 square well from Kinesis (Cambridgeshire, UK) or, for HPLC analysis, sealed with hard plastic ISOLUTE® pierceable sealing capmat for leak proof closure. The different cap mats are needed to prevent NH_3 to evaporate. Solid phase extraction (SPE) column plate, ISOLUTE®-96 ENV+ 50 mg fixed well plate, was from Biotage (Uppsala, Sweden).

2.2. Instrumentation

Quantitative analysis was conducted using a triple quadrupole linear ion trap mass spectrometer, equipped with TurboIonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system with two pumps (UFLC^{RX}, Shimadzu Corporation, Kyoto, Japan). Pure nitrogen was used as curtain gas and collision gas. Air was used as nebuliser and auxiliary gas. The temperature of the auxiliary gas was set at 650 °C and the ion spray voltage at 5500 V. The MS/MS analyses were carried out using selected reaction monitoring (SRM) in positive ionisation mode. To establish the appropriate SRM conditions, standard solutions were infused into the MS/MS for optimisation. Collision-induced dissociation (CID) of each $[M+H]^+$ was performed and the product ions giving the best signal to noise ratio were selected for the SRM analysis. All data acquisition and processing was performed using the Analyst 1.6.1 application software (Applied Biosystems, Foster City, CA, USA).

The solutions used for SPE extraction were added with a 96-multichannel pipette (Liquidator 96, Rainin pipetting 360°) from Rainin Instruments LLC, Mettler-Toledo International Inc., Greifensee, Switzerland. To accelerate the flow of the liquid through the SPE columns, positive pressure processing of the SPE-plates using nitrogen together with a multichannel pressure processor (CEREX 96 II multi-channel SPE) SPEware Corporation, Baldwin Park, CA, USA was used.

2.3. Preparation of calibration standards and quality control samples

The 5-OH-TBZ standard was purchased dissolved. Accurately weighed amount of IS [$^{13}C_2$, ^{15}N] 5-OH-TBZ was dissolved in methanol. The IS and standard stock solutions were diluted further in methanol and stored at −20 °C. Standard solutions were prepared in duplicates. For the calibration curve, 475 μL blank urine was spiked with 25 μL of the standard solutions and 25 μL of the IS solution, giving a urinary concentration between 0.05 and 100 ng 5-OH-TBZ/mL and 5 ng IS-5-OH-TBZ/mL urine. The calibration curve was corrected with the amount found in the chemical blank prepared from Millie-Q water and thereafter treated like the other samples. Urine blank samples and quality control (QC) samples were obtained from healthy volunteers at our laboratory. Blank urine samples were used for preparation of calibration curves and zero samples. As QC-samples, two authentic urine samples were pooled and then quantified to urinary concentrations 0.1, 1.0, 8.0 and 15 ng 5-OH-TBZ/mL urine. The QC-samples were divided into aliquots before stored at −20 °C.

2.4. Sample preparation

The urine samples and QC-samples were vortex-mixed after thawing and aliquots of 500 μL were transferred into a 96-well-plate and then 25 μL of IS solution, 150 μL 1 M ammonium acetate buffer pH 6.5 and 10 μL β-glucuronidase/arylsulphatase obtained from *H. pomatia* was added. The plate was sealed and mixed thoroughly for about 1 min before incubation. The enzyme incubation was performed at 37 °C with agitation at 400 rpm for about 18 h, i.e. overnight. After incubation, the samples were carefully mixed and transferred to a conditioned 96-SPE-plate using the Liquidator. The SPE-plate was conditioned in two steps with 1 mL of methanol and 1 mL of water. After the samples were applied, they were washed in three steps with 1 mL of water, 1 mL 40% methanol with 1% acetic acid and 1 mL acetonitrile. To elute 5-OH-TBZ and TBZ, 1 mL acetonitrile containing 5% NH_3 was manually added. The samples were eluted into a freeze-cooled 96-well-plate. Thus, the evaporation was minimised. The samples were gently mixed for 30 s and centrifuged for 10 min at 3000 × *g* immediately before analysis. The plate was stored in −20 °C if not analysed directly. If a sample concentration was above the linear range, a volume of 0.5 mL urine was diluted with Millie-Q water until an appropriate level was reached.

2.5. Analysis

The separation of the analytes was carried out, using a Poroshell 120EC-C18 column (4.6 × 233 mm, 2.7 μm, Agilent Technologies, Santa Clara, CA, USA). The two mobile phases used consisted of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v)