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Increased bioaccumulation of Urethane in CYP2E1-/- vs. CYP2E1+/+ Mice

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Abstract

Urethane is a fermentation by-product and a potent animal carcinogen. Human exposure to urethane occurs through consumption of alcoholic beverages and fermented foods. Recently, CYP2E1 was identified as the primary enzyme responsible for the metabolism of ^{14}C-carbonyl-labeled urethane. Subsequently, attenuation of urethane-induced cell proliferation and genotoxicity in CYP2E1-/- mice was reported. Present work compares the metabolism of single vs. multiple exposures of CYP2E1-/- and CYP2E1+/+ mice to ^{14}C-ethyl-labeled urethane. Urethane was administered as a single 10 or 100mg/kg gavage dose or at 100mg/kg/day for 5 consecutive days. CYP2E1+/+ mice administered single or multiple doses exhaled 78-88% of dose as $^{14}CO_2$/day. CYP2E1-/- mice eliminated 30-38% of a single dose as $^{14}CO_2$ in 24hr and plateaued after day 3 at ≈ 52% of dose/day. The concentrations of urethane-derived radioactivity in plasma and tissues were dose-dependent, increased as a function of the number of doses administered, and were significantly higher in CYP2E1-/- vs. CYP2E1+/+ mice. While urethane was the main chemical found in the plasma and tissues of CYP2E1-/- mice, it was not detectable in CYP2E1+/+ mice. In conclusion, multiple dosing led to considerable bioaccumulation of urethane in mice of both genotypes, however, greater retention occurred in CYP2E1-/- vs. CYP2E1+/+ mice. Further, greater bioaccumulation of ^{14}C-ethyl-labeled than ^{14}C-carbonyl-labeled urethane was observed in mice. Comparison of the metabolism of ethyl- vs. carbonyl-labeled urethane was necessary for tracing the source of CO_2 and led us to propose for the first time that C-hydroxylation is a likely pathway of urethane metabolism.

Introduction

Urethane is formed as a by-product of fermentation and is therefore found at appreciable levels in alcoholic beverages and foods, such as bread and cheese (Benson and Beland 1997). Earlier reports have suggested that the mean daily intake of urethane in adults ranges between 10-20 ng/kg b.wt. with bread as the primary source. Furthermore, the FDA has reported that toasting bread increases the level of urethane detected from 45 ng/slice to 117 ng/slice (Benson and Beland 1997). Depending on the extent and type of alcoholic beverage consumed, the mean daily intake of urethane can increase 3 to 60-fold (Zimmerli and Schlatter 1991). Urethane is also a component of tobacco leaves and mainstream smoke. Smoking 20 cigarettes/day can increase the daily intake of urethane by 2-fold (Zimmerli and Schlatter 1991). In the past, human exposure to urethane was also experienced through the use of pesticides, fumigants, cosmetics, and textiles (IARC 1974). In the 1940s, this chemical was used as a hypnotic and as an anti-neoplastic agent to treat chronic leukemia and multiple myeloma (Zimmerli and Schlatter 1991).

Urethane is a potent animal carcinogen that was classified as "reasonably anticipated to be a human carcinogen" (NTP 2000). Previous studies have shown that independent of animal species or strain and regardless of the route of exposure, urethane undergoes rapid systemic distribution and greater than 85% of an administered dose was eliminated in the expired air as CO_2, within 8 hours in genetically intact mice (Skipper *et al*. 1951; IARC 1974; Yamamoto *et al.* 1988, 1990; Nomier *et al.* 1989; Hoffler and Ghanayem 2003; Hoffler *et al*. 2003). Until recently, the accepted hypothesis regarding urethane metabolism presumed that enzymatic hydrolysis via esterase was the primary

metabolic route while N-hydroxylation and pathways mediated by cytochromes P450 (P450s) played minor roles (Figure 1). Recent studies conducted in this laboratory using CYP2E1-/- mice, however, demonstrated for the first time that cytochrome P4502E1 (CYP2E1), and not esterase, was the principal enzyme responsible for greater than 96% of ^{14}C-carbonyl-labeled urethane metabolism to $^{14}CO_2$ (Hoffler *et al.* 2003). It was also shown that P450s other than CYP2E1 and esterase contributed approximately 3 and less than 1%, respectively (Hoffler *et al*. 2003). In the absence of CYP2E1-mediated metabolism, the half-life of urethane was considerably increased (0.8 h in CYP2E1+/+ vs. 22h in CYP2E1-/- mice) and significantly higher blood and tissue concentrations of urethane-derived radioactivity were detected in CYP2E1-/- vs. CYP2E1+/+ mice (Hoffler *et al*. 2003).

An earlier study conducted by Lawson and Pound (1973) demonstrated that covalent binding of urethane to DNA *in vivo* requires the oxidation of the ethyl carbons. In turn, it was suggested that CYP2E1-mediated oxidation of the ethyl moiety of urethane results in the formation of vinyl carbamate and subsequently vinyl carbamate epoxide (Figure 1), leading to the formation of DNA, RNA, and protein adducts. It was hypothesized that the manifestation of these events led to urethane-caused mutagenesis and carcinogenesis. Therefore, the overall objective of our work is to assess the relationship between CYP2E1-mediated metabolism and urethane-induced mutagenicity and carcinogenicity. In particular, present studies were undertaken to compare the metabolism and disposition of single vs. multiple doses of ^{14}C-ethyl-labeled urethane in CYP2E1-/- and CYP2E1+/+ mice, compare the results of these studies with our earlier data on the metabolism and disposition of ^{14}C-carbonyl-labeled urethane, and assess the

potential bioaccumulation of ^{14}C-ethyl-labeled urethane-derived radioactivity as a result of multiple exposures and in comparison to the bioaccumulation of ^{14}C-carbonyl-labeled urethane (Hoffler *et al.* 2003). The use of ^{14}C-ethyl vs. -carbonyl-labeled urethane was necessary to identify the source of the urethane-derived CO_2 and characterize the retained metabolites.

Materials and Methods

Chemicals: [Ethyl-1-^{14}C] urethane, specific activity 56mCi/mmol was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Unlabeled urethane with purity greater than 98% was purchased from TCI America (Tokyo, Japan). Using high performance liquid chromatography (HPLC), the radiochemical purity of ethyl-labeled urethane was determined to be greater than 99%.

Animals and Treatment: Male CYP2E1+/+ (wild-type) and CYP2E1-/- (CYP2E1-null) mice were 8-9 months old and ranged in weight form 28-42g. Mice were obtained from a colony developed at the National Cancer Institute, Bethesda, MD and were re-derived and bred at Charles River Laboratories (Lee *et al*., 1996, Hoffler *et al*., 2003). Animals were individually housed in an animal facility with a 12-hr light-dark cycle and fed NIH #31 diet and water *ad libitum* throughout the study. All animal care and experimentation were conducted according to NIH guidelines (U.S. Department of Health and Human Services 1986). Dosing solutions were made in tap water using a mixture of radiolabeled and unlabeled urethane. All urethane-dosing solutions were made fresh and administered by gavage at either 10 or 100 mg/kg; delivering 100 μCi/kg in a dose volume of 10 ml/kg.

Experimental design: Groups (4-8 animals each) of CYP2E1-/- and CYP2E1+/+ mice were administered urethane by gavage as follows:

1. CYP2E1-/- and CYP2E1+/+ mice received a single 10 or 100 mg ^{14}C-ethyl-labeled urethane/kg and euthanized at 24 hr after urethane administration.
2. CYP2E1-/- mice received 100 mg ^{14}C-ethyl-labeled urethane/kg/day for 5 consecutive days and were euthanized at 24 hr after the last dose.

Immediately after urethane administration, mice were housed in individual glass metabolism cages (Wyse Glass Specialties, Inc. Freeland, MI) for 24 or 120 hr, which allowed for the separate collection of urine, feces, and exhaled radioactivity. A vacuum system was attached to the glass cages that permitted the passage of air through the cage at a flow rate of 0.6-0.8 L/min. Air passing through the cage was initially passed through solid calcium sulfate and soda lime to reduce moisture and CO_2 content, thus extending the efficiency of the trapping solutions over time. Air exiting the cage was passed through a series of traps. The first trap was an activated charcoal trap (SKC Inc., Eighty Four, PA) used to adsorb organic volatiles exhaled by urethane treated mice. A second trap containing 400 ml ethanol was used to capture exhaled organic volatiles that escape adsorption by the charcoal. Air was then passed through a trap containing approximately 400 ml of 7:3 (v/v) mixture of ethylene glycol monomethyl ether and ethanolamine for collection of expired $^{14}CO_2$. CO_2 traps were changed at 1, 2, 4, 6, 8, and 24 hours after urethane administration in the single dose study. For the 5day study, $^{14}CO_2$ traps were changed at 2, 4, 8, 16, and 24 hr post each daily dose of urethane. Ethanol traps were changed every 24 hours. Urine, feces and charcoal traps were collected every 24 hours after dosing. Urine and charcoal traps were stored at -80 ^{0}C to be analyzed at a later time. At the end of the holding periods, mice were euthanized by CO_2 asphyxiation, blood was collected from animals by cardiac puncture, and major tissues were dissected and stored at –60 to -80 ^{0}C for analysis at a later time. Tissue and whole blood samples weighing between 25 and 50 mg were sampled in triplicates and ^{14}C content was quantitated via oxidation to $^{14}CO_2$ using a Packard Tri-Carb sample oxidizer (Packard Instruments Co., Meriden, CT). Collected blood from 24 and 120 hours was centrifuged to separate

RBC's and plasma and urethane-derived radioactivity was also quantitated using the tissue oxidizer. Feces were air-dried, ground to a fine powder, weighed, and similarly analyzed in triplicate. Charcoal traps were opened; charcoal contents was weighed, and analyzed in triplicates using the sample oxidizer. Recovery of radioactivity from the sample oxidizer was greater than 95%. Oxidized samples as well as triplicate aliquots of the $^{14}CO_2$ and organic volatiles trapping solutions (1 ml) and urine (50 µl) were mixed with Ecolume (Cosa Mesa, CA) and counted using a Beckman Model LS 9800 scintillation counter (Beckman Instruments, Fullerton, CA).

HPLC Analysis of Plasma, Liver homogenates, and Urine: The metabolite profile of plasma, liver homogenates, and urine samples from CYP2E1+/+ and CYP2E1-/- mice treated with 100 mg ^{14}C-ethyl-labeled urethane/kg for 24 hr or daily for 5 consecutive days were analyzed by HPLC. Individual plasma, liver homogenates, and urine samples were centrifuged at 14,000x*g* for 20 min at 4°C and 100 µl of supernatant were directly injected into the HPLC. Small aliquots (10 µl) of plasma and urine supernatant were also analyzed for total radioactivity using a Beckman scintillation counter as previously described. The HPLC system consisted of a Waters 2690 Separations Module (Waters, Milford, MA) connected on line with a UV detector followed by a 515T Radiomatic Flow Scintillation Analyzer (Packard Instrument Co., Meriden, CT). Samples were analyzed using a 4.6 x 250 mm C18 Microsorb-MV column (Rainin Instrument Co., Woburn, MA) with Security guard column (Phenomenex, Torrance, CA) using a gradient consisting of 0.1% trifluroacetic acid to 70% acetonitrile over 25 min at a flow rate of 1 ml/min. UV-absorbing peaks were monitored at 210 nm. Radioactive peaks were detected using a 500 µl flow cell with Ultima Flo-M scintillation fluid (Packard

Instrument Co., Meriden, CT) at 3 ml/min. Parent urethane was identified in the urine, plasma, and liver homogenates by comparing the retention times of these biological samples to the retention time of ^{14}C-ethyl-labeled urethane standard. Further, biological samples were spiked with ^{14}C-ethyl-labeled urethane and analyzed using HPLC in order to confirm the presence of parent compound.

Statistical Analysis: Group mean comparisons were performed using Student's *t* test two-tailed assuming equal variances. Values were considered statistically significant at $P \leq 0.05$.

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Results

Metabolism and disposition of a single dose of ^{14}C-ethyl- labeled urethane:

Within twenty-four hours after a single administration of either 10 or 100 mg/kg ^{14}C-ethyl-labeled urethane, approximately 80% of the total administered dose was eliminated in the expired air of CYP2E1+/+ mice as $^{14}CO_2$ (Table 1, Figure 2). In contrast, CYP2E1-/- mice receiving the same treatment eliminated less than 40% of the total dose as $^{14}CO_2$ in 24 hours (Table 1, Figure 2). While ^{14}C-ethyl-labeled urethane-derived radioactivity eliminated as organic volatiles in CYP2E1+/+ mice comprised less than 1% of either the high or low doses; elimination in CYP2E1-/- mice via this route accounted for 3 and 4.2%, respectively (Table 1). In both CYP2E1+/+ and CYP2E1-/- mice, fecal excretion of ^{14}C-ethyl-labeled urethane-derived radioactivity was negligible and accounted for less than 1% of the total administered urethane doses (Table 1). No significant differences were evident in the % of dose excreted as ^{14}C-ethyl-labeled urethane-derived radioactivity in the urine regardless of the genotype or dose administered, 3.1 to 4.7% of dose (Table 1). However, HPLC analysis of urine identified greater than 62% of the ^{14}C-ethyl-labeled radioactivity as un-metabolized urethane in CYP2E1-null mice; while in CYP2E1+/+ mice, the majority of the urinary radioactivity (>70 %) was metabolites with parent urethane accounting for the remainder.

Notable differences and similarities were evident when comparing the current data on the metabolism and disposition of a single dose of ^{14}C-ethyl vs. our earlier work using ^{14}C-carbonyl-labeled urethane in CYP2E1-/- and CYP2E1+/+ mice (Hoffler et al. 2003). Regardless of the location of the radiolabel and independent of dose, exhalation of urethane-derived CO_2 in CYP2E1+/+ mice increased in a dose-dependent manner and

plateaued within 8 hours of exposure (Figure 2; Hoffler *et al*. 2003). An approximate 50% decrease in urethane-derived $^{14}CO_2$ exhalation was determined at both the 10 and 100 mg/kg doses of either ^{14}C-ethyl- or -carbonyl-treated CYP2E1-/- mice (Figure 2; Hoffler *et al*. 2003). Although, the average rate of $^{14}CO_2$ exhalation (% dose/hr) remained relatively constant in CYP2E1-/- mice during the entire 24 hrs after exposure to either the high or low ^{14}C-ethyl- or -carbonyl-labeled urethane doses, CYP2E1+/+ mice administered ^{14}C-ethyl-labeled urethane experienced a minor delay in the production of exhaled $^{14}CO_2$ in the first 4 hr after treatment (data not shown) in comparison to ^{14}C-carbonyl-labeled urethane (Hoffler *et al*. 2003). At the end of 24 hours it was evident that the cumulative % of doses eliminated as exhaled $^{14}CO_2$ slightly decreased in mice treated with ^{14}C-ethyl-labeled urethane in contrast to mice similarly treated with the ^{14}C-carbonyl-labeled chemical (Table 1; Hoffler *et al*. 2003). Elimination of urethane-derived radioactivity as exhaled organic volatiles, urinary, or fecal metabolites despite the location of the radiolabel were considered minor pathways. However, significant differences in the disposition of ^{14}C-ethyl-labeled urethane-derived radioactivity were observed in both CYP2E1+/+ and CYP2E1-/- mice administered 10 mg/kg in comparison to mice exposed to ^{14}C-carbonyl-labeled urethane at the same dose. CYP2E1+/+ mice treated with 10 mg/kg ^{14}C-ethyl-labeled urethane exhibited a 3-fold increase in the cumulative % of dose eliminated as organic volatiles in comparison to CYP2E1+/+ mice administered 10 mg/kg ^{14}C-carbonyl-labeled urethane (Table 1, Hoffler *et al*. 2003). Furthermore, 10 mg/kg ^{14}C-ethyl-treated CYP2E1+/+ mice eliminated 2-fold higher % of dose as urinary metabolites than its ^{14}C-carbonyl-treated counterparts. On the other hand, CYP2E1-/- mice administered the same ^{14}C-ethyl-labeled urethane treatment exhibited a

2-fold decrease in the % of dose eliminated via renal excretion in comparison to 10 mg/kg ^{14}C-carbonyl-treated CYP2E1-null mice (Table 1; Hoffler *et al*. 2003. In regards to the cumulative % of dose excreted as fecal metabolites, discernible differences were not apparent in mice treated with either ^{14}C-ethyl- or -^{14}C-carbonyl-labeled urethane (Table 1; Hoffler *et al*. 2003).

In general, the concentration of ^{14}C-ethyl-labeled urethane-derived radioactivity detected in the blood and tissues increased in a dose-dependent manner in both mouse genotypes, but were considerably higher in CYP2E1-/- mice independent of the location of the radiolabel (Table 2). Significant distinctions, however, were observed in the blood and tissues of CYP2E1+/+ mice. Twenty-four hours after exposure to ^{14}C-ethyl-labeled urethane, a 3-16-fold increase in the concentration of ^{14}C-ethyl-labeled urethane-derived radioactivity was detected in the blood and tissues of these mice vs. CYP2E1+/+ treated with ^{14}C-carbonyl-labeled urethane (Table 2, Hoffler *et al.* 2003).

Metabolism and disposition of ^{14}C-ethyl-labeled urethane administered daily for 5 consecutive days:

Inhibition of urethane metabolism to $^{14}CO_2$ continued after multiple doses of urethane (Figure 3). Exhalation of $^{14}CO_2$ in CYP2E1+/+ mice after each daily dose of 100 mg ^{14}C-ethyl-labeled urethane/kg exhibited a pattern similar to that seen after a single dose (Figure 3). Maximum $^{14}CO_2$ exhalation was achieved in CYP2E1+/+ mice after the second day of dosing and steady state levels were maintained with approximately 85% of the daily dose eliminated as respired $^{14}CO_2$. In CYP2E1-/- mice, maximum exhalation was delayed and observed after 3 days (approximately 52% of the daily doses) (Figure 3). An average of 28 and 13% of the 5 cumulative ^{14}C-ethyl-labeled

urethane doses was eliminated as urethane-derived radioactivity in the urine and organic volatiles of CYP2E1-/- mice, respectively. CYP2E1+/+ mice, however, eliminated merely 10 and 3.5% of the 5 doses, respectively, via these routes.

Although, elimination of urethane-derived radioactivity via renal excretion was considered a minor metabolic route, there were significant differences in the HPLC profiles of urinary metabolites from CYP2E1-/- and CYP2E1+/+ mice (Figure 4). Twenty-four hours after a single dose of ^{14}C-ethyl-labeled urethane was administered to CYP2E1+/+ mice, approximately 28% of the total radioactivity excreted in the urine was identified as un-metabolized urethane. In CYP2E1-/- mice, urethane comprised greater than 70% of the total radioactivity excreted in the urine (Figure 4). Furthermore, after Day 1, urethane constituted less than 10% of the total urinary radioactivity in CYP2E1+/+ mice, while in CYP2E1-/- mice it comprised 60-70% through Day 5 (Figure 4).

Plasma and tissue concentrations of urethane-derived radioactivity in both CYP2E1+/+ and CYP2E1-/- mice were markedly higher in mice administered 5 ^{14}C-ethyl-labeled urethane exposures than those receiving a single treatment (Table 2). HPLC analysis showed that most of the urethane-derived radioactivity detected in the plasma and liver homogenates of CYP2E1-/- mice that received a single ^{14}C-ethyl-labeled urethane dose was parent compound (Figure 5). In CYP2E1-/- mice administered 5 daily doses, approximately 83-88% of the urethane-derived radioactivity identified in the plasma and liver homogenates was un-metabolized urethane (Figure 5). Parent urethane was not detected in plasma and tissues of CYP2E1+/+ mice administered either a single or multiple urethane dose. In comparing the current data on the bioaccumulation

of ^{14}C-ethyl-labeled vs. ^{14}C-carbonyl-labeled urethane (Hoffler *et al.* 2003), it was clear that ^{14}C-ethyl-labeled urethane has a greater tendency to be retained in mice of both genotypes.

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Discussion

Numerous studies investigating urethane's metabolism and disposition have suggested the involvement of two pathways; the first entails hydrolysis via esterase and was thought to be responsible for greater than 90% of urethane's metabolism to $^{14}CO_2$ (Figure 1; Skipper *et al*.1951; Kaye 1960; Mirvish and Kaye 1964; Nomier *et al*. 1989; Salmon and Zeise ed. 1991). The second pathway which was considered minor, proposed dehydrogenation of urethane to form vinyl carbamate (VC) followed by P450-mediated oxidation of this intermediate to form vinyl carbamate epoxide (VCE) (Figure 1; Dahl *et al*. 1978, 1980; Guengerich and Kim 1991). Contrary to earlier assertions, a recent report from this laboratory using ^{14}C-carbonyl-labeled urethane showed for the first time that CYP2E1, and not esterase, was the principal enzyme responsible for greater than 96% of urethane's metabolism (Hoffler *et al*. 2003). The contributions of other P450s and esterase were estimated at approximately 3 and 0.5% of the dose, respectively (Hoffler *et al*. 2003). Lawson and Pound (1973) suggested that covalent binding to mouse liver DNA *in vivo* requires the oxidation of the ethyl moiety of urethane. This finding in addition to our recent report demonstrating an essential role for CYP2E1 in urethane metabolism and an increase in the half-life of urethane in CYP2E1-/- vs. CYP2E1+/+ (Hoffler *et al*. 2003) mice prompted this investigation. The objectives of the current studies included: 1) investigating the metabolism and disposition of ^{14}C-ethyl-labeled urethane in CYP2E1-/- and CYP2E1+/+ mice and to compare this data to our earlier results using ^{14}C-carbonyl-labeled urethane (Hoffler et al. 2003); 2) assess the consequences of lack of CYP2E1 on the metabolism and disposition of single vs.

multiple doses of ^{14}C-ethyl-labeled urethane in mice of both genotypes; and 3) characterize the bioaccumulation of ^{14}C-ethyl-labeled urethane in mice of both genotypes after multiple doses.

After gavage administration, urethane was rapidly absorbed and distributed to all major tissues. Current work showed that metabolism and elimination of ^{14}C-ethyl-labeled urethane via CO_2 was the predominant pathway in CYP2E1+/+ mice, however to a lesser measure than in CYP2E1+/+ mice treated with a similar dose of ^{14}C-carbonyl-labeled urethane (Hoffler *et al.* 2003). In CYP2E1+/+ mice administered ^{14}C-ethyl-labeled urethane, 73-77% of the dose was exhaled as $^{14}CO_2$ within 8 hours (Figure 2), while CYP2E1+/+ mice treated with ^{14}C-carbonyl-labeled urethane eliminated 90-94% of the dose via the same route in 8 hr (Hoffler *et al.* 2003). In CYP2E1-/- mice and regardless of the location of the label, animals exhaled 15-20% of the administered dose of urethane as $^{14}CO_2$ within 8hr after dosing. On average, 38% of a single ^{14}C-ethyl- or ^{14}C-carbonyl-labeled urethane dose was exhaled as $^{14}CO_2$ in 24hr after exposure of CYP2E1-/- mice. Variations in the % of dose eliminated as organic volatiles, urinary, and fecal metabolites were minor when comparing the disposition of ^{14}C-ethyl- vs. ^{14}C-carbonyl-labeled urethane. This data confirmed our earlier findings that CYP2E1 plays an essential role in the metabolism of urethane. Further, this data showed that a greater percentage of ^{14}C-carbonyl-labeled urethane doses were metabolized to CO_2 vs. ^{14}C-ethyl-labeled urethane. The decrease in CO_2 elimination was associated with greater retention of ^{14}C-ethyl-labeled urethane in blood and tissues of CYP2E1+/+ mice. In the current work, 24hrs after a single ^{14}C-ethyl-labeled urethane exposure of CYP2E1+/+ mice, the majority of tissues known to be targets of urethane had exceedingly higher

concentrations of radioactivity than in CYP2E1+/+ mice treated with ^{14}C-carbonyl-labeled urethane. These tissues included the brain (16-fold), spleen (14-fold), liver and thymus (13-fold), and lung (12-fold). These data are in agreement with Lawson and Pound's earlier work (1973), which concluded that metabolites of the ethyl- and not the carbonyl-carbons of urethane bind to mouse DNA *in vivo*.

Bioaccumulation of ^{14}C-ethyl-labeled urethane-derived radioactivity increased as a function of the number of doses administered to CYP2E1+/+ and CYP2E1-/- mice. However, in the absence of CYP2E1-mediated metabolism, a significantly greater retention of ^{14}C-ethyl-labeled urethane-derived radioactivity was detected in CYP2E1-/- vs. CYP2E1+/+ mice. HPLC analysis showed that while urethane was the primary chemical in the plasma and liver homogenates of CYP2E1-/- mice, it was not identifiable in CYP2E1+/+ mice. In a subsequent study using doses as high as 1000 mg/kg, significant prolongation of urethane-induced anesthesia was observed only in CYP2E1-/- mice administered urethane (unpublished data); confirming that the anesthesia was caused by um-metabolized urethane. Recent studies in this laboratory also demonstrated significant inhibition of urethane-induced genotoxicity and cell proliferation in CYP2E1-/- vs. CYP2E1+/+ mice (Hoffler *et al*. 2005). This supports the hypothesis that CYP2E1-mediated metabolism, presumably via epoxidation across the ethyl carbons, is a pre-requisite for the induction of genotoxicity and carcinogenicity by urethane.

Metabolism of both ^{14}C-ethyl- and ^{14}C-carbonyl-labeled urethane in CYP2E1+/+ mice produced considerable amounts of CO_2. However, until recently, it was thought enzymatic hydrolysis of urethane via esterase to ethanol and subsequent metabolism via alcohol and aldehyde dehydrogenases was the primary route for the formation of CO_2

(Figure 1). Because our recent findings indicated that the contribution of esterase to urethane metabolism was less than 1% of dose (Hoffler *et al*. 2003), it was concluded that esteric cleavage was insufficient to justify the high percentage of ^{14}C-ethyl-labeled urethane that converted to $^{14}CO_2$ as well as explain the potent carcinogenicity of this chemical. As a result, we proposed for the first time that C-hydroxylation, catalyzed primarily by CYP2E1 is an important contributor to urethane metabolism (Figure 1). This pathway explains $^{14}CO_2$ production in mice treated with ^{14}C-ethyl-labeled urethane and presents a second and/or alternate metabolic route for the generation of $^{14}CO_2$ from ^{14}C-carbonyl-labeled urethane (Figure 1). It was reported that P450s could oxidatively cleave esters, leading to the formation of aldehydes or ketones from the alcohol moiety of the C-hydroxylated metabolite (Guengerich 1987; Guengerich *et al*. 1988; Peng *et al*. 1995; Guengerich 2001). Cleavage of many alkyl esters such as ethyl acetate, ethyl formate, and ethyl propionate via CYP2E1 has also been reported (Peng *et al*. 1995). Additionally, C-hydroxylation may provide another route for the formation of vinyl carbamate via dehydration of α-hydroxyurethane (Figure 1).

Bioactivation of chemicals via dehydrogenation and subsequent P450-mediated oxidation is not unique to urethane. Aflatoxin B_2 and some alkenylbenzene carcinogens such as safrole are metabolized via this pathway (Miller and Miller, 1983). The dehydrogenation product of urethane, VC, was shown to be a more potent carcinogen and mutagen than either ethyl N-hydroxycarbamate (N-hydroxyurethane) or urethane (Dahl *et al*. 1978, 1980). Initial studies by Dahl *et al*. (1978, 1980) demonstrated difficulties in identifying VC as a metabolite of urethane either *in vivo* or *in vitro*. Subsequent studies using GC/MS, however, were successful in characterizing VC as a microsomal oxidation

product of urethane (Guengerich and Kim 1991). At present, isolation of VC *in vivo* has not been achieved. Oxidation of the ethyl moiety enables binding of the ethyl carbons to tissue macromolecules that may in turn foster adducts formation and subsequent mutagenesis and tumorigenesis presumably through VCE. Because of the highly reactive nature of epoxides, VCE has been termed as the ultimate carcinogenic metabolite of urethane. Numerous studies have identified etheno adducts of DNA and RNA in target tissues of urethane-induced carcinogenicity (Ribovich *et al.* 1982; Park *et al.* 1990; Fernando *et al.* 1996). In comparison to urethane, VC and VCE-derived adducts were increased in target vs. nontarget tissues (Miller and Miller 1983; Ribovich *et al.* 1982; Park *et al.* 1990; Fernando *et al.* 1996). Collectively these studies established that the formation of adducts resulted from the bioactivation of urethane at the ethyl carbons and not its hydrolysis product, ethanol.

In conclusion, current work supports our earlier findings demonstrating the predominance of CYP2E1 in urethane metabolism. In comparison to our earlier work using ^{14}C-carbonyl-labeled urethane (Hoffler *et al*. 2003), current data demonstrated that there is greater retention of ^{14}C-ethyl-labeled urethane-derived radioactivity in the blood and tissues of CYP2E1+/+ mice in association with a decrease in the exhalation of $^{14}CO_2$. Further, bioaccumulation of ^{14}C-ethyl-labeled urethane increased as a function of the number of administered urethane doses. Regardless of the position of the radiolabel or the number of urethane doses, there was significantly greater bioaccumulation in CYP2E1-/- vs. CYP2E1+/+ mice. While the majority of ^{14}C-ethyl-labeled urethane-derived radioactivity found in the blood and tissues of CYP2E1-/- mice was un-metabolized urethane, parent compound was not identified in CYP2E1+/+ mice. Collectively, our

current and earlier studies suggested that both carbonyl- and ethyl-carbons were extensively incorporated into urethane-derived CO_2. We therefore proposed for the first time that urethane undergoes CYP2E1-mediated C-hydroxylation. This pathway explains CO_2 production from the ethyl or carbonyl carbons of urethane and leads to the formation of VCE. Recent studies in this laboratory demonstrated significant attenuation of urethane-induced genotoxicity and cell proliferation in CYP2E1-/- vs. CYP2E1+/+ mice (Hoffler *et al.* 2005). Additionally, 1,N^6-ethenoadenosine adducts formation in the lungs of CYP2E1-/- mice exposed to urethane were reduced in comparison to urethane-treated CYP2E1+/+ mice (our unpublished data). These findings support the hypothesis that CYP2E1-mediated metabolism, presumably via the epoxidation across the ethyl carbons, is a pre-requisite for the induction of genotoxicity and carcinogenicity by urethane.

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References

Benson RW and Beland FA (1997) Modulation of urethane (ethyl carbamate) carcinogenicity by ethyl alcohol: A review. *International Journal of Toxicology* **16**(6): 521-544.

Dahl G, Miller JA, and Miller EC (1978) Vinyl Carbamate as a promutagen and a more carcinogenic analog of ethyl carbamate. *Cancer Research* **38**: 3793-3804.

Dahl G, Miller EC, and Miller JA (1980) Comparative carcinogenicities and mutagenicities of vinyl carbamate of vinyl carbamate, ethyl carbamate, and ethyl-N-hydroxycarbamate. *Cancer Research* **40**: 1194-1203.

Fernando RC, Nair J, Barbin A, Miller JA, and Bartsch H (1996) Detection of 1,N^6-ethenodeoxy-adenosine and 3,N^4-ethenodeoxycytodine by immunoaffinity/^{32}P-postalbelling in liver and lung DNA of mice treated with ethylcarbamate (urethane) or its metabolites. *Carcinogenesis* **17**: 1711-1718.

Guengerich FP (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Tox* **14**:612-640.

Guengerich FP and Kim DH (1991) Enzymatic oxidation of ethyl carbamate to vinyl carbamate and its role as an intermediate in the formation of 1, N^6- Ethenoadenosine. *Chem Res Toxicol* **4**(4): 413-421.

Guengerich FP, Peterson LA, and Böcker RH (1988) Cytochrome P450-catalyzed hydroxylation and carboxylic acid ester cleavage of hantzsch pyridine esters. *J Biol Chem* **263**:8176-8183.

Guengerich FP (1987) Oxidative cleavage of carboxylic esters by cytochrome P450. *J Biol Chem* **262**:8459-8462.

Hoffler U and Ghanayem BI (2003) Inhibition of urethane metabolism and bioaccumultion in cytochrome P4502E1-null mice. *Toxicol Sci* **72**:1546 Supplement

Hoffler U, El-Masri HA, and Ghanayem BI (2003) Cytochrome P4502E1 (CYP2E1) is the Principal Enzyme Responsible for Urethane Metabolism: Comparative Studies Using CYP2E1-/- and CYP2E1+/+CYP2E1+/+ Mice. *J Pharmocol Exp Therap* **305**:557-564.

Hoffler U, Dixon D, Peddada S, and Ghanayem BI (2005) Inhibition of Urethane-Induced Genotoxicity and Cell Proliferation in CYP2E1-/- Mice. *Mutat Res-Fund Mol Mutagen* 572:58-72.

IARC (1974) *Urethane. In monographs on the carcinogenic risk of chemicals to man: some antithyroid and related substances, nitrofurans and industrial chemicals.* Lyon, France, International Agency for Research on Cancer. **7:** 111-140.

Lawson TA and Pound AW (1973) The interaction of carbonyl-14 labelled alkyl carbamates labelled in the alkyl and carbonyl positions, with DNA *in vivo*. *Chem Biol Interact* 6:99-105

Lee SST, Buters JTM, Pineau T, Fernandez-Salguero P, and Gonzalez FJ. (1996) Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem.* 271:12063-12067.

Nomeir AA, Ioannou YM, Sanders JM, and Matthews HB (1989) Comparative metabolism and disposition of ethyl carbamate (urethane) in male Fischer 344 rats and male B6C3F1 mice. *Toxicol Appl Pharm* **97**(2): 203-215.

Miller JA and Miller EC (1983) The metabolic activation and nucleic acid adducts of naturally occurring carcinogens: recent results with ethyl carbamate and the spice flavors safrole and estragole. *Br J Cancer* **48**(1):1-15.

NTP (2000) *Report on Carcinogens*, 9th Edition. RTP, NC, National Institute of Environmental Health Sciences, National Institutes of Health.

Page DA and Carlson GP (1994) The effect of pyridine on the *in vitro* and *in vivo* metabolism of ethyl carbamate (urethane) by rat and mouse. *Carcinogenesis* **15**(10): 2177-2181.

Park KK, Surh YJ, Stewart BC, and Miller JA (1990) Synthesis and properties of vinyl carbamate epoxide, a possible ultimate electrophilic and carcinogenic metabolite of vinyl carbamtae and ethyl carbamate. *Biochem. Biophys. Res. Commun.* **169**:1094-1098.

Peng HM, Raner GM, Vaz AND, and Coon MJ (1995) Oxidative cleavage of esters and amides to carbonyl products by cytochrome P450. *Arch Biochem Biophys* **318**:333-339.

Ribovich ML, Miller JA, Miller EC, and Timmins LG (1982) Labeled 1,N^6-ethenoadenosine and 3,N^4-ethenocytidine in hepatic RNA of mice given [ethyl-1,2-^{3}H or ethyl-1-^{14}C] ethyl carbamate (urethane). *Carcinogenesis* **3**:593-546.

Skipper HE, Bennett LL Jr, Bryan CE, White L Jr, Newton MA, Simpson L (1951). Carbamates in the chemotherapy of leukemia. VIII. Over-all tracer studies on carbonyl-labelled urethan, methylene- labelled urethane and methylene- labelled ethyl alcohol. *Cancer Res* **11**: 46-51.

U.S. Department of Health and Human Services, National Institutes of Health (1986) *Guide of the Care and Use of Laboratory Animals.* NIH Publication no.86-23, National Institutes of Health, Bethesda, MD.

Yamamoto T, Pierce WM Jr, Hurst HE, Chen D, and Waddell WJ (1988) Inhibition of the metabolism of urethane by ethanol. *Drug Metab Dispos* **16**(3): 355-358.

Yamamoto T, Pierce WM Jr, Hurst HE, Chen D, and Waddell WJ (1990) Ethyl carbamate metabolism – *in vivo* Inhibitors and *in vitro* enzymatic systems. *Drug Metab Dispos* **18**(3): 276-280.

Zimmerli B and Schlatter J (1991). Ethyl Carbamate: analytical methodology, occurance, formation, biological activity and risk assessment. *Mutation Res* 259: 325-350.

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Figure Legend

Figure 1. A proposed scheme showing the metabolism of (*)ethyl- and (+)carbonyl-labeled urethane.

Figure 2. Effects of dose and time on cumulative $^{14}CO_2$ exhalation in CYP2E1-/- and CYP2E1+/+ mice after gavage administration of ^{14}C- ethyl-labeled urethane. Values are presented as cumulative % of dose and are the mean ± SE of 4-8 mice. **a&b** Denotes significant differences in $^{14}CO_2$ elimination by the 2 genotypes of mice at 100 and 10mg/kg; **c** denotes a significant difference of $^{14}CO_2$ elimination at the 2nd hour between the 10 and 100 mg/kg treated CYP2E1+/+ mice.

Figure 3. Effects of dose and time on the daily pattern of cumulative $^{14}CO_2$ exhalation in CYP2E1-/- and CYP2E1+/+ mice after gavage administration of 100 mg/kg^{14}C-ethyl-labeled urethane/day for 5 consecutive days. Values are presented as % of the cumulative doses administered and are the mean ± SE of 4-8 mice. All values from CYP2E1-/- mice are significantly different in comparison to CYP2E1+/+ values.

Figure 4. Representative HPLC radiochromatograms of ^{14}C-ethyl-labeled urethane-derived radioactivity in the urine of CYP2E1-/- and CYP2E1+/+ mice after daily gavage administration of 100 mg urethane/kg for 5 days.

Figure 5. Representative HPLC radiochromatograms of ^{14}C-ethyl-labeled urethane-derived radioactivity in the plasma and liver homogenates of CYP2E1-/- mice 24 hr after either a single or daily gavage administration of 100 mg urethane/kg for 5 days.

Table 1: Summary of Ethyl-labeled Urethane (U) Disposition 24 hours after 10 or 100mg/kg Administration by Gavage

	100mg/kg [ethyl-1-^{14}C]U		***10mg/kg [ethyl-1-^{14}C]U***	
	CYP2E1 +/+	*CYP2E1 -/-*	*CYP2E1 +/+*	*CYP2E1 -/-*
CO_2	87.5±2.9	38.0±2.2[a]	78.5±3.6	30.0±4.2[a]
Organic Volatiles	0.7±0.1	3.0±0.5[a]	0.6±0.5	4.2±0.4[a]
Urine	3.1±0.5	4.7±0.8	3.7±0.3	3.2±0.6
Feces	*neg*	*neg*	*neg*	*neg*
Total % of dose	**91.3**	**45.7**	**82.8**	**37.4**

Neg (neglible)- value was less than 1%.
Values are presented as cumulative % of dose and are the mean±SE of 3-8 mice. [a] denotes CYP2E1-/- values that are statistically different from corresponding CYP2E1+/+ values (P≤0.05).

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Table 2. Concentration of Urethane-Derived Radioactivity in tissues of mice Treated with 10 and 100 mg 1-^{14}C-Ethyl-labeled Urethane/kg once or Daily for 5 Consecutive Days

	Single Dose				***5 Daily Doses***	
	10mg/kg		***100mg/kg***		***100mg/kg***	
Tissues	***CYP2E1*** +/+	***CYP2E1*** -/-	***CYP2E1*** +/+	***CYP2E1*** -/-	***CYP2E1*** +/+	***CYP2E1*** -/-
Blood	0.53±0.1	4.83±0.3	7.43±0.9	58.93±0.9	17.36±1.2	129±10.8
Brain	0.31±0.1	4.88±06	11.68±0.9	52.55±0.7	42.70±4.3	124.34±7.8
Fat	0.19±0.1	1.08±0.0	2.93±0.6	10.16±1.4	9.28±0.0	46.42±11.2
Forestomach	1.52±0.2	3.10±0.6	24.65±1.2	47.64±3.0	81.12±0.6	161.32±3.5
Glandular stomach	0.87±0.3	2.99±0.8	16.86±1.1	38.48±3.6	57.24±2.8	136.15±9.3
Lg. intestine	0.98±0.1	4.02±0.1	12.59±2.9	31.99±5.6	63.73±6.3	98.15±6.5
Sm. intestine	1.05±0.1	3.41±0.6	25.66±1.0	35.08±4.8	59.89±3.5	107.22±8.8
Kidneys	1.23±0.3	5.79±0.4	18.51±1.3	55.61±3.9	50.01±0.9	149.13±9.5
Liver	1.50±0.1	5.59±0.1	32.42±5.7	56.31±4.5	56.42±1.6	131.76±9.0
Lung	1.00±0.3	4.92±0.3	18.25±1.7	49.87±4.5	48.20±5.8	133.12±7.5
Muscle	0.42±0.5	3.64±0.5	3.47±0.3	39.70±3.6	10.28±0.6	98.78±11.8
Skin	0.46±0.2	3.15±0.2	8.11±0.9	45.86±1.4	20.61±1.5	102.61±11.5
Spleen	0.52±0.6	5.98±0.4	22.29±2.1	55.51±4.2	51.66±0.7	146.78±9.5
Testes	0.36±0.4	4.53±0.3	9.34±1.4	42.48±2.6	27.53±0.6	126.99±9.9
Thymus	0.40±0.1	3.35±0.2	18.01±4.7	53.14±5.9	41.77±3.8	105.67±13.6
Urinary bladder	1.10±1.0	4.76±1.3	15.31±2.5	52.78±2.8	37.68±3.0	145.40±11.9

Each value represents the mean±S.E. of at least 3 mice expressed as µg urethane equivalents/ g tissue.
All tissue concentrations of urethane-derived radioactivity are statistically different in single and multi-dosed ethyl-labeled CYP2E1-/- vs. similarly treated CYP2E1+/+ mice
All tissue concentrations of urethane-derived radioactivity are statistically different in both genotypes of mice given a single ethyl-labeled dose vs. mice receiving 5 daily urethane doses.

Figure 1

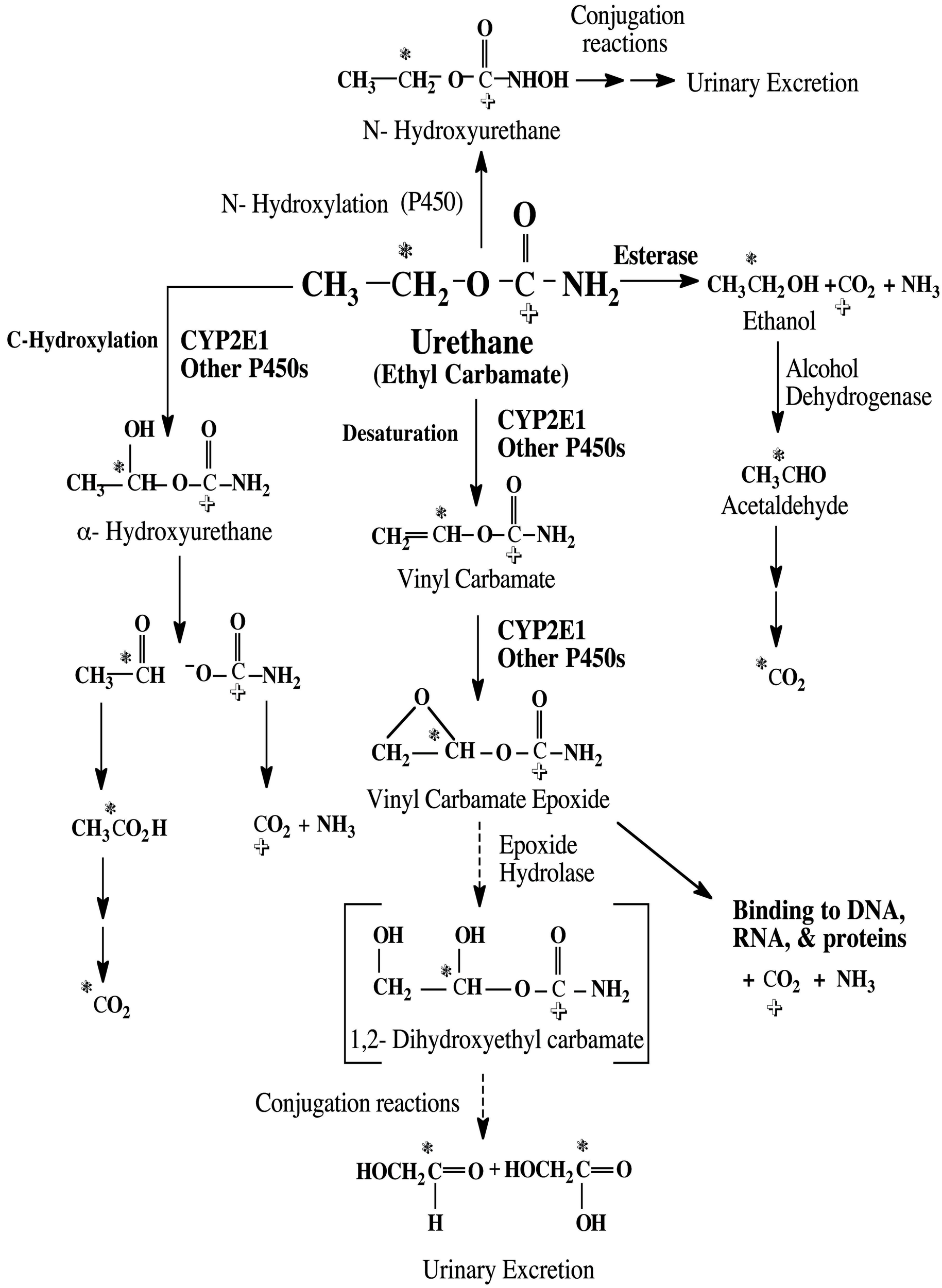

Figure 2

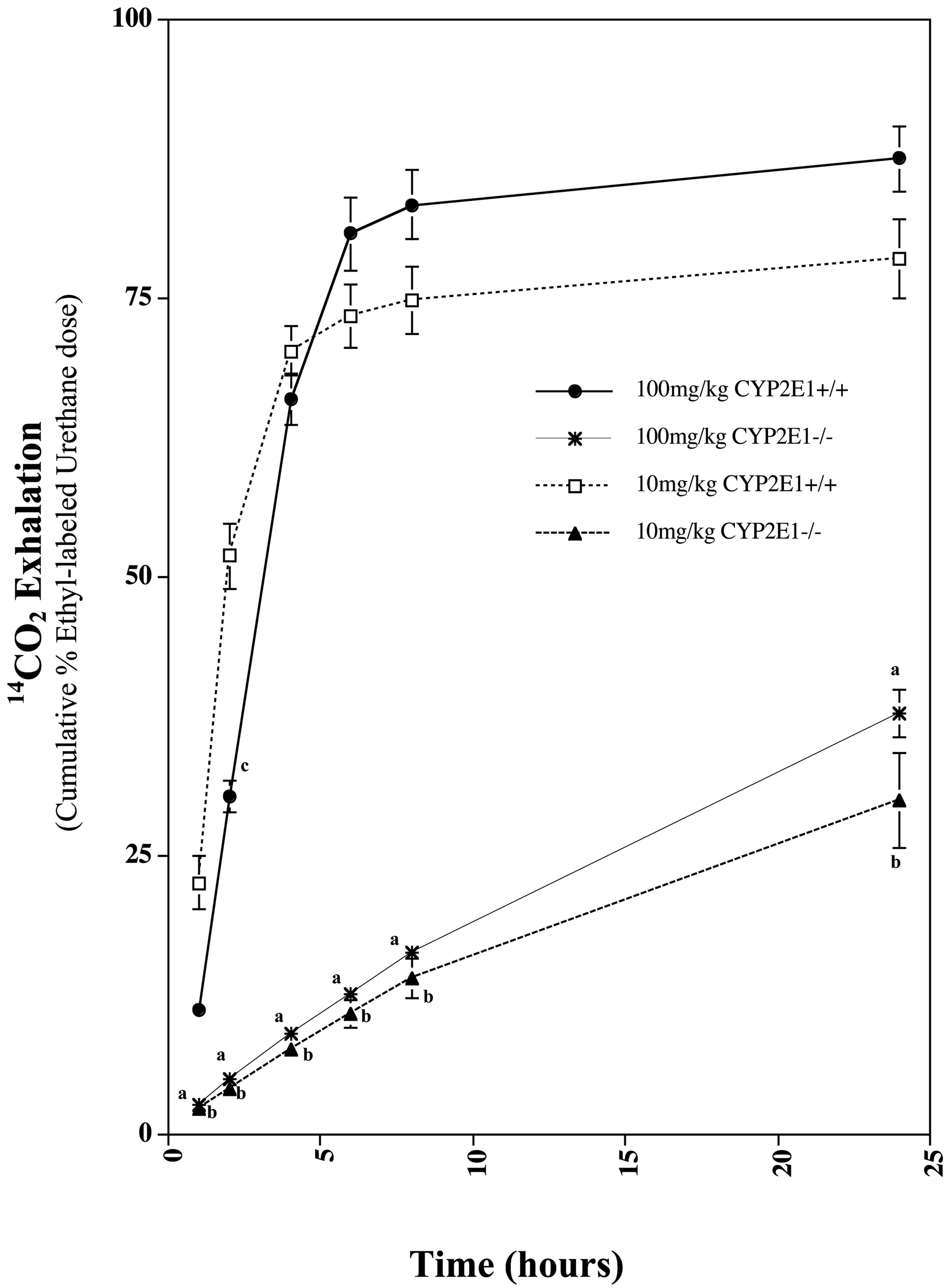

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Figure 3

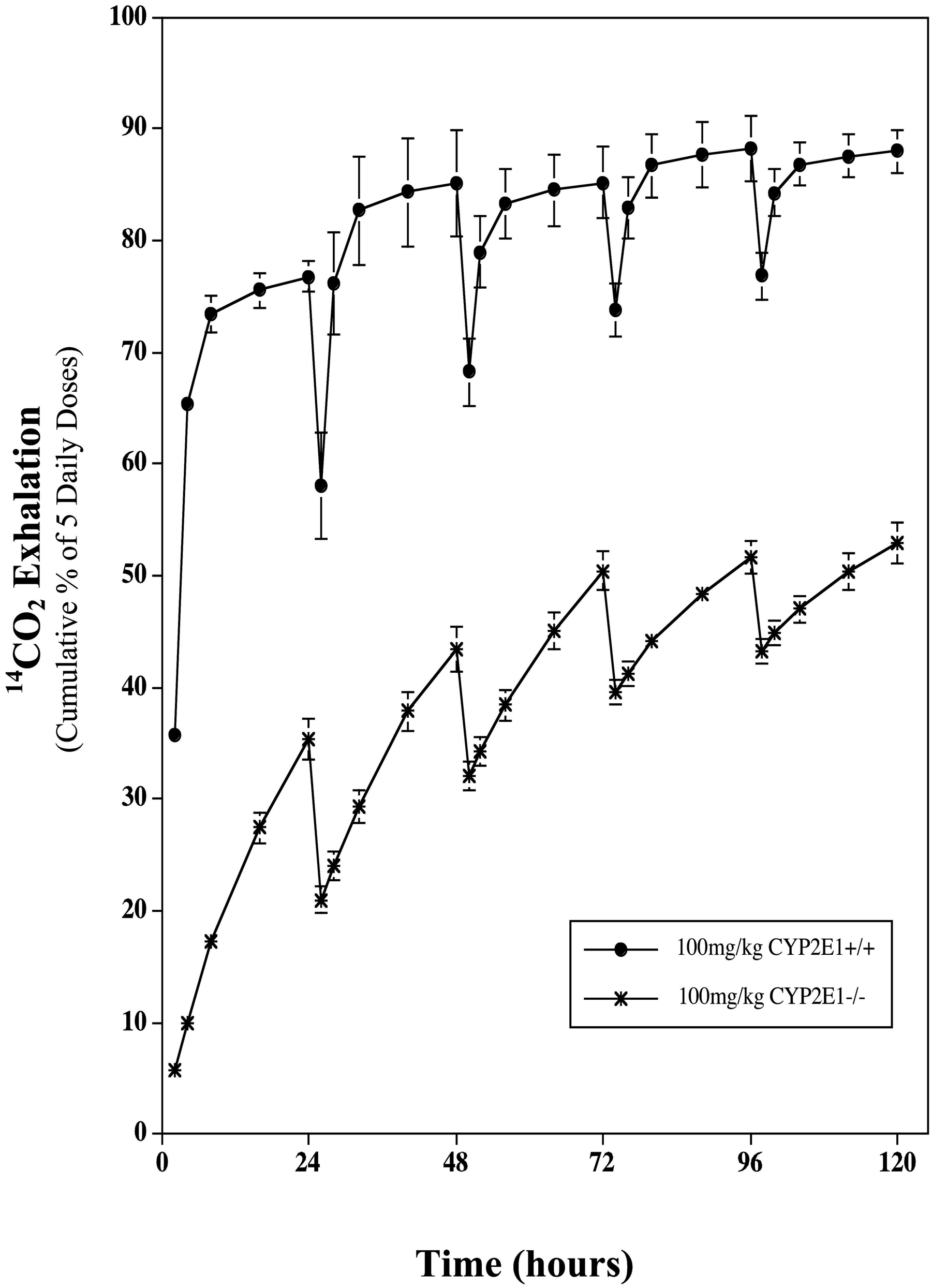

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Figure 4

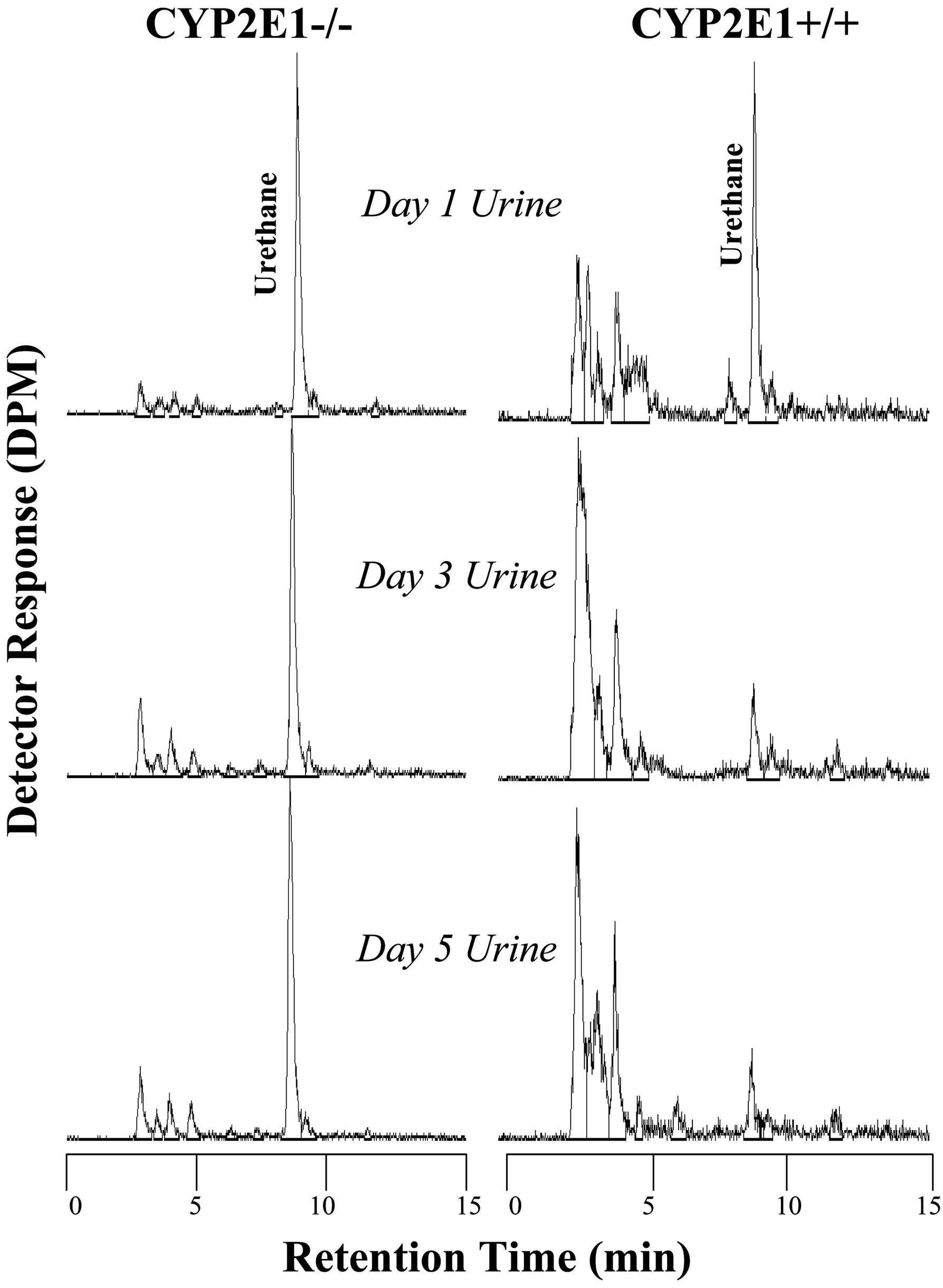

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Figure 5