Toluene and methylethylketone: effect of combined exposure on their metabolism in rat

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Multiple exposures are ubiquitous in industrial environments. In this article, we highlight the risks faced by workers and complete the data available on the metabolic impact of a common mixture: toluene (TOL) and methylethylketone (MEK).

Rats were exposed by inhalation under controlled conditions either to each solvent individually, or to mixtures of the two. How the interaction between the two solvents affected their fate in the blood and brain, their main relevant urinary metabolites (o-cresol, benzylmercapturic acid for TOL and 2,3-butanediols for MEK) and their hepatic metabolism were investigated.

Although the cytochrome P450 concentration was unchanged, and the activities of CYP1A2 and CYP2E1 isoforms were not additively or synergistically induced by co-exposure, TOL metabolism was inhibited by the presence of MEK (and vice versa). Depending on the relative proportions of each compound in the mixture, this sometimes resulted in a large increase in blood and brain concentrations. Apart from extreme cases (unbalanced mixtures), the amount of o-cresol and benzylmercapturic acid (and to a lesser extent 2,3-butanediols) excreted were proportional to the blood solvent concentrations.

In a co-exposure context, ortho-cresol and benzylmercapturic acid can be used as urinary biomarkers in biomonitoring for employees to relatively accurately assess TOL exposure.

Keywords: toluene, methylethylketone, ortho-cresol, benzylmercapturic acid, mixed exposure, metabolic interaction, occupational recommendations

Introduction

The ubiquity of solvents in manufacturing industries makes them the most frequent environmental hazards for workers. Among them, toluene (TOL), an aromatic hydrocarbon, is undoubtedly one of the most heavily and widely used. TOL is a major component in adhesives, paints, varnishes, printing inks, degreasers, fuel additives, glues, and thinners (Nagasawa et al., 2011, Estevan et al., 2012) and it is also used as feedstock in the manufacture of numerous products (ATSDR, 2015).

Chronic exposure to this aromatic solvent has been shown to affect the central nervous system (CNS), with the strongest evidence available in cases of solvent abuse (Lazar et al., 1983, Yamanouchi et al., 1995, Greenberg, 1997). In humans (but also in rats), neurological effects have been reported, including altered color vision, dizziness, fatigue, headache, and decreased performance in neurobehavioral tests (US Environmental Protection Agency, USEPA, 2005). TOL is also considered to be ototoxic and capable of modifying the effects of traumatic noise upon combined TOL-noise exposure (Loquet et al., 1999, Campo et al., 2007). All these potential toxic effects combined with regular reports of visual impairments led the American Conference of Governmental Industrial Hygienists (ACGIH) to recommend a time-weighted average threshold limit value for TOL of 20 ppm for an 8-h working day. Since 2012, 20 ppm (76.8 mg/m³) is also the 8h-occupational exposure limit (8h-OEL) for TOL in France (previously it was 50 ppm).

Recently, interactions between TOL and methylethylketone (MEK) (also known as butanone), a presumed non-toxic (or low-toxic) solvent used in large amounts in factories (8h-OEL = 200 ppm = 600 mg/m³), were highlighted as potentially leading to differences between blood concentrations of the parent compounds and levels of their metabolites excreted in urine (Cosnier et al., 2014). This observation was of concern because these solvents are frequently used together in industry. Indeed, according to the COLCHIC database

(Mater et al., 2016), 9473 individual measurements of TOL were performed in 1209 companies in France between 2005 and 2014. Of these measurements, 64% revealed a multiple exposure context, and the TOL/MEK mixture was present in almost 44% of companies. Statistical analysis of long-term measurements in manufacturing industry (which covers the vast majority of measurements) revealed that the 8h-OEL for TOL and MEK were exceeded in 3.3% and 0.6% of cases, respectively, with peaks exceeding 1400 ppm in both cases.

Even though the extent to which the 8h-OEL were exceeded remains low, it must not be forgotten that most OEL are established from results of studies involving exposure to single pure substances, and these values might be inappropriate when subjects are exposed to several chemicals simultaneously (Viau, 2002). Consequently, several authors, and the ACGIH, proposed an additivity formula to assess the risks encountered by workers faced with combined exposure. Thus, when two or more hazardous substances which have similar effects on the same organs are present in workplaces, their effects should be considered to be additive, unless established otherwise. Some tools, like the MIXIE database (<http://www.inrs-mixie.fr>) (Vyskocil et al., 2007), apply this approach to help occupational physicians to predict the risks faced by mixture-exposed workers.

For a biometrological assessment, Ogata et al. (1993) proposed a means to use a single biomarker to monitor exposure to a mixture when it can be assumed that its components act additively and that there are no toxicokinetic interactions between the elements composing the mixture. If there was interaction, biomonitoring data could be misinterpreted, but no formal recommendation suggests a method to adjust the biological indicators. Although they remain specialist tools, when they are available, physiologically based pharmacokinetic (PBPK) models can be useful (Haddad et al., 2001, Price and Krishnan, 2011). Some PBPK models dealing with TOL-containing mixtures (with aromatic solvents such as benzene, styrene,

xylenes, ethylbenzene and/or chlorinated compounds such as dichloromethane, chloroform, trichloroethylene and tetrachloroethylene) have been developed (Haddad et al., 1999, Haddad et al., 2000). These models are often developed by linking models for the individual chemicals at the assumed point of interaction, frequently hepatic metabolism. Following validation of the assumed mechanism, through comparison of model predictions and experimental data, the PBPK model can be used to predict the effects of co-exposure in a range of exposure scenarios. However, these models are often “limited” to the estimation of the blood concentration of the compound itself. Although this concentration mirrors the CNS concentration, and is therefore very useful, blood concentrations are rarely routinely monitored in occupational settings. Urinary excretion of the compounds or their metabolites are more commonly used, but only a few PBPK models predict urinary excretion (Marchand et al., 2015). Thus, we still require data that would allow efficient predictive modeling of these occupational scenarios (ATSDR, 2004).

In this study, we examined the metabolic impact of co-exposure to TOL and MEK in adult Brown Norway rats following inhalation exposure. Data on the fate of TOL and MEK in the blood and brain of rats and how the main relevant urinary metabolites of both solvents were affected by co-exposure were gathered and analyzed. In addition, the impact of the interaction on hepatic metabolism was assessed by measuring the GSH/GSSG balance, the total concentration of P450 cytochromes (CYP), and the activities of CYP1A2, CYP2E1, and glutathione S-transferase (GST) - the main enzymes involved in TOL metabolism (ATSDR, 2015).

Materials and methods

Animals

Four-week-old male Brown Norway (BN) rats were purchased from Janvier breeders (Le Genest St Isle, France). Two months before starting experiments, animals arrived at the animal facility where they were housed in individual cages (350 x 180 x 184 mm) on irradiated cellulose BCell8 bedding (ANIBED, Pontvallain, France). A04 irradiated pelleted food (SAFE, Augy, France) and tap water were available ad libitum. Animals were maintained on a 12 h/12 h day/night cycle. Room temperature and relative humidity in the animal facility were maintained at 22 ± 2 °C and $55 \pm 10\%$, respectively. The animal facility has full accreditation (C54-547-10), and the research described in this article was conducted in line with the Guide for Care and Use of Laboratory Animals promulgated by the European parliament and council (Directive, 2010/63/EU, 22 September 2010). The present study was approved by the local ethics committee and was registered as CELMEA-2012-0029. Animals weighed 250 ± 25 g at the time of conducting experiments.

Test materials

Toluene [puriss. p.a., ACS reagent, reag. ISO, reag. Ph. Eur., $\geq 99.7\%$ (GC) (Riedel de Haën ref # 32249)] and methylethylketone [puriss. p.a., ACS reagent, $\geq 99.5\%$ (GC) (Fluka ref # 04380)] were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All other chemicals used were of analytical grade.

Inhalation exposure

Groups of rats were exposed to TOL and/or MEK vapors (Table 1) in 200 L glass/stainless steel inhalation chambers. The stability of vapor generation was monitored and adjusted throughout exposure. Details of how test substances were generated and concentrations

controlled can be found in Cosnier et al. (2014).

Normal exposure duration was 6 hours/day (from 9:00 am to 3:00 pm), 5 days/week for 4 weeks; for 3 mixtures, the exposure duration was reduced to 1 day (6 hours) (Table 1). Unexposed rats (controls) were maintained in similar chambers, but exposed to clean air only. During the 6-hour exposure period no food or water was available. All the exposure parameters: temperature (22 ± 2 °C), humidity ($55 \pm 10\%$), air flow in the inhalation chambers, test substance concentrations, etc. were determined in accordance with OECD guideline No. 412 for testing of chemical toxicity (Subacute Inhalation Toxicity: 28-Day Study). Thus, individual chamber concentration samples never deviated by more than $\pm 10\%$ from the mean chamber concentration.

For each 1-month exposure campaign, three groups of rats were studied. Urine was collected from the first group exposed to solvent(s) ($n = 12$); blood was collected and organs (liver, brain and kidneys) were harvested from the second group exposed to solvent(s) ($n = 2 \times 8$); blood and organs were also collected from the third group, control animals ($n = 2 \times 8$). For the 1-day inhalation exposures to mixtures, only a single group was analyzed ($n = 8$) based on assays of blood concentrations.

[Table 1 near here]

Urine collection

Five time points were considered for urine collection: the first sample was taken 3 days before starting the inhalation exposure (control time = d0) and subsequent samples were taken after the first (d1), the fifth (d5), the tenth (d10) and the twentieth (d20) days of exposure. For urine collection, animals were placed in individual metabolic-type stainless steel cages with free access to food and water from 3:00 pm to 9:00 am the following day. During this 18-h period, urine was collected and refrigerated by a cooling system surrounding the collection tubes. Urine samples were frozen immediately after collection and stored at -20 °C. They were

thawed just before analysis.

Urinary analysis

Creatinine concentrations in urine were determined by the Jaffe method using a creatinine test kit (Randox Laboratories, Roissy en France, France). For the purposes of this study, creatinine results were primarily used to identify possible extreme values of metabolite concentrations.

The different urinary metabolites - ortho-cresol (oCre) and benzylmercapturic acid (BMA) for TOL, and 2,3-butanediols ($\pm$ 23BD and meso23BD) for MEK - were measured as previously described (Cosnier et al., 2012, Cosnier et al., 2013, Cosnier et al., 2017).

Blood and organ collection

Blood samples were collected from eight rats each from the second and third groups, at d1 and d5, and from the remaining eight rats per group at d10 and d20. Rats were removed one by one from the inhalation chamber using an airlock while atmospheric concentrations were maintained constant. Blood collection was completed on one rat before removing another animal from the chamber. Briefly, blood was collected within 5 min from the tail vein of rats anesthetized with isoflurane. Blood samples ($\approx$ 0.8 mL) were dispensed into 2-mL heparinized (30 μ L/150 UI) vials and frozen at - 20 °C.

At d5 and d20, once blood collection had been completed, rats were sacrificed after inducing deep anesthesia by i.p. injection of a ketamine/xylazine mixture (45/5 mg/kg). Anesthetized animals were then intracardially perfused with heparinized physiological saline, exsanguinated and dissected to retrieve organs, including the liver. Organs were frozen in liquid nitrogen immediately after collection and then stored at -80 °C.

Solvent assays in blood and brain

Solvent concentrations in blood and brain were determined based on similar protocols

involving liquid/liquid extraction of the tissues (800 μ L of blood or half a brain) into dichloromethane (DCM) (500 μ L) using n-propylbenzene as an internal standard (10 μ L, 1.7 g/L in DCM). For brain samples, a homogenate was first prepared by grinding half-brains in 2 mL lysing matrix D tubes containing 1.4-mm ceramic beads (20 sec at 4 m/sec in a Fast Prep instrument, MP Biomedicals). After shaking for 30 min, samples were centrifuged for 10 min at 3000 g (or 4000 g for brain) and -4 °C. A portion (0.3 mL) of the lower DCM layer, containing the TOL or the MEK, was transferred to a vial containing a glass insert.

The sample (1 μ L) was quantified by Gas Chromatography–Mass Spectrometry (GC-MS) on a QP2010 Ultra chromatograph (Shimadzu) equipped with a split/splitless injector and an AOC20i auto-sampler, controlled by SU1 software (version 4.11). Samples were separated on an Rtx-1701 capillary column (30 m $\times$ 0.25 mm, film thickness 1 μ m with 4-m integra-guard column) (Restek, France) using helium as the carrier gas at a linear velocity of 40 cm/s. Each sample (1 μ L) was injected in split mode with a ratio of 1/20. The oven temperature was held at 40 °C for 4 min, then increased at a rate of 15 °C/min to 235 °C, the temperature was maintained for 1 min (total duration, 18 min). Temperatures of the injection port, transfer line and ion source were set at 240, 250 and 200 °C, respectively. The MS was operated by electron impact ionization (70 eV) in selected ion monitoring (SIM) mode. In these conditions, the limits of quantification for TOL were approximately 0.004 μ g/g whatever the tissue studied, and 0.054 and 0.180 μ g/g for MEK in blood and brain, respectively.

Liver assays

Biochemical assays were carried out on liver homogenates (reduced (GSH) and oxidized (GSSG) glutathione), liver microsomes (total cytochrome P450 (CYP) concentration and CYP1A2 and CYP2E1 activities), and cytosolic extracts (GST activity).

GSH was measured by a colorimetric method using 5,5'-dithiobis 2-nitrobenzoic acid. GSH was blocked with 2-vinylpyridine before measuring its oxidized form (GSSG) following reduction by glutathione reductase (Griffith, 1980).

The total cytochrome P450 concentration was determined by the Omura and Sato (1964) colorimetric method, by measuring absorption at 450 nm and calculating the difference in absorption between the reduced form of the pigment and the carbon monoxide-conjugated form in the presence of dithionite.

CYP2E1 activity was determined according to the colorimetric method described by Koop (1986), measuring 456-nm absorption of 4-nitrocatechol obtained from p-nitrophenol after oxidation (hydroxylation) catalyzed by CYP2E1. CYP1A2 activity was measured in a similar manner by monitoring O-deethylation of ethoxyresorufin.

GST was measured according to the method developed by Habig et al. (1974) using 1-chloro-2,4 dinitrobenzene as substrate.

For assays of GSH, GSSG, CYP and GST, the amounts measured were normalized based on the total protein content measured in the various samples by the Lowry assay (Lowry et al., 1951). Except for GSH and GSSG, all of the assays discussed above were carried out on a fully automated random access bench-top clinical chemistry analyzer (Randox RX-Daytona).

Statistical analysis

All statistical analyses were performed using Statgraphics Centurion XVI Software (Version 16.2.04) (StatPoint Technologies, Inc., Warrenton, VA). To analyze results, various statistical procedures were implemented. These procedures were based on analysis of variance (ANOVA) to test one or more possible effects (the day effect, the atmospheric concentration effect, the co-solvent effect or the group effect). When necessary, a general linear model including the different fixed effects and a random effect (subject) was used to take the non-

independent nature of data into account. Post hoc analysis was performed by the Bonferroni method. The 95% confidence interval was used as an indicator of statistical significance. In addition, a least squares regression procedure based on a multiplicative (log – log linear) model was used to analyze the blood/atmosphere, brain/blood or urinary metabolites/blood relationships. Assay linearity was also tested by ANOVA. These representations are useful to determine the prediction interval when comparing new observations.

Results

Animal and organ weights

None of the exposure conditions (single-compound exposure or binary exposure) affected weight-gain in animals. In addition, for all exposure conditions the relative weights of brain, lungs and kidneys (expressed as a proportion of body weight) between treated and control animals were not different; however, the relative liver weight was significantly lower in exposed animals than in controls (except for TOL500/MEK200).

Blood and brain concentrations

For each of the eleven 1-month inhalation exposure conditions (single-compound exposure and binary exposure), blood was collected at four time points. Statistical analyses revealed no day effect on the TOL and/or MEK blood concentrations measured.

Table 2 summarizes the blood concentrations measured on day 1 and the mean concentrations measured over the four collecting times. Because the results obtained on day 1 and days 5, 10 and 20 were very similar, the effect of mixtures was measured using data from samples collected on day 1.

[Table 2 near here]

The relationship between the blood TOL (TOL-Blood) and atmospheric TOL (TOL-A) concentrations is plotted in Figure 1. Data from single-compound exposures (white diamonds) could be fitted with a multiplicative model (log-log):

$$\ln \text{TOL-Blood } (\mu\text{g/g}) = -6.217 + 1.378 \times \ln \text{TOL-A (ppm)} \quad (r = 0.9907)$$

The black line plots this fitted regression model and the gray lines delimit the upper and lower 95% prediction intervals for new observations using these models.

[Figure 1 near here]

When TOL-A = 500 ppm, none of the blood concentrations measured following co-exposure with MEK exceeded the 95% prediction interval. Thus, at this TOL level, MEK has no incidence on TOL-Blood. In contrast, data from TOL50/MEK1400, TOL5/MEK1400 and most points from TOL5/MEK200 clearly exceeded the 95% prediction interval. The data points from TOL50/MEK200 and TOL5/MEK20 were also placed above the fit line. These results indicate that TOL-Blood increased significantly upon co-exposure to TOL5 and increasing atmospheric concentrations of MEK ($\times 1.4$ with MEK20 to $\times 17$ with MEK1400). The same effect was observed with TOL50 co-exposures with MEK200 ($\times 1.6$) and MEK1400 ($\times 4.2$) (Figure 2a).

Similarly, the presence of TOL modified the MEK blood concentration, which increased significantly for MEK20 and MEK200 co-exposures in the presence of TOL500 (Figure 2b). We were unable to fit the data corresponding to the blood MEK concentrations (MEK-Blood) plotted against atmospheric MEK (MEK-A) concentrations (Cosnier et al., 2017).

[Figure 2 near here]

As demonstrated in Figure 3, TOL and MEK brain concentrations (TOL-Brain and MEK-Brain) (measured on days 5 and 20) increased proportionally to the corresponding blood concentrations measured in single and/or binary exposure conditions. The relationship

between brain and blood concentrations (in $\mu\text{g/g}$) obtained for TOL or MEK single exposures were fitted by the following equations:

$$\ln \text{TOL-Brain} = 0.345 + 0.924 \times \ln \text{TOL-Blood} \quad (r = 0.9793) \text{ and}$$

$$\ln \text{MEK-Brain} = -0.958 + 0.874 \times \ln \text{MEK-Blood} \quad (r = 0.9361)$$

These equations remain relevant in binary conditions since almost all observations were within the 95% prediction intervals determined from observations of single-compound exposure conditions.

The majority of the data points for TOL were above the first bisector (Figure 3a), thus indicating that TOL-Brain concentrations were higher than TOL-Blood. Conversely, the MEK brain concentrations were consistently lower than the blood load (data points below the first bisector in Figure 3b).

[Figure 3 near here]

Liver assays (data shown in supplementary table)

No statistically significant difference was observed in the total CYP concentration between exposed and control groups, whatever the nature of exposure or the day considered (day 5 or day 20) (578 ± 127 pmol/mg protein on average).

The CYP2E1 and CYP1A2 activities measured in the liver of exposed rats were almost always greater than those in control animals (except for MEK20 for both CYP isoforms and TOL5 and TOL50/MEK20 for CYP2E1). In addition, no day effect was observed. On average, enzymatic activities measured in control livers (all groups and days combined) were equal to 17.1 ± 6.3 pmol/min/mg protein for γCYP1A2 and 0.31 ± 0.14 nmol/min/mg protein for γCYP2E1 . Maximum induction was observed with TOL500 on day 20 (ratio exposed/control = 2.2) for γCYP1A2 and with MEK1400 on day 20 (ratio exposed/control = 2.4) for γCYP2E1 ; thus, co-exposure had no effect on induction of these

enzymes ($1.1 \leq \text{ratio } \gamma\text{CYP1A2} \leq 1.8$ and $1.0 \leq \text{ratio } \gamma\text{CYP2E1} \leq 1.9$) or their dose/response relationships.

At the cytosolic level, except for a slight decrease in activity in animals exposed to MEK200 and MEK1400 (Cosnier et al., 2017), GST activity was not altered by the other exposure conditions (1629 ± 334 U/g protein on average).

Finally, the GSH and GSSG concentrations measured for control animals (23.2 ± 6.0 nmol/mg protein and 0.35 ± 0.19 nmol/mg protein, respectively) were always higher than those measured for treated animals (-17% ($\pm 9\%$) for GSH and -18% ($\pm 13\%$) for GSSG). However, no relationship between the nature of the exposure and the decrease in concentrations could be established.

Urinary analysis

Apart from with TOL5 or TOL5/MEK200 exposures, the amount of BMA and oCre excreted at the different times were not statistically different.

The relationship between the amount of oCre (in nmol) excreted during the 18-h post-exposure period on days 1, 5, 10 and 20 and the TOL-Blood (in $\mu\text{g/g}$) for TOL single-compound exposures was described by the following equation:

$$\ln \text{oCre} = 5.204 + 0.553 \times \ln \text{TOL-Blood} \quad (r = 0.9795) \quad (\text{Figure 4a})$$

Through a similar process, the equation describing the relationship between BMA excretion (measured in nmol) and TOL-Blood (in $\mu\text{g/g}$) is as follows:

$$\ln \text{BMA} = 4.462 + 0.751 \times \ln \text{TOL-Blood} \quad (r = 0.9935) \quad (\text{Figure 4b})$$

For oCre, all the data points obtained in co-exposure contexts were within the 95% prediction intervals determined from the observations of single-compound conditions (Figure 4a). The total oCre excreted in urine (over the 18-h post-exposure period) thus directly correlated with the TOL blood concentration. For BMA, almost all the “co-exposure” points

were well described by the equation; only TOL5/MEK200 and TOL50/MEK1400 co-exposures gave points below the lower 95% prediction limit (Figure 4b).

[Figure 4 near here]

Contrary to what was observed with BMA and oCre, urinary excretion of the metabolites of MEK ($\pm$ 23BDs and meso23BD) varied over time for a given exposure condition and no obvious relationship with exposure could be identified. 23BDs are naturally present in urine, over 18 h mean amounts measured in d0 samples were 14.9 ± 11.1 nmol and 88.6 ± 44.5 nmol for $\pm$ 23BDs and meso23BD, respectively. Despite these levels and variations, the relationship between the amount of $\pm$ 23BD (Figure 5a) or meso23BD (Figure 5b) (in nmol) excreted over the 18-h post-exposure period on days 1, 5, 10 and 20 and the MEK blood concentrations (in $\mu\text{g/g}$) for MEK single-compound exposures were well fitted by the following equations:

$$\ln(\pm 23\text{BDs}) = 4.471 + 0.979 \times \ln \text{MEK-Blood} \quad (r = 0.9886)$$

$$\ln(\text{meso23BD}) = 5.919 + 0.878 \times \ln \text{MEK-Blood} \quad (r = 0.9905)$$

Due to the endogenous excretion, the incidence of TOL50/MEK20 co-exposure on 23BDs excretion could not be assessed. Data points corresponding to excretion following co-exposure to TOL50/MEK200 and TOL500/MEK200 (dotted blue circles) were placed near or below the lower limit of the 95% prediction interval for both groups of 23BDs. Indeed, the equations failed to describe these data satisfactorily. Finally, co-exposure to TOL50 with MEK1400 had no effect on 23BDs excretions. Overall, the “co-exposure” points tended to sit below the black lines (corresponding to the fitted regression models) indicating a decrease in the amount of 23BDs excreted in co-exposure scenarios.

[Figure 5 near here]

Discussion

The large number of combinations of chemicals that may be encountered each day in

workplaces and everyday life makes the problem of mixed exposures multifaceted. It is therefore indisputable that most industrial workers will be exposed to mixtures of chemicals rather than to a single compound. In this article, we wished to highlight the risks run by workers in these scenarios and to complete the data available on the metabolic impact of binary exposure to a common mixture, such as TOL plus MEK. This assessment was done in rats exposed by inhalation under controlled conditions. Indeed, toxicokinetic data on TOL and MEK gathered from rat studies compare favorably with the information available from human studies (USEPA, 2003, ATSDR, 2015).

Special attention was paid to blood concentrations since the first evidence of interaction between compounds can be detected at this level. In addition, the toxic effects of TOL are mainly linked to the molecule itself rather than its metabolites (ATSDR, 2015). Blood samples were collected on days 1, 5, 10 and 20 and the TOL and MEK blood burdens were measured after single or binary exposure. These concentrations remained relatively constant throughout the study. Blood concentrations were therefore extensively analyzed based on samples from day 1.

The relationship between TOL-Blood and TOL-A when animals were exposed to TOL alone, based on data collected at the end of the first day of exposure, could be fitted by the following equation: $\ln \text{TOL-Blood } (\mu\text{g/g}) = -6.217 + 1.378 \times \ln \text{TOL-A (ppm)}$. This equation was quite similar to that reported by Benignus et al. (1984) [$\ln \text{TOL-Blood} = -6.53 + 1.356 \times \ln \text{TOL-A}$] when studying inhalation exposure in Long-Evans rats exposed for 3 h to 50, 100, 500 and 1000 ppm TOL, and to the one we reported with Sprague-Dawley (SD) rats following 6-h inhalation of 2 to 500 ppm TOL [$\ln \text{TOL-Blood} = -5.37 + 1.29 \times \ln \text{TOL-A}$] (Cosnier et al., 2013). These similarities suggest that there is no difference in absorption of TOL between the different strains.

TOL-Blood concentrations measured after co-exposure did not fit this equation (Figure 1). In these cases, the presence of MEK led to an increase in TOL-Blood. Reciprocally, the presence of TOL induced an increase in MEK-Blood. Similar to observations from Uaki et al. (1995), these increases were particularly marked with unbalanced mixtures (TOL-Blood $\times 4.2$ for TOL50/MEK1400 and $\times 17$ for TOL5/MEK1400, whereas MEK-Blood $\times 3.5$ for TOL500/MEK200 and $\times 10$ for TOL500/MEK20) (Figure 2).

The increase in the blood burden of both solvents could be due to inhibitory action of the co-solvent on the activity of the CYP enzyme family since the total CYP concentration was unaltered in these exposure conditions. However, the activities of CYP1A2 and CYP2E1, the isoforms specifically involved in TOL (and MEK) metabolism (ATSDR, 2015, Cosnier et al., 2017), were systematically higher in exposed animals than in controls. No additive or synergistic induction was detected (even after 1-month exposure) in the presence of MEK. These data support a hypothesis of inhibition, presumably competitive but the determination of the exact nature of the inhibition (competitive, uncompetitive or non-competitive) would require additional experimentations. Consequently, clearance of the solvents takes longer in cases of co-exposure, and the relative amount of both solvents determines their half-life in the blood (Vodickova et al., 1995). Based on the 8h-OEL for TOL before 2012 (50 ppm), we examined the maximum increase in TOL-Blood between TOL50 and TOL50/MEK1400. The results indicated a fold-increase close to that measured with SD rats ($\times 4.8$) (Cosnier et al., 2013); however, with the SD strain, a 4.3-fold increase was already observed at TOL50/MEK200 ($\times 1.7$ with TOL50/MEK20). Similarly, while TOL5/MEK200 induced a 5.0-fold increase in TOL-Blood (compared to TOL5 alone) in SD rats, only a $\times 2.2$ -fold increase was observed in BN rats. The incidence of MEK on TOL-Blood is thus less pronounced in BN rats than in SD rats. This difference could be explained by strain-specific

differences in CYP (Nishiyama et al., 2016). Similar differences might also modulate the response in humans, where metabolism is quite similar to that of rats.

The distribution of the solvents between brain and blood was relatively stable for all of the combinations tested in this study and strong linear relationships were found between blood and brain concentrations (Figure 3). Based on this relationship, exposure to a lipophilic solvent such as TOL appears to consistently result in higher concentrations in the brain than in blood. This accumulation in the CNS makes the brain a major target for many lipophilic xenobiotics. Consequently, neuropharmacological, or even neurotoxic, effects may emerge in complex exposure scenarios involving TOL or aromatic solvents generally.

Even if the blood concentration reveals objective information about interactions between solvents, due to its invasive nature, blood sampling is not used in occupational biomonitoring, and urine sampling is preferred.

All of the potential urinary biomarkers of TOL (hippuric acid (HA), o,m,p-cresols, BMA and o,m,p-toluymercapturic acids) and MEK (2-butanol, 3-hydroxy-2-butanone and 23BDs) were investigated for this study. Based on previous results, some were already known to be poor biomarkers due to their low specificity or high endogenous excretion (for example hippuric acid, m,p-cresols or 2-butanol) (Ikeda et al., 2008, Cosnier et al., 2013, Cosnier et al., 2017). In a co-exposure context, HA also failed to highlight interaction between TOL and MEK at exposure levels below the 8h-OEL (Ukai et al., 1994, Uaki et al., 1995). Thus, we chose to report results only for oCre and BMA for TOL. oCre is produced by CYP1A2-oxidation of TOL's aromatic ring, whereas BMA is produced by side-chain oxidation of TOL (with CYP2E1 generating benzyl alcohol) and the glutathione pathway (Truchon et al., 1999, Inoue et al., 2002, ATSDR, 2015). In rats, the best urinary biomarkers for MEK exposure are 23BDs (Cosnier et al., 2017).

The amount of oCre excreted over the 18-h post-exposure period correlated well with TOL-Blood. The amount of BMA excreted also mirrored TOL-Blood, except in the cases of some unbalanced mixtures (slight under-excretion observed with TOL5/MEK200 and TOL50/MEK1400 co-exposures). These results for oCre and BMA were in line with previous observations in SD rats (Cosnier et al., 2014); they suggest that MEK could be a potent inhibitor of the side-chain oxidation of TOL which produces BMA (after intervention of the glutathione pathway), but that it does not affect its ring oxidation to produce oCre. This hypothesis is supported by Liira et al. (1991), who came to a similar conclusion when observing the xylene metabolites excreted by rats exposed to m-xylene (300 ppm) and MEK (600 ppm), separately and in combination. In accordance with our observations, they also observed that both xylene and MEK inhalation trigger a slight (10%) decrease in liver GSH. However, MEK inhalation did not lead to enhanced excretion of thioethers. Thus, the BMA under-excretion observed was probably not linked to the decreased GSH concentrations measured in exposed animal. It is important to consider that in this study, the incidence on biomarkers was estimated from the total urine volume excreted over 18 h. We do not necessarily presume that similar concentrations would be detected in the spot urine samples commonly collected following exposure as part of biomonitoring. These spot urine samples are more sensitive to kinetic modulations; and many scenarios can be imagined, such as a delay in peak excretion for example.

Concerning 23BDs, the results were even more pronounced than those observed with BMA. Thus, the amount of 23BDs excreted following exposure to MEK levels near the 8h-OEL was reduced in the presence of TOL. At lower MEK exposure levels, observation of this effect was hampered by the endogenous excretion of these metabolites. Overall, evaluation of the received MEK dose by measuring these metabolites does not appear to be very robust.

In conclusion, the results of this study showed that binary exposure to TOL and MEK can lead to an increase in the blood burden of both substances and slowed clearance of solvents due to competitive inhibition. These increases are clearly observable when the level of exposure to at least one of the two solvents is close to or exceeds its OEL. This phenomenon is more limited at lower exposure concentrations; indeed, as shown by Marchand et al. (2016) in a study of humans exposed to mixtures of chloroform and three aromatic solvents, metabolic inhibition between chemicals during co-exposure is unlikely to occur or be detectable at concentrations equal to or lower than $\frac{1}{4}$ of the OEL. Nevertheless, under normal working conditions (exposure level below the OEL), it cannot be excluded that exposure to the TOL/MEK mixture might have more damaging effects than exposure to TOL alone due to a reduction in the concentration from which potential neurotoxicity might appear. The situation could be further exacerbated if we consider additional non-work exposure to chemicals such as alcohol or drugs (Campo et al., 1998, Lee et al., 2005). In such conditions, the additivity hypothesis and its consequences on the reduction of the OEL (divided by two for a binary exposure) for both solvents should be considered to preserve workers' health. In commonly encountered co-exposure conditions (non-extreme atmospheric concentrations), urinary biomarkers oCre and BMA should allow biomonitoring of employees and provide a good estimate of exposure to TOL. We therefore recommend that those responsible for prevention consider measuring suitable urinary metabolites of all the main chemical products used in workplaces to get a true idea of the hazards present in the work environment. However, it is important to keep in mind that extrapolation from these measurements to determine the exposure concentrations must be done carefully, taking the impact of mixtures on the kinetics of metabolism into consideration.

PBPK models for inhaled TOL and MEK are available for rats and humans. For MEK, they are based on a limited number of datasets. Future work should test the predictive

capabilities of these models to describe the presented data (blood, brain and urinary) from single exposures (TOL or MEK) and the toxicokinetic interactions between both chemicals. Use of such models for risk assessment purposes could then be considered by substituting the rat parameter values by those of the human.

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Table 1. Composition of the test atmospheres generated

		TOL-A (ppm)			
		0	5	50	500
	0	⊙	⊙	⊙	⊙
MEK-A	20	⊙*	•	⊙	•
(ppm)	200	⊙*	⊙	⊙	⊙
	1400	⊙*	•	⊙	•

⊙ 1-month exposure (6 h/day, 5 days/week, 4 weeks)

• 6-hour exposure

* experiments described in (Cosnier *et al.*, 2017).

Table 2. Solvent blood concentration in $\mu\text{g/g}$ (mean $\pm$ standard deviation) measured on day 1 and mean concentration over the four collection times.

Exposure	TOL-Blood on day 1	Mean TOL-Blood	MEK-Blood on day 1	Mean MEK-Blood
MEK20*	/	/	0.19 ± 0.04	0.20 ± 0.05
MEK200*	/	/	2.78 ± 0.96	1.95 ± 0.80
MEK1400*	/	/	180.8 ± 33.6	162.2 ± 26.4
TOL5	0.03 ± 0.01	0.04 ± 0.01	/	/
TOL50	0.33 ± 0.08	0.35 ± 0.08	/	/
TOL500	11.84 ± 2.54	11.62 ± 2.98	/	/
TOL5/MEK20	0.04 ± 0.01	/	0.17 ± 0.02	/
TOL5/MEK200	0.07 ± 0.03	0.06 ± 0.02	3.36 ± 1.01	2.61 ± 0.88
TOL5/MEK1400	0.53 ± 0.12	/	118.4 ± 14.5	/
TOL50/MEK20	0.34 ± 0.07	0.42 ± 0.14	0.24 ± 0.07	0.23 ± 0.07
TOL50/MEK200	0.54 ± 0.09	0.55 ± 0.13	3.77 ± 1.000	3.43 ± 1.29
TOL50/MEK1400	1.40 ± 0.34	1.42 ± 0.28	154.9 ± 24.4	165.2 ± 21.7
TOL500/MEK20	12.21 ± 0.80	/	1.91 ± 0.20	/
TOL500/MEK200	7.08 ± 0.80	11.64 ± 3.58	9.77 ± 0.92	11.74 ± 2.08
TOL500/MEK1400	11.00 ± 0.95	/	124.2 ± 14.5	/

* data from (Cosnier et al., 2017)

Figure captions

Figure 1. TOL-Blood (in $\mu\text{g/g}$) directly correlates with TOL-A (ppm) on day 1 for TOL and/or MEK exposures. The black line shows the fit of the regression model (between TOL-Blood and TOL-A) obtained based on data from single-compound exposures. The gray lines delimit the 95% prediction interval for new observations using these models. The equation fitting the adjusted model is $\ln \text{TOL-Blood } (\mu\text{g/g}) = -6.217 + 1.378 \times \ln \text{TOL-A (ppm)}$ ($R^2 = 0.982$).

Figure 2. Co-exposure increases (a) TOL-Blood and (b) MEK-Blood concentrations.

Figure 3. Relationships between (a) TOL-Blood and TOL-Brain and (b) MEK-Blood and MEK-Brain concentrations (in $\mu\text{g/g}$) for TOL and/or MEK exposures. Black lines plot the fitted regression models (between TOL-Brain and TOL-Blood or MEK-Brain and MEK-Blood) obtained from data for single-compound exposures. The gray lines delimit the 95% prediction interval for new observations using these models. The equations for the adjusted models are $\ln (\text{TOL-Brain}) = 0.345 + 0.924 \times \ln (\text{TOL-Blood})$ ($R^2=0.959$) and $\ln (\text{MEK-Brain}) = -0.958 + 0.874 \times \ln (\text{MEK-Blood})$ ($R^2 = 0.876$).

Figure 4. Urinary oCre (a) and BMA (b) excreted (in nmol) correlate with TOL blood concentration (in $\mu\text{g/g}$) for TOL and/or MEK exposures. Each point represents paired data obtained from two animal groups (one for blood and one for urine); the error bars around the points (on the x- and y-axis) represent the standard deviations. Black lines plot the fit of regression models obtained for single exposures. The gray lines delimit the 95% prediction interval for new observations using these models. The equations describing the adjusted models are $\ln (\text{oCre}) = 5.204 + 0.553 \times \ln (\text{TOL-Blood})$ ($R^2=0.959$) and $\ln (\text{BMA}) = 4.4620 + 0.751 \times \ln (\text{TOL-Blood})$ ($R^2=0.987$).

Figure 5. Urinary $\pm 23\text{BDs}$ (a) and meso23BD (b) excreted (in nmol) correlate with the MEK blood concentration (in $\mu\text{g/g}$) except in some cases of co-exposure to TOL and MEK. Each point represents paired data obtained from two animal groups (one for blood and one for urine); the error bars around the points (on the x- and y-axis) represent the standard deviations. The black lines plot the fit of regression models obtained from data for single-compound exposures. The gray lines delimit the 95% prediction interval for new observations using these models. The equations for the adjusted models are $\ln (\pm 23\text{BDs}) = 4.471 +$

$0.979 \times \ln(\text{MEK-Blood})$ ($R^2=0.977$) and $\ln(\text{meso23BD}) = 5.919 + 0.878 \times \ln(\text{MEK-Blood})$ ($R^2=0.981$).

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Supplementary table. Liver assays (mean $\pm$ SD)

Exposure	Day	Group	[P450]	γ CYP1A2	γ CYP2E1	GSH	GSSG	GST
			pmol/mg proteins	pmol/min/mg proteins	nmol/min/mg proteins	nmol/mg proteins	nmol/mg proteins	U/g proteins
MEK20	5	Exposed	486 $\pm$ 68	21.2 $\pm$ 4.3	0.29 $\pm$ 0.06	17.9 $\pm$ 3.4	0.21 $\pm$ 0.05	1440 $\pm$ 373
MEK20	5	Control	489 $\pm$ 74	20.8 $\pm$ 4.0	0.22 $\pm$ 0.10	26.5 $\pm$ 7.4	0.25 $\pm$ 0.11	1522 $\pm$ 162
MEK20	20	Exposed	561 $\pm$ 50	19.4 $\pm$ 4.0	0.17 $\pm$ 0.05	19.2 $\pm$ 2.2	0.16 $\pm$ 0.03	1688 $\pm$ 239
MEK20	20	Control	544 $\pm$ 39	15.3 $\pm$ 2.2	0.20 $\pm$ 0.13	24.2 $\pm$ 2.8	0.18 $\pm$ 0.06	1526 $\pm$ 137
MEK200	5	Exposed	467 $\pm$ 52	21.0 $\pm$ 3.1	0.38 $\pm$ 0.22	21.0 $\pm$ 1.8	0.21 $\pm$ 0.02	1576 $\pm$ 128
MEK200	5	Control	431 $\pm$ 64	18.5 $\pm$ 2.3	0.22 $\pm$ 0.06	22.8 $\pm$ 3.4	0.35 $\pm$ 0.12	1713 $\pm$ 150
MEK200	20	Exposed	617 $\pm$ 40	16.8 $\pm$ 2.7	0.29 $\pm$ 0.08	22.5 $\pm$ 4.7	0.25 $\pm$ 0.02	1290 $\pm$ 109
MEK200	20	Control	526 $\pm$ 39	12.5 $\pm$ 2.1	0.11 $\pm$ 0.03	29.4 $\pm$ 9.4	0.31 $\pm$ 0.11	1777 $\pm$ 209
MEK1400	5	Exposed	422 $\pm$ 88	22.0 $\pm$ 3.4	0.38 $\pm$ 0.15	22.6 $\pm$ 3.5	0.37 $\pm$ 0.09	1325 $\pm$ 121
MEK1400	5	Control	451 $\pm$ 86	15.5 $\pm$ 3.2	0.35 $\pm$ 0.27	30.2 $\pm$ 8.4	0.59 $\pm$ 0.13	2222 $\pm$ 869
MEK1400	20	Exposed	623 $\pm$ 68	23.8 $\pm$ 4.0	0.49 $\pm$ 0.09	21.1 $\pm$ 1.3	0.37 $\pm$ 0.08	1360 $\pm$ 78
MEK1400	20	Control	588 $\pm$ 82	14.0 $\pm$ 2.8	0.29 $\pm$ 0.07	25.4 $\pm$ 2.3	0.48 $\pm$ 0.05	1698 $\pm$ 202
TOL5	5	Exposed	531 $\pm$ 122	29.5 $\pm$ 6.2	0.30 $\pm$ 0.04	16.6 $\pm$ 1.9	0.28 $\pm$ 0.04	1805 $\pm$ 192
TOL5	5	Control	585 $\pm$ 91	18.5 $\pm$ 2.2	0.36 $\pm$ 0.09	22.3 $\pm$ 1.8	0.39 $\pm$ 0.04	1642 $\pm$ 110
TOL5	20	Exposed	703 $\pm$ 102	24.3 $\pm$ 5.6	0.39 $\pm$ 0.06	15.2 $\pm$ 2.0	0.20 $\pm$ 0.03	1373 $\pm$ 123
TOL5	20	Control	622 $\pm$ 186	21.4 $\pm$ 3.1	0.36 $\pm$ 0.05	19.3 $\pm$ 1.6	0.29 $\pm$ 0.04	1576 $\pm$ 224
TOL50	5	Exposed	619 $\pm$ 29	14.7 $\pm$ 1.4	0.24 $\pm$ 0.06	19.7 $\pm$ 3.6	0.35 $\pm$ 0.10	1254 $\pm$ 151
TOL50	5	Control	544 $\pm$ 54	11.4 $\pm$ 2.5	0.16 $\pm$ 0.04	20.7 $\pm$ 3.3	0.38 $\pm$ 0.14	1370 $\pm$ 174
TOL50	20	Exposed	528 $\pm$ 61	10.6 $\pm$ 2.7	0.23 $\pm$ 0.06	21.8 $\pm$ 7.1	0.40 $\pm$ 0.16	1542 $\pm$ 475
TOL50	20	Control	510 $\pm$ 24	7.7 $\pm$ 0.7	0.19 $\pm$ 0.05	29.4 $\pm$ 5.5	0.53 $\pm$ 0.14	1339 $\pm$ 243
TOL500	5	Exposed	568 $\pm$ 102	19.5 $\pm$ 3.9	0.31 $\pm$ 0.08	18.6 $\pm$ 3.0	0.31 $\pm$ 0.06	1419 $\pm$ 118
TOL500	5	Control	569 $\pm$ 155	10.3 $\pm$ 1.6	0.22 $\pm$ 0.11	22.2 $\pm$ 6.3	0.34 $\pm$ 0.10	1468 $\pm$ 143
TOL500	20	Exposed	683 $\pm$ 34	21.1 $\pm$ 4.1	0.42 $\pm$ 0.07	19.4 $\pm$ 2.7	0.45 $\pm$ 0.09	1412 $\pm$ 330
TOL500	20	Control	620 $\pm$ 63	9.7 $\pm$ 1.9	0.31 $\pm$ 0.08	22.5 $\pm$ 3.3	0.46 $\pm$ 0.07	1308 $\pm$ 146

Exposure	Day	Group	[P450]	γ CYP1A2	γ CYP2E1	GSH	GSSG	GST
			pmol/mg proteins	pmol/min/mg proteins	nmol/min/mg proteins	nmol/mg proteins	nmol/mg proteins	U/g proteins
TOL5/MEK200	5	Exposed	622 ± 165	25.5 ± 5.3	0.40 ± 0.08	20.2 ± 1.3	0.18 ± 0.01	1780 ± 217
TOL5/MEK200	5	Control	599 ± 34	23.9 ± 3.1	0.36 ± 0.05	21.7 ± 2.1	0.26 ± 0.06	1713 ± 177
TOL5/MEK200	20	Exposed	626 ± 73	19.8 ± 5.8	0.47 ± 0.05	19.6 ± 1.8	0.20 ± 0.02	1614 ± 125
TOL5/MEK200	20	Control	657 ± 119	13.2 ± 3.1	0.39 ± 0.08	22.2 ± 2.2	0.22 ± 0.03	1532 ± 166
TOL50/MEK20	5	Exposed	589 ± 75	24.8 ± 2.8	0.42 ± 0.12	25.4 ± 1.7	0.22 ± 0.04	1685 ± 99
TOL50/MEK20	5	Control	546 ± 111	13.7 ± 2.6	0.44 ± 0.12	27.8 ± 2.8	0.29 ± 0.04	1824 ± 108
TOL50/MEK20	20	Exposed	838 ± 302	24.9 ± 6.2	0.60 ± 0.08	25.5 ± 3.2	0.24 ± 0.05	1622 ± 201
TOL50/MEK20	20	Control	562 ± 52	21.2 ± 4.1	0.55 ± 0.10	28.9 ± 3.6	0.27 ± 0.05	1681 ± 188
TOL50/MEK200	5	Exposed	420 ± 76	30.7 ± 6.8	0.52 ± 0.10	19.0 ± 2.6	0.16 ± 0.05	2160 ± 105
TOL50/MEK200	5	Control	419 ± 106	28.2 ± 7.5	0.39 ± 0.05	19.8 ± 2.4	0.20 ± 0.07	2002 ± 55
TOL50/MEK200	20	Exposed	632 ± 65	31.9 ± 3.6	0.49 ± 0.07	17.4 ± 1.3	0.20 ± 0.07	1763 ± 162
TOL50/MEK200	20	Control	628 ± 83	24.5 ± 4.3	0.38 ± 0.05	21.3 ± 2.8	0.22 ± 0.06	1803 ± 137
TOL50/MEK1400	5	Exposed	773 ± 47	34.8 ± 4.3	0.58 ± 0.10	16.3 ± 3.0	0.14 ± 0.05	2017 ± 148
TOL50/MEK1400	5	Control	454 ± 121	21.3 ± 5.4	0.48 ± 0.10	25.0 ± 4.4	0.20 ± 0.07	2004 ± 149
TOL50/MEK1400	20	Exposed	647 ± 51	36.8 ± 4.7	0.55 ± 0.05	16.5 ± 1.9	0.13 ± 0.04	1974 ± 99
TOL50/MEK1400	20	Control	523 ± 58	22.9 ± 4.0	0.29 ± 0.05	19.3 ± 4.1	0.16 ± 0.07	1884 ± 195
TOL500/MEK200	5	Exposed	618 ± 46	20.5 ± 2.3	0.40 ± 0.10	12.7 ± 3.5	0.62 ± 0.18	1196 ± 119
TOL500/MEK200	5	Control	644 ± 53	16.2 ± 2.1	0.30 ± 0.05	12.7 ± 5.0	0.54 ± 0.30	1278 ± 102
TOL500/MEK200	20	Exposed	660 ± 44	22.1 ± 2.7	0.41 ± 0.06	13.7 ± 2.6	0.75 ± 0.21	1747 ± 401
TOL500/MEK200	20	Control	614 ± 30	13.6 ± 2.4	0.34 ± 0.07	18.2 ± 2.1	0.77 ± 0.15	1618 ± 477