



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

ARTICLE INFO

Article history:

Received 9 May 2014

Accepted 4 October 2014

Available online 14 October 2014

Keywords:

Biomarker

Dermal

LC/MS/MS

Oral

Pesticide

Thiabendazole

ABSTRACT

Thiabendazole (TBZ) is widely used as a pre-planting and post-harvest agricultural fungicide and as an anthelmintic 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 administered 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.

© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/3.0/>).

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).

<http://dx.doi.org/10.1016/j.jchromb.2014.10.003>

1570-0232/© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/3.0/>).

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-hydroxythiabenzazole (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 sufficient 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 [$^{13}\text{C}_2$, ^{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) Riplat[®] 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 TurbolonSpray source (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system with two pumps (UFLC[®], 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 $[\text{M}+\text{H}]^+$ 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 [$^{13}\text{C}_2$, ^{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/arylsulfatase 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 $3000 \times g$ 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, $2.7 \mu\text{m}$, 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)

Table 1
Summary of the SRM transitions for 5-OH-TBZ and internal standard used in the LC/MS/MS analysis.

| Compound | Transitions (Da) | Collision energy (V) |
|---|------------------|----------------------|
| <i>Quantifier ions</i> | | |
| 5-OH-TBZ | 218.0 → 191.2 | 36 |
| [¹³ C ₂][¹⁵ N] 5-OH-TBZ | 221.0 → 194.0 | 35 |
| <i>Qualifier ions</i> | | |
| 5-OH-TBZ | 218.0 → 147.0 | 45 |
| [¹³ C ₂][¹⁵ N] 5-OH-TBZ | 221.0 → 150.0 | 45 |

formic acid in methanol (mobile phase B). An aliquot of 3.0 µL of the sample was injected on the column. The separation started with isocratic elution with 30% of mobile phase B for 1.0 min followed by a linear gradient of mobile phase B to 84% in 2.5 min, during which the analytes eluted. The column was washed with 95% mobile phase B during 1.0 min and then equilibrated with 30% mobile phase B during 1.5 min. Post-column, the effluent was diverted into the MS between 1.0 and 4.0 min. The total analytical run time per sample, including equilibration time, was 6.5 min. The flow rate was 0.7 mL/min and the column was maintained at 40 °C. The LC/MS/MS analysis was performed using SRM transitions. The quantifier- and qualifier-ions for 5-OH-TBZ and the IS are tabulated in Table 1. All samples were prepared in duplicates and analysed by single injections. Concentrations were determined by peak area ratios between analyte and IS. All values were corrected for the chemical blank.

2.6. Test of deconjugation by enzyme hydrolysis

The enzymes β-glucuronidase/arylsulfatase and β-glucuronidase were tested regarding hydrolysis of the 5-OH-TBZ conjugates. The described analytical method was used to analyse twenty authentic samples, ten from each volunteer participating in the oral and dermal exposure studies described below, either with 10 µL β-glucuronidase/arylsulfatase or with 20 µL of the β-glucuronidase enzyme. The concentration of 5-OH-TBZ was determined for each sample. The ratios were then calculated.

To determine an appropriate time for enzyme incubation, a pooled authentic urine sample was used: from each volunteer and study one sample was pooled. Aliquots from this sample were prepared in duplicates for nine time points between 0 and 48 h. The incubation starting point was scheduled for each aliquot so that enzyme incubation was completed at the same time, for all aliquots, and SPE sample preparation was performed for the aliquots together. The zero-sample aliquot was added with enzyme just before it was applied to the SPE-column. Then, sample preparation and analysis were performed according to the method. The standards and QC-samples were incubated for 18 h.

2.7. Validation of the analytical method

To assess limit of detection (LOD) and limit of quantification (LOQ) in urine matrix, seven blank authentic urine samples were used. LOD and LOQ were determined as the mean level of the peak area concentration at the same retention time as 5-OH-TBZ, plus three and ten times the standard deviation, respectively [16].

The linear range of the calibration curve was determined from ten concentration levels 0.1, 0.3, 0.5, 1.3, 2.5, 5.0, 10, 20, 50 and 100 ng 5-OH-TBZ/mL urine. The equation of the curve was calculated by linear regression and the correlation coefficient (*r*) was used as a measure of the fit of the curve.

The precision of the method was determined using three different approaches, within-run, between-run and between-batch precision. The precision was calculated as the coefficient of variation (CV) of repeated measurements. The within-run precision was

obtained from spiked urine at three concentration levels 0.1, 5.0 and 50 ng 5-OH-TBZ/mL urine. Each level was spiked in ten consecutive samples and prepared in one sample batch during one day. The mean concentrations at the three concentration levels were calculated.

The between-run precision was determined by including duplicates of the four QC-samples, containing 0.1, 1.0, 8.0 and 15 ng 5-OH-TBZ/mL urine, in seven analytical sample batches. The QC-samples are authentic urine samples. The batches were prepared and analysed on separate days during a period of four months. Thus, the standard deviation was calculated from fourteen individual results.

A between-batch precision of the method was determined by analysis of 110 urine samples collected in the dermal and oral exposure studies (see below). Each urine sample was divided into two aliquots which were then subjected to the entire analytical procedure. The samples were prepared and analysed in separate analytical batches and on different days. The CV was calculated from the differences between the duplicate samples as previously described [17].

The signal of the IS was used to determine the matrix effect. The ratio between the mean peak area of the IS in the chemical blanks (*n* = 6) and the mean peak area of the IS in authentic urine samples (*n* = 80) in an analytical batch was calculated. The geometric mean of the matrix ratio was calculated for two analytical batches.

The recovery of 5-OH-TBZ after SPE extraction was determined by comparing obtained concentrations in two sets of blank urine samples, spiked to a final concentration of 20 ng 5-OH-TBZ/mL. One set of samples was spiked before applied to the SPE columns. The other set of samples were also applied to the SPE columns but spiked after they had eluted. Addition of IS was made to all samples after they were eluted from the SPE columns. The urine samples spiked after eluting from the SPE columns were set as 100%. The recovery was calculated for three spiked blank urine samples.

The stability of the 5-OH-TBZ standard solutions in methanol was investigated by reanalysis after storage at –20 °C for 12 months and the authentic samples after storage at –20 °C for 5 months.

2.8. Ethical approval

These studies and the investigation of environmental exposure to TBZ in the general population in Sweden were ethically approved by The Regional Ethical Review Board in Lund, Lund University, Sweden (Dnr463/2005; Dnr2010/41; Dnr2010/465 and Dnr2013/6).

2.9. Human exposure studies

Two healthy volunteers, one female (age 67; weight 57 kg) and one male (age 42; weight 75 kg) had given their written informed consent to participate in the experimental studies. They minimised the intake of conventionally grown food a few days before as well as during the study.

2.9.1. Oral exposure study

The two healthy volunteers received one single oral dose corresponding to 50% of the accepted daily intake (ADI) of TBZ. The ADI for TBZ suggested by the European Union is 0.1 mg/kg/day [18]. The TBZ was dissolved in acetone to a concentration of 2.5 mg/mL and was then added to 250 mL organic orange juice. The final amounts of TBZ in the orange juice were 2.85 mg and 3.75 mg for the female and male, respectively. The first urine sample was collected immediately prior to the exposure. After exposure urine was voided in 1 h intervals for the first 8 h. Then, all urine voided, ad libitum for 4 days was collected.

2.9.2. Dermal exposure study

The dermal study was performed twelve weeks before the oral study. TBZ was administrated topically on an area of 75 cm² of the inner forearm of the two volunteers. The dose of TBZ corresponded to 30% and 25% of the ADI. An accurately weighed amount was dissolved in 6 mL of acetone and a volume of 600 μL containing 1.75 mg was administrated to the female and 640 μL containing 1.88 mg to the male volunteer. After administration, the vehicle evaporated to dryness and then the skin was occluded with aluminium foil. After 8 h of exposure, the remainder of the dose was wiped off with acetone, and the exposed area washed with soap and water. The first urine sample was collected immediately prior to the exposure. After exposure urine was voided in 2 h intervals for the first 12 h. Then, all urine voided, ad libitum for 6–7 days was collected.

2.10. Sample collection and adjustments for urinary dilution

The samples collected in the two studies were stored at –20 °C until analysis. Sample volume was determined and creatinine and density were determined in all samples to adjust for the urinary dilution. Creatinine was analysed with an enzymatic method [19] and density with a hand refractometer. The concentration adjusted for urinary density, C_d , was calculated according to $C_d = C(\text{observed}) \times (1.016 - 1) / (\rho - 1)$, where $C(\text{observed})$ is the obtained concentration in the urine sample, ρ is the measured specific density and 1.016 was used as the average reference urine density [20].

2.11. Calculation of half-lives

The half-life ($t_{1/2}$) of the elimination in urine was estimated from the slope of the curve in the natural logarithm linear (ln-linear) concentration versus time plot, where time is given as the mid time points between two sample collection time points.

2.12. Application of the analytical method in an environmental exposure study

Spot urine samples ($n = 285$) were collected from a general population in Sweden and the analytical method presented above was applied to determine 5-OH-TBZ. The population consisted mainly of men and women, living and working in cities or the countryside in the south of Sweden.

3. Results and discussion

3.1. Mass spectrometry and chromatography

A method for the analysis of 5-OH-TBZ in human urine using SPE and LC/MS/MS was developed. A large number of samples can efficiently be analysed with the analytical method, due to the use of 96-well plates throughout the sample preparation and LC/MS/MS analysis. The chosen analytical column provided consistent and reproducible chromatography and retained 5-OH-TBZ with stable retention times and good separation (supplementary data Fig. S1). Both 5-OH-TBZ and the IS showed an excellent sensitivity in ESI and positive ion mode. The transition giving the best to noise ratio signal was chosen as the quantifier ion for 5-OH-TBZ. A second SRM was chosen as a qualifier ion to strengthen the identity of the analyte. The transitions and optimum collision energies are tabulated in Table 1.

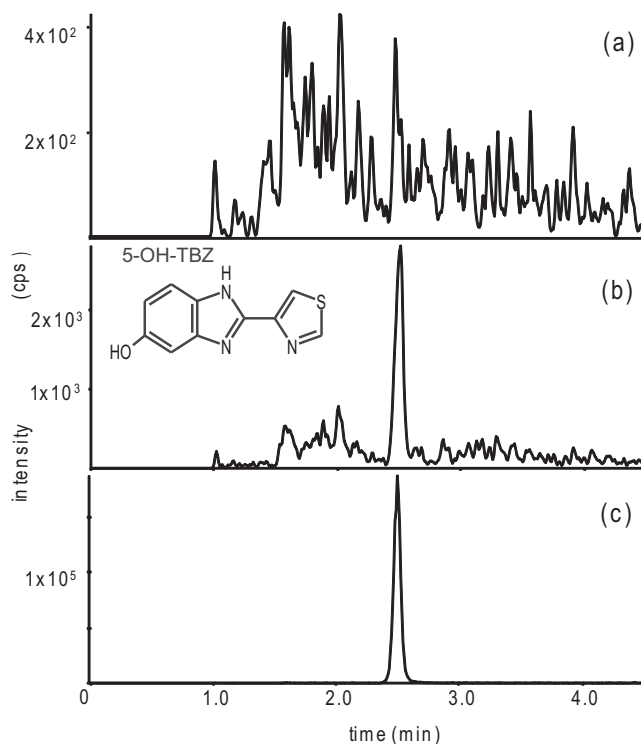


Fig. 1. LC/MS/MS SRM chromatogram showing (a) urinary blank, transition 218.0–191.2 Da (b) LOD in a urine sample spiked to a of 0.05 ng 5-OH-TBZ/mL urine, transition 218.0–191.2 Da. (c) Internal standard spiked to 5 ng/mL urine, transition 221.0–194.0 Da. Retention time of 5-OH-TBZ and IS is 5.2 min.

3.2. Deconjugation by enzyme hydrolysis

When the enzymes β -glucuronidase/arylsulfatase and β -glucuronidase were tested regarding hydrolysis of the 5-OH-TBZ conjugates, higher concentrations of 5-OH-TBZ were obtained when β -glucuronidase/arylsulfatase was used to hydrolyse the authentic urine samples. The concentrations increased about four times and almost two times in urine samples from the female and male volunteer, respectively (supplementary data Table S1). There seems to be differences between the two subjects regarding the level of conjugation; these results agree with earlier findings [12].

The optimal time for enzyme incubation was found to be 18 h (supplementary data Fig. S2).

3.3. Validation of the analytical method

The LOD was found to be 0.05 ng/mL and the LOQ 0.13 ng/mL. Chromatograms of a urine blank (a), a urine sample at LOD (b), and of the IS (c) are shown in Fig. 1. The LOD in urine is sufficiently low for the measurement of environmental human exposure.

The obtained calibration curves in the range 0.13–100 ng 5-OH-TBZ/mL urine showed excellent linearity with $r = 0.997$, a slope of 0.278 ± 0.015 (CV of 6.9%) and an intercept of 0.0395 ± 0.046 (CV of 14.7%) at 95% confidence level ($n = 6$).

The within-run, between-run and between-batch precisions are presented in Table 2.

The obtained concentrations at three concentration levels for spiked samples are shown in Table 2.

The assessment of the matrix effect was performed using [¹³C, ¹⁵N₂] 5-OH-TBZ which co-elutes with 5-OH-TBZ [21]. The geometric mean of the ratio between the IS in the chemical blanks and the IS in authentic urine samples was 0.99. The minor matrix effect probably is explained by the use of SPE, the use of a [¹³C, ¹⁵N₂] IS

Table 2

Precision of the method at different concentration levels. The within-run precision was calculated from spiked urine samples and the between-run and batch precision from authentic urine samples.

| | 5-OH-TBZ concentration (ng/mL) | n | Mean obtained 5-OH-TBZ concentration (ng/mL) | CV (%) |
|-------------------------|--------------------------------|-----|--|--------|
| Within-run precision | 0.13 | 10 | 0.14 | 7.0 |
| | 5.0 | 10 | 5.0 | 5.0 |
| | 50 | 10 | 47 | 3.6 |
| Between-run precision | 0.1 | 14 | 0.2 | 7.8 |
| | 1.0 | 14 | 1.2 | 7.7 |
| | 8.0 | 14 | 7.9 | 7.5 |
| | 15 | 14 | 16 | 6.8 |
| Between batch precision | Range 0.1–64 | 110 | 8.2 | 8.6 |

Table 3

Estimated half-life of 5-OH-TBZ and conjugates in urine of the two volunteers orally exposed to TBZ. The dose was 0.05 mg TBZ/kg of b.w (50% of ADI) for both volunteers. For the calculation values after 101 h for the female and 90 h for the male were excluded due to levels below LOD.

| Oral route | ^{a,b} $t_{1/2}$ (h) creatinine | | ^{a,b} $t_{1/2}$ (h) density | | ^{a,b} $t_{1/2}$ (h) unadjusted | | ^c C_{\max} (nmol/mmol creatinine) | $T_{C_{\max}}$ (h) |
|------------|---|---------|--------------------------------------|---------|---|---------|--|--------------------|
| | Phase 1 | Phase 2 | Phase 1 | Phase 2 | Phase 1 | Phase 2 | | |
| Female | 2 | 12 | 2 | 12 | 2 | 12 | 2385 | 1 |
| Male | 2 | 9 | 1 | 9 | 1 | 9 | 1729 | 1 |

^a The half-life of elimination ($t_{1/2}$) in urine estimated from the slope of the curve in the natural logarithm-linear concentration versus mid time plot.

^b Correlation coefficient (r) for the regression line of adjusted $t_{1/2}$ were 0.94–0.98 and 0.90–0.94 for unadjusted.

^c C_{\max} urinary concentration creatinine adjusted values.

and the analytical column, giving a good separation of the analyte from the matrix.

The recovery in spiked urine determined after SPE elution was found to be 100.3% ($n = 3$).

The standard solutions of 5-OH-TBZ dissolved in methanol were found to be stable for 12 months and the authentic urine samples for at least 5 months when stored at -20°C .

3.4. Oral exposure to TBZ

In urine collected before the exposure, the 5-OH-TBZ levels were below LOD in both volunteers. After the oral exposure to TBZ, the urinary levels of 5-OH-TBZ increased rapidly and the urinary C_{\max} was reached after 1 h, see Table 3 and Fig. 2a. The urinary elimination can be described by a two-compartment model and both the first rapid and the second slower elimination phase followed first-order kinetics; see the excretion curve in Fig. 3a. The half-lives of 5-OH-TBZ with correlation coefficients are shown in Table 3. The first rapid phase was found to be only 2 h. This

short half-life of 5-OH-TBZ (Table 3) limits its use as a biomarker and when collecting samples for biomonitoring, the toxicokinetics should be considered [22].

In chromatograms from the oral study, two small peaks with the same transitions as 5-OH-TBZ eluting close to the 5-OH-TBZ peak was observed (supplementary data Fig. S1). Thus, samples were screened for other metabolites using a compound-specific, predicted SRM/Information-Dependent Acquisition method (supplementary data Figs. S3 and S4).

The main metabolite was 5-OH-TBZ with glucuronide and sulphate conjugation. Other minor metabolites were OH-TBZ isomers, di-hydroxy-TBZ and TBZ. The metabolic specificity was confirmed since the concentrations increased after exposure to TBZ and decreased as the metabolites were rapidly excreted in the urine (supplementary data Fig. S3).

The amount of TBZ recovered in the study samples, was quantified and found to be less than 0.05% with regard to the administered dose in both volunteers. Hence, TBZ was not included in the validation of the method.

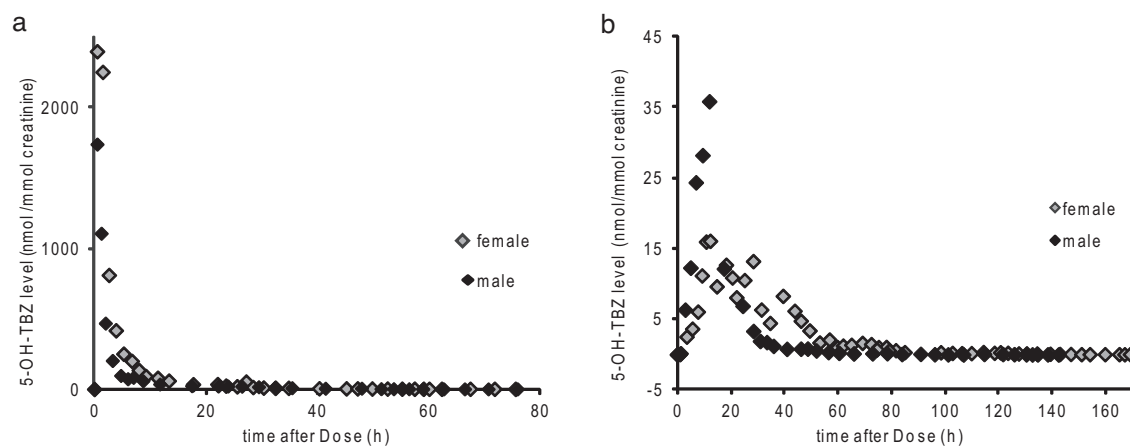


Fig. 2. The concentration versus time urinary excretion curves of 5-OH-TBZ for the female and male volunteer after oral (a) and dermal (b) exposure to TBZ. The creatinine adjusted urinary levels (nmol/mmol creatinine) were plotted versus the mid time points. (a) The oral dose corresponded to 50% of the accepted daily intake (ADI) mixed in 250 mL of organic orange juice. In both studies the first two urine samples were collected just before and 1 h after administration of the dose. (b) The volunteers were dermally exposed on the inner forearm for 8 h with a dose corresponding to 25% of the ADI (0.1 mg TBZ/kg b.w.).

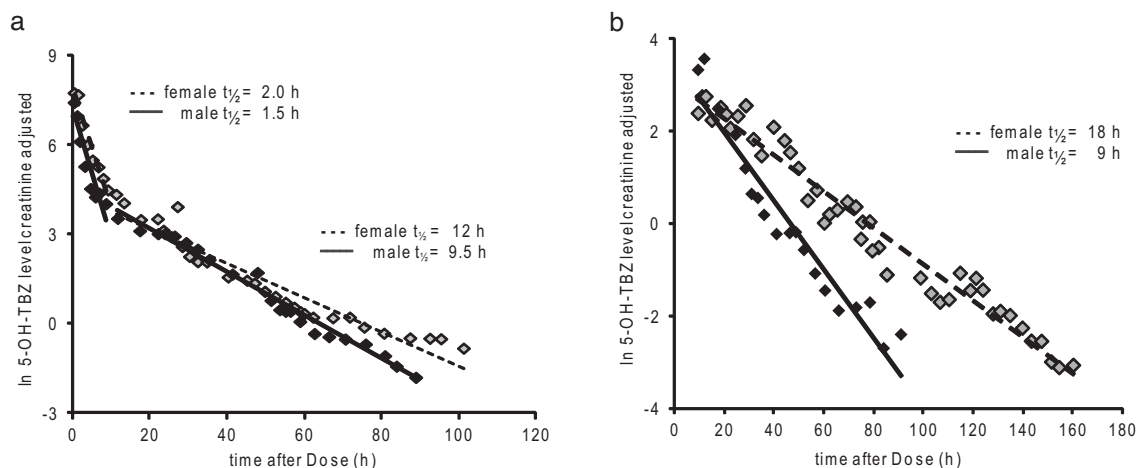


Fig. 3. Semi logarithmic urinary elimination curves of 5-OH-TBZ for the female and male volunteer after oral (a) and dermal (b) exposure to TBZ. The oral dose corresponded to 50% of the accepted daily intake (ADI) (0.1 mg TBZ/kg b.w.) and the dermal dose corresponded to 25% of the ADI. The half-life of elimination ($t_{1/2}$) in urine is estimated from the slope of the log-lin concentration versus time plot. The natural logarithm of the creatinine adjusted urinary levels (nmol/mmol creatinine) were plotted versus the mid time points.

In urine, the total recovery, measured as 5-OH-TBZ, was found to be 21–24% of the dose of TBZ. Of the recovered dose, 96% was found within 24 h. In an earlier study of oral exposure to ^{14}C -TBZ in human volunteers, 87% of the radioactivity was found in urine, of which 38% was recovered as 5-OH-TBZ conjugates, after 5 days [12]. The difference in uptake of TBZ may be due to the difference in doses administered (3 mg versus 1 g) or that the dosage form (solution versus suspension) to administrate the substance, differed. An oral study in mice has shown that the vehicle has great influence on the uptake of TBZ. When the mice were given ^{14}C ring radiolabeled TBZ in olive oil, instead of aqueous solution, TBZ was absorbed 12 times faster and the plasma levels became 5 times higher [23]. The solubility of TBZ may be the dependent factor. Moreover, metabolites not found by the analytical method are likely to be formed; since the benzene ring in TBZ can undergo ring cleavage and, sulfoxidation has also been suggested [12].

3.5. Dermal exposure to TBZ

Before the experimental exposure, the 5-OH-TBZ levels in urine were below LOD in both volunteers. The limited solubility of TBZ in acetone and the limited volume possible to disperse evenly on the application site resulted in a lower dose than in the oral study above. After the dermal exposure of TBZ, the urinary levels increased slowly. The urinary C_{max} was reached after 13 h in both volunteers compared to 1 h by the oral route (Table 4). After 9 h, the level of 5-OH-TBZ in the female urine reached a balance between absorption and elimination, followed by a plateau (Fig. 2b). No plateau was seen in the male volunteer. The urinary elimination of 5-OH-TBZ seemed to follow first-order kinetics and a one-compartment model (Fig. 3b). Also, in other studies of dermal

exposure to pesticides in humans, a plateau in the urinary excretion curve has been observed [24–29]. After 13 h, the levels started to decrease in both volunteers, probably reflecting the end of exposure. If there is a first rapid elimination phase it was not observed, probably obscured by the absorption phase. The dermal half-life is within the same range as that of the second slower phase in the oral study (Tables 3 and 4).

Of the administered dermal dose, the total recovery of 5-OH-TBZ in urine was 1% for both volunteers. The results indicate a low uptake of TBZ. Acetone was chosen as vehicle, because of its ability to dissolve TBZ and because it is commonly used in dermal experimental exposure studies. The choice of vehicle and use of occlusion may affect the absorption. The permeability of the skin can be increased by occlusion through increased hydration and temperature of the skin. Also the washing after exposure has been shown to enhance the dermal absorption [11]. The inner forearm was chosen as site of exposure as it is the most commonly used anatomic site in dermal experimental exposure studies [28,30]. However, it has been shown that the forehead and the neck are more permeable than the forearm. Thus, the absorption is probably higher, if other more permeable areas of the body are exposed [31]. This should be considered in studies of exposure and effect.

The toxicokinetic profiles were different between the oral and dermal administration routes. This may be because the uptake is both slower and lower in the dermal experiment. After dermal uptake, the substance enters the blood directly, i.e., the whole system, without passing the liver first, as it does when entering via the gastro intestinal tract. Moreover, the toxicokinetic profiles seemed to differ between the two individuals in the dermal study. This is not surprising, since it is well known that xenobiotic metabolism and excretion kinetics vary greatly depending on, i.e., age, sex, and activity level [11].

Table 4
Estimated half-life of 5-OH-TBZ and conjugates in urine for the two volunteers dermally exposed to TBZ for 8 h on an area of 75 cm². The dose was 0.030 and 0.025 mg TBZ/kg of b.w. (ca 25% of ADI 0.1 mg/kg of b.w.) for the female and male respectively. For the calculation values after 160 h for the female and 91 h for the male were excluded due to levels below LOD.

| Dermal route | ^{a,b} $t_{1/2}$ (h) creatinine | ^{a,b} $t_{1/2}$ (h) density | ^{a,b} $t_{1/2}$ (h) unadjusted | ^c C_{max} (nmol/mmol creatinine) | T_{Cmax} (h) |
|--------------|---|--------------------------------------|---|--|-----------------------|
| Female | 18 | 19 | 20 | 16 | 13 |
| Male | 9 | 9 | 10 | 17 | 13 |

^a The half-life of elimination ($t_{1/2}$) in urine estimated from the slope of the curve in the natural logarithm–linear concentration versus mid time plot.

^b Correlation coefficient (r) for the regression line of adjusted $t_{1/2}$ were 0.94–0.98 and 0.90–0.94 for unadjusted.

^c C_{max} urinary concentration creatinine adjusted values.

3.6. Adjustments for urinary dilution

The toxicokinetic data in this study indicate that an adjustment for the urinary dilution should be recommended and that adjustment for creatinine rather than for density should be applied. However, creatinine levels are affected by several factors (such as gender, age, muscle mass, and meat consumption) and when comparing individuals, groups or populations of both sexes and various ages, with large differences in muscle mass and meat intake, density adjustment may be the best choice.

3.7. Application of the analytical method in an environmental exposure study

Sample concentrations in the population samples ranged from <LOD to 33 ng 5-OH-TBZ/mL urine and 52% were above the LOD. The median was 0.05 ng/mL and the 90th percentile was 1.6 ng/mL.

The between-run precisions determined from QC-samples were CV=4.9% at 1 ng/mL ($n=21$) CV=3.9% at 5 ng/mL ($n=21$) and CV=2.7% at 10 ng/mL ($n=21$). The samples were prepared and analysed during 4 months. Thus, the method is applicable for running large sample batches. More detailed data from this study will be published elsewhere.

4. Conclusions

The metabolite 5-OH-TBZ was used as a biomarker of exposure to TBZ and a LC/MS/MS method for the quantification of 5-OH-TBZ in human urine was developed. The sample through put is high as all sample preparation is performed in 96-well plates.

The biomarker is conjugated to glucuronide and sulphates in urine. Thus enzyme hydrolysis using glucuronidase/arylsulfatase is suggested.

The method has excellent precision and a low LOD which enables detection of 5-OH-TBZ in environmentally exposed populations. When applying the method in a general Swedish population, 52% had concentration levels above LOD.

The method was applied in one oral and one dermal human experimental exposure study in two individuals. After oral exposure to TBZ, 21–24% was recovered as 5-OH-TBZ in urine, which was excreted in two phases with a rapid elimination half-life of 2 h and a slower of 9–12 h. Moreover, 96% of the recovered dose was found within 24 h. For a correct exposure assessment of TBZ, the fast elimination requires consideration of the time of sampling.

After dermal exposure to TBZ, 1% was recovered as 5-OH-TBZ in urine with an elimination half-life of 9–18 h. Although these studies are limited to two individuals, the data provide new information regarding the toxicokinetics of TBZ after oral and dermal exposure and will make the interpretation of the biomarker levels and, thus, risk assessment for TBZ easier.

Acknowledgements

This work was supported by the Swedish Environmental Protection, Agency; Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning; The Swedish Agency for International Development Cooperation; The Department for Research Cooperation; Swedish council for working life and social

research; Skåne Regional Council; and the Medical Faculty at Lund University, Sweden.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jchromb.2014.10.003>.

References

- [1] EU. Commission Regulation (EC) No 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I. Available: <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32008R0149:EN:NOT> (accessed 09.01.14).
- [2] EU. Review report for the active substance thiabendazole. Finalised in the Standing Committee on Plant Health at its meeting on 12 December 2000 in view of the inclusion of thiabendazole in Annex I of Directive 91/414/EEC, 7603/V1/97-final, European Commission, 2001.
- [3] G.C. Cook, Parasitol. Today 6 (1990) 133–136.
- [4] R. Portugal, R. Schaffel, L. Almeida, N. Spector, M. Nucci, Haematologica 87 (2002) 663–664.
- [5] Z. Bisoffi, D. Buonfrate, A. Angheben, M. Boscolo, M. Anselmi, S. Marocco, G. Monteiro, M. Gobbo, G. Bisoffi, F. Gobbi, PLoS Negl. Trop. Dis. 5 (2011) e1254.
- [6] É. Bion, É.A. Pariente, F. Maître, J. Hepatol. 23 (1995) 762–763.
- [7] Y. Tada, T. Fujitani, N. Yano, K. Yuzawa, A. Nagasawa, M. Yoneyama, Toxicology 162 (2001) 1–10.
- [8] A. Ogata, H. Ando, Y. Kubo, K. Hiraga, Food Chem. Toxicol. 22 (1984) 509–520.
- [9] Y. Tada, T. Fujitani, N. Yano, K. Yuzawa, A. Nagasawa, N. Aoki, A. Ogata, M. Yoneyama, Toxicology 169 (2001) 163–176.
- [10] G.R. Lankas, D.L. Wise, Food Chem. Toxicol. 31 (1993) 199–207.
- [11] M.A. Ngo, M. O'Malley, H.I. Maibach, J. Appl. Toxicol.: JAT 30 (2010) 91–114.
- [12] D.J. Tocco, C. Rosenblum, C.M. Martin, H.J. Robinson, Toxicol. Appl. Pharmacol. 9 (1966) 31–39.
- [13] M. Manno, C. Viau, J. Cocker, C. Colosio, L. Lowry, A. Mutti, M. Nordberg, S. Wang, Toxicol. Lett. 192 (2010) 3–16.
- [14] M.T. Watts, V.A. Raisys, L.A. Bauer, J. Chromatogr.: Biomed. Sci. Appl. 230 (1982) 79–86.
- [15] D.J. Tocco, R.P. Buhs, H.D. Brown, A.R. Matzuk, H.E. Mertel, R.E. Harman, N.R. Trenner, J. Med. Chem. 7 (1964) 399–405.
- [16] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, 5 edn., Pearson Education Limited, Harlow, UK, 2005.
- [17] C.H. Lindh, M. Littorin, G. Johannesson, B.A.G. Jönsson, Rapid Commun. Mass Spectrom. 22 (2008) 2573–2579.
- [18] EU. European Commission Health and Consumers, in: SANCO 3010 rev. 02/10/2007. Available: <http://ec.europa.eu/food/plant/protection/evaluation/stat.active.subs.3010.en.pdf> (accessed 08.01.14).
- [19] B.C. Mazzachi, M.J. Peake, V. Ehrhardt, Clin. Lab. 46 (2000) 53–55.
- [20] M.F. Boeniger, L.K. Lowry, J. Rosenberg, Am. Ind. Hyg. Assoc. J. 54 (1993).
- [21] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, J. Chromatogr. B 877 (2009) 2198–2207.
- [22] D. Paustenbach, D. Galbraith, Environ. Health Perspect. 114 (2006) 1143–1149.
- [23] M. Yoneyama, A. Ogata, T. Fujii, K. Hiraga, Food Chem. Toxicol.: Int. J. Publ. Br. Ind. Biol. Res. Assoc. 22 (1984) 731–735.
- [24] P. Griffin, H. Mason, K. Heywood, J. Cocker, Occup. Environ. Med. 56 (1999) 10–13.
- [25] S.J. Garfitt, K. Jones, H.J. Mason, J. Cocker, Toxicol. Lett. 134 (2002) 105–113.
- [26] S.J. Garfitt, K. Jones, H.J. Mason, J. Cocker, Toxicol. Lett. 134 (2002) 115–118.
- [27] W.J. Meuling, L.C. Ravensberg, L. Roza, J.J. van Hemmen, Int. Arch. Occup. Environ. Health 78 (2005) 44–50.
- [28] A. Berthet, M. Bouchard, D. Vernez, J. Appl. Toxicol. 32 (2012) 202–209.
- [29] E. Ekman, M. Maxe, M. Littorin, B.A.G. Jonsson, C.H. Lindh, J. Chromatogr. B – Anal. Technol. Biomed. Life Sci. 934 (2013) 53–59.
- [30] WHO, JMPR, IPCS, Inventory of IPCS and other WHO pesticide evaluations and summary of toxicological evaluations performed by the Joint Meeting on Pesticide Residues (JMPR) through 2010, http://www.who.int/foodsafety/chem/jmpr/publications/jmpr_pesticide/en/index.html (accessed 30.11.12), 10 edn., World Health Organisation (WHO), 2010.
- [31] R.A. Fenske, Scand. J. Work Environ. Health 31 (Suppl. 1) (2005) 67–73, discussion 63–65.