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Sex differences in urinary levels of several biological indicators of exposure:
a human volunteer study.

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Abstract

The aim of the study was to quantify the variability on biological indicators of exposure between men and women for three well known solvents: methyl ethyl ketone, 1-methoxy-2-propanol and 1,1,1-trichloroethane. Another purpose was to explore the effect of selected CYP2E1 polymorphisms on the toxicokinetic profile.

Controlled human exposures were carried out in a 12 m^3 exposure chamber for each solvent separately, during six hours and at half of the threshold limit value. The human volunteers groups were composed of ten young men and fifteen young women, including ten women using hormonal contraceptive.

An analysis of variance mainly showed an effect on the urinary levels of several biomarkers of exposure among women due to the use of hormonal contraceptive, with an increase of more than 50 % in metabolites concentrations and a decrease of up to 50 % in unchanged substances concentrations, suggesting an increase in their metabolism rate. The results also showed a difference due to the genotype CYP2E1*6, when exposed to methyl ethyl ketone, with a tendency to increase CYP2E1 activity when volunteers were carriers of the mutant allele.

Our study suggests that not only physiological differences between men and women but also differences due to sex hormones levels can have an impact on urinary concentrations of several biomarkers of exposure. The observed variability due to sex among biological exposure indices can lead to misinterpretation of biomonitoring results. This aspect should have its place in the approaches for setting limits of occupational exposure.

Keywords: Human Biomonitoring – Biological indicators of exposure – Sex differences – Toxicokinetics – Organic solvents

1. Introduction

Due to their chemical properties, organic solvents are commonly used worldwide in different industrial sectors as degreasing and diluting agents. They can be mainly classified as aliphatic hydrocarbons, cyclic hydrocarbons, aromatic hydrocarbons, halogenated hydrocarbons, ketones, amines, esters, alcohols, aldehydes, ethers and glycol ethers. Their production and use increased mainly in the latter half of the 19th century due to the coal-tar industry.

During the 1980's, a major concern for occupational hygienists and physicians was the risk assessment of occupational exposure to volatile organic compounds (Droz et al., 1983) as their neurotoxicity represented one of the most important emerging issues in the field of occupational health (Baker 1988).

Biological monitoring is a widely used method in the assessment of occupational exposure. It often relies on measurements of the chemical or its metabolites in biological fluids, identified as biomarkers of exposure. Recommended biological limits for chemical substances, most based on a direct correlation with the corresponding threshold limit values, are published periodically in Switzerland and in other countries for interpretation and decision-making.

Recently, much more attention has been devoted to variability associated with biological monitoring results (Tardif et al., 2002; Pierrehumbert et al., 2002; Truchon et al., 2006; Thomson et al., 2010). Several studies in volunteers and with toxicokinetic models have now shown that biological results follow approximatively log-normal distributions, characterized by geometric standard deviations ranging from about 1.3 to 2.0 (Droz, 1992), equivalent to a coefficient of variation between 30 and 100 %. This large biological variability was already obvious to drug therapists (Meibohm et al., 2002; Schwartz, 2003), and adaptation of dosage is normal current practice, at least for drugs with narrow therapeutic ranges.

Among others, sex is an easily identifiable determinant which could provide an important contribution to biological variability. The influence of sex on toxicokinetics may involve female-male differences in physical constitution (body water space, muscle mass, body fat, and blood flow), physiology (menopause and menstruation cycle), hormones (contraceptive pill) and metabolising enzymes (Löf et al., 1998). Lipophilic substances will tend to have a greater distribution volume in women, hydrophilic substances will show the opposite (Schwartz, 2003). The binding in plasma to proteins is also influenced by sex hormones, which can lead to toxicokinetic differences (Gandhi et al., 2004). Cytochrome P450 dependent mono-oxygenases are the most prominent enzymes involved in solvent metabolism. Isoenzymes belonging to the first four gene families (CYP 1-4) are involved in the metabolism of xenobiotics (Löf et al., 1998). The metabolism of many solvents includes oxidation by the CYP2E1 (Nakajima et al., 1997). This specific isoform, probably among others, is known to exhibit sex differences and to show a polymorphism (Bebia et al, 2004).

There are only a few studies evaluating possible sex differences in the toxicokinetics of occupational chemicals in humans. Swedish researchers exposed male and female volunteers to m-xylene and 2-propanol under controlled conditions (Ernstgård et al., 2003a,b) and found toxicokinetic modifications for both chemicals, which they attributed mainly to anatomical differences between sexes. Small changes in acute health effects were also identified in these experiments (Ernstgård et al, 2002).

Although one can establish a list of contributing factors (Gochfeld, 2007), their relative importance for different chemicals of occupational interest is unknown. Several authors recently recommended that more toxicokinetic studies be undertaken to better characterize the influence of sex (Arbuckle, 2006; Messing, 2006).

The main objective of this study was therefore to quantify the variability on biological indicators of exposure between

men and women for three well known solvents, which are methyl ethyl ketone, 1-methoxy-2-propanol and 1,1,1-trichloroethane. Another purpose was to explore the effect of selected CYP2E1 polymorphisms on the toxicokinetic profile. Some of the results of this study have been previously reported in the form of an abstract (Tomicic et al., 2010).

2. Material and methods

2.1. Human volunteers

The experimental protocol was approved by the Clinical Research Ethics Committee of the Faculty of Biology and Medicine at the University of Lausanne, according to the Declaration of Helsinki.

Ten men and fifteen women participated in the study. Each volunteer had to undergo a medical examination including a health questionnaire, a general physical examination, standard clinical blood and urine analyses, an electrocardiogram and a spirometry. Body fat was measured the day of exposure with a body fat monitor using bioelectric impedance (Omron BF 300, Omron Healthcare Europe).

The following inclusion criteria were used to select the participants: Caucasian, age between 20 and 25 years, body mass index between 18 and 28, non-smoker, no history of chronic or allergy disease, no occupational exposure to organic solvents, no chronic use of drugs and no pregnancy.

2.2. Chemicals

Three chemicals have been selected for their relatively low toxicity as well as their metabolic and physiological properties: methyl ethyl ketone (MEK), watersoluble and highly metabolized, 1-methoxy-2-propanol (1M2P), amphiphile and highly metabolized, and 1,1,1-trichloroethane, liposoluble and poorly metabolized.

MEK (> 99.5 %) and 1M2P (> 98 %) were obtained from Sigma-Aldrich (Buchs, Switzerland) and 111TCE (> 99.5 %) from Merck (VWR International AG, Schlieren, Switzerland).

The three solvents are mainly absorbed by inhalation and metabolized via the cytochrome P450 mixed-function oxidase system.

MEK is metabolized to 2,3-butanediol, 2-butanol and 3-hydroxy-2-butanone (major metabolite), all excreted in urine. A low fraction of unchanged solvent is excreted in the exhaled air and in urine. The urinary concentration of MEK at the end of the shift has been recommended as the most appropriate biological exposure indicator.

1M2P belongs to the family of the propylene glycol ethers (PGE) which exists under the form of two isomers, alpha-isomer and beta-isomer. The latter one, considered as an impurity in commercial use, is first transformed as all primary alcohols into 2-alkoxy acetaldehydes by alcohol deshydrogenases, and then into alkoxyacetic acids by aldehyde deshydrogenases, a toxic metabolite. Alpha-PGE are primarily metabolized to propylene glycol, carbon dioxide and glucuronide as well as sulfate conjugates of the parent compound. The urinary 1M2P concentration at the end of the shift corresponds as the appropriate biomarker.

1,1,1-trichloroethane is metabolised to trichloroethanol (TCE) and trichloroacetic acid (TCA), both excreted in urine. Other minor metabolites (carbon dioxide, acetylene) are excreted in the exhaled air. The corresponding biological exposure indices are the urinary concentration of both metabolites and the blood concentration of the parent compound.

The 111TCE concentration in the exhaled air can also be considered as a biomarker of exposure.

Table 1 summarizes useful information about existing threshold limit values and biological exposure indices regarding the biological determinants corresponding to the three studied organic solvents.

Table 1. Summary of the selected organic solvents with occupational exposure limits (OELs in ambient air) and corresponding biomarkers of exposure,for different countries (USA, Germany, Switzerland)

		BEI*/BAT/VBT***		
Biological determinant	**TLV*/MAK**/VME*** (ppm)**	***urine* (mg/l)**	***blood* (µg/l)**	***expired air* (ppm)**
Methyl ethyl ketone (MEK)	200/200/200	2/5/5 (ES[a])	-	-
1-Methoxy-2-propanol (1M2P)	100/100/100	-/15/20 (ES)	-	-
1,1,1-Trichloroethane (111TCE)	350/200/200	-	-/550/550 (PS[c], EW)	40/-/- (PS, EW)
Trichloroethanol (TCE)		30/-/- (ES, EW[b])	-	-
Trichloroacetic acid (TCA)		10/-/- (EW)	-	-

* The American Conference of Industrial Hygienists (ACGIH) sets threshold limit values (TLV) and biological exposure indices (BEI).

** The German Research Foundation (Deutsche Forschungsgemeinschaft - DFG) sets “Maximale Arbeitsplatz-Konzentrationen” (MAK) and “Biologische Arbeitsstoff-Toleranzen” (BAT).

*** The Swiss National Accident Insurance Fund (Schweizerische Unfallversicherungsanstalt - Caisse nationale suisse d'assurance en cas d'accidents - Istituto nazionale svizzero di assicurazione contro gli infortuni - SUVA) sets “valeurs (limites) moyennes d’exposition” (VME) and “valeurs biologiques tolérables” (VBT).

[a] ES: end of shift [b] EW: end of week [c] PS: prior to shift

2.3. Exposure conditions and experimental design

The experiments were carried out in a 12 m^3 air-conditioned exposure chamber and were conducted over 37 separate sessions. The chamber allows the simultaneous exposure of three volunteers to vapors. The responsible physician’s main duties were to check the participant before exposure, to make sure that inclusion criteria were still respected, and to see the volunteer after and the day following exposure to assess any health outcome due to the exposure session. Exposures lasted for six hours at rest, at half of the threshold limit value of the used solvent. The intended exposure levels were based on the Swiss OELs and corresponded to 100 ppm for MEK and 111TCE, and to 50 ppm for 1M2P. Each volunteer was separately exposed to the three solvents, with a minimum of two weeks (based on a 100 hr half-time) between 2 exposure scenarios to avoid cross-cumulation. The temperature in the chamber was maintained at about 24 °C and the air renewal was between 12 and 15 per hour. A peristaltic pump pushed the solvent to a heated aluminium plate (kept at a temperature about 10 °C above the boiling point of the considered solvent) where it evaporated to be immediately injected into the ventilation of the chamber. The solvent generation was controlled by the LabVIEW software (National Instruments Corporation, Texas, USA).

2.4. Air monitoring

The solvent concentration in the chamber was continuously monitored by two independent methods: a portable FTIR (Fourier Transform Infrared Spectroscopy) gas analyzer for ambient air analysis (Gasmet™ DX4015, Gasmet Technologies Oy, Helsinki, Finland) and a Perkin Elmer gas chromatograph (GC), with a flame ionization detector (FID), connected to a Hewlett Packard 3395 integrator. With the latter one, a measurement was taken every half an hour. Solvent concentration in the exposure chamber was additionally assessed by active personal air sampling. Solvents were collected on tubes containing activated charcoal as adsorbent, with air samplings rates of 40 ml/min. After desorption with carbon disulfide (in the case of MEK and 111TCE) or dichloromethane (in the case of 1M2P), analysis were done by GC using a flame ionization detector.

2.5. Biological monitoring

Sampling: Urine samples were collected before, during (with a predefined timing and in a solvent-free area) and after (collected *ad lib*) exposure. The samples were kept in polyethylene bottles or in brown borosilicate flasks directly after reception and stored refrigerated at 4 °C. When kept in polyethylene bottles, samples were aliquoted and stored frozen (-20 °C) at the end of the exposure session. Blood samples were taken before and after the exposure, as well as during the following day when the volunteers came back to bring the urine samples. Venous blood was collected in 9-ml vacuum tubes with an anticoagulant comprising ethylenediaminetetraacetic acid (EDTA) and stored in a refrigerator at 4 °C until analysis. Expired air was sampled immediately after the exposure by taking at least 3 points during the hour following the exposure.

Analytical methods: Analytical methods for exposure measurements and biological determinations in urine, blood and expired air are mostly under control at the Institute for Work and Health (IST) in Lausanne, Switzerland, in an ISO 17025 certification scheme. Parent compounds and metabolites have been analysed by gas chromatography according to published schemes. The determination of all the defined biomarkers of exposure was based on the headspace gas chromatography technique. As recommended since 1981 by Sedivec et al. (1981) and largely experimented by our team for alcohol determination in urinary samples (Sethre et al., 2000), the addition of a saturating amount of Na_2SO_4 has an important effect on the partition coefficient of solvents, in our case of MEK and 1M2P. An existing analytical method for the determination of urinary 1M2P including a solid-phase extraction and derivatization before analysis is available (Devanthéry et al., 2000) but presents some disadvantages. In the frame of this study, an alternative method based on the headspace gas chromatography technique has been developed (Tomicic et al., 2010). The quantity of conjugated 1M2P has been determined after an acidic hydrolysis, realized by adding hydrochloric acid to the samples and by keeping them at 100°C overnight.

Regarding the 111TCE metabolites, urinary TCE and urinary TCA have been analysed after methylation, transforming TCA to its corresponding and more volatile ester, and after hydrolysis, in order to assess total TCE as it can be present under its conjugated form (Breimer et al., 1974).

As recommended for spot urine specimen, creatinine adjustment was performed for each sample to correct for urine dilution. Creatinine concentration was determined by a modified Jaffe reaction using a deproteinisation before the addition of picric acid. The colorimetric intensity of the creatinine-picrate complex was measured at 520 nm. Normal creatinine values are between 0.3 and 3.0 g/l.

The apparatus used for the urine analysis was an Agilent 6890 gas chromatograph (Agilent Technologies AG, Urdorf,

Switzerland) equipped with a Gerstel Multipurpose Sampler (Gerstel AG, Sursee, Switzerland) operated in headspace mode and with a 2.5 mL tight gas syringe. The flame ionization detector (FID) has been used for the determination of the alcohols and the micro electron capture detector (μ-ECD) in the case of the chlorinated compounds.

The analyses of the solvents in the end-expired air were performed by using a specially designed sampling valve (Droz et al., 1986; Devanthéry et al., 2002), linked to a carbon dioxide detector and using a Perkin Elmer GC connected to a Hewlett Packard 3395 integrator.

2.6. Identification of genotypes

The most studied genetic variants CYP2E1*5B and CYP2E1*6 (Prieto-Castelló et al., 2010) were explored. Genotypes of the 25 participants have been identified by using PCR-based RFLP methods, as described by Prieto-Castelló et al. (2010). DNA extracted from blood was amplified with specific primers (QIAamp DNA Kit, Qiagen).

The primers used for CYP2E1*5B are described by Cai et al. (2005) and digestion occured during 3 hours at 37 °C with Pst I. Non-digested 553 bp fragments represented c1/c1 wild-type allele, fragments of 435 + 118 bp represented the c2/c2 genotype and fragments of 553 + 435 + 118 bp represented the c1/c2 genotype.

The primers used for CYP2E1*6 are described by Prieto-Castelló et al. (2010) and digestion was carried out during 3 hours at 37 °C with Dra I. Non-digested 373 bp fragments represented homozygous AA, fragments of 249 + 124 bp represented homozygous wild-type TT and fragments of 373 + 249 + 123 bp represented heterozygous TA.

2.7. Symptom questionnaire

No side effects are expected under these exposure conditions. However, irritation and central nervous system effects typical of some solvent exposures cannot be excluded. These possible symptoms were self-reported by the volunteers by filling a standard symptom questionnaire at different exposure times. The questionnaire has been taken from a Swedish study (Ernstgård et al., 2002), where the ratings were performed using a 0-100 mm visual analogue scale, graded from “not at all” (corresponding to 0 mm) through “hardly at all” (6 mm), “somewhat” (26 mm), “rather” (48 mm), “quite” (71 mm), “very” (90 mm) to “almost unbearable” (100 mm). The symptoms asked for were: discomfort in the eyes, in the nose or in the throat or airways, breathing difficulty, solvent smell, headache, fatigue, nausea, dizziness and feeling of intoxication.

2.8. Statistical analysis

The data were first analysed for normality using the Shapiro-Wilk test. Most observations were log normally distributed. Besides several studies in volunteers and with toxicokinetic models showed that biological results follow approximate lognormal distributions (Droz, 1992). Statistical calculations were therefore performed after logarithmic transformation of the dependent variables.

Statistical comparisons between mean values obtained at the end of exposure were made by using the Student’s two-sided t-test. The F-test was used to compare variances between groups.

Univariate analysis of covariance (ANCOVA) was also performed in order to identify potential confounders (body mass index, body fat, genotype). Correlations between mean urinary concentrations and potential confounders were evaluated

by using the Pearson correlation test. The significance level was set at α=0.05 and statistical analyses were performed using the software package STATA Version 10.

Ratings obtained from the symptom questionnaires were not normally distributed. Therefore, the Mann-Whitney U test was used to test for differences in symptom ratings between men and women.

3. Results

3.1. Human volunteers

Among all volunteers, only one woman with hormonal contraceptive declined to participate to her last exposure session, which concerned 111TCE. The characteristics of each group of human volunteers are shown in Table 2. There were no statistically significant differences in age and body mass index between the different groups. Body weight and body height were higher for men while body fat was higher in women (men *vs* women with hormonal contraceptive: $p = 0.02$, $p < 0.001$ and $p = 0.001$ respectively ; men *vs* women without hormonal contraceptive: $p = 0.01$, $p < 0.001$ and $p < 0.001$ respectively). When comparing women with hormonal contraceptive to those without, the only statistically significant difference was for body fat ($p = 0.008$).

Table 2. Characteristics of each volunteer group. Arithmetic mean values (± SD: standard deviation) are indicated; n = number of volunteers.

	Men (n=10)	**Women (n=15)**	***Women with hormonal contraceptive (n=10)***	***Women without hormonal contraceptive (n=5)***
Age (years)	22.4 ± 2.2	23.2 ± 2.9	22.6 ± 2.1	24.4 ± 4.2
Body weight (kg)	70.7 ± 10.3	61.9 ± 8.7	60.1 ± 7.2	65.6 ± 11.2
Body height (cm)	177.5 ± 3.2	165.8 ± 3.7	167 ± 3.8	163.4 ± 2.3
Body Mass Index (-)	22.3 ± 2.6	22.5 ± 3.2	21.6 ± 2.6	24.5 ± 3.7
Body fat (% of body weight)	11.3 ± 5.2	22.1 ± 6.9	19.8 ± 4.6	26.6 ± 8.9

3.2. Air monitoring

The air concentrations of the selected solvents in the exposure chamber remained very stable over time. Slight variations in solvent air concentrations were possible when volunteers went out of the chamber for urine sampling. But these variations had little impact on the mean air concentrations as the chamber door stayed open for very short moments. All methods of air monitoring agreed within 5 %. Air sampling using the tubes containing activated charcoal as adsorbent gave following solvents level: 99.15 (± 5.29) ppm for MEK, 53.22 (± 3.04) ppm for 1M2P and 102.55 (± 3.19) ppm for 111TCE, which corresponded to the intended ones.

3.3. Biological monitoring

Figures 1-4 show the mean urinary concentrations of the different biomarkers of exposure over time for the three groups of human volunteers. Toxicokinetic profiles were similar among all groups of volunteers, with differences in the maximal concentration reached. Urinary levels of biomarkers of exposure measured among women with hormonal

contraceptive were in general close to the ones measured among men. Moreover urinary levels were higher in women without hormonal contraceptive when biomarkers of exposure corresponded to the unchanged substances and the opposite was observed when biological indicators of exposure corresponded to the metabolites.

During exposure, urine was collected every 2 hours. In general, mean values were calculated from concentrations obtained for each human volunteer but as urine samples have been collected *ad lib* after exposure, a reduced number of volunteers contributed to the mean urinary levels at 8, 10, 12 and 14 hours from the beginning of the exposure (for details see captions of Figures 1-4).

For urinary MEK and urinary 1M2P, adjustment to one g of creatinine was not necessary due to their passive process of elimination in the kidney. In this case compounds secretions vary with urine flow rate and creatinine adjustment would not correct for urine dilution. Rather, in the case of urinary TCE and urinary TCA, corrections by creatinine gave less variability in the toxicokinetic profiles, showing that compensation to urine dilution was necessary.

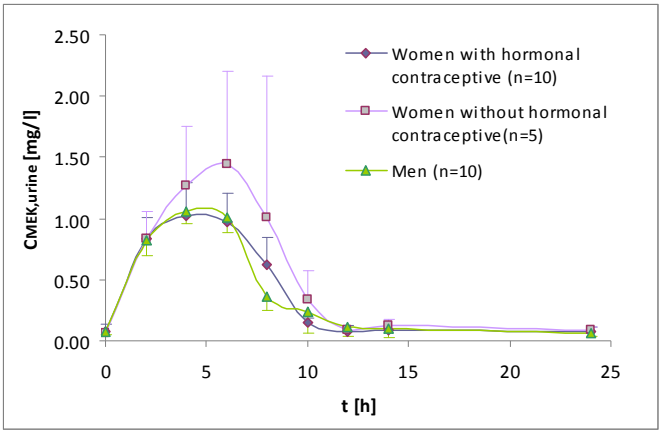

Figure 1 : Urinary MEK concentration as function of time after 6 hours of exposure to 99.15 (± 5.29) ppm MEK. Arithmetic mean values (± SD: standard deviation) are indicated; n = number of volunteers.

Mean urinary levels at 8, 10, 12 and 14 hours from the beginning of the exposure with:

n = 4, 6, 3 and 6 for women with hormonal contraceptive,

n = 4, 2, 1 and 4 for women without hormonal contraceptive,

n = 5, 6, 5 and 6 for men.

(a) (b)

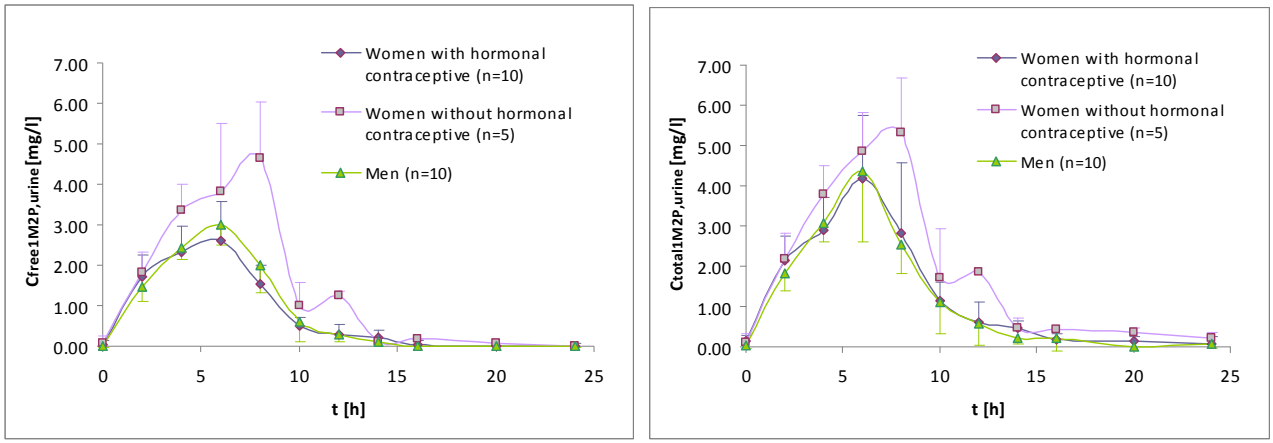

Figure 2 : Urinary free 1M2P (a) and urinary total 1M2P (b) concentrations as function of time after 6 hours of exposure to 53.22 (± 3.04) ppm of 1M2P. Arithmetic mean values (± SD: standard deviation) are indicated; n = number of volunteers.

Mean urinary levels at 8, 10, 12 and 14 hours from the beginning of the exposure with:

n = 4, 8, 5 and 3 for women with hormonal contraceptive,

n = 3, 4, 1 and 2 for women without hormonal contraceptive,

n = 6, 4, 7 and 5 for men.

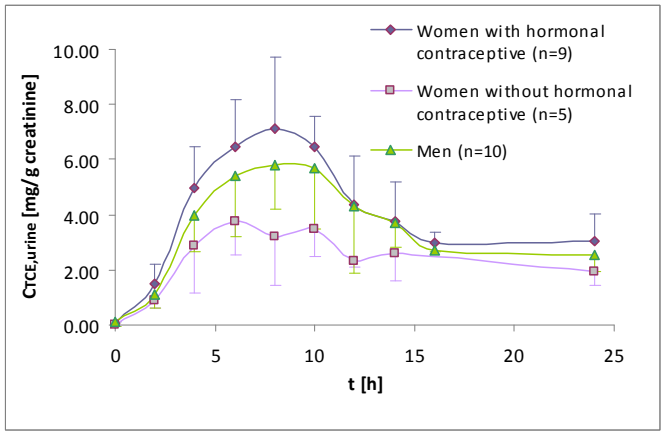

Figure 3 : Urinary TCE concentration as function of time after 6 hours of exposure to 102.55 (± 3.19) ppm of 111TCE. Arithmetic mean values (± SD: standard deviation) are indicated; n = number of volunteers.

Mean urinary levels at 8, 10, 12 and 14 hours from the beginning of the exposure with:

n = 5, 3, 5 and 6 for women with hormonal contraceptive,

n = 2, 4, 2 and 4 for women without hormonal contraceptive,

n = 4, 5, 7 and 3 for men.

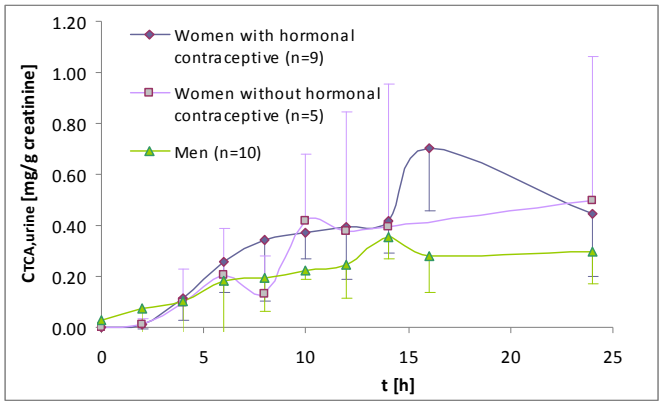

Figure 4 : Urinary TCA concentration as function of time after 6 hours of exposure to 102.55 (± 3.19) ppm of 111TCE. Arithmetic mean values (± SD: standard deviation) are indicated; n = number of volunteers.

Mean urinary levels at 8, 10, 12 and 14 hours from the beginning of the exposure with:

n = 5, 3, 5 and 6 for women with hormonal contraceptive,

n = 2, 4, 2 and 4 for women without hormonal contraceptive,

n = 4, 5, 7 and 3 for men.

End-exposure levels for the different urinary biomarkers of exposure, expressed as mean values (± SD), are summarized in Table 3. Student's tow-tailed t-tests showed statistically significant differences between women with and without hormonal contraceptive for urinary MEK, urinary free 1M2P and urinary TCE. For the latter one, statistically significant differences were also observed between men and women without hormonal contraceptive. Differences in urinary TCA levels were statistically significant between men and women with hormonal contraceptive. From the levels of free (aglycone) and total urinary 1M2P, we can deduce that urinary 1M2P is present up to 60 % in the conjugated form.

111TCE blood concentrations were determined and showed high interindividual variability for all the groups. Before exposure, values obtained for men, women with and without hormonal contraceptive, respectively were 0.60 (± 0.44), 1.00 (± 0.75) and 0.58 (± 0.33) µg/l, end-exposure levels were 362.97 (± 91.05), 306.53 (± 152.27) and 371.13 (± 240.64) µg/l and measured values at the following morning were 16.03 (± 7.71), 10.77 (± 4.46) and 17.86 (± 10.19) µg/l.

Measurements in exhaled air showed that for MEK and 1M2P, mean end-exposure levels were very low (about 2 to 3 % of the exposure concentration) with no statistically significant differences between the different groups.

In the case of exhaled 111TCE, mean end-exposure levels corresponded to 27.31 (± 11.09), 28.28 (± 10.36) and 27.47 (± 6.93) ppm for men, women with and without hormonal contraceptive, respectively. The following day, values dropped to 1.98 (± 3.11), 0.69 (± 0.27) and 0.88 (± 0.25) ppm.

Table 3. Summary of the different biomarkers of exposure values obtained at the end of exposure. Arithmetic mean values (± SD: standard deviation) are indicated; n = number of volunteers.

Biological indicator	Men (n=10)	Women with hormonal contraceptive (n=10)	Women without hormonal contraceptive (n=5)	p-value
Methyl ethyl ketone				
Urinary MEK at the end of exposure (mg/l)	1.00 ± 0.13	0.97 ± 0.23	1.44 ± 0.76	ns*/ns**/0.09***
1-Methoxy-2-propanol				
Free urinary 1M2P at the end of exposure (mg/l)	2.99 ± 0.49	2.61 ± 0.96	3.81 ± 1.68	ns/ns/0.1
Total urinary 1M2P at the end of exposure (mg/l)	4.36 ± 1.76	4.17 ± 1.58	4.85 ± 0.97	ns/ns/ns
Mean percentages of conjugated 1M2P (%)	26.62 ± 16.77	31.18 ± 34.16	22.70 ± 22.75	ns/ns/ns
1,1,1-Trichloroethane				
Urinary TCE at the end of exposure (mg/l)	5.66 ± 2.20	3.83 ± 1.26	2.09 ± 1.24	ns/0.01/0.03
Urinary TCE at the end of exposure (mg/g creatinine)	5.42 ± 2.19	6.46 ± 1.73	3.77 ± 1.24	ns/0.08/0.01
Urinary TCA at the end of exposure (mg/l)	0.20 ± 0.23	0.16 ± 0.08	0.11 ± 0.09	ns/ns/ns
Urinary TCA at the end of exposure (mg/g creatinine)	0.18 ± 0.20	0.26 ± 0.12	0.21 ± 0.18	0.08/ns/ns
111TCE in blood at the end of exposure (μg/l)	362.97 ± 91.05	306.53 ± 152.27	371.13 ± 240.64	ns/ns/ns
Exhaled 111TCE at the end of exposure (ppm)	27.31 ± 11.09	28.28 ± 10.36	27.47 ± 6.93	ns/ns/ns

* Student's two-tailed t-test between men and women with hormonal contraceptive

** Student's two-tailed t-test between men and women without hormonal contraceptive

*** Student's two-tailed t-test between women with and without hormonal contraceptive

with ns: not significant ($p > 0.1$)

3.4. Genotypes

Genotyping results for the CYP2E1*5 allele showed that among the participants, only one person presented the heterozygous type variant (c1/c2). Concerning the CYP2E1*6 allele, results showed that 21 volunteers (84 %) had the wild type variant (TT) and four (16 %) the heterozygous one (TA). Among the carriers of the mutant allele, one belonged to the group of men, another to the group of women with hormonal contraceptive and two to the group of women without hormonal contraceptive. For the latter, one of both was also the carrier of the heterozygous type variant (c1/c2) for genotype CYP2E1*5.

In view of the fact that the number of volunteers participating to the study is low, the number of persons carrying the mutant alleles of the CYP2E1 genotypes is even lower. Thus it was not possible to perform a statistical analysis on the genotype CYP2E1*5. Table 4 summarizes the results obtained after performing an ANCOVA analysis on the different biomarkers of exposure levels obtained for the studied volunteers groups.

Table 4. Results (F-ratios and p-values obtained from ANCOVA analysis) in order to identify potential confounders in evaluating differences, when exposed to organic solvents, between men and women with hormonal contraceptive (abbreviated in the table as horm. contr.), between men and women without hormonal contraceptive and between women with hormonal contraceptive and those without. Potential confounders considered: Body Mass Index (BMI), body fat (BF) and genotype CYP2E1*6.

Independent variables	Dependent variables									
	$C_{MEK,urine}$ (mg/l)		$C_{free1M2P,urine}$ (mg/l)		$C_{total,1M2P,urine}$ (mg/l)		$C_{TCE,urine}$ (mg/ g creatinine)		$C_{TCA,urine}$ (mg/ g creatinine)	
	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value
					Men vs women with hormonal contraception					
sex	4.61	0.05	0.83	0.38	0.99	0.34	0.25	0.62	0.79	0.39
BMI	1.63	0.22	0.21	0.65	2.94	0.11	0.02	0.88	0.10	0.76
BF	12.03	0.003*	4.06	0.06**	0.92	0.35	0.11	0.74	0.05	0.83
*CYP2E1*6*	6.11	0.03	0.19	0.67	1.82	0.19	0.00	0.98	1.72	0.21
					Men vs women without hormonal contraception					
sex	4.06	0.07	0.75	0.41	0.02	0.88	0.76	0.40	0.02	0.90
BMI	0.34	0.57	0.18	0.68	0.27	0.61	0.08	0.78	0.17	0.69
BF	0.24	0.64	0.26	0.62	0.09	0.77	0.05	0.83	0.25	0.63
*CYP2E1*6*	1.38	0.27	0.94	0.36	0.29	0.59	0.91	0.36	0.83	0.38
					Women with hormonal contraception vs women without					
horm. contr.	11.89	0.006	16.71	0.002	1.23	0.29	8.88	0.01	2.51	0.15
BMI	0.31	0.59	0.18	0.68	0.77	0.40	0.32	0.59	0.02	0.89
BF	1.25	0.29	15.11	0.003***	0.01	0.93	0.02	0.89	0.00	0.97
*CYP2E1*6*	0.41	0.54	6.99	0.02	1.81	0.21	0.01	0.93	0.61	0.45

* Pearson correlation matrix (correlation coefficient: -0.55, p = 0.01)
** Pearson correlation matrix (correlation coefficient: -0.58, p = 0.01)
*** Pearson correlation matrix (correlation coefficient: -0.35, p = 0.19)

The statistical analysis showed that body fat and genotype could be potential confounders when differences in urinary levels of biomarkers of exposure are observed between men and women.

When comparing men with women using hormonal contraceptives, body fat clearly influenced urinary concentrations for MEK and 1M2P. The Pearson correlation matrix confirmed a negative association in both cases. The genotype CYP2E1*6 when considered as covariate also showed a statistically significant p-value but the fact that only two persons were carrier of the mutant allele among the compared groups makes it difficult to deduce any affirmation. Urinary MEK concentrations for both volunteers in question were 0.93 mg/l and 0.75 mg/l, respectively. For the other two persons who belonged to the group of women without hormonal contraception, the measured urinary MEK concentrations were 0.73 mg/l and 1.35 mg/l, respectively, the latter one being the volunteer carrying also the heterozygous type variant (c1/c2) for genotype CYP2E1*5. Thus, when comparing these values with the mean urinary MEK levels obtained for each group of participants (see Table 3), there may be a tendency to have a higher CYP2E1 activity when the participant is carrier of the mutant allele of the genotype CYP2E1*6 only.

When comparing men with women without hormonal contraception, results showed no statistically significant p-value.

Finally, when comparing women using hormonal contraceptives with those who are not under hormonal contraception, results showed an effect due to the use of exogenous hormones for the three studied solvents. Moreover, in the case of 1M2P, body fat and genotype seem to be potential confounders. But the Pearson correlation matrix did not confirm an association between body fat and our dependent variable. For the CYP2E1*6 genotype, it is once again difficult to deduce something relevant due to the low number of participants carrying the mutant allele. The woman using hormonal contraceptives had a value of 3.05 mg/l of free 1M2P in urine. For the two women without hormonal contraception, the urinary free 1M2P concentrations were 2.86 mg/l and 4.07 mg/l, respectively, the latter one being the volunteer carrying also the heterozygous type variant (c1/c2) for genotype CYP2E1*5. The only man carrying the genetic variant of CYP2E1*5 had a value of 3.11 mg/l of free 1M2P in urine. Here, when comparing these values with

the mean urinary free 1M2P levels obtained for each group of participants (see Table 3), any tendency cannot be deduced.

3.5. Symptom questionnaire

The self-reported symptom questionnaire was distributed every 2 hours during exposure. The medical responsible saw the volunteers for possible symptoms before exposure, after exposure and in the morning of the following day.

Highest ratings were obtained for methyl ethyl ketone followed by 1-methoxy-2-propanol and the lowest one for 1,1,1-trichloroethane. Considering only the irritation symptoms (eyes, nose, throat), we can observe statistically significant differences between men and women, the latter ones giving higher ratings. The Mann-Whithey U test gave p values lower than 0.1 for discomfort in the eyes for exposure to MEK (after two and four hours) and to 1M2P (after two hours), for discomfort in the nose after six hours of exposure to 1M2P and to 111TCE, and for discomfort in the throat after four hours of exposure to 1M2P. The solvent smell was perceived by most of the volunteers at the beginning of the expoure. It seems that men got used more to the solvent odour than women who gave still a rather high score at the end of exposure ($p = 0.01$ after four hours of exposure to MEK and $p = 0.06$ after six hours of exposure to 1M2P). There were also higher ratings among women concerning headache, after four and six hours of exposure to MEK ($p = 0.04$ and $p = 0.06$, respectively). Women were more tired than men after six hours of exposure to MEK ($p = 0.06$) and the opposite was observed after four hours of exposure to 1M2P ($p = 0.03$). Finally, women had a higher feeling of intoxication than men at the end of exposure of MEK and 1M2P ($p = 0.09$ in both cases).

4. Discussion

Our study suggests that not only physiological differences between men and women but also differences due to sex hormones levels can have an impact on urinary concentrations of several indicators of exposure. The measured values are of the same order of magnitude as data found in literature (Caperos et al., 1982; Devanthéry et al., 2002; Imbriani and Ghittori, 2004). When comparing to the biological exposure indices, we can estimate, by taking into account the exposure conditions, that we generally do not exceed them. In the case of TCA, values are very low, which is due to its long half-life time. Accumulation is possible during the week and thus, it is not an appropriate biomarker for reflecting a daily exposure.

Univariate analysis of variance, performed after logarithmic transformation of the independent variables and by adjusting for potential confounders (body mass index, body fat, genotype), showed the following main observations:

- a negative correlation between body fat and urinary MEK as well as urinary free 1M2P concentrations when comparing men with women using hormonal contraceptives;
- a difference due to the genotype CYP2E1*6, when exposed to methyl ethyl ketone, with a tendency to increase CYP2E1 activity when volunteers are carrier of the mutant allele;
- an effect among women due to the use of hormonal contraceptive on the urinary levels of several biomarkers of exposure, with an increase of more than 50 % in metabolites concentrations and a decrease of up to 50 % in unchanged substances concentrations, suggesting an increase in their metabolism rate.

Body composition can influence the toxicokinetic behaviour of a substance (Sato et al., 1991). Men have generally higher lean body mass and women have higher body fat. The distribution of lipophilic compounds can especially be modified, as higher body fat increases the distribution volume for a lipophilic substance. Thus a decrease in the excretion rate of both the substance itself and its metabolites can be a consequence. Final urinary concentrations should

also be lower in women than in men. But when looking at the measured values of the different biological indicators of exposure, we can see that mean values obtained for women can be higher than those obtained for men as it is the case when the unchanged substance is determined. Moreover, among women, there is an obvious difference between those under hormonal contraceptive and those not. Thus parameters other than the physiological ones could explain the differences observed between the volunteer groups.

According to several authors (Tanaka, 1999; Clewell et al, 2002; Parkinson et al., 2004), enzymatic activities of CYP1A2, CYP3A4, CYP2D6, and CYP2E1 are usually higher in males than in females, a tendency reflected by our results but which can not be confirmed as the volunteers' phenotypes have not been measured.

Concerning genetic polymorphism in CYP2E1, Prieto-Castello et al. (2010) observed a reduced activity of CYP2E1 in the presence of CYP2E1*5 and CYP2E1*6 variant alleles. Our results showed the opposite, which could be explained by the very low number of carriers of the variant allele or by an eventual high interindividual variability in CYP2E1 enzyme activity (Bebia et al., 2004).

A recent publication about gender differences in drug toxicity (Nicolson et al., 2010) supports the idea that hormonal interaction could play a key role. Actually, the effect due to hormonal contraceptive was the most outstanding from the ANOVA analysis albeit the number of women for this comparison was low.

In a recent review of Kennedy (2008) about hormonal regulation of hepatic drug-metabolizing enzyme activity, it is has been shown that estrogens decrease hepatic microsomal activity and drug clearance in rodents. Evidence from clinical studies (Scandlyn et al., 2008) suggests that the CYP2E1 enzyme activity is lower in females than in men. The author states that CYP2E1 appears to be estrogen-regulated and that a reduced activity has been described during periods when estrogen levels remain elevated (pregnancy) or during changes in hormone levels (puberty, exogenous hormone administration).

In fact, combined oral contraceptives increase the estrogen and progesterone levels while decreasing the gonadotropins level (Kennedy, 2008). The increase in estrogen has as consequence an increase in the sex hormone-binding globulin (SHBG) level (Panzer et al., 2006), which has a high affinity and a low capacity for testosterone and estradiol (Pugeat et al., 2009). Albumin regulates the access of steroids to their receptors (Baker, 2002). The increase in SHGB level can enable steroids, bound with low affinity to albumin, to bind to SHGB. These variations in protein binding levels will have an impact on the balance between bound and unbound substances, and thus on hormonal activity. Xenoestrogens, as ethinylestradiol in hormonal contraceptive, bind to SHGB with low affinity, and are generally bounded to albumin.

One possible consequence of this imbalance in protein levels due to hormonal contraceptives could be a lower free estradiol level. Actually, free estradiol level is lower in women under hormonal contraceptive than women without hormonal contraceptive (Bjornerem et al., 2004). Thus, when combining Kennedy's hypothesis with our observations, we conclude that the use of hormonal contraceptives like the combined contraceptive pill may increase CYP2E1 activity. Moreover, it seems to be more correct to say that the free estradiol level influences CYP2E1 activity instead of the estrogen level.

When comparing results obtained for free and total urinary 1M2P, we observed that an important part of the 1M2P is present in its conjugated form. Thus it is relevant to determine the total urinary 1M2P concentration when assessing human exposure to 1M2P. Besides, women on hormonal contraceptives appeared to excrete a higher fraction as conjugate than those not taking hormonal contraception. This may indicate that sex hormones levels also influence the enzyme activity of phase II reactions (Miners et al., 1983; Kennedy, 2008).

Creatinine correction is usually used in spot urine analysis and accounts for differences in urine flow and concentration. However, creatinine excretion depends on the muscle mass, which is higher in men than in women. In fact, when urinary TCE and TCA concentrations were adjusted to creatinine, sex differences became more pronounced.

Regarding the average symptom ratings obtained for each group of volunteers, we noticed that women rated irritating symptoms generally higher than men. It is interesting to see that even at half the threshold limit value, volunteers perceived irritating symptoms, solvent smell and headache. Fatigue belonged also to the high rated symptoms, but as it has been shown in a previous study (Ernstgård et al., 2002), there was in general no significant difference between volunteers exposed to clean air and those exposed to a solvent, making this symptom unspecific for solvent exposure.

As volunteers of this study belonged to a narrow range of age, the biological variability due to age was limited. However, due to the low number of participants, as it is often the case for studies with human volunteers, we observed an important interindividual variability among the participants groups, especially among women who did not take hormonal contraceptive. Despite a severe volunteer profile, some participants groups were less homogenous than others. Several factors could thus contribute to the observed variability within a group, as for example body fat or the CYP2E1 activity itself (Bolt et al., 2003). But regarding women without hormonal contraceptives, this variability could also be related to the hormonal variation during the menstrual cycle, as they were exposed at any point of their cycle. The hormonal fluctuations are characterized by high estrogen levels during ovulation and high progesterone levels during the luteal phase. Nevertheless, it was possible to underline some statistically significant differences between the studied human volunteer groups.

In conclusion, our results suggest that there exist differences between men and women when exposed to organic solvents which could be explained not only by physiological differences but also by differences in CYP2E1 enzyme activity. For same exposure conditions, biological exposure indices can differ between men and women, which can lead to a misinterpretation of biomonitoring results. This aspect should have its place in the approaches for setting limits of occupational exposure as the existing recommended biological limits for chemical substances are most based on a direct correlation with the corresponding threshold limit values.

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