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Characterization of inter-tissue and inter-strain variability of TCE glutathione conjugation metabolites DCVG, DCVC, and NAcDCVC in the mouse

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Abstract

Trichloroethylene (TCE) is a ubiquitous environmental toxicant that is a liver and kidney carcinogen. Conjugation of TCE with glutathione (GSH) leads to formation of nephrotoxic and mutagenic metabolites postulated to be critical for kidney cancer development; however, relatively little is known regarding their tissue levels as previous analytical methods for their detection lacked sensitivity. Here, an LC-MS/MS-based method for simultaneous detection of S-(1,2-dichlorovinyl)-glutathione (DCVG), S-(1,2-dichlorovinyl)-L-cysteine (DCVC), and N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NAcDCVC) in multiple mouse tissues was developed. This analytical method is rapid, sensitive (limits of detection (LOD) 3–30 fmol across metabolites and tissues), and robust to quantify all three metabolites in liver, kidneys and serum. The method was used to characterize inter-tissue and inter-strain variability in formation of conjugative metabolites of TCE. Single oral dose of TCE (24, 240 or 800 mg/kg) was administered to male mice from 20 inbred strains of Collaborative Cross. Inter-strain variability in the levels of DCVG, DCVC, and NAcDCVC (GSD=1.6–2.9) was observed. Whereas NAcDCVC was distributed equally among analyzed tissues, highest levels of DCVG were detected in liver and DCVC in kidneys. Evidence indicated that inter-strain variability in conjugative metabolite formation of TCE might affect susceptibility to adverse health effects and that this method might aid in filling data gaps in human health assessment of TCE.

Keywords

Trichloroethylene (TCE); S-(1,2-dichlorovinyl)-glutathione (DCVG); S-(1,2-dichlorovinyl)-L-cysteine (DCVC); N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NAcDCVC); LC-MS/MS; inter-individual variability

Introduction

Trichloroethylene (TCE), a ubiquitous environmental toxicant, was shown to produce liver cancer in mice and kidney cancer in rats and humans (IARC, 2014; U.S. EPA, 2011b).

Trichloroethylene remains a top 10 priority chemical of concern for human health (U.S.

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EPA, 2017). Upon absorption, TCE is metabolized through cytochrome P450 oxidation and glutathione (GSH) conjugation pathways in both rodents and humans (Lash et al., 2000). Glutathione conjugation of TCE (Figure 1) occurs primarily via glutathione-S-transferases (GSTs) in liver to form DCVG. DCVG might undergo metabolism via liver or kidney gamma-glutamyl transferase and di-peptidase to generate DCVC, which is then further transformed into NAcDCVC via *N*-acetyl transferase. In addition, NAcDCVC may be deacetylated by acylase to regenerate DCVC. Aside from the *N*-acetylation, DCVC might be further metabolized by cysteine conjugate β -lyase to form reactive thioketenes, or undergo sulfoxidation by flavin-containing monooxygenase to form a reactive DCVC-sulfoxide (Lash et al., 2001b; Sausen & Elfarra, 1990).

Metabolites that are generated through both oxidation and GSH conjugation pathways are thought to be involved in organ-specific toxicity of TCE (Cichocki et al., 2016; Lash et al., 2014). While the effects of oxidative metabolites of TCE have been widely studied (Rusyn et al., 2014; Corton et al., 2014), the metabolites generated by GSH conjugation pathway received less attention attributed to low abundance and lack of sensitive analytical methods (Chiu et al., 2009). However, these metabolites are believed to be the cause of kidney cancer in rats and humans (Guha et al., 2012; Chiu et al., 2013). Glutathione conjugation metabolites of TCE are cytotoxic *in vitro* and nephrotoxic *in vivo* as well as mutagenic in bacterial and mammalian model systems (Irving & Elfarra, 2013; Lash et al., 2001a; 2001b; 2007; 2014; Chiu et al., 2013; Guha et al., 2012; Dekant, 2003). Still, the relationship between tissue levels of GSH conjugation metabolites of TCE and cancer remains unclear (Yoo et al., 2015b; Green et al., 1997).

Previous studies examined the flux of TCE through GSH conjugation pathway in tissues of TCE-exposed animals and humans (Lash et al., 1999; 2006; Kim et al., 2009a; Bloemen et al., 2001; Yoo et al., 2015b; 2015a). The analytical techniques ranged from LC-UV, to GC-MS and LC-MS/MS, and limits of detection (LOD) varied from 25 to 50,000 fmoles for DCVG and DCVC. One study reported a method for NAcDCVC with a LOD of 80 fmoles (Bloemen et al., 2001). Most sensitive was the LC-MS/MS-based method for analysis of DCVG and DCVC in mouse tissues (Kim et al., 2009a; Yoo et al., 2015a; 2015b); however, extraction protocols inconsistency confounded cross-tissue comparisons.

Because of the complexity of metabolism-related effects of TCE, inter-individual variability in pharmacokinetics may play a major role in the susceptibility to toxicity and tissue specificity (Chiu et al., 2006). Previously Bradford et al (2011) in mice showed considerable inter-strain variability in DCVG and DCVC in the liver; there was a greater than 10-fold inter-strain variability in flux of TCE metabolism through GSH conjugation (Chiu et al., 2014). This study aimed to further characterize inter-individual variability in TCE metabolism through GSH conjugation, and to extend the quantitative estimates to other tissues. To achieve this, a sensitive analytical method for simultaneous detection of DCVG, DCVC, and NAcDCVC in various tissues was developed using ultra-high performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry (LC-MS/MS). Further, inter-strain variability in levels of TCE conjugation metabolites in various tissues was characterized using 20 inbred strains of Collaborative Cross mice.

Materials and Methods

Chemicals

S-(1,2-dichlorovinyl)-glutathione (DCVG, purity: 98.9%), 2-amino-5-[(2-((¹³C)carboxy(¹³C)methyl)(¹⁵N)amino)-1-([1,2-dichloroethenyl]sulfanyl)-2-oxoethyl)amino]-5-oxopentanoic acid (DCVG*, purity: 94.4%, isotopic enrichment: 99.0%), S-(1,2-dichlorovinyl)-L-cysteine (DCVC, purity: 98.4%), and 2-(¹⁵N)amino-3-([1,2-dichloroethenyl]sulfanyl)(¹³C₃)propanoic acid (DCVC*, purity: 96.9%, isotopic enrichment: 99.0%) were obtained from TLC pharmaceutical standards (Vaughan, Canada). N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine (NAcDCVC, purity: 99.8%) and 3-([1,2-dichloroethenyl]sulfanyl)-2-[(1-¹³C, d₃)ethanoylamino]propanoic acid (NAcDCVC*, purity: 97.6%, isotopic enrichment: 99%) were purchased from Toronto Research Chemicals (Toronto, Canada). Acetic acid (>99.7%), TCE (>99.5%), and chloroform (>99.9%) were obtained from Sigma-Aldrich (St. Louis, MO). Methanol (>99.9%) was purchased from Fischer Chemicals (Fair Lawn, NJ). De-ionized water was generated by Arium®Pro Ultrapure Water System (Goettingen, Germany).

Animals and treatments

Two animal studies were conducted, both used male mice since TCE is more efficiently metabolized in male, as compared to female, mice (National Toxicology Program, 1990). The first aim was to demonstrate application of the validated LC-MS/MS method in an *in vivo* study. Second was to characterize inter-individual variability of tissue levels of DCVG, DCVC, and NAcDCVC using a panel of mouse strains. All animal treatments and procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University and the University of North Carolina at Chapel Hill.

First study was conducted in male B6C3F1/J mice (6–8 weeks old) obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in standard polycarbonate shoebox mouse cages with air filtration system on a 12:12 hr light/dark cycle with free access to food (standard laboratory mouse chow) and filtered tap water. After a week-long acclimation, mice (n=3/group) were dosed with 0, 24, 240 or 800 mg/kg of TCE (5 ml/kg in 5% alkamus-EL620 in saline) via oral gavage. The aqueous emulsion vehicle was used to aid oral absorption of TCE (Lee et al., 1996; 2000). Dose range was selected based upon previous acute and sub-chronic mouse studies showing that these amounts are well-tolerated; doses also corresponded to the range used in both 90-day and 2-year mouse studies (Yoo et al., 2015b; Buben & O'Flaherty, 1985; National Toxicology Program, 1990). It is also noted that mice form approximately 100 fold less GSH conjugation pathway metabolites of TCE as compared to humans (Chiu et al., 2009) which necessitated the use of doses that are higher than those expected in human exposures.

Second study was conducted in male mice (8–12 weeks old) from 20 Collaborative Cross strains. The Collaborative Cross (CC) is a panel of inbred mouse strains that were recently developed through a community effort (Threadgill & Churchill, 2012). The CC addresses many short-comings of other available mouse populations such as limited genetic diversity and a non-ideal population structure. The CC strains were derived from an 8 way cross using

a set of founders that include three wild-derived strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/HILtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) to capture nearly 90% of the known variation present in lab mice. The goal of this study was to test inter-strain variability; therefore, the number of CC strains employed is comparable to previous studies of inbred mouse panels (Bradford et al., 2011). Mice were acquired from the University of North Carolina Systems Genetics Core (Chapel Hill, NC). Housing and treatment conditions were identical to those detailed above. Mice were orally administered a single dose of TCE (800 mg/kg) prepared in 5% Alkamuls EL-620. In both studies, mice were euthanized with pentobarbital (50 mg/kg, *i.p.*) 2 hr after dosing and tissues immediately dissected for collection of liver, kidneys, and blood. Tissues were snap-frozen in liquid nitrogen and stored at -80°C until analyses. The time point for tissue collection was based on a toxicokinetic study of TCE metabolism that illustrated peak levels of GSH conjugates several hr after dosing (Kim et al., 2009b).

The potential for *ex vivo* formation of conjugation metabolites of TCE was tested utilizing serum (50 μl) and liver (50 mg) samples from untreated mice (male, B6C3F1/J). TCE (5 μM) was reacted with GSH (5 mM) in saline, serum, or liver homogenates for 1 hr at 37°C . Some reactions were carried out with or without protein inactivation (by heating for 10 min at 90°C), or GSH depletion through addition of 2,4-dinitrochlorobenzene (0.2 mM). Upon completion of these incubations, samples were extracted and analyzed as detailed below.

Sample preparation

Serum (50 μl) samples were spiked with 10 μl internal standard mixture (5 μM for each analyte), mixed with 100 μl methanol to precipitate protein, and then vortexed and centrifuged (10,000 g, 10 min, room temperature). Thereafter, the supernatant was diluted with 850 μl distilled deionized water for solid phase extraction (SPE). Liver or kidney (50 mg) samples were spiked with 10 μl internal standard mixture (5 μM for each analyte), and then homogenized in 200 μl methanol and 200 μl chloroform using stainless steel beads in a Bead Ruptor 24 (Omni Inc, Kennesaw, GA) for 30 sec at 4 m/sec. Homogenates were centrifuged (10,000g, 5 min, room temperature), the supernatant was diluted with 1 ml distilled deionized water, and diluent used for SPE. Strata-X-AW 96-well SPE plate (cat no. 8E-SO38-TGB, sorbent lot no. S308-0066; Phenomenex, Torrance, CA) was employed. The SPE procedure was slightly modified from our previously published method (Luo et al., 2017). In the final step, the dried residue was reconstituted in 30 μl water:methanol (9:1, v/v) containing 0.1% acetic acid. Samples were then transferred to an autosampler vial with a 200 μl insert, and stored at -20°C prior to analysis.

LC-MS/MS Method

Samples (10 μl) were automatically injected and chromatographed on a ZORBAX SSHD Eclipse Plus C18 column (3.0 $\times$ 50 mm, 1.8 μm , cat no. 959757-302; Agilent, Santa Clara, CA) with a guard column (2.1 $\times$ 5 mm, 1.8 μm , cat no.821725-901; Agilent) via a Waters Acquity H-class LC system (Waters, Milford, MA). Column temperature was maintained at 25°C . Initial chromatographic condition was maintained at 90% solvent A (water with 0.1% acetic acid, v/v) and 10% solvent B (methanol with 0.1% acetic acid, v/v) for 1 min, then increased to 90% solvent B by 3 min, then to 98% solvent B by 4 min, and then returned to

initial condition until 7 min for sufficient equilibration prior to next run. Flow rate was set at 0.4 ml/min. All analyses were performed using Agilent 6740 triple-quadrupole mass spectrometer. The optimal MS/MS parameters were obtained via direct infusion of standards and isotopically-labeled internal standards in mobile phase at the flow rate of 0.5 ml/min (1:1 methanol:water, v/v, containing 0.1% formic acid). An Agilent Optimizer Tool (MassHunter, ver B.08.00) was utilized to finalize the optimal parameters of the fragmentor, collision energy, cell accelerator voltage and mass transition for each analyte (Table 1). Along with the real chromatographic condition, capillary voltage was optimal at 3500V, sheath gas pressure and sheath gas temperature were optimal at 35 psi and 350°C, and gas temperature was optimal at 300°C (data not shown).

Method validation

Selectivity of the method was determined by using pooled tissues and/or serum of untreated male animals of the same strain. Sensitivity was defined by both the LOD the concentration with signal to noise ratio of 3 and limit of quantification (LOQ) the concentration with signal to noise ratio of 10. Inter-channel cross talk and sample-to-sample carry over effects were evaluated by using the highest concentration in the calibration curve. Calibration curves of DCVG, DCVC, and NAcDCVC were prepared in liver, kidneys, or serum samples obtained from untreated mice. Blank liver, kidneys, or serum (50 mg or µl) was spiked with standard mixture at 0, 0.25, 0.5, 1.25, 2.5, 6.25, 18.75, or 31.25 pmoles for each analyte. All standard solutions were also spiked with 50 pmole of each internal standard. Peak area ratio of standard and internal standard was employed for quantitation of DCVG, DCVC, and NAcDCVC.

Intra-day precision and accuracy (6 replicates per matrix) were assessed in liver, kidneys, and serum of untreated mice and in artificial urine (Surine™ negative urine control, Cerilliant, Round Rock, TX) after spiking in 2.5 pmole for each analyte. Inter-day precision and accuracy were evaluated over 6 consecutive days. Extraction performance was evaluated by recovery, matrix effect, and process efficiency as described elsewhere (Matuszewski et al., 2003). Freeze and thaw stability (three freeze-thaw cycles on dry ice), short-term stability (storing samples at room temperature for 4 hr), long-term stability (storing samples at -20°C for 30 days), and on-tray stability (storing samples at 4°C for 8 hr) of DCVG, DCVC, and NAcDCVC were evaluated by comparing extracted samples before and after each test condition. Stability of the stock solutions was evaluated after they were stored at room temperature for 6 hr.

Statistical analyses

One-way ANOVA and post-hoc tests were conducted by using GraphPad Prism (ver. 5, GraphPad Software, La Jolla, CA). A p-value of less than 0.05 was considered significant in all analyses.

Calculation of uncertainty factors for toxicokinetic variability

To avoid underestimation of toxicokinetic variability, concentrations for peaks between LOD and LOQ were estimated via extrapolation from the calibration curves. Values below LOD were replaced by ½ of the LOD, as recommended by US EPA (EPA, 2000). For kidney

DCVC, values below the background concentration were replaced by the background concentration derived from matrix-matched calibration curve.

The uncertainty factor for human toxicokinetic variability ($UF_{H,TK}$) can be expressed as the ratio between the internal dose in a “sensitive” individual (e.g., 95th or 99th percentile) to that in a “typical” individual (e.g., median) (WHO/IPCS, 2005). For a number of chemicals, including TCE, population PBPK modeling has been used to estimate this ratio (U.S. EPA, 2011a; 2011b). For TCE, Chiu et al. (2014) demonstrated that mouse inter-strain variability is in agreement with human inter-individual variability. Whereas previously, $UF_{H,TK}$ was estimated only for the overall flux of GSH conjugation, here one was able to extend this to tissue- and metabolite-specific dose metrics. Therefore, for each tissue and metabolite, given an inter-strain variance σ^2 , the $UF_{H,TK}$ was estimated for the 95th and 99th percentile as $UF_{H,TK,95} = \exp(z_{95} \sigma)$ and $UF_{H,TK,99} = \exp(z_{99} \sigma)$, respectively, where z_{95} and z_{99} are the z-scores for the corresponding percentiles.

The inter-strain variability of GSH conjugation metabolites of TCE was estimated by decomposing the total observed variance across CC strains into two components: intrinsic inter-strain heterogeneity and intra-strain variability due to environmental factors and measurement error. Only one sample from each CC strain was available, so the intra-strain variability was estimated by the observed variation across B6C3F1 mice, as follows:
 $\sigma^2_{\text{inter-strain}} = \sigma^2_{\text{total}} - \sigma^2_{\text{intra-strain}}$; σ^2_{total} = variance of log-transformed data across 20 CC mice; $\sigma^2_{\text{intra-strain}}$ = variance of log-transformed data across B6C3F1 mice.

The uncertainty factor of the inter-strain variability in toxicokinetics (UF_{TKvar}) is calculated from $UF_{TKvar} = e^{z^* \sigma}$, where $z = Z$ statistics and σ represents the square root of variance. The UF_{TKvar} of GSH conjugation metabolites are derived at 95 and 99%, respectively.

Population PBPK modeling

For comparison, mouse population PBPK modeling was used to estimate of serum concentrations of DCVG and DCVC was generated from 100 different populations (500 individuals/population) as detailed in (Chiu et al., 2014).

Results

LC-MS/MS method for detection of DCVG, DCVC, and NAcDCVC

Figure 2 presents the LC-MS/MS chromatogram of DCVG, DCVC, and NAcDCVC in blank kidney tissue spiked with 0.25 pmoles of standards and 50 pmoles of isotopically labeled internal standards. Representative retention time was 2.5 min for DCVC and DCVC*, 3.35 min for DCVG and DCVG*, and 3.79 min for NAcDCVC and NAcDCVC*.

Selectivity test showed that a peak at 2.75 min in the mass transition of m/z 220.0→201.9 and a peak at 2.55 min in the mass transition of m/z 216.0→82.9 commonly appeared in liver, kidney, and serum. A peak at 4.05 min in the mass transition of m/z 258.0→179.9 and m/z 262.0→216.9 specifically appeared in serum. Even though the peaks of the target analytes were chromatographically separated from the aforementioned peaks, these mass transitions as quantifiers were not used to minimize the potential influences on our

quantitative results. Calibration curves across different tissue matrices demonstrated excellent linearity ($r^2 > 0.998$), and relative standard deviations of the slopes were less than 3% (1.1% for DCVG, 2.5% for DCVC, and 0.8% for NAcDCVC; Figure 3). No inter-channel cross talk between standards and isotopically labeled standards occurred during analysis. The sample-to-sample carry over effect at the highest concentration of calibration curve was less than 0.08% for DCVG, 0.03% for DCVC, and 1.4% for NAcDCVC. Interestingly, a positive intercept was observed in the calibration curve of DCVC in kidney and serum, this background signal disappeared in the absence of isotopically labeled internal standards (Supplemental Figure S1). However, no background was detected in DCVC signal in liver, even in presence of isotopically labeled internal standards. It is likely that a small amount of the unlabeled portion of the internal standards (-dichloroethyl group) is released from the parent compound, and then reacts with the endogenous unbound S-cysteine, which is abundant in kidney and plasma, to generate a minute amounts of DCVC as background signal. Therefore, a matrix-matched calibration curve is highly recommended to subtract the background signals specifically in kidney or serum, even though the calibration curve in water showed excellent consistency with matrix-matched calibration curves. To test whether these metabolites can form *ex vivo*, TCE (5 μ M) was reacted with GSH (5 mM) in saline, as well as serum and liver homogenates. These reactions were carried out with or without protein inactivation or GSH depletion. No *ex vivo* formation of DCVG, DCVC or NAcDCVC was noted at levels exceeding LOD (data not shown).

Table 2 shows the method performance of DCVG, DCVC, and NAcDCVC in multiple mouse tissues. In serum, recovery of DCVG (43–73%) and DCVC (10–53%) was approximate 3-fold higher than previously reported (Kim et al., 2009a). The low recovery of DCVC in liver and kidneys likely results from sample loss during liquid-liquid extraction; however, to the best of our knowledge, the liquid-liquid extraction is required to minimize the matrix interference especially in liver and kidneys. The ion suppression of DCVG, DCVC, and NAcDCVC was most pronounced (40–50%) in liver, it was more variable in serum [22% DCVG, 10% DCVC, and 40% NAcDCVC], and minimal in kidneys (10–15%). The matrix effects on DCVG and DCVC reported herein were less pronounced as compared to those reported in (Kim et al., 2009a) (up to 45% for DCVG and up to 78% for DCVC). Even though ion suppression effect occurs during the analysis, this method is accurate (bias <15%) and reliable (precision <15%) in liver, kidneys, and serum by using isotopically-labeled internal standards (Table 2). The stability of DCVG, DCVC, and NAcDCVC was assessed with stock solutions and extracted samples under various storage conditions. Stock solutions were stable at room temperature for up to 8 hr (DCVG: 98.3%, DCVC: 102.5%, NAcDCVC: 94.8%). Analytes in extracted samples were stable in liver, kidneys, and serum when subjected to on-tray, short-term, long-term, and freeze-thaw storage conditions (Table 2).

Toxicokinetic profiling of TCE glutathione conjugates across tissues and strains

The levels of DCVG, DCVC, and NAcDCVC were quantified in multiple tissues of B6C3F1 mice treated with single dose of TCE (24, 240, or 800 mg/kg) 2 hr after oral gavage (Figure 4). DCVG and NAcDCVC were most abundant in liver, while DCVC was most abundant in kidneys. Significant dose-response in formation of conjugates was observed for all tissues

and metabolites where levels were detectable in at least two dose groups, except for DCVC in kidneys where non-linear kinetics was noted.

The levels of DCVG, DCVC, and NAcDCVC were also successfully quantified in multiple tissues of CC mice treated with TCE (single dose, 800 mg/kg). Figure 5 shows that in liver, DCVG exhibited the highest detection rate (>LOD, 100%) as compared to DCVC (65%) and NAcDCVC (85%). Similarly, the detection rate of DCVG (89%) in serum was higher than that DCVC (42%) and NAcDCVC (63%). NAcDCVC was most detectable in kidneys (74%), followed by DCVG (68%) and DCVC (63%). Levels of GSH conjugation metabolites in B6C3F1 mice were among the highest as compared to those in 20 CC strains.

Pairwise correlation analysis was conducted for all metabolites and tissues using data from 20 CC strains (Figure 6 and Supplemental Figure S2). Strong positive correlations were found between liver DCVG and NAcDCVC ($r=0.89$), serum DCVG and liver DCVG ($r=0.68$), and liver NAcDCVC and kidney NAcDCVC ($r=0.64$). Medium positive correlations were observed between kidney DCVG and serum DCVC ($r=0.57$), liver DCVG and kidney DCVG ($r=0.49$), liver DCVG and serum DCVG ($r=0.54$), kidney DCVG and DCVC ($r=0.50$), kidney DCVG and NAcDCVC ($r=0.53$). Interestingly, the correlation between liver DCVG and kidney DCVC was not statistically significant.

As postulated by Chiu et al (2014), inter-strain variability was used as a surrogate for human inter-individual variability to estimate uncertainty factors human toxicokinetic variability (UF_{H-TK}) for DCVG, DCVC, and NAcDCVC in liver, kidneys and serum (Table 3). As described in Methods, the variance obtained from B6C3F1 mice was utilized to estimate the intra-strain variability in a single CC strain, which was then subtracted from the overall observed variance across CC strains. The values of UF_{H-TK} for the 95th percentile were 3.3–5.8 for DCVG, 2.1–6.7 for DCVC, and 2.7–4.6 for NAcDCVC. Values for the 99th percentile were correspondingly higher. DCVG and NAcDCVC showed the highest UF_{H-TK} in liver, while DCVC showed the highest UF_{H-TK} in kidneys.

The extent of variability in the levels of DCVG in serum was less than the population variability estimates from a PBPK model (Chiu et al., 2014) (Figure 5). Specifically, using the Chiu et al (2014) PBPK model, $UF_{H-TK,95}$ for DCVG and DCVC in serum had 95% confidence intervals of (16–110) and (5–26), respectively, whereas the values estimated here were 5.8 and 4.5 (Table 3). In addition, model-derived estimates for DCVC in serum were lower than the measured values (Figure 5). These discrepancies may result from the relatively low detection rate of DCVC in serum (42%). About half of the model estimates were below the LOD, which is also consistent with % undetectable samples.

Discussion

Characterization of tissue levels of nephrotoxic GSH metabolites of TCE is an important challenge in regulatory toxicology, compounded by lack of sensitive analytical methods. Several analytical methods are available for analysis of DCVG, DCVC, and NAcDCVC (Table 4). In general, LC-MS/MS-based methods (Kim et al., 2009a; Yoo et al., 2015a; 2015b) exhibited superior sensitivity for DCVG and DCVC. The LC-MS/MS based method

for simultaneous detection of DCVG and DCVC was developed for mouse serum (Kim et al., 2009a); however, applicability of this method to parenchymal tissues such as liver and kidney, especially at lower doses of TCE, was questionable. Yoo et al (2015a; 2015b) tailored extraction methods to quantify DCVG and DCVC in liver and kidney, respectively; but differences in extraction methods may still result in a systematic bias (Luo et al., 2017). In this study, tissue extraction protocols were optimized, the sensitivity of detection for DCVG and DCVC across tissues was further enhanced, and the method extended to quantification of NAcDCVC.

The method was employed to characterize tissue-specific variation in formation of GSH conjugation metabolites of TCE. Consistent with our knowledge of the metabolic pathways for GSH metabolism of halogenated solvents, the most abundant metabolites of TCE were the GSH conjugates in liver and the cysteine conjugate in kidney (Whitfield, 2001; Krzysik & Adibi, 1977; Moron et al., 1979). It is noteworthy that our study enabled addressing several additional important gaps in our knowledge with respect to GSH conjugation metabolism of TCE.

It is interesting that NAcDCVC was most abundant in liver. Formation of mercapturic acids from GSH conjugates involves both liver and kidney (Inoue et al., 1984; 1987; Lash et al., 1998; Hinchman & Ballatori, 1994). DCVG synthesized within hepatocytes may be secreted across the canalicular membrane into bile, degraded to form DCVC, reabsorbed and n-acetylated to form NAcDCVC in liver. No *ex vivo* formation of GSH conjugates from TCE was observed. When quantified across tissues and individual strains, high correlations were found for liver DCVG and serum DCVG, and for liver DCVG and NAcDCVC which suggests that DCVG might be directly transported from liver into blood. Rapid conversion from DCVG to NAcDCVC is also plausible in liver. It is of interest that liver DCVG and NAcDCVC were not correlated with kidney DCVC, but associated with kidney NAcDCVC. These findings suggest that i) formation and elimination of DCVC in kidney may be a critical source of inter-individual variability among strains, and ii) liver may be the primary site for generation of NAcDCVC.

This study enabled quantitative comparisons of conjugative metabolism pathways between TCE and tetrachloroethylene (PERC). Levels of GSH conjugation metabolites in all tissues examined were lower upon exposure to TCE at the equivalent molar dose (6 mmole/kg) and time point compared to PERC (Luo et al., 2017). Specifically, concentrations of DCVG were lower by 3.9-fold in liver, 2.4-fold in kidneys, and 3.3-fold in serum as compared to *S*-(1,2,2-trichlorovinyl) glutathione (TCVG). Concentrations of DCVC were 2.6-fold lower in kidneys and 5.3-fold in serum, while were 1.2-fold higher in liver, compared to *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC). These findings are consistent with reports that in the absence of hepatic metabolism of GSH conjugates, PERC is more cytotoxic than TCE to isolated kidney cells (Lash et al., 2007), and that DCVC is less nephrotoxic than TCVC *in vivo* (Birner et al., 1997). However, kidney cancer hazard evidence in humans is more potent for TCE than PERC (Guha et al., 2012), indicating that bioactivation to downstream reactive sulfoxides, DCVCSO and NAcDCVCSO, may be most critical part of cancer etiology in the kidneys (Irving et al., 2013; Lash et al., 1994). It is also plausible that lower levels of DCVC and NAcDCVC, as compared to those of TCVC and NAcTCVC, are the product of a higher

conversion rate to their corresponding sulfoxides, a process mediated by hepatic cytochrome P450 3A enzymes (Werner et al., 1996).

This investigation characterized inter-strain variability in toxicokinetics of TCE GSH conjugates. Inter-strain variability in toxicokinetics of DCVG was greatest in liver (GSD=2.9, 44-fold differences). Corresponding values for serum and kidneys were 2.5 (6-fold) and 2.1 (5-fold). These differences may be attributed to inter-strain variability in enzyme activities, or in other PK modifiers such as absorption and elimination efficiency of TCE, cellular transporters, and plasma protein levels. The inter-individual variability in human enzyme activities related to formation of DCVG, DCVC, and NAcDCVC was reported for GSTs (4–92 fold differences), gamma-glutamyl transferase (3-fold), n-acetyltransferase (70-fold), and acylase (7.4-fold), as well as flavin monooxygenase 3 (10-fold), beta-lyase (4-fold), and CYP3A (40-fold) (Gul Altuntas & Kharasch, 2002; Slone et al., 1995; McCarthy et al., 1994; Lamba et al., 2002; Helander et al., 1998; Stormer et al., 2000). The importance of both toxicokinetic (TK) and toxicodynamic (TD) variability is further supported by observations that allelic variations in human enzymes involved in both TK and TD, such as GST θ and renal β lyase, were associated with increased TCE-mediated renal cancer risk (Moore et al., 2010; Spearow et al., 2017).

Quantitative estimates of variability among strains and tissues have important implications for risk assessment because in the absence of chemical-specific data a default “uncertainty factor” of $10^{0.5}$ (3.16) is utilized for inter-individual variability in TK. Although intended to be “conservative,” it is common for variability to exceed this value. In the most recent analysis by WHO/IPCS (Chiu & Slob, 2015), the GSD for TK variability has a 90% confidence interval of 1.2 to 2.6, which corresponds to $UF_{H-TK,99}$ protecting 99% of the population of 1.4 to 8.9, with a median of 3.6. Thus, the “default” value is actually closer to a central estimate of TK variability, suggesting that chemical-specific data are needed. Indeed, consistent with previous analyses by Chiu et al. (2009; 2014), data for GSH conjugation metabolites of TCE suggest that for most tissues and metabolites, a factor of 3.16 is not sufficient to cover 99% of the population variability in TK (Table 3).

There are several key limitations to this study. First, the analyte recovery of DCVC is inadequate (10~15%) as compared to those of DCVG and NAcDCVC. Improvements in sample preparation techniques are needed to further enhance the sensitivity of DCVC detection. Second, as only a single sample from each CC strain was available, inter-strain variability was estimated by subtracting total observed variability from intra-strain variability in B6C3F1 mice. Future studies may benefit from characterization of intra-strain variability in CC strains. Third, data in CC mice was only at a single dose and time point. Future investigations need to include time-course data at multiple dose levels to improve the estimates of tissue-specific GSH conjugation dosimetry, particularly if incorporated into a population PBPK model. To date, the PBPK modeling of inter-strain variability in oxidative metabolism of TCE has been well characterized, but the PBPK compartments in conjugative metabolism of TCE, especially at target organs, are yet to be fully established (Chiu et al., 2014).

Conclusions

In summary a LC-MS/MS method that simultaneously detects DCVG, DCVC, and NAcDCVC in multiple mouse tissues was successfully developed, and applied in animal studies. With this method, for the first time, the inter-individual variability in conjugative metabolism of TCE was characterized in multiple tissues of a panel of genetically diverse CC mice. Despite some study limitations, data suggest that for TCE, the default uncertainty factor of 3.16 for TK variability might be inadequate to protect 99% of the population for organ-specific toxicity mediated by DCVG, DCVC, and NAcDCVC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Birner G, Bernauer U, Werner M, Dekant W. Biotransformation, excretion and nephrotoxicity of haloalkene-derived cysteine S-conjugates. *Arch Toxicol.* 1997; 72:1–8. [PubMed: 9458184]
- Bloemen LJ, Monster AC, Kezic S, Commandeur JN, Veulemans H, Vermeulen NP, Wilmer JW. Study on the cytochrome P-450- and glutathione-dependent biotransformation of trichloroethylene in humans. *Int Arch Occup Environ Health.* 2001; 74:102–108. [PubMed: 11317702]
- Bradford BU, Lock EF, Kosyk O, Kim S, Uehara T, Harbourn D, DeSimone M, Threadgill DW, Tryndyak V, Pogribny IP, Bleyle L, Koop DR, Rusyn I. Interstrain differences in the liver effects of trichloroethylene in a multistrain panel of inbred mice. *Toxicol Sci.* 2011; 120:206–217. [PubMed: 21135412]
- Buben JA, O'Flaherty EJ. Delineation of the role of metabolism in the hepatotoxicity of trichloroethylene and perchloroethylene: A dose-effect study. *Toxicol Appl Pharmacol.* 1985; 78:105–122. [PubMed: 2994252]
- Chiu WA, Caldwell JC, Keshava N, Scott CS. Key scientific issues in the health risk assessment of trichloroethylene. *Environ Health Persp.* 2006; 114:1445–1449.
- Chiu WA, Campbell JL, Clewell HJ, Zhou YH, Wright FA, Guyton KZ, Rusyn I. Physiologically-based pharmacokinetic (PBPK) modeling of inter-strain variability in trichloroethylene metabolism in the mouse. *Environ Health Persp.* 2014; 122:456–463.
- Chiu WA, Jinot J, Scott CS, Makris SL, Cooper GS, Dzubow RC, Bale AS, Evans MV, Guyton KZ, Keshava N, Lipscomb JC, Barone S, Fox JF, Gwinn MR, Schaum J, Caldwell JC. Human health effects of trichloroethylene: Key findings and scientific issues. *Environ Health Persp.* 2013; 121:303–311.
- Chiu WA, Okino MS, Evans MV. Characterizing uncertainty and population variability in the toxicokinetics of trichloroethylene and metabolites in mice, rats, and humans using an updated database, physiologically based pharmacokinetic (PBPK) model, and Bayesian approach. *Toxicol Appl Pharmacol.* 2009; 241:36–60. [PubMed: 19660485]
- Chiu WA, Slob W. A unified probabilistic framework for dose-response assessment of human health effects. *Environ Health Persp.* 2015; 123:1241–1254.

- Cichocki JA, Guyton KZ, Guha N, Chiu WA, Rusyn I, Lash LH. Target organ metabolism, toxicity, and mechanisms of trichloroethylene and perchloroethylene: Key similarities, differences, and data gaps. *J Pharmacol Exp Ther*. 2016; 359:110–123. [PubMed: 27511820]
- Corton JC, Cunningham ML, Hummer BT, Lau C, Meek B, Peters JM, Popp JA, Rhomberg L, Seed J, Klaunig JE. Mode of action framework analysis for receptor-mediated toxicity: The peroxisome proliferator-activated receptor alpha (PPARalpha) as a case study. *Crit Rev Toxicol*. 2014; 44:1–49.
- Dekant W. Biosynthesis of toxic glutathione conjugates from halogenated alkenes. *Toxicol Lett*. 2003; 144:49–54. [PubMed: 12919723]
- EPA, US. Guidance for data quality assessment: Practical methods for data analysis. Washington, DC: 2000.
- Green T, Dow J, Ellis MK, Foster JR, Odum J. The role of glutathione conjugation in the development of kidney tumours in rats exposed to trichloroethylene. *Chem Biol Interact*. 1997; 105:99–117. [PubMed: 9251723]
- Guha N, Loomis D, Grosse Y, Lauby-Secretan B, El Ghissassi F, Bouvard V, Benbrahim-Tallaa L, Baan R, Mattock H, Straif K. International Agency for Research on Cancer Monograph Working Group. Carcinogenicity of trichloroethylene, tetrachloroethylene, some other chlorinated solvents, and their metabolites. *Lancet Oncol*. 2012; 13:1192–1193. [PubMed: 23323277]
- Gul Altuntas T, Kharasch ED. Biotransformation of L-cysteine S-conjugates and N-acetyl-L-cysteine S-conjugates of the sevoflurane degradation product fluoromethyl-2,2-difluoro-1-(trifluoromethyl)vinyl ether (compound A) in human kidney in vitro: interindividual variability in N-acetylation, N-deacetylation, and beta-lyase-catalyzed metabolism. *Drug Metab Dispos*. 2002; 30:148–154. [PubMed: 11792683]
- Helander A, Vabo E, Levin K, Borg S. Intra- and interindividual variability of carbohydrate-deficient transferrin, gamma-glutamyltransferase, and mean corpuscular volume in teetotalers. *Clin Chem*. 1998; 44:2120–2125. [PubMed: 9761244]
- Hinchman CA, Ballatori N. Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. *J Toxicol Environ Health*. 1994; A 41:387–409.
- IARC. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans (Vol. 106): Trichloroethylene, Tetrachloroethylene and Some Other Chlorinated Agents. 2014:106.
- Inoue M, Okajima K, Morino Y. Hepato-renal cooperation in biotransformation, membrane transport, and elimination of cysteine S-conjugates of xenobiotics. *J Biochem*. 1984; 95:247–254. [PubMed: 6706912]
- Inoue M, Okajima K, Nagase S, Morino Y. Inter-organ metabolism and transport of a cysteine-S-conjugate of xenobiotics in normal and mutant albuminemic rats. *Biochem Pharmacol*. 1987; 36:2145–2150. [PubMed: 3606632]
- Irving RM, Elfarra AA. Mutagenicity of the cysteine S-conjugate sulfoxides of trichloroethylene and tetrachloroethylene in the Ames test. *Toxicology*. 2013; 306C:157–161.
- Irving RM, Pinkerton ME, Elfarra AA. Characterization of the chemical reactivity and nephrotoxicity of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide, a potential reactive metabolite of trichloroethylene. *Toxicol Appl Pharmacol*. 2013; 267:1–10. [PubMed: 23253325]
- Kim S, Collins LB, Boysen G, Swenberg JA, Gold A, Ball LM, Bradford BU, Rusyn I. Liquid chromatography electrospray ionization tandem mass spectrometry analysis method for simultaneous detection of trichloroacetic acid, dichloroacetic acid, S-(1,2-dichlorovinyl)glutathione and S-(1,2-dichlorovinyl)-L-cysteine. *Toxicology*. 2009a; 262:230–238. [PubMed: 19549554]
- Kim S, Kim D, Pollack GM, Collins LB, Rusyn I. Pharmacokinetic analysis of trichloroethylene metabolism in male B6C3F1 mice: Formation and disposition of trichloroacetic acid, dichloroacetic acid, S-(1,2-dichlorovinyl)glutathione and S-(1,2-dichlorovinyl)-L-cysteine. *Toxicol Appl Pharmacol*. 2009b; 238:90–99. [PubMed: 19409406]
- Krzysek BA, Adibi SA. Cytoplasmic dipeptidase activities of kidney, ileum, jejunum, liver, muscle, and blood. *Am J Physiol*. 1977; 233:E450–E456. [PubMed: 23010]
- Lamba JK, Lin YS, Schuetz EG, Thummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev*. 2002; 54:1271–1294. [PubMed: 12406645]

- Lash LH, Sausen PJ, Duescher RJ, Cooley AJ, Elfarrar AA. Roles of cysteine conjugate beta-lyase and S-oxidase in nephrotoxicity: studies with S-(1,2-dichlorovinyl)-L-cysteine and S-(1,2-dichlorovinyl)-L-cysteine sulfoxide. *J Pharmacol Exp Ther*. 1994; 269:374–383. [PubMed: 8169843]
- Lash LH, Qian W, Putt DA, Desai K, Elfarrar AA, Sicuri AR, Parker JC. Glutathione conjugation of perchloroethylene in rats and mice in vitro: Sex-, species-, and tissue-dependent differences. *Toxicol Appl Pharmacol*. 1998; 150:49–57. [PubMed: 9630452]
- Lash LH, Putt DA, Brashear WT, Abbas R, Parker JC, Fisher JW. Identification of S-(1,2-dichlorovinyl)glutathione in the blood of human volunteers exposed to trichloroethylene. *J Toxicol Environ Health A*. 1999; 56:1–21. [PubMed: 9923751]
- Lash LH, Fisher JW, Lipscomb JC, Parker JC. Metabolism of trichloroethylene. *Environ Health Persp*. 2000; 108(Suppl 2):177–200.
- Lash LH, Hueni SE, Putt DA. Apoptosis, necrosis, and cell proliferation induced by S-(1,2-dichlorovinyl)-L-cysteine in primary cultures of human proximal tubular cells. *Toxicol Appl Pharmacol*. 2001a; 177:1–16. [PubMed: 11708895]
- Lash LH, Qian W, Putt DA, Hueni SE, Elfarrar AA, Krause RJ, Parker JC. Renal and hepatic toxicity of trichloroethylene and its glutathione-derived metabolites in rats and mice: Sex-, species-, and tissue-dependent differences. *J Pharmacol Exp Ther*. 2001b; 297:155–164. [PubMed: 11259540]
- Lash LH, Putt DA, Parker JC. Metabolism and tissue distribution of orally administered trichloroethylene in male and female rats: identification of glutathione- and cytochrome P-450-derived metabolites in liver, kidney, blood, and urine. *J Toxicol Environ Health A*. 2006; 69:1285–1309. [PubMed: 16754541]
- Lash LH, Putt DA, Huang P, Hueni SE, Parker JC. Modulation of hepatic and renal metabolism and toxicity of trichloroethylene and perchloroethylene by alterations in status of cytochrome P450 and glutathione. *Toxicology*. 2007; 235:11–26. [PubMed: 17433522]
- Lash LH, Chiu WA, Guyton KZ, Rusyn I. Trichloroethylene biotransformation and its role in mutagenicity, carcinogenicity and target organ toxicity. *Mutat Res Rev Mutat Res*. 2014; 762:22–36. [PubMed: 25484616]
- Lee KM, Bruckner JV, Muralidhara S, Gallo JM. Characterization of presystemic elimination of trichloroethylene and its nonlinear kinetics in rats. *Toxicol Appl Pharmacol*. 1996; 139:262–271. [PubMed: 8806842]
- Lee KM, Muralidhara S, White CA, Bruckner JV. Mechanisms of the dose-dependent kinetics of trichloroethylene: Oral bolus dosing of rats. *Toxicol Appl Pharmacol*. 2000; 164:55–64. [PubMed: 10739744]
- Luo YS, Cichocki JA, McDonald TJ, Rusyn I. Simultaneous detection of the tetrachloroethylene metabolites S-(1,2,2-trichlorovinyl) glutathione, S-(1,2,2-trichlorovinyl)-L-cysteine, and N-acetyl-S-(1,2,2-trichlorovinyl)-L-cysteine in multiple mouse tissues via ultra-high performance liquid chromatography electrospray ionization tandem mass spectrometry. *J Toxicol Environ Health A*. 2017; 80:513–524. [PubMed: 28696834]
- Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem*. 2003; 75:3019–3030. [PubMed: 12964746]
- Mccarthy RI, Lock EA, Hawksworth GM. Cytosolic C-S lyase activity in human kidney samples - Relevance for the nephrotoxicity of halogenated alkenes in man. *Toxicol Ind Health*. 1994; 10:103–112. [PubMed: 7570611]
- Moore LE, Boffetta P, Karami S, Brennan P, Stewart PS, Hung R, Zaridze D, Matveev V, Janout V, Kollarova H, Bencko V, Navratilova M, Szeszenia-Dabrowska N, Mates D, Gromiec J, Holcatova I, Merino M, Chanock S, Chow WH, Rothman N. Occupational trichloroethylene exposure and renal carcinoma risk: Evidence of genetic susceptibility by reductive metabolism gene variants. *Cancer Res*. 2010; 70:6527–6536. [PubMed: 20663906]
- Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim Biophys Acta*. 1979; 582:67–78. [PubMed: 760819]

- National Toxicology Program. Carcinogenesis Studies of Trichloroethylene (Without Epichlorohydrin) (CAS No. 79-01-6) in F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser. 1990; 243:1–174. [PubMed: 12750750]
- Rusyn I, Chiu WA, Lash LH, Kromhout H, Hansen J, Guyton KZ. Trichloroethylene: Mechanistic, epidemiologic and other supporting evidence of carcinogenic hazard. *Pharmacol Therap.* 2014; 141:55–68. [PubMed: 23973663]
- Sausen PJ, Elfarrar AA. Cysteine conjugate S-oxidase. Characterization of a novel enzymatic activity in rat hepatic and renal microsomes. *J Biol Chem.* 1990; 265:6139–6145. [PubMed: 2318851]
- Slone DH, Gallagher EP, Ramsdell HS, Rettie AE, Stapleton PL, Berlad LG, Eaton DL. Human variability in hepatic glutathione S-transferase-mediated conjugation of aflatoxin B1-epoxide and other substrates. *Pharmacogenetics.* 1995; 5:224–233. [PubMed: 8528269]
- Spearow JL, Gettmann K, Wade M. Review: Risk assessment implications of variation in susceptibility to perchloroethylene due to genetic diversity, ethnicity, age, gender, diet and pharmaceuticals. *Human Ecol Risk Assess.* 2017; 23:1466–1492.
- Stormer E, Roots I, Brockmoller J. Benzydamine N-oxidation as an index reaction reflecting FMO activity in human liver microsomes and impact of FMO3 polymorphisms on enzyme activity. *Br J Clin Pharmacol.* 2000; 50:553–561. [PubMed: 11136294]
- Threadgill DW, Churchill GA. Ten years of the Collaborative Cross. *Genetics.* 2012; 190:291–294. [PubMed: 22345604]
- U.S. EPA. Toxicological Review of Dichloromethane (Methylene Chloride) (CAS No. 75-09-2). Support of Summary Information on the Integrated Risk Information System (IRIS). 2011a
- U.S. EPA. Toxicological Review of Trichloroethylene (CAS No. 79-01-6). Support of Summary Information on the Integrated Risk Information System (IRIS). 2011b
- U.S. EPA. [cited May 19, 2017] EPA Names First Chemicals for Review Under New TSCA Legislation. 2017. Available from <https://www.epa.gov/newsreleases/epa-names-first-chemicals-review-under-new-tsca-legislation>
- Werner M, Birner G, Dekant W. Sulfoxidation of mercapturic acids derived from tri- and tetrachloroethene by cytochromes P450 3A: A bioactivation reaction in addition to deacetylation and cysteine conjugate beta-lyase mediated cleavage. *Chem Res Toxicol.* 1996; 9:41–49. [PubMed: 8924615]
- Whitfield JB. Gamma glutamyl transferase. *Crit Rev Clin Lab Sci.* 2001; 38:263–355. [PubMed: 11563810]
- WHO/IPCS. Chemical-specific adjustment factors for interspecies differences in human variability: Guidance document for use of data in dose/concentration-response assessment. Geneva, Switzerland: 2005.
- Yoo HS, Bradford BU, Kosyk O, Shymonyak S, Uehara T, Collins LB, Bodnar WM, Ball LM, Gold A, Rusyn I. Comparative analysis of the relationship between trichloroethylene metabolism and tissue-specific toxicity among inbred mouse strains: liver effects. *J Toxicol Environ Health A.* 2015a; 78:15–31. [PubMed: 25424544]
- Yoo HS, Bradford BU, Kosyk O, Uehara T, Shymonyak S, Collins LB, Bodnar WM, Ball LM, Gold A, Rusyn I. Comparative analysis of the relationship between trichloroethylene metabolism and tissue-specific toxicity among inbred mouse strains: kidney effects. *J Toxicol Environ Health A.* 2015b; 78:32–49. [PubMed: 25424545]

Figure 1. GSH conjugation pathway of TCE metabolism

Abbreviation: GST, glutathione S transferase; GSH, glutathione; GGT, gamma-glutamyl transferase; DP, dipeptidase; FMO, Flavin-containing monooxygenase; CCBL, cysteine S-conjugate beta lyase; NAT, n-acetyl transferase; CYP, cytochrome P450s; DCVT, 1,2-dichlorovinylthiol; CTK, chlorothioketene; CTAC, chlorothionoacetyl chloride; DCVCSO, DCVC sulfoxide; NAcDCVCSO, NAcDCVC sulfoxide.

Figure 2. Representative LC-MS/MS chromatograms and mass transitions for parent compounds (A) and isotopically labeled standards (B) of DCVG, DCVC, and NAcDCVC in kidney

The spiked amount on-column was 0.25 pmoles for standards and 50 pmoles for internal standards.

Figure 3. Matrix-matched calibration curves of DCVG, DCVC, and NAcDCVC in mouse tissues
 The influence of matrix effects on quantitative analysis has been minimized by using isotopically labeled internal standards, with a relative standard deviation of slopes less than 3%.

Figure 4. Dose-dependent increases of (A) DCVG, (B) DCVC, and (C) NAcDCVC in liver, kidney, and serum of B6C3F1 mice treated with TCE (n=3/group)

The red dashed line indicates the instrumental LODs; the blue and red triangles locate the matrix-matched LODs (S/N=3) and LOQs (S/N=10), respectively. One-way ANOVA and post hoc test for a linear trend was used to test the dose-response relationships in each tissue (* $p < 0.05$). +The bar is shown as 1/2 LOD if an analyte was below the LOD. #Measurements between LOD and LOQ are expressed as estimated values.

Figure 5. Inter-individual variability of conjugative metabolites of TCE in (A) liver, (B) kidney, and (C) serum of mice at 2 hour after dosing with TCE (800 mg/kg, oral gavage)
 Each data point represents the value reported in a CC mouse strain. The data point is shown as 1/2 LOD if an analyte was below the LOD. Data points with red color refer to the metabolite levels reported in male B6C3F1 mice. Middle line indicates the mean concentration among 20 CC strains. The box and whiskers plots (Min to Max) show the PBPK modeling estimates for serum levels of DCVG and DCVC based on Chiu et al 2014. The estimates were generated from the median distribution out of 100 different populations, with 500 individuals in each population. Different letters indicate different statistical groups, as determined by ANOVA with Turkey's post hoc test ($p < 0.05$).

Figure 6. Pairwise correlation analysis of DCVG, DCVC, and NAcDCVC across tissues

The correlation results are expressed in Spearman coefficient (ρ) and Pearson coefficient (r).

A p-value less than 0.05 was considered significant across all analyses. ns, not significant.

Table 1

Quantifiers and qualifiers for analysis of DCVG, DCVC, and NAcDCVC in multiple tissue.

Analytes	Ion transition (<i>m/z</i>)	Collision energy (V)	Fragmentor
DCVG	402.0→272.9	13	110
	402.0→169.9 [†]	25	110
	402.0→133.9	37	110
DCVG*	409.0→173.9 [†]	25	110
	405.0→169.9	25	110
DCVC	216.0→198.9 [†]	8	70
	216.0→126.9	25	70
	216.0→82.9	45	70
DCVC*	222.0→128.9 [†]	25	80
	220.0→201.9	10	80
NAcDCVC	258.0→215.9	9	90
	258.0→198.8 [†]	17	90
	258.0→179.9	5	90
NAcDCVC*	262.0→216.9	9	90
	262.0→198.8 [†]	17	90

[†] Selected quantifier for analysis.

Alternative quantifier of DCVC* in serum. Dwell time and cell accelerator voltage were set at 25 ms and 2 V.

Table 2

Comparative analysis of method performance of DCVG, DCVC, and NAcDCVC in multiple tissues

	Liver			Kidney			Serum		
	DCVG	DCVC	NAcDCVC	DCVG	DCVC	NAcDCVC	DCVG	DCVC	NAcDCVC
RE(%) [†]	73	15	58	43	10	57	69	53	83
ME(%)	60	62	50	85	85	90	78	90	60
PE(%)	44	9	29	37	8	52	54	48	50
Accuracy(%)	-3.4	+5.8	-0.3	-4.2	+13.6	-5.4	-3.3	-3.5	-5.4
Precision(%)	1.4	7.9	3.0	1.5	7.5	3.0	2.0	6.3	1.6
Accuracy(%)	-1.9	-2.4	-4.7	-4.2	+8.0	-4.5	-2.8	-9.9	-5.7
Precision(%)	2.5	7.5	3.5	0.5	10.0	3.8	2.0	5.2	2.1
On-tray stability (%) [*]	99.6±16.5	116.8±5.4	94.3±4.9	107.5±2.7	101.2±5.4	102.0±1.9	97.1±0.6	99.8±1.4	101.3±2.2
Short-term stability (%)	98.5±1.3	100.6±13.8	101.9±1.5	97.4±1.9	106.3±4.2	107.1±3.4	99.9±2.1	93.6±6.5	104.1±2.7
Long-term stability (%)	102.3±1.7	109.1±13.3	101.8±1.6	99.7±1.0	89.0±2.8	100.2±5.1	99.3±0.7	94.1±6.8	99.7±1.4
Freeze-thaw stability (%)	100.4±0.9	101.0±0.3	100.9±5.0	98.7±0.9	96.8±3.1	97.7±1.8	97.3±2.8	113.0±3.0	100.3±5.6

[†] Method validation for analysis of DCVG, DCVC, and NAcDCVC was compared at level of 50 pmole/g tissue or mL serum across different tissues. Recovery (RE), matrix effect (ME), and process efficiency (PE) were determined from n=5.

Inter-day (n=6) and intra-day (n=6) accuracy (%) are expressed as bias deviated from the nominal concentration. Precision (%) is expressed as relative standard deviation.

^{*} Results of stabilities are expressed in average±standard deviation (n=3).

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Table 3

Derivation of uncertainty factors for inter-strain variability in toxicokinetics of DCVG, DCVC, and NAcDCVC in multiple mouse tissues.

	DCVG			DCVC			NAcDCVC		
	Liver	Kidney	Serum	Liver	Kidney	Serum	Liver	Kidney	Serum
GM	20.3	1.5	1.1	2.0	10.5	0.5	4.4	3.3	2.1
GSD	2.9	2.1	2.9	1.6	3.3	2.5	2.5	2.2	1.8
σ^2_{total} *	1.22	0.57	1.15	0.23	1.06	0.86	0.86	0.61	0.37
$\sigma^2_{intra-strain}$	0.09	0.05	0.02	0.02	0.02	0.04	0.001	0.0007	0.012
$\sigma^2_{inter-strain}$	1.12	0.52	1.13	0.20	1.04	0.82	0.86	0.61	0.36
UF _{H-TK,95}	5.72	3.27	5.75	2.10	5.4	4.45	4.61	3.60	2.66
UF _{H-TK,99}	11.84	5.35	11.93	2.87	10.9	8.28	8.71	6.15	4.01

* GM= geometric mean, pmole/g tissue or mL serum; GSD= geometric standard deviation; Var_{total}= Variance of log-transformed data of 20 CC study; Var_{intra-strain}= Variance of log-transformed data of B6C3F1 study; Var_{inter-strain} = Var_{total} -Var_{intra-strain}; UF_{TKvar}. Uncertainty factors for inter-strain variability in toxicokinetics of DCVG, DCVC, and NAcDCVC.

Table 4
Comparative analysis of sensitivities for DCVG, DCVC, and NAcDCVC between tissues and studies.

Reference	Matrix	Extraction method*	Detect Method	DCVG [†] (fmole)	DCVC (fmole)	NAcDCVC (fmole)
This study	Liver	LL-SPE	LC-MS/MS	8.7	26.3	20.6
	Kidney			11.7	10.8	24
	Serum	PP-SPE		2.9	5.3	24.1
	Instrumental LOD			0.8	1.2	11
Kim et al., 2009	Serum	LL-SPE	LC-MS/MS	37.5	37.5	N/R
Lash et al., 1999	Blood/urine	PP-derivatization with iodoacetate and 1-fluoro-2,4-dinitrobenzene	LC-UV	50,000	5,000	N/R
Yoo et al., 2015	Kidney	LL-SPE	LC-MS/MS	25 [⊥]	250	N/R
Yoo et al., 2015	Liver	SPE	LC-MS/MS	100 [‡]	1,000	N/R
Lash et al., 2006	Blood/urine	Derivatization with iodoacetate and 1-fluoro-2,4-dinitrobenzene	LC-UV	110 [⌋]	110	N/R
	Liver			1,100	1,100	N/R
	Kidney			2,200	2,200	N/R
Bloemen et al., 2001	Urine	Derivatization with HCl and diethyl ether	GC-MS	N/R	N/R	80 [⌋]

[†] Limit of detection (LOD) is defined as the tested concentration with the signal-to-noise ratio equals to 3, and expressed with the on-column amount of analyte.
N/R: Not reported.

Instrumental LOD was tested in de-ionized water.

* LL-SPE: liquid-liquid extraction coupled with SPE; PP-SPE: protein precipitation coupled with SPE.

[⊥] Estimated on-column amount from LLOQ of 1 pmole/g kidney with starting tissue materials of 50 mg tissue.

[‡] Estimated on-column amount from LLOQ of 2 pmole/g liver with starting tissue materials of 100 mg tissue.

[⌋] Estimated on-column amount from LODs of urine/blood (1.1 pmol/ml), liver (11 pmol/g tissue), and kidney (22 pmol/g tissue) with starting tissue materials of 0.5 ml/500 mg tissue.

[⌋] Estimated on-line amount from LOD of 0.04 µmol/L.